“Drug Effects on the Excretory Ductal System of the Prostate, Seminal Vesicle and Epididymis”

INAUGURAL DISSERTATION

submitted in partial fulfillment of the requirements for the award of the degree of

Dr. rer. nat. /Ph.D.

as part of the joint award Ph.D. program of the

Justus-Liebig-University, Giessen, Germany and

Monash University, Clayton, Australia

Submitted by

Mathias Seidensticker

M. Sc. (Biology), Essen

Giessen, 2019

From the Institute of Anatomy and Cell Biology
Signaling group
Faculty of Medicine
Justus-Liebig-University, Giessen, Germany
Supervisor: **Prof. Dr. Ralf Middendorff**

(External) Supervisor and Committee Member/Reviewer: Prof. Dr. Ralf Middendorff
(Internal) Supervisor and Committee Member/Reviewer: PD Dr. Ellen Kauschke
Committee Member/Reviewer: Prof. Dr. Tina E. Trenczek
Committee Member/Reviewer: Prof. Dr. Florian Wagenlehner

Date of Disputation: 17.01.2020

Mit Genehmigung des Fachbereiches 08 - Biologie und Chemie
der Justus-Liebig-Universität Giessen
Dekan/Dean: Prof. Dr. Jürgen Janek: _____

and

The Anatomy and Developmental Biology
Monash Biomedicine Discovery Institute
Faculty of Medicine, Nursing and Health Sciences
Monash University
Clayton, Victoria, Australia
Supervisor: **Prof. Dr. Gail Risbridger**

Supervisor and Committee Member/Reviewer: Prof. Dr. Gail Risbridger
Committee Member/Reviewer: Dr. Donna Sellers
Committee Member/Reviewer: Dr. Scott Smid

I. Table of Contents

I. TABLE OF CONTENTS ..... **III**

II. DECLARATION ..... **VI**

III. PREFACE ..... **VIII**

IV. ACKNOWLEDGMENTS ..... **IX**

V. LIST OF FIGURES ..... **XI**

VI. LIST OF TABLES ..... **XIII**

VII. LIST OF ABBREVIATIONS ..... **XIV**

VIII. UNITS OF MEASUREMENT ..... **XVII**

IX. LIST OF PUBLICATIONS AND PRESENTATIONS ..... **XVIII**

X. ABSTRACT ..... **XXI**

XI. ZUSAMMENFASSUNG ..... **XXIII**

CHAPTER 1 - GENERAL INTRODUCTION **1**

1.1 Overview of the Male Reproductive Organs 1
1.2 Overview of Muscle Tissue and Smooth Muscle Contraction 4
1.2.1 The Muscular System *4*
1.2.2 Smooth Muscle Cells: Properties, Function and Contractility *5*
1.3 The Prostate Gland 10
1.3.1 Anatomy *10*
1.3.2 Function *15*
1.3.3 Prostate Innervation and Contractility *16*
1.3.4 Smooth Muscle Cells and α_1-adrenoceptors *18*
1.3.5 Hormonal Regulation of the Prostate Gland *21*
1.4 Benign Prostatic Hyperplasia 24
1.4.1 Etiology *26*
1.4.2 Pathophysiology *27*
1.4.3 Lower Urinary Tract Symptoms Associated with Benign Prostatic Hyperplasia *29*
1.4.4 Pharmacological Treatment Options and Side Effects *33*
1.4.5 Surgical Treatment Options *38*
1.4.6 The Rat Prostate Gland Animal Model *40*
1.5 Seminal Vesicles 43
1.5.1 Anatomy *44*
1.5.2 Function *45*
1.5.3 Smooth Muscle Cells and Contraction *45*
1.6 Epididymis 47
1.6.1 Anatomy *47*
1.6.2 Function *49*
1.6.3 Smooth Muscle Cells and Contraction *51*
1.7 Nitric Oxide/cGMP Pathway and Signaling Components 53
1.7.1 Transmembrane Guanylyl Cyclases and cGMP Function *53*
1.7.2 Soluble Guanylyl Cyclase and Nitric Oxide *55*
1.7.3 NO/cGMP Pathway in the Prostate Gland, Seminal Vesicles and Epididymis *56*
1.7.4 Pharmacological Inhibitors of PDE5 ..... *59*
1.8 Aims 61
1.8.1 Significance of the Project *61*

1.8.2 Aims *62*

CHAPTER 2 - MATERIALS AND METHODS **63**

2.1 TISSUES 63
2.1.1 Human Prostate Gland *63*
2.1.2 Rat Prostate, Seminal Vesicle and Epididymis *64*
2.2 TIME-LAPSE IMAGING 66
2.3 HISTOCHEMISTRY 68
2.3.1 Fixation *68*
2.3.2 Azan Staining *68*
2.3.3 Immunostaining *68*
2.4 CLEAR LIPID-EXCHANGED ACRYLAMIDE-HYBRIDIZED RIGID IMAGING/ IMMUNOSTAINING/ IN SITU HYBRIDIZATION-COMPATIBLE TISSUE-HYDROGEL (CLARITY) 71
2.5 TENSION RECORDING EXPERIMENTS 73
2.5.1 Human Prostate Gland *73*
2.6 CELL CULTURE EXPERIMENTS 75
2.6.1 Tissue Digestion *76*
2.6.2 qRT-PCR *77*
2.6.3 Western Blot *79*
2.7 STATISTICAL ANALYSIS 80
2.7.1 Time-lapse Imaging Analysis *80*
2.7.2 Tension Recording Analysis *80*
2.7.3 Cell Culture Experiments Analysis *81*

CHAPTER 3 - NOVEL IMAGING OF THE PROSTATE REVEALS SPONTANEOUS GLAND CONTRACTION AND EXCRETORY DUCT QUIESCENCE TOGETHER WITH DIFFERENT DRUG EFFECTS (FASEBJ, 2018) **82**

3.1 ABSTRACT 83
3.2 INTRODUCTION 84
3.3 MATERIAL AND METHODS 86
3.3.1 Tissues *86*
3.3.2 Time-lapse Imaging *86*
3.3.3 Immunostaining *87*
3.3.4 Clear lipid-exchanged acrylamide-hybridized rigid imaging/ immunostaining/ in-situ hybridization-compatible tissue-hydrogel *87*
3.3.5 Statistical Analysis *88*
3.4 RESULTS 89
3.4.1 All SMC Compartments of the Prostate Show PDE5 Expression *89*
3.4.2 Relaxing Effects of PDE5 Inhibition can be Directly Visualized in Human Prostate *92*
3.4.3 Isolated Prostate Glands Show Spontaneous Contractions that are Inhibited by PDE5 Inhibition *95*
3.4.4 Prostate Ducts do not Contract Spontaneously. Contractions Induced by NA, Mediating Emission and Ejaculation, are not Disturbed by PDE5 Inhibition *98*
3.5 DISCUSSION 101
3.6 ACKNOWLEDGEMENTS 104

CHAPTER 4 - TIME-LAPSE IMAGING REVEALS THAT THE ALPHA$_1$-ADRENOCEPTOR BLOCKER TAMSULOSIN BUT NOT THE PDE5 INHIBITOR TADALAFIL DIRECTLY DISTURBS THE SECRETORY FUNCTION OF THE SEMINAL VESICLES, PROSTATE AND SPERM RELEASE FROM THE EPIDIDYMIS (SUBMITTED) **105**

4.1 ABSTRACT 106
4.2 INTRODUCTION 107
4.3 MATERIALS AND METHODS 109
4.3.1 Tissues *109*
4.3.2 Time-lapse Imaging *109*

4.3.3 Statistical Analysis *110*
4.4 RESULTS 111
4.4.1 NA-Induced Secretion in Seminal Vesicles was Disturbed by Tamsulosin but not Tadalafil *111*
4.4.2 In Seminal Vesicles, NA-Induced Contractions were Abolished by Tamsulosin, but Unaffected by Tadalafil *114*
4.4.3 Tamsulosin Abolished NA-Induced Contractions in Prostatic Ducts, Different to Tadalafil *117*
4.4.4 In the Sperm Storing Part of the Epididymal Duct, Tadalafil did not Change Contractile Responses Induced by NA, in Contrast to Tamsulosin *120*
4.4.5 In the Sperm Storing Part of the Epididymal Duct, NA-Induced Contraction Frequencies are not Disturbed by Tadalafil Pre-Treatment, in Contrast to Tamsulosin *123*
4.5 DISCUSSION 126
4.6 CONCLUSIONS 128
4.7 ACKNOWLEDGEMENTS 128
4.8 TAKE HOME MESSAGE 128

CHAPTER 5 – INVESTIGATION OF A NOVEL NO DONOR AS A POTENTIAL PHARMACOLOGICAL TREATMENT OPTION FOR BPH 129

5.1 INTRODUCTION 129
5.2 MATERIALS AND METHODS 132
5.2.1 Tissues *132*
5.2.2 Tension Recording Experiments *132*
5.2.3 Cell Culture Experiments *133*
5.2.4 Synthesis of NO-Releasing Core Cross-Linked Star Polymer Conjugated GSNO *133*
5.2.5 Statistical Analysis *134*
5.3 RESULTS 135
5.3.1 Western Blot Analyses of cGMP Downstream Proteins Showed Changes in PDE5 and PKG1 Expression after Treatment with NO Donors *135*
5.3.2 Treatment of Human Prostate SMCs with GSNO Affected RNA Levels of cGMP Signaling Pathway Molecules *137*
5.3.3 Tension Recording Experiments Showed the Influence of GSNO and star-GSNO on Human Prostate Specimens by Altering Basal Tension, Amplitude, and Frequency of Contractions *140*
5.4 DISCUSSION 145

CHAPTER 6 - GENERAL DISCUSSION 148

6.1 STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE HUMAN AND RAT PROSTATE WITH FOCUS ON PDE5 INHIBITORS SILDENAFIL AND TADALAFIL 149
6.2 A NEWLY ESTABLISHED MODEL SYSTEM FOR THE EMISSION PHASE OF EJACULATION REVEALS ACTION OF PHARMACOTHERAPIES USED IN THE TREATMENT OF BPH 152
6.3 THE NEWLY SYNTHESIZED NO-RELEASING DONOR STAR-GSNO UPREGULATES CGMP PATHWAY COMPONENTS AND PROMOTES SMC RELAXATION 154

REFERENCES 157

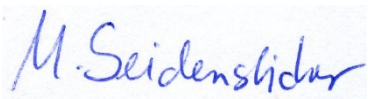
APPENDIX 178

II. Declaration

II.1 Thesis

I hereby declare, that I have completed this dissertation single-handedly without unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and cited all text passages that are derived verbatim from or are based on the content of published work of others, and all information relating to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus-Liebig-University Giessen 'Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis' in carrying out the investigations described in the dissertation. I consent to the use of an anti-plagiarism software to check my thesis according to § 25 Abs. 6 of the "Allgemeine Bestimmungen für modularisierte Studiengänge".

Hiermit versichere ich, die vorgelegte Thesis selbstständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt zu haben, die ich in der Thesis angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Thesis erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der ‚Satzung der Justus-Liebig-Universität zur Sicherung guter wissenschaftlicher Praxis' niedergelegt sind, eingehalten. Gemäß § 25 Abs. 6 der Allgemeinen Bestimmungen für modularisierte Studiengänge dulde ich eine Überprüfung der Thesis mittels Anti-Plagiatssoftware.

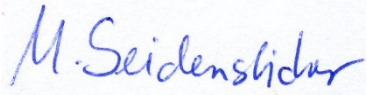


Giessen, 29.03.2019 Mathias Seidensticker

II.2 Published works

This thesis includes 1 (one) original paper published in a peer-reviewed journal (**Chapter 3**) and 1 (one) submitted publication in a peer-reviewed journal (**Chapter 4**). The core theme of the thesis is smooth muscle contractility and pharmacological responses in the human and rat prostate as well as rat seminal vesicle and epididymis. The ideas, development and writing up of the paper in Chapter 3 of the thesis were the principal responsibility of Robert Kügler, Andrea Mietens and myself, the student (shared first-authorships), and in chapter 4 myself, the student, working within the group signal transduction in the Anatomy and Cell Biology of the Justus-Liebig-University Giessen under the supervision of Professor Ralf Middendorff. The inclusion of co-authors reflects the fact that the work came from and active collaboration between researchers and acknowledges input into team-based research.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature:  Date: 29.03.2019

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions in this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: Date: 29.03.2019

III. Preface

This Ph.D. thesis was conducted within the Ph.D. joint program of the IRTG 1871 (International Research Training Group) between The Justus-Liebig-University Giessen Germany and Monash University, Clayton, Australia, and funded by the German Research Foundation (DFG). The supervisor arrangements are as follows:

Justus-Liebig-University	Monash University
Prof. Dr. Ralf Middendorff – 35% Main supervisor Faculty 11 – Medicine Institute of Anatomy and Cell Biology, Signal transduction	**Dr. Betty Exintaris – 35%** Main Supervisor Monash Institute of Pharmaceutical Sciences, Drug Discovery Biology
PD Dr. Ellen Kauschke – 15% Co-Supervisor Faculty 08 – Biology and Chemistry Department of Zoology and Developmental Biology, Human Biology	**Prof. Dr. Gail Risbridger – 15%** Co-Supervisor Faculty of Medicine, Nursing and Health Sciences, Anatomy and Developmental Biology

Experiments and data collection summarized in Chapter 3 and 4 was conducted in Prof Ralf Middendorff's laboratories in the Institute of Anatomy and Cell Biology of the Justus-Liebig-University Giessen within the first two years of candidature.

Experiments and data collection summarized in Chapter 5 was conducted in Dr. Betty Exintaris' laboratory at The Monash Institute of Pharmaceutical Sciences of the Monash University, Parkville, Australia and in Prof. Dr. Gail Risbridger's laboratory at The Institute of Anatomy and Developmental Biology of the Monash University, Clayton, Australia.

IV. Acknowledgments

Firstly, I would like to thank my main supervisor Prof. Ralf Middendorff for giving me the chance to work in his group at the Justus-Liebig-University Giessen as a doctoral candidate to fulfill my dream of achieving the degree as “Dr. rer. nat. /Ph.D.”. Your supervision, trust, knowledge, and support during this time couldn’t be appreciated more.

The same gratefulness goes to my main supervisor at Monash University, Dr. Betty Exintaris. Not only is your guidance as a supervisor greatly appreciated, but also the opportunities you made possible, be it the many presentations I was able to give at conferences or to fulfill the position as a teaching associate at the Monash Institute of Pharmaceutical Sciences at the Parkville Campus.

I also want to thank Dr. Ellen Kauschke and Prof. Dr. Gail Risbridger for their supervision, confidence, and resources.

Next, I would like to thank all my (post-) doctoral colleagues, technicians, and especially people I am proud to call friends now, both in Germany and Australia, for teaching me and brainstorming about the things I know now and the skills I acquired. Due to excellent cooperation, the present work has been possible to conduct with your support.

My appreciation also goes to everyone involved in the project of the International Research Training Group (IRTG), a collaboration between Justus-Liebig-University Giessen and Monash University. Being part of this project made it possible for me to get to know national and international experts in the field of the pathogenesis of male reproductive organs, share ideas and conduct research for one year abroad in Australia.

I would like to thank the team of the Giessen Graduate Centre for the Life Sciences (GGL) for supporting young researchers like me during the time as a doctoral researcher. I have made many important and useful experiences while participating in the programs offered, starting from networking to writing scientific texts, over to analyzing data and presenting thereof, up to join a mentoring program to support my future career paths.

Finally, and to me most importantly, I like to thank my family and close friends, of which the latter is regarded as part of the first, for all their support, motivation, open ears and minds, help, quality time, conversations, input, understanding, mindfulness and in general being there for me when I needed them, asked or unasked, in all the fun but also hard times throughout the three years of my PhD. I would not be where I am today without you. I will always remember this, and I could have not asked for more.

This study was funded by the Deutsche Forschungsgemeinschaft (DFG) Grant GRK 1871 and funding from Monash University, Australia, to The International Research Training Group (IRTG), a collaboration between Justus-Liebig-University Giessen and Monash University.

Project 6: “Hormonal regulation of cGMP pathways in the prostate - Impact for sperm transport”

V. List of Figures

Fig.1: Sagittal section of the male pelvis showing the reproductive organs.

Fig.2: Smooth Muscle Tissue.

Fig.3: Regulation of smooth muscle contraction.

Fig.4: Relaxation of smooth muscle.

Fig.5: The zonal anatomy of the human prostate gland.

Fig.6: Distribution of cell types within the prostate gland.

Fig.7: Innervation of the Prostate.

Fig.8: Intracellular signal transduction in prostate SMCs.

Fig.9: Influence of testosterone in epithelial and stromal cell growth of the prostate.

Fig.10: Comparison of the normal and enlarged prostate gland.

Fig.11: The dual pathogenesis hypothesis of BPH etiology.

Fig.12: International Prostate Symptom Score (IPSS).

Fig.13: Prevalence of moderate to severe BPH symptoms.

Fig.14: Overview of the male rat reproductive and urinary organs.

Fig.15: Overview of the reproductive organs in man.

Fig.16: Overview of the anatomical structures of the human testis and epididymis.

Fig.17: Overview of the NO/cGMP signaling pathway and components regulating cGMP action.

Fig.18: Localization of PDE5 in SMCs of glands, vessels, and ducts of the human prostate.

Fig.19: Visualization of spontaneous contractility of human prostate tissue sensitive to PDE5 inhibition.

Fig.20: Visualization of contractile pattern in rat prostate gland and PDE5 localization in glandular, vascular, and ductal SMCs.

Fig.21: Prostate duct showed absence of spontaneous contractions and no sildenafil effects on NA-induced contractions.

Fig.22: Tadalafil pre-treatment did not affect NA-induced secretion and therefore weight changes in seminal vesicles, but tamsulosin did.

Fig.23: Tadalafil did not affect NA-induced contractions in seminal vesicles. Tamsulosin, on the other hand, abolished NA-induced contractions.

Fig.24: Tadalafil did not change NA-induced contractions in prostatic ducts, but tamsulosin did.

Fig.25: Analysis of NA-induced contractions in the distal part of cauda epididymis in tadalafil and tamsulosin pre-treated tissue.

Fig.26: Investigation of NA-induced contraction frequency in Tadalafil and Tamsulosin pre-treated parts of the distal cauda part of the epididymal duct.

Fig.27: Western Blot analysis showed a different level of protein expression in cell lines treated with GSNO or star-GSNO.

Fig.28: qRT-PCR analyses showed upregulated RNA expression for genes encoding for sGC, PDE5, and PKG1 after 24h treatment with GSNO or star-GSNO.

Fig.29: Tension recording experiments for vehicle showed a decrease of basal tension over time.

Fig.30: Incubation of human prostate specimens with GSNO showed a gradual decrease of basal tension, amplitude, and frequency.

Fig.31: Tension recording experiments of human prostate specimens treated with the star polymer GSNO showed a significant decrease in basal tension.

VI. List of Tables

Table 1: Overview of current FDA approved medications in the treatment of BPH.

Table 2: Hydrogel and buffer composition.

Table 3: RIMS buffer composition for optical clearance enhancement.

Table 4: Components of SMC media.

Table 5: Human-specific primer descriptions.

Table 6: Template for qRT-PCR loading.

Table 7: Template for qRT-PCR conditions.

VII. List of Abbreviations

α-SMA	α-smooth muscle actin
3D	three dimensional
5AR	5α-reductase
ADP	adenosine 5’-diphosphate
AFMS	anterior fibromuscular stroma
ANP	atrial natriuretic peptide
AR	androgen receptor
ATP	adenosine-5'-triphosphate
AUA	American Urological Association
BNP	brain natriuretic peptide
BOO	bladder outlet obstruction
BPE	benign prostatic enlargement
BPH	benign prostatic hyperplasia
BPO	benign prostatic obstruction
BSA	bovine serum albumin
CaP	prostate cancer
CDK8	cyclin-dependent kinase 8
cDNA	complementary deoxyribonucleic acid
CNP	C-type natriuretic peptide
cGMP	cyclic guanosine monophosphate
CZ	central zone
DAB	3,3'-diaminobenzidine
DAG	diacylglycerol
DAPI	4’,6’-diamidino-2-phenylindole
DRE	digital rectal examination
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DHT	dihydrotestosterone
DMSO	dimethyl sulfoxide
EAU	European Association of Urology
EDTA	ethylenediaminetetraacetic acid

ERα	estrogen receptor α
ERβ	estrogen receptor β
FCS	fetal calve serum
FDA	US food and drug administration
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GC-A	guanylyl cyclase type A
GC-B	guanylyl cyclase type B
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GSNO	S-Nitrosoglutathione
GTP	guanosine-5'-triphosphate
Gα/β/γ	G protein subunits α/β/γ
HRE	hormone response element
HRP	horseradish peroxidase
ICC	interstitial cells of Cajal
IPSS	International Prostate Symptom Score
IP_3	inositol-1,4,5-triphosphate
LH	luteinizing hormone
LUTS	lower urinary tract symptoms
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
MLC	myosin light chain
MLCK	myosin light chain kinase
NA	noradrenaline
NO	nitric oxide
NOS	nitric oxide synthase
P_i	phosphate
PAP	prostatic acid phosphatase
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Triton
PDE5	cGMP-specific phosphodiesterase type 5
PFA	paraformaldehyde

PIC	prostate interstitial cells
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PKG1	cGMP-dependent protein kinase type 1
PLC	phospholipase C
PSA	prostate-specific antigen
PSS	physiological saline solution
PVC	polyvinyl chloride
PZ	peripheral zone
qRT-PCR	quantitative reverse transcription polymerase chain reaction
QoL	quality of life
RIMS	refractory index matching solution
RNA	ribonucleic acid
SR	sarcoplasmic reticulum
sGC	soluble guanylyl cyclase
SMA	smooth muscle actin
SMC	smooth muscle cell
SPI	Symptom Problem Index
T	testosterone
TRUS	transrectal ultrasound
TUNA	transurethral needle ablation
TUMT	transurethral microwave thermotherapy
TUR-P	transurethral monopolar electroresection of the prostate
TZ	transition zone

VIII. Units of Measurement

%	percent
cc	cubic centimeter
cm	centimeter
g	gram
h	hour(s)
M	molar
mg	milligram
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
mN	millinewton
mV	millivolt
ng	nanogram
p	p-value
pH	acidity or basicity of an aqueous solution
s	second(s)
°C	degree Celsius
μg	microgram
μm	micrometer
μM	micromolar

IX. List of Publications and Presentations

Published Journal Articles

Kügler R*, Mietens A*, Seidensticker M*, Tasch S, Wagenlehner FM, Kaschtanow A, Tjahjono Y, Tomczyk CU, Beyer D, Risbridger GP, Exintaris B, Ellem SJ, Middendorff R “Novel imaging of the prostate reveals spontaneous gland contraction and excretory duct quiescence together with different drug effects”, *equal contribution Epub 2018 Jan 3. doi: 10.1096/fj.201700430R (FASEB J. 2018 Mar;32(3):1130-1138)

Published Conference Abstracts

Oral Presentations

Seidensticker M, Kügler R, Mietens A, Tasch S, Wagenlehner FM, Risbridger GP, Ellem SJ, Exintaris B, Middendorff R, 2018 “Treatment of BPH and Disturbed Ejaculation: Abnormal function of accessory sex glands by alpha1-adrenoceptor blocker tamsulosin but not by PDE5 inhibitor tadalafil”, 10th National Symposium on Advances in Gastrointestinal & Urogenital Research 2018, November 26th 2018, The Stamford Grand Adelaide, Glenelg, Adelaide, Australia

Lee S, Hammar J, Van Gramberg J, Papargiris M, Seidensticker M, Risbridger GP, Whittaker M, Ellem SJ, Middendorff R, Exintaris B, 2018 “Targeting oxytocin for the treatment of bladder outlet obstruction due to benign prostatic hyperplasia”, International Continence Society (ICS) 48th Annual Meeting, August 28th – August 31st 2018, Pennsylvania Convention Centre, Philadelphia, USA

Freihat LA, Seidensticker M, Exintaris B, McIntosh MP, Short JL, 2018 “Incorporating new practical classes to the first year physiology unit a student and demonstrator perspective”,WCP2018 IUPHAR Pharmacology Education Section Satellite, Educating scientists and healthcare professionals for 21st century pharmacology, June 30th 2018 – July 1st 2018, Kyoto International Conference Center, Kyoto, Japan

Poster Presentations

Seidensticker M, Exintaris B, Middendorff R, 2018 “Treatment of BPH and Disturbed Ejaculation: Abnormal function of accessory sex glands by alpha1-adrenoceptor blocker tamsulosin but not by PDE5 inhibitor tadalafil”, IRTG Milestone Meeting 2018, December 10th – December 12th 2018, Monash University Law Chambers (MULC), 555 Lonsdale Street, Melbourne, Vic 3000, Australia

Seidensticker M, Kügler R, Mietens A, Tasch S, Wagenlehner FM, Risbridger GP, Ellem SJ, Exintaris B, Middendorff R, 2018 “Treatment of BPH: Disturbed function of prostate ducts by the alpha1-adrenergic receptor blocker tamsulosin but not by PDE5 inhibitors”, Continence Foundation of Australia (CFA), 27th National Conference on Incontinence 2018, October 24th – October 27th 2018, Grand Chancellor Hotel Hobart, Hobart, Tasmania

Seidensticker M, Mietens A, Tasch S, Exintaris B, Middendorff R, 2018 “A new model to visualize emission-related contractions in the epididymis and beyond”, Society for the study of Reproduction (SSR), 7th International Conference on the Epididymis, September 20th – September 23rd 2018, Plaza Centre-Ville, Montreal, Quebec, Canada

Seidensticker M, Exintaris B, Kügler R, Mietens A, Tasch S, Wagenlehner FM, Risbridger GP, Middendorff R, 2018 “Novel imaging techniques reveal disturbed function of prostate ducts by alpha1-adrenergic receptor blocker but not by PDE5-inhibitors in the treatment of BPH”, International Continence Society (ICS) 48th Annual Meeting, August 28th – August 31st 2018, Pennsylvania Convention Centre, Philadelphia, USA

Seidensticker M, Kügler R, Mietens A, Tasch S, Wagenlehner F, Risbridger G, Exintaris B, Ellem S, and Middendorff R, 2017. “Ejaculation disorders and treatment of BPH: Disturbed function of prostate ducts by the alpha1-adrenergic receptor blocker tamsulosin but not by PDE5 inhibitors”, 7.DVR Kongress München, December 7th – December 9th 2017, Holiday Inn Munich City Centre, Hochstraße 3, 81669 Munich

Seidensticker M, Kügler R, Mietens A, Tasch S, Wagenlehner F, Risbridger G, Exintaris B, Ellem S, and Middendorff R, 2017. “Visualization of PDE5 inhibitor effects in prostate tissue: no evidence for disturbances of ejaculation”, GGL Annual Conference, September 27th 2017, Heinrich-Buff-Ring 14, Giessen

Seidensticker M, Kügler R, Mietens A, Tasch S, Wagenlehner F, Risbridger G, Exintaris B, Ellem S, and Middendorff R, 2017. “Visualization of PDE5 inhibitor effects in prostate tissue: no evidence for disturbances of ejaculation”, 112th Annual Meeting of “Anatomische Gesellschaft”20th – 22nd September 2017, Würzburg

Seidensticker M, Ellem S, and Middendorff R, 2017. “Visualization of PDE5 inhibitor effects in the prostate: no evidence for disturbances of ejaculation” IRTG on-site review, March 22nd 2017, Institut für Anatomie & Zellbiologie, Aulweg 123, Giessen

Seidensticker M, Mietens A, Tasch S, Tomczyk C, Beyer D, Wagenlehner F, Risbridger G, Ellem S, Exintaris B, and Middendorff R, 2016. “Visualization of PDE5 inhibitor effects in the prostate: no evidence for disturbances of ejaculation”, GGL Annual Conference, September 21st 2016, Heinrich-Buff-Ring 14, Giessen

Awards

“Best Oral Presentation”, 10th National Symposium on Advances in Gastrointestinal & Urogenital Research 2018, November 26th 2018, The Stamford Grand Adelaide, Glenelg, Adelaide, Australia

3rd place “Best Poster Award”, GGL Annual Conference, September 27th 2017, Heinrich-Buff-Ring 14, Giessen

“Best Poster Award”, 7.DVR Kongress München, December 7th – December 9th 2017, Holiday Inn Munich City Centre, Hochstraße 3, 81669 Munich

Travel Grants

Central and Departmental (ADB Travel Grant) Monash Travel Grant (Study away) for the ICS2018 congress in Philadelphia, USA

X. Abstract

An increase in smooth muscle tone and enlargement of the human prostate gland are major components of benign prostatic hyperplasia (BPH) and the associated pathophysiology of lower urinary tract symptoms (LUTS). BPH is an extremely common disorder of the aging male population and the development can be categorized into two components; the dynamic and static component. An increase of the dynamic component is composed of the neurogenic tone and the myogenic tone of the prostate and leads to an increase in smooth muscle contractility and tone. The static component comprises the stromal proliferation of prostatic tissue and leads to an increase in prostate size and occurs due to age-dependent changes in hormone levels. Therefore, current pharmacological treatment options target to reduce either smooth muscle tone (dynamic component) or to decrease the prostate size (static component). Pharmacotherapies used to reduce the prostate size include 5α-reductase (5AR) inhibitors. Classical treatment options to reduce smooth muscle tone and contractility are performed with α_1-adrenoceptor antagonists like tamsulosin but often result in adverse effects like abnormal ejaculation, having an impact on the quality of life and thus potentially leading to discontinuation of therapy. The exact causes and mechanisms of action leading to ejaculation disorders due to treatment with tamsulosin are still a matter of debate. Although inhibitors of the cyclic guanosine monophosphate (cGMP)-hydrolyzing enzyme phosphodiesterase type 5 (PDE5) are now regularly used for the treatment of BPH, detailed information about their cellular site of action and function within the prostate is missing. Another key component regulating levels of cGMP within smooth muscle cells (SMCs) is nitric oxide (NO), a free radical gas that promotes SMC relaxation by causing an increase of cGMP levels within the cells.

In prostate tissue of rat and man, PDE5 was shown to be highly expressed in interstitial and vascular SMCs but not in epithelial cells of the gland. Investigation of the effects of various PDE5 inhibitors (tadalafil and sildenafil) on the smooth muscle tissue of the prostate was performed for rat and human tissue utilizing a novel time-lapse imaging approach.

Moreover, the structural and functional architecture of rat prostate tissue was defined which allowed separate investigations of the single glands, producing prostatic fluid, and the excretory ducts, transporting prostatic fluid to the urethra during ejaculation. In rat prostate, spontaneous contractility was only observed in terminal prostatic glands but not in proximal ducts. Recordings showed that PDE5 inhibitors reduced the myogenic tone (spontaneous contractions) of prostate tissue in rat and man.

Analysis of adverse effects of the α_1-adrenoceptor antagonist tamsulosin on contractile tissues responsible for ejaculation, specifically the excretory prostate ducts, seminal vesicles and the sperm-storing part of the cauda epididymal duct, was performed utilizing the novel time-lapse imaging approach. This model system visualized and revealed adverse effects of tamsulosin on different tissues involved in ejaculation and showed that these adverse effects were not present under the influence of the PDE5 inhibitor tadalafil.

Aside from already approved pharmacotherapies to treat BPH, research for novel pharmacological options to relax smooth muscle tissue is crucial. S-nitrosoglutathione (GSNO), a NO donor, was tested (i) as its common construct and (ii) conjugated to the core of a cross-linked star polymer in tension recording experiments as well as *in vitro* experiments of primary human prostate SMCs. Experiments showed an upregulation of cGMP pathway components on RNA and protein levels as well as relaxing effects on contractility, especially reduction of basal tension of tissues following incubation with either variant. Nevertheless, further studies are necessary to confirm the observed results due to differences in patient parameters like age, previous medication, and individual lifestyles.

XI. Zusammenfassung

Ein erhöhter Tonus der glatten Muskulatur und eine generelle Vergrößerung des Prostatagewebes sind die hauptsächlichen Faktoren, die bei der benignen Prostatahyperplasie (BPH) und die damit verbundene Pathophysiologie der Symptomatik des unteren Harnwegtraktes (LUTS) bestimmen. BPH ist eine sehr häufig auftretende Krankheit der alternden männlichen Population und kann in zwei Komponenten kategorisiert werden; die dynamische und die statische Komponente. Eine Erhöhung der dynamischen Komponente beinhaltet den neurogenen und myogenen Tonus der Prostata und führt zu einer Erhöhung von Tonus und Kontraktilität der glatten Muskelzellen. Die statische Komponente umfasst u.a. die Proliferation des Prostata-Stromas und führt zu einer Vergrößerung der Prostata, welche durch altersbedingte Veränderungen von Androgen- und anderen Hormonspiegeln auftritt. Daher zielen heutige pharmakologische Behandlungen darauf ab, den Muskeltonus (dynamische Komponente) bzw. die Größe des Prostatagewebes (statische Komponente) zu reduzieren. Medikamente, welche die Größe der Prostata reduzieren, sind 5α-Reduktase-Inhibitoren. Die klassische Behandlungsmethode, um den Muskeltonus zu reduzieren, wird mit α_1-Adrenozeptor-Antagonisten wie Tamsulosin durchgeführt, welche jedoch oft mit Nebenwirkungen wie Ejakulationsstörungen verbunden sind und somit einen erheblichen Einfluss auf die Lebensqualität haben, was oft zum Abbruch der Therapie führt. Der Wirkmechanismus und die genauen Ursachen, die zu Ejakulationsstörungen bei der Behandlung mit Tamsulosin führen, sind nicht bekannt. Obwohl Inhibitoren des cyclischen Guanosinmonophosphat (cGMP)-hydrolisierenden Enzyms Phosphodiesterase Typ 5 (PDE5) mittlerweile in der Behandlung von BPH genutzt werden, sind genaue Informationen über den zellulären Wirkungsort und die Funktion in der Prostata nicht bekannt. Ein weiteres Schlüsselmolekül in der Regulation von cGMP-Spiegeln in glatten Muskelzellen, welches die Relaxation des Gewebes fördert, ist Stickstoffmonoxid (NO), ein freies radikales Gas. Dieses führt zur Relaxation von glatten Muskelzellen. PDE5 fand sich im hohen Maße innerhalb der glatten Muskelzellen des interstitiellen Gewebes und der Gefäße der Prostata beim Menschen und der Ratte, aber nicht in Epithelzellen.

Die Untersuchung verschiedener PDE5-Inhibitoren (Sildenafil und Tadalafil) und deren Einfluss auf glatte Muskelzellen in der Prostata wurden mit einem neu etablierten Modellsystem (*Time-lapse Imaging*) für Gewebe aus Mensch und Ratte durchgeführt. Weiterhin wurde die strukturelle und funktionelle Architektur der Ratten-Prostata definiert, welche eine isolierte Untersuchung der einzelnen Prostatadrüsen, zuständig für die Produktion des Sekretes der Prostata, und der Ausführungsgänge der Prostata, zuständig für den Ausstoß des Sekretes während der Ejakulation in die Urethra, zulässt. Spontane Kontraktionen wurden nur in den Drüsen der Prostata beobachtet, allerdings nicht in den Ausführungsgängen. Inkubation des Gewebes mit PDE5-Inhibitoren führte zu einer signifikanten Abnahme des myogenen Tonus (Spontankontraktionen) des Gewebes der Prostata bei Mensch und Ratte.

Die Untersuchung der Nebenwirkungen, hervorgerufen durch den α_1-Adrenozeptor-Antagonisten Tamsulosin in den Ausführungsgängen der Prostata, der Samenblase und dem Spermien speichernden Teil des distalen Nebenhodens wurde mit dem neuen Modell des *Time-lapse Imaging* durchgeführt. Dieses Modellsystem visualisierte und offenbarte die negativen Effekte von Tamsulosin auf die Gewebe, welche für die Ejakulation zuständig sind und zeigte, dass diese negativen Effekte bei Behandlung mit dem PDE5-Inhibitor Tadalafil nicht auftraten.

Abgesehen von bereits genehmigten Medikamenten in der Behandlung von BPH, ist die Forschung nach neuen Relaxanzien glatter Muskelzellgewebe von hoher Bedeutung. S-Nitrosoglutathion (GSNO), ein Stickstoffmonoxid (NO)-Donor, wurde in gebräuchlicher Form oder als modifiziertes Konstrukt mit dem Organbad und *in vitro* untersucht. Die Experimente zeigten eine Hochregulierung von cGMP Signalwegskomponenten auf RNA- und Protein-Ebene. Zusätzlich wurden in Organbadexperimenten relaxierende Effekte auf die Kontraktionskraft insbesondere in Bezug auf die Grundspannung des Gewebes, für beide GSNO-Varianten festgestellt. Trotzdem sind weitere Studien notwendig, um diese Ergebnisse zu bestätigen aufgrund von Unterschieden der Patienten-Parameter wie Alter, vorherige Medikamentation und unterschiedliche Lebensweisen.

Chapter 1 - General Introduction

1.1 Overview of the Male Reproductive Organs

The male reproductive system in mammals comprises various organs, each with specific functions contributing to sexual reproduction. The organs of the male reproductive system include the testes, a system of ducts including the epididymis, ductus deferens, ejaculatory ducts and urethra, the penis, and the accessory sex glands which include the seminal vesicles, the prostate and the bulbourethral gland (Cowper's gland) (Sharma et al. 2017) (Figure 1).

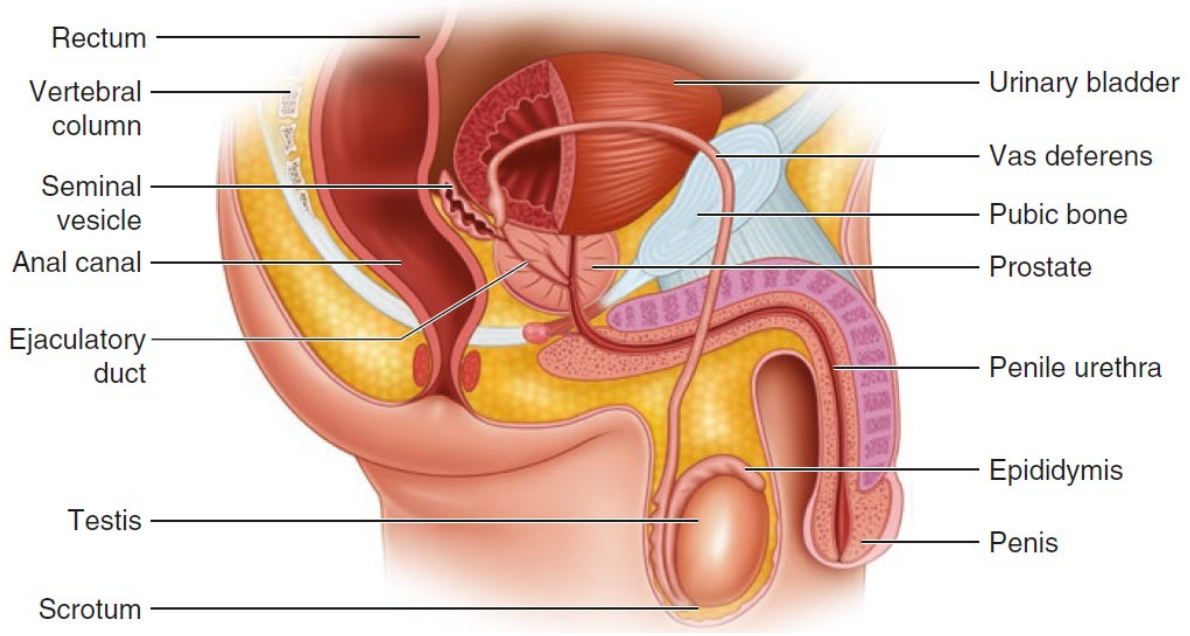


Figure 1: Sagittal section of the male pelvis showing the reproductive organs. The figure shows the penis, testis, epididymis, ejaculatory duct and penile urethra. The accessory sex glands are also shown which includes the vas deferens, seminal vesicle, and the bulbourethral gland just under the prostate (Sharma et al. 2017).

The testes, or testicles (male gonads), are paired oval-shaped glands contained within an extension of the abdomen called the scrotum outside of the body. The average size of a male testicle is about 5cm long and 2.5cm in diameter. Their function is to produce sperm (spermatogenesis) and androgens, primarily testosterone (Jenkins et al. 2009). The outer membranous fibrous tunica albuginea covers very fine coiled tubes, the seminiferous tubules, where spermatogenesis takes place through spermatogenic cells.

The other cell type inside the seminiferous tubules is the Sertoli cells. Sertoli cells are essential for testis formation and spermatogenesis. Sertoli cells facilitate the progression of germ cells to spermatozoa by controlling the environmental milieu within the seminiferous tubules. In between adjacent seminiferous tubules located are the Leydig cells, secreting the most prevalent androgen (male sex hormone) testosterone. It has long been known that testosterone promotes the development of male sexual characteristics and libido (Mooradian et al. 1987).

The sperm develop through the seminiferous tubules to the rete testis and are transported to the efferent ducts into the epididymis, where newly created sperm mature and acquire motility (De Jonge and Barratt 2006). The epididymis lies on the posterior border of each testis and is divided into three regions: the caput (head), corpus (body) and cauda (tail) and is about 4cm in length. The matured sperm are stored in the most distal part of the caudal epididymal duct and are released during the emission phase of ejaculation to the vas deferens via contractions of smooth muscle tissue leading to the ejaculatory duct. The ejaculatory duct is formed by the excretory ducts of each seminal vesicle together with the vas deferens just superior of the prostate. The ejaculatory duct terminates in the urethra, where the sperm, seminal vesicle and prostatic secretions are combined, forming the semen which is released to the exterior.

Sexual stimulation leads to an erection, the enlargement, and stiffening of the penis caused by smooth muscle cell (SMC) relaxation of the walls of arterioles supplying erectile tissue in the penis. This leads to the dilation of blood vessels, increased blood flow and widening of blood sinuses which results in an erection.

The sexual climax and orgasm commonly accompany the release of semen, called ejaculation, from the penis and is a sympathetic reflex coordinated by the lumbar portion of the spinal cord. During emission, before ejaculation occurs, the urinary bladder closes, preventing urine from being expelled and semen entering the bladder. Additionally, through contractions of smooth muscle tissue, the epididymis, vas deferens, seminal vesicles, ejaculatory ducts, and the prostate propel semen into the penile portion of the urethra before the release to the exterior.

Sympathetic nerve fibers, originating from the pelvic plexus, terminate as postganglionic fibers that innervate the bladder neck, prostate, vas deferens and seminal vesicles responsible for the emission phase of ejaculation, such as bladder neck closure and seminal vesicle emission (Lipshultz et al. 2009).

1.2 Overview of Muscle Tissue and Smooth Muscle Contraction

1.2.1 The Muscular System

Muscle tissue is one of the four primary tissue types (epithelial, connective, muscular and nervous tissue) of the body and the muscular system in vertebrates consists of three types of muscle tissue: skeletal, smooth and cardiac muscle cells. All types of muscle tissues share the function of permitting movement (of the body or organs), maintaining posture and the circulation of blood through the body. They all exhibit the quality of electrical excitability, contractility, extensibility, and elasticity because of the contractile myofilaments actin and myosin. Although they share some properties, they differ in their microscopic anatomy, location, organization of their contractile proteins and control by the nervous and endocrine system.

Skeletal muscles, or striated muscles, are under the voluntary control of the somatic nervous system (Birbrair et al. 2013). The name originates from the function to move bones of the skeleton for voluntary movement of the body. Aside from that, they are also controlled subconsciously, e.g. the diaphragm for breathing or other muscles to maintain posture and stability (Jenkins et al. 2009). Next to producing body movements and stabilizing body position, the skeletal muscles are responsible for storing and moving substances within the body, e.g. swallowing, urination, defecation and generating heat to maintain homeostasis through the release of energy when adenosine triphosphate (ATP) is broken down for muscle contraction, e.g. while shivering. Cardiac muscle tissue is only found in the heart. The function of cardiac muscle cells is to pump blood into the vessels of the circulatory system. It is striated, organized into sarcomeres, has shorter muscle fibers compared to skeletal muscles and contains only one nucleus. Contractions to control the heartbeat are performed by specialized cardiac muscle cells called pacemaker cells and cannot be consciously controlled.

In this thesis, the investigation of SMCs is the main object of interest and therefore are described more in detail in the following.

1.2.2 Smooth Muscle Cells: Properties, Function and Contractility

Sheets or layers of SMCs (Figure 2) are present in all viscera, walls of hollow organs like the urinary bladder, uterus, stomach, intestines, and tubular organs including blood vessels (arteries and veins), the tracts of the respiratory, urinary, and reproductive systems. A detailed description of SMC structures and functions in the male reproductive tract, especially the prostate, the seminal vesicles, and the epididymis is described in the following subchapters (see Chapters 1.3, 1.5 and 1.6). SMCs are also present in the eyes and skin (Webb 2003). Their function varies widely in different tissues, yet the variation of the appearance of SMCs is small in comparison (Szekeres and Papp 1994). Properties that are common to all SMCs include being uninuclear, having cell-to-cell and cell-to-stroma junctions, and an absence of striated banding patterns opposite to skeletal or cardiac muscle. Differences that vary from organ to organ are related to different functions of each muscle and can be characterized by the three-dimensional (3D) arrangements, layers, bundles, cords, packing density, occurrence and distribution of non-muscle cells, cytological characteristics and in the distribution of proteins involved in contraction, cytoskeleton, cell membrane and stroma.

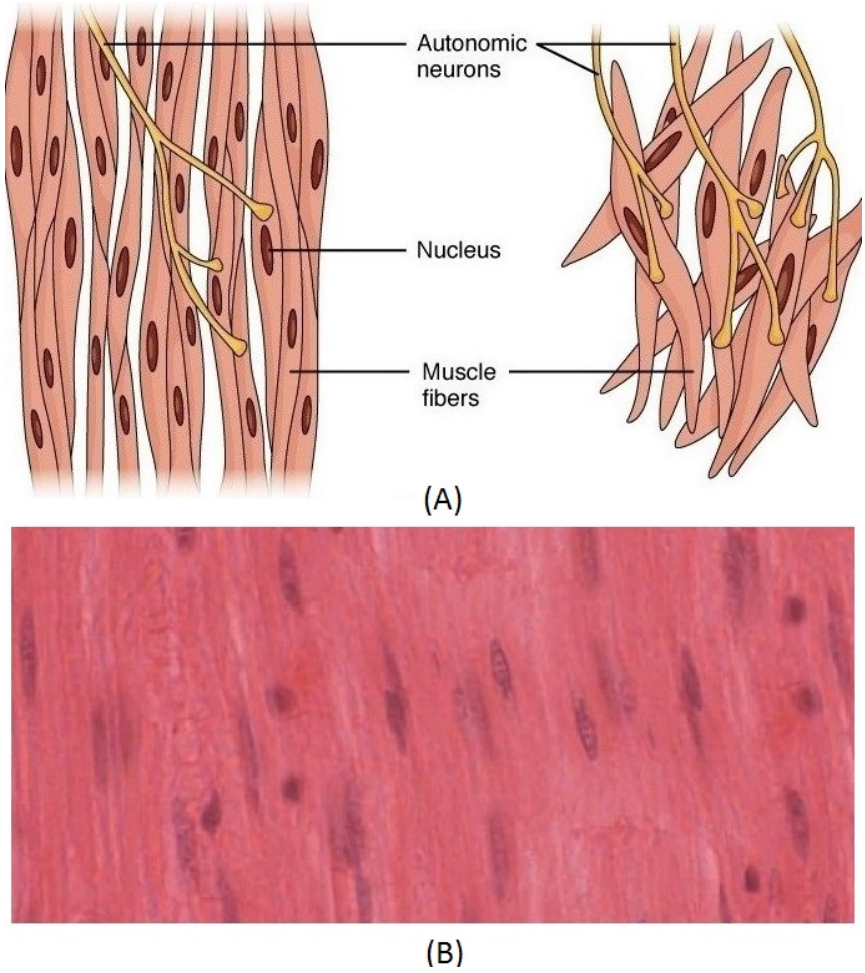


Figure 2: Smooth Muscle Tissue.
A: Spindle-shaped cells containing a single nucleus and lack of striation. Smooth muscle tissue is found around different organs which include the digestive and reproductive tracts. **B:** Micrograph photo of a sheet of smooth muscle (LMx1600). (Micrograph modified after the Regents of University of Michigan Medical School © 2012). Adapted from (Betts et al. 2016).

The types of SMCs of interest that are also highly abundant are SMCs among the male reproductive organs. In general, SMCs are spindle-shaped cells, 30 to 200μm in size and 20-100 times as long as they are wide at their widest point (Figure 2B). Although SMCs do not have striations and sarcomeres, the fibers have actin and myosin as contractile proteins and thick and thin filaments. The cell membrane is occupied by caveolae, which are flask-shaped in pockets of the cell membrane, rich in proteins and lipids with several functions in signal transduction. Interactions between cells are mediated through gap junctions. Gap junctions allow a direct and non-selective exchange of ions and small molecules between cells. Cell-to-cell junctions are very abundant in smooth muscle tissues and serve two main functions: mechanical coupling and excitation coupling (Szekeres and Papp 1994).

The former allows the contraction of adjacent cells to the one originally contracting. The latter allows the simultaneous activation of many muscle cells, e.g. from excitations by nerve endings or pacemaker cells. Contractions of SMCs (Figure 3) are regulated by the autonomic nervous system (e.g. due to the firing of action potentials) and are characterized by a shortening of the muscle. The contractile state is controlled by hormones, autocrine/paracrine agents and other local chemical signals. Regardless of the stimulus, the initiation of contractions follows the same principle of cross-bridge cycling between actin and myosin to develop force and the requirement of calcium (Ca^{2+}) ions to initiate contraction (Webb 2003).

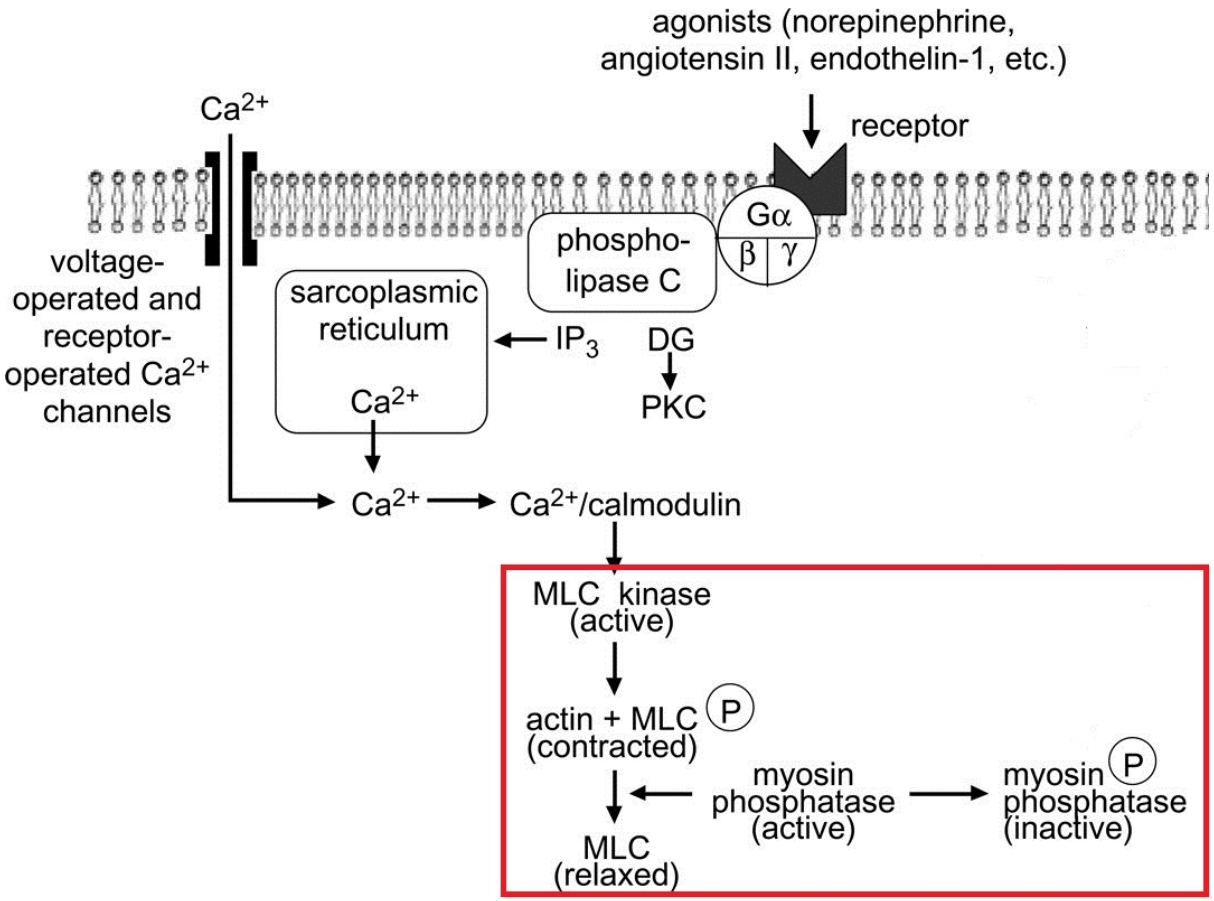


Figure 3: Regulation of smooth muscle contraction.
Contractions of SMCs are regulated by the autonomic nervous system. Various agonists activate contraction of smooth muscle through the binding to specific receptors. This activation causes an increase in phospholipase C activity via coupling through a G protein. Phospholipase C goes on to produce membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP3). IP3 causes the release of calcium (Ca^{2+}). Together DG and Ca^{2+} activate PKC, which functions in phosphorylating target proteins. Ca^{2+} binds to calmodulin which leads to the activation of myosin light chain kinase (MLC kinase). MLC kinase phosphorylates the light chain of myosin, in combination with actin, cross-bridge cycling occurs, which initiates shortening of the smooth muscle cell. Modified after (Webb 2003).

The process of SMC contraction is regulated by receptors and mechanical (stretch) stimuli (stretch-dependent ion channels), finally leading to the activation of the contractile proteins myosin and actin. The cross-bridge formation of these filaments is regulated by the protein calmodulin. Actin and myosin build two of three classes of filaments in SMCs. The arrangement of myosin filaments builds the thick filaments and actin the thin filaments. The third class comprises the intermediate filaments, a cytoskeletal component involved in contraction. The thin filaments are anchored by dense bodies. A dense body is analogous to the Z-discs of skeletal and cardiac muscles and act as an anchoring point of the actin filaments. Upon activation of a SMC, influx of extracellular Ca^{2+} ions through opened Ca^{2+} channels operate by changes in the surface membrane potential, in the sarcolemma, or the release of Ca^{2+} from intracellular stores of the sarcoplasmic reticulum (SR), and ultimately leads to an increase in free intracellular Ca^{2+}. Ca^{2+} then combines with the protein calmodulin building the Ca^{2+}-calmodulin complex which in turn activates an enzyme called myosin light-chain kinase (MLCK). The activation of MLCK results in the phosphorylation of the myosin heads through the breakdown of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate (P_i), with the P_i attaching to the myosin head. Following this step, the heads then attach to actin binding sites and pull on the thin filaments. When the thin filaments slide past the thick filaments they pull on the dense bodies, which in turn pull on the intermediate filaments' network leading to the contraction of the entire SMC in a manner whereby the ends are pulled towards the center, causing the midsection to bulge. The influx of Ca^{2+} from the extracellular space happens through receptor-operated Ca^{2+} channels. Agonists like noradrenaline (NA), angiotensin II etc. bind to receptors coupled to a heterotrimeric G protein that stimulates phospholipase C (PLC) activity. This enzyme is responsible for phosphatidylinositol 4,5-biphosphate (PIP_2) to catalyze the formation of the two second messengers inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to receptors on the SR, resulting in Ca^{2+} release. Together with DAG, Ca^{2+} activates protein kinase C (PKC) which phosphorylates specific target proteins such as Ca^{2+} channels to allow more Ca^{2+} influx into the cell for cross-bridge cycling.

SMC relaxation requires a decrease of intracellular Ca^{2+} concentrations (Figure 4) and is promoted by mechanisms involving the decrease of intracellular Ca^{2+} levels (e.g. active transport of Ca^{2+} into the SR by ATP-dependent Ca^{2+} pumps) following the dephosphorylation of MLCK by the enzyme myosin light-chain (MLC) phosphatase, or myosin phosphatase, which removes the high-energy phosphate from the light-chain of myosin (Webb 2003). This happens either as a result of the removal of the contractile stimulus or by the direct action of a substance that stimulates inhibition of the contractile mechanism. During relaxation, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane close, which results in a reduced entry of Ca^{2+} into the cell (Webb 2003).

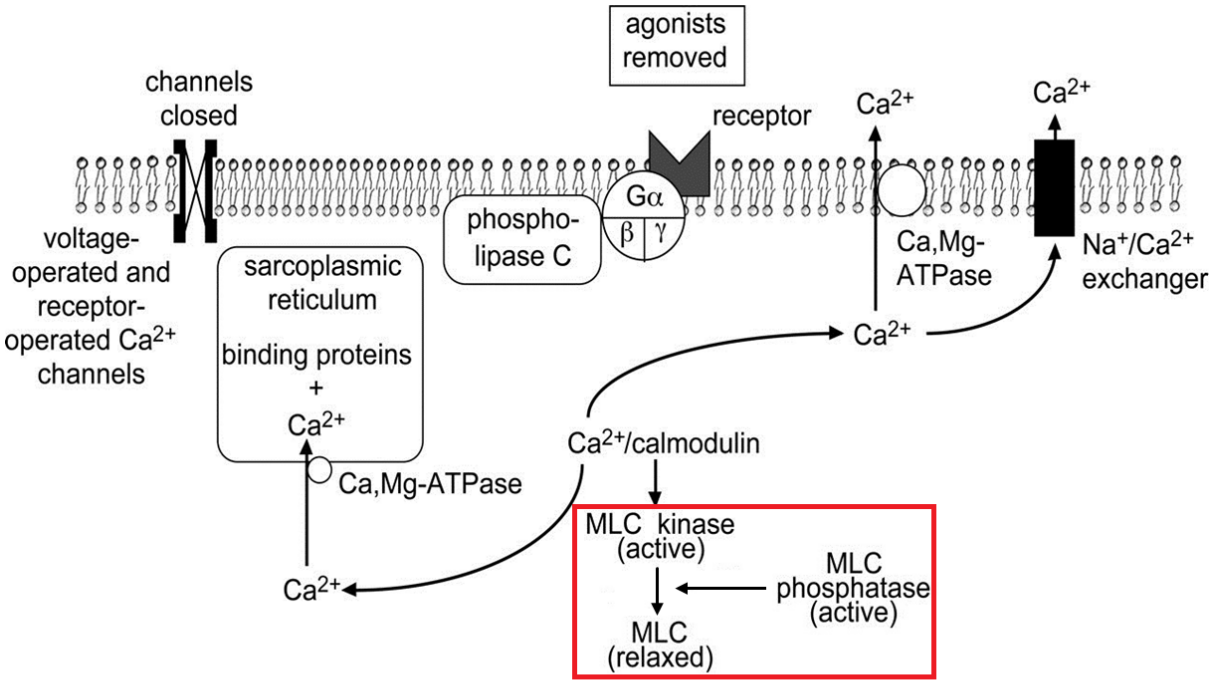


Figure 4: Relaxation of smooth muscle.
Smooth muscle relaxation requires a decreased intracellular Ca^{2+} concentration with increased MLC kinase activity. During relaxation, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane close, resulting in a decrease in Ca^{2+} entry into the cell. The dephosphorylation of MLC kinase by the enzyme myosin light-chain (MLC) phosphatase removes the high-energy phosphate from the light-chain of myosin. Modified after (Webb 2003).

1.3 The Prostate Gland

The prostate is an exocrine musculo-glandular organ of the male reproductive tract and is the largest male accessory sex organ. It is present in all male mammals with a considerable inter-species diversity in anatomy, biochemistry, and pathology. The name ‘prostate’ is derived from the Greek word ‘*prohistani*’, which means ‘to stand in front of’. The prostate produces and secretes a fluid that comprises about 20-30% of the semen playing an important role in fertilization. The growth and development of the prostate begins with the formation of prostatic buds from the fetal urogenital sinus and is complete at sexual maturity (Marker et al. 2003). The buds form solid branching cords developing a lumen rising (by birth) to a network of tubules and alveoli. As some of the apical cells become polarized, they start gaining secretory activity and the organ develops a stroma containing a large proportion of SMCs while the organ differentiates further developing ducts, acini and secretory epithelial cells (Shapiro et al. 1992).

1.3.1 Anatomy

The male adult prostate gland is located between the inferior urinary bladder, surrounding its neck, and the external urethral sphincter, surrounding the proximal (prostatic) male urethra (McNeal 1980, McNeal and Bostwick 1984). The nomenclature that is most commonly used to describe the zones and lobes of the human prostate is that of John McNeal (McNeal 1984) (see below, 1.3.1.1).

1.3.1.1 Macroscopic Anatomy

The prostate is about the size of a walnut, with an ovoid shape and weighs about 20g measuring approximately 4cm (transverse) x 3cm (vertical) x 2cm (anteroposterior). The prostate is shaped with a narrowed apex inferiorly and a broad base superiorly (Wein et al. 2011) and is enclosed by a prostatic fibromuscular capsule (McNeal 1981). However, there is no distinct fibromuscular capsule where the apex extends into the striated urethral sphincter muscle and where the base separates from the bladder, leading to a more recent suggestion that the prostate does not possess a ‘true’ capsule (Wein et al. 2011). The prostate is believed to develop from discrete lobes, although there are no discernible lobes present in the mature gland.

The anterior lobe lies in front of the prostatic urethra, the median lobe behind the urethra but in front of the ejaculatory ducts and the posterior lobe behind the median lobe and below the ejaculatory ducts (Sharma et al. 2017).

McNeal describes the prostate gland as being composed of approximately 70% glandular elements and 30% fibromuscular stroma (McNeal 1981). Furthermore, the prostate includes four anatomically distinct zones: the peripheral zone (PZ), central zone (CZ), transition zone (TZ) and the periurethral gland, or anterior fibromuscular stroma (AFMS) (Figure 5) (McNeal 1981). This anatomical description is a clinically important way of describing prostatic morphology.

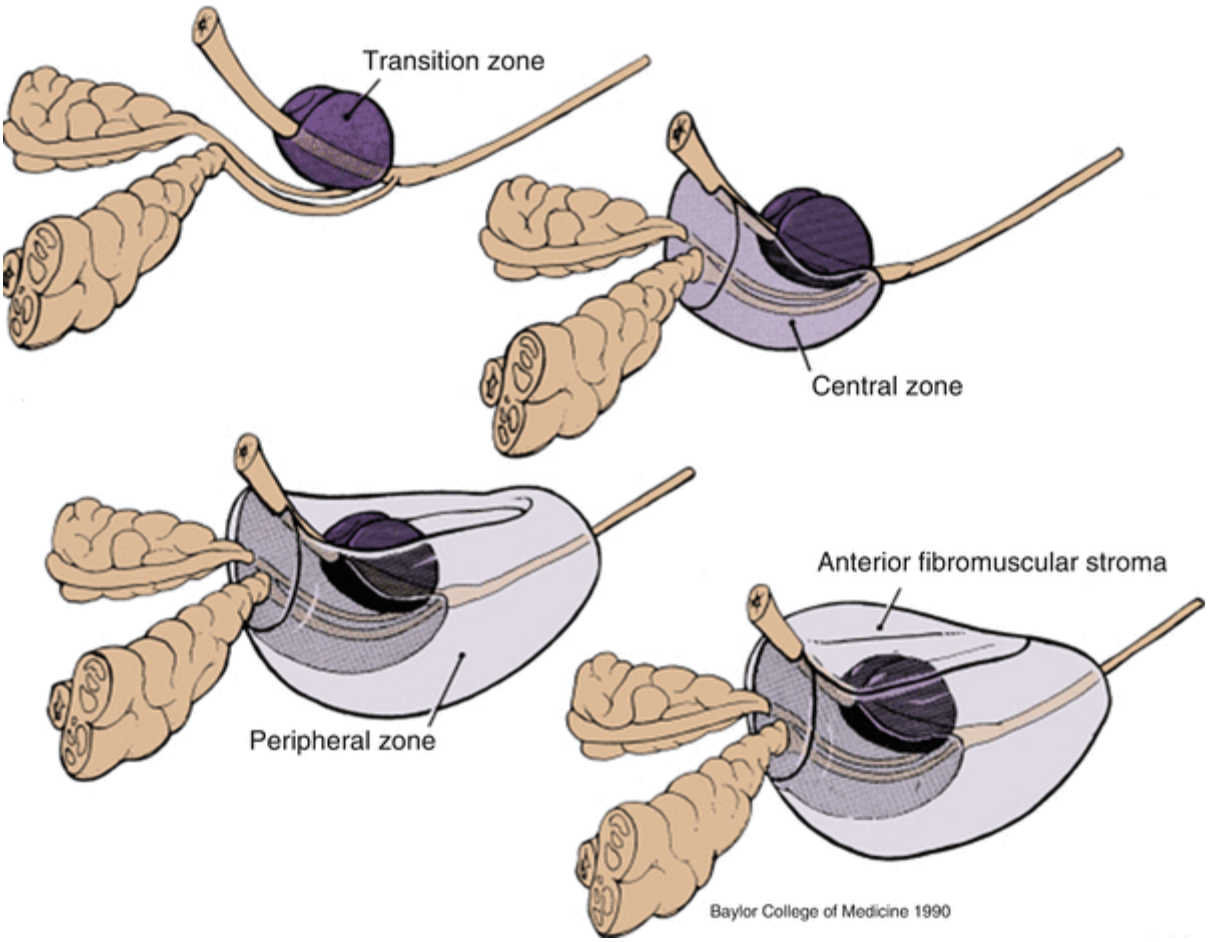


Figure 5: The zonal anatomy of the human prostate gland.
The figure shows the transition, central and peripheral zone as well as the anterior fibromuscular stroma. From Campbell-Walsh Urology, 10th Edition, 2011 (Wein et al. 2011)

The PZ, representing approximately 70% of the total glandular volume, is the outermost zone and encapsulates the CZ posterolaterally reaching from the apex of the prostate to surround the distal part of the urethra. It is the part where most commonly adenocarcinomas of the prostate arise (McNeal 1981), accommodating 70% of the carcinomas (CaP) (Wein et al. 2011) and is most affected by prostatic chronic inflammation (prostatitis) (Wein et al. 2011).

The CZ, representing approximately 25% of the total glandular volume (McNeal 1981), sits posteriorly to the TZ surrounding the ejaculatory ducts. In this zone, the prostatic ducts arise circumferentially around the openings of the ejaculatory ducts. Hyperplasia or adenocarcinomas are rare in this zone constituting only 1-5% of all prostate adenocarcinomas (Wein et al. 2011). The CZ constitutes most of the base of the prostate and surrounds the ejaculatory ducts.

The TZ represents 5-10% of the total glandular volume (McNeal 1988) and surrounds the distal part of the prostatic urethra near the apex of the CZ and ejaculatory ducts. It is the site where benign prostatic hyperplasia (BPH) most commonly arises (McNeal 1978) whereas less commonly around 20% of prostatic adenocarcinomas arise (Wein et al. 2011).

The non-glandular anterior fibromuscular stroma fills the space from the bladder neck to the proximal urethra covering the anterior lobe of the prostate (McNeal 1981). It consists of collagen, elastin, and SMCs fusing with the capsule and the glandular zone (McNeal 1988). It is predominantly fibromuscular with little (less than 1% of the total glandular volume) or no glandular structures (Gillenwater 2002, Lee et al. 2011).

1.3.1.2 Microscopic Anatomy

The human prostate has two main generic cell types: epithelial and stromal cells (Figure 6). Epithelial cells are mainly found in the tubuloalveolar glands of the organ. In the human prostate, there are 30-50 tubuloalveolar glands (Sharma et al. 2017) each of which is composed of secretory acini, consisting of lined columnar epithelial cells that comprise the lumen. This lumen is filled with the prostatic fluid produced by the epithelial cells and is mostly located in the PZ, whereas the (prostatic) excretory ducts are mainly found within the CZ leading to the prostatic urethra, which is in turn surrounded mostly by the TZ.

Two different cell types of epithelial cells can be distinguished; the basal epithelial and glandular epithelial cells. The basal epithelial cells synthesize and secrete components for the membrane of the acini and are located in between adjacent glandular epithelial cells, resting on the basement membrane (Bonkhoff and Remberger 1996). The function of the basal epithelial cells is not fully understood, but some of them are thought to function as stem cells for the production of glandular epithelial cells and could potentially have a role in cancer development in this region (Bonkhoff and Remberger 1996, De Marzo et al. 1998, De Marzo et al. 1998). The glandular epithelial cells, attached to the basal cells, extend to the acinar lumen and have a secretory activity forming the prostatic fluid.

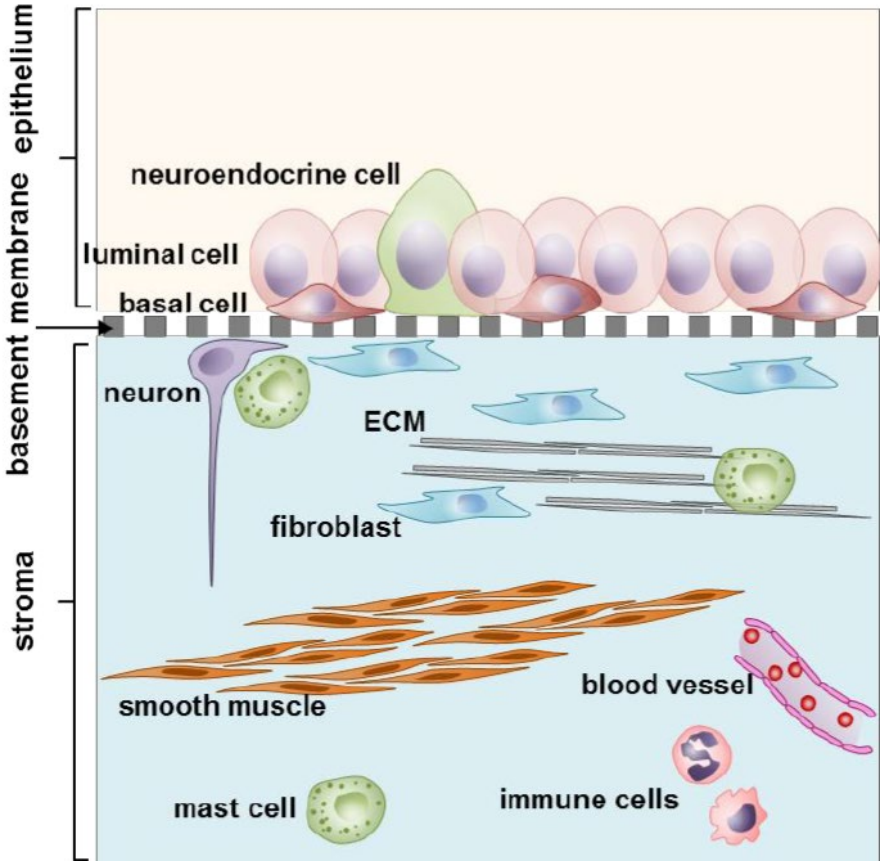


Figure 6: Distribution of cell types within the prostate gland.
Cell types consist of an epithelial and stromal compartment, separated by a basement membrane. Adapted from (Ellem et al. 2014)

Each acinus is surrounded by stromal tissue containing fibroblasts and SMCs (Nemeth and Lee 1996). The prostatic fluid is transported via the contraction of the stromal SMCs into a system of branching ducts that end in the prostatic urethra. Each prostatic gland opens into the urethra through a prostatic duct. In general, contractions of the prostatic SMCs help in forcing the secretions into the urethra during ejaculation (Ross et al. 1995, Standring 2005). In general, SMCs located in the fibromuscular stroma around the acinar structure and the capsule are mainly involved in contractions of the prostate gland (Wein et al. 2011). A basement membrane surrounds the acini and separates the epithelial acini and ducts from the fibromuscular stromal tissue, creating the interface between the glandular and stromal compartments.

The fibromuscular stroma of the adult human prostate is composed of SMCs, fibroblasts, subepithelial cells and includes blood vessels, capillary and lymphatic endothelial cells, peripheral nerves and ganglia, and tissue infiltrating white blood cells, e.g. mast cells and lymphocytes (Figure 6) (Aumüller 1983, Liu and True 2002, Ellem et al. 2014). SMCs are the cell types with the highest counts and their function is to generate muscle tone within the prostate to facilitate the movement of secretions from the glandular and ductal structures to the proximal urethra during ejaculation (see 1.3.3). Fibroblasts are located between prostatic ducts and can be separated in fibroblast and myofibroblast populations (Toivanen and Shen 2017). Subepithelial cells generating electrical activity leading to spontaneous contractions of the tissue have partly been identified as interstitial Cajal-like cells, located adjacent to the basal membrane, and were mentioned as prostate interstitial cells (PICs) (Huizinga et al. 1992) or interstitial cells of Cajal (ICCs) (Shafik et al. 2005) and may have slow wave properties.

The detailed physiological functions of the individual cell types have not been defined in detail. Investigations of the properties of SMCs and contractility are one of the research topics of this thesis (see below and 1.8).

1.3.2 Function

The prostate is the largest male accessory sex organ and the function is to produce and secrete a milky fluid that comprises part of the semen. The secretions contribute up to 30% of the total semen volume. Briefly, during ejaculation, sperm move from the distal part of the epididymis through the vas deferens to the ejaculatory duct in the area of the prostate and are combined with the prostatic and seminal fluid. Contractions of the muscular structures of the prostate help to expel the fluid and sperm through the urethra during ejaculation. The slightly acidic (pH 6.5) prostatic fluid nourishes and protects sperm to optimize conditions for fertilization by buffering the sperm in the vaginal environment. It also enhances sperm motility, viability and sperm transport in the male and female reproductive tracts, hence directly affecting male fertility (Wein et al. 2011).

The fluid contains several secretory products like prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), citrate, zinc, soluble fraction proteins, carbohydrates, electrolytes, polyamines like spermine, hormones, lipids and growth factors (Fair and Cordonnier 1978, Weidner et al. 1991, Zaichick et al. 1996, Sadava et al. 2008, Costello and Franklin 2009).

PSA belongs to the gene family of human tissue kallikrein. It is an androgen-regulated serine protease and its main function is to keep the semen liquid by cleaving the semenogelins (Neal et al. 1992). PSA is produced by the secretory epithelial cells in the acini of the prostate. For patients suspected to suffer from prostate cancer, most doctors consider measuring PSA levels before taking prostate biopsies to determine whether prostate cancer is present in patients (with a PSA level ≥4.0ng/ml) or not (normal PSA levels below 4.0ng/ml). Using PSA as a marker of prostate cancer is controversially regarded because, in addition to prostate cancer, a number of benign conditions can cause a rise in PSA levels like prostatitis or BPH. There is no evidence that prostatitis or BPH leads to prostate cancer, but it is possible for patients to suffer from one or both of these conditions and to develop prostate cancer as well. Moreover, recent studies have shown that some patients with PSA levels below 4.0ng/ml suffer from prostate cancer and that many patients with higher levels do not have prostate cancer (Thompson et al. 2004), leading to the conclusion that various factors can cause PSA level fluctuations and are not a reliable indicator for prostate cancer.

PAP is a dimeric glycoprotein enzyme and is synthesized by the epithelial cells of the prostate. Like PSA, it is androgen-regulated, and the function is to catalyze the hydrolysis of various phosphate esters thus releasing energy. It is thought to have a role in fertility by increasing sperm motility through the release of energy but this role is controversial (Kong and Byun 2013).

Epithelial cells in the prostate are also known as ‘citrate-producing cells’ (Sharma et al. 2017) because levels of citrate in the prostatic fluid are 400-1500 times higher than in blood plasma. Citrate is crucial in the citric acid cycle for the production of ATP, the "molecular currency" of intracellular energy transfer. Citrate in the prostate is thought to maintain osmotic/electrolyte equilibrium in the semen.

Zinc helps to keep up the major physiological function of citrate production and secretion. The decrease of zinc and citrate in the prostate plays an important role in the development of malignancies. The exact mechanism of zinc uptake and regulation in prostatic cells still needs to be explored (Franklin et al. 2005). Spermine is a polyamine secreted by the prostate gland. It stimulates ribonucleic acid (RNA) and protein synthesis by binding, e.g. to ribosomes (Pegg 2014).

1.3.3 Prostate Innervation and Contractility

As described before, the prostate gland is a fibromuscular organ and its function is to secrete a fluid to protect and mobilize the sperm during ejaculation. The regulation of prostate function, especially the expulsion of prostatic fluid, is dependent on the contractions of the whole organ. These contractions are regulated by a supply of mixed autonomic postganglionic neurons. These nerve fibers descend via the hypogastric and pelvic nerve, respectively (Vaalasti and Hervonen 1980, McVary et al. 1998, Rodrigues et al. 2002) (Figure 7). The prostate gland receives sympathetic input via the hypogastric nerves and parasympathetic input via the pelvic nerves. Aside from sympathetic and parasympathetic nerve input regulating contractions of the prostate gland, the prostate receives non-adrenergic non-cholinergic nerve input, e.g. from nitrergic nerves releasing nitric oxide (NO).

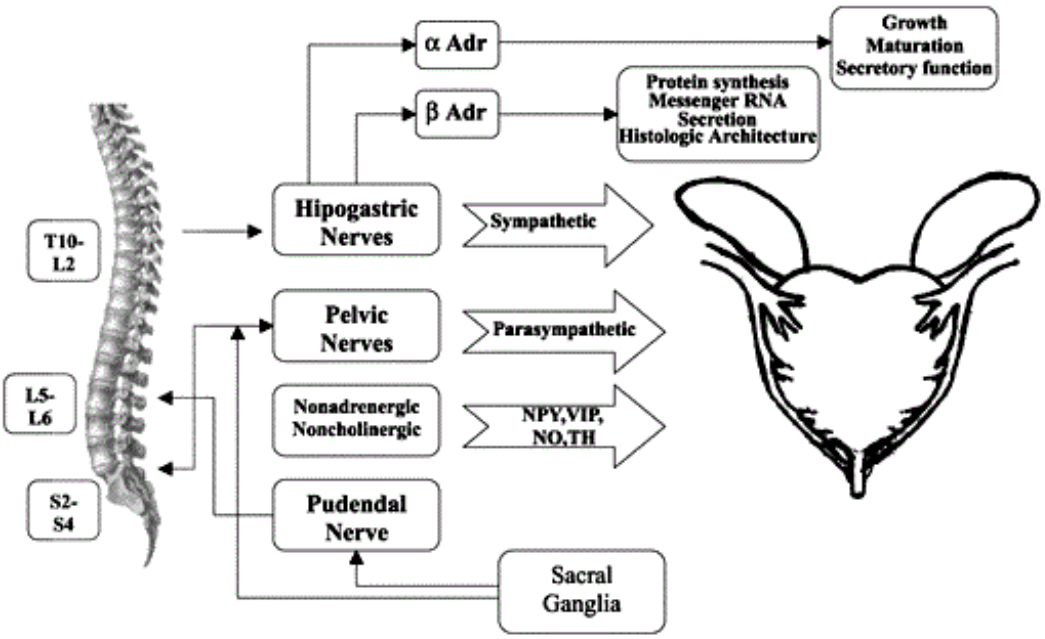


Figure 7: Innervation of the Prostate.
Contractions of the prostate are regulated by a supply of mixed autonomic postganglionic neurons. The prostate gland receives sympathetic input via the hypogastric nerves and parasympathetic input via the pelvic nerves. The prostate also receives non-adrenergic non-cholinergic nerve input associated with neuropeptide Y, vasoactive intestinal polypeptides, tyrosine hydroxylase, and also the release of nitric oxide (NO). Modified after (Rodrigues et al. 2002).

Contractions of prostatic SMCs are predominantly regulated by sympathetic nerve fibers and are activated during ejaculation (see 1.3.4). These fibers terminate at SMCs around the prostatic capsule and the stroma of each acinus (Higgins and Gosling 1989). SMCs surrounding the acini in the PZ of the prostate gland contribute to the production and movement of the prostatic fluid from the glandular epithelial cells into the lumen until expulsion out of the prostate is required during the emission phase of ejaculation (Ichihara et al. 1978). The function of the prostatic ducts is to transport the fluid into the urethra, where the prostatic contribution to the ejaculate is combined with the products of the ejaculatory ducts that comprise the sperm and the seminal fluid. The ductal system is mainly localized in the CZ of the prostate gland. The regulation of the ductal system, however, is not yet well defined.

The predominant parasympathetic innervation is received from cholinergic nerves, which play roles in secretory and contractile function through the use of acetylcholine as an agonist for muscarinic receptors. The non-adrenergic non-cholinergic innervation of the prostate gland comprises nitrergic innervation, regulating SMC tone by promoting SMC relaxation through the release of NO from nitric oxide synthase (NOS)-containing nerves (see 1.7).

Aside from activation by nerve fibers to induce contractions in the rat and human prostate, SMCs show spontaneous contractile activity in the fibromuscular stroma (Lee et al. 2017, Kügler et al. 2018). It is suggested that this spontaneous activity serves to mix and as such is required to keep the fluid in the lumen liquid. In contrast to the single glands of the prostate in the rat, the prostatic ducts do not contract spontaneously and contractions only occur upon noradrenergic activation for the expulsion of the prostatic fluid during ejaculation (Kügler et al. 2018).

An age-related decrease of prostate innervation can occur in the aging male combined with an accelerated growth of the prostate at approximately age 50 (Swyer 1944, Chow et al. 1997, White et al. 2013). The latter may cause an abnormal enlargement of the prostate and may, therefore, lead to BPH, together with LUTS (see 1.4).

The changes of innervation and the fact that classical pharmacological treatment targets the sympathetic signal transduction pathway, such as α_1-adrenoceptor antagonists (see 1.4.4), successful treatment of BPH varies among patients, and especially depends on the age of the patient.

1.3.4 Smooth Muscle Cells and α_1-adrenoceptors

The activation of SMCs during ejaculation is caused by the stimulation of sympathetic nerves which release NA at the adrenergic nerve endings. NA then binds to the adrenoceptors of SMCs and activates especially the α_1-adrenoceptor subtype that is widely distributed in organs such as the prostate (Michel and Vrydag 2006), the seminal vesicles (Shima 1993), the epididymis (Queiroz et al. 2008) and the vas deferens (Honner and Docherty 1999) (see below). NA belongs to the family of catecholamines and acts as an agonist at adrenoceptors which are highly abundant in the organs of the male reproductive system.

Adrenoceptors are a class of G protein-coupled receptors (GPCR) and are found in many organs, including the genitourinary tract. GPCRs are transmembrane receptors and detect signals outside a cell in the form of molecules and upon binding those molecules, start an internal signal transduction pathway leading to cellular responses.

Cellular responses are dependent on the G protein that binds to the receptor intracellularly. Binding of a ligand on the extracellular site leads to conformational changes of the GPCR that activates and causes dissociation of the G protein from the receptor leading to intracellular responses. The G proteins consist of $G\alpha$ and $G\beta\gamma$ subunits. The $G\alpha$ subunit mechanism is divided into classes such as G stimulatory ($G\alpha_s$), G inhibitory ($G\alpha_i$), G other ($G\alpha_o$), $G\alpha_q$ and $G\alpha_{12/13}$ (Preininger et al. 2003).

There are two main groups of adrenoceptors, α and β, with 9 subtypes in total. α-adrenoceptors are divided into α_1 ($G\alpha_q$ coupled receptor) and α_2 ($G\alpha_i$ coupled receptor). α_1-adrenoceptors consist of the subtypes α_{1A}, α_{1B} and α_{1D} and α_2-adrenoceptors have the subtypes α_{2A}, α_{2B} and α_{2C}. β-adrenoceptors are divided into β_1, β_2, and β_3. All three β-adrenoceptors are coupled to $G\alpha_s$ proteins. NA released from nerves can act on all classes of α- and β-adrenoceptors (Jenkinson and Morton 1967, Rang and Dale 2016).

In the human prostate, all types of adrenoceptors are expressed (Michel and Vrydag 2006). Of special interest and clinical importance, being the main target in pharmacological treatment for BPH and highly abundant in the prostate, are the α_1-adrenoceptors.

Radioligand binding studies have localized α_1-adrenoceptors to human prostatic SMCs, which couple to $G\alpha_q$ proteins and are likely to activate the signaling pathway for PLC. In the process, PIP_2 is cleaved into DAG and IP_3. DAG remains bound to the membrane, and IP_3 is released into the cytosol. IP_3 then diffuses through the cytosol to bind to IP_3 receptors, particularly Ca^{2+} channels in the SR. These channels are specific to Ca^{2+}, causing SMC contraction via an increase in intracellular Ca^{2+} (Alberts et al. 2002) (Figure 8).

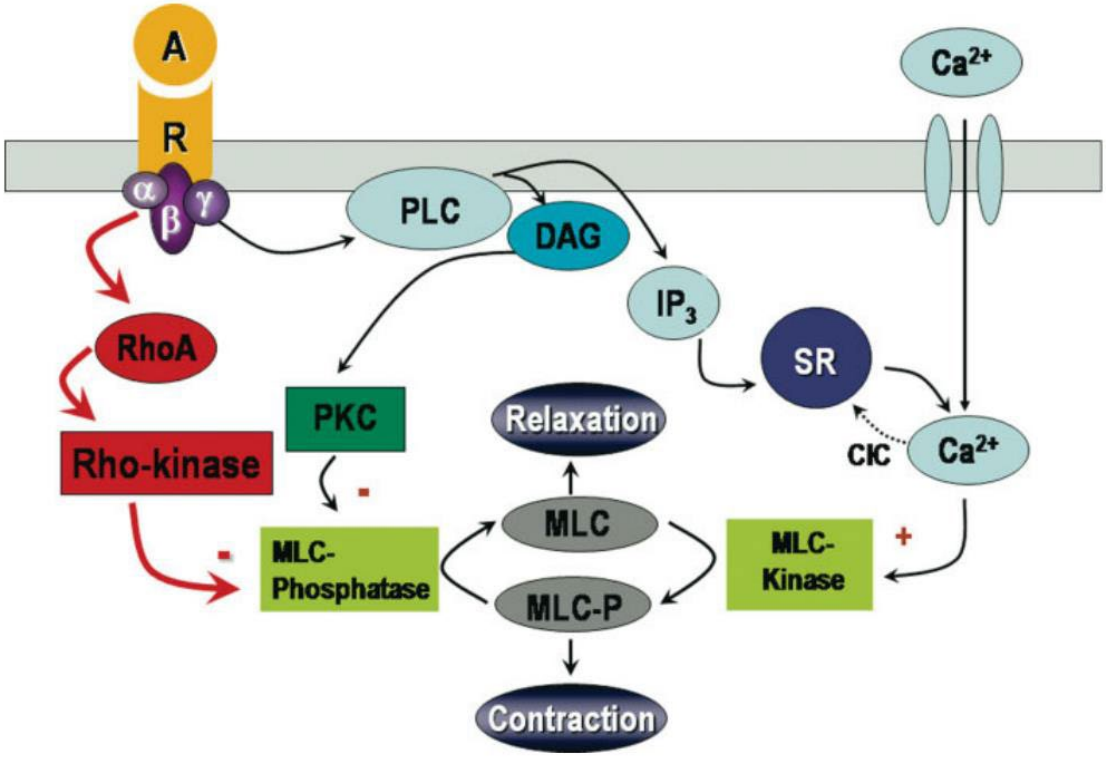


Figure 8: Intracellular signal transduction in prostate SMCs.
Activation of the signaling pathway activates PLC, releasing DAG and IP_3. IP_3 is released into the cytosol to bind to IP_3 receptors like Ca^{2+} channels in the SR, causing SMC contraction via an increase in intracellular Ca^{2+}. (+): stimulatory effect, (-): inhibitory effect, SR= sarcoplasmic reticulum, PLC=phospholipase C, DAG= diacylglycerol, PKC= protein kinase C, IP_3= inositol-1,4,5-triphosphate, MLC= myosin light-chain, CIC= Ca^{2+}-dependent chloride channel, A= agonist, R= receptor (Christ and Andersson 2007)

The α_{1A}-adrenoceptor is the dominant subtype of the α_1-adrenoceptors in the prostate gland and is primarily localized to the prostatic stroma (Kobayashi et al. 1993, Walden et al. 1997, Walden et al. 1999), whereas the α_{1B}-adrenoceptor subtype is located in the glandular epithelium and the α_{1D}-adrenoceptor subtype is located on blood vessels within the prostatic stroma (Walden et al. 1999). The abundance of α_2-adrenoceptors is primarily in the glandular epithelium and vascular tissue (Chapple et al. 1989, James et al. 1989, Michel and Vrydag 2006), but are not believed to play a role in prostate contractions. The three subtypes of β-adrenoceptors may also be involved in contractile function of the prostate, since β-adrenoceptor agonists inhibit the activity of α-adrenoceptors by activating the β-subtype-specific G protein signaling pathway (Goepel et al. 1997, Haynes 2007, Calmasini et al. 2015), therefore mediating SMC relaxation in the prostate.

The β_3-adrenoceptor agonist mirabegron was shown to relax isolated prostate specimens from human and rat after electrical field stimulation, providing support for further research of pharmacotherapies used to relax SMCs in the treatment of BPH (Calmasini et al. 2015)

1.3.5 Hormonal Regulation of the Prostate Gland

Hormones are molecules produced by glands in the body and function as chemical signals. They regulate cell and tissue function throughout the whole body and reach their targets by traveling through the bloodstream. Male sex hormones, androgens, are a class of hormones that control the development, secretory function, and maintenance of the human prostate. Testosterone (T) and dihydrotestosterone (DHT) are the most abundant androgens in men. Testosterone is biosynthesized in several steps from cholesterol (Nieschlag et al. 2012). The response of the prostate to androgens is mediated through the androgen receptor (AR), which is expressed in both prostatic epithelial and stromal cells (Cano et al. 2007, Nieschlag et al. 2012). Testosterone is mainly synthesized in the testis, but also by adrenal glands, and can diffuse into the prostatic epithelium and stroma. In stromal cells, the majority of testosterone is reduced to DHT in the stromal cells by the enzyme 5α-reductase (5AR). DHT binds to the same ARs, but with a five times higher androgenic potency and is more concentrated in the prostate gland (Farnsworth and Brown 1963, Shimazaki et al. 1965, Breiner et al. 1986, Wilson 2011), being the major androgen regulating growth, differentiation and function (Cunha et al. 1987, Prins and Putz 2008).

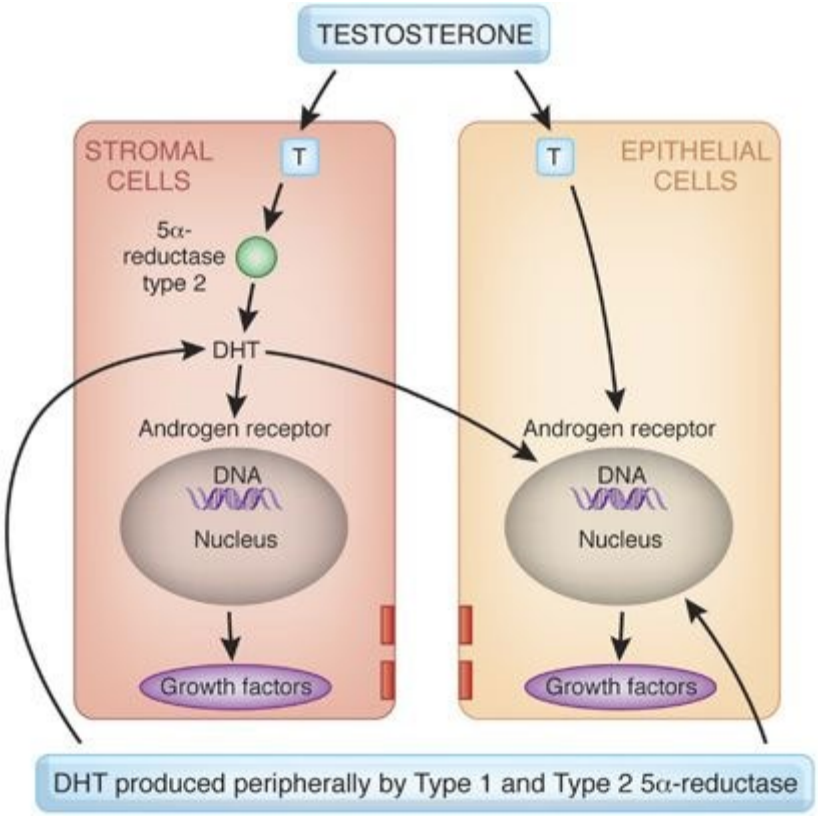


Figure 9: Influence of testosterone in epithelial and stromal cell growth of the prostate.
Testosterone (T) diffuses into the prostate epithelial and stromal cells. T can interact directly with the androgen (steroid) receptors bound to the promoter region of androgen-regulated genes. In the stromal cell, a majority of T is converted into the much more potent androgen dihydrotestosterone (DHT), which can act in an autocrine manner in the stromal cell or in a paracrine manner by diffusing into epithelial cells in close proximity (Roehrborn 2008)

Binding to ARs in the prostate affects growth and development. Testosterone and DHT are required for normal growth and function, but also promote the growth of cancerous prostate cells (Massie et al. 2011). Upon binding of androgens to the AR, an internal signaling cascade leads to the activation of the general transcription machinery in the nucleus. The areas of binding are called hormone response elements (HREs) and influence transcriptional activity of certain genes. AR-regulated target genes include PSA and PAP, the major secretory proteins of the prostate (see 1.3.2), but also cyclin-dependent kinase 8 (CDK8) and other proteins. CDK8 is involved in the cell cycle regulation and collectively, the activation of these genes by androgens leads to cell growth, survival and increased production of the secretory proteins (Feldman and Feldman 2001, Taplin 2007, Sharma et al. 2017).

The amount of testosterone that is synthesized is regulated by the hypothalamus, pituitary gland and the testis, called the hypothalamic-pituitary-testicular axis (Swerdloff et al. 1992). Low levels of testosterone lead to the release of the gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH stimulates the pituitary gland and releases follicle stimulating hormone (FSH) and luteinizing hormone (LH). These two hormones lead to the stimulation of the testis to synthesize testosterone in the Leydig cells (Wein et al. 2011). Through a negative feedback loop, increasing levels of testosterone act on the hypothalamus and pituitary to inhibit the release of GnRH and of FSH and LH.

Until reaching puberty, the prostate weighs only 2g due to low levels of androgens (Berry et al. 1984, de Klerk and Human 1985). During puberty, the prostate grows rapidly due to an increase of androgens until the age of 20 (Berry et al. 1984, de Klerk and Human 1985, Hayward and Cunha 2000). After that, androgen levels stabilize. However, around the age of 50, a shift in the ratio of androgens (T-DHT) leads to a slowing of prostate enlargement again. Abnormal growth can lead to BPH associated LUTS (Swyer 1944, Rosen et al. 2003). BPH affects approximately 50% of men in their 50s and up to 90% of men by the age of 80 (Berry et al. 1984).

Aside from testosterone, estrogen is the other active metabolite present in the prostate gland. Aromatase (CYP19) is an enzyme involved in the production of estrogen that acts by catalyzing the conversion of testosterone to estradiol. In males, this process takes place in several tissues, including the prostate (Hiramatsu et al. 1997, Negri-Cesi et al. 1999, Takase et al. 2006).

The effects of estrogens are mediated through two different estrogen receptors (ERα and ERβ), both expressed in the human prostate (Kuiper et al. 1996). In particular, ERα, expressed in prostatic stroma, has implications for stromal cell proliferation and growth (Untergasser et al. 2005, Prins and Putz 2008, Ho and Habib 2011). Other factors potentially regulating prostate function and growth include growth factors, prolactin, oxytocin and various paracrine, intracrine and autocrine factors (Wein et al. 2011).

1.4 Benign Prostatic Hyperplasia

There are three well-studied conditions that affect the prostate; BPH, prostate cancer and prostatitis. BPH is an age- and androgen-dependent, non-malignant enlargement of the prostate and occurs due to histological changes of prostatic tissue, consisting of the proliferation of stromal and epithelial cells affecting the transition and periurethral zones of the prostate (McNeal 1988). Although an increase in both, stromal and epithelial elements of the prostate, occurs in BPH, the major increase in prostate volume is due to an increased number of stromal SMCs (Rumpold et al. 2002). This increase in cells can, over time, create fairly large, discrete nodules in the periurethral region of the prostate. BPH is a non-malignant disease and therefore not life-threatening, but it is a very common troublesome disease affecting elderly males (Parsons 2010), varying from 32-52% in the age group 51-60 years to 77-99% of 81 years and older (Cockett et al. 1993) and associated with lower urinary tract symptoms (LUTS). BPH can lead to benign prostatic obstruction (BPO) which can lead to LUTS; however, it is important to mention that a number of other conditions can cause LUTS in men. The European Association of Urology (EAU) and the American Urology Association (AUA) publish evidence-based guidelines on these subjects (McVary et al. 2011, Gratzke et al. 2015). For diagnosis, different techniques are necessary for the assessment of different prostatic pathologies. Anatomical benign prostatic enlargement (BPE) can be measured by digital rectal examination (DRE) and/or transrectal ultrasound (TRUS) (Sech et al. 2001). The mechanical entity BPO can be measured by urodynamics, especially pressure flow studies with a commonly accepted maximum flow rate (Q_{max}) (Wein et al. 2011), while the histologic entity BPH can only be diagnosed microscopically and is therefore not applicable in a clinical setting. In early stages, the enlargement of the prostate may cause pressure on the urethra, resulting in an increased resistance to urinary flow, constriction of the urethra leading to urinary obstruction and possible renal failure in end stages of the disease (Figure 10) (Braeckman and Denis 2017).

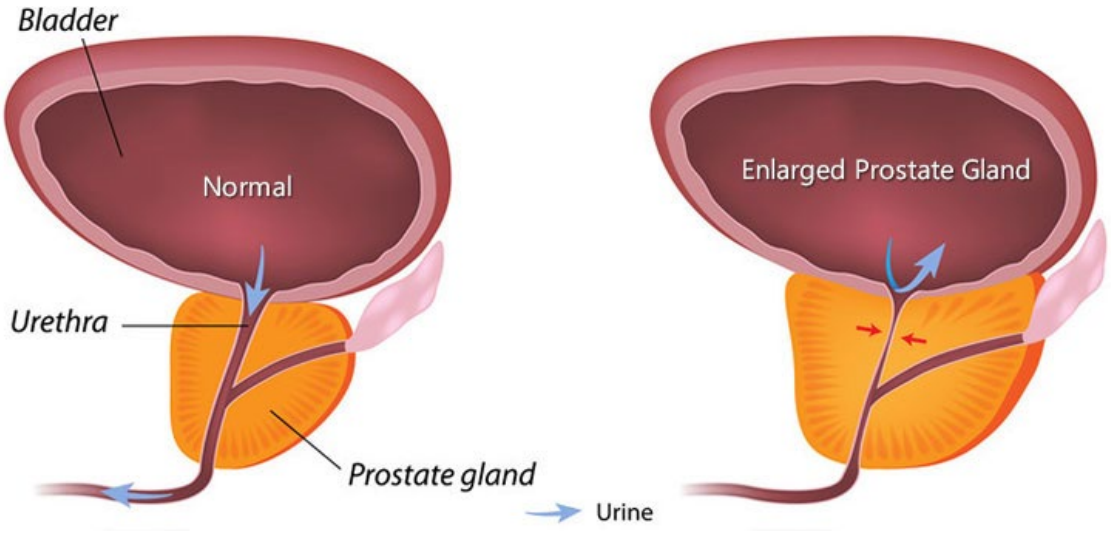


Figure 10: Comparison of the normal and enlarged prostate gland.
Comparison of sizes of normal prostate (left) and enlarged prostate gland (right) with indicated urine outflow resistance (adapted from: Prostate Conditions Education Council, https://www.prostateconditions.org/about-prostate-conditions/prostate-health-conditions/enlarged-prostate-bph)

Different approaches to treat BPH include a change of lifestyle comprising a healthy diet and exercise, different pharmacological treatments but also surgical treatment options. In the research of the causes and treatment of BPH, especially new pharmacological treatment options, different animal models have emerged as suitable candidates for the characterization of signaling pathways for SMCs and mechanisms of action of pharmacological treatments in those cells. In this thesis, the focus is on human and rat prostatic tissue.

Since increasing age is a main factor in the development of BPH combined with an aging society, its incidence will continue to increase significantly and the need for suitable treatment options will continue to be crucial to help clinicians in their decision for optimal treatment of patients.

1.4.1 Etiology

The exact pathogenesis of BPH is unknown, but a role of androgens, especially DHT, and age in causing pathological growth has been demonstrated (McNeal 1978). The development of BPH requires the presence of testicular androgens, as men who are castrated before puberty, or have androgen deficiency, do not develop BPH (Steers 2001, Carson and Rittmaster 2003). Androgens in the prostate are not only involved in cellular proliferation and growth, but also in preventing cell death (Carson and Rittmaster 2003). The enzyme 5AR plays a key role in the conversion of testosterone to DHT in the prostate, leading to hyperplasia (Bechis et al. 2014). Two subtypes of 5AR are known (5AR1 and 5AR2) with subtype-2 (5AR2) being the predominant subtype in the stromal compartment (Figure 9) and regarded as more important in the prostate, especially in BPH (Imperato-McGinley et al. 1992, Silver et al. 1994, Rittmaster 1997, Pelletier et al. 1998, Thomas et al. 2008). Another indicator that DHT plays a crucial role in the development of BPH is that with increasing age testosterone levels decline, but the level of DHT remains consistently high (Walsh et al. 1983) causing a shift in the ratio of testosterone to DHT in the prostate. The physiological interactions of testosterone and DHT in stromal and epithelial cells of the prostate are displayed in Figure 9 (Roehrborn 2008). Since age plays an important factor for BPH, this shift might be the reason. Androgens also regulate many growth factors and their receptors, which mediate prostatic growth through autocrine and paracrine pathways (Rennie et al. 1988, Wein et al. 2012). Another study showed that prostate adenoma is the cause of clinical BPH, resulting in a varying degree of obstruction with or without symptoms (Luo et al. 2013). If the obstruction is severe, it may eventually harm the bladder and the kidneys leading to e.g. kidney failure including diminished urine volume, fluid retention, chest pain, fatigue and nausea. In severe cases, kidney failure can lead to life-threatening situations like seizures or even coma.

Studies in canine BPH models showed that the condition can be induced with a combination of DHT and estradiol (Walsh and Wilson 1976, DeKlerk et al. 1979). Similar to the changes of the testosterone to DHT levels with age, a similar change is seen regarding the ratio of testosterone to estradiol.

While levels of testosterone decrease by about 35% between the age of 21 to 85, there is a constant level of estradiol. This change of the ratio is suggested to promote the development of BPH (Xiang-Yun et al. 2010, Nicholson and Ricke 2011). However, since these changes are not significant until after the first initiation of the disorder, their relationship to its induction can be questioned (Wilson 1980).

Nevertheless, in BPH tissue, epithelial AR and ER expression increases and expression of both receptors on the same cell is more prevalent in BPH tissue, indicative of a role for estrogens in the pathogenesis of BPH (Nicholson et al. 2013), because estrogens can affect subsequent proliferation of the prostate gland (Nicholson and Ricke 2011).

Aside from hormonal reasons as causes for the development of BPH, involvement of stromal-epithelial interaction (Cunha et al. 1983), prostatic stem cells (Isaacs 2008), inflammation (Kramer et al. 2007) and especially changes in innervation (dynamic component) (Yamada et al. 1987, Kondo et al. 1993, Nasu et al. 1996, Klotz et al. 1999) have been suggested as causes for BPH. Clearly, BPH is a multifactorial, complex condition that requires further investigation.

1.4.2 Pathophysiology

The hyperplastic process begins in the periurethral region of the TZ. Multiple factors that were mentioned before (see 1.4.1) lead to an increase in the number and size of epithelial and stromal cells through increased proliferation leading to glandular and stromal nodules, followed by a significant increase in large nodules (McNeal 1990). This proliferative process leads to tight packing of glands within a given area, along with hypertrophy of individual cells (Wein et al. 2012). The hypertrophic process takes place mainly in the stromal compartment compared to the epithelial compartment, suggesting that hypertrophy, next to hyperplasia of the stroma, results in a greater proportion of SMCs relative to the glandular epithelium, representing SMCs as the significant volume of the prostate gland in BPH (Shapiro et al. 1992, Shapiro et al. 1992).

As the prostate enlarges, intrusion into the urethral lumen or bladder neck can change the bladder outlet resistance by causing mechanical obstruction. The prostatic capsule plays a key role in the development of LUTS by transmitting the pressure of tissue expansion to the urethra and increasing urethral resistance (Roehrborn 2008). Therefore, BPH associated LUTS are only observed in humans. Although dogs develop naturally occurring BPH, 95% of the dogs with BPH do not express the symptoms of prostatic syndrome (Berry et al. 1986, Adel and Khadidja 2017). Additionally, reports suggest that transurethral incisions of the capsule result in significant improvements of BPH associated symptoms without affecting the size of the prostate (Ehrlich et al. 2010, Wein et al. 2012).

Still, the size of the prostate does not necessarily correlate closely with the symptoms of BPH, since patients with clinically diagnosed BPH can show the same LUTS without an enlarged prostate (Barry et al. 1993). Clinical BPH results from a dynamic and static component (Caine 1986, Von Heland and Casale 1994) (Figure 11). The static component of symptoms associated with BPH is attributed to the anatomic obstruction of the bladder neck and urethra which consists of stromal hyperplasia or hypertrophy, leading to increased prostate size, regulated by androgens. The dynamic component is mediated by the increase in tension, or tone, in prostatic SMCs via the sympathetic innervation of these cells (neurogenic tone) and an increase in spontaneous contractility of the prostate (myogenic tone) (Lepor 2009). An increase in SMC tone results in BPO of the urethra, and subsequent bladder outlet obstruction (BOO), all of which contribute to LUTS in aging men. Because the proliferative process of BPH involves both SMC and epithelial hyperplasia (Shapiro et al. 1992), it is reasonable to assume that both histologic elements contribute to the underlying pathophysiology of BOO and BPH (Shapiro et al. 1992).

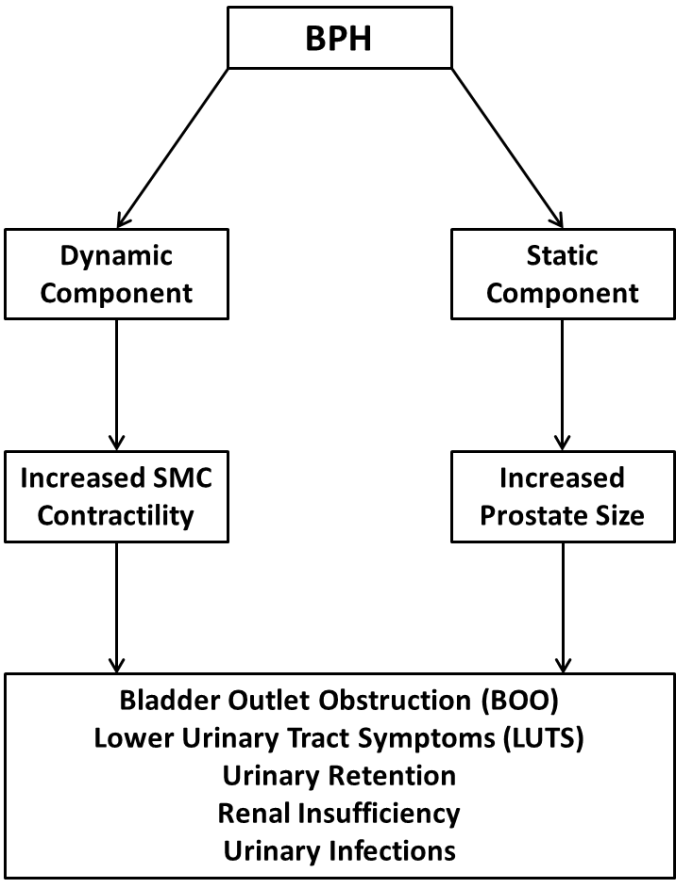


Figure 11: The dual pathogenesis hypothesis of BPH etiology.
The static component leads to an increased prostate size. The dynamic component is mediated by an increase in tension, or tone of prostatic SMCs causing an increase in spontaneous contractility of the prostate (myogenic tone), therefore leading to urinary tract complications.

1.4.3 Lower Urinary Tract Symptoms Associated with Benign Prostatic Hyperplasia

The term lower urinary tract symptoms has been introduced to cover all voiding and storage symptoms (Abrams 1994) regardless of etiology or gender, since the same urinary symptoms can occur in men and women, therefore regardless of prostatic presence (Chancellor and Rivas 1993, Lepor and Machi 1993). In men with LUTS associated BPH voiding symptoms are very prevalent, but storage symptoms like urgency and nocturia appear to be the most bothersome (Eckhardt et al. 2001). However, occurring symptoms are not necessarily specific due to BPH and obstruction could also be due to other diseases of the lower urinary tract (Chai et al. 1993, Chancellor and Rivas 1993, Lepor and Machi 1993, Chancellor et al. 1994, Yalla et al. 1995, Stoevelaar et al. 1996).

Aside from already mentioned urinary symptoms and BOO, there are several other clinical manifestations of BPH, e.g. acute urinary retention, inability to void, recurrent urinary tract infections, bladder decompensation, bladder stones, and renal failure. The total inability to void requires immediate intervention through catheterization (Stoevelaar et al. 1996). There is no commonly accepted definition of clinical BPH because several studies have focused on different aspects of BPH (the enlargement, the symptoms and the outflow obstruction). Therefore, it is not possible to establish general prevalence figures. For the diagnosis of BPH related symptoms, questionnaires (Figure 12) about the existence of particular voiding problems are used. In general, this questionnaire is divided between obstructive symptoms including weak urinary stream, the sensation of incomplete emptying of the bladder and post void dribbling, and irritative symptoms such as increased frequency of voiding and a sudden urge to void.

To unify measurements, the American Urological Association developed and validated a symptom questionnaire that was accepted as the International Prostate Symptom Score (IPSS) at the first World Health Organization Consultation on BPH in Paris 1991 (Cockett 1991). The IPSS is a self-administered screening tool that includes a total of seven urinary symptom questions and one quality of life question to quantify the prevalence of symptoms (Barry et al. 1992, Barry et al. 2017). More specifically, the questions relate to incomplete emptying, frequency, intermittency, urgency, weak urinary stream, hesitancy, and nocturia. Each question is assigned six answers indicating the severity of the particular symptom and can be answered on a scale from 0 (not at all) to 5 (almost always). The sum of the scores, varying from 0 to 35, forms the index (symptom score index). The total scores are divided into three categories: mild (0-7), moderate (9-19) and severe (20-35). Overall, the IPSS quantifies symptoms whereas for treatment it is of more importance to have a qualified statement (Figure 12) (Barry et al. 1992).

	Not at All	Less Than 1 Time in 5	Less Than Half the Time	About Half the Time	More Than Half the Time	Almost Always
1. Over the past month, how often have you had a sensation of not emptying your bladder completely after you finished urinating?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5
2. Over the past month, how often have you had to urinate again less than 2 hours after you finished urinating?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5
3. Over the past month, how often have you found you stopped and started again several times when you urinated?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5
4. Over the past month, how often have you found it difficult to postpone urination?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5
5. Over the past month, how often have you had a weak urinary stream?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5
6. Over the past month, how often have you had to push or strain to begin urination?	❑ 0	❑ 1	❑ 2	❑ 3	❑ 4	❑ 5

7. Over the past month, how many times did you most typically get up to urinate from the time you went to bed at night until the time you got up in the morning?

❑ 0 none ❑ 1 1 time ❑ 2 2 times ❑ 3 3 times ❑ 4 4 times ❑ 5 5 or more times

AUA symptom score = sum of questions 1 to 7.

From Barry MJ et al. *J Urol.* 1992;148:1549–1557.[14]

Figure 12: International Prostate Symptom Score (IPSS).
The seven symptom questions constitute a scale initially developed by the American Urological Association (AUA). The eighth question about the quality of life (QoL) is scored separately (Barry et al. 1992)

Therefore the AUA committee also developed a Symptom Problem Index (SPI) with 7 bothersomeness questions, of which each is regarding a question from the IPSS (Barry et al. 1995). Each question is answered on a scale of 0 (not at all) to 4 (very much), resulting in an index varying from 0 to 28. The quality of life question states: “If you were to spend the rest of your life with your urinary condition just the way it is now, how would you feel about that?” It can be scored from 0 (delighted) to 6 (terrible) (Barry et al. 1992, Barry et al. 2017), and is often used as a valuable starting point for counseling the patient. A clear trend towards an increase in IPSS with advancing age has been reported in patients from various populations around the world (Figure 13) (Stoevelaar et al. 1996) for samples in the United States (Chute et al. 1993), the Netherlands (Bosch et al. 1995), New Zealand (Nacey et al. 1995), Canada (Norman et al. 1994), France (Sagnier et al. 1994) and the United Kingdom (Hunter et al. 1994).

It is difficult to say whether the variations between the countries reflect real differences in symptoms or are due to differences in the composition of the respective study populations. Also, it is likely that cultural factors influence the perception of symptoms and answer categories.

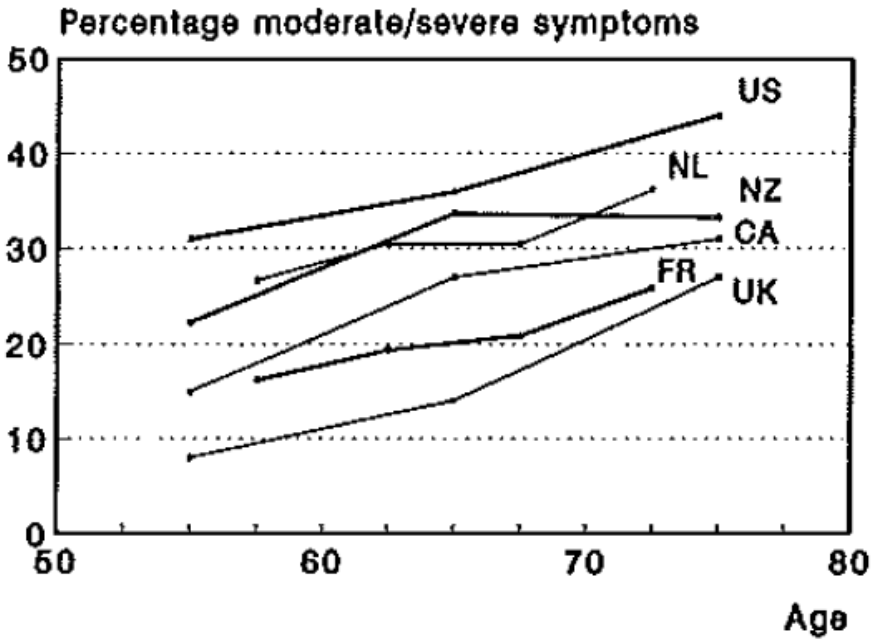


Figure 13: Prevalence of moderate to severe BPH symptoms.
Community-based samples in the United States, Netherlands, New Zealand, Canada, France and the UK. A clear trend towards an increase in IPSS with advancing age is seen in patients from various populations around the world. (Stoevelaar et al. 1996)

Prostatic enlargement does not necessarily lead to urinary symptoms. It is estimated that about half of all men with prostate enlargement will develop symptoms (Isaacs and Coffey 1989). Although occurring less frequently, there are patients with prostate enlargement and physiologic obstruction but without symptoms, called ‘silent' BPH.

On the contrary, various other studies have shown that the severity of the symptoms often does not correlate well with the presence of an obstruction (Bosch et al. 1995, Sirls et al. 1996, Wadie et al. 2001). In a study of 717 patients, it was found that patients with an IPSS of 0 to 7, 49% were not obstructed and 51% were obstructed, those with an IPSS of 20 to 35, 63% were obstructed and 37% were not (Rosier et al. 1996).

This leads to the conclusion that the IPSS has a poor correlation to obstruction and should not be used alone for further management of male LUTS associated with BPH, because using IPSS/QoL alone in the guidance of treatment could result in over-/or undertreatment.

1.4.4 Pharmacological Treatment Options and Side Effects

The primary aims of BPH therapy in patients are to alleviate distressing LUTS, improve the QoL, and prevent further complications such as urinary retention, upper urinary tract dilation, and urinary infections. In clinical practice, the need for intervention depends on whether the disease is: first, life-threatening; second, affecting the functions of organs; and last, affecting the patient's QoL, in order of priority.

Since the reasons for BPH associated LUTS vary, many options are available for today's treatment in men. To find the right individual treatment for patients, many factors are taken into account like the 'bothersomeness' of the symptoms and clinical parameters like the prostate volume and obstruction. In general, there are three approaches when it comes to treating BPH: (i) watchful waiting/conservative treatment, (ii) pharmacological treatment and (iii) open transurethral surgery (McVary et al. 2011, Oelke et al. 2013). Many patients refuse medical or surgical treatment in cases of BPH because of reasons that the symptoms are not bothersome enough or due to possible complications during surgery or side effects of pharmacological treatments. In a Dutch study, 41% of patients suffering from LUTS elected watchful waiting instead of another treatment (Stoevelaar et al. 1999). Recommendations in regards of watchful waiting include education, reassurance, lifestyle advice for the patient like regular exercise and a healthy diet, and monitoring of symptoms (McVary et al. 2011, Oelke et al. 2013). Medical therapy applies to men with bothersome symptoms in the absence of complicating factors. Medical treatment of BPH started in the early 1970s when first trials were investigating blockade of α-adrenoceptors (Caine et al. 1976) and androgen deprivation therapy (Caine et al. 1975).

Although these studies were very limited due to a small number of subjects and not using validated self-administered questionnaires and uroflowmetry to assess symptom improvements, observations showed that both therapies supported the paradigm that clinical BPH resulted from a dynamic and static pathway as described before (Caine 1986).

The first multicenter, randomized, double-blind, placebo-controlled studies were performed in the 1990's and confirmed the clinical effectiveness of α-adrenoceptor blockers (Lepor et al. 1992) and androgen deprivation therapy (Stoner et al. 1993) to treat BPH. In these studies, selective α_1-blockers and 5AR inhibitors (5ARIs) were used. Further trials to compare the effectiveness of both drugs alone and together showed that 5ARIs only work in BPH patients with an enlarged prostate, whereas α_1-blockers alone, or in combination with 5ARIs, were always effective regarding symptom improvements and increasing urinary peak flow rate (Lepor et al. 1996, Lepor et al. 1998).

Therefore, treatment options for BPH include 5ARIs like finasteride or dutasteride, of which the most used is the selective type 2 5ARI finasteride (dutasteride inhibits type 1 and type 2 5AR), but these drugs are only prescribed for an enlarged prostate (>30ml) (Naslund et al. 2008). However, 5ARIs may be more efficacious than placebo for prostate volumes of >40ml (Boyle et al. 1996). 5ARIs act by inhibiting the enzyme 5AR responsible for the hydrolysis of testosterone to the more potent DHT, which is involved in cell proliferation and therefore in the growth of prostatic tissue. The rationale for using 5ARIs is to reduce prostate size. A significant advantage of treatment with 5ARIs is the slow but sustained reduction of the volume of the prostate mitigating symptoms continuously leading to fewer risks of urinary retention and less risk for surgery (Roehrborn et al. 2002, McConnell et al. 2003, Roehrborn 2008). Improvements in symptoms take a minimum of 6-12 months to manifest (Oelke et al. 2013).

Studies show that finasteride reduces prostatic volume between 19-23% (Beisland et al. 1992, Andersen et al. 1995), IPSS score is reduced 2 points, maximum flow rate Q_{max} is increased 1.7ml/s and the requirement for prostate surgery is reduced by 55% compared to placebo with moderate LUTS (Andersen et al. 1997). Also, it is well tolerated and has a good safety profile (Moore et al. 1995).

Still, adverse side effects may occur and include impotence, loss of libido and ejaculation disorders (McConnell et al. 2003, Roehrborn et al. 2010) leading to discontinuation of therapy by many patients.

On the other hand, α_1-blockers, especially α_{1A}-adrenoceptor selective antagonists, provide a quick symptomatic relief which unfortunately reduces in effectiveness after a few years (Braeckman and Denis 2017). α_1-adrenoceptor antagonists are prescribed irrespective of prostate size (Lepor 2007) and are the first-line drug treatment for symptomatic relief of BPH due to the reasonably good efficacy. They relieve symptoms by blocking α_1-adrenoceptors and thereby reduce SMC tone in the prostate leading to a decreased resistance in urinary flow. The α_{1A}-subtype is predominant in tissues of the male reproductive tract like the prostate (Michel and Vrydag 2006) and other organs. Overall, there are 5 different α_1-adrenoceptor antagonists approved in the treatment of BPH/LUTS: terazosin, doxazosin, tamsulosin, alfuzosin and silodosin (McVary et al. 2011). While alfuzosin, doxazosin, and terazosin are all non-selective α_1-adrenoceptor antagonists, tamsulosin and silodosin are α_{1A}-subtype specific (Schwinn and Roehrborn 2008) and have emerged as the most effective drugs to treat BPH/LUTS by decreasing SMC tone (Michel and Vrydag 2006) and therefore improving urinary flow (Lepor 1990, Chapple 1996).

Multicenter, randomized, double-blind, placebo-controlled studies have demonstrated the safety and efficacy of α-blockers like tamsulosin. Symptom reduction is in the range of 1-4 IPSS points and maximum flow increase is about 1-4ml/s (Lepor et al. 1996, Buzelin et al. 1997, Narayan and Tewari 1998), but improvements in symptoms generally take a few weeks to develop. However, there is an overall unpredictable effectiveness associated with current drug therapies.

A study shows that as many as 30% of patients who receive α-blockers or 5ARIs alone or in combination for BPH report less than sufficient improvements in symptoms (Roehrborn 2011).

Table 1 gives an overview of the current, FDA approved pharmacological treatment choices for patients suffering from BPH.

Table 1: Overview of current FDA approved medications in the treatment of BPH.

α-Blocker	5α-Reductase Inhibitors	PDE5 Inhibitors
Alfuzosin (Uroxatral)	Finasteride (Propecia)	Tadalafil (Cialis)
Doxazosin (Cardura)	Dutasteride (Avodart)	
Prazosin (Minipress)		
Silodosin (Rapaflo)		
Tamsulosin (Flomax)		
Terazosin (Hytrin)		

Adverse effects in the treatment of BPH with α-blockers include orthostatic hypotension, headaches, dizziness, nasal congestion, drowsiness, fatigue (Chang and Campbell 2005) and more severe side effects like abnormal ejaculation, including reported retrograde ejaculation or even anejaculation (van Dijk et al. 2006).

In general, ejaculation is not to be confused with orgasm. Orgasm is a central nervous system phenomenon and is a distinct entity from ejaculation characterized by sensations experienced at the peak of sexual arousal.

Ejaculation can be divided into two phases: emission and expulsion. Emission is a physiologic process involving the distal part of the epididymis, seminal vesicles, prostate and adjacent structures like the vas deferens, prostatic urethra and bladder neck. Activation of the sympathetic nervous system and release of the neurotransmitter NA initially leads to the closure of the bladder neck to prevent retrograde ejaculation followed by mixture of secretions from the seminal vesicles, prostate, vas deferens (from the distal epididymis) and Cowper's glands in the urethra (Revenig et al. 2014). The neural control of emission originates from the thoracolumbar spine at T10-L2 and coordinates emission. Sympathetic efferent fibers merge into lumbar ganglia and continue to form the superior hypogastric plexus. Postganglionic fibers travel to the above-mentioned target organs to control the emission phase of ejaculation (Giuliano and Clement 2005, Lipshultz 2009).

Evidences of the incidences of ejaculatory dysfunctions in patients treated with α-blockers are described in many clinical studies and discussed throughout this work. As mentioned before, the exact mechanism behind ejaculatory dysfunctions remain unclear but it was suggested that it involves retrograde ejaculation due to the relaxation of the bladder neck by the blockade of the α-adrenoceptors or anejaculation or loss of seminal fluid by the blockade of a-adrenoceptors in the structures involved in the emission phase (see chapter 4).

Studies show variabilities of the occurrences of ejaculation disorders between different agents such as that treatment with silodosin shows higher incidences than with tamsulosin. Tamsulosin, on the other hand, shows higher incidences than e.g. alfuzosin. This might be to a higher specificity of silodosin to α_{1A}-adrenoceptors compared to tamsulosin, suggesting ejaculation disturbances are mainly affected by the α_{1A}-adrenoceptors subtype (Torre et al. 2015).

The lack of clarity of the precise effects of these agents on ejaculation also leads to the inconsistencies about the definition of the term “ejaculation disorders” or “ejaculatory dysfunction”, which are believed to be “anejaculation”, “retrograde ejaculation” or “absence of seminal emission”. Therefore, chapter 4 of this thesis elaborates on the clarification on the mechanisms behind ejaculation dysfunction in structures involved in the emission phase of ejaculation due to α_1-adrenoceptor antagonists used to treat BPH.

Although being the first-line treatment, α-blockers do not reduce urinary retention and therefore do not reduce the risk for further invasive surgical intervention, demonstrating that the risk of disease progression is not reduced (McConnell et al. 2003, Roehrborn et al. 2010). Also, recent research shows that only 40% of patients commenced on these medications remain on treatment 6 months later due to side effects (Schoenfeld et al. 2014). In organ bath contractility studies of human BPH tissue, Lee and colleagues (Lee et al. 2017) found age-related differences in responsiveness to tamsulosin, where tamsulosin was more effective in older patients and men with larger prostate volumes.

These studies clearly show that there is no universal pharmacological treatment option for patients suffering from BPH and that each treatment has to be individualized with many factors taken into account, e.g. age, prostate volume, obstruction, tolerability, side effects, effectiveness, disease reduction etc. The research for new, alternative pharmacological treatments aside from α-blockers and 5ARIs is crucial and of clinical importance to find the optimal treatment for patients.

New, recently approved pharmacotherapies are inhibitors of phosphodiesterases, especially subtype 5 (PDE5 inhibitors), in the treatment for BPH associated LUTS, like tadalafil (McVary et al. 2011). PDE5 catalyzes the hydrolysis of cGMP, an intracellular second messenger, promoting the relaxation in SMCs. Inhibiting PDE5 action with e.g. tadalafil, will lead to a prolonged activity of intracellular cGMP and therefore promote SMC relaxation in prostatic tissue. The most common indication for this medication is the treatment of erectile dysfunction (ED), but the same trials have shown improvement in BPH symptoms (Oelke et al. 2015). Long-term effects on the prostate gland are lacking, with the exact mechanism of action remaining unknown; additionally, adverse effects are still unclear (Vignozzi et al. 2013). Investigation about this pharmacotherapeutic approach was conducted in this thesis (detailed information provided below).

1.4.5 Surgical Treatment Options

In general, surgical treatment is always a measure of last resort. Conservative or pharmacological treatment options are first-line treatments for every disease if surgery can be avoided. The same principle goes for the treatment of BPH associated LUTS. But, if these strategies are ineffective, not tolerated or if further complications like acute urinary retention occur, or the patient exhibits severe symptoms, surgical treatment is the recommended option. For many decades, surgery has been the most important treatment for BPH.

In the first half of the 20th century, BPH surgery was still a lethal challenge because of a range of health care limitations. However, developments in antibiotics, anesthesia, increased knowledge of the pathophysiology regarding LUTS and improved procedures, especially the transurethral resection of the prostate (TUR-P), led to safer treatment outcomes (Braeckman and Denis 2017).

Aside from endoscopic surgery options, including TUR-P and transurethral incision of the prostate, open prostatectomy was available to most patients as a standard procedure of cure for BPH until medical treatment options (α-blockers and 5ARIs) were introduced.

Minimally invasive procedures include transurethral needle ablation (TUNA) of the prostate and transurethral microwave thermotherapy (TUMT) (McVary et al. 2011, Oelke et al. 2013).

The open prostatectomy was one of the first urological interventions and complete resection of the hyperplastic adenoma could be achieved, with maximum relief in obstruction. The most important differences to TUR-P are the abdominal incision and longer hospitalization. Prostatectomy is more advantageous over TUR-P in prostates with a size bigger than 80-100ml. Before the TUR-P procedure became the gold standard in the 1960s, open prostatectomy was the most often performed surgical procedure for clinical BPH in the first half of the last century.

Nowadays, TUR-P is the second most frequently performed intervention in adult male patients worldwide and is equally safe and effective in large size prostate as well as in small size (Kim et al. 2015) of up to <80ml. For prostates larger than 80-100ml, open prostatectomy is the only surgical option. Since the 1960s it is still regarded as the gold standard procedure for BPH.

For the procedure, a resectoscope is introduced transurethrally with a semicircular wire, the resection loop, at the end. Electricity flows through this loop and the current density around the small surface of the active electrode causes a direct cutting effect with only minimal tissue changes in the surrounding tissue. Cells within a range of a few millimeters do not clot (Honig 1975).

Although surgery results in high symptomatic improvements, the procedures are associated with severe adverse effects including impotence and ejaculatory dysfunction. Also, incidences of secondary operations have been reported and surgical treatments are expensive compared to drug treatments (McVary et al. 2011, Oelke et al. 2013).

1.4.6 The Rat Prostate Gland Animal Model

The prostate gland is an accessory gland of the male reproductive tract that is found only in mammals (Holstein 1994, Marker et al. 2003). Therefore, only mammalian animal models can be considered to study the mechanism underlying diseases like BPH. Comparisons among species are found in mammalian prostate morphogenesis, but the variability of the anatomy in different species is significant. Different to humans, the rat and mouse prostate, is not merged into one compact anatomical structure. The rodent prostate is composed of four distinct lobular structures. The individual lobes are defined according to their anatomical position: the anterior lobe, also known as the coagulating gland, the ventral, dorsal and lateral lobes (the last two collectively termed dorsolateral prostate) (Sugimura et al. 1986, Holstein 1994). These lobes exist as pairs on the left and right sides loosely bound together by connective tissue. Similar to the human prostate, the ventral lobes of the rat prostate gland are located below the urinary bladder on the ventral side of the urethra. The lateral lobes lie between the seminal vesicles and coagulation glands (anterior lobes) (Hayashi et al. 1991, Roy-Burman et al. 2004). The dorsal lobes are located posterior to the urinary bladder, behind and below the coagulation glands.

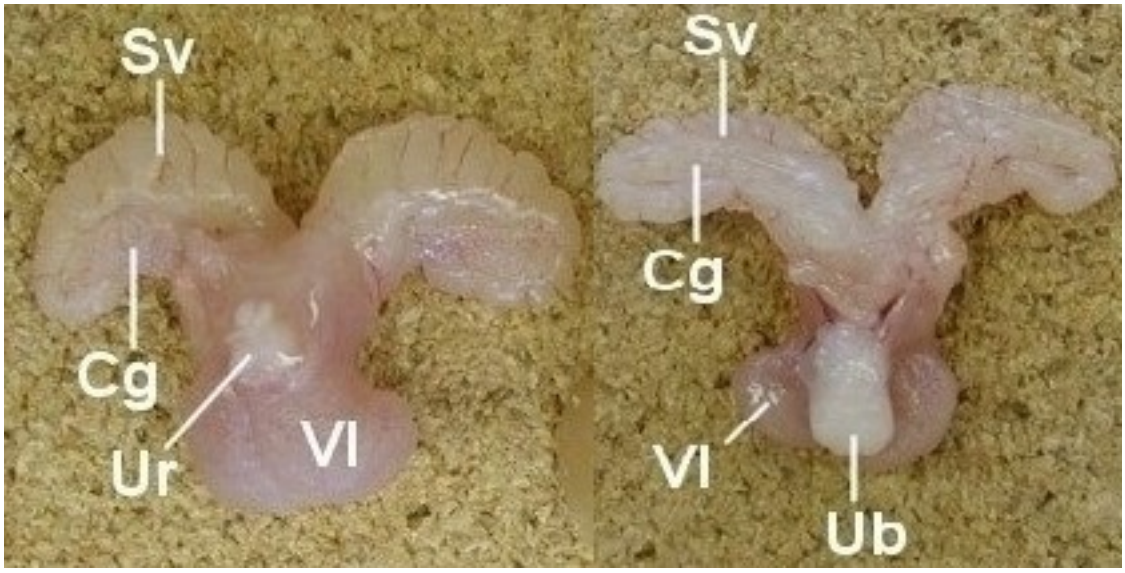


Figure 14: Overview of the male rat reproductive and urinary organs.
Indicated are seminal vesicles (Sv), coagulation glands (Cg), urethra (Ur), urinary bladder (Ub) and ventral lobes of the prostate (Vl). Modified after: (Kittel et al. 2004)

Although anatomically distinct, the human and rodent prostate glands are alike at the cellular level. Similar to the human prostate, the rodent prostate tissue consists of a glandular epithelium with basal and luminal secretory cells (Hayward et al. 1996) and a stromal compartment.

The secretory epithelial cells synthesize and secrete proteins into the glandular lumen with a similar composition as in humans (McNeal 1988). The stroma contains a large proportion of SMCs. Fibroblasts, elastin, and collagen are also abundant (Hayashi et al. 1991, Hayward et al. 1996, Kinbara and Cunha 1996). Additionally, growth and development of the rat prostate are regulated by the same factors as in humans and dependent especially on androgens and estrogens, where the reduced metabolite DHT is considered to be more potent than testosterone in maintaining secretory activity (Wright et al. 1996).

As mentioned above, the research for new pharmacological alternatives for the treatment of BPH is crucial since today's first-line treatments come with many adverse effects. Additionally, it is difficult to obtain long-term follow-up data as well as follow-up biopsy tissues. Factors like this motivate studies with animal models in order to test new potential BPH medications for *in vivo* efficacy. However, the only other species, aside from humans, that were reported to develop BPH naturally are dogs and chimpanzees (DeKlerk et al. 1979, Steiner et al. 1999). Due to the low availability and high cost of dogs or chimpanzees, *in vivo* experimental BPH models must be performed in other species. If necessary, BPH can be induced in various species by hormonal induction (growth regulated by androgens and estrogens), xenografting or transgenic methods (Mahapokai et al. 2000).

Despite the differences of anatomical and physiological features of the prostate in humans and rodents, the rodent prostate gland proved to be a viable model in the research of BPH and most of the *in vivo* studies are conducted in these animals. Advantages for such research in mice or rats include the small body size, easy breeding, short gestation time, cost-effectiveness, the similarity with the human genome (for rats approximately 96% identical) and, importantly, ease of genetic manipulation.

Therefore, studies on BPH are performed either after inducing BPH genetically, hormonally or in the untreated animal to conduct and analyze responses of appropriate pharmacological treatments. This is possible due to the same factors affecting growth and development and also the composition of cell types of the prostate in rat and human. The main focus lies on the responses regarding the contractility of SMCs. In addition, immunohistochemical and functional studies have suggested a role similar to the human prostate gland, with respect to adrenergic innervation and contribution of especially α_1-adrenoceptors, the main target of first-line pharmacotherapies like α-blockers (see above).

Overall, the rat, used as a model for research on BPH, has provided extensive insight into mechanisms involved in the regulation of prostatic SMC tone, which is a major component in the pathophysiology of LUTS associated with BPH.

1.5 Seminal Vesicles

The seminal vesicles (*glandulae vesiculosae*), also known as the vesicular or seminal glands, are a pair of glands found in male mammals, which function to produce and secrete a fluid that partly composes the semen. In humans, they are located posteroinferiorly to the urinary bladder within the pelvis and lateral to the vas deferens. The development of the seminal vesicles begins approximately 12 weeks of fetal age from the mesonephric or the Wolffian duct (Holstein 1994). The developing morphology and maintenance, as well as their secretion and size/weight, are highly dependent on testicular androgens (Jost et al. 1977, Gonzales 2001, Fey et al. 2012).

In the clinic, the physical examination of seminal vesicles is difficult. Therefore, laboratory examination of seminal fluid is preferred and requires a semen sample. To measure seminal vesicle function, fructose levels are measured and can give results as to obstruction or inflammation, mainly caused by bacterial infections (Nickel 1999). Abnormal ejaculation as a side effect of pharmacological treatment of BPH patients with α_1-blockers directly affects seminal vesicle function in a negative way (Hisasue et al. 2006, Nagai et al. 2008). Investigation of alternative treatment options is crucial to sublate disturbed ejaculation as a side effect of BPH treatment.

The basic organization of the seminal vesicle is identical in different species. Therefore, rat seminal vesicles are a suitable comparative model organism for further investigations. As described below (see 1.5.1), it comprises a motor portion, the muscular walls, and a secretory compartment, the epithelium. They are supplemented and surrounded with known auxiliary structures like vessels, nerve fibers, and connective tissue. Another common feature of this structure is the sensitivity to steroid hormones (Fukazawa 2009).

1.5.1 Anatomy

Each seminal vesicle consists of a single, coiled tube with several irregular pouches. It is about 3-5cm in length and 1cm in diameter in its curled up state inside the gland's structure. The uncoiled length is about 10cm. In rodents, each gland is up to 25mm long. Their most important anatomical relation is with the vas deferens, which combines with the duct of the seminal vesicles to form the ejaculatory duct, which subsequently drains into the prostatic urethra. These ducts open into the prostatic urethra on either side, pairwise (Figure 15).

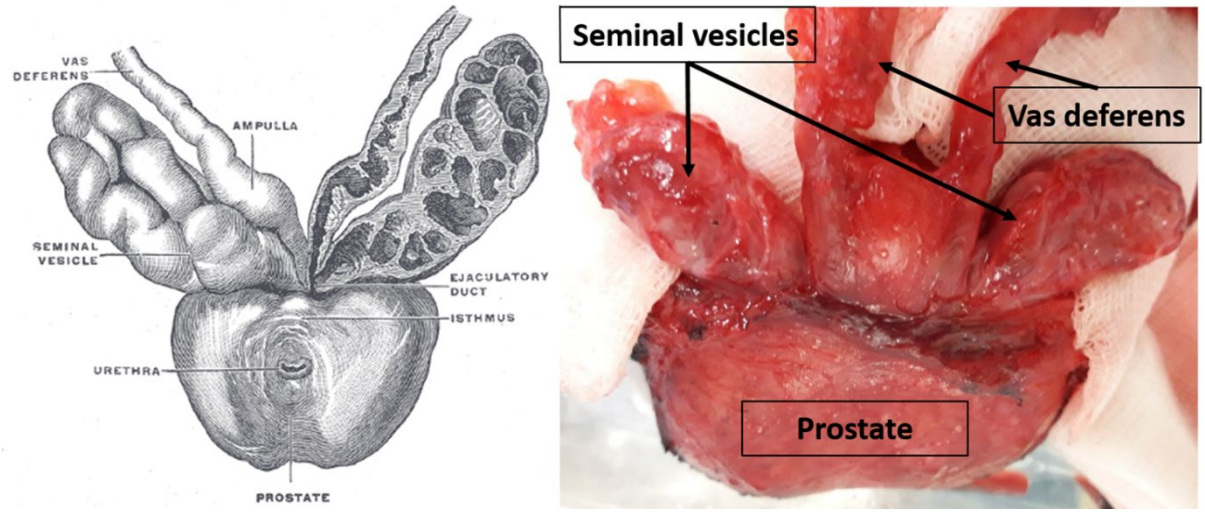


Figure 15: Overview of the reproductive organs in man.
Special focus on seminal vesicles. Left: schematic drawing (Gray and Lewis 1918), right: isolated human prostate, seminal vesicles and vas deferens (picture taken at the UKGM, Giessen, Germany)

Histologically, the seminal vesicles are composed of 3 layers: an inner mucosal layer, a muscular layer and an outer adventitial layer composed of loose fibrotic tissue containing blood and lymphatic vessels and nerve fibers. The inner mucosal layer consists of epithelial cells and a lamina propria. The epithelium is pseudostratified columnar, similar to other tissues in the male reproductive system. The lamina propria contains small blood vessels and lymphatics. The muscular layer has a distinct arrangement of SMCs; composed in an inner circular and outer longitudinal arrangement.

1.5.2 Function

The function of the seminal vesicles is to produce and secrete a fluid upon ejaculation that contributes around 70% of the fluid that will become semen (Kierszenbaum and Tres 2015). During ejaculation, the first fractions of expelled semen contain mainly spermatozoa and prostatic secretions; the fluids from the seminal vesicles are included in the late ejaculate fractions. The fluid consists of components that are important for semen functioning and survival and is slightly alkaline in nature. This alkaline property functions as a buffer for sperm survival in the acidic environment of the vaginal tract, prolonging the lifespan of sperm (Huggins et al. 1942). The Inner mucosal layer, with its glandular epithelial cells, secretes the fluids contributed by the seminal vesicles. The secretory products of the seminal vesicles contain enzymes, vitamin C, ions like potassium (K^+), low molecular weight substances such as fructose and prostaglandins, peptides, e.g. endorphin and proteins such as normal plasma proteins like transferrin, lactoferrin, and fibronectin, but also specific proteins like semenogelin.

Semenogelin is an important seminal vesicle protein that results in the formation of a gel-like matrix that encases ejaculated spermatozoa, causing semen to become sticky and jelly-like after ejaculation (Druart and de Graaf 2018, Wang and Wang 2018). Fructose provides an energy source for spermatozoa, the prostaglandins play a role in suppressing the female immune response to the foreign semen and are believed to aid fertilization by causing the mucous lining of the cervix to be more receptive to sperm, as well as by aiding the movement of the sperm toward the ovum by inducing peristaltic contractions of the uterus and fallopian tubes.

1.5.3 Smooth Muscle Cells and Contraction

The seminal vesicles are secretory organs under sympathetic and parasympathetic control. The parasympathetic nerve supply of the seminal vesicles is derived from the inferior hypogastric plexus. The sympathetic supply is from the superior lumbar and hypogastric nerves and terminates in the seminal vesicles.

The secretory glandular epithelium is controlled by parasympathetic innervation where cholinergic nerve endings are located. The secretory activity of the seminal vesicles determines testosterone supplementation to the epithelium.

SMC contraction is controlled by sympathetic and parasympathetic influences (McKay and Sharma 2018). Contractions of the muscular walls occur under the influence of excitatory adrenergic nerve fibers (Aumüller and Riva 1992) and the adrenergic innervation to the seminal vesicles is distributed throughout the SMC layers. Emitted seminal fluid can be stored within the lumen of the seminal vesicles until the emission phase of ejaculation occurs. During the emission phase of ejaculation, mediated through sympathetic fibers from the hypogastric nerves leading to contractions of the seminal vesicles along with the prostate, the fluid is transported into the ejaculatory ducts via strong contractions of SMCs resulting in the expulsion of spermatozoa and seminal fluid into the prostatic urethra.

The seminal vesicles are supplied with α- and β-adrenergic receptors of the SMCs. Characterization of adrenergic receptors in membranes from rat seminal vesicles indicates the distribution of β_2-adrenoceptors and α_1-adrenoceptors, of which mainly the α_{1A}-subtypes are found (Shima 1993). Studies in human seminal vesicles also showed that the α_{1A}-subtype was predominant in seminal vesicles (Hisasue et al. 2006). This knowledge is essential to explain the mechanism of adverse effects like abnormal ejaculation in the case of pharmacological treatment of BPH using a selective α_1-adrenergic antagonist such as tamsulosin.

1.6 Epididymis

The epididymis is derived from the Wolffian duct and at birth consists mainly of mesenchymal tissue. The epididymis undergoes considerable remodeling including duct elongation and convolution, so that by puberty the epididymis has acquired its fully differentiated state lined by epithelial cells (Robaire et al. 2002).

The most important part of the epididymis is a long, highly convoluted duct, the epididymal duct, where spermatozoa, released from the testes, undergo maturation and acquire motility and the ability to fertilize ova. Spermatozoa are stored in the most distal part of the duct until the emission phase of ejaculation. It bears some superficial resemblance to the testes, but the epididymis is smaller and the tubes of the epididymal duct are larger and less densely packed. The long duct is the connection between the testes and the vas deferens. It is present in male mammals, birds, and reptiles. The epididymis performs an important role in the maturation of spermatozoa including their acquisition of progressive motility and fertilizing ability (Plant and Zeleznik 2014).

1.6.1 Anatomy

The two epididymides develop in close proximity to the two testes. Unlike the seminiferous tubules of the testes, the epididymis consists of a single duct through which all spermatozoa pass. The epididymis is in close contact with the testis and descends into the scrotum. In humans, the uncoiled length is 6-7m but packed to about 4cm. In the rat, the epididymis is about 3m in length (Graham et al. 2010, Hinton et al. 2011). The epididymis can be divided into three main regions: the head (*caput*), the body (*corpus*) and the tail (*cauda*) (Figure 16) and is further subdivided into segments (F. Holstein 1969). The segments are separated by connective tissue septa (Benninghoff et al. 1994, Turner et al. 2003, Stammler et al. 2015, Domeniconi et al. 2016). The caput of the epididymis receives spermatozoa via efferent ducts from the testis. While spermatozoa mature throughout the epididymis, they are transported through the corpus to the cauda.

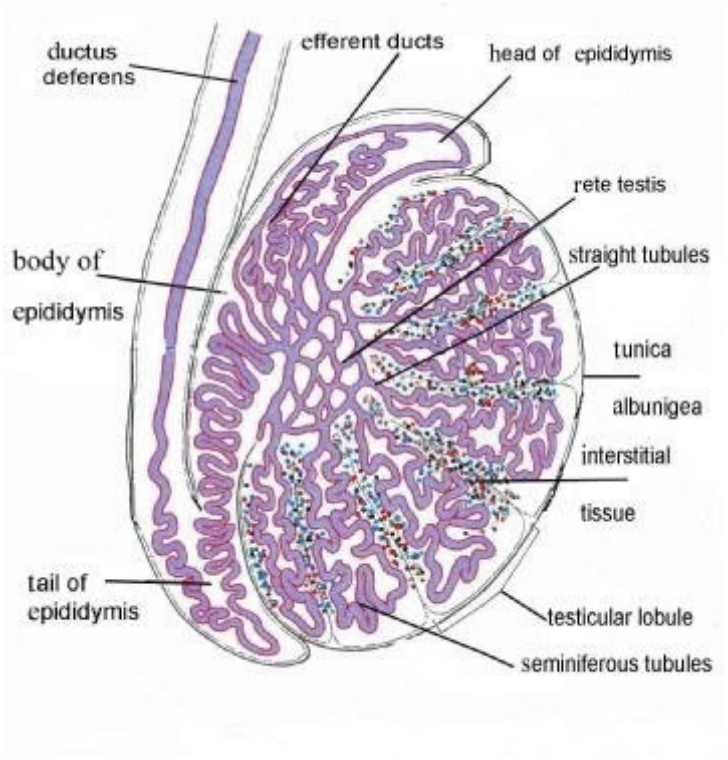


Figure 16: Overview of the anatomical structures of the human testis and epididymis. The epididymis consists of a single duct through which all spermatozoa pass and is in close contact with the testis descending into the scrotum. The epididymis is divided into three main regions: the head (caput), the body (corpus) and the tail (cauda). Modified after (Abd-Elmaksoud 2019)

The cauda epididymis is the primary storage site for mature sperm and is continuous with a highly muscular duct, the vas deferens. The diameter of the SMC layer of rat epididymal duct increases in the direction from caput to cauda. Histologically, the caput is characterized by a thick epithelium and few SMCs. The arrangement of SMCs in the surrounding layer of SMCs is in a circular manner. In the corpus, the epithelium decreases to an intermediate amount and SMC thickness increases. This is continued to the cauda, which is characterized by the thinnest epithelium and the highest quantity of SMCs, resulting in a complete further SMC layer with a predominantly longitudinal orientation of the cells (F. Holstein 1969, Baumgarten et al. 1971, Bacha and Bacha 2012). In this layer, SMCs are larger and more differentiated than the circular SMCs (Holstein 1994).

The epithelium is classified as a pseudostratified epithelium, with non-motile stereocilia pointing in the direction of the lumen which are long in the caput and shorter in the cauda (Kierszenbaum and Tres 2015).

The role of ciliated cells of the efferent ducts is not clear. A role in the transport of spermatozoa was recently suggested (Hinton and Robaire 2015). The major cell types in the epithelium of the epididymal duct are columnar principal cells that, together with the basal cells, form the majority of the epithelium. They extend from the lumen to the basal lamina (Kierszenbaum and Tres 2015). Other cell types of the epithelium include basal cells, apical cells, clear cells (Kierszenbaum and Tres 2015), intraepithelial lymphocytes and macrophages (Da Silva et al. 2011, Shum et al. 2014). Additionally, collagen fibers and true fibroblasts were also described (Holstein 1969, Baumgarten et al. 1971).

1.6.2 Function

Testicular spermatozoa are functionally immature in that they cannot fertilize ova and are immotile. The epididymis has four basic functions; (i) it serves as a sperm reservoir; (ii) discards old and superfluous sperm; (iii) is the site of spermatozoa maturation; and (iv) is responsible for the composition of the fluid surrounding epididymal spermatozoa. The main function is to oversee and facilitate the maturation of spermatozoa, which are transported from the testis into the proximal part of the epididymis, called caput, progress to the corpus, and finally reach the cauda region, where they are stored. It is now known that specific maturational changes occur in spermatozoa during epididymal transit which results in their developing the ability to fertilize ova (Jones 1999).

The epididymis manages this process by secreting substances that help the sperm survive and mature. During ejaculation, sperm flows from the cauda epididymides into the vas deferens, where they are expelled by peristaltic contractions of SMC layers in the wall of the vas deferens and are mixed with the fluids of the prostate, seminal vesicles, and other accessory glands prior to ejaculation, forming semen.

It is probable that the relatively small tubular lumen and large wall volume with plentiful epithelial cells in the caput region provide the necessary basis for biochemical interaction in the process of sperm maturation between the epithelium and sperm.

In contrast, the large lumen and small epithelium of the tubular wall of the caudal region are more suited for the storage of spermatozoa.

The transport of spermatozoa through the segments of the epididymis is thought to be regulated by contractions of SMCs around the whole epididymis. The layers of SMCs, consisting of thin circular SMCs and longitudinally bundles of thick SMCs around the circular SMC layer, were described above (see 1.6.1) (Elfgen et al. 2018). These thin SMCs are thought to be spontaneously contracting (Holstein 1994), whereas the large SMCs that are also found in the vas deferens might not contract spontaneously which seem to be important in the context of ejaculation, similar to the contraction patterns of SMCs surrounding the single prostate glands (spontaneously contracting) and excretory ducts (no spontaneous contractions) (Kügler et al. 2018). The spontaneous contractions are thought to be important mainly for the transport of spermatozoa through the duct. Important indications for this are peristaltic movements of the duct that have been observed in all parts of the epididymis (Risley and Skrepetos 1964, Hib and Caldeyro-Barcia 1974, Talo et al. 1979, Studdard et al. 2002, Mietens et al. 2014). From caput to cauda, the contractile frequency of SMCs decreases while amplitude increases (Mewe et al. 2006).

The secretory epithelium consists of several cell types, each of which has a specific function to contribute to the correct functioning of the duct. The primary cell type throughout the duct is the columnar principal cell, which makes up to 80% of the secretory epithelium. It is responsible for the amount of proteins that are secreted into the lumen (Cornwall 2009).

Other cell types include narrow, apical and clear cells. Little is known about the function, but they secrete protons into the lumen and thus participate in its acidification (Pietrement et al. 2006, Kujala et al. 2007). Basal cells are in close association with the overlying principal cells and thus may regulate their function (Veri et al. 1993, Seiler et al. 1999). Halo cells appear to be the primary immune cells in the epididymis, while apical cells may also endocytose luminal components.

In the process of sperm maturation, region-dependent expression of proteins and other substances contribute to distinctive luminal profiles within each epididymal duct region. It was demonstrated by Turner *et al*. (Turner et al. 2003) that the presence of connective tissue septa further subdivides the caput, corpus and cauda into intra-regional segments and that gene expression may, in fact, be highly ordered, region-specific and compartmentalized within these segments.

Thus, the epididymal duct is a highly ordered and segmented organ with each segment representing a unique physiological compartment. Each compartment possesses distinctive gene expression profiles within the epithelium that dictate segment-specific secretion of proteins into the luminal fluid, directly or indirectly affecting sperm maturation (Sipila and Bjorkgren 2016).

The maintenance of epididymal structure and function is dependent on androgens, especially DHT, as is the process of epididymal maturation of the sperm. This is demonstrated by experiments involving temporary deletion of the Leydig cells by ethane dimethane sulfonate (EDS), which revealed a reduced volume of the epididymis and epididymal duct potentially resulting in impaired sperm transport (Yang et al. 2006). In addition to epithelial cells, classic androgen receptors were described in SMCs (Zhou et al. 2002, Trybek et al. 2005). This might explain a role of androgens not only for secretions from epithelial cells but also for contractions of the duct in the transport of spermatozoa (Hib and Ponzio 1977, Din-Udom et al. 1985).

Nevertheless, contractions of the caudal part of the epididymal duct are mainly controlled by sympathetic innervation via adrenergic nerve fibers (Elfgen et al. 2018).

1.6.3 Smooth Muscle Cells and Contraction

Knowledge about SMC function of the epididymis is only well described for the cauda region, which is responsible for storage and emission of sperm. Information about the other parts is sparse. In general, neuronal input differs in the three parts of the epididymis. The cauda part is innervated by a dense supply of nerve fibers whereas the caput and corpus are supplied by only few nerves (Mitchell 1935). This finding fits with the function of the cauda to instantaneously contribute to emission.

Nerve fibers of the epididymis consist of mainly sympathetic and parasympathetic innervation, but peptidergic, purinergic or nitrergic neurotransmitter fibers also play a role (Mitchell 1935, Kuntz and Morris 1946, Risley and Skrepetos 1964, Baumgarten et al. 1968, Ventura and Pennefather 1991, Dun et al. 1996).

The sympathetic innervation of the epididymis is mediated through adrenergic input, as in the rest of the male reproductive system (described above). In human and rat, activation of adrenoceptors, especially α_1-adrenoceptors through adrenaline and NA, is responsible for initiating the emission phase of ejaculation (Graham et al. 1996, Kaplan 2009). The neurotransmitters predominantly bind to the α_{1A}-adrenoceptor subtype which is localized in blood vessels, epithelial cells and SMCs (Queiroz et al. 2002, Queiroz et al. 2008). A higher abundance of α_{1A}-adrenoceptors in SMCs in the caudal region of the epididymis was found (Queiroz et al. 2002) and shows the relevance of α_{1A}-mediated signaling during emission. Still, the exact contribution of different α-adrenoceptors in signaling, especially during emission, remains a topic of research and needs further clarification (Mewe et al. 2006, Mewe et al. 2007, Mietens et al. 2014). Different to the cauda, agonists and antagonists of α_2-adrenoceptors also showed an influence on contraction, especially in proximal parts of the epididymal duct (Chaturapanich et al. 2002, Mewe et al. 2007).

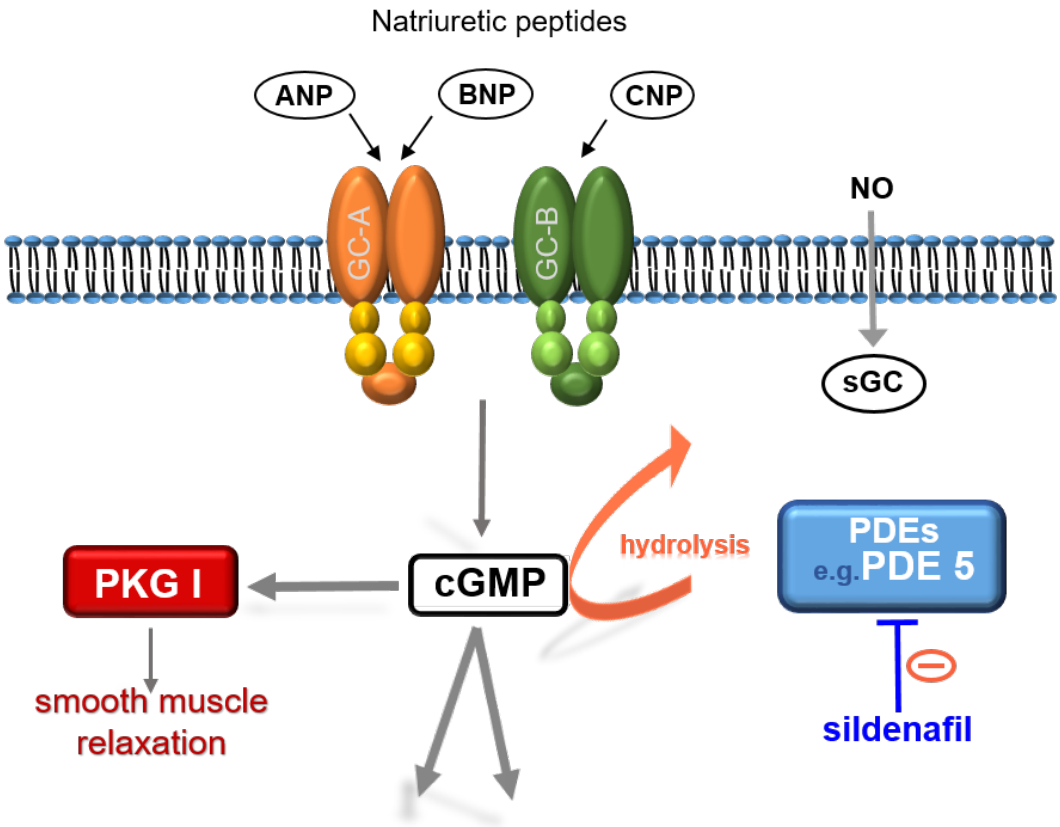
Blocking α_{1A}-adrenoceptors specifically with e.g. tamsulosin in the treatment for BPH has therefore not only potential adverse effects on organs like the prostate or seminal vesicles but also on the epididymal duct, especially in the distal part of the caudal region. This may lead to abnormal ejaculation since these three organs are mainly involved in the emission phase of ejaculation. Investigating mechanisms of α_{1A}-subtype signaling is crucial in the research to exclude side effects in the treatment of BPH for better tolerability. Parasympathetic input is mediated by cholinergic nerve fibers and although a potential effect in mediating SMC contraction (Laitinen and Talo 1981, Pholpramool and Triphrom 1984, Siu et al. 2006) is suggested, the functional relevance of cholinergic input on contractions of the epididymis is unclear.

1.7 Nitric Oxide/cGMP Pathway and Signaling Components

There are two well-known types of guanylyl cyclases (GCs) that catalyze the conversion of guanosine triphosphate (GTP) to 3',5'-cyclic guanosine monophosphate (cGMP); the classical transmembrane guanylyl cyclase and the soluble guanylyl cyclase (sGC). cGMP is an important intracellular second messenger that mediates several physiological processes in eukaryotic cells. In mammals, cGMP regulates processes as diverse as cellular growth, SMC relaxation, cardiovascular homeostasis, vasodilation, neuronal plasticity and learning (Lucas et al. 2000). Once formed, cGMP can be degraded by cyclic nucleotide phosphodiesterases ending cGMP signaling by catalyzing the hydrolysis of cGMP. The protein family of phosphodiesterases (PDEs) is comprised of 12 different types, namely PDE1-PDE12 in mammals that degrade the phosphodiester bond in the molecules cAMP and cGMP. PDEs are therefore important regulators of signal transduction mediated by these second messenger molecules (Rang 2016).

1.7.1 Transmembrane Guanylyl Cyclases and cGMP Function

The structural organization, biological function and regulation of the transmembrane GCs are well documented (Potter et al. 2009, Misono 2011, Potter 2011) (Figure 17). The mammalian genome encodes seven transmembrane GCs; GC-A to GC-G, which mainly modulate submembrane cGMP microdomains. These GCs share a unique topology, comprising an extracellular ligand binding domain and two intracellular domains: the catalytic cGMP-forming guanylyl cyclase domain as well as a protein kinase-homology domain. A short transmembrane section bisects the extracellular and intracellular domain. Atrial (A-) and B-type natriuretic peptides bind to GC-A which regulates arterial blood pressure and volume. GC-B is activated by C-type natriuretic peptides. GC-C mediates the paracrine effects of guanylin on intestinal ion transport and epithelial turnover. GC-E and GC-F are expressed in photoreceptor cells of the retina, and their activation by intracellular Ca^{2+} regulated proteins is essential for vision. GC-D and GC-G are olfactory cyclases in rodents (Potter 2011).



GC-A	guanylyl cyclase A	**sGC**	soluble guanylyl cyclase	**cGMP**	cyclic guanosine monophosphate
GC-B	guanylyl cyclase B	**PDE**	phosphodiesterases	**PKG I**	cGMP-dependent protein kinase I

Figure 17: Overview of the NO/cGMP signaling pathway and components regulating cGMP action.
Once activated by their specific ligand, classical transmembrane guanylyl cyclases and the soluble guanylyl cyclases (sGC) catalyze the formation of cGMP. cGMP mediates several physiological processes, such as the activating of PKG1, leading to smooth muscle relaxation. cGMP can be degraded by phosphodiesterases (PDEs), e.g. PDE5. This can be inhibited by PDE5 inhibitors, for example sildenafil.

The main function of cGMP is to activate intracellular protein kinases, especially cGMP-dependent protein kinase, or protein kinase G (PKG), to execute intracellular responses like SMC relaxation. In blood vessels, relaxation of vascular SMCs leads to vasodilation and increased blood flow. It also directly regulates cyclic nucleotide-gated ion channels to control intracellular Ca^{2+} levels. cGMP also plays a role in phototransduction.

Two PKG genes, coding for PKG type I (PKG1) and type II (PKG2), have been identified in mammals. PKG1 is a dimer consisting of one catalytic and one regulatory unit. The regulatory units block the active sites of the catalytic unit. cGMP activates the catalytic unit of the protein by binding on the regulatory units of PKG1, enabling the protein to phosphorylate their substrates. The execution of the physiological function of PKG1 is especially involved in the regulation of SMC relaxation, including vascular SMCs and in platelets. Lower levels are present in vascular endothelium, cardiomyocytes, fibroblasts and specific regions of the nervous system. Specifically, in SMCs, PKG1 phosphorylates the IP_3 receptor, leading to a decrease in Ca^{2+} concentration and, ultimately to SMC relaxation.

1.7.2 Soluble Guanylyl Cyclase and Nitric Oxide

The second type of GCs is the sGC. sGC is the only conclusively proven receptor for NO (Figure 17). sGC is a heterodimeric protein composed of α- and β-subunits: in addition, it is a hemoprotein. It is abundant in the cytoplasm of cells. NO is a free radical gas that can pass the cell membrane and exhibits its function on sGCs by binding to the prosthetic heme on the β-subunit of sGC (Ignarro et al. 1982, Stone and Marletta 1996). Activation of sGC by NO increases the conversion of GTP to cGMP, resulting in an elevation of cGMP, initiating the cGMP signaling pathway of e.g. cGMP-dependent protein kinase, cGMP-gated cation channel activity and cGMP-regulated phosphodiesterases, resulting in subsequent physiological changes (Waldman and Murad 1988, Furchgott and Jothianandan 1991, Bryan et al. 2009). Relaxation of vascular and gastrointestinal SMCs, inhibition of platelet aggregation, blunting of cardiac hypertrophy and improvement of cognitive function are some of the physiological processes regulated by NO-induced elevation of cGMP.

Most of these effects are mediated through the activation of PKG1 (Hofmann 2005, Lohmann and Walter 2005, Lincoln et al. 2006, Müllershausen et al. 2006, Hofmann et al. 2009).

NO is synthesized by the catalytic action of a family of NO synthases that convert the precursor amino acid, L-arginine, to NO and L-citrulline (Ignarro 1999, Ignarro 2005, Madhusoodanan and Murad 2007, Bryan et al. 2009). As mentioned before, NO signaling controls processes in the cardiovascular, neuronal and gastrointestinal systems (Garthwaite 2008, Friebe and Koesling 2009).

Three families of NO synthases produce NO. The inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS). iNOS is expressed upon inflammation and the production of NO is important against pathogens. nNOS was first found in neuronal tissue and produced. NO functions as a neurotransmitter, especially in non-adrenergic non-cholinergic nerves and is released in response to various depolarizing stimuli. It also has a role in long term potentiation and depression (Warner et al. 1994, Jaffrey and Snyder 1995). eNOS is highly expressed in endothelial cells, which is apparently the major source of plasma NO (Walter and Gambaryan 2009) which acts as a paracrine signal in a number of systems, including the vasculature regulating blood pressure (Warner et al. 1994). Its production is increased in response to the mechanical stress of blood passing over the cell surface. Activation of sGC leads to a subsequent rise in cGMP. Dissociation of NO from sGC or change in the redox status of the heme moiety reverses sGC activation.

1.7.3 NO/cGMP Pathway in the Prostate Gland, Seminal Vesicles and Epididymis

Classical treatment of BPH with the α-blocker tamsulosin has adverse effects like ejaculation disorders because it is likely to have negative effects on the prostate, but also on the organs responsible for ejaculation like seminal vesicles and epididymis. Therefore, it is of interest if emerging pharmacotherapies like PDE5 inhibitors differ to tamsulosin. To conduct functional studies, the abundance of cGMP signaling pathway components needs to be verified, not only in the prostate but also in the organs involved in ejaculation, especially the emission phase.

Components of the NO/cGMP signaling pathway are not only abundant in organs and tissues described above (see 1.7), but also in organs of the male reproductive tract like the prostate, seminal vesicles, and the epididymis, and more specifically in SMCs.

The NO/cGMP signaling pathway, including NO synthases, PKG and PDE5 in prostate tissue was first reported detecting PDE activity in the human prostate in 1970 (Kuciel and Ostrowski 1970). Additionally, all three forms of NO synthases were localized in prostatic tissue from BPH patients and display NO synthase activity (Hedlund et al. 1997, Gradini et al. 1999). In further studies, NO donors showed the relaxation of human, dog and rabbit prostate strips, suggesting an enhancement of cGMP formation (Hedlund et al. 1997).

More recently, PKG1 was also localized in human prostates, especially at the site where BPH is most likely to occur, the TZ (Waldkirch et al. 2007). Of the 12 different subtypes of PDEs, PDE5, which is specific for cGMP breakdown, is of high interest in the research of cGMP-mediated relaxation of SMCs in male reproductive organs and in clinical use for various conditions. Ückert *et al*. identified PDE mRNAs, especially from PDE5 in the human prostate (Ückert et al. 2001), which shows that important signaling components of the NO/sGC-mediated cGMP pathway are abundant in prostate SMCs. In more functional studies, Ückert *et al*. also showed the relaxation of human prostate tissue by inhibition of PDE5 with zaprinast or sildenafil (Viagra™) (Ückert et al. 2001). Clinical use of inhibitors of PDE5 that promote SMC relaxation is described below (see 1.7.4). Immunostaining against PDE5 in the human prostate suggested the localization of the protein mainly to be in the fibromuscular stroma as well as in the periurethral region and the TZ (Ückert et al. 2006). Detailed information, however, is still lacking.

This expression pattern in the TZ of human prostate tissues implies a functional role for PDE5 in the regulation of SMC tone and may be a therapeutic target in the treatment of BPH associated LUTS (see 1.7.4). The activity of PDE5 and NOS in seminal vesicles has been reported previously (Djoseland et al. 1980, Jen et al. 1997) and the expression and distribution was defined for PDE5 (Ückert et al. 2011). Additionally, studies have shown that an increase in cGMP levels in seminal vesicles can cause SMC relaxation (Bialy et al. 1996).

Studies demonstrated that NO and NO donors can inhibit electrically induced contractions of human seminal vesicle strips *in vitro* (Heuer et al. 2002), indicating that the relevant cGMP pathway components are abundant, controlled by NO/sGC in seminal vesicles and play a role in SMC relaxation.

In the bovine epididymis NO was shown as a modulator for spontaneous contractions of SMCs by Mewe *et a*l. who also showed the expression of eNOS in SMCs and epithelial cells of the epididymal duct (Mewe et al. 2006). In addition to eNOS, sGC is also mainly expressed in SMCs of the epididymal duct (Mewe et al. 2006, Mietens et al. 2012). Therefore, sGC mediates relaxing effects in the epididymal duct when stimulated by NO (Mewe et al. 2006).

Also in rat tissue, NOS distribution was found in all three regions (Burnett et al. 1995) and is highly abundant in the cauda (Dun et al. 1996). The key enzyme to mediate cGMP-dependent SMC relaxation is PKG1, which has been detected in all parts of the bovine and rat epididymis (Mewe et al. 2006, Müller et al. 2011). PDE5 was exclusively localized to the SMC layer in human and rat epididymal duct at mRNA and protein levels (Mietens et al. 2012). A study with a castration model of rats by Chamness *et al*. suggests that NOS expression in the epididymis is androgen-regulated because, after castration, NOS expression was significantly reduced in all regions of the epididymis (Chamness et al. 1995). In another functional study, inhibition of NOS increased the spontaneous SMC contractility of the epididymis (Mewe et al. 2006). Together, the NOS/NO/cGMP pathway in SMCs might provide a crucial role in sperm maturation and carrying out their physiological function is highly dependent on the regulation of contractility and androgen-dependent development, similar to other male reproductive organs like the prostate or seminal vesicles. Interference in contractile activity was demonstrated by Mietens *et al*., who showed that the clinically used PDE5 inhibitor sildenafil inhibits spontaneous SMC contractility in the rat epididymal duct (Mietens et al. 2012), which is similar to the studies of Kügler *et al.* who showed that sildenafil also inhibits spontaneous SMC contractions in the human and rat prostate gland (Kügler et al. 2018). This demonstrates that PDE5 inhibitors play a key role in regulating SMC tone and provides crucial insights for the research of new pharmacological treatment options for BPH.

1.7.4 Pharmacological Inhibitors of PDE5

The discovery of the NO/cGMP pathway provided many insights into several physiological functions and pathophysiological processes. The understanding that PDEs regulates the action of cGMP and ultimately PKG1, the most important effector protein, led to the development of inhibitors of PDE. The original discovery and pharmacological introduction, especially for inhibitors of type 5 phosphodiesterases (PDE5 inhibitors), showed effectiveness in pulmonary hypertension and other cardiovascular diseases (Chrysant 2013, Schellack and A 2014). Several studies have proven their effects in improving cardiac function, exercise capacity and increased cardiac output (Kukreja et al. 2011). One of the common side effects of this medication was that it caused erections in male patients and was subsequently approved by the US Food and Drug Administration (FDA) for the primary indication of ED replacing ineffective and unsafe medications as well as invasive therapies such as penile prosthetic surgery (Hellstrom et al. 2003, Ferguson and Carson 2013). Five years after official approval, three further inhibitors of PDE5 were approved for the treatment of ED: vardenafil, avanafil, and tadalafil (Ferguson and Carson 2013, Evans and Hill 2015). Sildenafil, vardenafil, and tadalafil are equally efficacious in mild to moderate ED. They are usually considered to be first-line therapy for ED (Rossiter et al. 2014, Yee et al. 2016).

The main pharmacotherapies used for the treatment of LUTS associated with BPH are α_1-adrenoceptor antagonists (e.g. tamsulosin) or 5ARIs (e.g. dutasteride), either alone or in combination (Bullock and Andriole 2006). However, the use of α-blockers is associated with adverse effects like sexual dysfunction and ejaculatory disorders (Gacci et al. 2011). Nowadays, several clinical studies have assessed PDE5 inhibitors including vardenafil, sildenafil, and tadalafil in reducing LUTS; however, tadalafil is the only PDE5 inhibitor approved by the FDA in 2011 for the treatment of signs and symptoms of BPH (Carson et al. 2014). Furthermore, tadalafil demonstrated significant improvements in the total IPSS, BPH impact index, and International Index of Erectile Function (IIEF) (Carson et al. 2014).

Combination therapy using PDE5 inhibitors and α_1-blockers resulted in additive favorable effects in men with LUTS associated with BPH when compared with PDE5 inhibitor monotherapy, suggesting that α-blockers may enhance the efficacy of PDE5 inhibitors, which is beneficial for the treatment of LUTS (Yan et al. 2014). Additionally, similar results were obtained in combination therapy of tadalafil and finasteride (5ARI), where a study showed that co-administration of these medications provided improvements in LUTS associated with BPH (Casabe et al. 2014). Nevertheless, the exact mechanism of action of PDE5 inhibitors, especially tadalafil in the treatment of BPH is not known. Therefore, long-term studies to evaluate safety are of interest combined with basic studies on potential adverse effects with PDE5 inhibitors that are present with the α-blocker tamsulosin. The reasons behind the occurrence of disturbed ejaculation and adverse effects in patients treated for BPH are not clarified and are still a matter of debate. The following results give crucial insights about the exact mechanism of action and contribute to discover new pharmacotherapies for BPH, avoiding adverse effects.

1.8 Aims

1.8.1 Significance of the Project

Research on new pharmacotherapies for BPH should focus on the function and responses of SMCs. SMC tone (dynamic component) and an increased number of stromal cells (static component) containing mainly SMCs play a crucial role in the pathophysiology of BPH, although the reasons for the increase in both components remain poorly understood. Generally, it is known that treating BPH symptoms effectively involves the reduction of SMC tone in the prostate and decreasing the actual size of the organ. The reduction of SMC tone is achieved by interfering with signaling pathways involved in SMC contraction and/or relaxation. The two main signaling pathways that can be modulated include the activation or inhibition of especially α_1-adrenoceptors and the accumulation of the molecule cGMP (e.g. with inhibitors of phosphodiesterase type 5), which activates the effector protein PKG1 that ultimately leads to SMC relaxation.

A wide variety of treatment options are available for patients suffering from BPH that severely affects the quality of life in men. They vary from conservative treatments (e.g. change of diet, exercise) to classical pharmacological treatments (e.g. α_1-blockers, 5α-reductase inhibitors, PDE5 inhibitors, combination drug therapy) and include surgery (e.g. TUR-P). Established pharmacological treatment options like α_1-blockers can induce many different side effects because of the wide distribution of α_1-adrenoceptors throughout the whole body, especially in the reproductive organs. Therefore, patients might experience global effects when treated with α_1-blockers. One of the best examples indicating that α_1-blockers affect many organs of the male reproductive system is the common side effect of disturbed ejaculation documented in men treated with agents such as tamsulosin or silodosin. These drugs might not only affect the prostate, but also other organs like the seminal vesicle or the epididymis. However, the exact mechanisms of adverse effects of tamsulosin on these tissues are not clarified. It is still a matter of debate what the term “abnormal” or “disturbed” ejaculation comprises. For an effective treatment of BPH with less or even without any side effects, i.e. before new treatment options can be introduced, the underlying pathognomonic features associated with BPH need to be investigated in more detail.

1.8.2 Aims

1) To clarify the abundance and distribution of SMCs and the localization of PDE5 within SMCs in the human and rat prostate using immunostaining and our modified CLARITY approach. This initial analysis of the abundance and distribution allows subsequent investigations of the specific effects of PDE5 inhibitors. Physiological investigation of different pharmacotherapies, like the PDE5 inhibitors sildenafil and tadalafil, on physiological spontaneous contractile activity (myogenic tone) in the human and rat prostate gland, was performed utilizing our novel time-lapse imaging approach.

2) To characterize the architectural properties and functional units of human and rat prostatic tissue, especially the single glands and excretory ducts that are mainly involved in the production and secretion of prostatic fluid upon the emission phase of ejaculation. This aim was addressed by performing Heidenhain's azan staining.

3) To test and understand the role of the α_{1A}-adrenoceptor antagonist tamsulosin, which is the gold standard treatment for BPH, and the newly emerged treatment option tadalafil, a PDE5 inhibitor, on tissues involved in ejaculation (in rat prostate ducts, seminal vesicle, and epididymis) to explain the adverse effects in these tissues regarding ejaculation disorders in comparison to tadalafil. For this purpose, our novel time-lapse imaging approach was applied. Sympathetic activity, initiating the emission phase of ejaculation was simulated using NA.

4) To establish primary cell lines of human prostate specimens for *in vitro* experiments, comparing effects of the NO donors S-Nitrosoglutathione (GSNO) (i) alone or (ii) conjugated in the core of a macromolecular carrier (star polymer) on the expression of cGMP pathway components (mRNA and protein levels).

5) To test and compare both NO donors in a physiological setting using the organ bath technique to investigate effects on contractility (myogenic tone) in isolated human prostate specimens.

Chapter 2 - Materials and Methods

2.1 Tissues

2.1.1 Human Prostate Gland

Human tissue samples collected in Germany for time-lapse imaging (see 2.2) and immunostaining (see 2.3.3) originated from patients (range 60-79 years, median 71.7±6.5) undergoing transurethral monopolar electroresection of the prostate (TUR-P) for BPH or radical prostatectomy for prostate cancer at the Department of Urology, Pediatric Urology and Andrology of the Justus-Liebig-University Giessen. Specimens obtained from radical prostatectomy were taken from normal non-malignant regions of the prostate gland. Tissue samples from TUR-P were collected from the periurethral region. The use of human prostate tissue was approved by the ethics committee of the Medical Faculty, Justus-Liebig-University Giessen, Germany (ethical vote: 49/05, 2005) and all patients were briefed about the nature and possible consequences of participating in this study before giving written informed consent. After radical prostatectomy, the excised tissue was placed in a sterile screw-capped jar and transported from the operating theatre to the pathologist in a physiological medium on ice. At pathology, the location of the tumor was identified by palpation and histopathology reports from previous biopsies taken from the same patient. A transverse slice from the posterior to the anterior side of the prostate was performed and regions of benign tissue were dissected from regions of interest (transition zone). Obtained tissue was transported on ice to the laboratory for further investigation. For time-lapse imaging, tissue pieces were dissected under a binocular microscope and cut into thin small pieces for transillumination microscopy (see 2.2). Prostate tissue that was not used for time-lapse recordings was fixed in 4% paraformaldehyde (PFA) or Bouin solution for histological and anatomical analysis (see 2.3).

The collection of human prostate specimens in Australia for tension recording experiments (see 2.5.1) was achieved together with the Australian Prostate Cancer BioResource (APCB) Coordinator Dr. Melissa Papargiris. Prior to surgery (radical prostatectomy), the coordinator obtained biopsy results from clinicians.

Collection and use of human specimens were in approval and compliance with the Cabrini Human Research Ethics Committee (13-14-04-08) and Monash University Human Research Ethics Committee (2004/145). Furthermore, patients undergoing surgery were informed about the study and gave written consent for human prostate specimen collection before surgery. Obtained tissue was transported in a screw-capped jar on ice to *TissuPath*, a registered private pathology company. Non-malignant tissue was dissected from the prostate and transported to the laboratory facilities to conduct tension recording experiments. A frozen histological section following routine hematoxylin and eosin (H&E) staining was performed to confirm non-malignancy. Tissue with evidence of malignancy was excluded from further studies.

2.1.2 Rat Prostate, Seminal Vesicle and Epididymis

Male rat reproductive organs obtained in Germany, including the prostate, the seminal vesicle, and the epididymis were collected from adult Wistar rats at full sexual maturity (>12 weeks old). The animals were housed in the animal facilities of Justus-Liebig-University Giessen, with access to food and water *ad libitum*. Housing, animal care, and all procedures were conducted according to the guidelines for animal care and approved by the committee for laboratory animals of Justus-Liebig-University Giessen (JLU no. 469_M and 510_M). Rats were anesthetized with 5% isoflurane and sacrificed via cervical dislocation. The subsequent preparation of the different tissues investigated was performed within the next 1-2h.

The organs of interest were carefully collected under a binocular microscope using fine surgical scissors. For collection, an abdominal incision was performed and the urogenital and reproductive organs were removed and transferred to minimal essential medium (MEM) for further dissection. Prostatic tissue from ventral lobes was divided and distinguished into the single glands and the excretory ducts. For this, the prostate lobes were carefully separated from any attached anatomical structures, connective tissue and adipose tissue using fine surgical scissors and tweezers.

For the dissection of the sperm-storing part of the cauda epididymal duct, the same procedure as for the prostate tissue was performed and the isolated epididymal duct cut into smaller pieces. The seminal vesicles are organs that are not embedded in any other surrounding tissues and the excretory part directly leads to the urethra.

Therefore, incisions to isolate the tissue were performed at the most distal excretory part, flanking the urethra. Obtained tissues were further processed for time-lapse imaging recordings (see 2.2). Prostate tissue that was not used for time-lapse imaging recordings was fixed in 4% PFA or Bouin solution for histological and anatomical analysis (see 2.3).

2.2 Time-Lapse Imaging

For the functional investigation of the effect of different drug classes on the contractility of SMCs in reproductive organs including the rat prostate glands, prostate ducts, seminal vesicles, and the cauda epididymal duct and human prostate specimens, time-lapse imaging was performed in combination with transillumination microscopy for visualization. For this, the tissue of the obtained human prostate specimens and the reproductive organs from rat were embedded in a collagen suspension to ensure tissue immobilization. Collagen was collected from tails of adult rats and suspended in 10xDMEM/F12 (Gibco, Invitrogen, Karlsruhe, Germany), together with 0.1% glacial acetic acid, 0.5M sodium hydroxide (NaOH) and 1M HEPES. Prostate samples were placed inside the viscous collagen solution and mounted at the bottom of a Delta T dish (Bioptechs, Buttler, USA). At 37°C collagen was polymerized and covered by 1ml MEM for optimal tissue supply. Time-lapse recording was performed using a BX50WI transillumination microscope (Olympus, Tokyo, Japan) equipped with UMPlan FI10x/0,5W and UMPlanFI20x/0,5W objectives that were attached to a Till-Imago QE CCD camera (Photonics, Gräfelfing, Germany). Camera input was recorded by TillVision software (Version 4.0, Photonics). Pictures were taken automatically to produce virtual time stacks. The temperature was set constantly to 34°C during the whole recording time. This procedure applied to all tissues investigated. Pictures were taken for prostate tissue (human and rat) and seminal vesicles (rat) every 2s and in the epididymal duct (rat), pictures were taken every second.

The experimental setup for all investigated tissues followed the same principle. After starting the recording, the tissues were left untreated for 15-30min to characterize spontaneous activity of the tissue, followed by drug treatment with either one of the PDE5 inhibitors [sildenafil (5μM, Pfizer, New York, USA) or tadalafil (2.5μM, Lilly Icos LLC, Indianapolis, USA)], the α_1-blocker tamsulosin (5μM, Sigma-Aldrich, Steinheim, Germany) or vehicle for another 15min. Any response to NA (10μM, Sigma-Aldrich, Steinheim, Germany) was recorded for an additional 15min to prove for tissue vitality. All substances investigated were directly dripped into the dish containing the tissue. Sample recordings without any visible response to NA were excluded from statistics.

The experimental setups described in Chapters 3 and 4 were adjusted to achieve the most reliable investigation of the tissues in regard to the aims set for this study. For details of specific adjustments in experimental procedures see Chapters 3 and 4. Briefly, for the setup described in chapter 3, spontaneous contractility was investigated for the first 30min of each recording and used as positive control, followed by 15min of treatment with the PDE5 inhibitor sildenafil in all tissues and tadalafil for rat prostate glands. For proof for tissue vitality, any response to NA was recorded for an additional 15min. In a final step, the histological determination of all observed tissues was confirmed by paraffin-embedded azan staining (see 2.3.2). Digital time stack processing was performed using ImageJ 1.50e (public domain software, NIH, Bethesda, USA, download at http://imagej.hih.gov/ij) to visualize tissue contractions. The built-in tool “Reslice” was used to set regions of interest that translated all contractions into visible peaks on a fixed timescale and could be followed over time. Contrast enhancement was adjusted if necessary, to improve the final outcome. Contraction frequency could then be calculated as the number of peaks over time. The first 10min of spontaneous contractions and first 4min after drug addition were taken as residence time and excluded from calculations. Sample recordings without any visible response to NA were excluded from statistics. Time-lapse imaging recordings for the studies in Chapter 4 were adjusted accordingly to the aims of the study. Drugs used included the α_1-blocker tamsulosin (5μM), the PDE5 inhibitor tadalafil (2.5μM) and NA (10μM). The latter was used to simulate the emission phase of ejaculation in the investigated tissues. Different approaches to analyze recordings were applied. Responses to NA in all tissues investigated (prostate ducts, seminal vesicles, and epididymal duct) after different drug pre-treatments (tamsulosin, tadalafil) were analyzed and compared to vehicle with measuring the area of the observed tissue before and after the addition of NA. Changes in size of the observed tissue showed the effect of drug pre-treatment on tissue contractility and any potential interference with sympathetic stimulation.

Additionally, contraction frequencies for epididymal ducts in response to NA, as well as weight changes in seminal vesicles before and after the addition of NA, indicating expulsion of secretion, were measured. Sample recordings without any visible response to NA were excluded from statistics. Digital time stack processing was performed using ImageJ 1.50e.

2.3 Histochemistry

2.3.1 Fixation

Human and rat prostate tissue samples were fixed in Bouin solution for 24h. Following this, ascending alcohol concentrations were used for dehydration of the tissue. Afterwards, tissues were embedded in paraffin and allowed to harden completely before slicing using a RM2255 microtome (Leica, Wetzlar, Germany). Slicing thickness was set to 5μm and slices were put on microscopic slides and kept at room temperature.

2.3.2 Azan Staining

For the investigated human and rat prostatic tissue the Azan trichrome staining was used to analyze the general histology of the investigated tissue. In this study, the Azan staining according to Heidenhain was performed, resulting in dark red staining of cell nuclei, whereas collagen was colored in blue. The deparaffination of microscopic slides was achieved with a descending alcohol series. Afterwards, cell nuclei and the surrounding cytoplasm were stained with acetocarmine solution at 56°C for 10-15min under visual control. Phosphotungstic acid binds the dye to the tissue, followed by the second step of staining with aniline blue, which stained collagen fibers. Tissue sections were dehydrated in isopropanol and xylene, embedded in mounting medium (Eukitt; Fluka, Buchs, Switzerland) and protected by Automat Star coverslips (Engelbrecht, Edermünde, Germany) for long-term storage.

2.3.3 Immunostaining

Immune reactive staining was performed with special interest on the spatial arrangement of SMCs in rat and human prostatic tissue, especially around single glands and prostatic excretory ducts for comparison. First, sections were deparaffinized in descending alcohol series, including a step of incubation in 1.2% H_2O_2 (dissolved in methanol) to block endogenous peroxidase activity.

Primary antibodies targeting α-smooth muscle actin (α-SMA) (mouse monoclonal, 1:1000; Sigma-Aldrich, Steinheim, Germany), PKG1 (rabbit polyclonal, 1:1000, Enzo Life Sciences, Lörrach, Germany) and PDE5 (rabbit polyclonal, 1:1000; gift from Laurinda Jaffe, University of Connecticut Health Center, Farmington, USA) were diluted in phosphate-buffered saline (PBS) with 0.2% bovine serum albumin (BSA) and 0.1% sodium azide (NaN_3) and incubated at 4°C overnight to ensure a slow penetration of the antibody into the tissue. For the overnight incubation, slides were transferred to humidity chambers. Sections without primary antibodies served as negative control. On second day of the immunostaining, tissue sections were washed several times in PBS before adding horseradish peroxidase (HRP)-labeled polymer (DAKO, Hamburg, Germany) to each section at room temperature for 30min. After washing with PBS and 0.1M PB, peroxidase activity was detected by 3,3'-diaminobenzidine (DAB). The reaction was initiated by nickel-glucose oxidase amplification and stopped under visual control. For storage, all sections were dehydrated by ascending alcohol series and xylene, and mounted in Eukitt Quick-hardening mounting medium (Merck, Darmstadt, Germany), and then protected with Automat Star coverslips

To investigate the co-localization of two different proteins of interest in single SMCs, immunofluorescence double staining in the same paraffin-embedded tissue sections was performed. Deparaffinization in descending alcohol series, including the incubation in 1.2% H_2O_2, was followed by washing steps in PBS. After this, sections were transferred to 2% normal goat serum for 1h at room temperature to block unspecific binding sites. Primary antibodies targeting α-SMA (1:1000) and PDE5 (1:1000) were diluted in PBS with 0.2% BSA and 0.1% NaN_3 and applied to tissue sections for overnight incubation in humidity chambers at 4°C. Sections without primary antibodies served as negative control. On the second day, sections were washed with PBS and incubated with fluorescence-labeled secondary antibodies (Cy3 anti-rabbit IgG, 1:500; Jackson ImmunoResearch, West Grove, USA respectively Alexa Fluor 488 anti-mouse IgG, 1:500; Thermo Fisher Scientific, Waltham, USA) together with 4',6'-diamidino-2-phenylindole (DAPI) (1:1250; Merck, Darmstadt, Germany) for 1h at room temperature.

To avoid a loss of fluorescence by UV influence from daylight, all steps on the second day were performed in darkness. Final washing steps with PBS were followed by mounting the slides under coverslips with buffered glycerol for long-term storage at 4°C. Conventional light and fluorescence microscopy served for documentation of all performed stainings using an Axioplan 2 microscope and for imaging the Axiovision LE software (Zeiss, Munich, Germany).

2.4 Clear lipid-exchanged acrylamide-hybridized rigid imaging/ immunostaining/ in situ hybridization-compatible tissue-hydrogel (CLARITY)

Clear lipid-exchanged acrylamide-hybridized rigid imaging/ immunostaining/ in situ hybridization-compatible tissue-hydrogel (CLARITY) improves common histological techniques by generating 3D images of whole tissues (Chung and Deisseroth 2013). This technique allows conserving biological tissue by transforming it into a translucent hydrogel-tissue hybrid. The structural framework is preserved via mounting biomolecules in a hydrogel matrix while unattached lipids will be washed away. This technique was originally developed for the clearance of brain tissue (Chung and Deisseroth 2013). Therefore, no generally accepted protocol for prostate tissue was available and instructions by Chung & Deisseroth (Chung and Deisseroth 2013) had to be adapted. The protocol was used for prostate tissue samples dissected from rat ventral lobes or human prostate tissue.

Tissue sections used were approximately 1mm^3 in size and were fixed in 4% PFA for 24h at 4°C. Incubation in hydrogel solution (Table 2) for 24h at 4°C was followed by its polymerization at 37°C for 3h forming a hydrogel mesh of the prostatic tissue. The remaining hydrogel excess was carefully removed. In a subsequent step, tissue samples were incubated in clearing solution for a minimum of 5 days under visual control to wash out most lipids. Removal of lipid components is crucial to avoid light scattering for optimal tissue opacity.

Table 2: Hydrogel and buffer composition.

Hydrogel solution components	**Final concentration**
Acrylamide	4%
Bisacrylamide	0.05%
2,2′-Azobis(2-methylpropionamidine)dihydrochloride (AAPH)	0.25%
Clearing solution components	**Final concentration**
Sodium dodecyl sulfate (SDS)	10%
Boric acid	200mM

Extensive washing steps with 0.1% PBS-Triton (PBST) were performed before and after adding primary antibodies targeting α-SMA (1:100), PKG1 (1:100), PDE5 (1:100), calponin-1 (rabbit monoclonal, 1:100; Epitomics, Burlingame, USA), Ki67 (1:100, rabbit polyclonal, Novocastra, Newcastle, UK) as well as α-tubulin (mouse monoclonal, 1:100, Sigma-Aldrich, Steinheim, Germany) and fluorescence-labeled secondary antibodies (Cy3 anti-rabbit IgG,1:200 and/or Alexa Fluor 488 anti-mouse IgG, 1:200). All antibodies were diluted in 0.1% PBST and incubated for at least 5 days at room temperature. Staining was performed either as single antibody staining or double antibody staining. Additionally, DAPI staining (1:400) helped to distinguish between glandular epithelium and stromal SMCs. A 24h washing step with 0.1% PBST was executed before the tissue was transferred into a refractory index matching solution (RIMS, Table 3) for 24h at room temperature. RIMS buffer allows optical enhancement of clearance and is caused by closely approximated refractory indexes of the translucent sample and its embedding solution. The embedding solution facilitates light to pass through with neither refraction nor reflection.

Table 3: RIMS buffer composition for optical clearance enhancement.

Refractory index matching solution components	Final concentration
Histodenz	88%
Tween-20	0.1%
Sodium azide (NaN_3)	0.01%

Samples were mounted in RIMS buffer on microscopic slides. The sample thickness could reach 2mm and more; therefore, a gap between slide and coverslip could occur and was bridged by a ring of blue tack (Bostik, Wauwatosa, USA). Documentation of all samples was performed on a LSM710 confocal laser scanning microscope (Observer.Z1, Zeiss, Munich, Germany), capturing z-stacks with slice distances between 1.5-5µm. Reconstruction of z-stacks was performed with the open source program ImageJ 1.50e. With the integrated program tool “3D projects” a simple setup for interpolated 3D reconstructions was possible to achieve. If necessary, automatic contrast enhancement was applied.

2.5 Tension Recording Experiments

In tension recording experiments, non-malignant human prostate specimens from patients undergoing radical prostatectomy (see 2.1.1) were included.

2.5.1 Human Prostate Gland

Obtained human specimens from the TZ of the prostate were transported to laboratory facilities for tension recording experiments. Microdissections were undertaken under a SMZ800 stereoscopic zoom microscope (Nikon Instruments INC., New York, USA). For each recording, one piece of tissue was prepared for approximately 2x5mm in size. This piece was tied and knotted on each end with suture cotton thread to place into a mini organ bath (Monash Facility for Instrumentation and Technology Development, Melbourne, Australia) with a total volume of 1ml and the base lined with Sylgard184 (Dow Corning, Midland, USA) for tension recording experiments. The cotton thread was tightened around each end of the tissue. With additional knots in the thread the tissue could be placed in between two forked metal hooks in the mini organ bath, of which the right one was attached to a force transducer (Monash Facility for Instrumentation and Technology Development, Melbourne, Australia) extended to a ML221 Bridge AMP (ADInstruments Pty Ltd, Bella Vista, Australia) connected to a high-performance data acquisition system, PowerLab® 4/30 (ADInstruments Pty Ltd, Bella Vista, Australia). Once the tissue was put into place, this experimental setup was able to record changes in tension, indicating contractions of the tissue preparation, using an application program on a personal computer, named Chart Pro® v5.5.6 for Windows (ADInstruments Pty Ltd, Bella Vista, Australia). The organ bath was then mounted onto a wooden stage within a Faraday cage to reduce vibrational interference from the environment. Physiological saline solution (PSS) was bubbled with carbogen (95% O_2, 5% CO_2) in a 100ml beaker and was circulated through the mini organ bath using a Minipuls®3 peristaltic pump (Gilson, Inc., Middleton, USA) at a rate of 3-4ml/min using polyvinyl chloride (PVC) tubes, consisting of a diameter of 2.79mm.

PSS was heated to a physiological temperature of 37°C using an in-line solution heater SH-27B (Warner Instruments LLC, Hamden, USA) before reaching the organ bath and the tissue of interest, respectively.

For equilibration, an initial tension of 25-30mN was applied to the tissue and the tissue was left to equilibrate for 60min. The tension that followed the equilibration period was defined as 'basal tension'. This tension was not readjusted to a predefined baseline to allow spontaneous contractions to emerge. Following the equilibration period, the preparation was incubated with ascending concentrations (1μM, 3μM and 10μM) with either S-Nitrosoglutathione (GSNO), a NO donor, alone or conjugated in the core of a macromolecular carrier, the star-polymer conjugated GSNO (see 5.2), prepared in fresh PSS for 10min of each concentration, or vehicle (0.1% DMSO in PSS) for 30min alone (for further details see Chapter 5). After a washout period of at least 5min with PSS, the tissue was exposed to a high concentrated potassium chloride (KCl) solution (40mM) to assess the viability of the tissue. It induced a reliable and robust contraction in viable preparations.

2.6 Cell Culture Experiments

For *in vitro* experiments of human prostate tissue, specimens from patients undergoing radical prostatectomy due to prostate cancer were obtained from non-malignant regions of the TZ (see 2.1.1). Obtained tissue was digested (see 2.6.1) following a setup in specific SMC media to promote SMC growth (Wang et al. 2007) (Table 4). The aim was to isolate SMCs of the whole tissue of the human prostate and conduct a variety of different experiments to investigate different concentrations of two variants of S-Nitrosoglutathione (GSNO) (see Chapter 5), and its effect on the cGMP signaling pathway components, especially PKG1 and PDE5, on RNA and protein levels.

All cells were cultured at 37°C, under 5% O_2 and 5% CO_2 in 15ml SMC specific growth medium (Table 4) in T75 flasks. The medium was renewed every 2-3 days. If cell growth reached at least 90% confluence, cells were reseeded in T175 flasks. Next time when cell growth reached total confluence, cells were passaged. Passaging always included the following steps. First, the old growth medium was aspirated, followed by a washing step with 5ml PBS+EDTA for 5min at room temperature. 2ml of trypsin (0.1%) was added and incubated for 5min at 37°C. Trypsin was neutralized by adding of 5ml medium with fetal calf serum (FCS) and this cell suspension was then transferred into a 15ml tube and spun down at 1000rpm for 5min at room temperature. The supernatant was aspirated, and the remaining cell pellet was resuspended in 1ml medium. After cell counting with the help of a TC10 automated cell counter (Bio-Rad, Munich, Germany), the cell suspension was split in half and added to 15ml medium within two new T75 flasks, containing at least $1x10^6$ cells. Cell isolation, as well as all necessary cell culture experiments, were performed at the facilities of the Department of Anatomy and Developmental Biology, Monash University, Melbourne, Australia.

Table 4: Components of SMC media.

SMC media components	Final concentration
Horse Serum	15%
HEPES	10mmol/l
P/S Antibiotics	1%
non-essential amino acids	1%
Insulin	5μg/ml
Transferrin	10μg/ml
Sodium selenite (Na_2SeO_3)	5ng/ml
Estradiol	0.1μmol/l
Dexamethasone	0.1μmol/l

2.6.1 Tissue Digestion

Patient-derived primary SMCs were isolated from human prostate tissue using an established methodology described by Lawrence and colleagues (Lawrence et al. 2013). Briefly, tissue was collected as mentioned above, placed in transfer media, and weighed. First, the obtained tissue was washed in PBS supplemented with gentamicin (100μg/ml) and cut into approximately 1mm^2 pieces using a sterile scalpel blade. The dissected tissue was placed into RPMI1640 media containing Penicillin and Streptomycin (P/S), 5% FCS, gentamicin, 225U/ml collagenase and 125U/ml hyaluronidase. The incubation of the tissue was performed in an oven with a rotator for 16h. Following incubation, the tissue was processed into a single cell suspension by mechanical digestion with pipette tips, and centrifugation. The remaining cell pellet was resuspended in RPMI1640 media containing 5% FCS and P/S antibiotics and placed in a T25 flask. Following the initial passage, cells were cultured in specific SMC media (Table 4) for the purity of cells isolated and utilized at passages 3-5. The setup of the SMC media and utilization at passages 3-5 followed a protocol established by Wang and colleagues (Wang et al. 2007).

2.6.2 qRT-PCR

RNA was collected from cells treated with vehicle (0.1% DMSO in PSS) or one of the two different NO donors (GSNO) at a final concentration of 10μM (see Chapter 5), for 24h. RNA was extracted using the RNeasy Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's protocol. cDNA synthesis was performed with 500ng of RNA each, and generated using Superscript III (Roche, Basel, Switzerland) following routine "First Strand cDNA synthesis Protocol". Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using cDNA generated as described above. Primers utilized in this experiment are listed in Table 5. Except for α-SMA, all primers were self-designed by using the following procedure. The complete nucleotide sequence was searched online from the NCBI database. PCR analysis allows multiplying a DNA sequence of interest, targeted by specific primer pairs. Here, qRT-PCR was performed using the QuantiTect SYBR Green Kit (Qiagen). qRT-PCR products were labeled with fluorescence markers to quantify the amplified amount of DNA. Each gene was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and compared with vehicle controls using the $2^{-\Delta\Delta Ct}$ method, and fold change was calculated relative to vehicle control of independent cell lines.

Table 5: Human-specific primer descriptions.

Gene name	Primer position	Primer sequence	Annealing temp.	PCR product length [bp]	Origin
sGC (β1)	1470 1611	(fwd) 5'-TGCTGGCCAGGTTCAAGTAG-3' (rev) 5'-TGTTCGGCTTGTGAGGTTGA-3'	59,96 59,82	142	self-designed
PDE5	2835 3024	(fwd) 5'-TATGCCTAGTTTCTTACACACTGTC-3' (rev) 5'-GTGCTAACAGTGGATGTTGTTGAT-3'	58,6 59,78	190	self-designed
PKG1	1330 1447	(fwd) 5'-GTTGGAGGTTTCGGACGAGT-3' (rev) 5'-GGATGTGCTCCTGCTGTCTT-3'	59,97 60,04	118	self-designed
α-SMA	537 648	(fwd) 5'-TGTAAGGCCGGCTTTGCT-3' (rev) 5'-CGTAGCTGTCTTTTTGTCCCATT-3'	59,57 59,5	112	(Madar et al. 2009)

qRT-PCR was always performed with the following composition (Tables 6 and 7).

Table 6: Template for qRT-PCR loading.

Component	Volume [µl] for 1 sample
Power SYBR Green Master Mix	12.5
10µM forward primer	0.5
10µM reverse primer	0.5
Template	5
Water	6.5
Total	25

Table 7: Template for qRT-PCR conditions. (50 cycles)

Temperature [°C]	Duration [s]
95	600
95	20
60	20
72	20
50	30
20	∞

2.6.3 Western Blot

Analysis of proteins of the cGMP signaling pathway, including PKG1 (primary antibody: anti-PKG1, rabbit polyclonal, Enzo Life Sciences, Lörrach, Germany) and PDE5 (primary antibody: anti-PDE5, rabbit polyclonal, Laurinda Jaffe, University of Connecticut, Health Centre, Farmington, USA) in prostatic SMCs was performed with Western Blot analysis after incubation of the cells with vehicle (0.1% DMSO in PSS) or one of the two different GSNO variants (see chapter 5) to investigate if NO release in SMCs affects the expression of the cGMP signaling pathway downstream of PKG1 and PDE5. Incubation with antibodies targeting β-actin (primary antibody: anti-β-actin, mouse polyclonal, Sigma-Aldrich, St. Louis, USA) was used as positive control. For this, $1x10^6$ cells were seeded in a 60x15mm dish. After 24h incubation, cells were incubated with one of the two variants of the NO donor GSNO or vehicle and left for another 24h. After treatment, the medium was aspirated, followed by washing steps with PBS. 200µl of lysis buffer was added for 1min.

Afterwards, cells were scraped off the plate, collected in tubes and left on ice for 30min. Lysates were further processed for Western Blot analysis or frozen at -80°C. Setup of the Western Blot was performed using an established protocol modified after Rebello and colleagues (Sipila and Bjorkgren 2016). Briefly, protein concentrations were determined (method after Bradford) using the detergent-compatible protein assay kit (Bio-Rad, Hercules, USA). Equal amounts of protein (25µg) were separated by SDS-PAGE on a 10% gel and transferred to PVDF membranes (Millipore, Burlington, USA). The primary antibody incubation of the membranes was performed overnight at 4°C. After washing, secondary antibody incubation with either polyclonal goat α-rabbit immunoglobulins/HRP (1:10,000, Dako, Glostrup, Denmark) or polyclonal goat α-mouse immunoglobulins/HRP (1:10,000, Dako, Glostrup, Denmark) in 5% blocking solution for 1h at room temperature. Chemiluminescence was achieved using Amersham Hyperfilm MP autoradiography film (GE Healthcare, UK).

2.7 Statistical Analysis

2.7.1 Time-lapse Imaging Analysis

For all time-lapse imaging experiments, only vital samples (confirmed by a visible response to NA) were included. Normal distribution of the data was checked by the Kolmogorov-Smirnov test. Data sets were analyzed using a one-tailed t-test in case of normal distribution, otherwise Wilcoxon matched-pairs signed rank test was applied. Analysis of data, statistics and graphs were performed with the statistics program GraphPad Prism (version 6.02 for Windows, GraphPad Software, La Jolla, USA, www.graphpad.com) where $p<0.05$ was considered to be statistically significant. Measured percentages of the area or weight changes and contraction frequencies measured as [contr/min] are displayed as mean±standard error of the mean (SEM). For in detailed data pairing for the different recordings regarding contraction frequencies, area changes or weight changes in tissues please refer to Chapters 3 and 4.

2.7.2 Tension Recording Analysis

Tension recording data was analyzed using Chart Pro® v5.5.6 for Windows (ADInstruments Pty Ltd, Bella Vista, Australia). Unless noted, paired one-way ANOVA with Dunnett's post hoc test was used for statistical analysis of the data, and was represented as a mean±SEM where $p<0.05$ was considered to be statistically significant. Analysis of data, statistics and graphs were performed with the statistics program GraphPad Prism. Analysis of tension recordings to define spontaneous contractions is focused on three parameters: the resting basal tension (mN), amplitude (N/g) and frequency (contr/min) of contractions. These parameters were measured for 5 consecutive contractions for spontaneous occurring contractions (set as matched control), for each concentration (1μM, 3μM, 10μM) of one of the two GSNO variants and for vehicle. The parameters measured after the addition of a drug were averaged, and then compared to the averaged parameters of the matched control (spontaneous contractions). Experiments were repeated, with n representing the number of patients.

2.7.3 Cell Culture Experiments Analysis

Unless noted, all data are displayed as mean±SEM, with statistical analysis performed by a t-test, one-way ANOVA and Tukey's post hoc test, or two-way ANOVA and Tukey's post hoc test, using the statistics program GraphPad Prism where $p<0.05$ was considered to be statistically significant. An Extreme Studentized Deviate (ESD) test with an Alpha level of 0.05 was performed to exclude outliers.

Chapter 3 - Novel Imaging of the Prostate Reveals Spontaneous Gland Contraction and Excretory Duct Quiescence Together with Different Drug Effects (FASEBJ, 2018)

Authors

Robert Kügler[1]*, Andrea Mietens[1]*, Mathias Seidensticker[1]*, Sabine Tasch[1], Florian M. Wagenlehner[2], Andre Kaschtanow[1], Yudy Tjahjono[1], Claudia U. Tomczyk[1], Daniela Beyer[1], Gail P. Risbridger[3], Betty Exintaris[4], Stuart J. Ellem[3], and Ralf Middendorff[1]

*These authors contributed equally to this work

Affiliations

[1]Justus-Liebig-University Giessen, Institute of Anatomy and Cell Biology, Giessen, Germany

[2]Justus-Liebig-University Giessen, Department of Urology, Pediatric Urology and Andrology, Giessen, Germany

[3]Department of Anatomy and Developmental Biology, Biomedicine Discovery Institute, Monash University, Clayton, Victoria, Australia

[4]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Melbourne, Victoria, Australia

Author Contributions

R. Kügler, M. Seidensticker, and S. Tasch performed the experiments; A. Kaschtanow and C. U. Tomczyk performed the CLARITY imaging; R. Kügler, A. Mietens, M. Seidensticker, and D. Beyer contributed to the statistical analysis; F. M. Wagenlehner provided material support; F. M. Wagenlehner, Y. Tjahjono, G. P. Risbridger, B. Exintaris, and S. Ellem contributed valuable advice to the redaction of the manuscript; R. Kügler, A. Mietens, and R. Middendorff wrote the manuscript; and R. Middendorff designed the study and directed the project.

Published in: **The FASEB Journal**, Published date: 9th March 2018

DOI: https://doi.org/10.1096/fj.201700430R

Subchapters within Chapter 3 follow the regulations of the according Journal for submission.

3.1 Abstract

Prostate carcinoma and benign prostate hyperplasia (BPH) with associated lower urinary tract symptoms (LUTS) are among the most prevalent and clinically relevant diseases in men. BPH is characterized by an enlargement of prostate tissue associated with increased tone of SMCs which surround the single glands composing the prostate. Secretions of the glands leave the prostate through local excretory ducts during the emission phase of ejaculation. Pharmacological treatment of BPH suggests different local drug targets based on reduction of prostate SMC as the main effect and disturbed ejaculation as a common side effect. This highlights the need for detailed investigation of single prostate glands and ducts. We combined structural and functional imaging techniques-notably, clear lipid-exchanged, acrylamide-hybridized rigid imaging/immunostaining/*in situ* hybridization–compatible tissue-hydrogel (CLARITY) and time-lapse imaging and defined glands and ducts as distinct SMC compartments in human and rat prostate tissue. The single glands of the prostate (comprising the secretory part) are characterized by spontaneous contractions mediated by the surrounding SMCs, whereas the ducts (excretory part) are quiescent. In both SMC compartments, phosphodiesterase (PDE)-5 is expressed. PDE5 inhibitors have recently emerged as alternative treatment options for BPH. We directly visualized that the PDE5 inhibitors sildenafil and tadalafil act by reducing spontaneous contractility of the glands, thereby reducing the muscle tone of the organ. In contrast, the ductal (excretory) system and thus the prostate's contribution to ejaculation is unaffected by PDE5 inhibitors. Our differentiated imaging approach reveals new details about prostate function and local drug actions and thus may support clinical management of BPH.

3.2 Introduction

Benign prostatic hyperplasia (BPH) is a highly prevalent, non-malignant disease that affects aging men and results in serious lower urinary tract symptoms (LUTS) (Roehrborn 2008). Morphologically, BPH is mainly characterized by an increase in interstitial stroma, leading to an increase in prostate size. In the human prostate, SMCs are an important component of the interstitial stroma among the glands. Blood vessels and the prostate excretory ducts are additional prostate structures that are characterized by copious amounts of SMCs (Ichihara et al. 1978, Lee et al. 1990, Nemeth and Lee 1996). The ducts function to transport secretions from individual glands of the prostate, which are responsible for one-third of the ejaculate (Plant and Zeleznik 2014), toward the urethra and are of particular importance for the emission phase of ejaculation.

Most of the drugs used for the treatment of LUTS and BPH target SMC function (McNeal and Company 1983). α_1-adrenergic blockers (e.g., tamsulosin and silodosin) are well-established drugs for BPH treatment (McVary et al. 2011), but are associated with significant side effects such as ejaculation disorders (Tatemichi et al. 2012, Bozkurt et al. 2015, Capogrosso et al. 2015). Phosphodiesterase (PDE)-5 inhibitors (e.g., tadalafil) are promising new therapeutic options for the treatment of BPH (Ückert et al. 2001, Fibbi et al. 2010, Gacci et al. 2012, Oelke et al. 2013, Wang et al. 2014, Wang et al. 2015). PDE5 hydrolyzes the second messenger cGMP, which is produced by binding of NO and natriuretic peptides to specific guanylyl cyclases (Rybalkin et al. 2002). PDEs essentially regulate the duration of cGMP action (Juilfs et al. 1999). In their most recent review, Gacci *et al.* (Gacci et al. 2016) describe a series of cGMP/PDE5 actions in LUTS and BPH such as modulation of oxygenation, inflammation, proliferation, and nerve activity. However, the most established mechanism of action of PDE5 inhibitors is SMC relaxation leading to reduced muscle tone (Ückert et al. 2008, Gacci et al. 2016). PDE5 inhibitors have been shown to relax isolated prostate strips in numerous species (Ückert et al. 2001, Ückert et al. 2008, Angulo et al. 2012, Dey et al. 2012).

Information on the spatial arrangement of the SMCs and PDE5-expressing cells in the stromal compartment is scarce, whereas direct visualization and demonstration of the effects of PDE5 inhibition have not been shown. Moreover, knowledge about PDE5 expression in the ducts and their susceptibility to PDE5 inhibitors is completely lacking. These data are of particular importance, especially since a characteristic adverse side effect of other BPH therapeutics, such as α_1-adrenergic blockers, is abnormal and decreased ejaculation (Chapple 2004, Kaplan 2009). In this study, we used novel imaging techniques to examine the effects of PDE5 inhibitors on the different muscular structures of the prostate, including those involved in ejaculation.

3.3 Material and Methods

3.3.1 Tissues

Human tissue samples originated from patients (age range, 60-79 years; median, 71.7±6.5) undergoing transurethral monopolar electroresection of the prostate for BPH or radical prostatectomy for prostate cancer. Use of human prostate tissue was approved by the ethics committee of the Medical Faculty, Justus-Liebig-University Giessen, Germany (ethical vote 49/05, 2005), and all patients gave written informed consent.

Rat prostate tissue was obtained from adult Wistar rats housed in the animal facility of Justus-Liebig-University Giessen. Housing, animal care, and all procedures were conducted according to the guidelines for animal care and approved by the committee for laboratory animals of Justus-Liebig-University Giessen (JLU no. 469_M and 510_M).

Tissues were fixed in 4% paraformaldehyde for histologic analysis or immediately processed for further investigation. Rat tissue was carefully dissected under a binocular microscope by using fine surgical scissors to isolate single prostate glands and ducts from the ventral lobes (Wang et al. 2015).

3.3.2 Time-lapse Imaging

Tissue was immobilized for transillumination microscopy by embedding in collagen gel (Mietens et al. 2014) and maintained in MEM (Thermo Fisher Scientific, Waltham, USA). Frames were captured every 2s. Sildenafil, tadalafil, and NA were used at a final concentration of 5, 2.5 and 10µM, respectively, to ensure diffusion into the tissue. The duration of the treatments was 12-15min. In all series of experiments (total tissue, glands, and ducts), between 5 and 8 different individual samples from 5 -8 patients or animals were investigated.

To visualize contractile activity, time-lapse images were treated as a stack of frame shots taken at regular time intervals (time stack). By placing a virtual section through the time stack, a specific region (indicated as a blue bar in the relevant overview pictures) can be followed over time (Mietens et al. 2014).

Contractions become visible as twitches or little peaks and were marked by arrows, the contractions were counted and analyzed with ImageJ 1.50e (public domain software; U.S. National Institutes of Health, Bethesda, MD, USA). Contrast enhancement was used if necessary to improve discrimination of twitches. NA-induced contraction in prostate ducts was quantified by comparing ductal areas before and after addition of NA (see Fig. 21D). Afterward, the tissue used in the assays was paraffin embedded for azan staining.

3.3.3 Immunostaining

For immunofluorescence staining of paraffin sections we used monoclonal mouse primary antibodies targeting α-SMA (1:1000; Millipore-Sigma, St. Louis, USA), rabbit polyclonal targeting PDE5 (1:1000; generous gift from Laurinda Jaffe, University of Connecticut Health Center, Farmington, CT, USA) and the fluorescence-labeled secondary antibodies Cy3 anti-mouse IgG (1:500; Jackson ImmunoResearch, West Grove, USA) and Alexa Fluor 488 anti-rabbit IgG (1:500; Thermo Fisher Scientific, Waltham, USA).

3.3.4 Clear lipid-exchanged acrylamide-hybridized rigid imaging/ immunostaining/ in-situ hybridization-compatible tissue-hydrogel

Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue-hydrogel (CLARITY) (Chung and Deisseroth 2013) is a spatial advancement of common histochemical techniques and allows generating a 3-dimensional image of whole tissues. It offers the possibility of transforming intact biologic tissue into a translucent hydrogel-tissue hybrid by removing lipid components responsible for light scattering and thus tissue opacity. In this form, the hybrid is permeable to agents such as specific antibodies, and imaging against a translucent background can be greatly enhanced.

Patient tissue samples and rat tissue of ~1mm^3 were treated as described by Yang *et al.* (Yang et al. 2014) with a few modifications (Saboor et al. 2016). Tissue sections were fixed in 4% paraformaldehyde for 24h at 4°C. Prostate tissue was incubated in hydrogel-forming solution [acrylamide 4% in PBS, bis-acrylamide 0.05% in PBS and 0.25% 2,2'-azobis(2-methylpropionamidine) dihydrochloride] for 24h at 4°C followed by polymerization at 37°C for 3h.

Tissue sections were cleared in a solution containing SDS 10% and boric acid 200mM (pH 7.4) in water, for at least 5d. Extensive washing steps with PBS-Triton 0.1% were performed before and after adding primary antibodies. Primary and secondary antibodies were the same as mentioned above (see immunostaining), but at dilutions of 1:100 and 1:200, respectively. Antibodies were diluted in PBS-Triton 0.1% and incubated for at least 5d at room temperature. After further washing steps (24h), tissue was transferred into a refractory index–matching buffer: Histodenz 88% (D2158; Millipore-Sigma) in 0.02 M phosphate buffer with 0.1% Tween-20 and 0.01% sodium azide (pH 7.5) for 24h at room temperature. Documentation was performed on an LSM710 Confocal Laser Scanning Microscope (Zeiss, Jena, Germany), z-stacks were captured every 1.5–5µm and reconstructed in ImageJ by using the command “3D projects,” allowing interpolation and contrast auto enhancement if necessary.

3.3.5 Statistical Analysis

Only vital samples (confirmed by a visible response to NA) were included. Normal distribution of the data was checked by the Kolmogorov-Smirnov test. Contraction frequencies were analyzed by paired one-tailed t-tests for normally distributed data; otherwise, the Mann-Whitney U test was applied. For NA effects in the ducts, assessed as areas before and after treatment, paired two-tailed t-tests were used. All statistical procedures were performed with GraphPad Prism software (version 6.02 for Windows, GraphPad Software, La Jolla, USA, www.graphpad.com).

3.4 Results

3.4.1 All SMC Compartments of the Prostate Show PDE5 Expression

Prostate tissue comprises glands and their ducts (Fig. 18A, B) which transport glandular secretions toward the urethra. Both are encompassed by interstitial tissue including also blood vessels (Fig. 18A). In the human prostate, SMCs (marked by SMA immunostaining) are found, not only in the interstitial tissue around prostate glands (Fig. 18C, F) and in blood vessels (Fig. 18H), but also in the ducts (Fig. 18J, L, M). Larger ducts (Fig. 18J, L) are distinguished by several SMC layers surrounding the epithelial layer (Fig. 18J, L, M). In our efforts to reveal the prostate targets of drugs used for BPH treatment, we localized PDE5, the enzyme affected by PDE5 inhibition. PDE5 immunoreactivity was regularly observed in interstitial SMCs, confirmed by double staining of SMA and PDE5 (Fig. 18C-E). CLARITY visualized the 3-dimensional arrangement of PDE5-expressing SMCs, thereby providing a better understanding of the spatial architecture of these cells which fill the interstitial compartments and are orientated in various directions (Fig. 18F, G and suppl. movies 1 and 2). PDE5 is also expressed in vascular SMCs (Fig. 18H, I) and in the SMCs of the larger (Fig. 18J-L) and smaller (Fig. 18M) prostatic ducts.

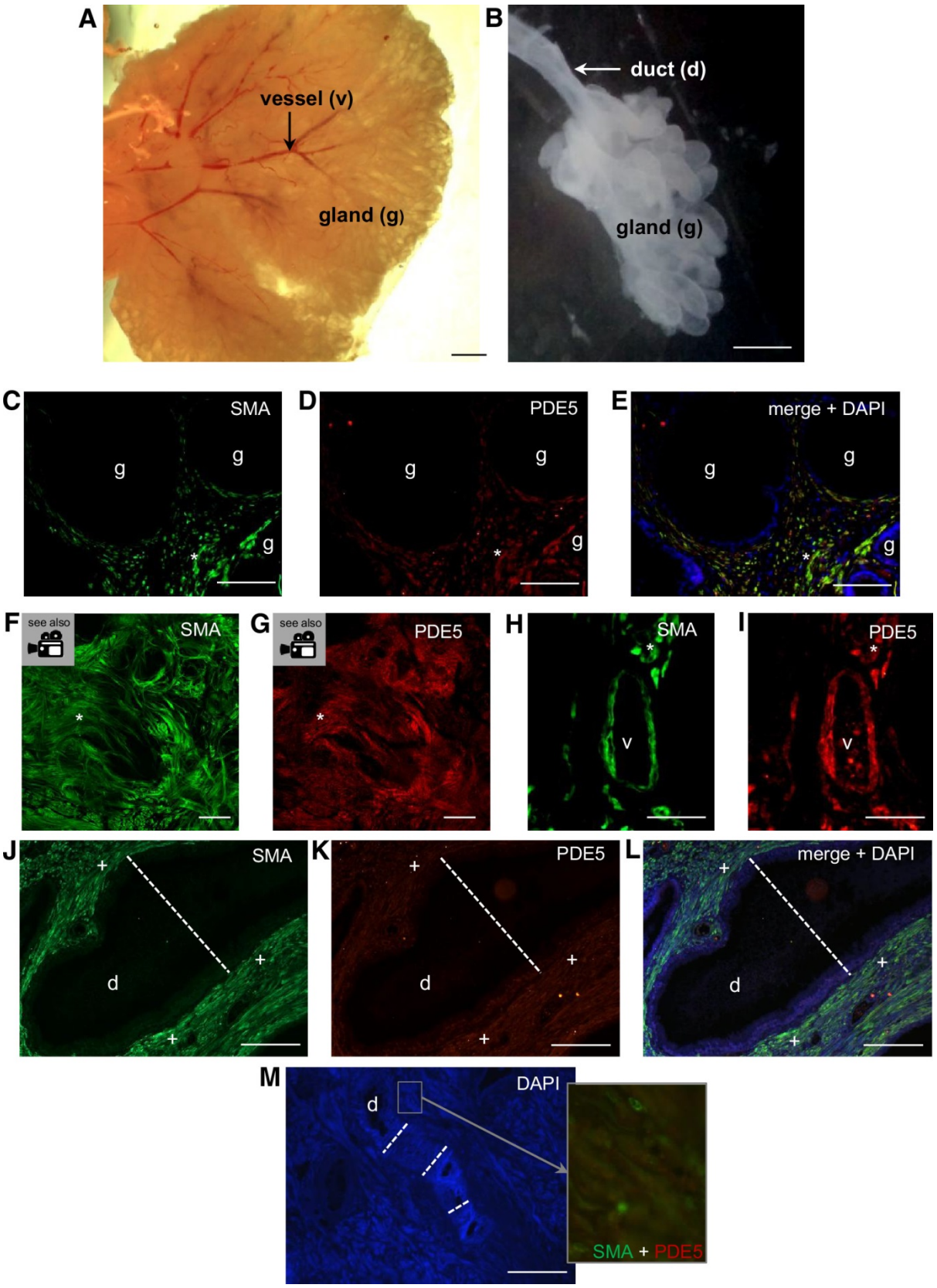

A
vessel (v)
gland (g)
B
duct (d)
gland (g)
C
SMA
D
PDE5
E
merge + DAPI
F
see also
SMA
G
see also
PDE5
H
SMA
I
PDE5
J
SMA
K
PDE5
L
merge + DAPI
M
DAPI
SMA + PDE5

Figure 18: **Localization of PDE5 in SMCs of glands, vessels, and ducts of the human prostate.**

A, B: Preparation of glandular structures of the prostate showed glands (g) and ducts (d) to be the components of the secretory and transport system in addition to vessels (v) as part of the interstitial tissue. SMC localization of PDE5 in human prostate.

C-G: PDE5 in human interstitial SMCs. In the human prostate, interstitial SMCs were identified by SMA expression in the stromal compartment (*) around the glands (g). PDE5 expression was co-localized to these SMA-positive cells, as shown in the merged image.

E: DAPI staining helped to identify the location of the epithelium.

F, G: Movie frame shots from double staining of SMA and PDE5 in the human prostate using a 3-dimensional CLARITY approach showed congruent localization of both antigens. Please refer to the corresponding suppl. movies 1 and 2 to appreciate the spatial architecture.

H, I: PDE5 in human vascular SMCs. Immunostaining with SMA and PDE5 confirmed an equal distribution in vascular (v) contractile cells in human prostate, stromal interstitial cells are also visible (*).

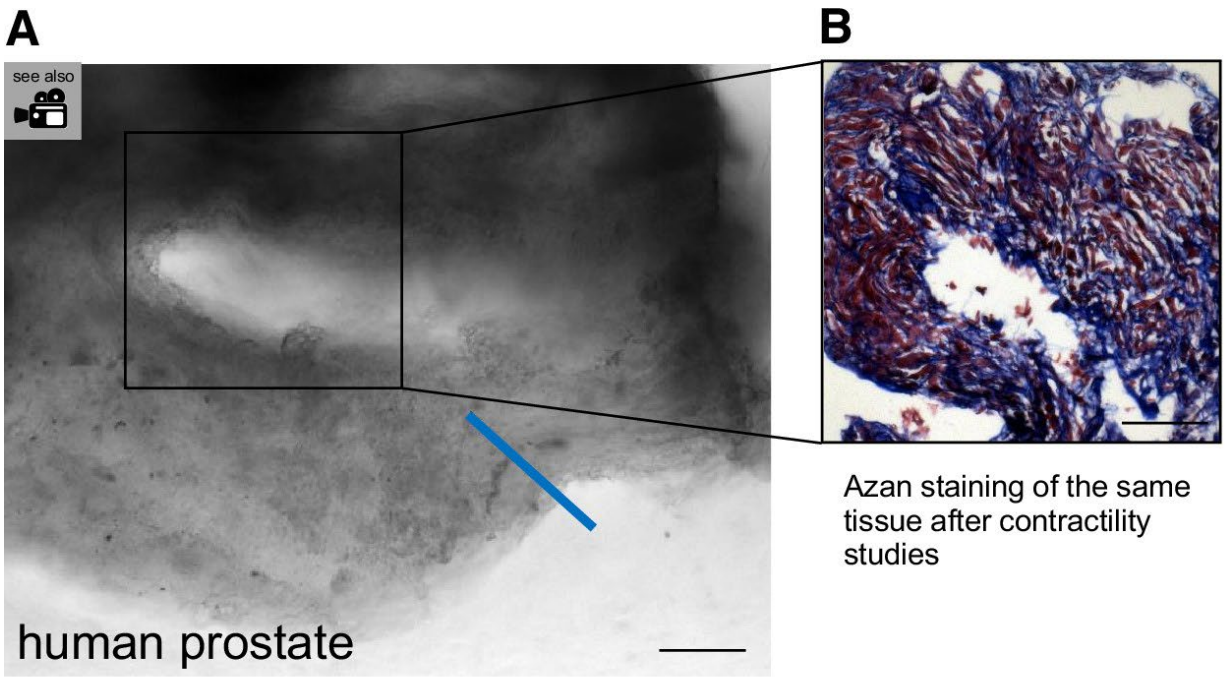
J-L: PDE5 in SMCs of a big human prostate duct (d). It shows several layers of SMCs (+) that express SMA and PDE5, as confirmed in the merged image. Dotted lines: the lumen and epithelium of the duct directly surrounded by SMCs (+).

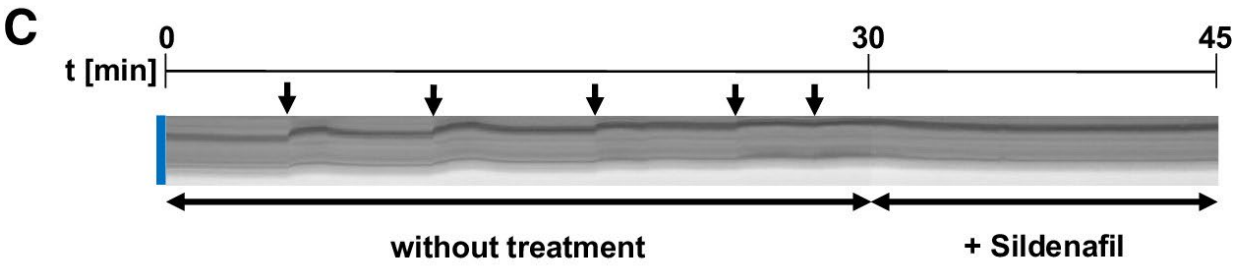
M: Smaller human prostate duct. DAPI staining shows a longitudinal section of a prostate duct (d). Dotted lines: the diameter (lumen and epithelium). Inset: co-localization of SMA and PDE5 in the SMCs surrounding the epithelium.

Scale bars: (**A**) 1mm; (**B**) 100µm; (**C-E**) 75µm; (**F, G**) 200µm; (**H-I**) 25µm; (**J-M**) 50µm.

3.4.2 Relaxing Effects of PDE5 Inhibition can be Directly Visualized in Human Prostate

Time-lapse imaging of human prostate tissue was performed to directly visualize contractions of interstitial SMCs in their regular environment and to monitor effects of PDE5 inhibition. Slow spontaneous contractions of the tissue were readily observed. The PDE5 inhibitor sildenafil markedly reduced spontaneous contractile frequency (Fig. 19A and suppl. movie 3). Further treatment with NA confirmed functional integrity and viability of the tissue sample. Figure 19C gives a visual impression of contractions and sildenafil effects; in this example, sildenafil even abolished spontaneous contractions. Statistical analysis showed that PDE5 inhibition by sildenafil significantly reduced spontaneous contractile frequency (Fig. 19D). After time-lapse imaging, histologic evaluation of the tissue showed SMCs oriented in the various directions in the interstitial compartment around glandular tissue (Fig. 19B).


A
see also
human prostate
B
Azan staining of the same tissue after contractility studies


C
t [min]
0
30
45
without treatment
+ Sildenafil

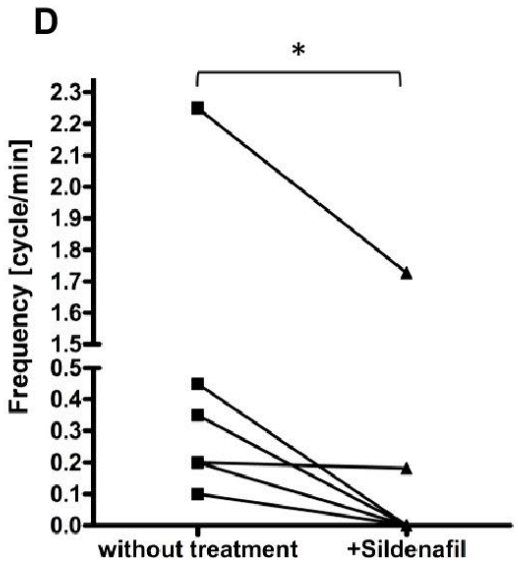

D
*
Frequency [cycle/min]
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
0.5
0.4
0.3
0.2
0.1
0.0
without treatment
+Sildenafil

Figure 19: Visualization of spontaneous contractility of human prostate tissue sensitive to PDE5 inhibition.

A: Frame shot from suppl. movie 3 shows a piece of human prostate tissue by transillumination imaging and indicates the location of the virtual slice (blue line) which is followed through the time stack in **C**. Scale bar, 75µm.

B: Azan staining of one 6µm section from the tissue used in (**A**). Interstitial SMCs stained red. Scale bar, 75µm.

C: Contractions elicited small movements of the human prostate tissue and became visible as little twitches (marked by vertical arrows for better visibility). Slow, spontaneous, irregular contractions are visible, and their frequency is reduced (or even abolished in this example) after the addition of the PDE5 inhibitor sildenafil.

D: Statistical analysis of 6 human samples showed a significant reduction of spontaneous contractile frequency by sildenafil. *$p \leq 0.05$.

3.4.3 Isolated Prostate Glands Show Spontaneous Contractions that are Inhibited by PDE5 Inhibition

Our CLARITY approach 3-dimensionally demonstrated that the interstitial periglandular SMCs in rat prostate (Fig. 20A and suppl. movie 4) were arranged tighter around the glands, than in the human prostate, where SMCs were widely dispersed in the interstitium (see Fig. 18F and suppl. movie 1). This structural difference enabled visualization and analysis of the contractile pattern of single, isolated glands with their musculature. Movies revealed spontaneous contractility occurring in a slightly irregular pattern (Fig. 20B and suppl. movie 5). Following wall movements through the time stack at the indicated position (Fig. 20B), the contractile pattern and the drug effects were analyzed (Fig. 20C). Addition of sildenafil resulted in a clearly visible (Fig. 20B, C and suppl. movie 5) and significant (Fig. 20D) reduction of contractile frequency. The response to NA confirmed viability of the tissue (not displayed). Analogous experiments were performed using the PDE5 inhibitor tadalafil. Our findings showed a reduction of spontaneous contractile frequency in rat prostate glands by tadalafil (Fig. 20E), as seen before by sildenafil.

In line with our functional studies, PDE5 was found in SMCs surrounding rat prostate glands, as indicated by SMA and PDE5 double staining (Fig. 20F, G). Corresponding to the human tissue (Fig. 18) PDE5 was also found in SMCs of blood vessels (Fig. 20F-K and suppl. movie 6) and prostate ducts (Fig. 20I-K). Interestingly, the composition of prostate ducts in rat (Fig. 20I) and human (Fig. 18J) and their PDE5 expression in surrounding SMCs (Fig. 20I-K vs. Fig.18J-M) is comparable.

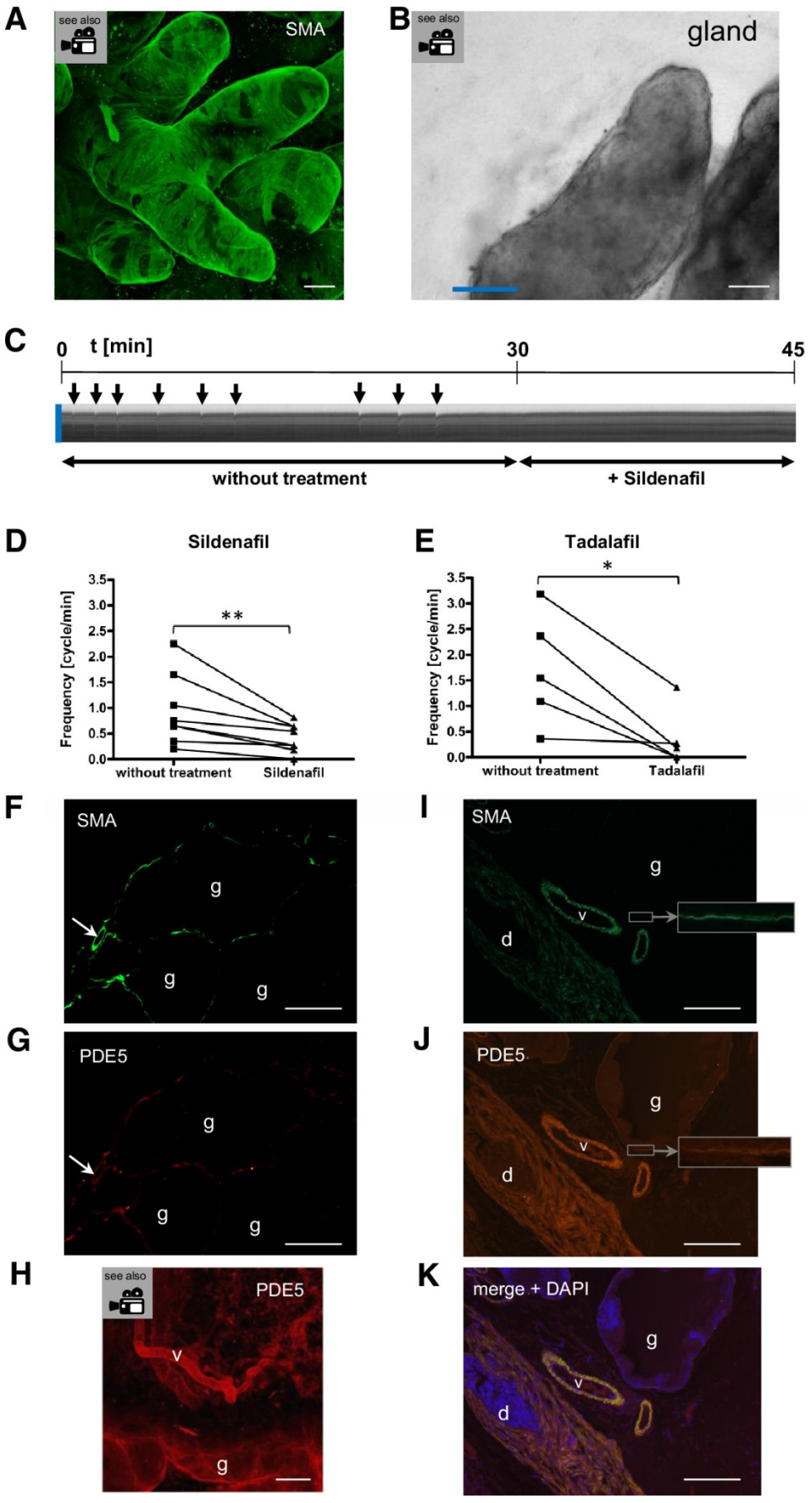

A
see also
SMA
B
see also
gland
C
0
t [min]
30
45
without treatment
+ Sildenafil
D
Sildenafil
Frequency [cycle/min]
**
without treatment
Sildenafil
E
Tadalafil
Frequency [cycle/min]
*
without treatment
Tadalafil
F
SMA
g
g
g
I
SMA
g
v
d
G
PDE5
g
g
g
J
PDE5
g
v
d
H
see also
PDE5
v
g
K
merge + DAPI
g
v
d

Figure 20: Visualization of contractile pattern in rat prostate gland and PDE5 localization in glandular, vascular, and ductal SMCs.

A: Frame shot from suppl. movie 4 shows SMCs (SMA+) surrounding rat prostate glands. Visualization by the 3-dimensional CLARITY approach allows appreciation of a tight association of SMCs around the prostate glands. (See suppl. movie 4 for 3-dimensional view).

B: Frame shot from suppl. movie 5 shows a transilluminated view of an isolated prostate gland used for time-lapse imaging. The line at the left lower margin indicates the position of the virtual slice that is followed over time in **C**. Scale bars: (**A, B**: 100µm).

C: Time-lapse imaging at the position indicated *in Bas* a blue line is followed through the time stack. Contractions are visible as twitches (arrows). Spontaneous contractions occur in a slightly irregular pattern. Sildenafil reduced (or, in this example, abolished) contractile frequency.

D: Sildenafil significantly reduced contractile frequency in rat prostate glands (n=8) **p<0.01.

E: Comparable results were visible with tadalafil (n=5). *p<0.05.

F, G: SMA and PDE5 immunostaining showed corresponding localizations in periglandular (g) SMCs. A positive vessel was also visible (arrow).

H: Frame shot of PDE5 expression detected by the CLARITY approach. PDE5 expression is localized to glands (g) and along vessels (v) of the rat. Also refer to suppl. movie 6 for 3-dimensional appreciation. Scale bar, 75µm.

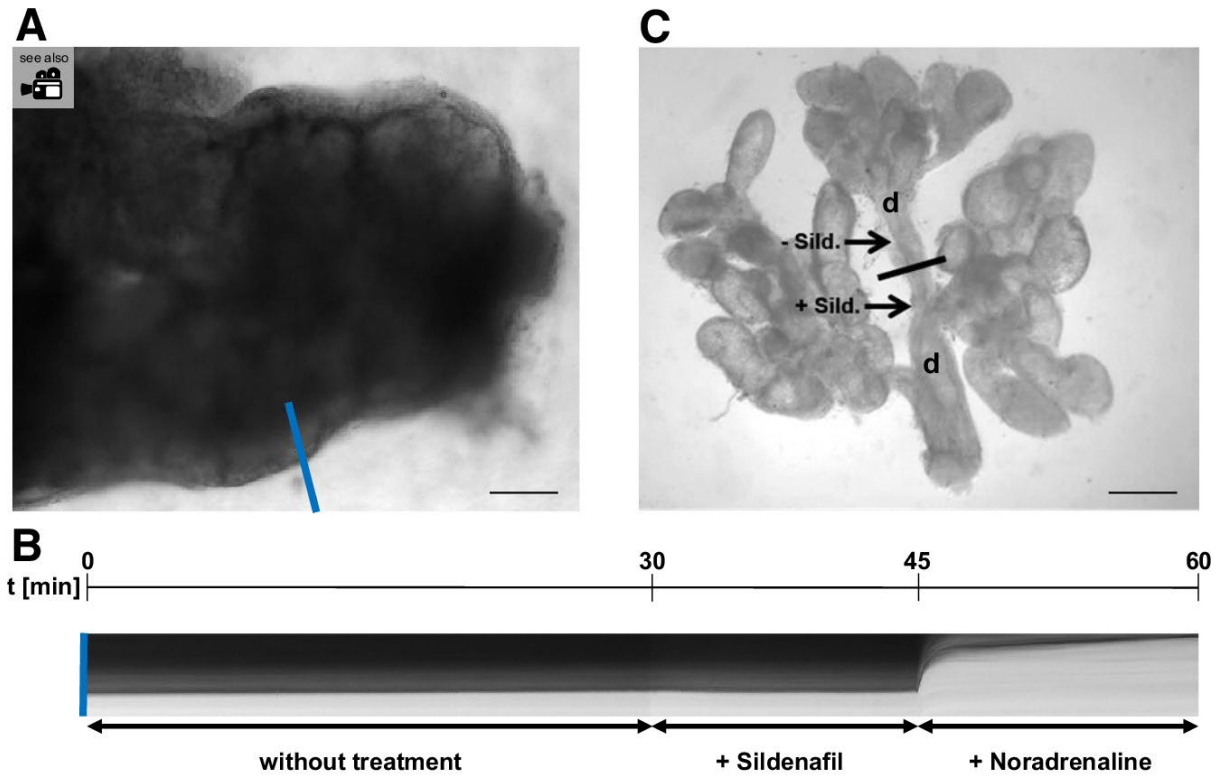
I–K: Double staining of SMA and PDE5 in gland (g), vessel (v), and duct (d) show co-localization in all contractile cells (merged image). Insets: enlargements of the periglandular muscle layer. Nuclear counterstain: DAPI. Scale bars, 50µm.

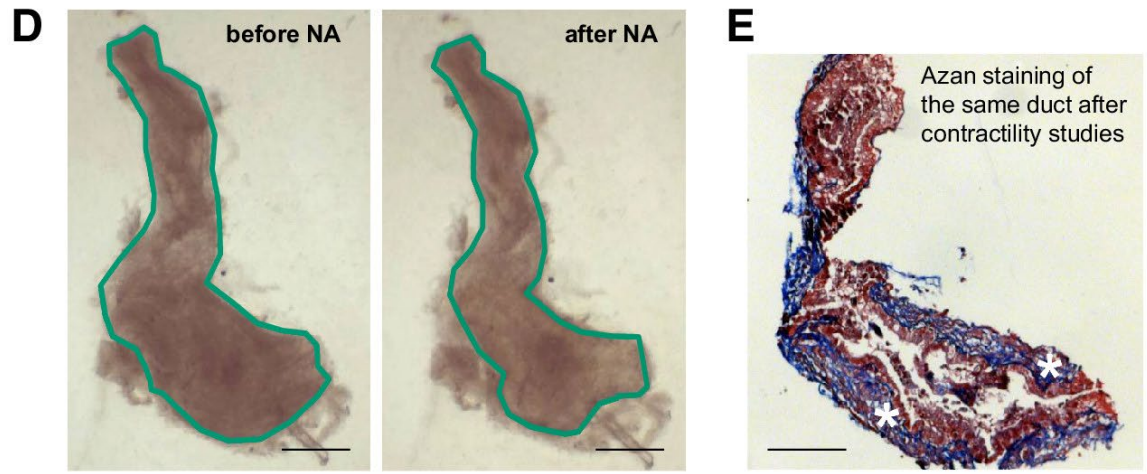
3.4.4 Prostate Ducts do not Contract Spontaneously. Contractions Induced by NA, Mediating Emission and Ejaculation, are not Disturbed by PDE5 Inhibition

Whereas the role of PDE5 inhibitors in vasculature has already been discussed (Gacci et al. 2016), nothing is known about the functional relevance of PDE5 inhibitors in prostate ducts with a large amount of PDE5-expressing SMCs. Therefore, prostate ducts were isolated and investigated by our life-imaging approach *ex vivo* (Fig. 21A). As can be seen in suppl. movie 7 and the corresponding time stack analyses (Fig. 21B) the duct did not show (spontaneous) contractions without treatment, which contrasts with the regular observation of spontaneous contractility in glands (see Fig. 19 and 20). No obvious sildenafil effect was visible in the duct samples, in particular, no dilatation was seen. NA, however, when used to test the viability of the tissue and to mimic sympathetic stimulation of the prostate during ejaculation, resulted in a rapid and strong contraction of the whole duct (Fig. 21B and suppl. movie 7), also illustrated by a distinct area change (Fig. 21D). Spillage of secretions from the duct during the NA-induced contraction was also noted (suppl. movie 7). In contrast, the glands contracted only slightly, and no relevant area change was visible.

The PDE5 inhibitors used to treat BPH interfered with the prostatic component of ejaculation. We therefore investigated systematically whether (pre-) treatment of the prostate duct with sildenafil changes the contractile response to NA, the key player for the initiation of ejaculation. For this, we cut prostate ducts in two parts (Fig. 21C). Each duct served as its own control (Fig. 21C) and was exposed to sildenafil (+Sild.) or vehicle (−Sild.). To assess and quantify NA-induced contractions, we used frame shots from movies before and after the addition of NA and compared the area occupied by the tissue as a surrogate parameter for the contraction (Fig. 21D). To confirm that the samples used in the contraction assay were correctly classified as prostate ducts, we used azan-stained histologic sections (Fig. 21E) and found the typical architecture of prostate ducts consisting of several SMC layers around the epithelium. NA-induced contractions of the isolated duct segments occurred irrespective of sildenafil pretreatment and in both cases, the area after NA treatment was ~80%, compared with the area before treatment (Fig. 21F).

Sildenafil did not significantly delay the time from addition of the drug to onset of contractions; rather, a prompt reaction was seen (Fig. 21G).


A
see also
C
d
- Sild.
+ Sild.
d
B
t [min]
0
30
45
60
without treatment
+ Sildenafil
+ Noradrenaline


D
before NA
after NA
E
Azan staining of the same duct after contractility studies

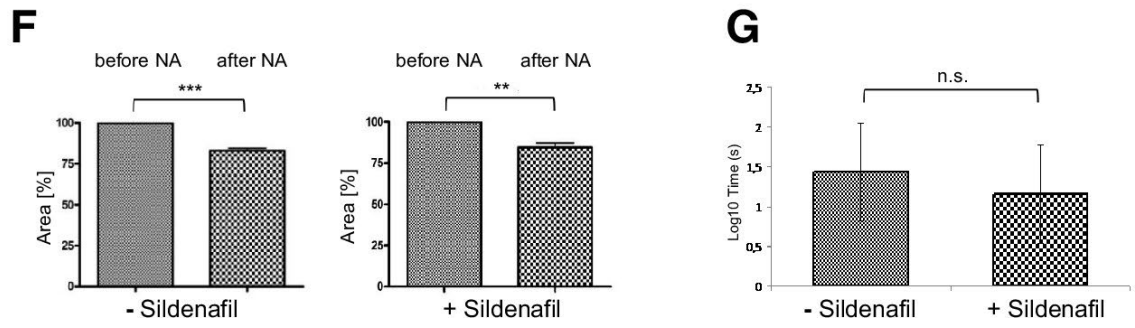

F
before NA
after NA
***
Area [%]
- Sildenafil
before NA
after NA
**
Area [%]
+ Sildenafil
G
n.s.
Log10 Time (s)
- Sildenafil
+ Sildenafil

Figure 21: Prostate duct showed absence of spontaneous contractions and no sildenafil effects on NA-induced contractions.

A: Frame shot from suppl. movie 7 shows the transillumination image of an isolated duct and the position of the virtual section being followed over time (**B**). Scale bar, 75µm.

B: Absence of twitches illustrates missing spontaneous activity and no sildenafil effect. When NA is added, the contraction is large and therefore appears as a sharp peak. Scale bar, 200µm.

C: Illustration of the procedure used to assess potential effects of sildenafil pre-treatment on NA-induced duct contractions. Ducts (d) were cut in two parts and exposed to either sildenafil (+Sild.) or vehicle (-Sild.).

D: Transillumination images of a prostate duct before and after the addition of NA illustrate area reduction as a means to assess and quantify the contraction. Scale bar, 100µm.

E: Corresponding azan staining of the material used in **D** confirms that visualization was performed in a duct portion of prostate tissue. Asterisks: characteristic SMC layers of the duct. Scale bar, 150µm.

F: Contraction after the addition of NA occurred irrespective of sildenafil pretreatment. Area before addition of NA was set as 100%. **p<0.01; ***p<0.005.

G: No significant difference (n.s.) was found in the time delay from addition of NA to the beginning of the contraction.

3.5 Discussion

In the present study, we combined structural and functional imaging of intact prostate tissue to characterize SMC compartments and their function within the prostate. Our approach using isolated prostate glands and ducts largely maintains tissue architecture and allows assessment of pharmacologic effects. It is therefore suitable for systematic testing of drugs assumed to affect prostate function or the prostatic component of the emission phase of ejaculation. Beside the vessels, we discriminated distinct SMC compartments in glands and ducts with different spatial architecture, function, and pharmacologic response to PDE5 inhibition. In prostate ducts which were characterized by high amounts of PDE5-expressing SMCs we directly uncovered the absence of spontaneous contractions and ensuing lack of sildenafil effects. Interestingly, data also showed that NA-induced contractions of prostatic ducts, as found during ejaculation, remain unaltered in the presence of sildenafil treatment.

Prostate ducts lacked spontaneous contractions, whereas isolated prostate glands exhibited spontaneous contractile activity. These contractions may provide a basal muscular tone and could be important for agitation of secretions in the glandular lumen. This finding supports the idea of pacemaker cells which generate contractile activity in human and guinea pig prostate cells independent of neuronal activation (Nguyen et al. 2011). Because NA effects on glandular acini were relatively weak, contractions of the secretory system (glands) may be of limited importance in the context of ejaculation physiology, when compared to the transport system (excretory ducts). Periglandular SMCs were shown to express PDE5, and the addition of sildenafil or tadalafil resulted in diminished contractile frequency. It would be interesting to know whether long-term treatment with PDE5 inhibitors results in an increasing number of prostatic contractions, caused by reduced agitation of secretions. Currently, long-term experience with PDE5 inhibitors in men with LUTS is limited to one trial with tadalafil with only a 1-year follow-up (Donatucci et al. 2011). Because of the different architecture of interstitial SMCs in the prostate, functionally intact glands can be isolated only in rodents. Intact human tissue pieces and isolated rat glands showed spontaneous contractility in a slightly irregular pattern at a similar range of frequencies, and reactions to drugs like sildenafil and NA were comparable.

Our data suggest that in human stromal tissue (with its high amounts of SMCs around glandular structures), the physiologic role of spontaneous contractions may also be associated with glandular function.

Prostate ducts may be seen as a conduit serving to expel secretions during ejaculation which is highlighted by our finding of powerful contractions observed upon NA exposure, whereas in unstimulated conditions, no spontaneous contractile activity was detected. To our knowledge, this study is the first to investigate the effects of a PDE5 inhibitor on prostate ducts. PDE5 inhibitors did not affect NA-induced contractions that mediate ejaculation. Thus, PDE5 inhibitor-induced disturbances of prostate secretion during ejaculation are unlikely. This finding is of clinical relevance, given that drugs alternatively used for the treatment of BPH such as α_1-adrenergic blockers are known to negatively affect ejaculation (Chapple 2004, Kaplan 2009). Whereas α_1-adrenergic receptor inhibitors and ejaculation-mediating NA bind to the same membrane receptor (Andersson and Gratzke 2007), PDE5 inhibitors do not; they inhibit hydrolysis of intracellular cGMP by PDE5 (Corbin and Francis 1999). The insensitivity to PDE5 inhibitors in the ducts cannot be explained by missing PDE5 in these structures, as PDE5 was readily detected in SMCs of prostate ducts from both human and rat. However, the precise role of PDE5 in prostate ducts is not clear. It is plausible that (basal) PDE5 activity limits cGMP levels and thus reduces SMC relaxation. Thereby a basic tension of the duct musculature may be maintained to ensure the rapid catecholamine-induced contractions that are necessary for ejaculation.

In comparison to sole analyses of ejaculation and ejaculate, our data give much more information about the physiology of the prostatic component of ejaculation. Different from organ bath studies with strips of the whole prostate (Ückert et al. 2001, Ückert et al. 2008, Angulo et al. 2012, Dey et al. 2012), our methodology allows discrimination between effects on SMCs of the ducts and the interstitial periglandular compartment.

The CLARITY approach (Chung and Deisseroth 2013, Saboor et al. 2016) added the third dimension to the usual histologic techniques and valuable information to our understanding of the spatial arrangement and function of SMCs around the prostate glands. In human prostate, SMCs not only surrounded the epithelial layer of the glands, but were detectable in all parts of the interstitial compartment with different orientation.

In the rat, the 3-dimensional projection of the SMCs showed a bandage-like arrangement around the glands, with regions devoid of SMCs consistent with the observation of “patchy” contractile activity in our functional studies.

Besides PDE5 (Gacci et al. 2016), other components of cGMP pathways now represent potential targets for therapeutic intervention. These include other PDEs (Hennenberg et al. 2016), as well as cGMP-generating guanylyl cyclases (Müller et al. 2011, Dey et al. 2012, Calmasini et al. 2016). In all cases, the beneficial cGMP effect for patients with BPH may be the reduction of muscular tone in the pathologically enlarged interstitial compartment, along with the maintenance of the NA-induced contractile function in the ducts.

In summary, our study advances our understanding of different SMC compartments in the prostate with respect to their distinct spatial arrangement, physiologic function, and pharmacologic responses.

3.6 Acknowledgements

The authors thank Dr. Laurinda Jaffe (University of Connecticut Health Center, Farmington, CT), for providing us with anti-PDE5 antibodies; Dr. Dieter Müller (Justus-Liebig-University Giessen, Germany), for helpful discussions; and Ingrid Schneider-Hüther, Tania Bloch, and Kerstin Wilhelm (Justus-Liebig-University), for excellent technical assistance. Funding was provided by the Deutsche Forschungsgemeinschaft Grant GRK 1871 and funding from Monash University, Australia, to the International Research Training Group (IRTG), a collaboration between Justus-Liebig-University Giessen and Monash University. The authors declare no conflicts of interest.

For a copy of the original publication, please refer to the appendix as well as for the hardcopy of suppl. movies 1-7.

This article includes supplemental data. Please visit http://www.fasebj.org.ezproxy.lib.monash.edu.au to obtain this information.

Chapter 4 - Time-Lapse Imaging Reveals that the alpha$_1$-adrenoceptor Blocker Tamsulosin but not the PDE5 Inhibitor Tadalafil Directly Disturbs the Secretory Function of the Seminal Vesicles, Prostate and Sperm Release from the Epididymis

(submitted)

Authors

Mathias Seidensticker[1], Sabine Tasch[1], Betty Exintaris[2], Ralf Middendorff[1]

Affiliations

[1]Justus-Liebig-University Giessen, Institute of Anatomy and Cell Biology, Giessen, Germany

[2]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Melbourne, Victoria, Australia

Author Contributions

M. Seidensticker and S. Tasch performed the experiments; M. Seidensticker analyzed data; B. Exintaris contributed valuable advice to the redaction of the manuscript; M. Seidensticker, R. Middendorff, B. Exintaris wrote the manuscript; R. Middendorff designed the study and directed the project.

Submitted to: **European Urology**, submission date: 11th February 2019

Subchapters within Chapter 4 follow the regulations of the according Journal for submission.

4.1 Abstract

Background: Structures responsible for the emission phase of ejaculation are the excretory prostate ducts, the most distal part of the cauda epididymis and the excretory part of seminal vesicles. Underlying mechanisms of ejaculative dysfunction due to established and emerging pharmacological strategies in BPH treatment, such as tamsulosin or tadalafil, on these areas of the male reproductive tract are unknown. A differential analysis potentially will help clinicians directly to guide and optimize their treatment choices for BPH patients.

Objective: To visualize and compare effects of tamsulosin and tadalafil on contractility in the relevant muscular structures during the emission phase of ejaculation.

Design, Setting and Participants: Differential investigation of the most important tissues responsible for ejaculation can only be performed in rodents but not in man, due to embedding of the prostate ducts in the surrounding tissue. However, SMC architecture and function of human prostate ducts can be translated to the rodent, where isolated investigation is possible, therefore providing a suitable model to predict pharmacological responses.

Outcome, Measurements and Statistical Analysis: Using a novel time-lapse imaging approach, physiological and pharmacological properties of prostate ducts, cauda epididymis and seminal vesicles were systematically investigated for the first time.

Results and Limitations: NA-induced contractions, imitating emission phase of ejaculation by the sympathetic nervous system, were abolished with tamsulosin pre-treatment in prostate ducts and seminal vesicles and significantly decreased in the epididymal duct. These adverse effects were not observed with tadalafil.

Conclusions: Our model system reveals the mechanism of tamsulosin resulting in adverse effects during ejaculation in patients treated for BPH. These adverse effects on contractility do not apply to tadalafil treatment. This new knowledge translates directly to clinical medicine.

Patient summary: PDE5 inhibitors like tadalafil are a good alternative therapy in BPH for patients experiencing abnormal ejaculation due to treatment with tamsulosin.

4.2 Introduction

Benign prostatic hyperplasia (BPH) is one of the most common non-malignant diseases in aging men, affecting approximately 50% of men in their 50s and up to 90% of men by the age of 80 (Berry et al. 1984). BPH arises predominantly in the periurethral and transition zone of the prostate. It is characterized by an enlargement especially of the stroma, containing SMCs, together with an increase of SMC tone (McNeal and Company 1983, Von Heland and Casale 1994). BPH associated lower urinary tract symptoms (LUTS) include storage and voiding symptoms and bladder outlet obstruction due to anatomical obstruction and increased muscle tone (Lepor 2004, Speakman et al. 2015). Treatment options for BPH include e.g. 5ARIs or α_1-adrenoceptor antagonists (Lepor 2007, Naslund et al. 2008). In general, α_1-adrenoceptors divide into three subtypes: α_{1A}, α_{1B}, α_{1D} (Michel et al. 1995) of which the α_{1A}-subtype is predominant in smooth muscle tissues of the male reproductive tract like the prostate (Michel and Vrydag 2006), seminal vesicles (Shima 1993), epididymis (Queiroz et al. 2008) and vas deferens (Honner and Docherty 1999). Tamsulosin and silodosin are α_{1A}-subtype-specific antagonists (Schwinn and Roehrborn 2008) and have emerged as effective drugs to treat BPH/LUTS by decreasing SMC tone (Michel and Vrydag 2006) and therefore improving urinary flow (Lepor 1990, Chapple 1996). Tamsulosin is the most frequently prescribed (Lepor 2007, McVary et al. 2011) because it is well tolerated and does not require dose titration (Lepor 1990, Chapple 1996, Lee 2000, Lepor 2007). While effective, treatment with tamsulosin or silodosin often results in minor side effects like dizziness, headache, flu-like symptoms and more serious side effects such as abnormal ejaculation (Lee 2000). There are inconsistencies in the literature regarding the definition of the term ‘abnormal ejaculation’ which can include ‘retrograde’ ejaculation, reduced ejaculate volume, absence of seminal emission and ‘anejaculation’, each term describing a different process. This shows that the mechanism of action behind the side effects of abnormal ejaculation is not definitively clarified (Lee 2000, Hellstrom and Sikka 2006, Roehrborn et al. 2011). These side effects negatively impact quality of life, results in discontinuation of therapy and poor compliance; therefore, need for other treatment options becomes more important.

Inhibitors of phosphodiesterase type 5 (PDE5) like tadalafil and sildenafil have emerged as alternative treatment options for BPH/LUTS (McVary et al. 2007, McVary et al. 2007, Gacci et al. 2012, Oelke et al. 2013, Gacci et al. 2016). PDE5 is a part of the cGMP signaling pathway in SMCs and is responsible for the hydrolysis of the second messenger cGMP, a molecule that plays a key role in SMC relaxation. Inhibitors of PDE5 inhibit the hydrolysis of cGMP and thus lead to an increase of cGMP levels, which is responsible for SMC relaxation and reduced muscle tone (Juilfs et al. 1999, Rybalkin et al. 2002). Sildenafil and tadalafil are mainly used to treat ED but are now also recommended for the treatment of BPH/LUTS (Oelke et al. 2013).

In a previous study, we were able to separate and distinguish rat prostatic tissue in two different functional units, the single glands, which produce the prostatic fluid, and the excretory ducts, which play an important role in transporting prostatic fluid during the emission phase of ejaculation (Kügler et al. 2018).

In this study, we used a novel time-lapse imaging approach as a model system to investigate and compare the effects of the α_1-adrenoceptor antagonist tamsulosin and PDE5 inhibitor tadalafil on the tissues of the male reproductive tract involved in ejaculation. The emission phase during ejaculation was simulated with NA, which plays a key role in the process of ejaculation. We investigated tissue of the prostate, especially the excretory ducts, which contributes up to 30% of the whole volume of the ejaculate; the seminal vesicles, which contribute up to 60-70% of the whole ejaculate volume and the distal part of the cauda epididymal duct that stores and releases the sperm to the urethra during ejaculation.

4.3 Materials and Methods

4.3.1 Tissues

Prostate, seminal vesicles and epididymis were obtained from adult Wistar rats housed in the animal facility of Justus-Liebig-University Giessen. Housing, animal care and all other procedures were conducted according to the guidelines for animal care and approved by the committee for laboratory animals of Justus-Liebig-University Giessen (JLU no. 469_M and 510_M). Tissues were fixed in 4% paraformaldehyde for histological analyses or immediately processed for further investigation. Rat tissue was carefully dissected under a binocular microscope using fine surgical scissors to isolate single prostate ducts, seminal vesicles and the most distal part of the epididymal duct.

4.3.2 Time-lapse Imaging

Tissue was immobilized for transillumination microscopy by embedding in collagen gel (Mietens et al. 2014) and maintained in MEM (Invitrogen, Karlsruhe, Germany). Frames were captured every 2s for seminal vesicles and prostatic ducts. For epididymal duct recordings, frames were captured every second to ensure reliable analyses of contraction frequencies (see below). Tamsulosin and tadalafil were used at a final concentration of 5µM and 2.5µM and NA at 10µM, respectively. Duration of each drug treatment was 12min for prostatic ducts and seminal vesicles and 15min for epididymis recordings. In all series of experiments 5-7 different individual samples, each from one animal was investigated. For the most reliable comparison we isolated one prostatic duct and cut it into two parts (Kügler et al. 2018). One part was exposed to the drug of interest, being tadalafil (+Tadalafil) or tamsulosin (+Tamsulosin), respectively and the other part of the same duct served as its own control (labeled “-Tadalafil” or “-Tamsulosin”) and was exposed to vehicle (MEM). NA-induced contractions in all tissues were quantified by comparing tissue areas before and after addition of NA for uniform analyses. Movie stills were taken directly after the start and at the very end of the recording for vehicle and drug treated tissue.

Regions of interest were drawn around the whole prostate and epididymal duct observed and the tissue in focus of seminal vesicles to determine the area size. The area size was measured with ImageJ 1.50e.

The size of the tissue at the beginning of the experiment was set to 100% and compared to the size after the application of NA. Additionally, weight changes were measured for seminal vesicles before and after the experiment, to quantify the expulsion of seminal fluid by NA-induced contractions. Quantification of NA-induced contractions of the epididymal duct was performed by analyzing the frequency of contractions. This was performed by placing a virtual section through the tissue recorded, so that a specific region could be followed over time (time stack). Contractions become visible as small peaks or twitches, and were counted and analyzed using ImageJ for the quantification of contractile patterns. Contrast enhancement was used if necessary to improve discrimination of twitches (Mietens et al. 2014). Afterwards, the tissue used in the assays was paraffin-embedded for azan staining (Kügler et al. 2018).

4.3.3 Statistical Analysis

Only vital samples (confirmed by a visible response to NA) were included. Normal distribution of the data was checked by the Kolmogorov-Smirnov test. For NA-induced area changes in the prostate ducts, seminal vesicles and epididymal duct, paired one-tailed t-tests were used for normally distributed data, otherwise the Wilcoxon matched-pairs signed rank test was applied. Weight changes in seminal vesicles, assessed before and after the experiment and contraction frequencies for epididymal ducts were analyzed with paired one-tailed t-tests for normally distributed data, otherwise the Wilcoxon matched-pairs signed rank test was applied. Measured percentages of area or weight changes and contraction frequencies measured as [contr/min] are displayed as mean±SEM. The comparison of analyzed data was significantly different for $p<0.05$. All statistical procedures were performed using GraphPad Prism software.

4.4 Results

4.4.1 NA-Induced Secretion in Seminal Vesicles was Disturbed by Tamsulosin but not Tadalafil

Rat seminal vesicles were isolated and investigated by time-lapse imaging *ex vivo*. Tissue was either pre-treated with tadalafil (Fig. 22A, +Tadalafil) or vehicle (Fig. 22A, -Tadalafil) and tamsulosin (Fig. 22C, +Tamsulosin) or vehicle (Fig. 22C, -Tamsulosin) before the addition of NA. NA was used to simulate sympathetic stimulation during emission phase of ejaculation. The resulting weight change was used to indicate the degree of NA-induced secretion. Weight was recorded before (set to 100%, Fig. 22A, C, dotted line) and after the addition of NA. Analysis showed that weight changes for vehicle (Fig. 22A, white boxplot) and tadalafil (Fig. 22A, grey boxplot) pre-treated tissue were not significantly different after NA-induced contractions. No weight reduction was observed with tamsulosin pre-treatment (Fig. 22C, grey boxplot), while in vehicle (Fig. 22C, white boxplot) the weight was reduced after NA-addition, resulting in significant differences. Movie stills of tissue pre-treated with tadalafil (Fig. 22B suppl. movie 8) showed clear expulsion of secretion upon NA-addition. Tissue pre-treated with tamsulosin did not lead to expulsion of secretions (Fig. 22D suppl. movie 9).

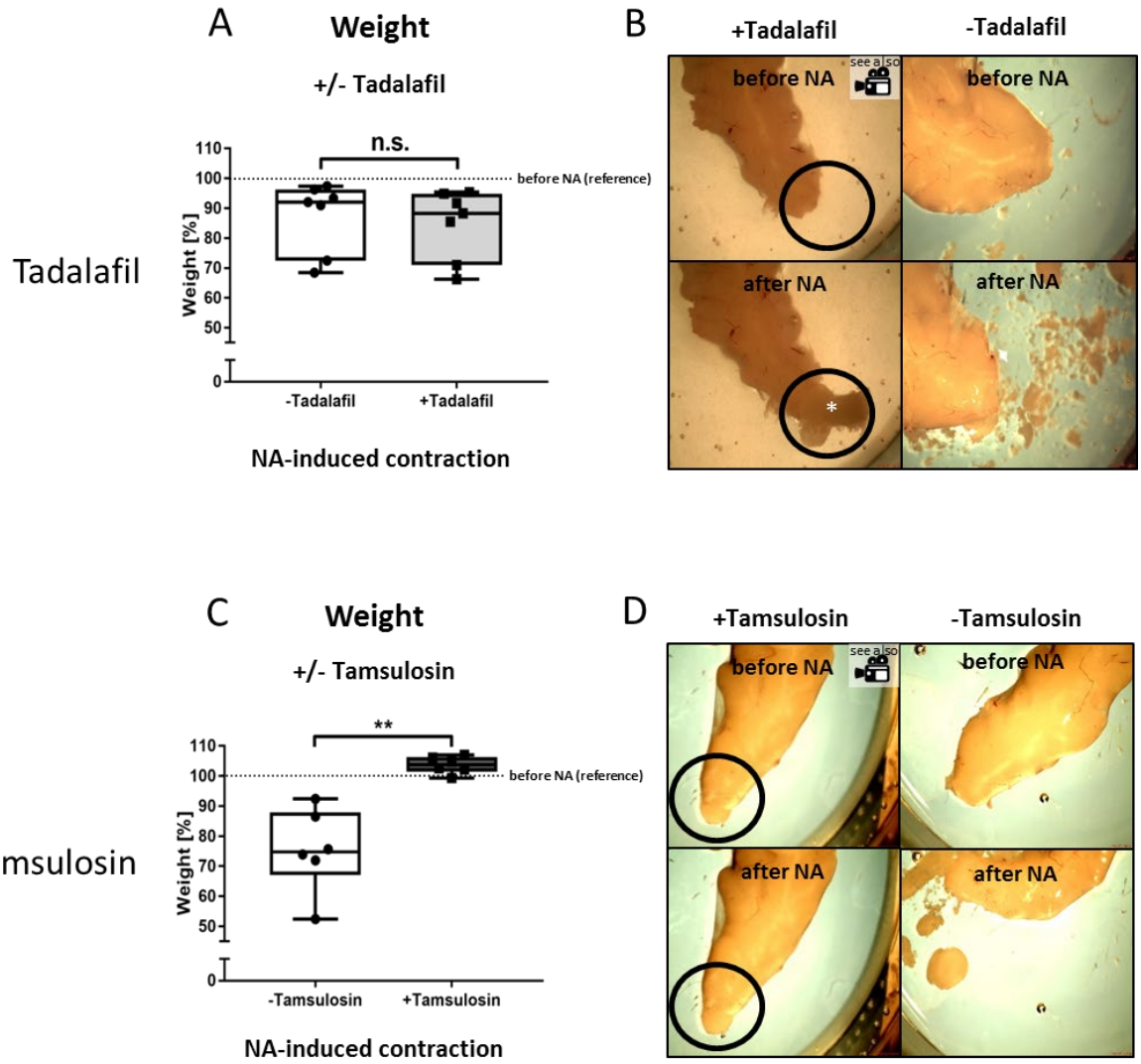

A
Weight
+/- Tadalafil
Tadalafil
n.s.
before NA (reference)
Weight [%]
110
100
90
80
70
60
50
0
-Tadalafil
+Tadalafil
NA-induced contraction
B
+Tadalafil
-Tadalafil
see also
before NA
before NA
after NA
after NA
C
Weight
+/- Tamsulosin
Tamsulosin
**
before NA (reference)
Weight [%]
-Tamsulosin
+Tamsulosin
NA-induced contraction
D
+Tamsulosin
-Tamsulosin
see also
before NA
before NA
after NA
after NA

Figure 22: Tadalafil pre-treatment did not affect NA-induced secretion and therefore weight changes in seminal vesicles, but tamsulosin did.

A: Weight changes of vehicle (-Tadalafil) and tadalafil pre-treated (+Tadalafil) seminal vesicles were compared after NA-induced contractions. No significant differences were measured (p=0.0547, n.s.). The changes in weight were relative to weight measurements before the experiments (set to 100%, dotted line). In tadalafil pre-treated tissue the size was reduced to 84.72±4.39% and for vehicle to 87.32±4.45% upon NA-induced contractions.

B: Movie stills showed that in the presence of tadalafil (+Tadalafil, see also suppl. movie 8, *) expulsion of secretion is not disturbed after NA application as for vehicle treatment (-Tadalafil).

C: Significant differences in weight changes (paired t-test, one-tailed, p=0.0014, **) between vehicle (-Tamsulosin) and tamsulosin pre-treated (+Tamsulosin) seminal vesicles were observed in NA-induced contractions. No weight reduction of tamsulosin treated seminal vesicles was observed after addition of NA, the weight even increased to 103.7±1.17% compared to the reference (100%, dotted line, data not shown). Control experiments (not displayed) showed that incubating the tissue in MEM alone also leads to an increased weight, suggesting the tissue soaked up some Medium. The weight for vehicle treated tissue was decreased to 75.49±5.64%.

D: Movie stills showed an absence of expulsion of fluid in tamsulosin treated seminal vesicles (+Tamsulosin, see also suppl. movie 9) after the addition of NA contrasting vehicle treatment (-Tamsulosin).

4.4.2 In Seminal Vesicles, NA-Induced Contractions were Abolished by Tamsulosin, but Unaffected by Tadalafil

In seminal vesicle, contractions induced by NA were also compared between pre-treatment with tadalafil (Fig. 23A, +Tadalafil) or vehicle (Fig. 23A, -Tadalafil) and tamsulosin (Fig. 23C, +Tamsulosin) or vehicle (Fig. 23C, -Tamsulosin) via analyzing area changes of the observed tissue. Areas before (set to 100%, Fig. 23A, C, dotted line) and after the experiment were measured by placing a virtual line around the tissue in focus during time-lapse recordings. Area changes after NA-addition showed no significant differences for tadalafil pre-treatment (Fig. 23A, grey boxplot) compared to vehicle (Fig. 23A, white boxplot). In tamsulosin experiments, vehicle (Fig. 23C, white boxplot) diminished the area of the initial size after NA-addition, while in tamsulosin (Fig. 23C, grey boxplot) pre-treated tissue contractions were abolished. Time-lapse recordings revealed that tadalafil pre-treatment did not interfere with NA-induced contractions (Fig. 23B suppl. movie 8). In contrast, tamsulosin pre-treatment resulted in an absence of contractions after addition of NA (Fig. 23D suppl. movie 9).

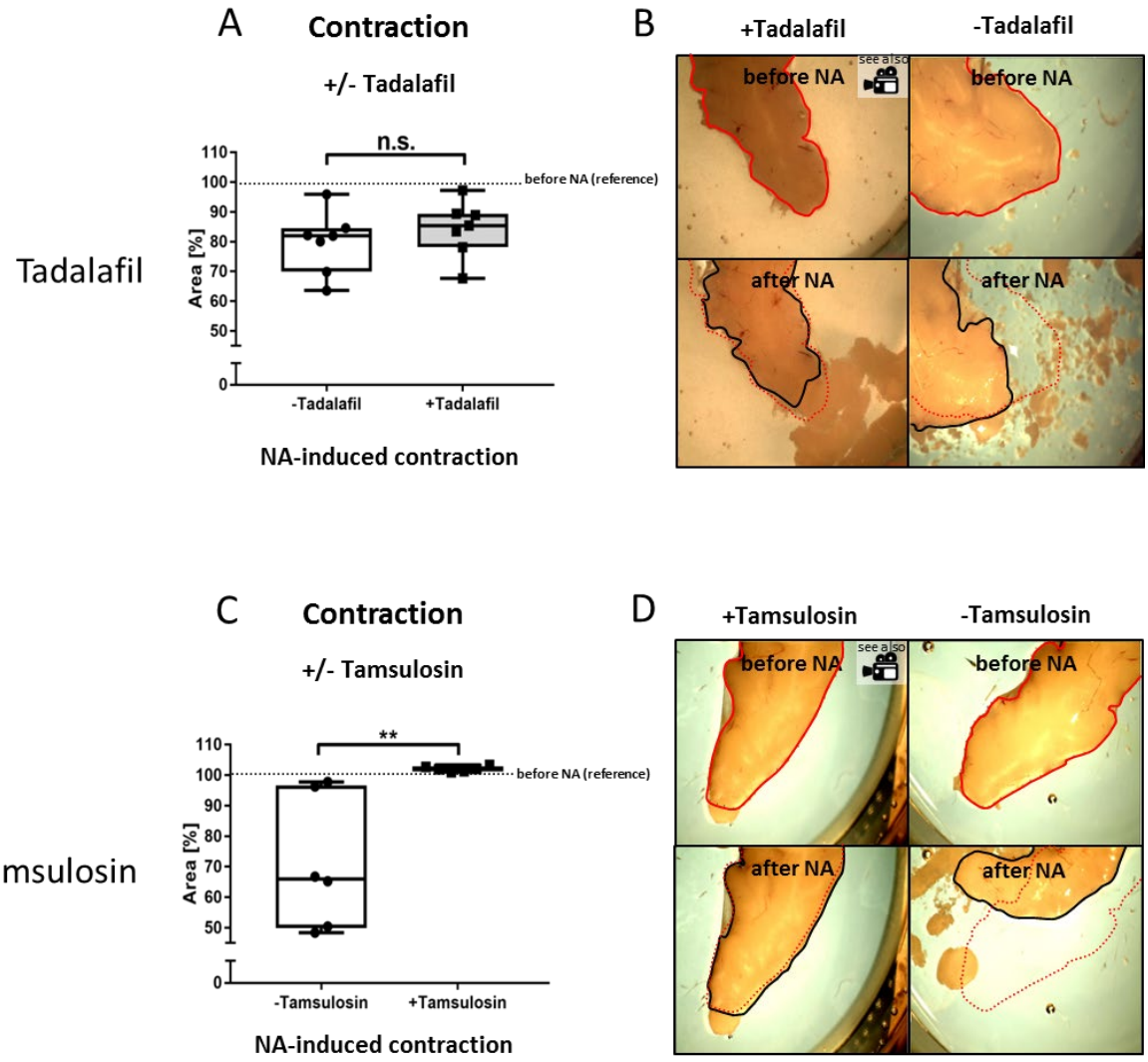

A
Contraction
+/- Tadalafil
Tadalafil
n.s.
before NA (reference)
Area [%]
110
100
90
80
70
60
50
0
-Tadalafil
+Tadalafil
NA-induced contraction
B
+Tadalafil
-Tadalafil
see also
before NA
before NA
after NA
after NA
C
Contraction
+/- Tamsulosin
Tamsulosin
**
before NA (reference)
Area [%]
110
100
90
80
70
60
50
0
-Tamsulosin
+Tamsulosin
NA-induced contraction
D
+Tamsulosin
-Tamsulosin
see also
before NA
before NA
after NA
after NA

Figure 23: Tadalafil did not affect NA-induced contractions in seminal vesicles. Tamsulosin, on the other hand, abolished NA-induced contractions.

A: No significant differences (p=0.2169, n.s.) in change of area size after NA-induced contractions for vehicle (-Tadalafil) and tadalafil (+Tadalafil) treated seminal vesicles was analyzed. Changes of size were measured in percentage compared to size before the experiment (set to 100%, dotted line). Measured areas were decreased to 79.75±3.95% for vehicle and 84.33±3.56% for tadalafil pre-treated tissue, respectively.

B: Movie stills showed clear expulsion of seminal fluid after the addition of NA in vehicle (-Tadalafil) and tadalafil (+Tadalafil, see also suppl. movie 8) treated seminal vesicles.

C: Addition of NA in vehicle treated seminal vesicles (-Tamsulosin,) resulted in a clear reduction of size to 70.82±8.84% compared to reference (set to 100%, dotted line). Tamsulosin (+Tamsulosin) abolished NA-induced contractions and thus resulted in a significant difference compared to vehicle (p=0.0082, **). The size of the tissue increased to 102.1±0.36%.

D: Movie stills of time-lapse recordings showed the absence of contractions in tamsulosin (+Tamsulosin) treated tissue after the addition of NA and clearly visible contractions (see also suppl. movie 9) and a reduction of size in vehicle (-Tamsulosin).

4.4.3 Tamsulosin Abolished NA-Induced Contractions in Prostatic Ducts, Different to Tadalafil

Next, we investigated the drug-induced contractions of prostatic ducts, which are responsible for the expulsion of secretions from the single glands of the prostate (Kügler et al. 2018). We compared the area of prostatic ducts before (set to 100%, Fig. 24A, C, dotted line) and after the experiment for tadalafil (Fig. 24A, +Tadalafil) or vehicle (Fig. 24A, -Tadalafil), and tamsulosin (Fig. 24C, +Tamsulosin) or vehicle (Fig. 24C, -Tamsulosin) pre-treated tissue. No significant differences in size reduction induced by NA were observed for tadalafil pre-treated (Fig. 24A, grey boxplot) prostatic ducts compared to vehicle (Fig. 24A, white boxplot). Tamsulosin pre-treatment nearly completely prevented NA-induced tissue contraction (Fig. 24C, grey boxplot) but vehicle did not (Fig. 24C, white boxplot), resulting in significant differences. Contractions of ducts induced by NA occurred irrespective of tadalafil pre-treatment (Fig. 24B suppl. movie 10). In contrast, contractions were abolished by tamsulosin (Fig. 24D suppl. movie 11).

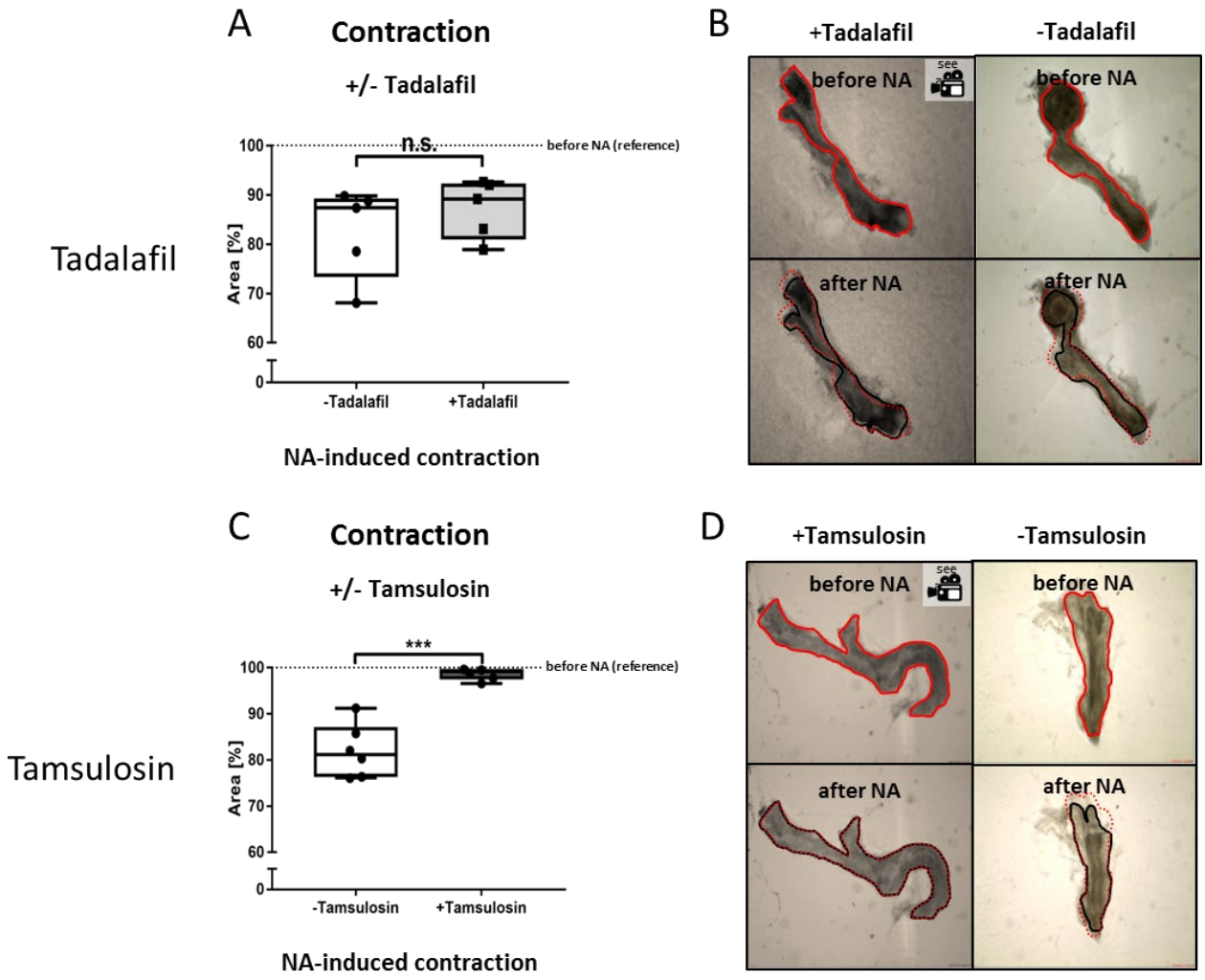

A
Contraction
+/- Tadalafil
Tadalafil
n.s.
before NA (reference)
100
90
80
70
60
0
Area [%]
-Tadalafil
+Tadalafil
NA-induced contraction
B
+Tadalafil
-Tadalafil
before NA
after NA
C
Contraction
+/- Tamsulosin
Tamsulosin
***
-Tamsulosin
+Tamsulosin
NA-induced contraction
D
+Tamsulosin
-Tamsulosin

Figure 24: Tadalafil did not change NA-induced contractions in prostatic ducts, but tamsulosin did.

A: The change of size in tadalafil (+Tadalafil) pre-treated prostatic ducts after NA-addition was not significantly different (p=0.1830, n.s.) compared to vehicle (-Tadalafil). The change in size of the ducts was relative to the size measured before the experiment (set to 100%, dotted line) and was reduced to 82.53±4.12% for vehicle and 87.2±2.68% in tadalafil pre-treated tissue.

B: Movie stills showed area changes after the addition of NA and were compared between vehicle (-Tadalafil) and tadalafil treated (+Tadalafil, see also suppl. movie 10) prostatic ducts.

C: Analysis showed that the changes of size in tamsulosin pre-treated (+Tamsulosin) ducts to 98.72±0.54% was significantly smaller after addition of NA compared to vehicle (-Tamsulosin) which were reduced to 81.96±2.37% (p=0.0010, ***).

D: Nearly no changes in the size of the duct were observed with tamsulosin pre-treatment (+Tamsulosin, see also suppl. movie 11) after the addition of NA which was in contrast to the observations in vehicle (-Tamsulosin) where a distinct area change was observed.

4.4.4 In the Sperm Storing Part of the Epididymal Duct, Tadalafil did not Change Contractile Responses Induced by NA, in Contrast to Tamsulosin

Statistical analysis of area changes (Fig. 25A) in tadalafil pre-treated tissue (+Tadalafil, grey boxplot) showed no significant differences in NA-induced contractions compared to vehicle (-Tadalafil, white boxplot). In both treatments, the size of the observed tissue decreased after the addition of NA compared to the size at the very beginning of the recording (set to 100%, Fig. 25A, C, dotted line). Tamsulosin pre-treated tissue (Fig. 25C, +Tamsulosin, grey boxplot) resulted in a significant difference of area reduction compared to vehicle (Fig. 25C, -Tamsulosin, white boxplot) after NA-induced contraction. Recordings visualize differences in NA-induced contractions between tadalafil (Fig. 25B suppl. movie 12) and tamsulosin (Fig. 25D suppl. movie 13) pre-treatment.

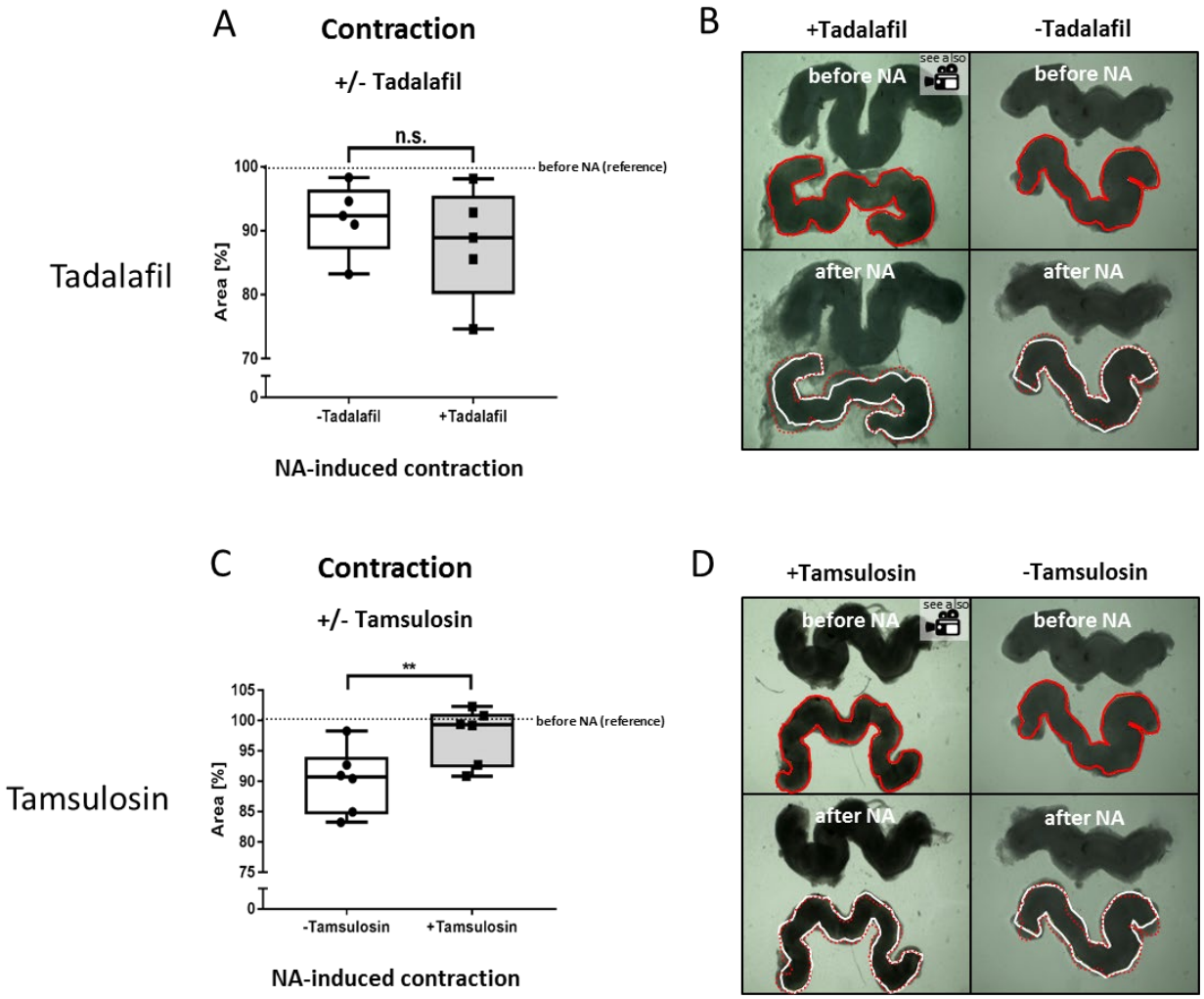

A
Contraction
+/- Tadalafil
Tadalafil
n.s.
before NA (reference)
Area [%]
100
90
80
70
0
-Tadalafil
+Tadalafil
NA-induced contraction
B
+Tadalafil
-Tadalafil
see also
before NA
before NA
after NA
after NA
C
Contraction
+/- Tamsulosin
Tamsulosin
**
before NA (reference)
Area [%]
105
100
95
90
85
80
75
0
-Tamsulosin
+Tamsulosin
NA-induced contraction
D
+Tamsulosin
-Tamsulosin
see also
before NA
before NA
after NA
after NA

Figure 25: Analysis of NA-induced contractions in the distal part of cauda epididymis in tadalafil and tamsulosin pre-treated tissue.

A: Statistical analysis of area changes were measured in percentage compared to size before the experiment (set to 100%, dotted line). Tadalafil (+Tadalafil) pre-treated epididymal tissue, which was reduced to 87.98±3.95% upon NA-induced contractions, showed no significant differences compared to vehicle (-Tadalafil) which was reduced to 91.86±2.5% in size (p=0.2534, n.s.).

B: Movie stills of recordings of either tadalafil (+Tadalafil, see suppl. Movie 12) pre-treated tissue or vehicle (-Tadalafil) before and after addition of NA for comparison of area changes showed no differences in contractility of the tissue.

C: Tamsulosin (+Tamsulosin) pre-treated epididymal tissue was only slightly reduced to 97.52±1.89% after addition of NA which was significantly different in the magnitude of area changes compared to vehicle (-Tamsulosin) which was reduced to 90.07±2.23% (p=0.0097, **).

D: Movie stills showed differences in contractions of tamsulosin (+Tamsulosin, see suppl. movie 13) pre-treated and vehicle (-Tamsulosin) tissue before and after NA-induced contractions.

4.4.5 In the Sperm Storing Part of the Epididymal Duct, NA-Induced Contraction Frequencies are not Disturbed by Tadalafil Pre-Treatment, in Contrast to Tamsulosin

NA-induced contraction frequencies [contr/min] were analyzed with setting a virtual section through the observed tissue which could be followed over time (time stack, Fig. 26A red line, E). Contractions occurred as little peaks or twitches (Mietens et al. 2014) (Fig. 26B, F). Only upon addition of NA, rapid contractions occurred and were analyzed (Fig. 26C, G). The number of peaks were counted and compared for pre-treatment with tadalafil (Fig. 26C, D, +Tadalafil) or vehicle (Fig. 26D, -Tadalafil) as well as tamsulosin (Fig. 26G, H, +Tamsulosin) or vehicle (Fig. 26H, -Tamsulosin). The NA-induced frequency of contractions in tadalafil (Fig. 26D, grey boxplot) pre-treated tissue was not significantly different compared to vehicle (Fig. 26D, white boxplot). A significant decrease by approximately 80% in the frequency of NA-induced contractions occurred in tamsulosin (Fig. 26H, grey boxplot) pre-treated tissue compared to vehicle (Fig. 26H, white boxplot).

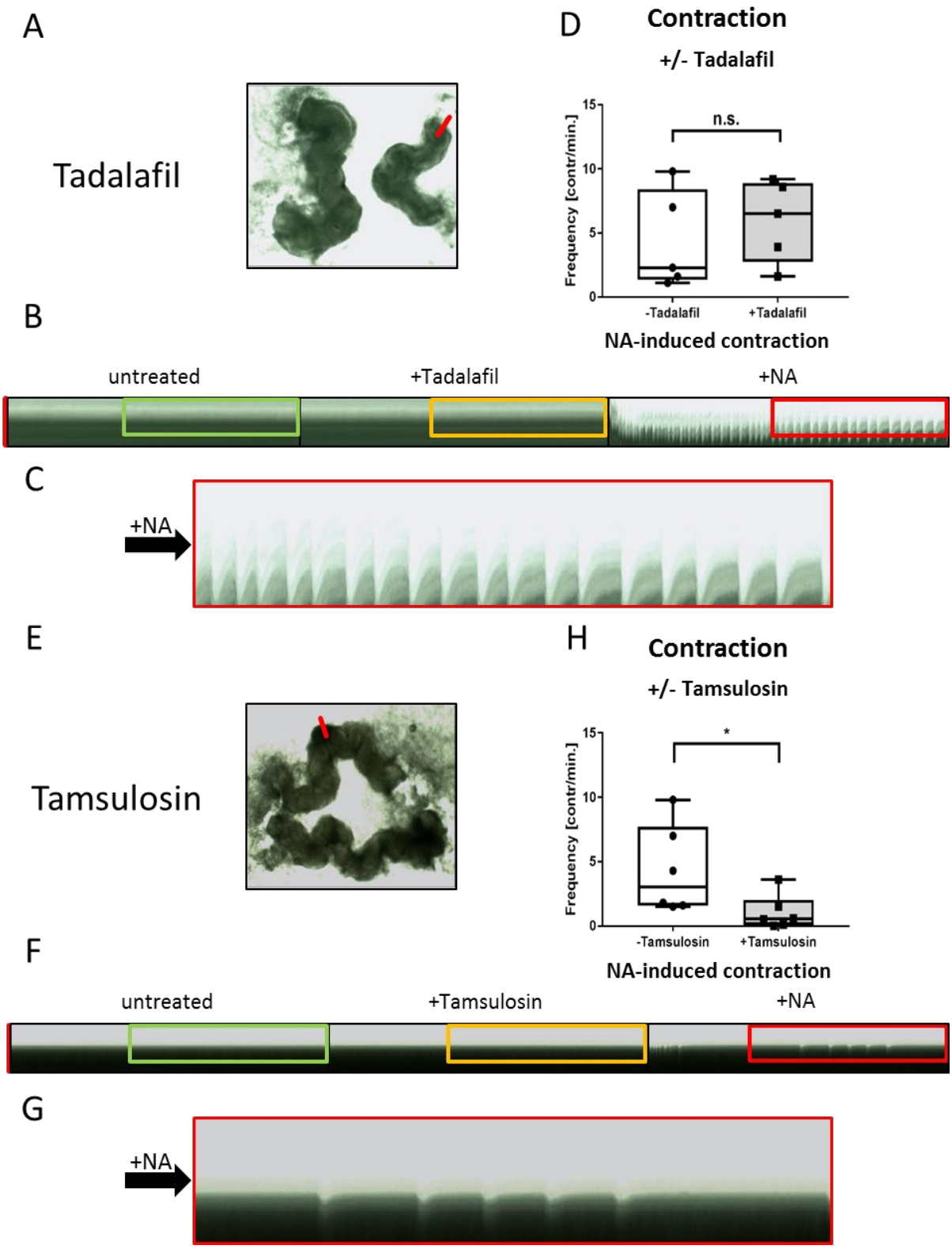

A
Tadalafil
D
Contraction
+/- Tadalafil
n.s.
Frequency [contr/min.]
15
10
5
0
-Tadalafil
+Tadalafil
NA-induced contraction
B
untreated
+Tadalafil
+NA
C
+NA
E
Tamsulosin
H
Contraction
+/- Tamsulosin
*
Frequency [contr/min.]
15
10
5
0
-Tamsulosin
+Tamsulosin
NA-induced contraction
F
untreated
+Tamsulosin
+NA
G
+NA

Figure 26: Investigation of NA-induced contraction frequency in Tadalafil and Tamsulosin pre-treated parts of the distal cauda part of the epididymal duct.

Analysis of time-lapse recordings for the caudal part of the epididymis for contraction patterns was performed by placing virtual slices (Reslices) through the observed tissue.

A: Movie still of recordings of the distal part of the cauda epididymal duct was taken and indicates the position of the time stack (red line).

B: Time stacks according to movie still allowed analysis of untreated activity (green box), response to drug treatment (+Tadalafil, orange box) and addition of NA (red box) for tadalafil pre-treated tissue.

C: Magnification of the time stacks of NA-induced contractions displayed in **B**.

D: Analysis of tadalafil (+Tadalafil) pre-treated tissue showed no significant differences in NA-induced contractions compared to vehicle (-Tadalafil) ($p=0.0772$, n.s.). Contraction frequencies for tadalafil pre-treated tissue were 5.96±1.43 contr/min and 4.36±1.72 contr/min for vehicle.

E: Movie still of tamsulosin pre-treated tissue showed the position of the time stack for analysis of contraction frequencies (red line).

F: Time stack according to movie still showed untreated tissue (green box), the response to tamsulosin treatment (orange box) and response to addition of NA (red box).

G: Magnification of the time stacks of NA-induced contractions displayed in **F**.

H: Analysis of tamsulosin (+Tamsulosin) pre-treated tissue showed a significant decrease in NA-induced contraction frequencies compared to vehicle (-Tamsulosin) ($p=0.0273$, *). In tamsulosin pre-treatment 1.05±0.55 contr/min occurred upon NA-addition. For vehicle, 4.33±1.4 contr/min. was analyzed.

4.5 Discussion

Previous studies regarding α_1-adrenoceptor antagonist induced abnormal ejaculation show inconsistencies in definition. It is suggested that the loss of ejaculate volume can occur due to 'retrograde' ejaculation (Lee 2000, Drobnis and Nangia 2017), the absence of seminal emission (Roehrborn et al. 2011) or 'anejaculation' (Hellstrom and Sikka 2006). Understanding the underlying mechanism of action of abnormal ejaculation is crucial to improve current pharmacological treatments or for identifying new alternative treatment options. For this, a novel model system was successfully established in this study giving clear results about (i) the mechanism of tamsulosin action on emission and (ii) the lack of tadalafil effects on emission at the potential targets.

We visualized the effects of tadalafil and tamsulosin in seminal vesicles, the distal epididymis and prostate ducts, recently described as the most relevant structure of the prostate for the release of the fluid (Kügler et al. 2018). In ducts, the SMC layer was shown to behave completely different from the majority of glandular SMCs, including a different arrangement of SMCs around the duct (Kügler et al. 2018). The present study was conducted in the rat model. Recent analyses reveal that structural properties of human prostate ducts are comparable to rodents (Kügler et al. 2018) and therefore are a suitable model to be used to predict responses in human tissue. However, in man the ducts are directly embedded in the surrounding prostate tissue when compared to rodents, which does not allow an investigation of duct function directly.

Tamsulosin and tadalafil served as the clinically relevant model (Lepor 1990, Von Heland and Casale 1994, Lee 2000, Lepor 2007, McVary et al. 2007, McVary et al. 2011, Gacci et al. 2012, Oelke et al. 2013, Gacci et al. 2016) to elucidate and compare effects on the NA responses, thus mimicking the emission phase of ejaculation. Blocking α_1-adrenoceptors with tamsulosin in seminal vesicles and prostate ducts led to the abolishment of NA-induced contractions. Quantifying weight changes in seminal vesicles between the different drug treatments and vehicles similarly reinforced this notion.

In tamsulosin pre-treated tissue upon NA-addition, there was no change in weight. Importantly, this implies that treatment with tamsulosin may affect ejaculation by reducing the contribution of secretions by the prostate and seminal vesicle during ejaculation leading to the reduced volume of ejaculate or even 'anejaculation' supporting the idea of absence of seminal emission (Hellstrom and Sikka 2006).

For the distal part of the cauda epididymis, contractions were significantly decreased in tamsulosin pre-treated tissue compared to vehicle, as quantified with two different approaches (Figures 25 and 26). Time-lapse recordings of the epididymis still showed the release of sperm after addition of NA. These findings implicate disturbances but no abolishment in sperm release from the epididymis during ejaculation. This finding is in conclusion with studies that suggest a loss of sperm in abnormal ejaculation due to an impairment in transport by tamsulosin (Hellstrom and Sikka 2006, Hisasue et al. 2006, Michel 2007).

Pre-incubation of these different tissues with the PDE5 inhibitor tadalafil showed no significant differences in NA-induced contractions of the tissue compared to vehicle. Weight changes (seminal vesicles), area changes (prostate ducts, seminal vesicles and epididymis) or frequency of contractions (epididymis) remained unchanged. Our results clearly show that the PDE5 inhibitor tadalafil has no negative impact on the emission and expulsion of secretions during ejaculation.

These findings give new insight in the explanation of the mechanism of action of ejaculation disorders in patients treated with tamsulosin for BPH. Our results clearly show that abnormal ejaculation described in the literature involves a distinct absence of seminal and prostatic contribution to the ejaculate, which is the main reason for reduced ejaculate volume and reduced, but not absent, sperm amount.

At the same time, we were able to show that these differences in SMC contraction of the three different tissue types are unaffected by tadalafil. This suggests it to be free of ejaculation disturbances.

4.6 Conclusions

Our study presents a model system explaining in detail the adverse effects of tamsulosin on ejaculation in BPH patients. These negative effects do not apply for tadalafil treatment.

4.7 Acknowledgements

Funding was provided by the Deutsche Forschungsgemeinschaft (GRK 1871) and Monash University, Australia, to the International Research Training Group (IRTG) between Justus-Liebig-University Giessen and Monash University.

4.8 Take Home Message

Understanding the underlying mechanisms of action of α_1-adrenoceptor antagonists and PDE5 inhibitors allows us to focus on treatment options with fewer side effects to improve the quality of life for patients suffering from BPH.

For a hardcopy of the suppl. movies 8-13 please refer to the appendix.

Chapter 5 – Investigation of a Novel NO Donor as a Potential Pharmacological Treatment Option for BPH

5.1 Introduction

NO, a gaseous signaling molecule (SoRelle 1998), has a significant influence on components of the cGMP signaling pathway, especially in the accumulation of the second messenger cGMP, a molecule that regulates PKG1 function. The increase of intracellular levels of cGMP is achieved by the conversion of GTP to cGMP, catalyzed by sGC which in turn is activated by NO. Additionally, sGC is the only conclusively proven receptor for NO. NO itself is produced by the enzyme NOS from the amino acid L-arginine. NO exerts a central role in mediating the relaxation of SMCs. Functions of NO include the contribution to the regulation of vascular tone, especially vasodilation and the physiological regulation of the cardiovascular system (Moncada and Higgs 2006, Pacher et al. 2007). In organs like the prostate gland, bladder, bladder neck and urethra (Andersson and Wein 2004), NO results in the accumulation of intracellular cGMP levels, ultimately leading to relaxation of SMCs. In organs of the lower urinary tract and male reproductive system, studies have shown that NO is physiologically released from nitrergic nerve terminals and acts as a non-adrenergic, non-cholinergic neurotransmitter in SMCs of bladder (Andersson and Persson 1994), urethra (Andersson and Persson 1994) and prostate (Ehren et al. 1994).

The mechanism of PDE5 inhibitors leads to the same effect, the accumulation of intracellular cGMP levels leading to SMC relaxation, but exhibit their function by inhibiting the hydrolysis of cGMP catalyzed by PDEs, e.g. PDE5 (see 1.7.2 and 1.7.4). Therefore, NO provides a potential pharmacological treatment option for BPH associated LUTS. Additionally, nNOS expression was detected in the human prostate and biochemical analysis revealed NOS in SMC layers of the PZs and TZs of the stroma, the TZ being the predominant zone of the occurrence of BPH (Otunctemur et al. 2015). nNOS has been immunohistochemically localized to nerve fibers in subepithelial nerve plexuses in the human prostate (Burnett et al. 1995).

NO also contributes to the regulation of the central and peripheral nervous system, e.g. functioning as a neurotransmitter in learning and several motivated behaviors including sexual and aggressive behaviors as well as influencing gastrointestinal motility and defense mechanisms in infectious diseases. Therefore, NO also exerts its physiological functions in the nervous and immune system (Hibbs et al. 1988, Bredt et al. 1990, Bredt and Snyder 1992, Nelson et al. 1997, Bogdan 2001, Toda et al. 2012). Because of the wide variety of physiological functions and production in various organs by the three different types of NOS (eNOS, iNOS, and nNOS, see 1.7.2), pharmacological molecules that release NO, called NO donors, were developed for their potential benefits in the treatment of diseases, especially in the cardiovascular system including angina pectoris, peripheral arterial diseases, hypertension, stroke, atherosclerosis and other cardiovascular and pulmonary diseases (Loskove and Frishman 1995, Herman and Moncada 2005, Katsumi et al. 2007, Falconer et al. 2018). NO donors also provide a model in the research of carcinogenesis, used as potential anti-oncogenic agents to overcome tumor cell resistance to various cytotoxic stimuli (Huerta et al. 2008).

However, because of its various biological spectrum of activities, the action of NO is regarded with some contention as it acts as an anti-neoplastic as well as a pro-neoplastic reagent (Huerta et al. 2008). Advantages of synthetically produced NO donors in the treatment of diseases such as cancer or cardiovascular disease are due to their capabilities of (i) producing a sustained release of NO, (ii) releasing a predictable dose, (iii) known half-life of the compound, (iv) safety for clinical application and (v) multiple mechanisms of NO release such as spontaneous release of NO or enzymatic oxidation (Feelisch 1998, Wang et al. 2002, Huerta et al. 2008). Because of their various mechanisms of releasing NO, several NO donors were developed, each exhibiting desirable properties. Reports by Wang *et al.* and Feelisch and Stamler provide an in-depth review of the chemistry of the various classes of NO donors and their preparation (Feelisch 1998, Wang et al. 2002).

In this study, we utilized an exogenously applied NO donor, S-nitrosoglutathione (GSNO), to investigate the effects of NO release from the donor on cGMP pathway components of SMCs and SMC relaxation in isolated, patient-derived prostatic SMCs via two different techniques; *in vitro* cell culture (see 2.6) and tension recording experiments of isolated specimens (see 2.5.1).

Two different types of the NO donor GSNO were used and effectiveness compared; (i) the commonly used donor molecule GSNO and (ii) a synthesized NO-releasing core cross-linked star polymer nanoparticle, in which GSNO was conjugated into the core of the star as a macromolecular carrier of GSNO (see 5.2.4).

The synthesis of a macromolecular carrier construct for GSNO was performed to stabilize and subsequently increase the control of the release of NO from the donor, since the direct use of NO and small molecule NO donors in biological systems has limitations, mainly involving poor stability, high reactivity and lack of specificity, resulting in poor pharmacokinetics and short half-life (Fast 2005). The incorporation of small molecule NO donors into macromolecular carriers has been shown to overcome some of these problems (Duong et al. 2015).

GSNO alone and the GSNO-conjugated core cross-linked star polymer were provided in collaboration with Dr. Michael Whittaker (Senior Research Fellow at Drug Delivery Disposition & Dynamics, Monash University, Melbourne, Australia).

5.2 Materials and Methods

5.2.1 Tissues

Experiments conducted in Australia collected from human prostate specimens are described in Chapter 2.1.
Briefly, the collection of human samples was achieved together with the Australian Prostate Cancer BioResource (APCB) Coordinator Dr. Melissa Papargiris. Collection and use of human specimens were in approval and compliance with the Cabrini Human Research Ethics Committee (13-14-04-08) and Monash University Human Research Ethics Committee (2004/145). The obtained tissue was either directly transported to the laboratory facilities to conduct tension recording experiments (see 2.5 and below) or were processed for tissue digestion for cell culture experiments (see 2.6 and below).

5.2.2 Tension Recording Experiments

Human prostate specimens used for tension recording experiments were processed, dissected and prepared as described in Chapter 2.5.1 which followed a protocol to assess the myogenic tone of the tissue. The tissue was placed into the organ bath and an initial tension of 25-30mN was applied for at least 60min during equilibration. Spontaneous contractions (myogenic tone) of the tissue were recorded for at least 10 more minutes. Following this step, the recorded tissue was either treated with vehicle (0.1% DMSO in PSS) for 30min as control, or 3, increasing concentrations of one of the two NO Donors, (i) either the commonly used NO donor molecule GSNO or (ii) a synthesized core cross-linked star polymer nanoparticle serving as a macromolecular carrier in which GSNO was conjugated into the core of the star (see 5.2.4). The addition of each concentration was followed by an incubation time of 10min to analyze and compare the effects of the NO donors on the myogenic tone of the investigated tissue. The three different concentrations used included (1μM, 3μM, and 10μM) and the GSNO constructs were dissolved in 0.1% DMSO in PSS.
After a washout period of at least 5min with PSS, the tissue was exposed to a high concentrated KCl solution (40mM) to assess viability. KCl induces a reliable and robust contraction in viable preparations.

5.2.3 Cell Culture Experiments

The investigation of the core cross-linked star polymer conjugated GSNO and the commonly used GSNO for *in vitro* experiments was performed as described in Chapter 2.6 with isolated primary SMCs obtained from human prostate specimens. For experimental approaches, cells were incubated either with vehicle (0.1% DMSO in PSS) as control or with a concentration of 10μM with either of the two GSNO constructs.

5.2.4 Synthesis of NO-Releasing Core Cross-Linked Star Polymer Conjugated GSNO

The successful synthesis of polymers with known molecular architecture specifically with star, cyclic, linear, dendritic, comb, brush or hyperbranched structures is well-defined (Sumerlin and Vogt 2010, Boyer et al. 2011). For the synthesis of GSNO (Sigma-Aldrich, Steinheim, Germany) into a macromolecular carrier polymer, the star polymer was used (synthesis and supply is a generous gift and in collaboration with Dr. Michael Whittaker, Senior Research Fellow at Drug Delivery Disposition & Dynamics, Monash University, Melbourne, Australia).
The star polymer consists of several linear chains, called arms, and are linked together at a central core. The central core is functionally designed to allow the specific conjugation of GSNO. The advantage of the star construct over e.g. linear polymers is the lower viscosity and are therefore useful as rheology control agents (Fetters et al. 1993, Ren et al. 2016), finding increasing application in nanomedicine, as nanocontainers and nanoreactors (Gao 2012). The preparation of a star polymer can be achieved with different methods (Ren et al. 2016) of which the reversible addition-fragmentation chain transfer (RAFT) was used because of synthetic ease and high versatility (Hu et al. 2017).

For detailed information of the synthetic process of star polymers by RAFT polymerization for biomedical and drug delivery applications originating in the laboratory of Dr. Michael Whittaker, please refer to (Liu et al. 2012, Li et al. 2014, Hu et al. 2017, Yu et al. 2018).

5.2.5 Statistical Analysis

Statistical analysis of tension recordings and cell culture experiments were performed as described in Chapter 2.7.2 and 2.7.3, respectively.

Briefly, unless noted otherwise, paired one-way ANOVA with Dunnett's post hoc test was used for statistical analysis of the data where $p<0.05$ was considered to be statistically significant for tension recordings and data was represented as a mean±SEM. For qPCR analysis, two-way ANOVA and Tukey's post hoc test was used. In all cases, $p<0.05$ was considered to be statistically significant. Analyses were performed using the statistics program GraphPad Prism.

5.3 Results

5.3.1 Western Blot Analyses of cGMP Downstream Proteins Showed Changes in PDE5 and PKG1 Expression after Treatment with NO Donors

Changes of cGMP signaling pathway components, notably PDE5 and PKG1, were analyzed and compared on protein levels after 24h incubation with one of the two GSNO variants via Western blot analysis. Activation of sGC by NO leads to the intracellular accumulation of cGMP. cGMP, in turn, activates the downstream protein PKG1 resulting in SMC relaxation. The *PDE5A* gene encodes for PDE5. The physiological function of PDE5 is to catalyze the hydrolysis of cGMP and regulate cGMP signaling.

Analyses of Western blots showed a higher expression of PDE5 in GSNO and star-GSNO treated cells compared to vehicle for two of the four investigated cell lines (Figure 27, BPC126, BPC127). The expression of PKG1 in those two cell lines, on the other hand, was only upregulated in one of the two lines (BPC127) compared to control. For the other cell line (BPC126), expression levels remained the same in GSNO incubated cells compared to control whereas incubation with star-GSNO showed a decrease of protein levels of PKG1 in this cell line (Figure 27). Protein expression of PDE5 and PKG1 was slightly downregulated in comparison to control for the cell line BPC131 incubated with GSNO and remained unchanged when incubated with star-GSNO (Figure 27). Protein expression levels of PKG1 and PDE5 for cell line BPC129 were not affected by any of the two NO donors (Figure 27).

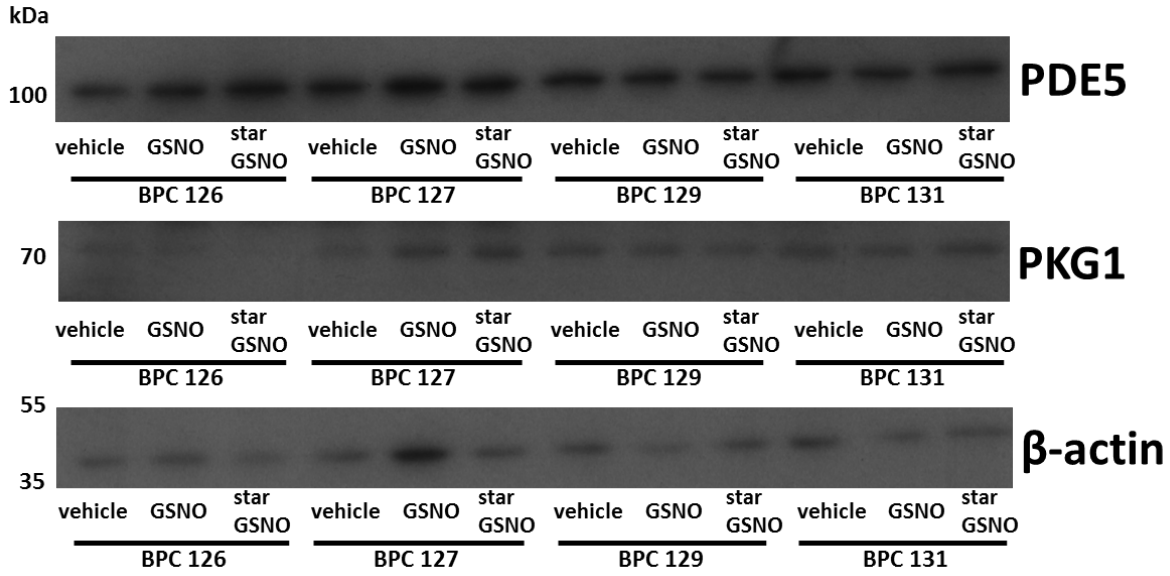


Figure 27: Western Blot analysis showed a different level of protein expression in cell lines treated with GSNO or star-GSNO.

Elevated protein levels for PDE5 were observed in BPC126 and BPC127 for both NO donors used. After incubation with each of the NO donors, PDE5 protein levels remained unchanged for BPC129 and differed only slightly for BPC131.

PKG1 expression after incubation with each of the donors was downregulated in BPC126 but upregulated in BPC127. PKG1 expression levels in BPC129 and BPC131 remained the same or change only slightly.

5.3.2 Treatment of Human Prostate SMCs with GSNO Affected RNA Levels of cGMP Signaling Pathway Molecules

To assess and compare the effects of the two architecturally different NO donors on cGMP signaling pathway components in human-derived primary SMCs, qRT-PCR analyses were performed.

For this, NO donors were exogenously applied at a concentration of 10µM and incubated for 24h. After incubation, RNA of cells was isolated followed by cDNA synthesis and qRT-PCR to assess fold changes of the genes *sGCbeta1*, *PDE5A,* and *PKG1*. *sGCbeta1* encodes for the beta1 subunit of sGC, present in all relevant heterodimers of sGC. Once activated, sGC mediates the conversion of GTP to cGMP which ultimately leads to an accumulation of cGMP. cGMP, in turn, activates the downstream protein PKG1. The *PDE5A* gene encodes for PDE5. The physiological function of PDE5 is to catalyze the hydrolysis of cGMP and regulate cGMP signaling.

Analyses showed that 24h treatment of GSNO alone in the four different cell lines led to a significant upregulation of RNA expression levels of PDE5 and PKG1 in at least one of the cell lines relative to control (BPC127, $p<0.0001$, ****, Figure 28B, C). In other cell lines, GSNO incubated cells showed a significant decrease of transcript expression for PDE5 and PKG1 as well (BPC129, $p<0.05$, *, Figure 28B, C). Expression levels for sGC RNA remained unchanged (Figure 28A) after GSNO incubation.

On the other hand, the treatment of cells with the star polymer GSNO showed a significant upregulation of RNA for all analyzed genes in at least one of the cell lines relative to control ($p<0.0001$, ****, Figure 28A, B, C). PKG1 RNA expression was downregulated in another cell line ($p<0.05$, *, Figure 28C) whereas the other cell lines remained unchanged relative to control.

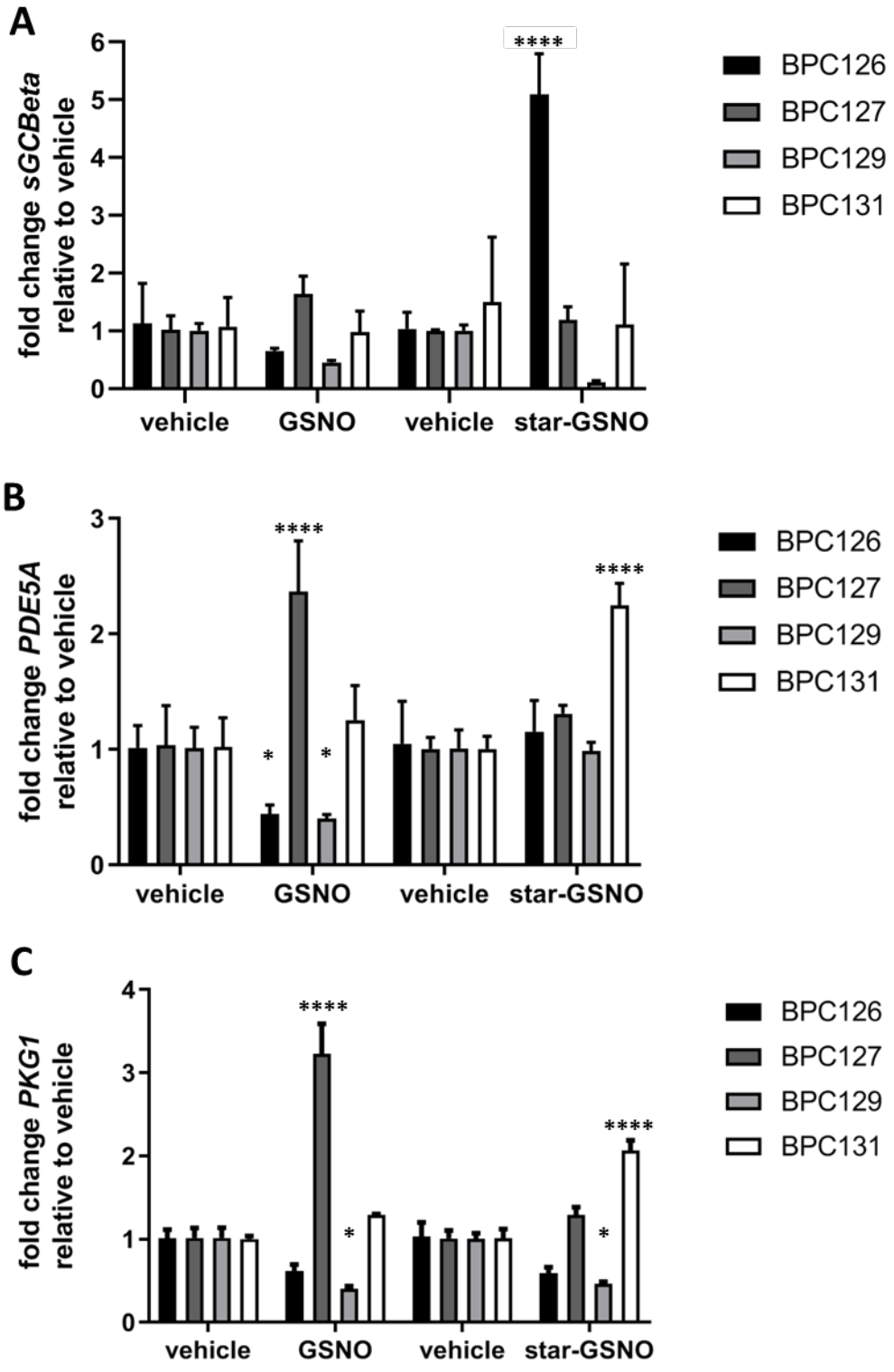

A
fold change sGCBeta relative to vehicle
6
5
4
3
2
1
0
****
vehicle
GSNO
vehicle
star-GSNO
BPC126
BPC127
BPC129
BPC131
B
fold change PDE5A relative to vehicle
3
2
1
0
****
*
*
****
vehicle
GSNO
vehicle
star-GSNO
BPC126
BPC127
BPC129
BPC131
C
fold change PKG1 relative to vehicle
4
3
2
1
0
****
*
*
****
vehicle
GSNO
vehicle
star-GSNO
BPC126
BPC127
BPC129
BPC131

Figure 28: qRT-PCR analyses showed upregulated RNA expression for genes encoding for sGC, PDE5, and PKG1 after 24h treatment with GSNO or star-GSNO.

A: A significant upregulation of RNA expression levels for sGC relative to control was observed for one cell line ($p<0.0001$, ****, BPC126) incubated with star-GSNO. Incubation with GSNO alone did not lead to significant changes of RNA levels for sGC.

B: PDE5 RNA expression was highly upregulated in one cell line ($p<000.1$, ****, BPC127) incubated with GSNO and significantly downregulated relative to control in two other cell lines ($p<0.05$, *, BPC126, BPC129).
Treatment of cells with the star polymer GSNO led to a significant upregulation of RNA expression in one cell line ($p<000.1$, ****, BPC131) and remained unchanged in the other cell lines.

C: PKG1 RNA expression levels showed a significant upregulation in one cell line ($p<0.0001$, ****, BPC127) treated with GSNO and a significant upregulation for another cell line ($p<0.0001$, ****, BPC131) treated with star-GSNO. RNA expression levels were significantly downregulated relative to control in one cell line for both GSNO treatments ($p<0.05$, *, BPC127).

5.3.3 Tension Recording Experiments Showed the Influence of GSNO and star-GSNO on Human Prostate Specimens by Altering Basal Tension, Amplitude, and Frequency of Contractions

Next, we investigated the effects of GSNO alone and star-GSNO in prostatic strips of human specimens on the contractility of the whole isolated tissue. The obtained tissue was set up in the organ bath and an initial tension of 25-30mN was applied as described above (see 2.5.1). The recording of spontaneously developing contractile activity was measured and set as control for each treatment; vehicle (n=4), GSNO (n=5) or star-GSNO (n=4). The tissue was incubated either with vehicle (0.1% DMSO in PSS) for 30min or increasing concentrations (1μM, 3μM and 10μM) with one of the two NO donors each for 10min. Contractile activity was recorded and analyzed after the experiment. For analyses, the changes in basal tension [mN], amplitude of contractions [N/g] and frequency of contractions [contr/min] were compared over time (vehicle) or for differing concentrations of the drug of interest (GSNO, star-GSNO).
Raw data of recordings in tissue incubated with vehicle showed a significant decrease in basal tension after 30min compared to control ($p<0.01$, **, Figure 28A, I) but no change of amplitude of contractions over the observed time (Figure 29A, II). Contraction frequency increased significantly after 20min compared to control ($p<0.05$, *, Figure 29A, III) but decreased again after 30min. Data of the percent change from baseline for the observed time points showed a significant decrease in basal tension from 10min to 30min (Figure 29B, I) but no significant changes were observed for the percent change of the amplitude (Figure 29B, II) or frequency of contractions (Figure 29B, III) for vehicle. However, both amplitude and frequency were slightly increased.
Raw data for tissue incubated with GSNO (Figure 30) showed a steady decrease of basal tension with increasing concentrations of GSNO which were significant for 3μM compared to control ($p<0.05$, *, Figure 30A, I) and 10μM compared to control ($p<0.05$, *, Figure 30A, I). A steady decrease of the amplitude of contractions was observed, which was significant for 10μM GSNO compared to control ($p<0.05$, *, Figure 30A, II) as well as a decrease of contraction frequency, significant for 3μM GSNO compared to control ($p<0.05$, *, Figure 30 A, III).
The percent changes from baseline showed the same tendency, a decrease of basal tension, amplitude and frequency, but was not significantly different compared to each other.

The analyses of the raw data for specimens treated with the star polymer GSNO showed a gradual decrease of basal tension with increasing concentrations of the polymer and was significantly decreased for concentrations of 3µM ($p<0.05$, *, Figure 31A, I) and 10µM ($p<0.01$, **, Figure 31A, I) compared to control. The amplitude of contractions (Figure 31A, II), as well as the frequency of contractions (Figure 31A, III) of the raw data, was gradually decreased with increasing concentrations of the star-GSNO compared to control for all observations (except for the amplitude after the incubation with 1µM star GSNO which was slightly increased) but no significant differences were observed. Analyses of percent changes from baseline for star-GSNO treated tissue showed a gradual decrease with increasing concentrations of the star polymer with significant differences between 1µM to 10µM ($p<0.01$, **, Figure 31B, I). After a slight increase of percent changes of the amplitude of contractions with 1µM star-GSNO (Figure 31B, II), the amplitude decreased with increasing concentrations of star-GSNO with a maximum decrease of about 25% for 3µM star-GSNO (Figure 31B, II). The decrease of frequency of contractions with the star polymer was observed with the concentration of 1µM, higher concentrations did not result in a further decrease of the frequency (Figure 31B, III).

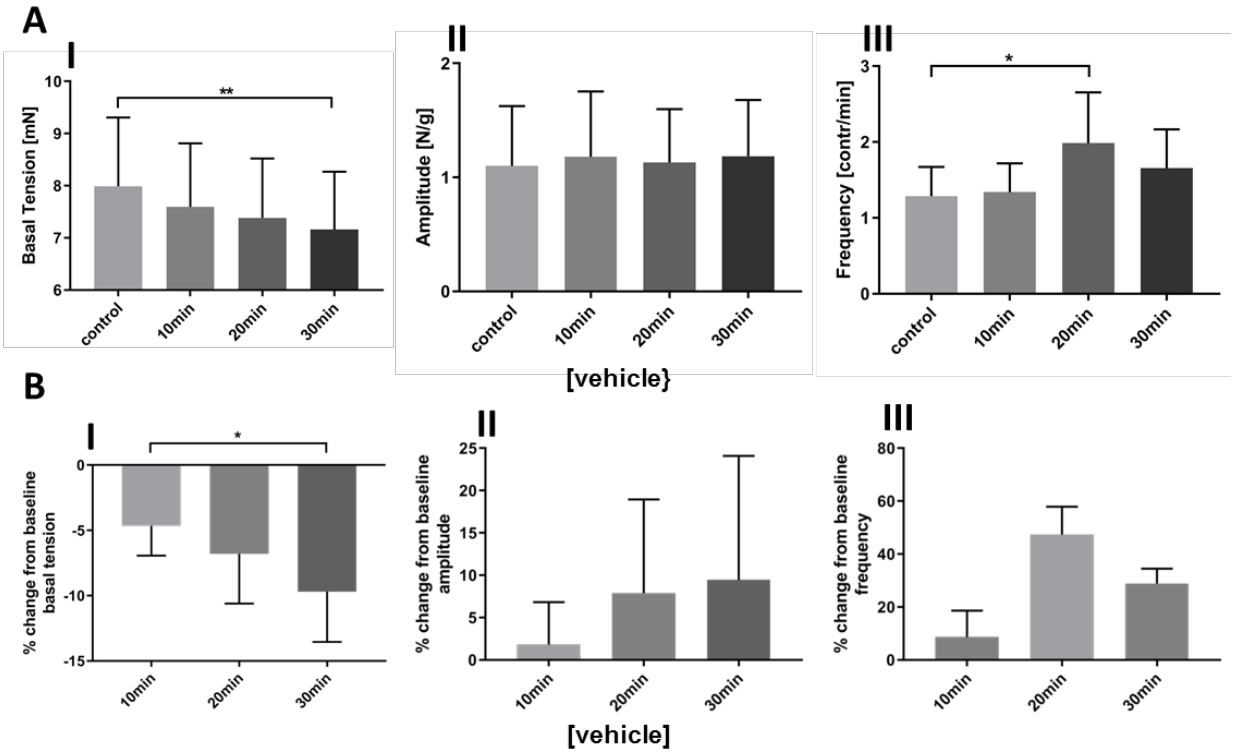


Figure 29: Tension recording experiments for vehicle showed a decrease of basal tension over time.

A: Analyses of raw data for basal tension (I), amplitude of contractions (II) and frequency of contractions (III) followed over time for vehicle. A significant decrease of basal tension ($p<0.01$, **) was observed after 30min compared to control, whereas the frequency of contractions was significantly increased after 20min of recording compared to control ($p<0.05$, *), but not after 30min.

B: The investigation of percent changes from baseline between the different time groups showed a significant decrease in basal tension from 10min to 30min ($p<0.05$, *, I).

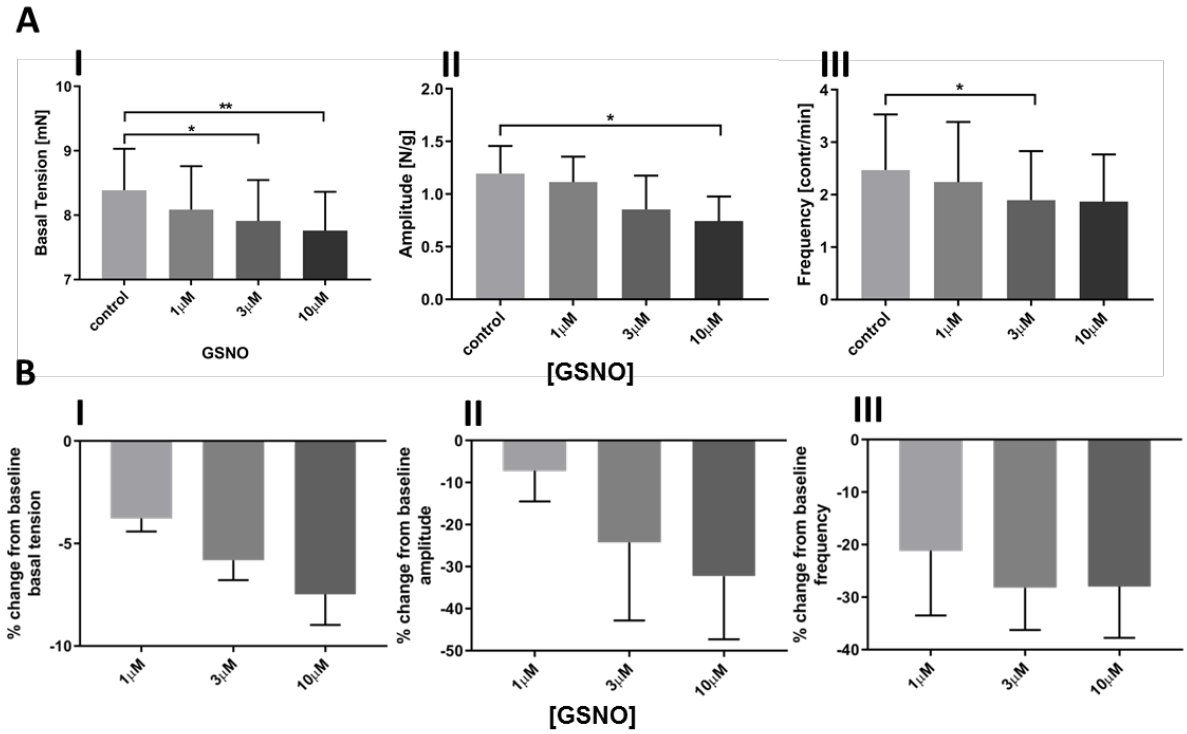


Figure 30: Incubation of human prostate specimens with GSNO showed a gradual decrease of basal tension, amplitude, and frequency.

A: Analyses of raw data showed significant decreases of basal tension for 3µM GSNO ($p<0.05$, *, I) and 10µM GSNO ($p<0.01$, **, I) compared to control as well as a significant decrease in amplitude (10µM, $p<0.05$, *, II) and frequency (3µM, $p<0.05$, *, III) of contractions.

B: No significant change, but a gradual decrease of percent change from baseline was observed for basal tension (I), amplitude (II) and frequency (III).

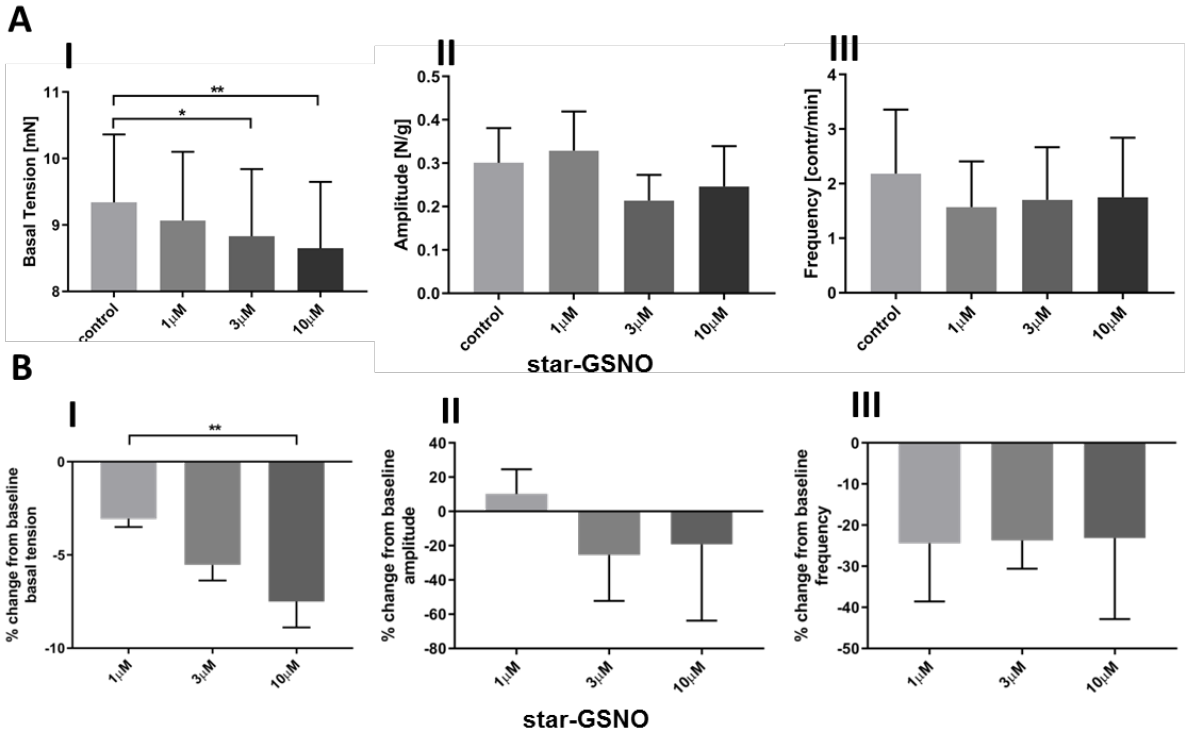


Figure 31: Tension recording experiments of human prostate specimens treated with the star polymer GSNO showed a significant decrease in basal tension.

A: A decrease of the basal tension was observed with increasing concentrations of the star polymer and was significant for concentrations of 3µM ($p<0.05$, *, I) and 10µM ($p<0.01$, **, II). A decrease of the amplitude of contractions (II) was observed as well as a decrease of the frequency of contractions (III) but was not significantly reduced.

B: Percent changes from baseline were observed for basal tension with a significant difference of 1µM of the star polymer compared to 10µM ($p<0.01$, **, I). No significant changes were observed for the amplitude of contractions (II) as well as for the frequency of contractions (III) where the maximum decrease of about 20% is observed with 1µM of the incubation with the star polymer.

5.4 Discussion

In this study, we investigated and established the properties of a novel star polymer construct conjugated NO donor GSNO in comparison to GSNO in different experimental setups, with *in vitro* and tension recording experiments on specimens that were obtained from non-malignant areas of the prostate TZ from patients undergoing radical prostatectomy. We showed that the treatment of human prostate primary SMCs with NO donors leads to an increase of transcripts of cGMP pathway components, namely sGC, PDE5, and PKG1 as well as increased protein levels in the same cell lines investigated for PDE5 and PKG1. Tension recording experiments showed that NO donors mainly have an effect on the reduction of the amplitude and contraction frequency when using GSNO alone. A significant reduction of the basal tension could not only be observed in the tissues treated with the NO donors but also in vehicle control recordings.

These novel findings have significant implications for the research of novel pharmacotherapies used in the treatment of BPH. Currently, α_1-adrenoceptor antagonists such as tamsulosin, 5ARIs such as finasteride and PDE5 inhibitors such as tadalafil are approved by the FDA in the treatment of BPH (Carson et al. 2014, Brasure et al. 2016, MacDonald et al. 2019). While classical treatment options potentially have significant side effects (e.g. disturbed ejaculation with tamsulosin) (Lee 2000), possibly leading to discontinuation of therapy, or are only prescribed under specific circumstances (5ARIs only with an enlarged prostate) (Kim et al. 2018), the limitations for PDE5 inhibitors are lack of data from clinical trials on long term effects of treatment. Nevertheless, PDE5 inhibitors could potentially be a preferred pharmacological treatment option, because it reduces myogenic tone of the prostate and demonstrates safety and tolerability with no severe side effects, such as disturbed ejaculation as seen from the results displayed in this thesis. Therefore, it seems logical to investigate further novel pharmacotherapies affecting the cGMP signaling pathway for SMC relaxation, like donors of NO. Drugs used to relax SMCs are a suitable pharmacologic treatment option, since myogenic tone is increased in the TZ of the prostate, where BPH occurs (Wasserman 2006).

The rationale behind the utilization of a NO donor is that NO activates sGC in SMCs which in turn catalysis the reaction that transforms GTP to cGMP, ultimately leading to SMC relaxation (Carvajal et al. 2000).

The intracellular accumulation of cGMP due to NO donors is a comparable effect as to PDE5 inhibitors which stop the hydrolysis of cGMP, ultimately leading to the accumulation of intracellular cGMP levels. GSNO, as well as the star polymer conjugated GSNO, are reagents that were mainly used in cancer research (Huerta et al. 2008). The synthesis of a macromolecular carrier has many advantages compared to the compound alone (see 5.1) and studies assessing properties of NO release showed a slower but stable and more controllable release of NO from the polymer compared to GSNO alone (data not shown, studies performed by Whittaker et al.). This knowledge could potentially explain the slight differences in the outcome observed in our studies. Short-term incubation with the NO donors in tension recording experiments suggests that GSNO alone has a higher impact on the decrease of the amplitude and frequency on contractions compared to star-GSNO, although both donors exhibited the same effects on basal tension. This might be because due to the slower release of NO from the star polymer, the total incubation time with the tissue (30min) is not sufficient to exhibit the full inhibition of myogenic tone of the samples. GSNO alone releases, on a short-term basis, more NO and significant effects occur faster. This explanation is reinforced with “long-term” incubation of 24h as evidenced through the results of the western blot analysis. Here, the protein expression of PDE5 at least in three of four patients was greater with the star-GSNO compared to control and GSNO alone. Still, this result is limited as an indirect effect and higher expression of PDE5 is related back to catalyze the hydrolysis of an increased amount of cGMP due to the activation of sGC by NO, which is mediated by the NO donor. A similar effect is seen in two of four patients for PKG1, a downstream protein of cGMP signaling. Higher intracellular amounts of cGMP due to a higher activation rate of sGC by NO leads to a higher expression of PKG1. That both NO donors have a significant impact on the transcription of the genes of PDE5 and PKG1 is observed in the qRT-PCR data, with 2-3-fold upregulation of those genes in one cell line each compared to control. A more direct effect on cGMP signaling is only observed with the star polymer GSNO.

Only here, a highly significant 5-fold upregulation relative to vehicle was observed in one cell line for the gene encoding for sGC. This effect is related back to the steady exposure to NO by the star polymer.

Although both NO donors investigated showed significant effects on SMC contractility and cGMP pathway components, the studies conducted are limited. Firstly, NO is a free radical gas with a short half-life and is highly reactive. This may lead to oxidation or reduction of the molecule NO. Consequently, it would not have any effects on components of the cGMP pathway anymore. Secondly, information about any of the two donors used for *in vitro/ex vivo* are very limited and tested for the first time in biological tissue settings conducted in this thesis. Only chemical properties were evaluated, and this data may not be reflective of their influence for *in vitro/ex vivo* experiments. Therefore, observed effects need to be repeated as well as different experimental setups tested to obtain reliable results. Still, obtained results show a certain number of effects that are encouraging further investigation as a potential treatment option for patients suffering from BPH.

Chapter 6 - General Discussion

Benign prostatic hyperplasia (BPH) is a highly prevalent disease in the aging male population. BPH is often associated with lower urinary tract symptoms (LUTS) and therefore has a high impact on the quality of life. The number of men diagnosed with BPH is increasing due to higher life expectancies of the population. Although a high percentage of men are most likely to experience BPH associated LUTS, the exact mechanism of BPH development is still unclear. It is suggested that androgens, especially testosterone and dihydrotestosterone (DHT) and age-related changes in the ratio of those androgens, as well as changes in the ratio of testosterone to estradiol, play a key role in the development of BPH. BPH is characterized by an enlargement of prostatic tissue due to abnormal cell proliferation (static component), especially in the stromal compartment, predominantly consisting of smooth muscle cells (SMCs). This is often accompanied by an increase of the myogenic tone and contractility of SMCs (dynamic component). The most common treatment option involves pharmacotherapeutic approaches with α_1-adrenoceptor antagonists such as tamsulosin or 5α-reductase inhibitors (5ARIs) such as finasteride. A newly emerged treatment option includes inhibitors of phosphodiesterase type 5 (PDE5) such as tadalafil and although effective, these treatments have limitations. Treatment with tamsulosin often results in severe side effects like disturbed ejaculation, 5ARIs are only prescribed for an enlarged prostate, the exact mechanism for PDE5 inhibitors used to treat BPH is still unclear and data on long-term effects of treatment are missing (Hatzimouratidis 2014).

Thus, investigation on pharmacotherapies used for BPH is crucial to explain the mechanism of action of emerging drugs (e.g. tadalafil) on contractility as well as to understand the adverse effects of established drugs (e.g. tamsulosin). With the gathered knowledge we aimed to find new, alternative mediators to relax SMCs without severe side effects. This outcome is coupled with the complexity and multifactional nature of factors underlying the initiation and progression of the disease.

This thesis consists of three chapters that are jointly investigating myogenic tone (contractility) and architectural structures in the prostate. Furthermore, this thesis reveals the adverse effects of disturbed ejaculation with standard pharmaceutical treatment like tamsulosin, as well as to visualize the effects of PDE5 inhibitors (sildenafil and tadalafil) on contractility and on the emission phase of ejaculation in organs involved in ejaculation such as the prostate, seminal vesicles, and epididymis. Studies conducted for this thesis reveal in detail the cellular arrangement of SMCs and the expression of PDE5 exclusively in SMCs of prostate glands and ducts. The last part of this thesis focused on introducing a novel molecule and exhibiting the potential role as an inhibitor of prostatic SMCs for the use as an alternative treatment option for patients suffering from BPH.

6.1 Structural and Functional Analysis of the Human and Rat Prostate with Focus on PDE5 Inhibitors Sildenafil and Tadalafil

The published work displayed in Chapter 3 of this thesis revealed the histological arrangement of SMCs around isolated rat prostate secretory glands and excretory ducts as well as in human prostate tissue. Our modified CLARITY approach demonstrated that the spatial arrangement and orientation of SMCs differ in single glands compared to excretory ducts, suggesting they have specific roles before and during ejaculation. The single glands of the prostate consist of a single layer of SMCs with multiple orientations. The excretory ducts have at least two layers of SMCs that are arranged in a circular manner around each duct. Aside from the spatial arrangement, our time-lapse imaging model system revealed spontaneous contractile activity in single glands, whereas excretory ducts were quiescent, suggesting different functional roles of the two prostatic structures. The differences in spatial arrangement and functional properties suggest that single glands of the prostate produce the prostatic fluid that comprises up to 30% of the whole semen, which is expelled during the emission phase of ejaculation through the excretory ducts into the urethra. Incubation of prostatic tissue with the sympathetic activator (NA) of SMCs during ejaculation showed strong contractions of the prostate ducts underlining the emission phase (Kügler et al. 2018).

We revealed the distribution of PDE5 in prostate SMCs of human and rat using the adapted 3D CLARITY approach, which facilitated our functional studies of the effect of PDE5 inhibitors on prostate tissue. PDE5 is part of the cGMP signaling pathway, which in turn is important in the process of SMC relaxation. PDE5 is known to catalyze the hydrolysis of cGMP and therefore diminishes cGMP effects. Another important effector protein in the cGMP signaling pathway is the cGMP-dependent protein kinase 1 (PKG1) that mediates SMC relaxation (Schlossmann et al. 2003, Francis et al. 2010, Wolfertstetter et al. 2013) which we found is expressed in SMCs in the prostatic stroma (and vasculature) in man and rat as previous studies have shown before (Wolfe et al. 1989, Francis and Corbin 1994).

The focus was to determine the effects of the PDE5 inhibitors sildenafil and tadalafil on contractility (myogenic tone) of prostate SMCs, which resulted in a significant decrease of myogenic tone in prostate tissue after PDE5 inhibitor incubation in man and rat.

While previous studies mainly focused on pre-stimulation of prostate tissue e.g. with electrical field stimulation or noradrenergic drugs like NA to investigate drug responses that decrease contractions (Angulo et al. 2012), fewer studies have focused on spontaneous contractile activity, mostly in guinea-pig prostate models, while the human prostate is only insufficiently described (Oh et al. 2003, Exintaris et al. 2006, Exintaris et al. 2009, Chakrabarty et al. 2015, Lee et al. 2017).

For the first time, sildenafil and tadalafil action was visualized on intact prostate tissue. Pre-treatment of prostate ducts with sildenafil showed no interference with NA-induced contractions, which showed for the first time that PDE5 inhibitors (here sildenafil) do not interfere with adrenergic activation of functionally important prostate excretory duct SMCs during the emission phase of ejaculation. These results clearly show that PDE5 inhibitors reduce SMC tone in the prostate without affecting noradrenergic activation of the tissue. This knowledge is important for the investigation of alternative treatment options for BPH, without adverse effects like disturbed ejaculation that occur with α_1-adrenergic antagonists such as tamsulosin.

Additionally, utilizing the time-lapse imaging approach was advantageous, since this technique allows investigations of tissues close to *in vivo* conditions respecting architectural integrity, and is less invasive regarding tissue preparation and experimental investigation. For further studies, it would be of interest to investigate the effects of other cGMP specific PDEs such as PDE6 and PDE9 and their effect on SMC contractility in prostate tissue of man and rat.

The 3D approach of CLARITY allowed us to visualize and broaden our understanding of the spatial arrangement and function of SMCs in prostate tissue (Kügler et al. 2018). We found that the human prostate SMCs show different arrangements compared to rat prostate tissue. The human prostate possesses SMCs in all parts of the prostate stroma, which itself is alternated by connective tissue. These differences occur due to the different anatomical features in both.

The human prostate tissue is tightly packed and appears as one strongly interconnected tissue, whereas the rat prostate tissue is rather loose, and each single gland is connected via sparse connective tissue. This might also affect the transport of prostatic fluid in both species.

The arrangement of rat prostatic SMCs leads to the suggestion that selective fluid transport is mediated through the size reduction of single glands. The fluid is transported to the prostatic ducts. Rat prostate ducts only contract upon activation of the SMC layers that are tightly packed around the duct, with a strong contraction of the duct leading to the expulsion of fluid out of the prostate. This idea is supported by previous studies on functional activities of the rat ductal system (Lee et al. 1990). In humans, the architectural structure and arrangement of SMCs are distributed over the whole prostate stroma and may lead to a more indirect type of prostate fluid secretion. Contractions of SMCs may cause a reduction in stromal tissue, which leads to a local reduction of the size of the prostate, which in turn would drain nearby glands. Because of the structure of the human prostate, distinguishing between single glands and excretory ducts is therefore only possible with investigations of spatial arrangements but not by functional time-lapse imaging studies.

Assuming the human prostate ducts exhibit the same properties as in rat, our studies demonstrate the importance of the individual structures between single glands and the excretory ducts, with implications for pharmacotherapeutical interactions by drugs used to treat BPH in these tissues.

6.2 A Newly Established Model System for the Emission Phase of Ejaculation Reveals Action of Pharmacotherapies Used in the Treatment of BPH

Published studies shown in Chapter 3 led us to the investigation of drug interactions on tissues involved in the emission phase of ejaculation including the prostate excretory ducts, seminal vesicles and the sperm-storing part of the cauda epididymides. Tissues were pre-treated with the α_1-adrenoceptor antagonist tamsulosin and the PDE5 inhibitor tadalafil respectively and responses to NA were compared with NA simulating the emission phase of ejaculation.

Tamsulosin is the classical pharmacological treatment choice and although often resulting in severe adverse effects like disturbed ejaculation, the exact mechanism behind abnormal ejaculation is unknown. This has led to inconclusive studies trying to define the process of abnormal ejaculation, suggesting retrograde ejaculation, the absence of seminal emission or anejaculation (Lee 2000, Hellstrom and Sikka 2006, Roehrborn et al. 2011)

Therefore, we utilized our novel time-lapse imaging approach to establish a model system for the investigation of drug responses in various organs. This visualization gave clear insights that explain the reason behind abnormal ejaculation in patients treated with tamsulosin. Our results suggest that blocking α_1-adrenoceptors with tamsulosin inhibits or even abolishes sympathetic stimulation of organs involved in the emission phase of ejaculation. Pre-treatment of tissues with tamsulosin abolished NA-induced contractions in seminal vesicles and prostate excretory ducts, and a significant decrease of contractions in the distal part of the cauda epididymis. These results demonstrate that treatment with tamsulosin leads to an absence of seminal and prostatic contribution to semen and a significant decrease of sperm release through the vas deferens into the urethra. Therefore, the volume of ejaculate that is released will be almost absent and would mainly contain sperm.

We showed that disturbed ejaculation is the absence of seminal emission including a significantly decreased release of sperm rather than retrograde ejaculation alone (of the whole semen), since contractions in the responsible organs are abolished and fluid is consequently not released.

Our study gives clear insights behind the reason of the occurrence of abnormal ejaculation in patients treated with tamsulosin. These findings are crucial for the investigation of new pharmacological treatment options for symptoms experienced with BPH in combination, as to avoiding severe side effects. We demonstrated that decreasing myogenic tone in prostate, seminal vesicle, and epididymis with PDE5 inhibitors did not affect NA-induced contractions and therefore does not interfere with the emission phase of ejaculation.

It is logical to pursue the investigation of further cGMP signaling pathway components in promoting SMC relaxation, since PDE5 inhibitors like tadalafil have proven efficacy and safety in the treatment of patients with BPH (Porst et al. 2011, Dong et al. 2013, Oelke et al. 2017).

The α_{1A}-adrenoceptor is abundantly expressed in the lower urinary tract and is the principal therapeutic target for the symptomatic treatment of lower urinary tract symptoms in men. Tamsulosin is a highly specific alpha$_{1A}$-antagonist and therefore this subclass of adrenoceptors was addressed. The main target of our studies was to visualize and explain the adverse effects of tamsulosin on organs of the reproductive system involved in the emission phase of ejaculation. However, another important aspect for future studies is the investigation of a subclass of the α_{1A}-adrenoceptor; the α_{1L}-adrenoceptor. This subclassified adrenoceptor is a product of the α_{1A}-adrenoceptor gene, but little is known about the mechanisms of how this altered phenotype is achieved (White et al. 2019). Although pharmacotherapies that target to reduce SMC contractility in the prostate specifically block the α_{1A}-subtype, it is the pharmacologically classified α_{1L}-adrenoceptor that is responsible for mediating the contractile responses in the human (Muramatsu et al. 1994, Israilova et al. 2004) and rat (Hiraoka et al. 1999). Therefore, further research of this subtype is useful for investigation of pharmacological BPH treatment. In the present work, we first wanted to investigate adverse effects of tamsulosin on the alpha$_{1A}$-adrenoceptors before addressing further aspects such as alpha$_{1L}$-adrenoceptors.

6.3 The Newly Synthesized NO-Releasing Donor Star-GSNO Upregulates cGMP Pathway Components and Promotes SMC Relaxation

For the investigation of alternative reagents to promote SMC relaxation in prostate tissue, the macromolecular carrier star polymer containing the NO donor S-Nitrosoglutathione (GSNO) was developed in collaboration with Dr. Michael Whittaker. While best known for its important signaling role in human physiologic functions, NO is also of therapeutic interest.

For this, nanoparticle-based systems such as the GSNO-conjugated core cross-linked star polymer enable the sustained exogenous delivery of NO. In general, these reagents are tested in various potential therapeutic applications (Quinn et al. 2015). NO plays an important role in the research of SMC contractility of the prostate stroma. It is a free radical gas that can pass the cell membrane to the intracellular side and can bind to sGC. sGC, in turn, leads to the accumulation of intracellular cGMP levels ultimately leading to SMC relaxation. This effect is comparable to PDE5 inhibitors, since the inhibition of PDE5 leads to higher availability of cGMP because it is not degraded by PDE5.

Therefore, the rationale is to utilize GSNO and the star-conjugated GSNO polymer to investigate the effects on patient-derived primary SMCs, with emphasis on cGMP signaling pathway components such as sGC, PKG1, and PDE5 *in vitro*. Additionally, we investigated the effects on the contractility (myogenic tone) of both donors in isolated tension recording experiments on vital human prostate specimens. Models established from human tissue were determined with a high clinical relevance because animal models are not capable of replicating the epithelial and stromal structure of the human prostate, together with the multifactorial nature of the pathogenesis of BPH. Vital tissue samples acquired and used in combination with organ bath experiments are, therefore, the closest it can be to physiological conditions. The organ bath used is a custom-made setup to assure that all myogenic contractions, which can be small in magnitude, are recorded.

Only samples from patients undergoing radical prostatectomy were included since samples obtained from TUR-P surgeries were determined to be non-viable for our experiments. The use of a laser to remove excessive prostate tissue results in small

TUR-P “chips” and is extremely damaged by the procedure as it gets burnt and distorted. For radical prostatectomy, the whole prostate is surgically removed and stays mostly intact for experiments. Primary SMCs derived from patients were obtained from radical prostatectomy, as well as the tissue used for tension recording experiments. Limitations of obtained results include the variability of the age of the patients as well as different previous medication prior to surgery. Lastly, every patient has an individual lifestyle, potentially leading to differences and variations of the experimental outcome.

Nevertheless, from the data we were able to acquire, changes in cGMP pathway signaling components on mRNA and protein levels were observed in cell lines incubated with the NO donors, such as increases in PKG1 and PDE5 expression. This indirect effect on the cGMP pathway can be reasoned due to the elevated exposure to NO released from the NO donor. Higher levels of NO lead to increased activity of sGC to mediate the conversion of GTP to cGMP, which ultimately leads to higher levels of the effector protein PKG1. To degrade the high levels of cGMP, more PDE5 is expressed to catalyze hydrolysis. This *in vitro* effect was investigated in the organ bath. Here, the main effect was the reduction of basal tension of the tissue for both donors.

Although the incubation of the tissue with both donors led to the decrease of contraction frequency and amplitude, only for the GSNO alone a significant decrease was observed. This observation can be explained due to the fact that the use of the star polymer GSNO results in a slower but steady release of NO from the core compared to GSNO alone. Therefore, short-term effects such as recordings in the organ bath (total of 30min incubation) have a more rapid effect observed with GSNO alone compared to the star molecule. Incubation with either one of the NO donors for *in vitro* studies was performed for 24h. Here, it seems that the slow but sustained release of NO from the star polymer has a higher impact in the upregulation of cGMP pathway gene transcripts and proteins than GSNO alone (e.g. a 5-fold increase of the transcript of *sGCbeta1* with the star polymer, whereas GSNO alone had no significant upregulation effect of this gene).

Our study shows the potential role of NO donors for the treatment of BPH. Currently, the therapeutic potential of NO is particularly focused and demonstrated in tumor cells

(Evig et al. 2004) and potentially useful in antimicrobial applications (Fang 1997). Therefore, physiological effects in SMCs in the male reproductive system, especially the prostate and for BPH treatment are still unknown. Combined with the limitations of the study mentioned above, more follow-up experiments need to be performed for reliable conclusions.

References

Abd-Elmaksoud, A. (2019). Morphological, Glycohistochemical, and Immunohistochemical Studies on the Embryonic and Adult Bovine Testis.
Abrams, P. (1994). "In support of pressure-flow studies for evaluating men with lower urinary tract symptoms." Urology **44**(2): 153-155.
Adel, A. and M. Khadidja (2017). Canine Prostatic Disorders.
Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2002). Molecular Biology of the Cell, Fourth Edition, Garland Science.
Andersen, J. T., P. Ekman, H. Wolf, H. O. Beisland, J. E. Johansson, M. Kontturi, T. Lehtonen and K. Tveter (1995). "Can finasteride reverse the progress of benign prostatic hyperplasia? A two-year placebo-controlled study. The Scandinavian BPH Study Group." Urology **46**(5): 631-637.
Andersen, J. T., J. C. Nickel, V. R. Marshall, C. C. Schulman and P. Boyle (1997). "Finasteride significantly reduces acute urinary retention and need for surgery in patients with symptomatic benign prostatic hyperplasia." Urology **49**(6): 839-845.
Andersson, K. E. and C. Gratzke (2007). "Pharmacology of alpha1-adrenoceptor antagonists in the lower urinary tract and central nervous system." Nat Clin Pract Urol **4**(7): 368-378.
Andersson, K. E. and K. Persson (1994). "Nitric oxide synthase and nitric oxide-mediated effects in lower urinary tract smooth muscles." World J Urol **12**(5): 274-280.
Andersson, K. E. and A. J. Wein (2004). "Pharmacology of the lower urinary tract: basis for current and future treatments of urinary incontinence." Pharmacol Rev **56**(4): 581-631.
Angulo, J., P. Cuevas, A. Fernandez, J. M. La Fuente, A. Allona, I. Moncada and I. Saenz de Tejada (2012). "Tadalafil enhances the inhibitory effects of tamsulosin on neurogenic contractions of human prostate and bladder neck." J Sex Med **9**(9): 2293-2306.
Aumüller, G. (1983). "Morphologic and endocrine aspects of prostatic function." Prostate **4**(2): 195-214.
Aumüller, G. and A. Riva (1992). "Morphology and functions of the human seminal vesicle." Andrologia **24**(4): 183-196.
Bacha, W. J. and L. M. Bacha (2012). Color Atlas of Veterinary Histology, Wiley.
Barry, M. J., A. T. Cockett, H. L. Holtgrewe, J. D. McConnell, S. A. Sihelnik and H. N. Winfield (1993). "Relationship of symptoms of prostatism to commonly used physiological and anatomical measures of the severity of benign prostatic hyperplasia." J Urol **150**(2 Pt 1): 351-358.
Barry, M. J., F. J. Fowler, Jr., P. O'Leary M, R. C. Bruskewitz, H. L. Holtgrewe, W. K. Mebust and A. T. Cockett (2017). "The American Urological Association Symptom Index for Benign Prostatic Hyperplasia." J Urol **197**(2s): S189-s197.
Barry, M. J., F. J. Fowler, Jr., M. P. O'Leary, R. C. Bruskewitz, H. L. Holtgrewe and W. K. Mebust (1995). "Measuring disease-specific health status in men with benign prostatic hyperplasia. Measurement Committee of The American Urological Association." Med Care **33**(4 Suppl): As145-155.
Barry, M. J., F. J. Fowler, Jr., M. P. O'Leary, R. C. Bruskewitz, H. L. Holtgrewe, W. K. Mebust and A. T. Cockett (1992). "The American Urological Association symptom index for benign prostatic hyperplasia. The Measurement Committee of the American Urological Association." J Urol **148**(5): 1549-1557; discussion 1564.
Baumgarten, H. G., B. Falck, A. F. Holstein, C. Owman and T. Owman (1968). "Adrenergic innervation of the human testis, epididymis, ductus deferens and prostate: a fluorescence microscopic and fluorimetric study." Z Zellforsch Mikrosk Anat **90**(1): 81-95.
Baumgarten, H. G., A. F. Holstein and E. Rosengren (1971). "Arrangement, ultrastructure, and adrenergic innervation of smooth musculature of the ductuli efferentes, ductus epididymidis and ductus deferens of man." Z Zellforsch Mikrosk Anat **120**(1): 37-79.

Bechis, S. K., A. G. Otsetov, R. Ge and A. F. Olumi (2014). "Personalized medicine for the management of benign prostatic hyperplasia." J Urol **192**(1): 16-23.
Beisland, H. O., B. Binkowitz, E. Brekkan, P. Ekman, M. Kontturi, T. Lehtonen, P. Lundmo, F. Pappas, E. Round, D. Shapiro and et al. (1992). "Scandinavian clinical study of finasteride in the treatment of benign prostatic hyperplasia." Eur Urol **22**(4): 271-277.
Benninghoff, A., D. Drenckhahn and W. Zenker (1994). Anatomie: makroskopische Anatomie, Embryologie und Histologie des Menschen, Urban und Schwarzenberg.
Berry, S. J., D. S. Coffey, P. C. Walsh and L. L. Ewing (1984). "The development of human benign prostatic hyperplasia with age." J Urol **132**(3): 474-479.
Berry, S. J., J. D. Strandberg, W. J. Saunders and D. S. Coffey (1986). "Development of canine benign prostatic hyperplasia with age." Prostate **9**(4): 363-373.
Betts, J. G., P. Desaix, J. E. Johnson, O. Korol, D. Kruse, B. Poe, J. A. Wise, M. Womble and K. A. Young (2016). Anatomy and Physiology, 12th Media Services.
Bialy, M., J. Beck, P. Abramczyk, A. Trzebski and J. Przybylski (1996). "Sexual behavior in male rats after nitric oxide synthesis inhibition." Physiol Behav **60**(1): 139-143.
Birbrair, A., T. Zhang, Z. M. Wang, M. L. Messi, G. N. Enikolopov, A. Mintz and O. Delbono (2013). "Role of pericytes in skeletal muscle regeneration and fat accumulation." Stem Cells Dev **22**(16): 2298-2314.
Bogdan, C. (2001). "Nitric oxide and the immune response." Nature Immunology **2**: 907.
Bonkhoff, H. and K. Remberger (1996). "Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model." Prostate **28**(2): 98-106.
Bosch, J. L., W. C. Hop, W. J. Kirkels and F. H. Schroder (1995). "The International Prostate Symptom Score in a community-based sample of men between 55 and 74 years of age: prevalence and correlation of symptoms with age, prostate volume, flow rate and residual urine volume." Br J Urol **75**(5): 622-630.
Boyer, C., M. H. Stenzel and T. P. Davis (2011). "Building nanostructures using RAFT polymerization." Journal of Polymer Science Part A: Polymer Chemistry **49**(3): 551-595.
Boyle, P., A. L. Gould and C. G. Roehrborn (1996). "Prostate volume predicts outcome of treatment of benign prostatic hyperplasia with finasteride: meta-analysis of randomized clinical trials." Urology **48**(3): 398-405.
Bozkurt, O., O. Demir, V. Sen and A. Esen (2015). "Silodosin causes impaired ejaculation and enlargement of seminal vesicles in sexually active men treated for lower urinary tract symptoms suggestive of benign prostatic hyperplasia." Urology **85**(5): 1085-1089.
Braeckman, J. and L. Denis (2017). "Management of BPH then 2000 and now 2016 - From BPH to BPO." Asian J Urol **4**(3): 138-147.
Brasure, M., R. MacDonald, P. Dahm, C. M. Olson, V. A. Nelson, H. A. Fink, M. Risk, B. Rwabasonga and T. J. Wilt (2016). AHRQ Comparative Effectiveness Reviews. Newer Medications for Lower Urinary Tract Symptoms Attributed to Benign Prostaic Hyperplasia: A Review. Rockville (MD), Agency for Healthcare Research and Quality (US).
Bredt, D. S., P. M. Hwang and S. H. Snyder (1990). "Localization of nitric oxide synthase indicating a neural role for nitric oxide." Nature **347**(6295): 768-770.
Bredt, D. S. and S. H. Snyder (1992). "Nitric oxide, a novel neuronal messenger." Neuron **8**(1): 3-11.
Breiner, M., G. Romalo and H. U. Schweikert (1986). "Inhibition of androgen receptor binding by natural and synthetic steroids in cultured human genital skin fibroblasts." Klin Wochenschr **64**(16): 732-737.
Bryan, N. S., K. Bian and F. Murad (2009). "Discovery of the nitric oxide signaling pathway and targets for drug development." Front Biosci (Landmark Ed) **14**: 1-18.
Bullock, T. L. and G. L. Andriole, Jr. (2006). "Emerging drug therapies for benign prostatic hyperplasia." Expert Opin Emerg Drugs **11**(1): 111-123.

Burnett, A. L., M. P. Maguire, S. L. Chamness, D. D. Ricker, M. Takeda, H. Lepor and T. S. Chang (1995). "Characterization and localization of nitric oxide synthase in the human prostate." Urology **45**(3): 435-439.
Burnett, A. L., D. D. Ricker, S. L. Chamness, M. P. Maguire, J. K. Crone, D. S. Bredt, S. H. Snyder and T. S. Chang (1995). "Localization of nitric oxide synthase in the reproductive organs of the male rat." Biol Reprod **52**(1): 1-7.
Buzelin, J. M., S. Roth, C. Geffriaud-Ricouard and M. C. Delauche-Cavallier (1997). "Efficacy and safety of sustained-release alfuzosin 5 mg in patients with benign prostatic hyperplasia. ALGEBI Study Group." Eur Urol **31**(2): 190-198.
Caine, M. (1986). "The present role of alpha-adrenergic blockers in the treatment of benign prostatic hypertrophy." J Urol **136**(1): 1-4.
Caine, M., S. Perlberg and R. Gordon (1975). "The treatment of benign prostatic hypertrophy with flutamide (SCH: 13521): a placebo-controlled study." J Urol **114**(4): 564-568.
Caine, M., A. Pfau and S. Perlberg (1976). "The use of alpha-adrenergic blockers in benign prostatic obstruction." Br J Urol **48**(4): 255-263.
Calmasini, F. B., E. C. Alexandre, F. H. Silva, G. De Nucci, E. Antunes, C. A. D'Ancona and F. Z. Monica (2016). "Soluble Guanylate Cyclase Modulators, BAY 41-2272 and BAY 60-2770, Inhibit Human and Rabbit Prostate Contractility." Urology **94**: 312.e319-312.e315.
Calmasini, F. B., T. Z. Candido, E. C. Alexandre, C. A. D'Ancona, D. Silva, M. A. de Oliveira, G. De Nucci, E. Antunes and F. Z. Monica (2015). "The beta-3 adrenoceptor agonist, mirabegron relaxes isolated prostate from human and rabbit: new therapeutic indication?" Prostate **75**(4): 440-447.
Cano, P., A. Godoy, R. Escamilla, R. Dhir and S. A. Onate (2007). "Stromal-epithelial cell interactions and androgen receptor-coregulator recruitment is altered in the tissue microenvironment of prostate cancer." Cancer Res **67**(2): 511-519.
Capogrosso, P., A. Serino, E. Ventimiglia, L. Boeri, F. Deho, R. Damiano, A. Briganti, F. Montorsi and A. Salonia (2015). "Effects of silodosin on sexual function - realistic picture from the everyday clinical practice." Andrology **3**(6): 1076-1081.
Carson, C., 3rd and R. Rittmaster (2003). "The role of dihydrotestosterone in benign prostatic hyperplasia." Urology **61**(4 Suppl 1): 2-7.
Carson, C. C., M. Rosenberg, J. Kissel and D. G. Wong (2014). "Tadalafil - a therapeutic option in the management of BPH-LUTS." Int J Clin Pract **68**(1): 94-103.
Carvajal, J. A., A. M. Germain, J. P. Huidobro-Toro and C. P. Weiner (2000). "Molecular mechanism of cGMP-mediated smooth muscle relaxation." J Cell Physiol **184**(3): 409-420.
Casabe, A., C. G. Roehrborn, L. F. Da Pozzo, S. Zepeda, R. J. Henderson, S. Sorsaburu, C. Henneges, D. G. Wong and L. Viktrup (2014). "Efficacy and safety of the coadministration of tadalafil once daily with finasteride for 6 months in men with lower urinary tract symptoms and prostatic enlargement secondary to benign prostatic hyperplasia." J Urol **191**(3): 727-733.
Chai, T. C., W. D. Belville, E. J. McGuire and L. Nyquist (1993). "Specificity of the American Urological Association voiding symptom index: comparison of unselected and selected samples of both sexes." J Urol **150**(5 Pt 2): 1710-1713.
Chakrabarty, B., A. Dey, M. Lam, S. Ventura and B. Exintaris (2015). "Tamsulosin modulates, but does not abolish the spontaneous activity in the guinea pig prostate gland." Neurourol Urodyn **34**(5): 482-488.
Chamness, S. L., D. D. Ricker, J. K. Crone, C. L. Dembeck, M. P. Maguire, A. L. Burnett and T. S. Chang (1995). "The effect of androgen on nitric oxide synthase in the male reproductive tract of the rat." Fertil Steril **63**(5): 1101-1107.
Chancellor, M. B. and D. A. Rivas (1993). "American Urological Association symptom index for women with voiding symptoms: lack of index specificity for benign prostate hyperplasia." J Urol **150**(5 Pt 2): 1706-1708; discussion 1708-1709.
Chancellor, M. B., D. A. Rivas, F. X. Keeley, M. A. Lotfi and L. G. Gomella (1994). "Similarity of the American Urological Association Symptom Index among men with benign prostate hyperplasia

(BPH), urethral obstruction not due to BPH and detrusor hyperreflexia without outlet obstruction." Br J Urol **74**(2): 200-203.
Chang, D. F. and J. R. Campbell (2005). "Intraoperative floppy iris syndrome associated with tamsulosin." J Cataract Refract Surg **31**(4): 664-673.
Chapple, C. R. (1996). "Selective alpha 1-adrenoceptor antagonists in benign prostatic hyperplasia: rationale and clinical experience." Eur Urol **29**(2): 129-144.
Chapple, C. R. (2004). "Pharmacological therapy of benign prostatic hyperplasia/lower urinary tract symptoms: an overview for the practising clinician." BJU Int **94**(5): 738-744.
Chapple, C. R., M. L. Aubry, S. James, P. M. Greengrass, G. Burnstock, R. T. Turner-Warwick, E. J. Milroy and M. J. Davey (1989). "Characterisation of human prostatic adrenoceptors using pharmacology receptor binding and localisation." Br J Urol **63**(5): 487-496.
Chaturapanich, G., S. Maythaarttaphong, V. Verawatnapakul and C. Pholpramool (2002). "Mediation of contraction in rat cauda epididymidis by alpha-adrenoceptors." Reproduction **124**(6): 887-892.
Chow, P. H., P. Dockery and A. Cheung (1997). "Innervation of accessory sex glands in the adult male golden hamster and quantitative changes of nerve densities with age." Andrologia **29**(6): 331-342.
Christ, G. J. and K. E. Andersson (2007). "Rho-kinase and effects of Rho-kinase inhibition on the lower urinary tract." Neurourol Urodyn **26**(6 Suppl): 948-954.
Chrysant, S. G. (2013). "Effectiveness and safety of phosphodiesterase 5 inhibitors in patients with cardiovascular disease and hypertension." Curr Hypertens Rep **15**(5): 475-483.
Chung, K. and K. Deisseroth (2013). "CLARITY for mapping the nervous system." Nat Methods **10**(6): 508-513.
Chute, C. G., L. A. Panser, C. J. Girman, J. E. Oesterling, H. A. Guess, S. J. Jacobsen and M. M. Lieber (1993). "The prevalence of prostatism: a population-based survey of urinary symptoms." J Urol **150**(1): 85-89.
Cockett, A. T. K. (1991). Proceedings : the International Consultation on Benign Prostatic Hyperplasia (BPH), Paris, June 26-27, 1991, [Paris], [Scientific Communication International].
Cockett, A. T. K., O. World Health, U. European Association of, S. Organisation Mondiale de la, O. World Health and H. International Consultation on Benign Prostatic (1993). The 2nd international consultation on benign prostatic hyperplasia (BPH), Paris, June 27-30, 1993 : proceedings. Jersey, Scientific Communication International.
Corbin, J. D. and S. H. Francis (1999). "Cyclic GMP phosphodiesterase-5: target of sildenafil." J Biol Chem **274**(20): 13729-13732.
Cornwall, G. A. (2009). "New insights into epididymal biology and function." Hum Reprod Update **15**(2): 213-227.
Costello, L. C. and R. B. Franklin (2009). "Prostatic fluid electrolyte composition for the screening of prostate cancer: a potential solution to a major problem." Prostate cancer and prostatic diseases **12**(1): 17-24.
Cunha, G. R., L. W. Chung, J. M. Shannon, O. Taguchi and H. Fujii (1983). "Hormone-induced morphogenesis and growth: role of mesenchymal-epithelial interactions." Recent Prog Horm Res **39**: 559-598.
Cunha, G. R., A. A. Donjacour, P. S. Cooke, S. Mee, R. M. Bigsby, S. J. Higgins and Y. Sugimura (1987). "The endocrinology and developmental biology of the prostate." Endocr Rev **8**(3): 338-362.
Da Silva, N., V. Cortez-Retamozo, H.-C. Reinecker, M. Wildgruber, E. Hill, D. Brown, F. K. Swirski, M. J. Pittet and S. Breton (2011). "A dense network of dendritic cells populates the murine epididymis." Reproduction (Cambridge, England) **141**(5): 653-663.
De Jonge, C. and C. L. R. Barratt (2006). The sperm cell: Production, maturation, fertilization, regeneration.
de Klerk, D. P. and H. J. Human (1985). "Fluctuations in prostatic glycosaminoglycans during fetal and pubertal growth." Prostate **6**(2): 169-175.

De Marzo, A. M., A. K. Meeker, J. I. Epstein and D. S. Coffey (1998). "Prostate stem cell compartments: expression of the cell cycle inhibitor p27Kip1 in normal, hyperplastic, and neoplastic cells." Am J Pathol **153**(3): 911-919.
De Marzo, A. M., W. G. Nelson, A. K. Meeker and D. S. Coffey (1998). "Stem cell features of benign and malignant prostate epithelial cells." J Urol **160**(6 Pt 2): 2381-2392.
DeKlerk, D. P., D. S. Coffey, L. L. Ewing, I. R. McDermott, W. G. Reiner, C. H. Robinson, W. W. Scott, J. D. Strandberg, P. Talalay, P. C. Walsh, L. G. Wheaton and B. R. Zirkin (1979). "Comparison of spontaneous and experimentally induced canine prostatic hyperplasia." The Journal of clinical investigation **64**(3): 842-849.
Dey, A., R. J. Lang and B. Exintaris (2012). "Nitric oxide signaling pathways involved in the inhibition of spontaneous activity in the guinea pig prostate." J Urol **187**(6): 2254-2260.
Din-Udom, A., S. Sujarit and C. Pholpramool (1985). "Short-term effect of androgen deprivation on intraluminal pressure and contractility of the rat epididymis." J Reprod Fertil **73**(2): 405-410.
Djoseland, O., J. O. Gordeladze, S. Hoglo, J. I. Halse and H. N. Haugen (1980). "Evidence for androgen-dependent phosphodiesterase activity in rat seminal vesicle and epididymis." Int J Androl **3**(4): 363-366.
Domeniconi, R. F., A. C. Souza, B. Xu, A. M. Washington and B. T. Hinton (2016). "Is the Epididymis a Series of Organs Placed Side By Side?" Biol Reprod **95**(1): 10.
Donatucci, C. F., G. B. Brock, E. R. Goldfischer, P. J. Pommerville, A. Elion-Mboussa, J. D. Kissel and L. Viktrup (2011). "Tadalafil administered once daily for lower urinary tract symptoms secondary to benign prostatic hyperplasia: a 1-year, open-label extension study." BJU Int **107**(7): 1110-1116.
Dong, Y., L. Hao, Z. Shi, G. Wang, Z. Zhang and C. Han (2013). "Efficacy and safety of tadalafil monotherapy for lower urinary tract symptoms secondary to benign prostatic hyperplasia: a meta-analysis." Urol Int **91**(1): 10-18.
Drobnis, E. Z. and A. K. Nangia (2017). "Cardiovascular/Pulmonary Medications and Male Reproduction." Adv Exp Med Biol **1034**: 103-130.
Druart, X. and S. de Graaf (2018). "Seminal plasma proteomes and sperm fertility." Anim Reprod Sci **194**: 33-40.
Dun, N. J., S. L. Dun, R. L. Huang, E. C. Dun, C. C. Lai, P. Y. Wong and U. Forstermann (1996). "Distribution of origin of nitric oxide synthase-immunoreactive nerve fibers in the rat epididymis." Brain Res **738**(2): 292-300.
Duong, H. T., Z. Dong, L. Su, C. Boyer, J. George, T. P. Davis and J. Wang (2015). "The use of nanoparticles to deliver nitric oxide to hepatic stellate cells for treating liver fibrosis and portal hypertension." Small **11**(19): 2291-2304.
Eckhardt, M. D., G. E. van Venrooij, H. H. van Melick and T. A. Boon (2001). "Prevalence and bothersomeness of lower urinary tract symptoms in benign prostatic hyperplasia and their impact on well-being." J Urol **166**(2): 563-568.
Ehren, I., J. Adolfsson and N. P. Wiklund (1994). "Nitric oxide synthase activity in the human urogenital tract." Urol Res **22**(5): 287-290.
Ehrlich, Y., R. S. Foster, R. Bihrle, L. Cheng, Y. Tong and M. O. Koch (2010). "Division of prostatic anterior fibromuscular stroma reduces urethral resistance in an ex vivo human prostate model." Urology **76**(2): 511.e510-513.
Elfgen, V., A. Mietens, M. Mewe, T. Hau and R. Middendorff (2018). "Contractility of the epididymal duct - function, regulation and potential drug effects." Reproduction.
Ellem, S. J., E. M. De-Juan-Pardo and G. P. Risbridger (2014). "In vitro modeling of the prostate cancer microenvironment." Adv Drug Deliv Rev **79-80**: 214-221.
Evans, J. D. and S. R. Hill (2015). "A comparison of the available phosphodiesterase-5 inhibitors in the treatment of erectile dysfunction: a focus on avanafil." Patient preference and adherence **9**: 1159-1164.

Evig, C. B., E. E. Kelley, C. J. Weydert, Y. Chu, G. R. Buettner and C. Patrick Burns (2004). "Endogenous production and exogenous exposure to nitric oxide augment doxorubicin cytotoxicity for breast cancer cells but not cardiac myoblasts." Nitric Oxide **10**(3): 119-129.
Exintaris, B., D. T. Nguyen, A. Dey and R. J. Lang (2006). "Spontaneous electrical activity in the prostate gland." Auton Neurosci **126-127**: 371-379.
Exintaris, B., D. T. Nguyen, M. Lam and R. J. Lang (2009). "Inositol trisphosphate-dependent Ca stores and mitochondria modulate slow wave activity arising from the smooth muscle cells of the guinea pig prostate gland." Br J Pharmacol **156**(7): 1098-1106.
F. Holstein, A. (1969). Morphologische Studien am Nebenhoden des Menschen.
Fair, W. R. and J. J. Cordonnier (1978). "The pH of prostatic fluid: a reappraisal and therapeutic implications." J Urol **120**(6): 695-698.
Falconer, D., N. Papageorgiou, K. Salem, W. Y. Lim, A. Katsargyris, E. Avgerinos and D. Tousoulis (2018). "Nitric oxide donors for peripheral artery disease." Curr Opin Pharmacol **39**: 77-85.
Fang, F. C. (1997). "Perspectives series: host/pathogen interactions. Mechanisms of nitric oxide-related antimicrobial activity." The Journal of Clinical Investigation **99**(12): 2818-2825.
Farnsworth, W. E. and J. R. Brown (1963). "TESTOSTERONE METABOLISM IN THE PROSTATE." Natl Cancer Inst Monogr **12**: 323-329.
Fast, W. (2005). "Nitric Oxide Donors: For Pharmaceutical and Biological Applications Edited by Peng George Wang, Tingwei Bill Cai, and Naoyuki Taniguchi. Wiley-VCH Verlag GmbH & Co. KgaA, Weinheim, Germany. 2005. xxi + 390 pp. 17.5 × 25 cm. ISBN 3-527-31015-0. $185.00." Journal of Medicinal Chemistry **48**(15): 5056-5056.
Feelisch, M. (1998). The use of nitric oxide donors in pharmacological studies.
Feldman, B. J. and D. Feldman (2001). "The development of androgen-independent prostate cancer." Nat Rev Cancer **1**(1): 34-45.
Ferguson, J. E., 3rd and C. C. Carson, 3rd (2013). "Phosphodiesterase type 5 inhibitors as a treatment for erectile dysfunction: Current information and new horizons." Arab J Urol **11**(3): 222-229.
Fetters, L. J., A. D. Kiss, D. S. Pearson, G. F. Quack and F. J. Vitus (1993). "Rheological behavior of star-shaped polymers." Macromolecules **26**(4): 647-654.
Fey, B., F. Heni, A. Kuntz, D. F. McDonald, L. Quenu, L. G. Wesson and C. Wilson (2012). Physiologie und Pathologische Physiologie / Physiology and Pathological Physiology / Physiologie Normale et Pathologique, Springer Berlin Heidelberg.
Fibbi, B., A. Morelli, L. Vignozzi, S. Filippi, A. Chavalmane, G. De Vita, M. Marini, M. Gacci, G. B. Vannelli, P. Sandner and M. Maggi (2010). "Characterization of phosphodiesterase type 5 expression and functional activity in the human male lower urinary tract." J Sex Med **7**(1 Pt 1): 59-69.
Francis, S. H., J. L. Busch, J. D. Corbin and D. Sibley (2010). "cGMP-dependent protein kinases and cGMP phosphodiesterases in nitric oxide and cGMP action." Pharmacol Rev **62**(3): 525-563.
Francis, S. H. and J. D. Corbin (1994). "Structure and function of cyclic nucleotide-dependent protein kinases." Annu Rev Physiol **56**: 237-272.
Franklin, R. B., B. Milon, P. Feng and L. C. Costello (2005). "Zinc and zinc transporters in normal prostate and the pathogenesis of prostate cancer." Front Biosci **10**: 2230-2239.
Friebe, A. and D. Koesling (2009). "The function of NO-sensitive guanylyl cyclase: what we can learn from genetic mouse models." Nitric Oxide **21**(3-4): 149-156.
Fukazawa, Y. (2009). Effects of Steroid Hormones and Growth Factors on the Development of the Male Mouse Reproductive Tract In Vitro.
Furchgott, R. F. and D. Jothianandan (1991). "Endothelium-dependent and -independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light." Blood Vessels **28**(1-3): 52-61.
Gacci, M., K. E. Andersson, C. Chapple, M. Maggi, V. Mirone, M. Oelke, H. Porst, C. Roehrborn, C. Stief and F. Giuliano (2016). "Latest Evidence on the Use of Phosphodiesterase Type 5 Inhibitors for the Treatment of Lower Urinary Tract Symptoms Secondary to Benign Prostatic Hyperplasia." Eur Urol **70**(1): 124-133.

Gacci, M., G. Corona, M. Salvi, L. Vignozzi, K. T. McVary, S. A. Kaplan, C. G. Roehrborn, S. Serni, V. Mirone, M. Carini and M. Maggi (2012). "A systematic review and meta-analysis on the use of phosphodiesterase 5 inhibitors alone or in combination with alpha-blockers for lower urinary tract symptoms due to benign prostatic hyperplasia." Eur Urol **61**(5): 994-1003.
Gacci, M., I. Eardley, F. Giuliano, D. Hatzichristou, S. A. Kaplan, M. Maggi, K. T. McVary, V. Mirone, H. Porst and C. G. Roehrborn (2011). "Critical analysis of the relationship between sexual dysfunctions and lower urinary tract symptoms due to benign prostatic hyperplasia." Eur Urol **60**(4): 809-825.
Gao, H. (2012). "Development of Star Polymers as Unimolecular Containers for Nanomaterials." Macromolecular Rapid Communications **33**(9): 722-734.
Garthwaite, J. (2008). "Concepts of neural nitric oxide-mediated transmission." Eur J Neurosci **27**(11): 2783-2802.
Gillenwater, J. Y. (2002). Adult and Pediatric Urology, Lippincott Williams & Wilkins.
Giuliano, F. and P. Clement (2005). "Neuroanatomy and physiology of ejaculation." Annu Rev Sex Res **16**: 190-216.
Goepel, M., A. Wittmann, H. Rubben and M. C. Michel (1997). "Comparison of adrenoceptor subtype expression in porcine and human bladder and prostate." Urol Res **25**(3): 199-206.
Gonzales, G. F. (2001). "Function of seminal vesicles and their role on male fertility." Asian J Androl **3**(4): 251-258.
Gradini, R., M. Realacci, A. Ginepri, G. Naso, C. Santangelo, O. Cela, P. Sale, A. Berardi, E. Petrangeli, M. Gallucci, F. Di Silverio and M. A. Russo (1999). "Nitric oxide synthases in normal and benign hyperplastic human prostate: immunohistochemistry and molecular biology." J Pathol **189**(2): 224-229.
Graham, R. M., D. M. Perez, J. Hwa and M. T. Piascik (1996). "alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling." Circ Res **78**(5): 737-749.
Graham, S. D., T. E. Keane and J. F. Glenn (2010). Glenn's Urologic Surgery, Wolters Kluwer Health/Lippincott Williams & Wilkins.
Gratzke, C., A. Bachmann, A. Descazeaud, M. J. Drake, S. Madersbacher, C. Mamoulakis, M. Oelke, K. A. O. Tikkinen and S. Gravas (2015). "EAU Guidelines on the Assessment of Non-neurogenic Male Lower Urinary Tract Symptoms including Benign Prostatic Obstruction." Eur Urol **67**(6): 1099-1109.
Gray, H. and W. H. Lewis (1918). Anatomy of the Human Body, Lea & Febiger.
Hatzimouratidis, K. (2014). "A review of the use of tadalafil in the treatment of benign prostatic hyperplasia in men with and without erectile dysfunction." Therapeutic advances in urology **6**(4): 135-147.
Hayashi, N., Y. Sugimura, J. Kawamura, A. A. Donjacour and G. R. Cunha (1991). "Morphological and functional heterogeneity in the rat prostatic gland." Biol Reprod **45**(2): 308-321.
Haynes, J. M. (2007). "beta(2) and beta(3)-adrenoceptor inhibition of alpha(1)-adrenoceptor-stimulated Ca(2+) elevation in human cultured prostatic stromal cells." Eur J Pharmacol **570**(1-3): 18-26.
Hayward, S. W., L. S. Baskin, P. C. Haughney, A. R. Cunha, B. A. Foster, R. Dahiya, G. S. Prins and G. R. Cunha (1996). "Epithelial development in the rat ventral prostate, anterior prostate and seminal vesicle." Acta Anat (Basel) **155**(2): 81-93.
Hayward, S. W. and G. R. Cunha (2000). "The prostate: development and physiology." Radiol Clin North Am **38**(1): 1-14.
Hedlund, P., P. Ekstrom, B. Larsson, P. Alm and K. E. Andersson (1997). "Heme oxygenase and NO-synthase in the human prostate--relation to adrenergic, cholinergic and peptide-containing nerves." J Auton Nerv Syst **63**(3): 115-126.
Hellstrom, W. J., M. Gittelman, G. Karlin, T. Segerson, M. Thibonnier, T. Taylor and H. Padma-Nathan (2003). "Sustained efficacy and tolerability of vardenafil, a highly potent selective phosphodiesterase type 5 inhibitor, in men with erectile dysfunction: results of a randomized, double-blind, 26-week placebo-controlled pivotal trial." Urology **61**(4 Suppl 1): 8-14.

Hellstrom, W. J. and S. C. Sikka (2006). "Effects of acute treatment with tamsulosin versus alfuzosin on ejaculatory function in normal volunteers." J Urol **176**(4 Pt 1): 1529-1533.
Hennenberg, M., M. Schott, A. Kan, P. Keller, A. Tamalunas, A. Ciotkowska, B. Rutz, Y. Wang, F. Strittmatter, A. Herlemann, Q. Yu, C. G. Stief and C. Gratzke (2016). "Inhibition of Adrenergic and Non-Adrenergic Smooth Muscle Contraction in the Human Prostate by the Phosphodiesterase 10-Selective Inhibitor TC-E 5005." Prostate **76**(15): 1364-1374.
Herman, A. G. and S. Moncada (2005). "Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis." Eur Heart J **26**(19): 1945-1955.
Heuer, O., S. Ückert, S. A. Machtens, C. G. Stief, D. Tsikas, J. C. Frolich and U. Jonas (2002). "Effects of various nitric oxide donating agents on the contractility and cyclic nucleotide turnover of human seminal vesicles in vitro." Urology **59**(6): 958-962.
Hib, J. and R. Caldeyro-Barcia (1974). "Neurohormonal control of epididymal contractions." Basic Life Sci **4**(Pt. B): 111-126.
Hib, J. and R. O. Ponzio (1977). "Effects of cyproterone acetate on spontaneous contractility of the rat epididymis." Andrologia **9**(4): 329-331.
Hibbs, J. B., R. R. Taintor, Z. Vavrin and E. M. Rachlin (1988). "Nitric oxide: A cytotoxic activated macrophage effector molecule." Biochemical and Biophysical Research Communications **157**(1): 87-94.
Higgins, J. R. and J. A. Gosling (1989). "Studies on the structure and intrinsic innervation of the normal human prostate." Prostate Suppl **2**: 5-16.
Hinton, B. and B. Robaire (2015). The Epididymis. **1:** 691-771.
Hinton, B. T., M. M. Galdamez, A. Sutherland, D. Bomgardner, B. Xu, R. Abdel-Fattah and L. Yang (2011). "How do you get six meters of epididymis inside a human scrotum?" J Androl **32**(6): 558-564.
Hiramatsu, M., I. Maehara, M. Ozaki, N. Harada, S. Orikasa and H. Sasano (1997). "Aromatase in hyperplasia and carcinoma of the human prostate." Prostate **31**(2): 118-124.
Hiraoka, Y., T. Ohmura, M. Oshita, Y. Watanabe, K. Morikawa, O. Nagata, H. Kato, T. Taniguchi and I. Muramatsu (1999). "Binding and functional characterization of alpha1-adrenoceptor subtypes in the rat prostate." Eur J Pharmacol **366**(1): 119-126.
Hisasue, S., R. Furuya, N. Itoh, K. Kobayashi, S. Furuya and T. Tsukamoto (2006). "Ejaculatory disorder caused by alpha-1 adrenoceptor antagonists is not retrograde ejaculation but a loss of seminal emission." Int J Urol **13**(10): 1311-1316.
Ho, C. K. and F. K. Habib (2011). "Estrogen and androgen signaling in the pathogenesis of BPH." Nat Rev Urol **8**(1): 29-41.
Hofmann, F. (2005). "The biology of cyclic GMP-dependent protein kinases." J Biol Chem **280**(1): 1-4.
Hofmann, F., D. Bernhard, R. Lukowski and P. Weinmeister (2009). "cGMP regulated protein kinases (cGK)." Handb Exp Pharmacol(191): 137-162.
Holstein, A. F. (1969). Morphologische Studien am Nebenhoden des Menschen. Stuttgart, Thieme.
Holstein, A. F. (1994). "E. Knobil, J. D. Neill (eds): The Physiology of Reproduction." Andrologia **26**(6): 357-357.
Honig, W. M. (1975). "The Mechanism of Cutting in Electrosurgery." IEEE Transactions on Biomedical Engineering **BME-22**(1): 58-62.
Honner, V. and J. R. Docherty (1999). "Investigation of the subtypes of alpha1-adrenoceptor mediating contractions of rat vas deferens." Br J Pharmacol **128**(6): 1323-1331.
Hu, J., R. Qiao, M. R. Whittaker, J. F. Quinn and T. P. Davis (2017). "Synthesis of Star Polymers by RAFT Polymerization as Versatile Nanoparticles for Biomedical Applications*." Australian Journal of Chemistry **70**(11): 1161-1170.
Huerta, S., S. Chilka and B. Bonavida (2008). Nitric oxide donors: Novel cancer therapeutics (Review).
Huggins, C., W. W. Scott and J. H. Heinen (1942). "CHEMICAL COMPOSITION OF HUMAN SEMEN AND OF THE SECRETIONS OF THE PROSTATE AND SEMINAL VESICLES." American Journal of Physiology-Legacy Content **136**(3): 467-473.

Huizinga, J. D., L. W. Liu, M. G. Blennerhassett, L. Thuneberg and A. Molleman (1992). "Intercellular communication in smooth muscle." Experientia **48**(10): 932-941.
Hunter, D. J., C. M. McKee, N. A. Black and C. F. Sanderson (1994). "Urinary symptoms: prevalence and severity in British men aged 55 and over." J Epidemiol Community Health **48**(6): 569-575.
Ichihara, I., M. Kallio and L. J. Pelliniemi (1978). "Light and electron microscopy of the ducts and their subepithelial tissue in the rat ventral prostate." Cell Tissue Res **192**(3): 381-390.
Ignarro, L. J. (1999). "Nitric oxide: a unique endogenous signaling molecule in vascular biology." Biosci Rep **19**(2): 51-71.
Ignarro, L. J. (2005). "Nitric oxide." Curr Top Med Chem **5**(7): 595.
Ignarro, L. J., K. S. Wood and M. S. Wolin (1982). "Activation of purified soluble guanylate cyclase by protoporphyrin IX." Proc Natl Acad Sci U S A **79**(9): 2870-2873.
Imperato-McGinley, J., R. S. Sanchez, J. R. Spencer, B. Yee and E. D. Vaughan (1992). "Comparison of the effects of the 5 alpha-reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: dose-response studies." Endocrinology **131**(3): 1149-1156.
Isaacs, J. T. (2008). "Prostate stem cells and benign prostatic hyperplasia." Prostate **68**(9): 1025-1034.
Isaacs, J. T. and D. S. Coffey (1989). "Etiology and disease process of benign prostatic hyperplasia." Prostate Suppl **2**: 33-50.
Israilova, M., T. Tanaka, F. Suzuki, S. Morishima and I. Muramatsu (2004). "Pharmacological characterization and cross talk of alpha1a- and alpha1b-adrenoceptors coexpressed in human embryonic kidney 293 cells." J Pharmacol Exp Ther **309**(1): 259-266.
Jaffrey, S. R. and S. H. Snyder (1995). "Nitric oxide: a neural messenger." Annu Rev Cell Dev Biol **11**: 417-440.
James, S., C. R. Chapple, M. I. Phillips, P. M. Greengrass, M. J. Davey, R. T. Turner-Warwick, E. J. Milroy and G. Burnstock (1989). "Autoradiographic analysis of alpha-adrenoceptors and muscarinic cholinergic receptors in the hyperplastic human prostate." J Urol **142**(2 Pt 1): 438-444.
Jen, P. Y., J. S. Dixon and J. A. Gosling (1997). "Co-localization of nitric oxide synthase, neuropeptides and tyrosine hydroxylase in nerves supplying the human post-natal vas deferens and seminal vesicle." Br J Urol **80**(2): 291-299.
Jenkins, G., C. Kemnitz and G. J. Tortora (2009). Anatomy and Physiology: From Science to Life, Wiley.
Jenkinson, D. H. and I. K. Morton (1967). "The role of alpha- and beta- adrenergic receptors in some actions of catecholamines on intestinal smooth muscle." The Journal of physiology **188**(3): 387-402.
Jones, R. C. (1999). "To store or mature spermatozoa? The primary role of the epididymis." Int J Androl **22**(2): 57-67.
Jost, A., J. Prepin and B. Vigier (1977). "Hormones in the morphogenesis of the genital system." Birth Defects Orig Artic Ser **13**(2): 85-97.
Juilfs, D. M., S. Soderling, F. Burns and J. A. Beavo (1999). "Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs)." Rev Physiol Biochem Pharmacol **135**: 67-104.
Kaplan, S. A. (2009). "Side Effects of alpha-Blocker Use: Retrograde Ejaculation." Rev Urol **11**(Suppl 1): S14-18.
Katsumi, H., M. Nishikawa and M. Hashida (2007). "Development of nitric oxide donors for the treatment of cardiovascular diseases." Cardiovasc Hematol Agents Med Chem **5**(3): 204-208.
Kierszenbaum, A. L. and L. Tres (2015). Histology and Cell Biology: An Introduction to Pathology E-Book, Elsevier Health Sciences.
Kim, E. H., J. A. Brockman and G. L. Andriole (2018). "The use of 5-alpha reductase inhibitors in the treatment of benign prostatic hyperplasia." Asian journal of urology **5**(1): 28-32.
Kim, K. S., S. W. Choi, W. J. Bae, S. J. Kim, H. J. Cho, S. H. Hong, J. Y. Lee, T. K. Hwang and S. W. Kim (2015). "Efficacy of a vaporization-resection of the prostate median lobe enlargement and vaporization of the prostate lateral lobe for benign prostatic hyperplasia using a 120-W GreenLight high-performance system laser: the effect on storage symptoms." Lasers Med Sci **30**(4): 1387-1393.

Kinbara, H. and G. R. Cunha (1996). "Ductal heterogeneity in rat dorsal-lateral prostate." Prostate **28**(1): 58-64.
Kittel, B., C. Ruehl-Fehlert, G. Morawietz, J. Klapwijk, M. R. Elwell, B. Lenz, M. G. O'Sullivan, D. R. Roth and P. F. Wadsworth (2004). "Revised guides for organ sampling and trimming in rats and mice – Part 2: A joint publication of the RITA1)and NACAD2)groups." Experimental and Toxicologic Pathology **55**(6): 413-431.
Klotz, T., M. J. Mathers, W. Bloch, W. Nayal and U. Engelmann (1999). "Nitric oxide based influence of nitrates on micturition in patients with benign prostatic hyperplasia." Int Urol Nephrol **31**(3): 335-341.
Kobayashi, S., R. Tang, E. Shapiro and H. Lepor (1993). "Characterization and localization of prostatic alpha 1 adrenoceptors using radioligand receptor binding on slide-mounted tissue section." J Urol **150**(6): 2002-2006.
Kondo, S., Y. Tashima and T. Morita (1993). "Quantitative analysis of adrenergic alpha-1 and alpha-2 receptors in human prostatic urethral tissue." Br J Urol **72**(1): 68-73.
Kong, H. Y. and J. Byun (2013). "Emerging roles of human prostatic Acid phosphatase." Biomolecules & therapeutics **21**(1): 10-20.
Kramer, G., D. Mitteregger and M. Marberger (2007). "Is benign prostatic hyperplasia (BPH) an immune inflammatory disease?" Eur Urol **51**(5): 1202-1216.
Kuciel, R. and W. Ostrowski (1970). "Phosphodiesterase from human prostate gland." Bull Soc Chim Biol (Paris) **52**(10): 1051-1060.
Kügler, R., A. Mietens, M. Seidensticker, S. Tasch, F. M. Wagenlehner, A. Kaschtanow, Y. Tjahjono, C. U. Tomczyk, D. Beyer, G. P. Risbridger, B. Exintaris, S. J. Ellem and R. Middendorff (2018). "Novel imaging of the prostate reveals spontaneous gland contraction and excretory duct quiescence together with different drug effects." Faseb j **32**(3): 1130-1138.
Kuiper, G. G., E. Enmark, M. Pelto-Huikko, S. Nilsson and J. A. Gustafsson (1996). "Cloning of a novel receptor expressed in rat prostate and ovary." Proc Natl Acad Sci U S A **93**(12): 5925-5930.
Kujala, M., S. Hihnala, J. Tienari, K. Kaunisto, J. Hastbacka, C. Holmberg, J. Kere and P. Hoglund (2007). "Expression of ion transport-associated proteins in human efferent and epididymal ducts." Reproduction **133**(4): 775-784.
Kukreja, R. C., F. N. Salloum, A. Das, S. Koka, R. A. Ockaili and L. Xi (2011). "Emerging new uses of phosphodiesterase-5 inhibitors in cardiovascular diseases." Experimental and clinical cardiology **16**(4): e30-e35.
Kuntz, A. and R. E. Morris, Jr. (1946). "Components and distribution of the spermatic nerves and the nerves of the vas deferens." J Comp Neurol **85**: 33-44.
Laitinen, L. and A. Talo (1981). "Effects of adrenergic and cholinergic drugs on electrical and mechanical activities of the rat cauda epididymidis in vitro." J Reprod Fertil **63**(1): 205-209.
Lawrence, M. S., P. Stojanov, P. Polak, G. V. Kryukov, K. Cibulskis, A. Sivachenko, S. L. Carter, C. Stewart, C. H. Mermel, S. A. Roberts, A. Kiezun, P. S. Hammerman, A. McKenna, Y. Drier, L. Zou, A. H. Ramos, T. J. Pugh, N. Stransky, E. Helman, J. Kim, C. Sougnez, L. Ambrogio, E. Nickerson, E. Shefler, M. L. Cortes, D. Auclair, G. Saksena, D. Voet, M. Noble, D. DiCara, P. Lin, L. Lichtenstein, D. I. Heiman, T. Fennell, M. Imielinski, B. Hernandez, E. Hodis, S. Baca, A. M. Dulak, J. Lohr, D. A. Landau, C. J. Wu, J. Melendez-Zajgla, A. Hidalgo-Miranda, A. Koren, S. A. McCarroll, J. Mora, B. Crompton, R. Onofrio, M. Parkin, W. Winckler, K. Ardlie, S. B. Gabriel, C. W. M. Roberts, J. A. Biegel, K. Stegmaier, A. J. Bass, L. A. Garraway, M. Meyerson, T. R. Golub, D. A. Gordenin, S. Sunyaev, E. S. Lander and G. Getz (2013). "Mutational heterogeneity in cancer and the search for new cancer-associated genes." Nature **499**(7457): 214-218.
Lee, C., J. A. Sensibar, S. M. Dudek, R. A. Hiipakka and S. T. Liao (1990). "Prostatic ductal system in rats: regional variation in morphological and functional activities." Biol Reprod **43**(6): 1079-1086.
Lee, C. H., O. Akin-Olugbade and A. Kirschenbaum (2011). "Overview of prostate anatomy, histology, and pathology." Endocrinol Metab Clin North Am **40**(3): 565-575, viii-ix.

Lee, M. (2000). "Tamsulosin for the treatment of benign prostatic hypertrophy." Ann Pharmacother **34**(2): 188-199.
Lee, S. N., B. Chakrabarty, B. Wittmer, M. Papargiris, A. Ryan, M. Frydenberg, N. Lawrentschuk, R. Middendorff, G. P. Risbridger, S. J. Ellem and B. Exintaris (2017). "Age Related Differences in Responsiveness to Sildenafil and Tamsulosin are due to Myogenic Smooth Muscle Tone in the Human Prostate." Sci Rep **7**(1): 10150.
Lepor, H. (1990). "Role of alpha-adrenergic blockers in the treatment of benign prostatic hyperplasia." Prostate Suppl **3**: 75-84.
Lepor, H. (2004). "Pathophysiology, epidemiology, and natural history of benign prostatic hyperplasia." Rev Urol **6 Suppl 9**: S3-S10.
Lepor, H. (2007). "Alpha blockers for the treatment of benign prostatic hyperplasia." Rev Urol **9**(4): 181-190.
Lepor, H. (2009). "Pathophysiology of benign prostatic hyperplasia: insights from medical therapy for the disease." Rev Urol **11**(Suppl 1): S9-s13.
Lepor, H., S. Auerbach, A. Puras-Baez, P. Narayan, M. Soloway, F. Lowe, T. Moon, G. Leifer and P. Madsen (1992). "A randomized, placebo-controlled multicenter study of the efficacy and safety of terazosin in the treatment of benign prostatic hyperplasia." J Urol **148**(5): 1467-1474.
Lepor, H. and G. Machi (1993). "Comparison of AUA symptom index in unselected males and females between fifty-five and seventy-nine years of age." Urology **42**(1): 36-40; discussion 40-31.
Lepor, H., W. O. Williford, M. J. Barry, M. K. Brawer, C. M. Dixon, G. Gormley, C. Haakenson, M. Machi, P. Narayan and R. J. Padley (1996). "The efficacy of terazosin, finasteride, or both in benign prostatic hyperplasia. Veterans Affairs Cooperative Studies Benign Prostatic Hyperplasia Study Group." N Engl J Med **335**(8): 533-539.
Lepor, H., W. O. Williford, M. J. Barry, C. Haakenson and K. Jones (1998). "The impact of medical therapy on bother due to symptoms, quality of life and global outcome, and factors predicting response. Veterans Affairs Cooperative Studies Benign Prostatic Hyperplasia Study Group." J Urol **160**(4): 1358-1367.
Li, Y., S. Laurent, L. Esser, L. V. Elst, R. N. Muller, A. B. Lowe, C. Boyer and T. P. Davis (2014). "The precise molecular location of gadolinium atoms has a significant influence on the efficacy of nanoparticulate MRI positive contrast agents." Polymer Chemistry **5**(7): 2592-2601.
Lincoln, T. M., X. Wu, H. Sellak, N. Dey and C. S. Choi (2006). "Regulation of vascular smooth muscle cell phenotype by cyclic GMP and cyclic GMP-dependent protein kinase." Front Biosci **11**: 356-367.
Lipshultz, L., Howards, S., & Niederberger, C. (2009). Infertility in the Male. Cambridge, Cambridge University Press.
Lipshultz, L. I., S. S. Howards and C. S. Niederberger (2009). Infertility in the Male, Cambridge University Press.
Liu, A. Y. and L. D. True (2002). "Characterization of prostate cell types by CD cell surface molecules." The American journal of pathology **160**(1): 37-43.
Liu, J., H. Duong, M. R. Whittaker, T. P. Davis and C. Boyer (2012). "Synthesis of functional core, star polymers via RAFT polymerization for drug delivery applications." Macromol Rapid Commun **33**(9): 760-766.
Lohmann, S. M. and U. Walter (2005). "Tracking functions of cGMP-dependent protein kinases (cGK)." Front Biosci **10**: 1313-1328.
Loskove, J. A. and W. H. Frishman (1995). "Nitric oxide donors in the treatment of cardiovascular and pulmonary diseases." Am Heart J **129**(3): 604-613.
Lucas, K. A., G. M. Pitari, S. Kazerounian, I. Ruiz-Stewart, J. Park, S. Schulz, K. P. Chepenik and S. A. Waldman (2000). "Guanylyl cyclases and signaling by cyclic GMP." Pharmacol Rev **52**(3): 375-414.
Luo, G. C., K. T. Foo, T. Kuo and G. Tan (2013). "Diagnosis of prostate adenoma and the relationship between the site of prostate adenoma and bladder outlet obstruction." Singapore Med J **54**(9): 482-486.

MacDonald, R., M. Brasure, P. Dahm, C. M. Olson, V. A. Nelson, H. A. Fink, M. C. Risk, B. Rwabasonga and T. J. Wilt (2019). "Efficacy of newer medications for lower urinary tract symptoms attributed to benign prostatic hyperplasia: a systematic review." Aging Male **22**(1): 1-11.
Madar, S., R. Brosh, Y. Buganim, O. Ezra, I. Goldstein, H. Solomon, I. Kogan, N. Goldfinger, H. Klocker and V. Rotter (2009). "Modulated expression of WFDC1 during carcinogenesis and cellular senescence." Carcinogenesis **30**(1): 20-27.
Madhusoodanan, K. S. and F. Murad (2007). "NO-cGMP signaling and regenerative medicine involving stem cells." Neurochem Res **32**(4-5): 681-694.
Mahapokai, W., F. J. Van Sluijs and J. A. Schalken (2000). "Models for studying benign prostatic hyperplasia." Prostate Cancer Prostatic Dis **3**(1): 28-33.
Marker, P. C., A. A. Donjacour, R. Dahiya and G. R. Cunha (2003). "Hormonal, cellular, and molecular control of prostatic development." Dev Biol **253**(2): 165-174.
Massie, C. E., A. Lynch, A. Ramos-Montoya, J. Boren, R. Stark, L. Fazli, A. Warren, H. Scott, B. Madhu, N. Sharma, H. Bon, V. Zecchini, D. M. Smith, G. M. Denicola, N. Mathews, M. Osborne, J. Hadfield, S. Macarthur, B. Adryan, S. K. Lyons, K. M. Brindle, J. Griffiths, M. E. Gleave, P. S. Rennie, D. E. Neal and I. G. Mills (2011). "The androgen receptor fuels prostate cancer by regulating central metabolism and biosynthesis." EMBO J **30**(13): 2719-2733.
McConnell, J. D., C. G. Roehrborn, O. M. Bautista, G. L. Andriole, Jr., C. M. Dixon, J. W. Kusek, H. Lepor, K. T. McVary, L. M. Nyberg, Jr., H. S. Clarke, E. D. Crawford, A. Diokno, J. P. Foley, H. E. Foster, S. C. Jacobs, S. A. Kaplan, K. J. Kreder, M. M. Lieber, M. S. Lucia, G. J. Miller, M. Menon, D. F. Milam, J. W. Ramsdell, N. S. Schenkman, K. M. Slawin and J. A. Smith (2003). "The long-term effect of doxazosin, finasteride, and combination therapy on the clinical progression of benign prostatic hyperplasia." N Engl J Med **349**(25): 2387-2398.
McKay, A. C. and S. Sharma (2018). Anatomy, Abdomen and Pelvis, Seminal Vesicle. StatPearls. Treasure Island (FL), StatPearls Publishing

StatPearls Publishing LLC.
McNeal, J. (1990). "Pathology of benign prostatic hyperplasia. Insight into etiology." Urol Clin North Am **17**(3): 477-486.
McNeal, J. E. (1978). "Origin and evolution of benign prostatic enlargement." Invest Urol **15**(4): 340-345.
McNeal, J. E. (1980). "Anatomy of the prostate: an historical survey of divergent views." Prostate **1**(1): 3-13.
McNeal, J. E. (1981). "The zonal anatomy of the prostate." Prostate **2**(1): 35-49.
McNeal, J. E. (1984). "Anatomy of the prostate and morphogenesis of BPH." Prog Clin Biol Res **145**: 27-53.
McNeal, J. E. (1988). "Normal histology of the prostate." Am J Surg Pathol **12**(8): 619-633.
McNeal, J. E. and D. G. Bostwick (1984). "Anatomy of the prostatic urethra." JAMA **251**(7): 890-891.
McNeal, J. E. and B. W. Company (1983). The Prostate Gland, Morphology and Pathobiology, Burroughs Wellcome Company.
McVary, K. T., K. E. McKenna and C. Lee (1998). "Prostate innervation." Prostate Suppl **8**: 2-13.
McVary, K. T., W. Monnig, J. L. Camps, Jr., J. M. Young, L. J. Tseng and G. van den Ende (2007). "Sildenafil citrate improves erectile function and urinary symptoms in men with erectile dysfunction and lower urinary tract symptoms associated with benign prostatic hyperplasia: a randomized, double-blind trial." J Urol **177**(3): 1071-1077.
McVary, K. T., C. G. Roehrborn, A. L. Avins, M. J. Barry, R. C. Bruskewitz, R. F. Donnell, H. E. Foster, Jr., C. M. Gonzalez, S. A. Kaplan, D. F. Penson, J. C. Ulchaker and J. T. Wei (2011). "Update on AUA guideline on the management of benign prostatic hyperplasia." J Urol **185**(5): 1793-1803.
McVary, K. T., C. G. Roehrborn, J. C. Kaminetsky, S. M. Auerbach, B. Wachs, J. M. Young, A. Esler, G. D. Sides and B. S. Denes (2007). "Tadalafil relieves lower urinary tract symptoms secondary to benign prostatic hyperplasia." J Urol **177**(4): 1401-1407.

Mewe, M., C. K. Bauer, D. Müller and R. Middendorff (2006). "Regulation of spontaneous contractile activity in the bovine epididymal duct by cyclic guanosine 5'-monophosphate-dependent pathways." Endocrinology **147**(4): 2051-2062.
Mewe, M., C. K. Bauer, J. R. Schwarz and R. Middendorff (2006). "Mechanisms regulating spontaneous contractions in the bovine epididymal duct." Biol Reprod **75**(4): 651-659.
Mewe, M., I. Wulfsen, R. Middendorff and C. K. Bauer (2007). "Differential modulation of bovine epididymal activity by oxytocin and noradrenaline." Reproduction **134**(3): 493-501.
Michel, M. C. (2007). "Alpha1-adrenoceptors and ejaculatory function." Br J Pharmacol **152**(3): 289-290.
Michel, M. C., B. Kenny and D. A. Schwinn (1995). "Classification of alpha 1-adrenoceptor subtypes." Naunyn Schmiedebergs Arch Pharmacol **352**(1): 1-10.
Michel, M. C. and W. Vrydag (2006). "Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate." Br J Pharmacol **147 Suppl 2**: S88-119.
Mietens, A., S. Tasch, C. Feuerstacke, G. Eichner, J. Volkmann, R. T. Schermuly, F. Grimminger, D. Muller and R. Middendorff (2012). "Phosphodiesterase 5 (PDE5) inhibition, ANP and NO rapidly reduce epididymal duct contractions, but long-term PDE5 inhibition in vivo does not." Mol Cell Endocrinol **349**(2): 145-153.
Mietens, A., S. Tasch, A. Stammler, L. Konrad, C. Feuerstacke and R. Middendorff (2014). "Time-lapse imaging as a tool to investigate contractility of the epididymal duct--effects of cGMP signaling." PLoS One **9**(3): e92603.
Misono, K. S. (2011). "Natriuretic peptides and their receptors." Febs j **278**(11): 1791.
Mitchell, G. A. (1935). "The Innervation of the Kidney, Ureter, Testicle and Epididymis." J Anat **70**(Pt 1): 10-32.15.
Moncada, S. and E. A. Higgs (2006). Nitric Oxide and the Vascular Endothelium. The Vascular Endothelium I. S. Moncada and A. Higgs. Berlin, Heidelberg, Springer Berlin Heidelberg**:** 213-254.
Mooradian, A. D., J. E. Morley and S. G. Korenman (1987). "Biological actions of androgens." Endocr Rev **8**(1): 1-28.
Moore, E., B. Bracken, W. Bremner, J. Geller, J. Imperato-McGinley, J. McConnell, J. Roy, L. Tenover, D. Vaughan and F. Pappas (1995). "Proscar: five-year experience." Eur Urol **28**(4): 304-309.
Müller, D., A. K. Mukhopadhyay, M. S. Davidoff and R. Middendorff (2011). "Cyclic GMP signaling in rat urinary bladder, prostate, and epididymis: tissue-specific changes with aging and in response to Leydig cell depletion." Reproduction **142**(2): 333-343.
Müllershausen, F., A. Lange, E. Mergia, A. Friebe and D. Koesling (2006). "Desensitization of NO/cGMP signaling in smooth muscle: blood vessels versus airways." Mol Pharmacol **69**(6): 1969-1974.
Muramatsu, I., M. Oshita, T. Ohmura, S. Kigoshi, H. Akino, M. Gobara and K. Okada (1994). "Pharmacological characterization of alpha 1-adrenoceptor subtypes in the human prostate: functional and binding studies." Br J Urol **74**(5): 572-578.
Nacey, J. N., P. Morum and B. Delahunt (1995). "Analysis of the prevalence of voiding symptoms in Maori, Pacific Island, and Caucasian New Zealand men." Urology **46**(4): 506-511.
Nagai, A., R. Hara, T. Yokoyama, Y. Jo, T. Fujii and Y. Miyaji (2008). "Ejaculatory dysfunction caused by the new alpha1-blocker silodosin: A preliminary study to analyze human ejaculation using color Doppler ultrasonography." Int J Urol **15**(10): 915-918.
Narayan, P. and A. Tewari (1998). "A second phase III multicenter placebo controlled study of 2 dosages of modified release tamsulosin in patients with symptoms of benign prostatic hyperplasia. United States 93-01 Study Group." J Urol **160**(5): 1701-1706.
Naslund, M., T. S. Regan, C. Ong and S. L. Hogue (2008). "5-Alpha reductase inhibitors in men with an enlarged prostate: an evaluation of outcomes and therapeutic alternatives." Am J Manag Care **14**(5 Suppl 2): S148-153.
Nasu, K., N. Moriyama, K. Kawabe, G. Tsujimoto, M. Murai, T. Tanaka and J. Yano (1996). "Quantification and distribution of alpha 1-adrenoceptor subtype mRNAs in human prostate:

comparison of benign hypertrophied tissue and non-hypertrophied tissue." Br J Pharmacol **119**(5): 797-803.
Neal, D. E., Jr., S. Clejan, D. Sarma and T. D. Moon (1992). "Prostate specific antigen and prostatitis. I. Effect of prostatitis on serum PSA in the human and nonhuman primate." Prostate **20**(2): 105-111.
Negri-Cesi, P., A. Colciago, A. Poletti and M. Motta (1999). "5alpha-reductase isozymes and aromatase are differentially expressed and active in the androgen-independent human prostate cancer cell lines DU145 and PC3." Prostate **41**(4): 224-232.
Nelson, R. J., L. J. Kriegsfeld, V. L. Dawson and T. M. Dawson (1997). "Effects of Nitric Oxide on Neuroendocrine Function and Behavior." Frontiers in Neuroendocrinology **18**(4): 463-491.
Nemeth, J. A. and C. Lee (1996). "Prostatic ductal system in rats: regional variation in stromal organization." Prostate **28**(2): 124-128.
Nguyen, D. T., A. Dey, R. J. Lang, S. Ventura and B. Exintaris (2011). "Contractility and pacemaker cells in the prostate gland." J Urol **185**(1): 347-351.
Nicholson, T. M. and W. A. Ricke (2011). "Androgens and estrogens in benign prostatic hyperplasia: past, present and future." Differentiation **82**(4-5): 184-199.
Nicholson, T. M., P. D. Sehgal, S. A. Drew, W. Huang and W. A. Ricke (2013). "Sex steroid receptor expression and localization in benign prostatic hyperplasia varies with tissue compartment." Differentiation **85**(4-5): 140-149.
Nickel, J. C. (1999). Textbook of Prostatitis, Taylor & Francis.
Nieschlag, E., H. M. Behre and S. Nieschlag (2012). Testosterone: Action, Deficiency, Substitution, Cambridge University Press.
Norman, R. W., J. C. Nickel, D. Fish and S. N. Pickett (1994). "'Prostate-related symptoms' in Canadian men 50 years of age or older: prevalence and relationships among symptoms." Br J Urol **74**(5): 542-550.
Oelke, M., A. Bachmann, A. Descazeaud, M. Emberton, S. Gravas, M. C. Michel, J. N'Dow, J. Nordling and J. J. de la Rosette (2013). "EAU guidelines on the treatment and follow-up of non-neurogenic male lower urinary tract symptoms including benign prostatic obstruction." Eur Urol **64**(1): 118-140.
Oelke, M., R. Shinghal, A. Sontag, S. K. Baygani and C. F. Donatucci (2015). "Time to onset of clinically meaningful improvement with tadalafil 5 mg once daily for lower urinary tract symptoms secondary to benign prostatic hyperplasia: analysis of data pooled from 4 pivotal, double-blind, placebo controlled studies." J Urol **193**(5): 1581-1589.
Oelke, M., A. Wagg, Y. Takita, H. Buttner and L. Viktrup (2017). "Efficacy and safety of tadalafil 5 mg once daily in the treatment of lower urinary tract symptoms associated with benign prostatic hyperplasia in men aged >/=75 years: integrated analyses of pooled data from multinational, randomized, placebo-controlled clinical studies." BJU Int **119**(5): 793-803.
Oh, S. J., K. M. Kim, Y. S. Chung, E. K. Hong, S. Y. Shin and S. J. Kim (2003). "Ion-channel currents of smooth muscle cells isolated from the prostate of guinea-pig." BJU Int **92**(9): 1022-1030.
Otunctemur, A., H. Besiroglu, M. Dursun, L. Ozcan, E. C. Polat, A. Somay, N. Ozbay, K. Ozer and E. Ozbek (2015). "The comparison of GLUT-4 and nNOS expression in diabetic and non-diabetic patients with BPH/LUTS." Int Urol Nephrol **47**(6): 899-904.
Pacher, P., J. S. Beckman and L. Liaudet (2007). "Nitric Oxide and Peroxynitrite in Health and Disease." Physiological Reviews **87**(1): 315-424.
Parsons, J. K. (2010). "Benign Prostatic Hyperplasia and Male Lower Urinary Tract Symptoms: Epidemiology and Risk Factors." Curr Bladder Dysfunct Rep **5**(4): 212-218.
Pegg, A. E. (2014). "The function of spermine." IUBMB Life **66**(1): 8-18.
Pelletier, G., V. Luu-The, X. F. Huang, H. Lapointe and F. Labrie (1998). "Localization by in situ hybridization of steroid 5alpha-reductase isozyme gene expression in the human prostate and preputial skin." J Urol **160**(2): 577-582.
Pholpramool, C. and N. Triphrom (1984). "Effects of cholinergic and adrenergic drugs on intraluminal pressures and contractility of the rat testis and epididymis in vivo." J Reprod Fertil **71**(1): 181-188.

Pietrement, C., G. H. Sun-Wada, N. D. Silva, M. McKee, V. Marshansky, D. Brown, M. Futai and S. Breton (2006). "Distinct expression patterns of different subunit isoforms of the V-ATPase in the rat epididymis." Biol Reprod **74**(1): 185-194.
Plant, T. M. and A. J. Zeleznik (2014). Knobil and Neill's Physiology of Reproduction, Elsevier Science.
Porst, H., E. D. Kim, A. R. Casabe, V. Mirone, R. J. Secrest, L. Xu, D. P. Sundin and L. Viktrup (2011). "Efficacy and safety of tadalafil once daily in the treatment of men with lower urinary tract symptoms suggestive of benign prostatic hyperplasia: results of an international randomized, double-blind, placebo-controlled trial." Eur Urol **60**(5): 1105-1113.
Potter, L. R. (2011). "Guanylyl cyclase structure, function and regulation." Cell Signal **23**(12): 1921-1926.
Potter, L. R. (2011). "Regulation and therapeutic targeting of peptide-activated receptor guanylyl cyclases." Pharmacol Ther **130**(1): 71-82.
Potter, L. R., A. R. Yoder, D. R. Flora, L. K. Antos and D. M. Dickey (2009). "Natriuretic peptides: their structures, receptors, physiologic functions and therapeutic applications." Handb Exp Pharmacol(191): 341-366.
Preininger, A., J. Vanhauwe, M. Medkova, T. O. Thomas, T. M. Cabrera-Vera, H. E. Hamm and M. R. Mazzoni (2003). "Insights into G Protein Structure, Function, and Regulation." Endocrine Reviews **24**(6): 765-781.
Prins, G. S. and O. Putz (2008). "Molecular signaling pathways that regulate prostate gland development." Differentiation **76**(6): 641-659.
Queiroz, D. B., F. R. Mendes, C. S. Porto and M. C. Avellar (2002). "Alpha1-adrenoceptor subtypes in rat epididymis and the effects of sexual maturation." Biol Reprod **66**(2): 508-515.
Queiroz, D. B., C. S. Porto, G. Grossman, P. Petrusz and M. C. Avellar (2008). "Immunolocalization of alpha(1A)-adrenoceptors in rat and human epididymis." Cell Tissue Res **332**(3): 509-522.
Quinn, J. F., M. R. Whittaker and T. P. Davis (2015). "Delivering nitric oxide with nanoparticles." J Control Release **205**: 190-205.
Rang, H. P. (2016). Rang & Dale's pharmacology 8th ed. [Philadelphia, PA?], Elsevier Churchill Livingstone, an imprint of Elsevier Limited.
Rang, H. P. and M. M. Dale (2016). Rang & Dale's pharmacology. Edinburgh, Elsevier/Churchill Livingstone.
Ren, J. M., T. G. McKenzie, Q. Fu, E. H. H. Wong, J. Xu, Z. An, S. Shanmugam, T. P. Davis, C. Boyer and G. G. Qiao (2016). "Star Polymers." Chemical Reviews **116**(12): 6743-6836.
Rennie, P. S., N. Bruchovsky and S. L. Goldenberg (1988). "Relationship of androgen receptors to the growth and regression of the prostate." Am J Clin Oncol **11 Suppl 2**: S13-17.
Revenig, L., A. Leung and W. Hsiao (2014). "Ejaculatory physiology and pathophysiology: assessment and treatment in male infertility." Transl Androl Urol **3**(1): 41-49.
Risley, P. L. and C. N. Skrepetos (1964). "HISTOCHEMICAL DISTRIBUTION OF CHOLINESTERASES IN THE TESTIS, EPIDIDYMIS AND VAS DEFERENS OF THE RAT." Anat Rec **148**: 231-249.
Rittmaster, R. S. (1997). "5alpha-reductase inhibitors." J Androl **18**(6): 582-587.
Robaire, B., B. Hinton and M. C. Orgebin-Crist (2002). The Epididymis: From Molecules to Clinical Practice: From Molecules to Clinical Practice : a Comprehensive Survey of the Efferent Ducts, the Epididymis and the Vas Deferens, Springer US.
Rodrigues, A. O., M. T. Machado and E. R. Wroclawski (2002). "Prostate innervation and local anesthesia in prostate procedures." Revista do Hospital das Clínicas **57**: 287-292.
Roehrborn, C. G. (2008). "BPH progression: concept and key learning from MTOPS, ALTESS, COMBAT, and ALF-ONE." BJU Int **101 Suppl 3**: 17-21.
Roehrborn, C. G. (2008). "Pathology of benign prostatic hyperplasia." Int J Impot Res **20 Suppl 3**: S11-18.
Roehrborn, C. G. (2011). "Male lower urinary tract symptoms (LUTS) and benign prostatic hyperplasia (BPH)." Med Clin North Am **95**(1): 87-100.

Roehrborn, C. G., P. Boyle, J. C. Nickel, K. Hoefner and G. Andriole (2002). "Efficacy and safety of a dual inhibitor of 5-alpha-reductase types 1 and 2 (dutasteride) in men with benign prostatic hyperplasia." Urology **60**(3): 434-441.
Roehrborn, C. G., S. A. Kaplan, H. Lepor and W. Volinn (2011). "Symptomatic and urodynamic responses in patients with reduced or no seminal emission during silodosin treatment for LUTS and BPH." Prostate Cancer Prostatic Dis **14**(2): 143-148.
Roehrborn, C. G., P. Siami, J. Barkin, R. Damiao, K. Major-Walker, I. Nandy, B. B. Morrill, R. P. Gagnier and F. Montorsi (2010). "The effects of combination therapy with dutasteride and tamsulosin on clinical outcomes in men with symptomatic benign prostatic hyperplasia: 4-year results from the CombAT study." Eur Urol **57**(1): 123-131.
Rosen, R., J. Altwein, P. Boyle, R. S. Kirby, B. Lukacs, E. Meuleman, M. P. O'Leary, P. Puppo, C. Robertson and F. Giuliano (2003). "Lower urinary tract symptoms and male sexual dysfunction: the multinational survey of the aging male (MSAM-7)." Eur Urol **44**(6): 637-649.
Rosier, P. F., M. J. de Wildt, H. Wijkstra, F. F. Debruyne and J. J. de la Rosette (1996). "Clinical diagnosis of bladder outlet obstruction in patients with benign prostatic enlargement and lower urinary tract symptoms: development and urodynamic validation of a clinical prostate score for the objective diagnosis of bladder outlet obstruction." J Urol **155**(5): 1649-1654.
Ross, M. H., E. J. Reith and L. Romrell (1995). Histology : A Text and Atlas.
Rossiter, D., U. o. C. T. D. o. C. Pharmacology, S. A. M. A. Health and M. P. Group (2014). South African Medicines Formulary, Health and Medical Publishing Group of the South African Medical Association.
Roy-Burman, P., H. Wu, W. C. Powell, J. Hagenkord and M. B. Cohen (2004). "Genetically defined mouse models that mimic natural aspects of human prostate cancer development." Endocr Relat Cancer **11**(2): 225-254.
Rumpold, H., K. Mascher, G. Untergasser, E. Plas, M. Hermann and P. Berger (2002). Trans - Differentiation of Prostatic Stromal Cells Leads to Decreased Glycoprotein Hormone α Production.
Rybalkin, S. D., I. G. Rybalkina, R. Feil, F. Hofmann and J. A. Beavo (2002). "Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells." J Biol Chem **277**(5): 3310-3317.
Saboor, F., A. N. Reckmann, C. U. Tomczyk, D. M. Peters, N. Weissmann, A. Kaschtanow, R. T. Schermuly, T. V. Michurina, G. Enikolopov, D. Muller, A. Mietens and R. Middendorff (2016). "Nestin-expressing vascular wall cells drive development of pulmonary hypertension." Eur Respir J **47**(3): 876-888.
Sadava, D. E., H. C. Heller, W. K. Purves, G. H. Orians and D. M. Hillis (2008). Life: The Science of Biology, W. H. Freeman.
Sagnier, P. P., G. MacFarlane, F. Richard, H. Botto, P. Teillac and P. Boyle (1994). "Results of an epidemiological survey using a modified American Urological Association symptom index for benign prostatic hyperplasia in France." J Urol **151**(5): 1266-1270.
Schellack, N. and A. A (2014). A review of phosphodiesterase type 5 inhibitors.
Schlossmann, J., R. Feil and F. Hofmann (2003). "Signaling through NO and cGMP-dependent protein kinases." Ann Med **35**(1): 21-27.
Schoenfeld, M. J., E. F. Shortridge, S. C. Gelwicks, Z. Cui and D. G. Wong (2014). "Treatment patterns in alpha-blocker therapy for benign prostatic hyperplasia." Am J Mens Health **8**(3): 267-272.
Schwinn, D. A. and C. G. Roehrborn (2008). "Alpha1-adrenoceptor subtypes and lower urinary tract symptoms." Int J Urol **15**(3): 193-199.
Sech, S., J. Montoya, C. J. Girman, T. Rhodes and C. G. Roehrborn (2001). "Interexaminer reliability of transrectal ultrasound for estimating prostate volume." J Urol **166**(1): 125-129.
Seiler, P., T. G. Cooper, C. H. Yeung and E. Nieschlag (1999). "Regional variation in macrophage antigen expression by murine epididymal basal cells and their regulation by testicular factors." J Androl **20**(6): 738-746.

Shafik, A., I. Shafik and O. el-Sibai (2005). "Identification of c-kit-positive cells in the human prostate: the interstitial cells of Cajal." Arch Androl **51**(5): 345-351.
Shapiro, E., M. J. Becich, V. Hartanto and H. Lepor (1992). "The relative proportion of stromal and epithelial hyperplasia is related to the development of symptomatic benign prostate hyperplasia." J Urol **147**(5): 1293-1297.
Shapiro, E., V. Hartanto and H. Lepor (1992). "Quantifying the smooth muscle content of the prostate using double-immunoenzymatic staining and color assisted image analysis." J Urol **147**(4): 1167-1170.
Shapiro, E., V. Hartanto and H. Lepor (1992). "The response to alpha blockade in benign prostatic hyperplasia is related to the percent area density of prostate smooth muscle." Prostate **21**(4): 297-307.
Sharma, M., S. Gupta, B. Dhole and A. Kumar (2017). The Prostate Gland. Basics of Human Andrology: A Textbook. A. Kumar and M. Sharma. Singapore, Springer Singapore**:** 17-35.
Shima, S. (1993). "Characterization of adrenergic receptors in membranes from the rat seminal vesicle." Jpn J Pharmacol **61**(2): 87-92.
Shimazaki, J., H. Kurihara, Y. Ito and K. Shida (1965). "Metabolism of testosterone in prostate. 2. Separation of prostatic 17-beta-ol-dehydrogenase and 5-alpha-reductase." Gunma J Med Sci **14**(4): 326-333.
Shum, W. W., T. B. Smith, V. Cortez-Retamozo, L. S. Grigoryeva, J. W. Roy, E. Hill, M. J. Pittet, S. Breton and N. Da Silva (2014). "Epithelial basal cells are distinct from dendritic cells and macrophages in the mouse epididymis." Biology of reproduction **90**(5): 90-90.
Silver, R. I., E. L. Wiley, A. E. Thigpen, J. M. Guileyardo, J. D. McConnell and D. W. Russell (1994). "Cell type specific expression of steroid 5 alpha-reductase 2." J Urol **152**(2 Pt 1): 438-442.
Sipila, P. and I. Bjorkgren (2016). "Segment-specific regulation of epididymal gene expression." Reproduction **152**(3): R91-99.
Sirls, L. T., A. K. Kirkemo and J. Jay (1996). "Lack of correlation of the American Urological Association Symptom 7 Index with urodynamic bladder outlet obstruction." Neurourol Urodyn **15**(5): 447-456; discussion 457.
Siu, E. R., F. Yasuhara, E. Marostica, M. C. Avellar and C. S. Porto (2006). "Expression and localization of muscarinic acetylcholine receptor subtypes in rat efferent ductules and epididymis." Cell Tissue Res **323**(1): 157-166.
SoRelle, R. (1998). "Nobel Prize Awarded to Scientists for Nitric Oxide Discoveries." Circulation **98**(22): 2365-2366.
Speakman, M., R. Kirby, S. Doyle and C. Ioannou (2015). "Burden of male lower urinary tract symptoms (LUTS) suggestive of benign prostatic hyperplasia (BPH) - focus on the UK." BJU Int **115**(4): 508-519.
Stammler, A., T. Hau, S. Bhushan, A. Meinhardt, D. Jonigk, T. Lippmann, A. Pilatz, I. Schneider-Huther and R. Middendorff (2015). "Epididymitis: ascending infection restricted by segmental boundaries." Hum Reprod **30**(7): 1557-1565.
Standring, S. (2005). Gray's Anatomy: The Anatomical Basis of Clinical Practice (ed 39th). Philadelphia.
Steers, W. D. (2001). "5alpha-reductase activity in the prostate." Urology **58**(6 Suppl 1): 17-24; discussion 24.
Steiner, M. S., R. C. Couch, S. Raghow and D. Stauffer (1999). "The chimpanzee as a model of human benign prostatic hyperplasia." J Urol **162**(4): 1454-1461.
Stoevelaar, H. J., C. Van de Beek, A. F. Casparie, J. McDonnell and H. G. Nijs (1999). "Treatment choice for benign prostatic hyperplasia: a matter of urologist preference?" J Urol **161**(1): 133-138.
Stoevelaar, H. J., C. van de Beek, A. F. Casparie, H. G. Nijs, J. McDonnell and R. A. Janknegt (1996). "[Variation in the diagnosis and treatment of benign prostatic hyperplasia in urological practice]." Ned Tijdschr Geneeskd **140**(15): 837-842.

Stoevelaar, H. J., C. van de Beek, H. G. Nijs, A. F. Casparie, J. McDonnell and R. A. Janknegt (1996). "The symptom questionnaire for benign prostatic hyperplasia: an ambiguous indicator for an ambiguous disease." Br J Urol **77**(2): 181-185.
Stone, J. R. and M. A. Marletta (1996). "Spectral and kinetic studies on the activation of soluble guanylate cyclase by nitric oxide." Biochemistry **35**(4): 1093-1099.
Stoner, E., A. I. Mocellini, R. Gardiner, V. Marshall, W. Johnson, G. Bartsch, C. P. Schmidbauer, H. Mossing, P. J. Van Cangh, L. J. Denis, S. Arap, G. C. Freire, D. De Latorre, H. Botto, F. Richard, M. Devonec, P. Teillac, G. Vallancien and Z. Braf (1993). Finasteride (MK-906) in the treatment of benign prostatic hyperplasia.
Studdard, P. W., J. L. Stein and M. J. Cosentino (2002). "The effects of oxytocin and arginine vasopressin in vitro on epididymal contractility in the rat." Int J Androl **25**(2): 65-71.
Sugimura, Y., G. R. Cunha, A. A. Donjacour, R. M. Bigsby and J. R. Brody (1986). "Whole-mount autoradiography study of DNA synthetic activity during postnatal development and androgen-induced regeneration in the mouse prostate." Biol Reprod **34**(5): 985-995.
Sumerlin, B. S. and A. P. Vogt (2010). "Macromolecular Engineering through Click Chemistry and Other Efficient Transformations." Macromolecules **43**(1): 1-13.
Swerdloff, R. S., C. Wang and S. Bhasin (1992). "Developments in the control of testicular function." Baillieres Clin Endocrinol Metab **6**(2): 451-483.
Swyer, G. I. (1944). "Post-natal growth changes in the human prostate." Journal of anatomy **78**(Pt 4): 130-145.
Szekeres, L. and J. Papp (1994). Pharmacology of Smooth Muscle.
Takase, Y., M. H. Levesque, V. Luu-The, M. El-Alfy, F. Labrie and G. Pelletier (2006). "Expression of enzymes involved in estrogen metabolism in human prostate." J Histochem Cytochem **54**(8): 911-921.
Talo, A., U. M. Jaakkola and M. Markkula-Viitanen (1979). "Spontaneous electrical activity of the rat epididymis in vitro." J Reprod Fertil **57**(2): 423-429.
Taplin, M. E. (2007). "Drug insight: role of the androgen receptor in the development and progression of prostate cancer." Nat Clin Pract Oncol **4**(4): 236-244.
Tatemichi, S., K. Kobayashi, R. Yokoi, K. Kobayashi, K. Maruyama, Y. Hoyano, M. Kobayashi, J. Kuroda and H. Kusama (2012). "Comparison of the effects of four alpha1-adrenoceptor antagonists on ejaculatory function in rats." Urology **80**(2): 486.e489-416.
Thomas, L. N., R. C. Douglas, C. B. Lazier, C. K. L. Too, R. S. Rittmaster and D. J. Tindall (2008). "Type 1 and Type 2 5α-Reductase Expression in the Development and Progression of Prostate Cancer." European Urology **53**(2): 244-252.
Thompson, I. M., D. K. Pauler, P. J. Goodman, C. M. Tangen, M. S. Lucia, H. L. Parnes, L. M. Minasian, L. G. Ford, S. M. Lippman, E. D. Crawford, J. J. Crowley and C. A. Coltman (2004). "Prevalence of Prostate Cancer among Men with a Prostate-Specific Antigen Level ⩽4.0 ng per Milliliter." New England Journal of Medicine **350**(22): 2239-2246.
Toda, N., K. Ayajiki and T. Okamura (2012). "Neurogenic and endothelial nitric oxide regulates blood circulation in lingual and other oral tissues." J Cardiovasc Pharmacol **60**(1): 100-108.
Toivanen, R. and M. M. Shen (2017). "Prostate organogenesis: tissue induction, hormonal regulation and cell type specification." Development **144**(8): 1382-1398.
Torre, A., G. Giupponi, D. Duffy, U. P. Conca, T. Cai and A. Scardigli (2015). "Sexual Dysfunction Related to Drugs: a Critical Review. Part V: α-Blocker and 5-ARI Drugs." Pharmacopsychiatry **49**.
Trybek, G., A. Kolasa, M. Marchlewicz, L. Wenda-Rozewicka and B. Wiszniewska (2005). "Immunolocalization of androgen receptor in the epididymis of rats with dihydrotestosterone deficiency." Reprod Biol **5**(3): 291-301.
Turner, T. T., D. Bomgardner, J. P. Jacobs and Q. A. Nguyen (2003). "Association of segmentation of the epididymal interstitium with segmented tubule function in rats and mice." Reproduction **125**(6): 871-878.

Ückert, S., A. Kuthe, U. Jonas and C. G. Stief (2001). "Characterization and functional relevance of cyclic nucleotide phosphodiesterase isoenzymes of the human prostate." J Urol **166**(6): 2484-2490.
Ückert, S., M. Oelke, C. G. Stief, K. E. Andersson, U. Jonas and P. Hedlund (2006). "Immunohistochemical distribution of cAMP- and cGMP-phosphodiesterase (PDE) isoenzymes in the human prostate." Eur Urol **49**(4): 740-745.
Ückert, S., M. Sormes, G. Kedia, F. Scheller, W. H. Knapp, U. Jonas and C. G. Stief (2008). "Effects of phosphodiesterase inhibitors on tension induced by norepinephrine and accumulation of cyclic nucleotides in isolated human prostatic tissue." Urology **71**(3): 526-530.
Ückert, S., E. S. Waldkirch, J. E. Sonnenberg, P. Sandner, M. A. Kuczyk and P. Hedlund (2011). "Expression and distribution of phosphodiesterase isoenzymes in the human seminal vesicles." J Sex Med **8**(11): 3058-3065.
Untergasser, G., S. Madersbacher and P. Berger (2005). "Benign prostatic hyperplasia: age-related tissue-remodeling." Exp Gerontol **40**(3): 121-128.
Vaalasti, A. and A. Hervonen (1980). "Autonomic innervation of the human prostate." Invest Urol **17**(4): 293-297.
van Dijk, M. M., J. J. de la Rosette and M. C. Michel (2006). "Effects of alpha(1)-adrenoceptor antagonists on male sexual function." Drugs **66**(3): 287-301.
Ventura, S. and J. N. Pennefather (1991). "Sympathetic co-transmission to the cauda epididymis of the rat: characterization of postjunctional adrenoceptors and purinoceptors." British journal of pharmacology **102**(2): 540-544.
Veri, J. P., L. Hermo and B. Robaire (1993). "Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the staining of epithelial cells of the testis, efferent ducts, and epididymis of the male rat." J Androl **14**(1): 23-44.
Vignozzi, L., M. Gacci, I. Cellai, A. Morelli, E. Maneschi, P. Comeglio, R. Santi, S. Filippi, A. Sebastianelli, G. Nesi, S. Serni, M. Carini and M. Maggi (2013). "PDE5 inhibitors blunt inflammation in human BPH: a potential mechanism of action for PDE5 inhibitors in LUTS." Prostate **73**(13): 1391-1402.
Von Heland, M. and P. Casale (1994). "Role of alpha blockers in the treatment of BPH: a critical review of clinical results." Arch Esp Urol **47**(9): 873-881; discussion 881-872.
Wadie, B. S., E. H. Ibrahim, J. J. de la Rosette, M. A. Gomha and M. A. Ghoneim (2001). "The relationship of the International Prostate Symptom Score and objective parameters for diagnosing bladder outlet obstruction. Part I: when statistics fail." J Urol **165**(1): 32-34.
Walden, P. D., M. M. Durkin, H. Lepor, J. M. Wetzel, C. Gluchowski and E. L. Gustafson (1997). "Localization of mRNA and receptor binding sites for the alpha 1a-adrenoceptor subtype in the rat, monkey and human urinary bladder and prostate." J Urol **157**(3): 1032-1038.
Walden, P. D., C. Gerardi and H. Lepor (1999). "Localization and expression of the alpha1A-1, alpha1B and alpha1D-adrenoceptors in hyperplastic and non-hyperplastic human prostate." J Urol **161**(2): 635-640.
Waldkirch, E. S., S. Ückert, K. Langnase, K. Richter, U. Jonas, G. Wolf, K. E. Andersson, C. G. Stief and P. Hedlund (2007). "Immunohistochemical distribution of cyclic GMP-dependent protein kinase-1 in human prostate tissue." Eur Urol **52**(2): 495-501.
Waldman, S. A. and F. Murad (1988). "Biochemical mechanisms underlying vascular smooth muscle relaxation: the guanylate cyclase-cyclic GMP system." J Cardiovasc Pharmacol **12 Suppl 5**: S115-118.
Walsh, P. C., G. M. Hutchins and L. L. Ewing (1983). "Tissue content of dihydrotestosterone in human prostatic hyperplasis is not supranormal." J Clin Invest **72**(5): 1772-1777.
Walsh, P. C. and J. D. Wilson (1976). "The induction of prostatic hypertrophy in the dog with androstanediol." The Journal of clinical investigation **57**(4): 1093-1097.
Walter, U. and S. Gambaryan (2009). "cGMP and cGMP-dependent protein kinase in platelets and blood cells." Handb Exp Pharmacol(191): 533-548.

Wang, C. Y., J. D. Shi, C. H. Yan, Q. Wu, H. Klocker, I. Park, C. Lee and J. Zhang (2007). "Development of a cell-isolation method for human prostatic smooth muscle cells based on cell type-specific activation of the SM22 gene promoter." BJU Int **99**(1): 183-188.
Wang, D. C. and J. Q. Wang (2018). "[Neurophysiological effects of seminal vesicles]." Zhonghua Nan Ke Xue **24**(4): 360-363.
Wang, L., X. Zhang, G. Wang, S. S. Visweswariah, G. Lin, Z. Xin, T. F. Lue and C. S. Lin (2015). "Lobe-specific expression of phosphodiesterase 5 in rat prostate." Urology **85**(3): 703.e707-713.
Wang, P. G., M. Xian, X. Tang, X. Wu, Z. Wen, T. Cai and A. J. Janczuk (2002). "Nitric Oxide Donors: Chemical Activities and Biological Applications." Chemical Reviews **102**(4): 1091-1134.
Wang, X., X. Wang, S. Li, Z. Meng, T. Liu and X. Zhang (2014). "Comparative effectiveness of oral drug therapies for lower urinary tract symptoms due to benign prostatic hyperplasia: a systematic review and network meta-analysis." PLoS One **9**(9): e107593.
Wang, X. H., X. Wang, M. J. Shi, S. Li, T. Liu and X. H. Zhang (2015). "Systematic review and meta-analysis on phosphodiesterase 5 inhibitors and alpha-adrenoceptor antagonists used alone or combined for treatment of LUTS due to BPH." Asian J Androl **17**(6): 1022-1032.
Warner, T. D., J. A. Mitchell, H. Sheng and F. Murad (1994). "Effects of cyclic GMP on smooth muscle relaxation." Adv Pharmacol **26**: 171-194.
Wasserman, N. F. (2006). "Benign prostatic hyperplasia: a review and ultrasound classification." Radiol Clin North Am **44**(5): 689-710, viii.
Webb, R. C. (2003). "Smooth muscle contraction and relaxation." Adv Physiol Educ **27**(1-4): 201-206.
Weidner, W., C. Jantos, H. G. Schiefer, G. Haidl and H. J. Friedrich (1991). "Semen parameters in men with and without proven chronic prostatitis." Arch Androl **26**(3): 173-183.
Wein, A. J., L. R. Kavoussi and M. F. Campbell (2012). Campbell-Walsh urology. 10th edition.
Wein, A. J., L. R. Kavoussi, A. C. Novick, A. W. Partin and C. A. Peters (2011). Campbell-Walsh Urology, Elsevier Health Sciences.
White, C. W., E. D. da Silva Junior, L. Lim and S. Ventura (2019). "What makes the alpha1A -adrenoceptor gene product assume an alpha1L -adrenoceptor phenotype?" Br J Pharmacol **176**(14): 2358-2365.
White, C. W., J. H. Xie and S. Ventura (2013). "Age-related changes in the innervation of the prostate gland: implications for prostate cancer initiation and progression." Organogenesis **9**(3): 206-215.
Wilson, J. D. (1980). "The pathogenesis of benign prostatic hyperplasia." Am J Med **68**(5): 745-756.
Wilson, J. D. (2011). "The critical role of androgens in prostate development." Endocrinol Metab Clin North Am **40**(3): 577-590, ix.
Wolfe, L., J. D. Corbin and S. H. Francis (1989). "Characterization of a novel isozyme of cGMP-dependent protein kinase from bovine aorta." J Biol Chem **264**(13): 7734-7741.
Wolfertstetter, S., J. P. Huettner and J. Schlossmann (2013). "cGMP-Dependent Protein Kinase Inhibitors in Health and Disease." Pharmaceuticals (Basel) **6**(2): 269-286.
Wright, A. S., L. N. Thomas, R. C. Douglas, C. B. Lazier and R. S. Rittmaster (1996). "Relative potency of testosterone and dihydrotestosterone in preventing atrophy and apoptosis in the prostate of the castrated rat." J Clin Invest **98**(11): 2558-2563.
Xiang-Yun, L., X. Ying-Wen, X. Chen-Jing, W. Jiu-Jiu, P. Qi, G. Bo and S. Zu-Yue (2010). "Possible mechanism of benign prostatic hyperplasia induced by androgen-estrogen ratios in castrated rats." Indian journal of pharmacology **42**(5): 312-317.
Yalla, S. V., M. P. Sullivan, H. S. Lecamwasam, C. E. DuBeau, M. A. Vickers and E. G. Cravalho (1995). "Correlation of American Urological Association symptom index with obstructive and nonobstructive prostatism." J Urol **153**(3 Pt 1): 674-679; discussion 679-680.
Yamada, S., N. Ashizawa, H. Ushijima, K. Nakayama, E. Hayashi and K. Honda (1987). "Alpha-1 adrenoceptors in human prostate: characterization and alteration in benign prostatic hypertrophy." J Pharmacol Exp Ther **242**(1): 326-330.
Yan, H., H. Zong, Y. Cui, N. Li and Y. Zhang (2014). "The efficacy of PDE5 inhibitors alone or in combination with alpha-blockers for the treatment of erectile dysfunction and lower urinary tract

symptoms due to benign prostatic hyperplasia: a systematic review and meta-analysis." J Sex Med **11**(6): 1539-1545.
Yang, B., J. B. Treweek, R. P. Kulkarni, B. E. Deverman, C. K. Chen, E. Lubeck, S. Shah, L. Cai and V. Gradinaru (2014). "Single-cell phenotyping within transparent intact tissue through whole-body clearing." Cell **158**(4): 945-958.
Yang, Z. W., L. S. Kong, Y. Guo, J. Q. Yin and N. Mills (2006). "Histological changes of the testis and epididymis in adult rats as a result of Leydig cell destruction after ethane dimethane sulfonate treatment: a morphometric study." Asian J Androl **8**(3): 289-299.
Yee, G. C., R. L. Talbert, G. R. Matzke, J. T. DiPiro, B. G. Wells and L. M. Posey (2016). Pharmacotherapy: A Pathophysiologic Approach, Tenth Edition, McGraw-Hill Education.
Yu, S. H., M. Patra, S. Ferrari, P. Ramirez Garcia, N. A. Veldhuis, L. M. Kaminskas, B. Graham, J. F. Quinn, M. R. Whittaker, G. Gasser and T. P. Davis (2018). "Linker chemistry dictates the delivery of a phototoxic organometallic rhenium(i) complex to human cervical cancer cells from core crosslinked star polymer nanoparticles." Journal of Materials Chemistry B **6**(47): 7805-7810.
Zaichick, V. Y., T. V. Sviridova and S. V. Zaichick (1996). "Zinc concentration in human prostatic fluid: normal, chronic prostatitis, adenoma and cancer." Int Urol Nephrol **28**(5): 687-694.
Zhou, Q., R. Nie, G. S. Prins, P. T. Saunders, B. S. Katzenellenbogen and R. A. Hess (2002). "Localization of androgen and estrogen receptors in adult male mouse reproductive tract." J Androl **23**(6): 870-881.

Appendix

I. Original copy of the published manuscript displayed in Chapter 3 entitled:

"Novel Imaging of the Prostate Reveals Spontaneous Gland Contraction and Excretory Duct Quiescence Together with Different Drug Effects"

The FASEB Journal article fj.201700430R. Published online October 31, 2017.

Novel imaging of the prostate reveals spontaneous gland contraction and excretory duct quiescence together with different drug effects

Robert Kügler,*,[1] Andrea Mietens,*,[1] Mathias Seidensticker,*,[1] Sabine Tasch,* Florian M. Wagenlehner,[†] Andre Kaschtanow,* Yudy Tjahjono,* Claudia U. Tomczyk,* Daniela Beyer,* Gail P. Risbridger,[‡] Betty Exintaris,[§] Stuart J. Ellem,[‡] and Ralf Middendorff*,[2]

*Institute of Anatomy and Cell Biology and [†]Department of Urology, Pediatric Urology, and Andrology, Justus-Liebig-University Giessen, Giessen, Germany; [‡]Department of Anatomy and Developmental Biology, Monash University, Melbourne, Victoria, Australia; and [§]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Melbourne, Victoria, Australia

ABSTRACT: Prostate carcinoma and benign prostate hyperplasia (BPH) with associated lower urinary tract symptoms (LUTS) are among the most prevalent and clinically relevant diseases in men. BPH is characterized by an enlargement of prostate tissue associated with increased tone of smooth muscle cells (SMCs) which surround the single glands composing the prostate. Secretions of the glands leave the prostate through local excretory ducts during the emission phase of ejaculation. Pharmacological treatment of BPH suggests different local drug targets based on reduction of prostate smooth muscle tone as the main effect and disturbed ejaculation as a common side effect. This highlights the need for detailed investigation of single prostate glands and ducts. We combined structural and functional imaging techniques—notably, clear lipid-exchanged, acrylamide-hybridized rigid imaging/immunostaining/*in situ* hybridization–compatible tissue-hydrogel (CLARITY) and time-lapse imaging—and defined glands and ducts as distinct SMC compartments in human and rat prostate tissue. The single glands of the prostate (comprising the secretory part) are characterized by spontaneous contractions mediated by the surrounding SMCs, whereas the ducts (excretory part) are quiescent. In both SMC compartments, phosphodiesterase (PDE)-5 is expressed. PDE5 inhibitors have recently emerged as alternative treatment options for BPH. We directly visualized that the PDE5 inhibitors sildenafil and tadalafil act by reducing spontaneous contractility of the glands, thereby reducing the muscle tone of the organ. In contrast, the ductal (excretory) system and thus the prostate's contribution to ejaculation is unaffected by PDE5 inhibitors. Our differentiated imaging approach reveals new details about prostate function and local drug actions and thus may support clinical management of BPH.—Kügler, R., Mietens, A., Seidensticker, M., Tasch, S., Wagenlehner, F. M., Kaschtanow, A., Tjahjono, Y., Tomczyk, C. U., Beyer, D., Risbridger, G. P., Exintaris, B., Ellem, S. J., Middendorff, R. Novel imaging of the prostate reveals spontaneous gland contraction and excretory duct quiescence together with different drug effects. FASEB J. 32, 000–000 (2018). www.fasebj.org

KEY WORDS: contractility · time-lapse imaging · 3D imaging · PDE5 inhibitors

Benign prostatic hyperplasia (BPH) is a highly prevalent, nonmalignant disease that affects aging men and results in serious lower urinary tract symptoms (LUTS) (1). Morphologically, BPH is mainly characterized by an increase in interstitial stroma, leading to an increase in prostate size. In the human prostate, smooth muscle cells (SMCs) are an important component of the interstitial stroma among the glands. Blood vessels and the prostate excretory ducts are additional prostate structures that are characterized by copious amounts of SMCs (2–4). The ducts function to transport secretions from individual glands of the prostate, which are responsible for one-third of the ejaculate (5), toward the urethra and are of particular importance for the emission phase of ejaculation.

Most of the drugs used for the treatment of LUTS and BPH target SMC function (6). α_1-Adrenergic blockers (*e.g.*, tamsulosin and silodosin) are well-established drugs for BPH treatment (7, 8), but are associated with significant

ABBREVIATIONS: BPH, benign prostatic hyperplasia; CLARITY, clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/*in situ* hybridization-compatible tissue-hydrogel; LUTS, lower urinary tract symptoms; PDE, phosphodiesterase; SMA, α-smooth muscle actin; SMC, smooth muscle cell

[1] These authors contributed equally to this work.

[2] Correspondence: Institute of Anatomy and Cell Biology, Justus-Liebig-University Giessen, Aulweg 123, D-35392 Giessen, Germany. E-mail: ralf.middendorff@anatomie.med.uni-giessen.de

doi: 10.1096/fj.201700430R
This article includes supplemental data. Please visit *http://www.fasebj.org* to obtain this information.

Downloaded from www.fasebj.org to IP 216.185.5.2.

side effects such as ejaculation disorders (9–11). Phosphodiesterase (PDE)-5 inhibitors (*e.g.*, tadalafil) are promising new therapeutic options for the treatment of BPH (12–17). PDE5 hydrolyzes the second messenger cGMP, which is produced by binding of NO and natriuretic peptides to specific guanylyl cyclases (18). PDEs essentially regulate the duration of cGMP action (19). In their most recent review, Gacci *et al.* (20) describe a series of cGMP/PDE5 actions in LUTS and BPH such as modulation of oxygenation, inflammation, proliferation, and nerve activity. However, the most established mechanism of action of PDE5 inhibitors is smooth muscle relaxation leading to reduced muscle tone (20, 21). PDE5 inhibitors have been shown to relax isolated prostate strips in numerous species (14, 21–23).

Information on the spatial arrangement of the SMCs and PDE5-expressing cells in the stromal compartment is scarce, whereas direct visualization and demonstration of the effects of PDE5 inhibition has not been shown. Moreover, knowledge about PDE5 expression in the ducts and their susceptibility to PDE5 inhibitors is completely lacking. These data are of particular importance, especially since a characteristic adverse side effect of other BPH therapeutics, such as α_1-adrenergic blockers, is abnormal and decreased ejaculation (24, 25). In this study, we used novel imaging techniques to examine the effects of PDE5 inhibitors on the different muscular structures of the prostate, including those involved in ejaculation.

MATERIALS AND METHODS

Tissues

Human tissue samples originated from patients (age range, 60–79 yr; median, 71.7 ± 6.5) undergoing transurethral monopolar electroresection of the prostate for BPH or radical prostatectomy for prostate cancer. Use of human prostate tissue was approved by the ethics committee of the Medical Faculty, Justus-Liebig-University Giessen, Germany (ethical vote 49/05, 2005), and all patients gave written informed consent.

Rat prostate tissue was obtained from adult Wistar rats housed in the animal facility of Justus-Liebig-University Giessen. Housing, animal care and all procedures were conducted according to the guidelines for animal care and approved by the committee for laboratory animals of Justus-Liebig-University Giessen (JLU no. 469_M and 510_M).

Tissues were fixed in 4% paraformaldehyde for histologic analyses or immediately processed for further investigation. Rat tissue was carefully dissected under a binocular microscope by using fine surgical scissors to isolate single prostate glands and ducts from the ventral lobes (26).

Time-lapse imaging

Tissue was immobilized for transillumination microscopy by embedding in collagen gel (27) and maintained in Minimal Essential Medium (Thermo Fisher Scientific, Waltham, MA, USA). Frames were captured every 2 s. Sildenafil, tadalafil, and noradrenaline were used at a final concentration of 5, 2.5 and 10 μM, respectively, to ensure diffusion into the tissue. The duration of the treatments was 12–15 min. In all series of experiments (total tissue, glands, and ducts), between 5 and 8 different individual samples from 5 to 8 patients or animals were investigated.

To visualize contractile activity, time-lapse images were treated as a stack of frame shots taken at regular time intervals (time stack). By placing a virtual section through the time stack, a specific region (indicated as a blue bar in the relevant overview pictures) can be followed over time (27). Contractions become visible as twitches or little peaks and were marked by arrows, the contractions were counted and analyzed with ImageJ 1.50e (public domain software; U.S. National Institutes of Health, Bethesda, MD, USA). Contrast enhancement was used if necessary to improve discrimination of twitches. Noradrenaline-induced contraction in prostate ducts was quantified by comparing ductal areas before and after addition of noradrenaline (see Fig. 4*D*). Afterward, the tissue used in the assays was paraffin embedded for azan staining.

Immunostaining

For immunofluorescence staining of paraffin sections we used monoclonal mouse anti-α-smooth muscle actin (SMA,1:1000; Millipore-Sigma, St. Louis, MO, USA), rabbit polyclonal anti-PDE5 (1:1000; generous gift from Laurinda Jaffe, University of Connecticut Health Center, Farmington, CT, USA) and the fluorescence-labeled secondary antibodies Cy3 anti-mouse IgG (1:500; Jackson ImmunoResearch, West Grove, PA, USA) and Alexa Fluor 488 anti-rabbit IgG (1:500; Thermo Fisher Scientific, Waltham, MA, USA).

Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/*in situ* hybridization-compatible tissue-hydrogel

Clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/*in situ* hybridization-compatible tissue-hydrogel (CLARITY) (28) is a spatial advancement of common histochemical techniques and allows generating a 3-dimensional image of whole tissues. It offers the possibility of transforming intact biologic tissue into a translucent hydrogel-tissue hybrid by removing lipid components responsible for light scattering and thus tissue opacity. In this form, the hybrid is permeable to agents such as specific antibodies, and imaging against a translucent background can be greatly enhanced .

Patient tissue samples and rat tissue of ~1 mm^3 were treated as described by Yang *et al.* (29) with a few modifications (30). Tissue sections were fixed in 4% paraformaldehyde for 24 h at 4°C. Prostate tissue was incubated in hydrogel-forming solution [acrylamide 4% in PBS, bis-acrylamide 0.05% in PBS and 0.25% 2,2′-azobis(2-methylpropionamidine) dihydrochloride] for 24 h at 4°C followed by polymerization at 37°C for 3 h. Tissue sections were cleared in a solution containing SDS 10% and boric acid 200 mM (pH 7.4) in water, for at least 5 d. Extensive washing steps with PBS-Triton 0.1% were performed before and after adding primary antibodies. Primary and secondary antibodies were the same as mentioned above (see immunostaining), but at dilutions of 1:100 and 1:200, respectively. Antibodies were diluted in PBS-Triton 0.1% and incubated for at least 5 d at room temperature. After further washing steps (24 h), tissue was transferred into a refractory index–matching buffer: Histodenz 88% (D2158; Millipore-Sigma) in 0.02 M phosphate buffer with 0.1% Tween-20 and 0.01% sodium azide (pH 7.5) for 24 h at room temperature. Documentation was performed on an LSM 710 Confocal Laser Scanning Microscope (Zeiss, Jena, Germany), *Z*-stacks were captured every 1.5–5 μm and reconstructed in ImageJ by using the command "3D projects," allowing interpolation and contrast autoenhancement if necessary.

Downloaded from www.fasebj.org to IP 216.185.5.2. *The FASEB Journal* Vol., No. , pp:, November, 2017

Statistical analysis

Only vital samples (confirmed by a visible response to noradrenaline) were included. Normal distribution of the data was checked by the Kolmogorov-Smirnov test. Contraction frequencies were analyzed by paired 1-tailed *t* tests for normally distributed data; otherwise, the Mann-Whitney *U* test was applied. For noradrenaline effects in the ducts, assessed as areas before and after treatment, paired 2-tailed *t* tests were used. All statistical procedures were performed with GraphPad Prism software (version 4.03; GraphPad Software, La Jolla, CA, USA).

RESULTS

All SMC compartments of the prostate show PDE5 expression

Prostate tissue comprises glands and their ducts (**Fig. 1*A*, *B***) which transport glandular secretions toward the urethra. Both are encompassed by interstitial tissue including also blood vessels (Fig. 1*A*). In the human prostate, SMCs (marked by SMA immunostaining) are found, not only in the interstitial tissue around prostate glands (Fig. 1*C, F*) and in blood vessels (Fig. 1*H*), but also in the ducts (Fig. 1*J, L, M*). Bigger ducts (Fig. 1*J, L*) are distinguished by several SMC layers surrounding the epithelial layer (Fig. 1*J, L, M*). In our efforts to reveal the prostate targets of drugs used for BPH treatment, we localized PDE5, the enzyme affected by PDE5 inhibition. PDE5 immunoreactivity was regularly observed in interstitial SMCs, confirmed by double staining of SMA and PDE5 (Fig. 1*C–E*). CLARITY visualized the 3-dimensional arrangement of PDE5-expressing SMCs thereby providing a better understanding of the spatial architecture of these cells which fill the interstitial compartments and are oriented in various directions (Fig. 1*F, G* and Supplemental Movies 1 and 2). PDE5 is also expressed in vascular SMCs (Fig. 1*H, I*) and in the SMCs of the larger (Fig. 1*J–L*) and smaller (Fig. 1*M*) prostatic ducts.

Relaxing effects of PDE5 inhibition can be directly visualized in human prostate

Time-lapse imaging of human prostate tissue was performed to directly visualize contractions of interstitial SMCs in their regular environment and to monitor effects of PDE5 inhibition. Slow spontaneous contractions of the tissue were readily observed. The PDE5 inhibitor sildenafil markedly reduced spontaneous contractile frequency (**Fig. 2*A*** and Supplemental Movie 3). Further treatment with noradrenaline confirmed functional integrity and viability of the tissue sample. Figure 2*C* gives a visual impression of contractions and sildenafil effects; in this example, sildenafil even abolished spontaneous contractions. Statistical analysis showed that PDE5 inhibition by sildenafil significantly reduced spontaneous contractile frequency (Fig. 2*D*). After time-lapse imaging, histologic evaluation of the tissue showed SMCs oriented in the various directions in the interstitial compartment around glandular tissue (Fig. 2*B*).

Isolated prostate glands show spontaneous contractions that are inhibited by PDE5 inhibition

Our CLARITY approach 3-dimensionally demonstrated that the interstitial periglandular SMCs in rat prostate (**Fig. 3*A*** and Supplemental Movie 4) were arranged tighter around the glands, than in the human prostate, where SMCs were widely dispersed in the interstitium (see Fig. 1*F* and Supplemental Movie 1). This structural difference enabled visualization and analysis of the contractile pattern of single, isolated glands with their musculature. Movies revealed spontaneous contractility occurring in a slightly irregular pattern (Fig. 3*B* and Supplemental Movie 5). Following wall movements through the time stack at the indicated position (Fig. 3*B*), the contractile pattern and the drug effects were analyzed (Fig. 3*C*). Addition of sildenafil resulted in a clearly visible (Fig. 3*B, C* and Supplemental Movie 5) and significant (Fig. 3*D*) reduction of contractile frequency. The response to noradrenaline confirmed viability of the tissue (not displayed). Analogous experiments were performed using the PDE5 inhibitor tadalafil. Our findings showed a reduction of spontaneous contractile frequency in rat prostate glands by tadalafil (Fig. 3*E*), as seen before by sildenafil.

In line with our functional studies PDE5 was found in SMCs surrounding rat prostate glands, as indicated by SMA and PDE5 double staining (Fig. 3*F, G*). Corresponding to the human tissue (Fig. 1) PDE5 was also found in SMCs of blood vessels (Fig. 3*F–K* and Supplemental Movie 6) and prostate ducts (Fig. 3*I–K*). Interestingly, the composition of prostate ducts in rat (Fig. 3*I*) and human (Fig. 1*J*) and their PDE5 expression in surrounding SMCs (Fig. 3*I–K vs.* Fig. 1*J–M*) is comparable.

Prostate ducts do not contract spontaneously. Contractions induced by noradrenaline, mediating emission and ejaculation, are not disturbed by PDE5 inhibition.

Whereas the role of PDE5 inhibitors in vasculature has already been discussed (20), nothing is known about the functional relevance of PDE5 inhibitors in prostate ducts with their large amount of PDE5-expressing SMCs. Therefore, prostate ducts were isolated and investigated by our life-imaging approach *ex vivo* (**Fig. 4*A***). As can be seen in Supplemental Movie 7 and the corresponding time stack analyses (Fig. 4*B*) the duct did not show (spontaneous) contractions without treatment, which contrasts with the regular observation of spontaneous contractility in glands (see Figs. 2 and 3). No obvious sildenafil effect was visible in the duct samples—in particular, no dilatation was seen. Noradrenaline, however, when used to test the viability of the tissue and to mimic sympathetic stimulation of the prostate during ejaculation, resulted in a rapid and strong contraction of the whole duct (Fig. 4*B* and Supplemental Movie 7), also illustrated by a distinct area change (Fig. 4*D*). Spillage of secretions from the duct during the noradrenaline-induced contraction was also noted (Supplemental Movie 7). In contrast, the glands

Downloaded from www.fasebj.org to IP 216.185.5.2.

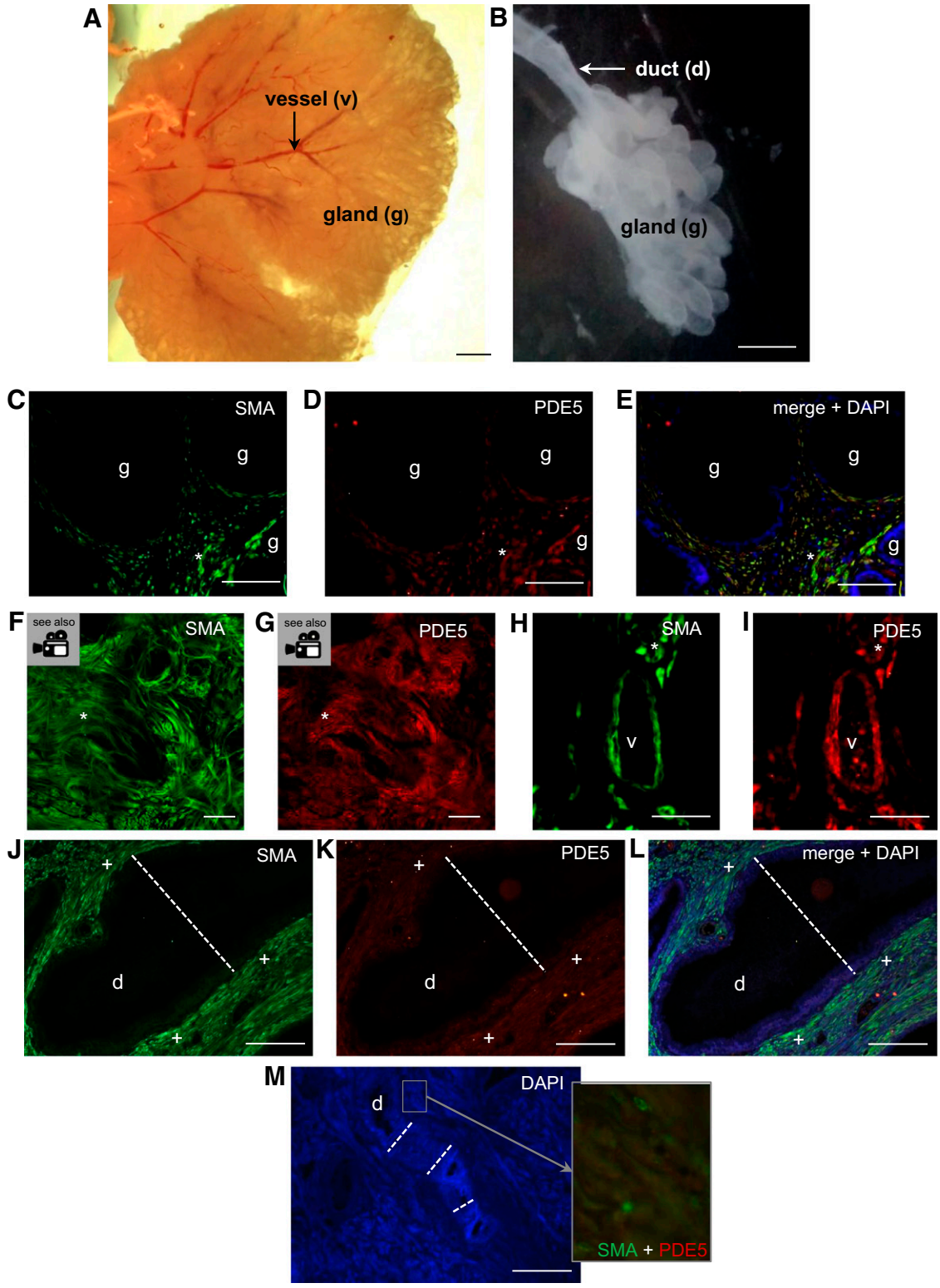


Figure 1. Localization of PDE5 in SMCs of glands, vessels, and ducts of the human prostate. *A*, *B*) Preparation of glandular structures of the prostate showed glands (g) and ducts (d) to be the components of the secretory and transport system in addition to vessels (v) as part of the interstitial tissue. SMC localization of PDE5 in human prostate. *C*–*G*) PDE5 in human interstitial SMCs. In the human prostate, interstitial SMCs were identified by SMA expression in the stromal compartment (*) around the glands (g). PDE5 expression was colocalized to these SMA-positive cells, as shown in the merged image. *E*) DAPI staining helped to identify the location of the epithelium. (*F*, *G*) Movie frame shots from double staining of SMA and PDE5 in the human prostate using a 3-dimensional CLARITY approach showed congruent localization of both antigens. Please refer to the corresponding Supplemental Movies 1 and 2 to appreciate the spatial architecture. *H*, *I*) PDE5 in human vascular SMCs. Immunostaining with SMA and PDE5 confirmed an equal distribution in vascular (v) contractile cells in human prostate, stromal interstitial cells are

(*continued on next page*)

Downloaded from www.fasebj.org to IP 216.185.5.2. *The FASEB Journal* Vol., No. , pp:, November, 2017

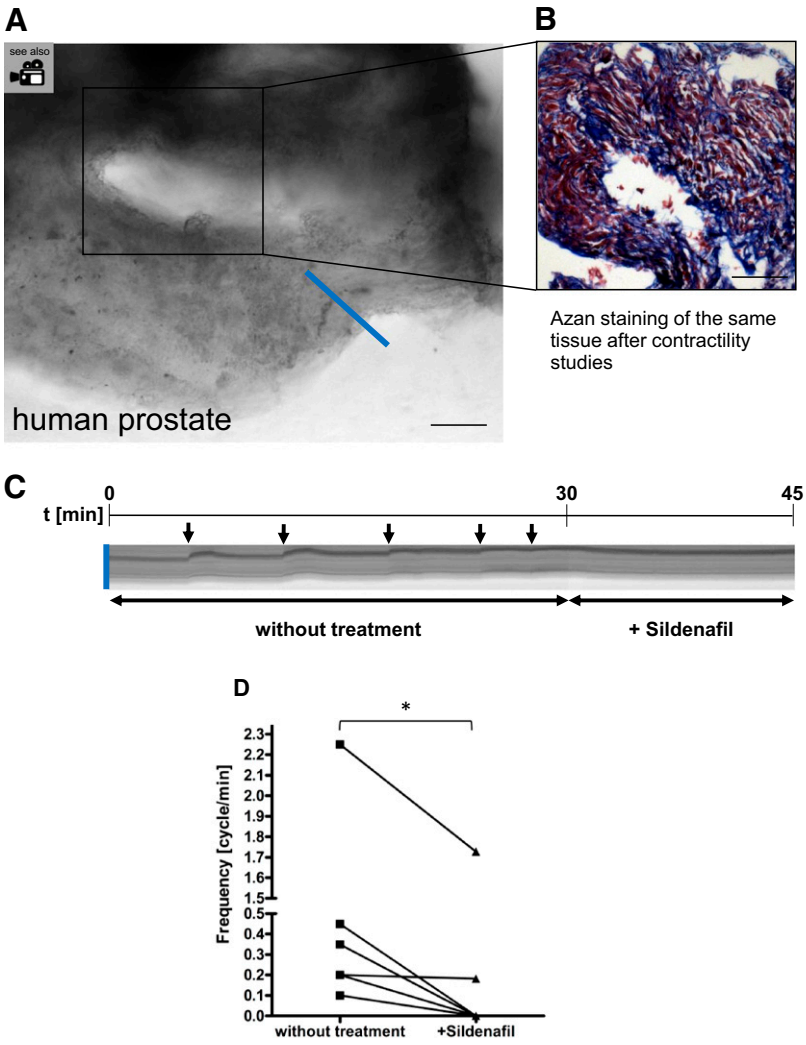


Figure 2. Visualization of spontaneous contractility of human prostate tissue sensitive to PDE5 inhibition. *A*) Frame shot from Supplemental Movie 3 shows a piece of human prostate tissue by transillumination imaging and indicates the location of the virtual slice (blue line) which is followed through the time stack in (*C*). Scale bar, 75 μm. *B*) Azan staining of one 6 μm section from the tissue used in (*A*). Interstitial SMCs stained red. Scale bar, 75 μm. *C*) Contractions elicited small movements of the human prostate tissue and became visible as little twitches (marked by vertical arrows for better visibility). Slow, spontaneous, irregular contractions are visible, and their frequency is reduced (or even abolished in this example) after the addition of the PDE5 inhibitor sildenafil. *D*) Statistical analysis of 6 human samples showed a significant reduction of spontaneous contractile frequency by sildenafil. $*P \leq 0.05$.

contracted only slightly, and no relevant area change was visible.

The PDE5 inhibitors used to treat BPH interfered with the prostatic component of ejaculation. We therefore investigated systematically whether (pre)treatment of the prostate duct with sildenafil changes the contractile response to noradrenaline, the key player for the initiation of ejaculation. For this, we cut prostate ducts in two parts (Fig. 4*C*). Each duct served as its own control (Fig. 4*C*) and was exposed to sildenafil (+Sild.) or vehicle (−Sild.). To assess and quantify noradrenaline-induced contractions, we used frame shots from movies before and after the addition of noradrenaline and compared the area occupied by the tissue as a surrogate parameter for the contraction (Fig. 4*D*). To confirm that the samples used in the contraction assay were correctly classified as prostate ducts, we used Azan-stained histologic sections (Fig. 4*E*) and found the typical architecture of prostate ducts consisting of several smooth muscle layers around the epithelium. Noradrenaline-induced contractions of the isolated duct segments occurred irrespective of sildenafil pretreatment and in both cases, the area after noradrenaline treatment was ~80%, compared with the area before treatment (Fig. 4*F*). Sildenafil did not significantly delay the time from addition of the drug to onset of contractions; rather, a prompt reaction was seen (Fig. 4*G*).

DISCUSSION

In the present study, we combined structural and functional imaging of intact prostate tissue to characterize SMC compartments and their function within the prostate. Our approach using isolated prostate glands and ducts largely maintains tissue architecture and allows assessment of pharmacologic effects. It is therefore suitable for systematic testing of drugs assumed to affect prostate function or

also visible (*). *J–L*) PDE5 in SMCs of a big human prostate duct (d). It shows several layers of SMCs (+) that express SMA and PDE5, as confirmed in the merged image. Dotted lines: the lumen and epithelium of the duct directly surrounded by SMCs (+). *M*) Smaller human prostate duct. DAPI staining shows a longitudinal section of a prostate duct (d). Dotted lines: the diameter (lumen and epithelium). Inset: colocalization of SMA and PDE5 in the SMCs surrounding the epithelium. Scale bars: (*A*) 1 mm; (*B*) 100 μm; (*C–E*) 75 μm; (*F, G*) 200 μm; (H–I) 25 μm; (*J–M*) 50 μm.

Downloaded from www.fasebj.org to IP 216.185.5.2.

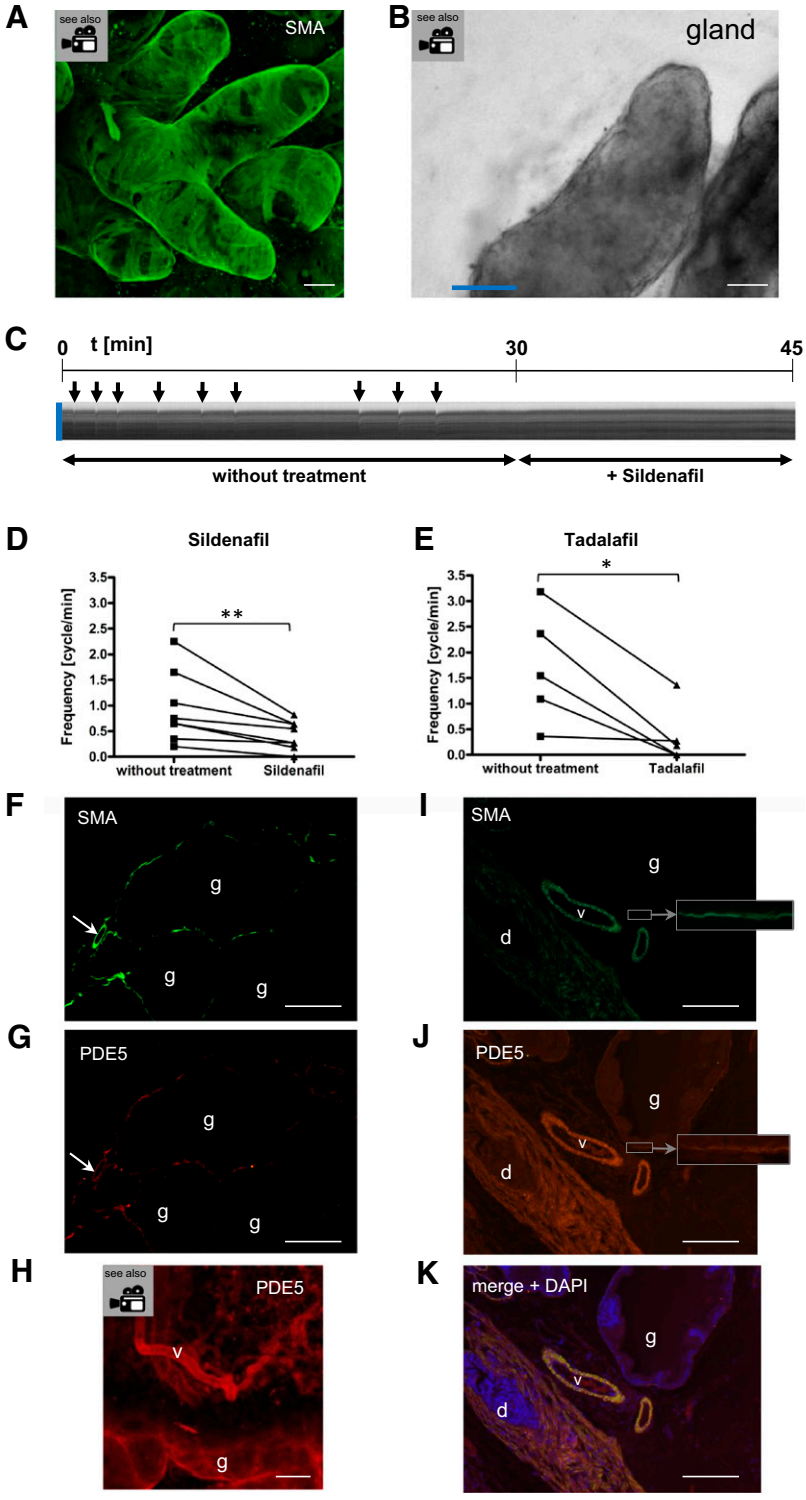


Figure 3. Visualization of contractile pattern in rat prostate gland and PDE5 localization in glandular, vascular, and ductal SMCs. *A*) Frame shot from Supplemental Movie 4 shows SMCs (SMA^+) surrounding rat prostate glands. Visualization by the 3-dimensional CLARITY approach allows appreciation of a tight association of SMCs around the prostate glands. (See Supplemental Movie 4 for 3-dimensional view.) *B*) Frame shot from Supplemental Movie 5 shows a transilluminated view of an isolated prostate gland used for time-lapse imaging. The line at the left lower margin indicates the position of the virtual slice that is followed over time in (*C*). Scale bars: (*A*, *B*) 100 μm. *C*) Time-lapse imaging at the position indicated in (*B*) as a blue line is followed through the time stack. Contractions are visible as twitches (arrows). Spontaneous contractions occur in a slightly irregular pattern. Sildenafil reduced (or, in this example, abolished) contractile frequency. *D*) Sildenafil significantly reduced contractile frequency in rat prostate glands ($n = 8$) $^{**}P < 0.01$. *E*) Comparable results were visible with tadalafil ($n = 5$). $^{*}P < 0.05$. *F*, *G*) SMA and PDE5 immunostaining showed corresponding localizations in periglandular (g) SMCs. A positive vessel was also visible (arrow). *H*) Frame shot of PDE5 expression detected by the CLARITY approach. PDE5 expression is localized to glands (g) and along vessels (v) of the rat. Also refer to Supplemental Movie 6 for 3-dimensional appreciation. Scale bar, 75 μm. *I–K*) Double staining of SMA and PDE5 in gland (g), vessel (v), and duct (d) shows colocalization in all contractile cells (merged image). Insets: enlargements of the periglandular muscle layer. Nuclear counterstain: DAPI. Scale bars, 50 μm.

the prostatic component of the emission phase of ejaculation. Beside the vessels, we discriminated distinct SMC compartments in glands and ducts with different spatial architecture, function, and pharmacologic response to PDE5 inhibition. In prostate ducts which were characterized by high amounts of PDE5-expressing SMCs we directly uncovered the absence of spontaneous contractions and ensuing lack of sildenafil effects. Interestingly, data also showed that noradrenaline-induced contractions of prostatic ducts, as found during ejaculation, remain unaltered in the presence of sildenafil treatment.

Prostate ducts lacked spontaneous contractions, whereas isolated prostate glands exhibited spontaneous contractile activity. These contractions may provide a basal muscular tone and could be important for agitation of secretions in the glandular lumen. This finding supports the idea of pacemaker cells which generate contractile activity, suggested in human and guinea pig

Downloaded from www.fasebj.org to IP 216.185.5.2. *The FASEB Journal* Vol., No. , pp:, November, 2017

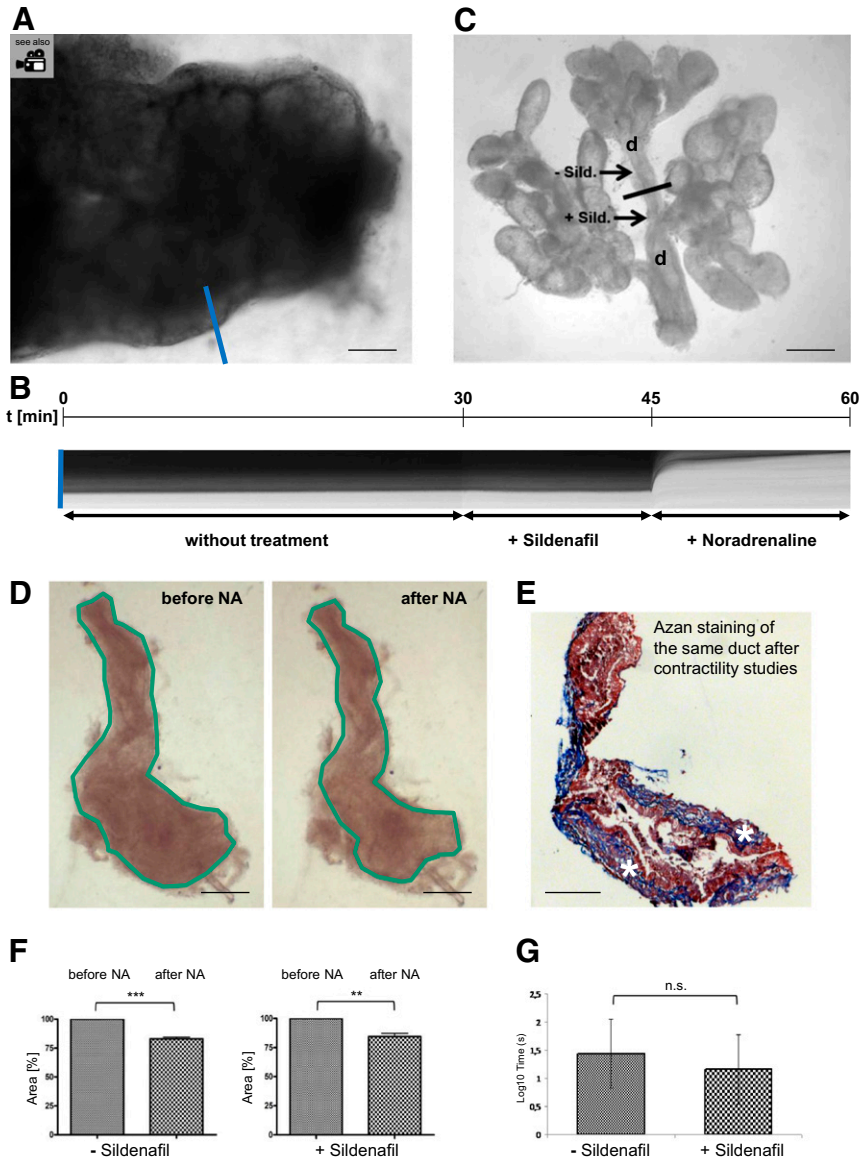


Figure 4. Prostate duct shows absence of spontaneous contractions and no sildenafil effects on noradrenaline-induced contractions. *A*) Frame shot from Supplemental Movie 7 shows the transillumination image of an isolated duct and the position of the virtual section being followed over time (*B*). Scale bar, 75 μm. *B*) Absence of twitches illustrates missing spontaneous activity and no sildenafil effect. When noradrenaline is added, the contraction is large and therefore appears as a sharp peak. Scale bar, 200 μm. *C*) Illustration of the procedure used to assess potential effects of sildenafil pretreatment on noradrenaline-induced duct contractions. Ducts (d) were cut in two parts and exposed to either sildenafil (+Sild.) or vehicle (−Sild.). *D*) Transillumination images of a prostate duct before and after the addition of noradrenaline illustrate area reduction as a means to assess and quantify the contraction. Scale bar, 100 μm. *E*) Corresponding azan staining of the material used in (*D*) confirms that visualization was performed in a duct portion of prostate tissue. Asterisks: characteristic smooth muscle layers of the duct. Scale bar, 150 μm. *F*) Contraction after the addition of noradrenaline occurred irrespective of sildenafil pretreatment. Area before addition of noradrenaline was set as 100%. $^{**}P < 0.01$; $^{***}P < 0.005$. *G*) No significant difference (n.s.) was found in the time delay from addition of noradrenaline to the beginning of the contraction.

prostate cells, independent of neuronal activation (31). Because noradrenaline effects on glandular acini were relatively weak, contractions of the secretory system (glands) may be of limited importance in the context of ejaculation physiology, different from the transport system (excretory ducts). Periglandular SMCs were shown to express PDE5, and the addition of sildenafil or tadalafil resulted in diminished contractile frequency. It would be interesting to know whether long-term treatment with PDE5 inhibitors results in an increasing number of prostatic concretions, caused by reduced agitation of secretions. Currently, long-term experience with PDE5 inhibitors in men with LUTS is limited to one trial with tadalafil with only a 1 yr follow-up (32).

Because of the different architecture of interstitial SMCs in the prostate, functionally intact glands can be isolated only in rodents. Intact human tissue pieces and isolated rat glands showed spontaneous contractility in a slightly irregular pattern at a similar range of frequencies, and reactions to drugs like sildenafil and noradrenaline are comparable. Our data suggest that in human stromal tissue (with its high amounts of SMCs around glandular structures), the physiologic role of spontaneous contractions may also be associated with glandular function.

Prostate ducts may be seen as a conduit serving to expel secretions during ejaculation which is highlighted by our finding of powerful contractions observed upon noradrenaline exposure, whereas in unstimulated conditions, no spontaneous contractile activity was detected. To our knowledge, this study is the first to investigate the effects of a PDE5 inhibitor on prostate ducts. PDE5 inhibitors did not affect noradrenaline-induced contractions that mediate ejaculation. Thus, PDE5 inhibitor-induced disturbances of prostate secretion during ejaculation are unlikely. This finding is of clinical relevance, given that drugs alternatively used for the treatment of BPH such as α_1-adrenergic blockers are known to negatively affect ejaculation (24, 25). Whereas α_1-adrenergic receptor inhibitors and ejaculation-mediating noradrenaline bind to the same membrane receptor (33), PDE5 inhibitors do not;

Downloaded from www.fasebj.org to IP 216.185.5.2.

they inhibit hydrolysis of intracellular cGMP by PDE5 (34). The insensitivity to PDE5 inhibitors in the ducts cannot be explained by missing PDE5 in these structures, as PDE5 was readily detected in SMCs of prostate ducts from both human and rat. However, the precise role of PDE5 in prostate ducts is not clear. It is plausible that (basal) PDE5 activity limits cGMP levels and thus reduces SMC relaxation. Thereby a basic tension of the duct musculature may be maintained to ensure the rapid catecholamine-induced contractions that are necessary for ejaculation.

In comparison to sole analyses of ejaculation and ejaculate, our data give much more information about the physiology of the prostatic component of ejaculation. Different from organ bath studies with strips of the whole prostate (14, 21–23), our methodology allows discrimination between effects on SMCs of the ducts and the interstitial periglandular compartment.

The CLARITY approach (28, 30) added the third dimension to the usual histologic techniques and valuable information to our understanding of the spatial arrangement and function of SMCs around the prostate glands. In human prostate, SMCs not only surrounded the epithelial layer of the glands, but were detectable in all parts of the interstitial compartment with different orientation. In the rat, the 3-dimensional projection of the SMCs showed a bandage-like arrangement around the glands, with regions devoid of SMCs consistent with the observation of "patchy" contractile activity in our functional studies.

Besides PDE5 [see Gacci *et al.* (20)], other components of cGMP pathways now represent potential targets for therapeutic intervention. These include other PDEs (35), as well as cGMP-generating guanylyl cyclases (23, 36, 37). In all cases, the beneficial cGMP effect for patients with BPH may be the reduction of muscular tone in the pathologically enlarged interstitial compartment, along with the maintenance of the noradrenaline-induced contractile function in the ducts.

In summary, our study advances our understanding of different SMC compartments in the prostate with respect to their distinct spatial arrangement, physiologic function, and pharmacologic responses. FJ

ACKNOWLEDGMENTS

The authors thank Dr. Laurinda Jaffe (University of Connecticut Health Center, Farmington, CT), for providing us with anti-PDE5 antibodies; Dr. Dieter Müller (Justus-Liebig-University Giessen, Germany), for helpful discussions; and Ingrid Schneider-Hüther, Tania Bloch, and Kerstin Wilhelm (Justus-Liebig-University), for excellent technical assistance. Funding was provided by the Deutsche Forschungsgemeinschaft Grant GRK 1871 and funding from Monash University, Australia, to the International Research Training Group (IRTG), a collaboration between Justus-Liebig-University Giessen and Monash University. The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

R. Kügler, M. Seidensticker, and S. Tasch performed the experiments; A. Kaschtanow and C. U. Tomczyk performed the CLARITY imaging; R. Kügler, A. Mietens, M. Seidensticker, and D. Beyer contributed to the statistical analyses; F. M. Wagenlehner provided material support; F. M. Wagenlehner, Y. Tjahjono, G. P. Risbridger, B. Exintaris, and S. Ellem contributed valuable advice to the redaction of the manuscript; R. Kügler, A. Mietens, and R. Middendorff wrote the manuscript; and R. Middendorff designed the study and directed the project.

REFERENCES

1. Roehrborn, C. G. (2008) Pathology of benign prostatic hyperplasia. *Int. J. Impot. Res.* **20**(Suppl 3), S11–S18
2. Ichihara, I., Kallio, M., and Pelliniemi, L. J. (1978) Light and electron microscopy of the ducts and their subepithelial tissue in the rat ventral prostate. *Cell Tissue Res.* **192**, 381–390
3. Lee, C., Sensibar, J. A., Dudek, S. M., Hiipakka, R. A., and Liao, S. T. (1990) Prostatic ductal system in rats: regional variation in morphological and functional activities. *Biol. Reprod.* **43**, 1079–1086
4. Nemeth, J. A., and Lee, C. (1996) Prostatic ductal system in rats: regional variation in stromal organization. *Prostate* **28**, 124–128
5. Plant, T. M., and Zeleznik, A. J. (2015) *Knobil and Neill's Physiology of Reproduction*, Elsevier Science, Burlington, MA, USA
6. McNeal, J. E. (1983) *The Prostate Gland, Morphology and Pathobiology*, Burroughs Wellcome Company, Princeton, NJ, USA
7. AUA Practice Guidelines Committee (2003) AUA guideline on management of benign prostatic hyperplasia (2003). Chapter 1: diagnosis and treatment recommendations. *J. Urol.* **170**, 530–547
8. McVary, K. T., Roehrborn, C. G., Avins, A. L., Barry, M. J., Bruskewitz, R. C., Donnell, R. F., Foster, H. E., Jr., Gonzalez, C. M., Kaplan, S. A., Penson, D. F., Ulchaker, J. C., and Wei, J. T. (2011) Update on AUA guideline on the management of benign prostatic hyperplasia. *J. Urol.* **185**, 1793–1803
9. Bozkurt, O., Demir, O., Sen, V., and Esen, A. (2015) Silodosin causes impaired ejaculation and enlargement of seminal vesicles in sexually active men treated for lower urinary tract symptoms suggestive of benign prostatic hyperplasia. *Urology* **85**, 1085–1089
10. Capogrosso, P., Serino, A., Ventimiglia, E., Boeri, L., Dehò, F., Damiano, R., Briganti, A., Montorsi, F., and Salonia, A. (2015) Effects of silodosin on sexual function: realistic picture from the everyday clinical practice. *Andrology* **3**, 1076–1081
11. Tatemichi, S., Kobayashi, K., Yokoi, R., Kobayashi, K., Maruyama, K., Hoyano, Y., Kobayashi, M., Kuroda, J., and Kusama, H. (2012) Comparison of the effects of four α1-adrenoceptor antagonists on ejaculatory function in rats. *Urology* **80**, 486.e9–486.e16
12. Wang, X.-H., Wang, X., Shi, M.-J., Li, S., Liu, T., and Zhang, X.-H. (2015) Systematic review and meta-analysis on phosphodiesterase 5 inhibitors and α-adrenoceptor antagonists used alone or combined for treatment of LUTS due to BPH. *Asian J. Androl.* **17**, 1022–1032
13. Oelke, M., Bachmann, A., Descazeaud, A., Emberton, M., Gravas, S., Michel, M. C., N'dow, J., Nordling, J., and de la Rosette, J. J.; European Association of Urology. (2013) EAU guidelines on the treatment and follow-up of non-neurogenic male lower urinary tract symptoms including benign prostatic obstruction. *Eur. Urol.* **64**, 118–140
14. Ückert, S., Küthe, A., Jonas, U., and Stief, C. G. (2001) Characterization and functional relevance of cyclic nucleotide phosphodiesterase isoenzymes of the human prostate. *J. Urol.* **166**, 2484–2490
15. Gacci, M., Corona, G., Salvi, M., Vignozzi, L., McVary, K. T., Kaplan, S. A., Roehrborn, C. G., Serni, S., Mirone, V., Carini, M., and Maggi, M. (2012) A systematic review and meta-analysis on the use of phosphodiesterase 5 inhibitors alone or in combination with α-blockers for lower urinary tract symptoms due to benign prostatic hyperplasia. *Eur. Urol.* **61**, 994–1003
16. Wang, X., Wang, X., Li, S., Meng, Z., Liu, T., and Zhang, X. (2014) Comparative effectiveness of oral drug therapies for lower urinary tract symptoms due to benign prostatic hyperplasia: a systematic review and network meta-analysis. *PLoS One* **9**, e107593

Downloaded from www.fasebj.org to IP 216.185.5.2. *The FASEB Journal* Vol., No. , pp:, November, 2017

17. Fibbi, B., Morelli, A., Vignozzi, L., Filippi, S., Chavalmane, A., De Vita, G., Marini, M., Gacci, M., Vannelli, G. B., Sandner, P., and Maggi, M. (2010) Characterization of phosphodiesterase type 5 expression and functional activity in the human male lower urinary tract. *J. Sex. Med.* **7**, 59–69
18. Rybalkin, S. D., Rybalkina, I. G., Feil, R., Hofmann, F., and Beavo, J. A. (2002) Regulation of cGMP-specific phosphodiesterase (PDE5) phosphorylation in smooth muscle cells. *J. Biol. Chem.* **277**, 3310–3317
19. Juilfs, D. M., Soderling, S., Burns, F., and Beavo, J. A. (1999) Cyclic GMP as substrate and regulator of cyclic nucleotide phosphodiesterases (PDEs). *Rev. Physiol. Biochem. Pharmacol.* **135**, 67–104
20. Gacci, M., Andersson, K.-E., Chapple, C., Maggi, M., Mirone, V., Oelke, M., Porst, H., Roehrborn, C., Stief, C., and Giuliano, F. (2016) Latest evidence on the use of phosphodiesterase type 5 inhibitors for the treatment of lower urinary tract symptoms secondary to benign prostatic hyperplasia. *Eur. Urol.* **70**, 124–133
21. Ückert, S., Sormes, M., Kedia, G., Scheller, F., Knapp, W. H., Jonas, U., and Stief, C. G. (2008) Effects of phosphodiesterase inhibitors on tension induced by norepinephrine and accumulation of cyclic nucleotides in isolated human prostatic tissue. *Urology* **71**, 526–530
22. Angulo, J., Cuevas, P., Fernández, A., La Fuente, J. M., Allona, A., Moncada, I., and Sáenz de Tejada, I. (2012) Tadalafil enhances the inhibitory effects of tamsulosin on neurogenic contractions of human prostate and bladder neck. *J. Sex. Med.* **9**, 2293–2306
23. Dey, A., Lang, R. J., and Exintaris, B. (2012) Nitric oxide signaling pathways involved in the inhibition of spontaneous activity in the guinea pig prostate. *J. Urol.* **187**, 2254–2260
24. Chapple, C. R. (2004) Pharmacological therapy of benign prostatic hyperplasia/lower urinary tract symptoms: an overview for the practising clinician. *BJU Int.* **94**, 738–744
25. Kaplan, S. A. (2009) Side effects of alpha-blocker use: retrograde ejaculation. *Rev. Urol.* **11**(Suppl 1), S14–S18
26. Wang, L., Zhang, X., Wang, G., Visweswariah, S. S., Lin, G., Xin, Z., Lue, T. F., and Lin, C.-S. (2015) Lobe-specific expression of phosphodiesterase 5 in rat prostate. *Urology* **85**, 703.e7–703.e13
27. Mietens, A., Tasch, S., Stammler, A., Konrad, L., Feuerstacke, C., and Middendorff, R. (2014) Time-lapse imaging as a tool to investigate contractility of the epididymal duct: effects of cGMP signaling. *PLoS One* **9**, e92603
28. Chung, K., and Deisseroth, K. (2013) CLARITY for mapping the nervous system. *Nat. Methods* **10**, 508–513
29. Yang, B., Treweek, J. B., Kulkarni, R. P., Deverman, B. E., Chen, C.-K., Lubeck, E., Shah, S., Cai, L., and Gradinaru, V. (2014) Single-cell phenotyping within transparent intact tissue through whole-body clearing. *Cell* **158**, 945–958
30. Saboor, F., Reckmann, A. N., Tomczyk, C. U., Peters, D. M., Weissmann, N., Kaschtanow, A., Schermuly, R. T., Michurina, T. V., Enikolopov, G., Müller, D., Mietens, A., and Middendorff, R. (2016) Nestin-expressing vascular wall cells drive development of pulmonary hypertension. *Eur. Respir. J.* **47**, 876–888
31. Nguyen, D.-T. T., Dey, A., Lang, R. J., Ventura, S., and Exintaris, B. (2011) Contractility and pacemaker cells in the prostate gland. *J. Urol.* **185**, 347–351
32. Donatucci, C. F., Brock, G. B., Goldfischer, E. R., Pommerville, P. J., Elion-Mboussa, A., Kissel, J. D., and Viktrup, L. (2011) Tadalafil administered once daily for lower urinary tract symptoms secondary to benign prostatic hyperplasia: a 1-year, open-label extension study. *BJU Int.* **107**, 1110–1116
33. Andersson, K.-E., and Gratzke, C. (2007) Pharmacology of alpha1-adrenoceptor antagonists in the lower urinary tract and central nervous system. *Nat. Clin. Pract. Urol.* **4**, 368–378
34. Corbin, J. D., and Francis, S. H. (1999) Cyclic GMP phosphodiesterase-5: target of sildenafil. *J. Biol. Chem.* **274**, 13729–13732
35. Hennenberg, M., Schott, M., Kan, A., Keller, P., Tamalunas, A., Ciotkowska, A., Rutz, B., Wang, Y., Strittmatter, F., Herlemann, A., Yu, Q., Stief, C. G., and Gratzke, C. (2016) Inhibition of adrenergic and non-adrenergic smooth muscle contraction in the human prostate by the phosphodiesterase 10-selective inhibitor TC-E 5005. *Prostate* **76**, 1364–1374
36. Müller, D., Mukhopadhyay, A. K., Davidoff, M. S., and Middendorff, R. (2011) Cyclic GMP signaling in rat urinary bladder, prostate, and epididymis: tissue-specific changes with aging and in response to Leydig cell depletion. *Reproduction* **142**, 333–343
37. Calmasini, F. B., Alexandre, E. C., Silva, F. H., De Nucci, G., Antunes, E., D'Ancona, C. A., and Mónica, F. Z. (2016) Soluble guanylate cyclase modulators, BAY 41-2272 and BAY 60-2770, inhibit human and rabbit prostate contractility. *Urology* **94**, 312.e9–312.e15

Received for publication June 18, 2017.
Accepted for publication October 16, 2017.

Downloaded from www.fasebj.org to IP 216.185.5.2.

THE FASEB JOURNAL
The Journal of the Federation of American Societies for Experimental Biology

Novel imaging of the prostate reveals spontaneous gland contraction and excretory duct quiescence together with different drug effects

Robert Kügler, Andrea Mietens, Mathias Seidensticker, et al.

FASEB J published online October 31, 2017
Access the most recent version at doi:10.1096/fj.201700430R

Supplemental Material http://www.fasebj.org/content/suppl/2017/10/30/fj.201700430R.DC1

Subscriptions Information about subscribing to *The FASEB Journal* is online at http://www.faseb.org/The-FASEB-Journal/Librarian-s-Resources.aspx

Permissions Submit copyright permission requests at: http://www.fasebj.org/site/misc/copyright.xhtml

Email Alerts Receive free email alerts when new an article cites this article - sign up at http://www.fasebj.org/cgi/alerts



© FASEB

Downloaded from www.fasebj.org to IP 216.185.5.2. *The FASEB Journal* Vol., No. , pp:, November, 2017

II. Hardcopy of suppl. movies mentioned in Chapter 3 (suppl. movies 1-7) and Chapter 4 (suppl. movies 8-13).

III. Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one (1*)* original papers published in a peer reviewed journal (Chapter 3) and one (1) submitted publication (Chapter 4). The core theme of the thesis is smooth muscle contractility and pharmacological responses in the normal and diseased human and rat prostate. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of Robert Kügler, Andrea Mietens and myself, the student (shared first-authorship), working within the group signal transduction in the Anatomy and Cell Biology of the Justus-Liebig-University Giessen under the supervision of Professor Ralf Middendorff.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 3 and 4 my contribution to the work involved the following:

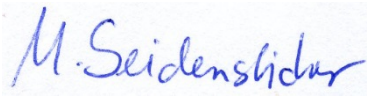
Thesis Chapter	**Publication Title**	**Status** *(published, in press, accepted or returned for revision, submitted)*	**Nature and % of student contribution**	**Co-author name(s) Nature and % of Co-author's contribution***	**Co-author(s), Monash student Y/N***
3	Novel Imaging of the Prostate Reveals Spontaneous Gland Contraction and Excretory Duct Quiescence Together with Different Drug Effects	*Published*	*Shared first author* *Performed experiments* *Conducted data analysis* *Drafted manuscript and incorporated feedback from all authors* *(20%)*	*Robert Kügler* - *Designed and performed initial experiments* - *Conducted data analysis* - *Drafted manuscript (20%)* *Andrea Mietens* - *Gave critical feedback on manuscript and data analysis (20%)* *Sabine Tasch* - *Performed experiments (5%)* *Florian Wagenlehner* - *Provided human prostate tissue from surgery, provided feedback on manuscript (2%)* *Andre Kaschtanow* - *Performed experiments (2%)* *Yudy Tjahjono* - *Performed initial experiments (2%)* *Claudia U. Tomczyk* - *Performed Immunostaining (2%)* *Daniela Beyer* - *Gave feedback on manuscript* - *Performed experiments (2%)* *Gail P. Risbridger* - *Gave feedback on manuscript (3%)*	*No* *No* *No* *No* *No* *No* *No* *No* *No*

				Betty Exintaris - *Gave feedback on manuscript (4%)* *Stuart J. Ellem* - *Gave feedback on manuscript (3%)* *Ralf Middendorff* - *Designed experiments* - *Gave critical feedback on manuscript and data analysis* - *Drafted manuscript (15%)*	*No* *No* *No*
4	*Time-Lapse Imaging Reveals that the α_1-adrenoceptor Blocker Tamsulosin but not the PDE5 Inhibitor Tadalafil Directly Disturbs the Secretory Function of the Seminal Vesicles, Prostate and Sperm Release from the Epididymis*	*submitted*	*First author* *Designed and performed experiments* *Conducted data analysis* *Drafted manuscript and incorporated feedback from all authors* *(50%)*	*Sabine Tasch* - *Conducted experiments (10%)* *Betty Exintaris* - *Designed experiments* - *Gave critical feedback regarding manuscript and data analysis (20%)* *Ralf Middendorff* - *Designed experiments* - *Gave critical feedback regarding manuscript and data analysis* - *Drafted manuscript (20%)*	No No No

**If no co-authors, leave fields blank*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Mathias Seidensticker

Student signature:  **Date: 16.05.2019**

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Prof. Ralf Middendorff

Main Supervisor signature: **Date: 16.05.2019**

IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission.

Reviewer 1:

Comments/Address #1

Correction/Addendum:

Chapter 1

*Page 7: "*superscript 2+ in all uses in this Figure Caption."

Answer: 2+ was superscripted in all uses in Figure 3.

Page 19:" this line does not link clearly with the preceding sentence. Clarification is needed."

Answer: Highlighted sentence was adapted for better understanding.

*Page 36: "*some/many/all?"

Answer: Changed "…by the patients…" to "…by many patients…"

*Page 41: "*Commence the next paragraph with this last sentence. It really forms a distinct narrative separate to the preceding text, instead introducing a basis for the use of animal models. On this, link it into the next sentence more clearly - are you trying to say that animal models are used to enable preclinical research to better inform new treatments?"

Answer: Changes were applied and link to next sentence was performed.

*Page 41: "*orphan paragraph - merge with following paragraph*."*

Answer: Changes were applied.

*Page 49: "*It is unclear what biochemical interaction means here. Please clarify of be more specific."

Answer: Further clarification was provided in the text.

*Page 58: "*italicize et al wherever it appears throughout remainder of text*"*

Answer: Changes were applied.

Chapter 2

*Page 63: "*Indicate the total number of specimens used for the research (i.e how many patients were used to obtain samples)."

Answer: The total number of specimens is mentioned in the following Chapter 3.

*Page 63: "*Indicate the total number of specimens used for the research (i.e how many patients were used to obtain samples)."

Answer: The total number of specimens is mentioned in the following Chapter 5.

*Page 66: "*How did you decide on the test concentration of each substance?*"*

Answer: In agreement with previous studies our pilot tests showed strong contractile responses in prostate ducts, seminal vesicles and the cauda epididymal duct with a final concentration of 10µM NA (data not shown). Therefore, 10µM NA was used in all experiments. Tamsulosin, sildenafil and tadalafil were used at a final concentration of 5µM and 2.5µM, respectively, ranging in common levels used for ex vivo/in vitro experiments.

*Page 79: "*State the type of protein assay if noted on the kit where possible*."*

Answer: The type of protein assay was stated in the document (Bradford method).

Chapter 3

*Page 88: "*Include version number of software here*"*

Answer: Version number was included.

Chapter 5

*Page 130: "*formatting of line breaks/spacing between paragraphs looks to be different than in previous chapters*"*

Answer: The settings were amended for the whole document.

*Page 130: "*omit breaks between paragraph for linked or related content here*."*

Answer: Changes were applied.

*Page 130: "*from same previous citation?*"*

Answer: Yes. The citation was moved to the end of the sentence.

*Page 131: "*references here*?"*

Answer: References are mentioned at the end of the paragraph/following sentence.

*Page 136: "*It is difficult to make the blots out: please enhance contrast*."*

Answer: For western blot analysis contrast enhancement of the western blot was performed (Fig.27.1).

Edited WB (Fig.27.1) referring to "Chapter 5.3: Results" (p.135):

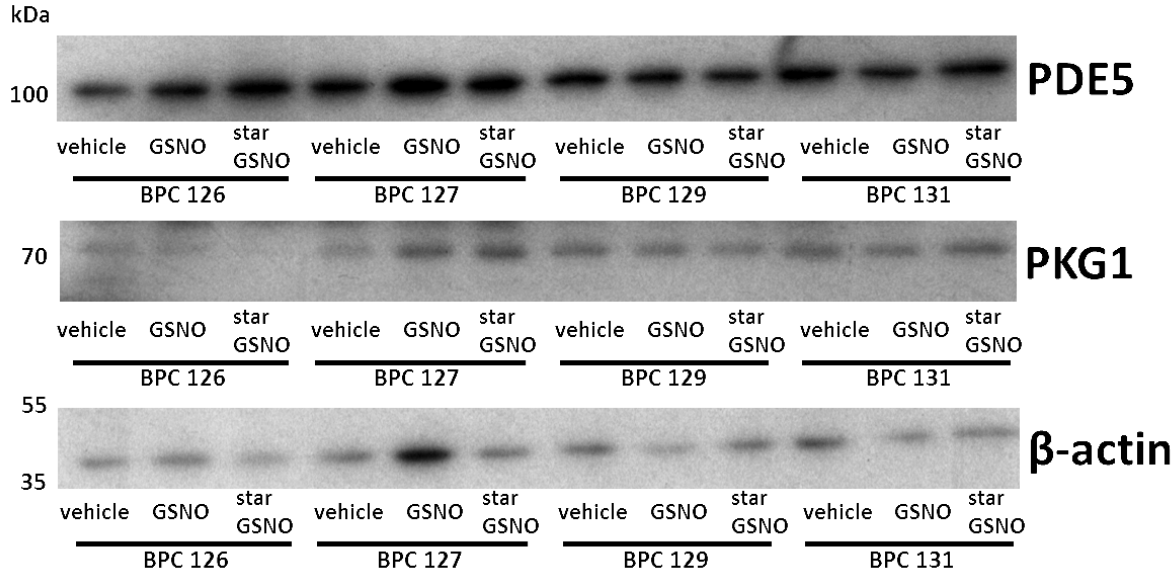


Figure 27.1: Western Blot analysis showed a different level of protein expression in cell lines treated with GSNO or star-GSNO.

*Page 137: "*Qualitative analysis of blot data is rarely valid; it is very difficult to make eyeball judgments on altered expression and makes invalid interpretations of up- or down-regulation. Provide densitometric ratios or expression relative to a housekeeping protein*."*

Answer: Densitometric analyses of the western blot were performed (Fig.27.2 – 27.4). The analysis did not show statistical differences in protein expression levels, but by displaying all the bands of the WB as displayed in the thesis document, we were able to directly visualize tendencies of differences in protein levels. In general, the obtained results, are preliminary and show limited informative value. Therefore, densitometric ratios are not suitable because of the few patients analyzed. Further experiments, especially increasing the number of patients, need to be performed for validation of observed tendencies. For statistical analysis, one-way ANOVA for multiple comparisons tests were performed, assuming normal distribution of data.

No significant differences (p<0.05) were overserved for any protein expression investigated. Densitometric analyses of protein expression levels referring to the displayed WB (Fig.27.1):

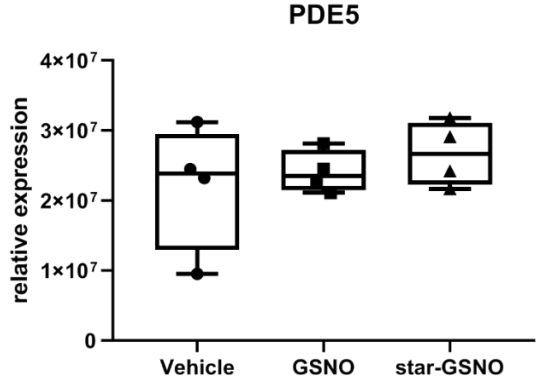


Figure 27.2: Western Blot analysis showed no significant differences of PDE5 protein expression in cell lines treated with vehicle, GSNO or star-GSNO (One-way ANOVA).

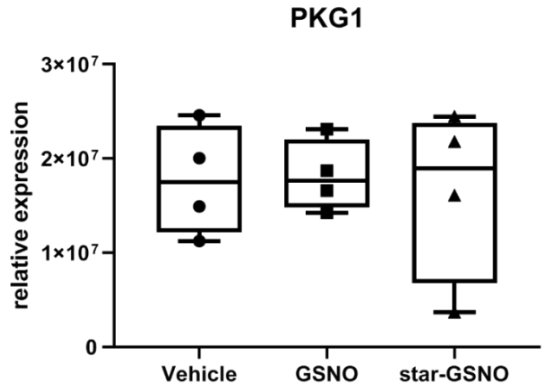


Figure 27.2: Western Blot analysis showed no significant differences of PKG1 protein expression in cell lines treated with vehicle, GSNO or star-GSNO (One-way ANOVA).

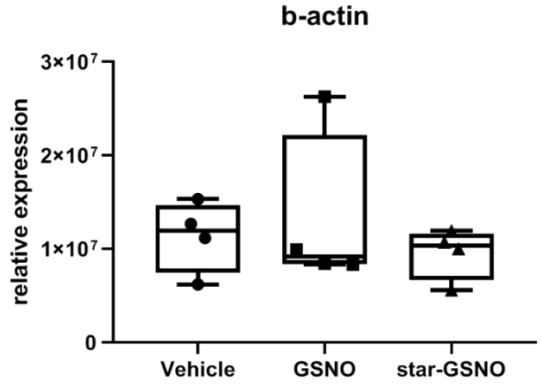


Figure 27.4: Western Blot analysis showed no significant differences of □-actin protein expression in cell lines treated with vehicle, GSNO or star-GSNO (One-way ANOVA).

*Page 139: "*Is the sGCBeta expression in BPC129 significantly lower with star-GSNO?*"*
Answer: No. All acquired datasets were tested for significant differences.

*Page 143: "*what are the n numbers here?*"*
Answer: vehicle: n=4, GSNO n=5, star-GSNO n=4

*Page 146: "*I did not understand the meaning of this text; did you mean 'overlooked data'*?"*
Answer: … lack of data… Changes included in thesis.

Comments/Address #2
Errata/Addendum:

All notations were amended within the thesis and under section "IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" on page 184 of the appendix.

Comments/Address #3
Errata/Addendum:

Additional paragraph on ATP as a co-transmitter during ejaculation ("IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" under "Comments/Address #3", page 188):
*"*Noradrenaline (NA) is a hormone and neurotransmitter and is produced and released from the brain as well as the adrenal glands. In general, it mobilizes the brain and body for action in situations of stress or danger in the so-called fight-or-flight response. NA also acts as the main mediator during the emission phase of ejaculation by binding to α-adrenoceptors located on the cell membrane of SMCs. Activation leads to contractions of structures relevant for the emission phase and expulsion of fluid during ejaculation.

Therefore, with this established drug, we aimed to establish our visualization approach for the three different emission-related structures investigated (prostate, seminal vesicles and epididymal duct) and to obtain most reliable results for the investigation of drug-related ejaculation dysfunctions during the treatment of patients suffering from BPH.

Aside from NA, co-transmitters play a crucial role in the process of ejaculation. One of the most important co-transmitters is ATP. ATP acts as an extracellular signaling molecule (purinergic signaling), as well as an intracellular energy source. The molecule, according receptors and signaling pathways are well-described and of high research interest because of their therapeutic potential for a wide range of diseases including thrombosis, stroke, dry eye, atherosclerosis, kidney failure, osteoporosis, bladder incontinence and neurodegenerative diseases (Burnstock 2018). ATP is released by sympathetic and parasympathetic nerves that can induce SMC contraction (or relaxation) in various organs via specific receptors (Burnstock 2004, Burnstock and Knight 2004). For example, ATP released as a co-transmitter from perivascular sympathetic nerves activate P2X receptors resulting in contraction of SMCs whereas ATP released from endothelial cells during changes in blood flow and hypoxia acts on P2X and P2Y receptors on endothelial cells, resulting in production of NO and relaxation (Burnstock 2018). The function of ATP as a co-transmitter during ejaculation is of additional interest since in this work the focus of the experimental setups were based solely on NA as a mediator of ejaculation processes, excluding the influence of other factors. But in physiological conditions, e.g. using *in vivo* approaches, it is relevant to include co-transmitter such as ATP during sympathetic activity. The vas deferens for example is rich in ATP in sympathetic nerves. Although the vas deferens is not directly involved in the emission phase of ejaculation, which is the focus in this work, it plays an important role during the expulsion of sperm released from the distal part of the epididymal duct. Purine and purinergic receptors as well as physiology and pathophysiology of purinergic neurotransmission was well described by Geoffrey Burnstock, giving insights for future clinical uses especially for ejaculatory dysfunction induced by blockade of α-adrenoceptors (Burnstock 2007, Burnstock 2018).”

Comments/Address #6
Errata/Addendum:

Please refer to Comments/Address #1 on page 184.

Comments/Address #7
Errata/Addendum:

Additional assessment and re-examination of protein expression levels (Fig.27, "IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" under "Comments/Address #7", page 190):

*"*But WB analyses did not only show an upregulated protein expression in the investigated samples after GSNO/star-GSNO treatment. The protein expression of PDE5 and PKG1 was variable across the four samples assessed, therefore being variably upregulated, unchanged or downregulated for control or 24h treatment with one of the two NO donors as mentioned before in the results. Because of this, obtained results are not reliable enough and the sample size is too small for an analysis of e.g., pooled data. On the other hand, investigation of these datasets for each patient individually allows to describe tendencies and display preliminary results. This notion is strengthened with densitometric analysis of the WBs (Fig. 27.2 – Fig 27.4). Analysis showed no significant differences in protein expression among the patients in addition to high variations of protein expression with or without drug pre-treatment. In a publication by Müller et al. (Muller et al. 2011) it was described and explained that especially because of the above mentioned unreliabilities, densitometric analyses are not performed in those cases. (Detailed explanations from this paper is found in the "Material & Methods" section under "Quantitative immunoblot analyses and densitometric measurements")."

Comments/Address #8
Errata/Addendum:

Statistical analysis of the organ bath studies was clarified within the thesis document under "2.7.2 Tension recording Analysis" (page 80) and "5.2.5 Statistical Analysis" (page 134) to the following:
*"*Briefly, unless noted otherwise, paired one-way ANOVA with Dunnett's post hoc test was used for statistical analysis of the data, and was represented as a mean±SEM where $p<0.05$ was considered to be statistically significant in tension recording experiments.*"*

Comments/Address #9
Errata/Addendum:

Please refer to thesis and the "Errata/Addendum" section of "Comments/ Address #10" for correction (page 191).

Comments/Address #10
Errata/Addendum:
Please refer to chapter "5.4 Discussion" (page 145) and the appendix section "IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" under "Comments/Address #10", page 191.
"Tension recording experiments showed that NO donors mainly have an effect on the reduction of the basal tension but also significantly decreases contraction frequency and amplitude when using GSNO alone."

was changed to:

*"*Tension recording experiments showed that NO donors mainly have an effect on the reduction of the amplitude and contraction frequency when using GSNO alone. A significant reduction of the basal tension could not only be observed in the tissues treated with the NO donors but also in vehicle control recordings."

Reviewer 2:

Comments/Address #1

Correction/Addendum:

Answer: The sentence "The core theme of the thesis is smooth muscle contractility and pharmacological responses in the normal and diseased human and rat prostate."

was changed to:

"The core theme of the thesis is smooth muscle contractility and pharmacological responses in the human and rat prostate as well as rat seminal vesicle and epididymis." *(see thesis page VII)*

Comments/Address #2

Correction/Addendum:

Answer: Additional image of the human prostate for illustration of histological aspects (Fig. 6.1 and Fig. 6.2 additionally to Chapter 1, page 14).

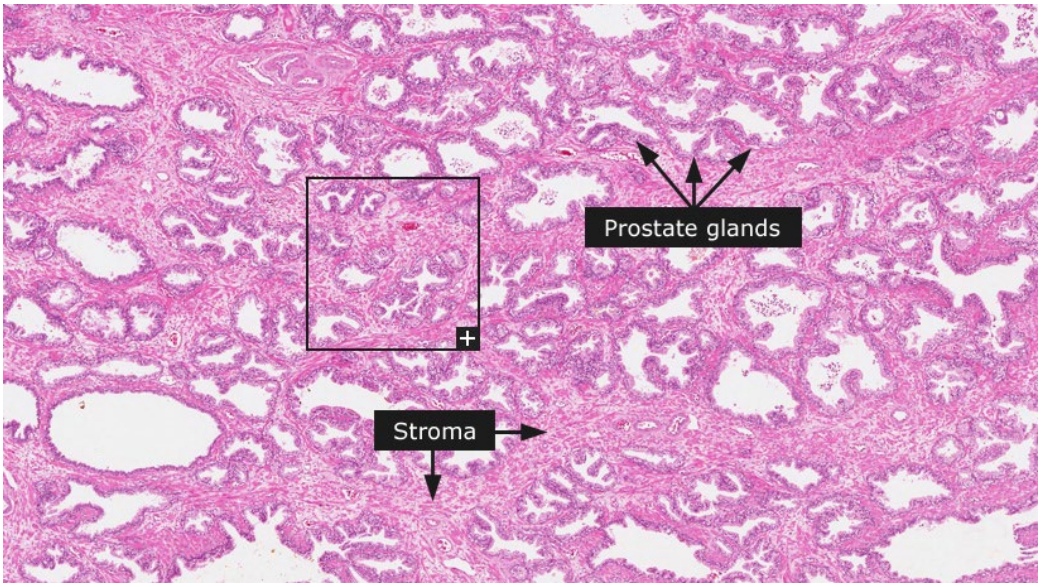


Figure 6.1: Histological overview of human prostate tissue indicating prostate single glands lined with epithelial cells facing the lumen as well as indicating interstitial stromal tissue containing SMCs. Magnification see Fig. 6.2.

(image modified afterhttps://www.proteinatlas.org/learn/dictionary/normal/prostate)

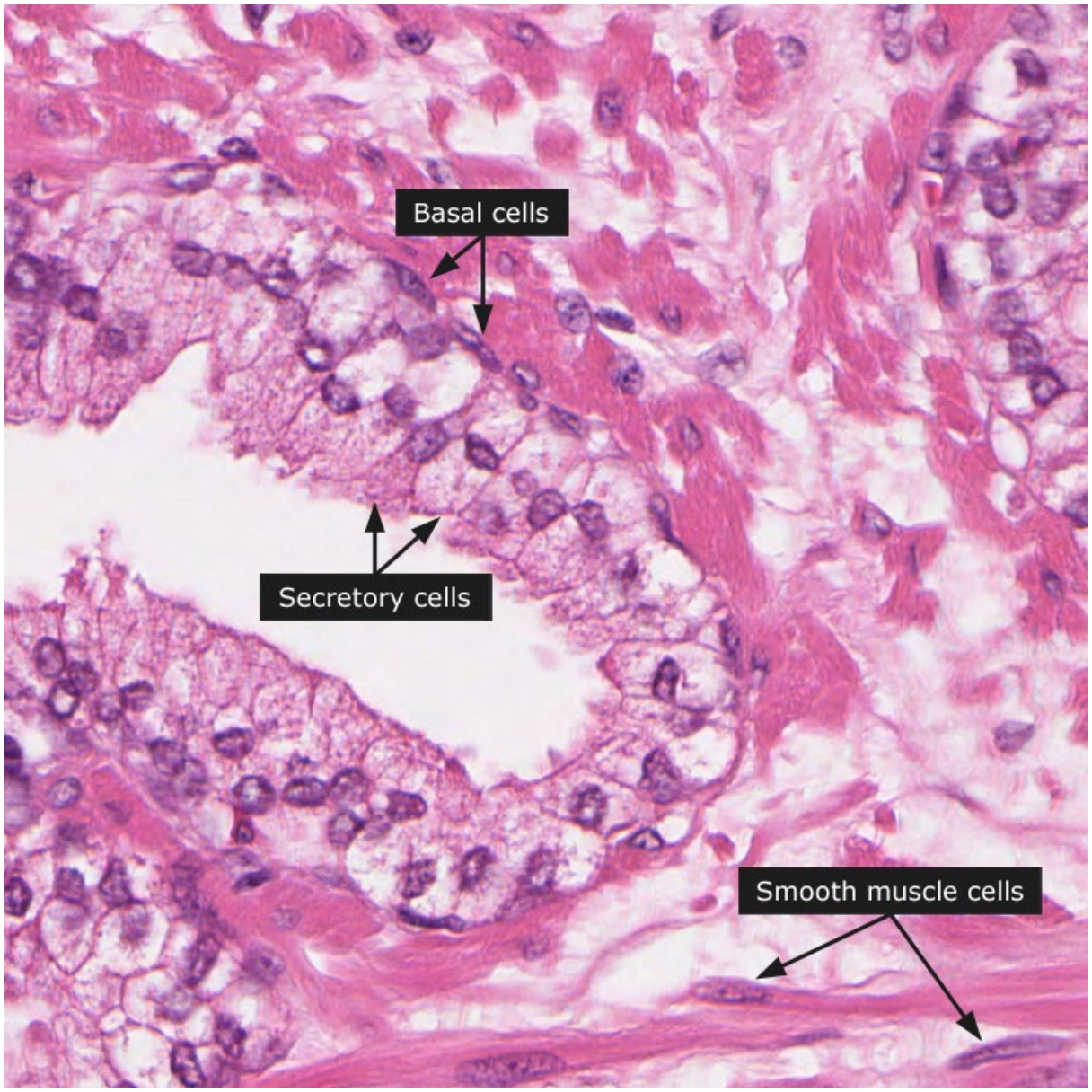


Figure 6.2: Display of magnification indicated in Fig. 6.1: Magnification shows different cell types of prostate tissue from the epithelial layer (basal cells and secretory luminal cells) and from the interstitial stroma (SMCs).

(image modified after https://www.proteinatlas.org/learn/dictionary/normal/prostate/detail+1/magnification+1)

Comments/Address #3

Correction/Addendum:

Answer: An additional section was included in the thesis document under chapter 1.4.4 (page 36) as followed:

*"*In general, ejaculation is not to be confused with orgasm. Orgasm is a central nervous system phenomenon and is a distinct entity from ejaculation characterized by sensations experienced at the peak of sexual arousal. Ejaculation can be divided into two phases: emission and expulsion. Emission is a physiologic process involving the distal part of the epididymis, seminal vesicles, prostate and adjacent structures like the vas deferens, prostatic urethra and bladder neck. Activation of the sympathetic nervous system and release of the neurotransmitter NA initially leads to the closure of the bladder neck to prevent retrograde ejaculation followed by mixture of secretions from the seminal vesicles, prostate, vas deferens (from the distal epididymis) and Cowper's glands in the urethra (Revenig et al. 2014). The neural control of emission originates from the thoracolumbar spine at T10-L2 and coordinates emission. Sympathetic efferent fibers merge into lumbar ganglia and continue to form the superior hypogastric plexus. Postganglionic fibers travel to the above-mentioned target organs to control the emission phase of ejaculation (Giuliano and Clement 2005, Lipshultz 2009).

Evidences of the incidences of ejaculatory dysfunctions in patients treated with α-blockers are described in many clinical studies and discussed throughout this work. As mentioned before, the exact mechanism behind ejaculatory dysfunctions remain unclear but it was suggested that it involves retrograde ejaculation due to the relaxation of the bladder neck by the blockade of the α-adrenoceptors or anejaculation or loss of seminal fluid by the blockade of a-adrenoceptors in the structures involved in the emission phase (see chapter 4).

Studies show variabilities of the occurrences of ejaculation disorders between different agents such as that treatment with silodosin shows higher incidences than with tamsulosin. Tamsulosin, on the other hand, shows higher incidences than e.g. alfuzosin. This might be to a higher specificity of silodosin to α_{1A}-adrenoceptors compared to tamsulosin, suggesting ejaculation disturbances are mainly affected by the α_{1A}-adrenoceptors subtype (Torre et al. 2015).

The lack of clarity of the precise effects of these agents on ejaculation also leads to the inconsistencies about the definition of the term “ejaculation disorders” or “ejaculatory dysfunction”, which are believed to be “anejaculation”, “retrograde ejaculation” or “absence of seminal emission”. Therefore, chapter 4 of this thesis elaborates on the clarification on the mechanisms behind ejaculation dysfunction in structures involved in the emission phase of ejaculation due to α_1-adrenoceptor antagonists used to treat BPH.”

Comments/Address #4
Correction/Addendum:

Answer: The idea to investigate the influence of alpha$_{1L}$-adrenoceptors in future studies was included in Chapter 6 (see page 153).
“The α_{1A}-adrenoceptor is abundantly expressed in the lower urinary tract and is the principal therapeutic target for the symptomatic treatment of lower urinary tract symptoms in men. Tamsulosin is a highly specific alpha$_{1A}$-antagonist and therefore this subclass of adrenoceptors was addressed. The main target of our studies was to visualize and explain the adverse effects of tamsulosin on organs of the reproductive system involved in the emission phase of ejaculation. However, another important aspect for future studies is the investigation of a subclass of the α_{1A}-adrenoceptors; the α_{1L}-adrenoceptor. This subclassified adrenoceptor is a product of the α_{1A}-adrenoceptor gene, but little is known about the mechanisms of how this altered phenotype is achieved (White et al. 2019). Although pharmacotherapies that target to reduce SMC contractility in the prostate specifically block the α_{1A}-subtype, the pharmacologically classified α_{1L}-adrenoceptor was described to be responsible for mediating the contractile responses in men (Muramatsu et al. 1994, Israilova et al. 2004) and rat (Hiraoka et al. 1999). Therefore, further research of this subtype is useful for the investigation of pharmacological BPH treatment.
In the present work, we first wanted to investigate adverse effects of tamsulosin on the alpha$_{1A}$-adrenoceptors before addressing further aspects such as alpha$_{1L}$-adrenoceptors.”

Comments/Address #10

Correction/Addendum:

Answer: Additional information about patient groups for the prostate tissues compared between Germany and Australia:

*"*Human tissue samples collected in Germany for time-lapse Imaging and Immunostaining originated from patients (range 60-79 years, median 71.7 ± 6.5) undergoing transurethral monopolar electroresection of the prostate (TUR-P) for BPH or radical prostatectomy for prostate cancer at the Department of Urology, Pediatric Urology and Andrology of the Justus-Liebig-University Giessen. Specimens obtained from radical prostatectomy were taken of normal, non-malignant peripheral regions, of the prostate gland. Tissue samples from TUR-P were collected from the periurethral region.

Human prostate specimen collection in Australia for tension recording experiments was achieved together with the Australian Prostate Cancer BioResource (APCB) Coordinator Dr. Melissa Papargiris. Prior to surgery (radical prostatectomy), the coordinator obtained biopsy results from clinicians. Patients were selected for study if they had a cytologically or histologically confirmed diagnosis of mild to moderate prostate cancer located in the peripheral zone with a Gleason score of ≤ 7. A Gleason Score of 8 or higher led to the exclusion of the study in order to select appropriate specimens for further investigations.

The Gleason score indicates the stage of prostate cancer to determine an appropriate treatment strategy and prognosis."

Additional information included in the appendix section under "IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" under "Comments/Address #10" (page 196).

Comments/Address #21

Correction/Addendum:

Answer: Additional information on NO donors for clinical use:

*"*NO has been the focus of immense scientific and medical research and is recognized as an important molecule in almost every physiological system: cardiovascular (Lundberg et al. 2015), immune (Bogdan 2001), central nervous system (Calabrese et al. 2007), and outflow physiology (Chang et al. 2015). NO plays a significant therapeutic role. Therefore, many efforts focus on developing means for an effective NO delivery system for various pathological conditions. However, there are only a few FDA approved products for NO delivery, e.g. nitroglycerine for the treatment of acute angina (Hambrecht et al. 2013), nitroprusside for the treatment of congestive heart failure and high blood pressure (Opasich et al. 2009) and inhaled NO for pulmonary treatments (Benza et al. 2010). Lower NO concentrations (nm) generally promote cell survival and proliferation, while higher concentrations (µm) lead to apoptosis and cell cycle arrest. Therefore, in cancer treatment, NO is regarded controversially (Yang et al. 2018). NO deficiency can lead to pathological conditions such as atherosclerosis (Tian et al. 2012), blood flow disturbances in the central nervous system (Toda et al. 2009), inflammation (Ying and Hofseth 2007), poor wound healing (Witte and Barbul 2002), and tumor progression (Fukumura et al. 2006). Bio(nano)materials are used as platforms to artificially deliver NO in a controlled and substantial manner and have emerged as promising approaches to overcome challenges associated with biological administration of NO and to promote the generation of physiologically relevant concentrations of NO in diverse biomedical applications for therapeutic use. Of special interest here is the NO delivery from small molecule NO donors. NO donors are pharmacologically active substances that carry NO to stabilize the radical until it needs to be released. There are several classes of NO donors and are categorized on their chemical reactivity or the mechanism of NO release from the carrier. The release of NO from donor molecules can be triggered by various factors such as light, heat, pH, or enzyme activity. At a different approach, NO can be released via chemical reactions of the donors with acids, alkalis, metals, or thiols (Wo et al. 2016). NO donors to note include nitrates, diazeniumdiolates (NONOates), and *S*-nitrosothiols (RSNOs).

NONOate is one of the most investigated NO donors due to its capability to release two molecules of NO per mole of donor at physiological conditions and its pH-dependent decomposition property (Yang et al. 2018). Laboratory generation of RSNOs require reactions between thiols and nitrosating agents. NO can be exhausted from RSNO by multiple triggers (i.e., heat or light). Two relatively stable compounds in this group, and most commonly used for in vivo preclinical studies, include *S*-nitroso-*N*-acetylpenicillamine (SNAP) and *S*-nitrosoglutathione (GSNO) (Naghavi et al. 2013). Still, information about properties in biological setups of the GSNO compound is sparse. Therefore GSNO and the newly synthesized star-GSNO are the choice of study for elucidation and evaluation in this work (see Chapter 5). Readers are referred to excellent reviews that cover detailed aspects of NO donors (Wang et al. 2002, Miller and Megson 2007, Naghavi et al. 2013).
Additional information was included in the appendix section under "IV. Thesis amendments in response to thesis reviewers' comments in accordance to the guidelines of Monash University for thesis submission" under "Comments/Address #21 on page 197.

Comments/Address #31
Correction/Addendum:
Answer: "highly decreased release of sperm" *was changed to* "significantly decreased release of sperm" *for clarification (see Chapter 6 on page 153).*

Comments/Address #33
Correction/Addendum:
Answer: Additional concluding paragraph for Chapter 6 on page 155:
"All in all, with the work that is presented in the Chapters 3 to 5, we systematically analysed structural and functional differences in the prostate and the role and distribution of cGMP signalling pathway components in these structures. In a subsequent step, our pharmacological approach also gave insights into the mechanisms of action of current and emerging drugs on various male reproductive organs including the explanation of unwanted side-effects in the investigated organs. Although we gathered reliable, extensive and robust data in the field of treating BPH, there were some limitations. Chapter 5 for example mainly consists of pilot studies.

These pilot studies need further repetitions, a much larger and more stable patient cohort, as well as a robust experimental design with determined factors like incubation time, used concentrations. to establish a reliable and working setup.
To evaluate effects of NO and NO delivery systems on the cGMP signalling pathway and components, different methods to directly examine cGMP effects need to be considered, e.g. cGMP ELISA. With our obtained results, we only observed an indirect effect of NO donors on the cGMP pathway and components.
cGMP ELISAs were planned and conducted for the experimental design in Chapter 5, but due to different factors we were not able to gather publish worthy results. For future references, research questions of interest include the establishment of a reliable NO donor system for the treatment of BPH. Only then we would gain a qualitative insight into the characteristics and effects of the NO donors used to ask the right questions for future directions. Also, with our newly established time-lapse imaging approach, to conduct further experiments with different SMC relaxing agents like mirabegron, a β_3-adrenoceptor-agonist.

Typographical/grammatical errors - pages 3, 4, 8, 10, 15, 16, 16, 17, 41, 60, 61, 102, 147:
Answer: All typographical/grammatical errors were corrected.

References

Benza, R. L., D. P. Miller, M. Gomberg-Maitland, R. P. Frantz, A. J. Foreman, C. S. Coffey, A. Frost, R. J. Barst, D. B. Badesch, C. G. Elliott, T. G. Liou and M. D. McGoon (2010). "Predicting Survival in Pulmonary Arterial Hypertension." Circulation **122**(2): 164-172.

Bogdan, C. (2001). "Nitric oxide and the immune response." Nature Immunology **2**(10): 907-916.

Burnstock, G. (2004). "Cotransmission." Curr Opin Pharmacol **4**(1): 47-52.

Burnstock, G. (2007). "Physiology and pathophysiology of purinergic neurotransmission." Physiol Rev **87**(2): 659-797.

Burnstock, G. (2018). "Purine and purinergic receptors." Brain and Neuroscience Advances **2**: 2398212818817494.

Burnstock, G. and G. E. Knight (2004). "Cellular distribution and functions of P2 receptor subtypes in different systems." Int Rev Cytol **240**: 31-304.

Calabrese, V., C. Mancuso, M. Calvani, E. Rizzarelli, D. A. Butterfield and A. M. Giuffrida Stella (2007). "Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity." Nature Reviews Neuroscience **8**(10): 766-775.

Chang, J. Y. H., W. D. Stamer, J. Bertrand, A. T. Read, C. M. Marando, C. R. Ethier and D. R. Overby (2015). "Role of nitric oxide in murine conventional outflow physiology." American Journal of Physiology-Cell Physiology **309**(4): C205-C214.

Fukumura, D., S. Kashiwagi and R. K. Jain (2006). "The role of nitric oxide in tumour progression." Nature Reviews Cancer **6**(7): 521-534.

Giuliano, F. and P. Clement (2005). "Neuroanatomy and physiology of ejaculation." Annu Rev Sex Res **16**: 190-216.

Hambrecht, R., K. Berra and K. J. Calfas (2013). "Managing Your Angina Symptoms With Nitroglycerin." Circulation **127**(22): e642-e645.

Hiraoka, Y., T. Ohmura, M. Oshita, Y. Watanabe, K. Morikawa, O. Nagata, H. Kato, T. Taniguchi and I. Muramatsu (1999). "Binding and functional characterization of alpha1-adrenoceptor subtypes in the rat prostate." Eur J Pharmacol **366**(1): 119-126.

Israilova, M., T. Tanaka, F. Suzuki, S. Morishima and I. Muramatsu (2004). "Pharmacological characterization and cross talk of alpha1a- and alpha1b-adrenoceptors coexpressed in human embryonic kidney 293 cells." J Pharmacol Exp Ther **309**(1): 259-266.

Lipshultz, L., Howards, S., & Niederberger, C. (2009). Infertility in the Male. Cambridge, Cambridge University Press.

Lundberg, J. O., M. T. Gladwin and E. Weitzberg (2015). "Strategies to increase nitric oxide signalling in cardiovascular disease." Nature Reviews Drug Discovery **14**(9): 623-641.

Miller, M. R. and I. L. Megson (2007). "Recent developments in nitric oxide donor drugs." British Journal of Pharmacology **151**(3): 305-321.

Muller, D., A. K. Mukhopadhyay, M. S. Davidoff and R. Middendorff (2011). "Cyclic GMP signaling in rat urinary bladder, prostate, and epididymis: tissue-specific changes with aging and in response to Leydig cell depletion." Reproduction **142**(2): 333-343.

Muramatsu, I., M. Oshita, T. Ohmura, S. Kigoshi, H. Akino, M. Gobara and K. Okada (1994). "Pharmacological characterization of alpha 1-adrenoceptor subtypes in the human prostate: functional and binding studies." Br J Urol **74**(5): 572-578.

Naghavi, N., A. de Mel, O. S. Alavijeh, B. G. Cousins and A. M. Seifalian (2013). "Nitric Oxide Donors for Cardiovascular Implant Applications." Small **9**(1): 22-35.

Opasich, C., G. Cioffi and A. Gualco (2009). "Nitroprusside in decompensated heart failure: What should a clinician really know?" Current Heart Failure Reports **6**(3): 182-190.

Revenig, L., A. Leung and W. Hsiao (2014). "Ejaculatory physiology and pathophysiology: assessment and treatment in male infertility." Transl Androl Urol **3**(1): 41-49.

Tian, X. Y., W. T. Wong, A. Xu, Y. Lu, Y. Zhang, L. Wang, W. S. Cheang, Y. Wang, X. Yao and Y. Huang (2012). "Uncoupling Protein-2 Protects Endothelial Function in Diet-Induced Obese Mice." Circulation Research **110**(9): 1211-1216.

Toda, N., K. Ayajiki and T. Okamura (2009). "Cerebral Blood Flow Regulation by Nitric Oxide: Recent Advances." Pharmacological Reviews **61**(1): 62-97.

Torre, A., G. Giupponi, D. Duffy, U. P. Conca, T. Cai and A. Scardigli (2015). "Sexual Dysfunction Related to Drugs: a Critical Review. Part V: α-Blocker and 5-ARI Drugs." Pharmacopsychiatry **49**.

Wang, P. G., M. Xian, X. Tang, X. Wu, Z. Wen, T. Cai and A. J. Janczuk (2002). "Nitric Oxide Donors: Chemical Activities and Biological Applications." Chemical Reviews **102**(4): 1091-1134.

White, C. W., E. D. da Silva Junior, L. Lim and S. Ventura (2019). "What makes the alpha1A -adrenoceptor gene product assume an alpha1L -adrenoceptor phenotype?" Br J Pharmacol **176**(14): 2358-2365.

Witte, M. B. and A. Barbul (2002). "Role of nitric oxide in wound repair." The American Journal of Surgery **183**(4): 406-412.

Wo, Y., E. J. Brisbois, R. H. Bartlett and M. E. Meyerhoff (2016). "Recent advances in thromboresistant and antimicrobial polymers for biomedical applications: just say yes to nitric oxide (NO)." Biomaterials Science **4**(8): 1161-1183.

Yang, T., A. N. Zelikin and R. Chandrawati (2018). "Progress and Promise of Nitric Oxide-Releasing Platforms." Advanced Science **5**(6): 1701043.

Ying, L. and L. J. Hofseth (2007). "An Emerging Role for Endothelial Nitric Oxide Synthase in Chronic Inflammation and Cancer." Cancer Research **67**(4): 1407-1410.