Some etiopathogenetic aspects of chronic prostatitis: mycoplasmas, coryneform bacteria and oxidative stress

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PREFACE

The core of the book consists of the results of experiments conducted during my PhD studies. The results of these studies were published in four papers [given as references 3, 6, 7 and 9 in the next page]. Time will tell whether the identification of some new probable pathogens contributes to effective treatment or cure. In addition, it seems that some pathogens may be rare to the extent where the usual statistical methods of identifying and association between a species and disease becomes impractical – it just seems that there are about four species of culturable ‘normal’ coryneform bacteria while the rest of these bacteria grow in significant quantities only in prostatitis patients. As such, these must be either suspect pathogens or markers of urogenital tract dysbacteriosis. I also felt that it is necessary to add some extra discussion dedicated to general problems of prostatitis, especially in the sense of addressing the topic of pain in addition to bacteria, inflammation and oxidative stress. I felt that if the field of prostatitis research has as if provided so many puzzle pieces (experimental data) without really solving the puzzle (understanding of pathogenesis), one must analyze what is the relevance and what is the likely place of our own experimental puzzle piece in the big picture of prostatitis research. The analysis of possible pathogenetic pathways resulted in identifying a couple of possible positive feedback loops, both of them associated with oxidative stress metabolites. It seems that systemic oxidative stress may exert positive feedback to prostate region through bioactive metabolites that secrete with urine. In addition, it seems that vulnerability to neuronal oxidative stress may be one of the key pathways of persisting pain. These pathogenetic pathways do have some limitations, implied by the as good as axiomatic observation that any combination of pain and oxidative stress does not necessarily cause pain. These patients must have either vulnerable urinary tract epithelium or lower tolerance to neuronal oxidative stress.

This book came originally into being as my PhD dissertation in University of Tartu, Estonia. The dissertation was commenced in November 2009 and a few clarifications were added in the late winter of 2011. The dissertation bases on the original research conducted in University of Tartu (departments of Microbiology and Biochemistry) as well as Andrology Centre of Tartu University Hospital. I am grateful to my supervisors Dr. Reet Mändar (Department of Microbiology, University of Tartu) and Dr. Tiiu Kullisaar (Department of Biochemistry, University of Tartu) as well as to our good collaborators in Andrology Centre at Tartu University Hospital Dr. Margus Punab (head of the Centre), Dr. Paul Korrovits and Dr Kristo Ausmees. I also wish to acknowledge Prof. Sandra Mazzoli (University of Florence and
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Silver Türk, Tartu, February 2011.
RESEARCH ON PROSTATITIS
AT UNIVERSITY OF TARTU


### ABBREVIATIONS

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>8-EPI</td>
<td>8-Isoprostanes</td>
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<td>8-OHdG</td>
<td>8-Hydroxy-2’-Deoxyguanosine</td>
</tr>
<tr>
<td>ABTS+</td>
<td>2’,2’-AzinoBis-Ethylbenzothiazoline 6-Sulfonate</td>
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<tr>
<td>ANF</td>
<td>Absolute Nonfermenter</td>
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<tr>
<td>API</td>
<td>Analytical Profile Index</td>
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<tr>
<td>AsA</td>
<td>Ascorbic Acid</td>
</tr>
<tr>
<td>BC</td>
<td>Before Christ</td>
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<tr>
<td>BEA</td>
<td>Bile Esculin Agar</td>
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<td>BPH</td>
<td>Benign Prostate Hyperplasia</td>
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<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) Ligand</td>
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<tr>
<td>CC</td>
<td>Current Contents</td>
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<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
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<tr>
<td>CFU</td>
<td>Colony Forming Unit</td>
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<tr>
<td>CGRP</td>
<td>Calcitonin Gene-Related Peptide</td>
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<td>CNS</td>
<td>Coagulase Negative Staphylococci</td>
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<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>CP/CPPS</td>
<td>Chronic Prostatitis / Chronic Pelvic Pain Syndrome</td>
</tr>
<tr>
<td>CPPS</td>
<td>Chronic Pelvic Pain Syndrome</td>
</tr>
<tr>
<td>CRP</td>
<td>C-Reactive Protein</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<tr>
<td>DC</td>
<td>Diene Conjugates</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>DRE</td>
<td>Digital Rectal Examination</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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<tr>
<td>EN</td>
<td>European Standard</td>
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<tr>
<td>EPS</td>
<td>Expressed Prostatic Secretion</td>
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<tr>
<td>GPSS</td>
<td>Giessen Prostatitis Symptom Score</td>
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<tr>
<td>GPX</td>
<td>Glu</td>
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<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized Glutathione</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>hsCRV</td>
<td>high-sensitivity C-Reactive Protein</td>
</tr>
<tr>
<td>IC</td>
<td>Interstitial Cystitis</td>
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<tr>
<td>ICP-AES</td>
<td>Inductively Coupled Plasma Atomic Emission Spectrometer</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-gamma</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPSS</td>
<td>International Prostate Symptom Score</td>
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<tr>
<td>iNOS</td>
<td>Inducible Nitric Oxide Synthetase</td>
</tr>
<tr>
<td>ISO</td>
<td>International Organization for Standardization</td>
</tr>
<tr>
<td>LA</td>
<td>Linolenic Acid</td>
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<tr>
<td>LOX</td>
<td>Lipoxygenase</td>
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<tr>
<td>LUTS</td>
<td>Lower Urinary Tract Symptoms</td>
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<tr>
<td>MA</td>
<td>Maryland</td>
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<tr>
<td>MIC</td>
<td>Minimal Inhibitory Concentration</td>
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<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
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MLSb  Macrolide-Lincosamide-Streptogramin B
MPO  Myeloperoxidase
MRS  de Man-Rogosa-Sharp
MUG  4-methylumbelliferyl β-D-glucuronide
NADPH  Nicotinamide Adenine Dinucleotide Phosphate
NGF  Nerve Growth Factor
NIH  National Institutes of Health
NIH-CPSI  National Institutes of Health Chronic Prostatitis Symptom Index
NSAID  Non-Steroidal Anti-Inflammatory Drug
OPDA  o-phenylenediamine
OxS  Oxidative Stress
PAP  Prostatic Acid Phosphatase
PBS  Painful Bladder Syndrome
PBP  Prostate Binding Protein
PCR  Polymerase Chain Reaction
PGE  Prostaglandin E
PGF\textsubscript{2α}  Prostaglandin F 2 alpha
PMN  Polymorphonuclear
PSA  Prostate-Specific Antigen
Q\textsubscript{10}  Coenzyme Q\textsubscript{10} (Ubiquinone)
ROS  Reactive Oxygen Species
rRNA  Ribosomal Ribonucleic Acid
SE  Standard Error
SIP  Stock Iso-osmotic Percoll
SOD  Superoxide Dismutase
STD  Sexually Transmitted Disease
TAA  Total Antioxidant Activity
TAS  Total Antioxidant Status
TBA  Tributyrin Acid
TEMPO  2,2,6,6-tetramethylpiperidine-1-oxy radical
TGF-β  Transforming Growth Factor Beta
TLR  Toll-Like Receptor
TMP/SMX  Trimethoprim/Sulfomethoxazole
TNF-α  Tumor Necrosis Factor Alpha
TNFR  Tumor Necrosis Factor Receptor
TRPV  Transient Receptor Potential Vanilloid
TUMT  Transurethral Microwave Therapy
TUNA  Transurethral Needle Ablation
TURP  Transurethral Resection of the Prostate
UK  United Kingdom
USA  United States of America
UTI  Urinary Tract Infection
UV  Ultraviolet
VB1  Voided Bladder 1, Initial-stream Urine
VB3  Voided Bladder 3, Post-prostate-massage Urine
WA  Washington
WBC  White Blood Cells
WC  Wilkins-Chalgren
WHO  World Health Organization
INTRODUCTION

Prostatitis are a puzzling set of clinical entities that have been grouped in National Institutes of Health (NIH) Prostatitis Classification (NIH Chronic Prostatitis Workshop in Bethesda, MD, 1995; Table 1). Bacterial etiology has been shown for two categories – acute bacterial or NIH I and chronic bacterial or NIH II that are caused by known urinary tract pathogens – *Escherichia coli* and other *Enterobacteriaceae* and enterococci. The etiology of remaining categories (NIH III and NIH IV) is largely unknown. Chronic Prostatitis/Chronic Pelvic Pain Syndrome (CP/CPPS) or NIH III is characterized mainly by long-lasting pelvic pain. This category is divided into inflammatory and noninflammatory subtypes, also known as NIH IIIA and NIH IIIB, according to presence or absence of white blood cells (WBC) in prostate secretion (EPS), semen or postmassage urine (VB3). Asymptomatic inflammatory prostatitis or NIH IV is a clinical entity that is only superficially investigated – due to lack of symptoms this condition is usually found by chance (Korrovits, 2008). While leukocytospermia has been studied in the context of infertility before, the category of NIH IV prostatitis was created about 15 years ago (NIH Chronic Prostatitis workshop in Bethesda, MD, 1995).

The problems with prostatitis of categories III and IV include inadequate understanding of etiology and pathogenesis, insufficient methods for diagnosing and subtyping patients as well as deficient treatment schemes. On one hand, there is a multitude of treatment options to choose, including several experimental treatment modes. On the other hand, there is a longtime tradition of treating prostatitis patients with antibiotics although the etiology remains mostly unknown. To date, it is not clear whether and which of the treatment modes are reliable (with a probable exception of α-blockers) since the etiopathogenesis is unclear. Nevertheless, any kind of prostatitis (from NIH I to NIH IV) is usually treated with fluoroquinolones but it is unknown whether and which bacteria are valid fluoroquinolone targets in case of category III or IV prostatitis.

In Estonia, prostatitis has been the subject of interdisciplinary research shared by Department of Microbiology in University of Tartu and Andrology Centre of Tartu University Hospital since 1999 with the support from Estonian Science Foundation (Kermes et al., 2003; Punab et al., 2003; Korrovits et al., 2006a; 2006b; 2008). The pertinent earlier studies of our workgroup have shown the presence of abundant polymicrobial communities in the semen of NIH III and NIH IV prostatitis patients (Kermes et al., 2003; Korrovits et al., 2006; Punab et al., 2003). However, the role of particular species belonging to this microbiota as possible causative agents as well as their association with inflammation has remained unclear. Therefore, we focused our research on two groups of microorganisms. First, we investigated coryneform bacteria (*Corynebacterium sp.* and morphologically related genera) because they are overlooked in routine diagnostics although some studies have associated them with pros-
tatitis. In order to supplement physicians with the data relevant to antibacterial treatment modes, we gathered also antibacterial susceptibility data. Second, we studied mycoplasmas because some of them are associated with urethritis while they remain undetected during routine prostatitis diagnostics. Third, we made a complex study where we intended to see how microbiota (especially coryneforms), microelements and semen parameters relate to oxidative stress (OxS) that accompanies inflammatory prostatitis.

All studies for this dissertation were carried out in the Department of Microbiology in University of Tartu, Department of Biochemistry in University of Tartu and Andrology Centre of Tartu University Hospital.
REVIEW OF LITERATURE

1. Prostate gland

1.1. Anatomy and histology

The prostate is a muscular pyramid-shaped exocrine gland lying on pelvic muscularfascial floor. It is inferior to urinary bladder and surrounds the first three centimeters of the urethra, and is connected to the bladder neck. The part of urethra that passes the prostate is three centimeters long, and ejaculatory ducts join the urethra at that section. The prostatic urethra is distal from the bladder. These glands and acini are lined by columnar secretory cells. The columnar secretory cells are separated from the stroma by a layer of basal cells, which belong to basement membrane. The prostate itself is fixed to puboprostatic ligaments, and surrounded by prostatic capsules referred to as “true” and “false” capsule. Puboprostatic ligaments connect the prostate with pubic bones. A layer of prostatic smooth muscles is continuous with the vesical muscles and sphincter urethrae. The glandular tissue consists of numerous follicles, which open to elongated canals, which join to form 15–20 excretory ducts; the ducts and canals are held together by loose connective tissue, muscular stroma and extensions of fibrous capsule. Canicular and follicular epithelium is columnar, but the prostatic ducts have another layer of cuboid epithelium under the columnar one. The prostate is 4 cm wide, 3 cm high and 2 cm deep (Churchill-Livingstone 1995).

Different regions of the prostate have different susceptibilities to pathologies and the histologists can observe regional differences in the structure and stroma as well. These regional differences are the reason behind a classification, which divides prostate to peripheral, central and transition zone. For example, the transition zone is susceptible to prostatic hyperplasia, peripheral zone to prostate cancer. According to Churchill-Livingstone (1995), an older classification system divides prostate into five lobes (anterior, posterior, median and two lateral lobes). Some researchers deny topographical lobation; others have not managed to agree upon topography and terminology (Churchill-Livingstone 1995; McNeal 1988).

1.2. Physiology

The smooth muscles of the prostate can contract like a sponge to squeeze the prostatic secretions from the acini via the ducts into the prostatic urethra (Carola et al., 1990). If the prostate would be removed, then ejaculate would be propelled into the bladder. The prostate has abundant innervation, sympathetic and parasympathetic. The nerves come from pelvic plexus and form a periprostatic plexus. Neuropeptide Y and vasointestinal polypeptide nerve fibers are localized in the subepithelial nerve tissue, smooth muscles and walls of blood vessels (Churchill-Livingstone 1995). The associations between prostatitis and
pain-related messenger molecules may be critical, the pain-related molecules being substance P, calcitonin gene-related peptide (CGRP), endorphins and heat receptor TRPV1 (Chen et al., 2005; Zhang et al., 2007; Tang et al., 2007; Meyer-Siegler et Vera 2004; Shahed et Shoskes 2001; Turini et al., 2006).

The prostatic secretion makes spermatozoa motile and helps to neutralize vaginal acidity. The prostatic secretion is a minor component in the urine and a major component (circa 20%...30%) in the ejaculate. The volume of the ejaculate ranges from two to six milliliters (McCance et Huether 2006). The secretion of healthy men’s prostate is slightly acidic (pH 6.2...6.5), in contrast to basic reaction of seminal vesicle secretion (White 1975); those combine with secretions of other glands into semen with normal pH range of 7.8...8.0 (Haugen et Grotmol 1998). There is a shift towards basic reaction of EPS (expressed prostatic secretions) in case of chronic prostatitis (Wagenlehner et al., 2005).

The molecular composition of prostate secretion is complex, and the list of its constituents is not limited to those discussed below. Transition metal zinc was known as ‘prostatic antibacterial factor’ before knowing that it was just zinc (Fair et Parrish 1981). Accumulation of Zn in the mitochondria of prostate inhibits citrate oxidation. Hence, unusually high citrate concentrations are characteristic for the prostate (Costello et Franklin 1998). Prostatic ascorbic acid is very important for protecting DNA from oxidative damage (Song et al. 2006). Ubiquitous polyamines (spermine, spermidine, and putrescine) of seminal fluid originate from prostate and these stress-induced molecules regulate gene expression, cell proliferation and signaling, function as reactive oxygen species (ROS) scavengers, chemical chaperones, contribute to acid tolerance and are essential to pathogen-host interactions (Rhee et al. 2007, Lynch et Nicholson 1997, Jakobsen et al., 1989; Lynch et al. 1994). 27 non-serum proteins were found from prostate fluid by Lee et al. (1986). Most pertinent EPS proteins of a healthy man include prostatic PAP (prostatic acid phosphatase), PBP (prostate binding protein) and PSA (prostate-specific antigen). These proteins are, respectively, an androgen-dependent cancer marker, an androgen-dependent marker of secretory function, and a favorite prostate cancer marker that was also the enzyme responsible for semen liquefaction (Lee et al., 1986; Lam et al., 1979, Seregni et al. 1996; Pelletier et al., 1988; Aumüller et al., 1985; Saito et al., 2007; Lukkarinen et al., 1993). The normal prostate fluid also contains extracellular messenger molecules like macrophage migration inhibitory factor and epidermal growth factor (Frenette et al., 2005; Fuse et al., 1992).

Prostate is the source of small (40–600 nm) membrane-bound vesicles (similar to synaptosomes) that can fuse with sperm – these particles are prostatosomes (Burden et al., 2006). Prostatosomes enrich spermatozoa with various proteins, zinc and calcium; these contribute to semen liquefaction, act against bacteria, influence immune system and, finally, stimulate the acrosome reaction (Stegmayr et Ronquist 1982, Arienti et al., 2004; Vivaqua et al., 2004, Oliw et al. 1993; Siciliano et al., 2008). Hence, the prostatosomes seem as if supplementing the spermatozoa during the period, starting with spermatozoa exposing to prostate secretion and ending with the fertilization.
In the present thesis, relation between leukocytospermia and inflammation of the prostate is an important topic. In that context, it is crucial to know that the leukocytes in semen seem to come normally mainly from epididymis while in case of pathology from prostate instead (Tsuboi et al., 2007; Wolff 1995; Haidl 1990; Simbini et al., 1998).

2. Prostatitis

2.1. Concept and classification

Prostatitis is an arbitrary term for a common but poorly understood concept. Evidence of forsaking logic and reason altogether is present as the diagnostic category of so-called “non-inflammatory prostatitis”. Prostatitis does have characteristic symptoms but these symptoms are far from unique in the sense of sensitivity and selectivity, so it is diagnosed when the patient does not fit into other categories. Diagnosis is made by assessing symptoms, inflammation, and presence of pathogens. Fortunately, the recently developed NIH-CPSI questionnaire has improved assessing the symptoms now. The symptoms assessed by NIH-CPSI are divided into three subcategories: pain, urination and quality of life.

Inflammation is measured by default from semen, postmassage urine (VB3) or EPS. If invasive methods are justified, then prostate biopsy may be chosen. The older prostatitis classification stands upon on the Meares-Stamey ‘four glass test’ and it divided patients into four categories. If a pathogen was present, then the prostatitis was either acute or chronic bacterial prostatitis. If the pathogens were not present then it depended upon whether there was inflammation (chronic non-bacterial prostatitis) or not (prostatodynia) (Drach et al., 1978; Meares et al., 1968).

Aiming to improve the diagnosis and treatment of prostatitis, the National Institutes of Health (NIH) established an International Prostatitis Collaborative Network. This group convened two consensus conferences (1995 and 1998) to establish a new definition and classification of prostatitis syndromes (Krieger et al., 1999). As a result, a new, NIH classification emerged along with the concept of CP/CPPS that stands for chronic prostatitis/chronic pelvic pain syndrome, which is a composite of prostatodynia and non-bacterial prostatitis from previous classification (NIH Chronic Prostatitis workshop in Bethesda, MD, 1995) (Table 1). Since enactment of new classification, there is a category for men with inflammation without prostatitis symptoms, which is NIH IV prostatitis. For sake of simplicity, CP/CPPS will hereunder be referred to as either simply ‘prostatitis’ or ‘CPPS’ – as is commonly done – so that every instance of simply ‘prostatitis’ or ‘CPPS’ alone refers to CP/CPPS. Twice as much men show up as ill if the new classification is used instead of the old one (due to the new category IV). In other aspects, it may be that the differences
between the old and the new classification are mostly semantic, as is the opinion of many physicians and scientists (Krieger et al., 2002) (Table 1).

**Table 1. National Institutes of Health Classification of the Prostatitis Syndromes**

<table>
<thead>
<tr>
<th>Category</th>
<th>Type</th>
<th>Description</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Acute bacterial prostatitis</td>
<td>Acute infection of the prostate gland</td>
<td>Acute febrile illness associated with perineal and suprapubic pain, dysuria, and obstructive voiding symptoms</td>
</tr>
<tr>
<td>II</td>
<td>Chronic bacterial prostatitis</td>
<td>Chronic infection of the prostate gland</td>
<td>Recurrent urinary tract infections with pain and voiding disturbances</td>
</tr>
<tr>
<td>III</td>
<td>Chronic prostatitis/chronic pelvic pain syndrome</td>
<td>Chronic genitourinary pain in the absence of uropathogenic bacteria localized to the prostate gland employing standard methodology</td>
<td>Chronic perineal, suprapubic, testicular, penile or ejaculatory pain associated with variable dysuria and obstructive and irritating voiding symptoms</td>
</tr>
<tr>
<td>IIIA</td>
<td>Inflammatory</td>
<td>Significant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen</td>
<td>See category III</td>
</tr>
<tr>
<td>IIIB</td>
<td>Non-inflammatory</td>
<td>Insignificant number of white blood cells in expressed prostatic secretions, post-prostatic-massage urine sediment, or semen</td>
<td>See category III</td>
</tr>
<tr>
<td>IV</td>
<td>Asymptomatic inflammatory prostatitis</td>
<td>White blood cells (and/or bacteria) in expressed prostatic secretions, post-prostatic-massage urine sediment, semen, or histological specimens of prostate gland</td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>

NIH I prostatitis is an acute bacterial inflammation due to acknowledged urinary tract pathogens. NIH II prostatitis is a chronic inflammation, also due to acknowledged urinary tract pathogens. NIH II is generally considered a recurrent infection. According to Naide et al. (2006) the stereotype of recurrent infection does not always fit, though, so a sub-categorization of NIH II into primary and recurrent was proposed.
NIH III prostatitis is poorly understood, as there is even no agreement whether it is principally a prostatitis (renaming prostatodynia to non-inflammatory prostatitis is a sign of that). As there are difficulties in determining objective criteria, CPPS is considered a symptom complex. The important clinical feature of NIH III to point out is lasting pain (>3 months). Other common complaints include sexual dysfunction and LUTS (lower urinary tract symptoms). NIH III divides into two subtypes: inflammatory NIH IIIA and non-inflammatory NIH IIIB. Inflammation must be confirmed by increased WBC count in biopsy, EPS or semen.

NIH IV is the new addition to prostatitis classification. If there is none of the symptoms but there is an inflammation in any prostate-specific specimen then NIH IV is diagnosed. NIH IV prostatitis is usually detected incidentally, most commonly as a ‘by-product’ of cancer or fertility testing. The body of knowledge regarding NIH IV prostatitis is quite small.

The field seems to be in a phase where improved detection and recognition of both inflammation and pathogens is sought. More data is necessary for evaluation of pertinent policies.

### 2.2. Clinical features

The characteristic symptoms of NIH I prostatitis are those of acute UTI (frequency and dysuria) and some display those of systemic infection (malaise, fever, myalgia). NIH II prostatitis is a chronic, usually recurrent UTI with the same persisting known uropathogen. NIH I and NIH II prostatites have an infectious etiology but are not nearly as prevalent as CPPS.

The lasting pain characteristic to NIH III has been described as pelvic, penile, suprapubic, penile, scrotal, lower back and postejaculatory. Tenderness and altered heat sensitivity is found in perineum as well as other body parts. Hyperexcitability of neurons located in the dorsal horn of spinal cord might be responsible for this sensitization (Yang et al., 2003; Berger et al., 2007; Shoskes et al., 2008). The men with prostatitis are sensitized not only to the heat but also to the opposite, the cold, as well (Hedelin et Jonsson, 2007). There is a link between premature ejaculation and CPPS, especially in case of the inflammatory form (Trinchieri et al. 2007; Shamloul et al., 2006). Lower urinary tract symptoms (LUTS – frequency, urgency, nocturia) may also be a concern in case of CPPS, to the extent that it becomes similar with painful bladder syndrome / interstitial cystitis (Hedelin et Jonsson, 2007). The prostate of a CPPS patient may be normal, tender or boggy; usually the prostate is not enlarged like in case of benign prostatic hyperplasia (Roberts et al., 1999).

The men with prostatitis have troubles with mood, personality and sexuality (Anderson et al., 2008; Mehik et al., 2001; McNaughton-Collins et al., 2001; Keltikangas-Järvinen et al., 1981 and 1982). The sexual problems are loss of libido, erectile dysfunction and decreased sexual activity. Psychical disorders include anxiety, depression, paranoia, compulsions, affect lability, weak
masculine identity and features suggesting of borderline, narcissistic and alexithymic personalities. Their increased stress is also reflected in heightened levels of awakening cortisol (Aubin et al., 2008).

2.3. Histopathological aspects

The diagnosis of NIH IIIA or NIH IV prostatitis is based on heightened amount of leukocytes in semen, expressed prostatic secretions (EPS) or in the prostate biopsy material. Of these, obtaining prostate biopsy has the greatest prostate-specificity but it is invasive; EPS, if obtainable, yields tiny amounts of quite specific material; semen is the least specific but it is the easiest to obtain, and in most cases, also most abundant.

Comparison of prostatitis symptoms with histologic inflammation has revealed controversial results. A prospective prostatitis study by True et al. (1999) revealed that only a third of CPPS patients had any histological inflammation (5% of them had moderate or severe glandular, periglandular or multifocal inflammation) while Schatteman et al. (2000) reported that almost every set of prostate biopsies contained inflammatory material. In some studies, leukocytospermia has shown poor correlation with histological inflammation and hyperemia (Tsuboi et al., 2007; Cho et al., 2000). In other studies, histological inflammation is commonly observed; especially the perivascular inflammation has been associated with elevated PSA levels that indicate tissue damage (Gümüş et al., 2004, Hasui et al., 1994). In a study of 5597 men with and without prostatitis-like symptoms by Nickel et al. (2007), significant correlations were found between average chronic inflammation, and total Chronic Prostatitis Symptom Index score and subscores for urinary symptoms and quality of life but the magnitude of these correlations was small. Histological inflammation is proved ubiquitous (98%) in men with BPH as well (Köhnen et Drach 1979).

Some researchers have investigated whether body parts other than prostate might play a role in the pathogenesis. Parsons (2007) has suggested a new paradigm of dysfunctional urothelium diseases, which would include prostatitis, urethritis and interstitial cystitis (or painful bladder syndrome). Urothelium (urinary epithelium) is a barrier between urine and other tissues. The protective barrier is formed of anionic mucus (glycosaminoglycans) and the disruption of that barrier allows migration of $K^+$ into interstitial space that depolarizes nerves and muscles, and cause tissue injury. Traditionally this potassium sensitivity is associated with the diagnostic window of painful bladder syndrome (also IC/CPPS – interstitial cystitis / chronic pelvic pain syndrome) but this potassium sensitivity is observed in prostatitis diagnostic window as well (Hassan et al.; 2007, Parsons 2007). Correlation between severe LUTS and positive $K^+$ sensitivity test in prostatitis patients show that urothelium is impaired (Hassan et al., 2007).
Finally, there is emerging evidence that inflammation of the prostate may contribute to either hyper- or neoplastic changes, thus leading to BPH or prostate cancer, respectively (Sciarra et al., 2008).

### 2.4. Defense mechanisms of a human

When the defense mechanisms of humans are considered, then it must be taken into account that the pathogenesis mechanisms of prostatitis are unclear, although the later research has granted some more insight. Finer defense mechanisms of the man against CP/CPPS are related to the theories of etiology and pathogenesis. Three main theories (autoimmune, infectious and neuromuscular) point out their respective causes of CP/CPPS: (1) the immune system’s liability to develop autoimmune disease; (2) infectious agents; (3) sensitization towards neuromuscular pain.

Well-known defense factors related to male lower genitourinary tract of macro-organism include the presence of T-cells and tissue macrophages in normal prostate and the capability to recruit other immune cells. One possible mechanism for prostate to recruit macrophages, neutrophils and mast cells is that they are summoned by chemokine IL-15 (Handisurya et al., 2001, Brzezińska-Blaszczyk et Misiak-Tłoczek 2007). IL-15 is only one element of an immunologic cascade that includes IL-18, leukotrienes, TNFR-1, MIP-1α (CCL3), MIP-2, 5-LOX, TNF-α, and TLR4, while IFN-γ is not part of that particular cascade (Verri et al., 2007).

Prostate secretes antibacterial zinc-containing proteins. In healthy men, the average concentration of zinc in EPS was 448 µg/ml; in prostatitis patients it was 50 µg/ml (Fair et al., 1976). The antibacterial activity of Zn was shown in a straightforward study by Cho et al. (2002) where the Zn²⁺ solution was injected directly into the prostates of the rats. The distribution of Zn²⁺ is influenced by stress, adrenergic signals or glucocorticoids in one way (intracellular Zn²⁺↑), and by cholinergic signal in another (intracellular Zn²⁺↓) (Berehova et al., 2007). Anti-infectious properties have also been attributed to Surfactant Protein D, which protects epithelial cells against Chlamydia trachomatis and is upregulated during prostatitis (Oberley et al., 2005).

Normal flow of the urine, its acidity, high osmolarity and antibacterial factors, longer urethra in males, intact urothelium and secretion of IgA to mucosal surfaces have protect against infections as well.
2.5. Epidemiology

2.5.1. Types of studies

Epidemiology of prostatitis has been researched using questionnaires in the following types of studies: cross-sectional study (Ejike et al. 2008), cohort study (Shoskes et al. 2008), and case-control study (Bartoletti et al., 2007). The methods of delivering questions includes direct delivery (Ejike et al. 2008), regular mail (Mehik et al. 2000), recruiting urologists randomly (Nickel et al. 2005) and by using the Internet (Mazzoli et al. 2007). In addition, database research is a mean for epidemiological study (Clemens et al., 2007). There are also epidemiological studies on prostatitis which are based on cooperation of multiple hospitals or research facilities (multi-centre studies); these are made in order to improve objectivity and remove bias (Rizzo et al., 2003). The benefits of getting answers from people by mail rather than telephone are wider range of responses and minimized "acquiescence biases, according to Hall (1995).

Several questionnaires have been used in order to assess symptoms of prostatitis (Schneider et al., 2003): Giessen Prostatitis Symptom Score (GPSS), International Prostate Symptom Score (IPSS), Chronic Prostatitis Symptom Index of the National Institutes of Health (NIH-CPSI). The latter is a valuable tool for both clinical and epidemiological studies but for the puroposes of gaining insight to pathogenetic pathways, perhaps the very recent DABREC phenotyping system will bring an improvement to the understanding of the pathogenesis (Allsop et al., 2011). Since questionnaires measure symptoms, laboratory tests must be used to find out the prevalence of NIH IV prostatitis.

2.5.2. Prevalence of prostatitis

The prevalence of CPPS (NIH IIIA and NIH IIIB combined) has ranged from 2.7% to 14.2% (Table 2). The incidence of CPPS is 33…37 per 10 000 person years while the prevalence of ejaculatory pain (a most characteristic specific symptom of CPPS) is from 1 to 9% in general population (58% in NIH IIIA or NIH II patients) (Mehik et al., 2000; Ilie et al., 2007; Clemens et al., 2005).

Table 2. Prevalence studies of prostatitis

<table>
<thead>
<tr>
<th>Prevalence</th>
<th>Study population</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.2%</td>
<td>1832 men from Finland, 20–59 years old. Cross-sectional postal survey, 75% response rate.</td>
<td>Mehik et al., 2000</td>
</tr>
<tr>
<td>13.8%</td>
<td>2006 men from 28 centers, 25–50 years old. Prospective case-control study of 28 urology clinics.</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>12.8%</td>
<td>8503 men from Italy, 16–83 years old.</td>
<td>Rizzo et al., 2003</td>
</tr>
<tr>
<td>Prevalence</td>
<td>Study population</td>
<td>Reference</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>12.2%</td>
<td>Cross-sectional study by 70 urologists.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1507 men from Nsukka, Nigeria, 20–70 years old. Random cross-sectional survey.</td>
<td>Ejike et al., 2008</td>
</tr>
<tr>
<td>9.7% (11.5% in the younger, 8.5% in the older subgroup)</td>
<td>868 men divided into subgroups of younger and older men, 20–50 and 51–74 years old, respectively. Cross-sectional postal survey.</td>
<td>Nickel et al., 2001</td>
</tr>
<tr>
<td>4.5%</td>
<td>Computer database research in USA. Prostatitis-patients were compared with age-matched controls.</td>
<td>Clemens et al., 2007</td>
</tr>
<tr>
<td>2.7%</td>
<td>1765 men, 20–79 years old</td>
<td>Marszalek et al., 2007</td>
</tr>
<tr>
<td>2.7%</td>
<td>6037 men, comparative prevalence study of prostatitis, interstitial cystitis and epididymitis. The patients were seen by randomly recruited urologists. Urology outpatient study.</td>
<td>Nickel et al., 2005</td>
</tr>
</tbody>
</table>

### 2.5.3. Risk factors of prostatitis

The researchers have found many epidemiological correlates for CP/CPPS (Table 3). For example, Pontari et al. (2005) estimated the risk factors and found that CP/CPPS patients had five times higher prevalence of cardiovascular disorders, triple prevalence of urethritis or neurological disease, two and half times greater prevalence of psychiatric conditions, double prevalence of haematopoietic, lymphatic or infectious diseases. It seems that bicycling is often assumed as a risk factor of prostatitis. There are some case reports of pudendal nerve injury along with otherwise interesting theory of pudendal nerve entrapment. Dangers of bicycling are reviewed by Asplund et al. (2007) and they stated that majority of bicycling injuries are due to overuse, improper equipment, technique, or training patterns. In addition, many papers provide casuistic evidence or mention the dangerous side of bicycling (Leibovitch et Mor 2005, De Rose et al., 2001; Antolak et al. 2002, Ramsden et al., 2003).

<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette smoking</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>High-calorie diet with low intake of fruits and vegetables</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Sexual relationship with more than one partner</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Coitus interruptus</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Frequent masturbation</td>
<td>Gao et al., 2007</td>
</tr>
<tr>
<td>Long-time urine holding</td>
<td>Gao et al., 2007</td>
</tr>
<tr>
<td>Sitting or driving</td>
<td>Gao et al., 2007; Chiappino</td>
</tr>
</tbody>
</table>

Table 3. Epidemiological correlates of CP/CPPS

22
<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constipation</td>
<td>et Pisani, 2003 ; Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Meteorism</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Slow digestion</td>
<td>Bartoletti et al., 2007</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>Clemens et al., 2007</td>
</tr>
<tr>
<td>History of UTI-s</td>
<td>Gao et al., 2007 ; Daniels et al., 2007 ; Pontari et al. 2005</td>
</tr>
<tr>
<td>Cold environment and stress</td>
<td>Gao et al., 2007; Mehik et al., 2000</td>
</tr>
<tr>
<td>Cardiovascular diseases</td>
<td>Pontari et al. 2005; Collins et al., 2002</td>
</tr>
<tr>
<td>Neurological disease</td>
<td>Pontari et al. 2005; Pontari et al. 2005; Clemens et al., 2007; Collins et al., 2002</td>
</tr>
<tr>
<td>Psychiatric conditions (mood, anxiety, other)</td>
<td>Pontari et al. 2005; Pontari et al. 2005; Clemens et al., 2007; Collins et al., 2002</td>
</tr>
<tr>
<td>Hematopoietic, lymphatic, infectious disease</td>
<td>Pontari et al. 2005; Pontari et al. 2005; Clemens et al., 2007; Collins et al., 2002</td>
</tr>
<tr>
<td>Esophageal reflux</td>
<td>Clemens et al., 2007</td>
</tr>
<tr>
<td>Being widowed</td>
<td>Mehik et al., 2000</td>
</tr>
<tr>
<td>Similar diseases (BPH)</td>
<td>Clemens et al., 2007; Collins et al., 2002</td>
</tr>
<tr>
<td>History of STD-s</td>
<td>Collins et al., 2002</td>
</tr>
</tbody>
</table>

### 2.5.4. Similar and comorbid diseases

Prostatitis shares a lot of similarity with other symptom complexes, especially with benign prostate hyperplasia (BPH) and painful bladder syndrome (PBS, also known as IC – interstitial cystitis). According to Barry et al. (2008), the population fitting into diagnostic window of NIH III shares almost half of its men with the diagnostic window of BPH. There is smaller but still remarkable overlap with the diagnostic windows of incontinence and that of IC or painful bladder syndrome (PBS).

CPPS is distinguished from BPH by pain, while the urinary symptoms may be similar. Especially the pain on ejaculation indicates prostatitis but not BPH. According to Nickel et al. (2005), about 20% of BPH patients have pain or discomfort on ejaculation. These BPH patients having prostatitis-like symptoms did clearly differ from the patients with just LUTS, because their LUTS were severe, they had higher prevalence of erectile dysfunction, and reduced ejaculation. Of immunological markers, elevated concentration of IL-8 in the semen is common in both BPH and NIH IIIA prostatitis (Penna et al., 2007). The treatment of BPH and prostatitis is overlapping as concerns α1- or α1A-adreno-blocking agents (Nickel 2008; Lee et al., 2007; Nickel et al., 2003; Dunn et al., 2002; Duclos et al., 2007).

Similarities between prostatitis and PBS (IC) include but are not limited to etiology or pathophysiology, treatment and positive K’ sensitivity test and im-
pact on quality of life (Hassan et al., 2007; Pontari 2006; Peters et al., 1999, Barry et al., 2007). IC patients have 40 times higher risk of also having (or “fitting into category of”) chronic prostatitis (Wu et al., 2006). Urinary cytokine profile of IC has been provided by pioneering work of Peters et al., (1999), rise of IL-2, IL-6 and IL-8 is a trait common with prostatitis. Further similarities between the symptom complexes cannot be assumed, because Khadra et al. (2006) investigated IL-8 from both urine and semen of CPPS patients and found IL-8 solely from semen, not urine. There is currently not enough information for a valid comparison between the cytokine profiles of IC and prostatitis.

In addition to similar diseases like BPH, IC and occasional pudendal nerve entrapment, there are other diseases reported as comorbid with prostatitis (Table 3). These diseases are allergies, anxiety disorders, depression, erectile dysfunction, ejaculatory dysfunction, gastrointestinal disorders, premature ejaculation, irritable bowel syndrome, rheumatologic diseases, sinusitis, psychiatric conditions and various soft tissue disorders (Antolak et al. 2002; Clemens et al. 2006; Clemens et al. 2007; Wu et al. 2006; Trinchieri et al. 2007; Li et al., 2002; Naughton-Collins et al. 2001; Pontari 2003).

2.6. Impact on life quality

Prostatitis seriously affects the quality of life (Wenninger et al., 1996; Walz et al., 2007; Smith et al., 2006). In a study of Walz et al. (2007), 10.5% of men had prostatitis-like symptoms, which adversely affected the quality of life in growing order of magnitude: urinary frequency, incomplete bladder emptying, pain frequency and pain intensity. According to Tripp et al. (2004), the main predictors of prostatitis patient’s life quality were LUTS, depressive symptoms and pain intensity. The negative impact of prostatitis is comparable to that of myocardial infarct or Crohn’s disease. Prostatitis is a problem not only to the patient but to his female partner, too.

2.7. History of prostatitis research

The pioneers of prostate research were Herophilus (circa 350 BC) and Nicola Massa (16th century) who elucidated prostate anatomy. Legneau described prostatitis syndrome in 1815. In the last two decades of 19th and in the three first decades of 20th century the bacteria were sought as etiological factor. Later on, the possibility of other etiological factors was taken more seriously, while some doctors and psychoanalysts denied the existence of chronic prostatitis at all (summarized by Mehik, 2001). The invention of four glass test (Meares et Stamey, 1968) and prostatitis classification (Drach 1978) fueled the following prostatitis research.

The last decade of 20th century saw the acceleration of prostatitis research, including increase in therapeutical options and a new prostatitis classification.
To improve the diagnosis and treatment of prostatitis, the National Institutes of Health (NIH) established an International Prostatitis Collaborative Network. This group convened 2 consensus conferences (1995 and 1998) to establish a new definition and classification of prostatitis syndromes (Krieger et al., 1999) (Table 1). It was speculated that a new era of prostatitis research begins, because of new classification, validated symptom assessment tool (NIH-CPSI) and consensus on the future of prostatitis research (Nickel 2000). The beginning of 21st century saw also a critical revision of extant treatment modes, ‘prostato-centric approach’ and infectious etiology (Potts et al., 2003). The rising activity in the field is evident: 2301 articles are available in PubMed in answer to a query of “chronic prostatitis” and 1090 of these have been published in the 21st century. Research involves continued search for pathogens, elucidating immune, neural and oxidative mechanisms behind prostatitis. In addition, several animal models have been developed, new epidemiologic surveys have been made and from the practical side there is an ongoing search for better diagnostic and treatment options. Incremental additions to mainstream theories are made steadily, and even the possibility of paradigm shift for the entire field has been discussed, and that is good, because the fundamental question why these patients suffer is still unanswered.

2.8. Theories of etiology and pathogenesis

Infectious, autoimmune and neuromuscular – these have been the main theories of etiology during last two decades. Additional etiopathogenesis theories include but are not limited to anatomic and traumatic (Vega 2000; Nickel 2002), prostatic stones (Geramoutsos et al., 2004), hormonal (Dimitrakov et al., 2008), urothelial dysfunction (Parsons, 2007), and most recently, dysbacteriosis (Liu et al., 2009). Theory of multifactorial cascade (Nickel, 2002) has attempted to tie some more specific mechanisms into a related complex form.

2.8.1. Theory of multifactorial cascade

Nickel (2002) has postulated that the pathogenesis of prostatitis may involve several inter-related pathogenetic pathways, starting in an initiating event and culminating in a neuropathic state (Fig. 1). These ‘initiators’ can be infection, high pressure dysfunctional voiding, trauma or toxin. This initiating event can result in either injury and/or inflammation. The injury could be to the local nerves and muscles or even the prostatic glandular or stromal tissue. Inflammation likely is initially restricted to the prostate and peri-prostatic area. The initial neuropathy or immunologic reaction may progress because of persistent initiating factors (persistence of bacteria, dysfunctional voiding or perineal trauma). Neuropathic and immunologic pathology can persist, even with eradication of or amelioration of the initiating factor, through a self-perpetuating
stimulatory loop. Inflammation can continue because of initiation of a new autoimmune mechanism. Inflammation in the prostatic and peri-prostatic area can promote a neurogenic reaction resulting in chronic neuropathy. It is also recognized that peripheral neuropathy can initiate and promote a progressive and durable inflammatory reaction. Up-regulation of the local pelvic neural loop perpetuates the neuropathic state. The result for the patient is pain in the perineum, pelvis and genitalia, abnormal voiding parameters and because of the proximity of the erectile mechanisms in the area, various degrees of sexual dysfunction. Therapies aimed at the initiating factors are important to eradicate potentiating agents but, in the long term, they may be ineffective, because the syndrome has progressed along the spectrum of diseases, where the initiating event may now be irrelevant. This is the rationale for the introduction of neurologic medications, neuromodulatory interventions and physical therapies.

As concerns the neuropathic state, it might be viewed as a vicious circle, since neural irritation of one pelvic organ could have radiated through spinal, maybe also supraspinal mechanisms into other pelvic organs due to pelvic cross-sensitization (Malykhina, 2007; Chen et al., 2005). Data support the idea that neural circuits operating in a pathogenic mode unleash and keep up an cascade of factors which influence not only one pelvic organ but are capable of dealing collateral damage to other organs as well (Meyer-Siegler et Vera, 2004; Vera et Meyer-Siegler 2004; Zhang et al. 2007).

Fig. 1. Scheme of multifactorial cascade in pathogenesis of prostatitis (Modified from Nickel et al., 2002).
2.8.2. Infectious

NIH I and NIH II prostatitis are considered infectious diseases because traditional uropathogens, like *E. coli* or enterococci, can be isolated employing routine tests (Nickel 2002). At the same time, NIH III prostatitis (CPPS) lacks ‘traditional’ uropathogens in prostate-specific materials (semen, VB3 or EPS). Hence, only routine cultures do not differentiate between healthy men and CPPS patients. This has been expressively shown by Nickel et al. (2003), who found controls and CPPS patients as microbiologically similar, and it did not make any difference whether the chosen specimen was semen, EPS or VB3. Microbial counts have not been found to correlate with symptoms, either (Schaeffer 2003; Shoskes et al., 2004).

Opinions about the plausibility of infectious etiology have ranged from one extreme to another: one group declared evidence non-existent (Potts et al., 2003); another suggested that the major cause was a single overlooked pathogen (Skerk et al., 2007); third was wary of extant but encouraging about future evidence (Pontari et Ruggieri, 2008). Whether Gram-positive organisms other than enterococci can be considered commensals or pathogens is a good question, and while the ‘final truth’ is not yet there, some preliminary answers have been provided by Tanner et al. (1999), Shahed et Shoskes (2000), Ivanov et al., (2008) and by our previous research (Kermes et al., 2003). Many groups have undertaken the task to improve detection limits or find out unusual pathogens (Tanner et al., 1999; Takahashi et al., 2003; Szöke et al., 1998; Skerk et al., 2004; Budía et al., 2008; Villanueva-Diaz et al., 1999; Krieger et al., 1996). Hua et al., (2005) reviewed the topic of unusual pathogens and concluded that specific microorganisms (atypical pathogens) explained prostatitis in up to tenth of the patients.

2.8.2.1. Microbiological studies using conventional methods

In order to discern pathology one must know the norm. In case of any prostate-specific material (EPS, semen or VB3), there is an inherent possibility of urethral contamination. Rehewy et al. (1979) have studied semen of healthy men and found *Staphylococcus epidermidis, Staphylococcus aureus, Corynebacterium sp, Mycoplasma hominis* and *Ureaplasma urealyticum*. The study by Willen et al. (1996) was an in-depth analysis of normal urogenital microflora. They studied nearly one hundred healthy men scheduled for vasectomy and compared microorganisms from vas deferens, EPS, semen and distal urethra (coronal sulcus) using aerobic and anaerobic cultures. Their results showed that CNS and streptococci (but not corynebacteria) were the dominant microbial groups, and the main source of these bacteria was urethra. According to studies of Montagnini-Spaine et al., (2000) in healthy urethra and prostate secretions the predominating microorganisms were CNS, viridans streptococci, *Corynebacterium sp.* and *Enterococcus sp.*
2.8.2.1.1. Expressed prostatic secretion

Most of the studies have implemented expressed prostatic secretions (EPS), a traditional sample in prostatitis studies. Bartoletti et al. (2007) used 152 probably infertile men as controls for 2006 prostatitis patients. Their microbiological study implemented traditional Meares-Stamey four-glass test that was positive in 13.3% of prostatitis patients and 2.9% of infertility patients. In addition, 6% of Meares-Stamey negative prostatitis patients carried agents of sexually transmitted disease in their urethras.

Shahed et Shoskes (2000) have considered any Gram-positive aerobic bacteria (mainly staphylococci, enterococci and corynebacteria) as pathogens if those were expressed specifically in EPS or were expressed in 100 times higher concentration in VB3 than in VB1. The most common Gram-positive microorganisms in their study were staphylococci, enterococci and corynebacteria. If these Gram-positive microorganisms were present in such quantities, then the authors diagnosed chronic bacterial prostatitis (NIH II).

Anaerobic bacteria have been seldom searched from prostate-specific specimens. Szöke et al., (1998) searched for anaerobic species using a cut-off value of $10^6$ CFU/ml and 6-day incubation. As a result, they observed that 18 of 50 patients were positive for anaerobes only, six of 50 were positive for both aerobic and anaerobic species and none was positive for solely aerobic bacteria. Nearly half of the patients (26 men) remained microbiologically negative at this cut-off value. Peptostreptococcus sp. was quite usual (54%), followed by Propionibacterium sp. (30%), Bacteroides ureolyticus (20%), Prevotella sp. (16%), Bifidobacterium sp. (14%), Eubacterium sp. (12%), Prevotella sp. (10%) and Veillonella sp. (10%). Disappearance of anaerobes from EPS was associated with therapeutic success.

2.8.2.1.2. Semen

Semen is clinical sample that is relatively easy to obtain, especially in younger men, and is relatively less time-consuming for physician than EPS. It has been approved for prostatitis diagnostics by NIH workshop on chronic prostatitis in Bethesda, MD, USA, 1995 (Executive Summary, 1995). Although some investigators have found the sensitivity of microbiological semen analysis inferior to that of EPS (Weidner, Anderson 2008), the others have claimed just the contrary results (Budia et al., 2006). Previous studies of our research group have supported the latter opinion indicating that from the microbiological viewpoint, semen is a suitable specimen differentiating well between prostatitis patients and controls (Kermes et al., 2003; Punab et al., 2003). In these studies, quantitative cultures of microorganisms, including anaerobic and microaerophilic species were employed, the most frequently found microorganisms being CNS, peptostreptococci, corynebacteria and anaerobic Gram-negative rods. The main differences between prostatitis patients and controls were quantitative – inflammatory chronic prostatitis patients harbored significantly higher total concentration of bacteria as well as higher number of different species in their semen than controls. In the study of Kermes et al. (2003) also suitability of semen
as specimen was proved comparing semen with first-catch urine that reveals urethral microflora. The microorganisms’ concentration and number of species were significantly higher in patients’ semen than in patients’ urine, and these specimens shared only one third of species showing that most of the semen microorganisms originate from upper genital tract. In an interesting study, Ivanov et al., (2008) have investigated anti-complement activity of semen microorganisms. They found that microorganisms isolated from patients revealed greater anti-complement phenotype than those isolated from control group. They suggested that characterizing prostatitis microbiota should be focused upon functional parameters (resistance to host defense mechanisms) rather than upon classical taxonomy. In fact, already in 1975 Mårdh and Colleen found that the semen of healthy men inhibited the growth of staphylococci more than the semen of prostatitis patients.

2.8.2.1.3. Prostate tissue
Physicians usually do not use biopsy to diagnose prostatitis. Prostate tissue has mainly been researched in association with prostate cancer diagnostics, and there are a few studies, which have used samples from organ donors. There are three types of prostate biopsy: transrectal, transperineal and transurethral. Usually, the physicians have stuck to the transrectal approach (Ravery et al., 2000). All these methods may be associated with contamination of specimen, especially transrectal, although double needle techniques can help to diminish it. Doble et al., (1989) found bacteria from only 15% of patients’ biopsy samples while Lee et al. (2003) found that this number would be approximately 37% in both patients and controls, and both groups used transperineal biopsy, which minimized bacterial contamination. Doble et al. (1989) treated their patients with antibiotics respective to these bacteria. The treatment failure led the authors to the idea that these bacteria were contaminants rather than causative agents. The authors also took special effort to clarify whether chlamydiae were behind prostatitis, but they did not find any active chlamydiae in the prostates nor did they find moderate or higher serum titers of Chlamydia trachomatis antibody. Matsumoto et al., (1992) reported that biopsy culture is seldom positive compared with EPS or semen. They reasoned that the infections could have been focal ones. Berger et al. (1997), on the contrary, could demonstrate that inflammatory EPS correlated with isolation of any bacteria, including anaerobes, as well as higher bacterial counts and more species isolated in biopsy material. The latter is in good correlation with our former study (Kermes et al., 2003).

2.8.2.2. Microbiological studies using genotypic methods
Researchers have used nucleic acid probes not only for detection of individual species but for detection of any prokaryotic species as well. That was possible by using sequences that coded 16S subunit of the ribosome, which has been relatively resistant to changes during the course of phylogenesis.
2.8.2.2.1. Expressed prostatic secretion

The EPS studies that implement genotype-based methods form a group of studies that point in the direction of infectious etiology. Tanner et al., (1999) found with 16S rRNA probe an unexpectedly diverse list of Corynebacterium species, some of them characteristic to men with prostatitis. These bacteria were often unculturable. Interestingly, 7 of 11 men who had bacteria in EPS were susceptible to treatment with antibiotics. Ribosomal DNA of ‘atypical pathogens’ (Chlamydia, Mycoplasma or Trichomonas) has been found as well – Krieger et al. (1996) found that 8% of CPPS patients had one of those, and Skerk et al., (2007) detected Chlamydia trachomatis in more than third of prostatitis patients (using both geno- and phenotypic methods). Liu et al. (2006) found traces of bacteria from EPS of all NIH II patients, 94% of NIH IIIA patients and 67% of NIH IIIB patients. Zhou et al. (2003) found that in 78% of patients the concentrations of prokaryotic DNA were a log higher in EPS than in VB1 urine, and patients with such bacterial signal did respond better to antibiotic treatment.

2.8.2.2.2. Semen

Microbiological studies of semen have been mostly conducted in context of infertility while less in prostatitis patients. Infertility has been frequently associated with leukocytospermia – that is also the basis to diagnose asymptomatic inflammatory prostatitis (NIH IV). By our best knowledge, there are only two studies of semen that have used metagenomic methods to detect wide spectrum of microorganisms (Jarvi et al., 1996; Kiessling et al., 2008).

Jarvi et al. (1996) investigated healthy culture-negative semen donors as well as infertile men. They found that an equal proportion, two thirds of donors or infertile men had at least 10^4 bacteria per ml by quantitative PCR. The most pertinent bacteria were Prevotella sp. Other bacteria included several anaerobic and aerobic species like Peptostreptococcus, Veillonella, Eubacterium, Corynebacterium group, Rubrivirax, Actinobacillus, Streptococcus, and Burkholderia.

Kiessling et al., (2008) investigated the presence of rDNA of microorganisms in the semen of men undergone fertility evaluation or vasectomy. Conditions of PCR were adjusted to detect only abundant organisms (>20 000 bacteria/mL). 65% of the men were positive. The most frequently found genera were Peptomiphilus, Anaerococcus, Finegoldia, Peptostreptococcus and Corynebacterium. Normal sperm forms were lower in the rDNA positive than negative subjects were. The authors concluded that abundant bacteria in semen are not commensals but arise from infection in the male genitourinary tract.

In addition to the two above-described studies, some scarce papers present data of certain species. Badalyan et al., (2003) investigated whether chronic prostatitis patients had Chlamydia sp. or Ureaplasma sp. in their semen and found one third of the both inflammatory and non-inflammatory prostatitis patients as PCR-positive.
2.8.2.3. Prostate Tissue

Hochreiter et al. (2000) and Xie et al. (2003) have investigated the autopsy material from apparently healthy men for the presence of prokaryotic nucleic acids. Xie et al. (2003) found that fifth of the normal and half of the inflammatory samples had traces of bacteria inside. Hochreiter et al. (2000) found traces of bacteria in association with inflammatory changes due to BPH or cancer, while healthy prostates were void of such traces.

In a series of studies (Krieger et al., 1996; Riley et al., 1998, Krieger et Riley, 2004), ¾ patients’ prostates have been positive for nonspecific probes, while 10% of patients had at least one of the following species: *Mycoplasma genitalium*, *Chlamydia trachomatis*, or *Trichomonas vaginalis*. The patient group of these studies was selected, though, by being refractory to multiple courses of antibiotics while lacking evidence of ‘true’ or ‘atypical’ pathogens. At the same time the results of Takahashi et al., (2003) and Leskinen et al., (2003) are less supportive of infectious etiology showing that 25% or 10% of prostatitis patients had traces of any bacteria in their prostates, respectively. The former study also revealed that 10% of patients were positive for *Escherichia coli*.

2.8.2.3. Animal studies

Animal models in prostatitis research have been used for immunological, microbiological, pharmacological and neurological studies. Rats, dogs, non-obese diabetes mice, guinea pigs and baboons have been used. Motrich et al., (2008) observed deterioration of semen quality due to OxS in rats with experimental autoimmune prostatitis. Phan et al., (2008) and Quintar et al. (2006) have investigated prostatic infections in rats and found that when they introduced a pathogen (*Proteus* or *Escherichia*), then an infection occurred, and that infection always included an acute component. In the rat model described by Nickel et al., (1990 and 1991) the infection of the prostate persisted in the form of sparse slime-protected microcolonies in prostatic ducts and acini. They found that the progression of the disease had striking similarities to natural progression of the disease.

2.8.2.4. Coryneform bacteria in case of prostatitis

Coryneform bacteria are aerobic, asporogenous, not acid-fast, irregular Gram-positive rods (Funke et al., 1997). They belong to the phylum *Actinobacteria*. Their classification has undergone dramatic changes – genus *Corynebacterium* has been defined more narrowly and many species now belong to other genera like *Arthrobacter*, *Cellulomonas* and *Rhodococcus*, instead. With a notorious exception of *C. diphtheriae*, the coryneform bacteria have been considered as part of the normal human flora or environmental contaminants, but were rec-
ognized increasingly as a cause of life-threatening diseases later (Bernasconi et al., 2004). In addition, new coryneform species were discovered frequently. One of them, C. seminale (also known as C. glucuronolyticum) was discovered first from prostatitis patients (Riegel et al., 1995).

As mentioned before, coryneform bacteria have been frequently found in male urogenital tract (Rehewy et al., 1979; Willen et al., 1996; Montagnini-Spaine et al., 2000) and some authors have associated these microorganisms with prostatitis (Drach, 1974; Domingue et Hellstrom, 1998; Tanner et al., 1999, Kermes et al., 2003). At the same time, these bacteria tend to be often overlooked, and the unculturable or fastidious coryneforms remain undetected during routine culturing measures. Upgrading from blood agar to enriched media and paying extra attention to microscopy has revealed the presence of those Gram-positive irregular rods.

The list of coryneforms found from healthy or prostatitis-associated male urogenital tract includes C. seminale (Riegel et al., 1995), C. afermentans, C. xerosis and Corynebacterium group ANF (Tanner et al., 1999), C. singulare (Riegel et al., 1997), C. freneyi (Renaud et al., 2001), C. striatum or C. amycolatum, C. macginley, C. jeikeium, Dermabacter hominis (Jedrzejezak et al., 2005), C. minutissimum (Domingue et al., 1997) and even the notorious C. diphtheriae (Machado et al., 1989). In addition, Gardnerella vaginalis (a catalase-negative, bacterial vaginosis-associated coryneform) has been found from male genital tract (Hillier et al. 1990).

Nucleotide-based studies by Tanner et al. (1999) and Lee et al. (2007) both showed that Corynebacterium sp. were the most common bacteria in the EPS or urine, respectively, among prostatitis patients. More specifically, Corynebacterium species were more prevalent, abundant and represented by higher number of species in prostatitis patients than controls. Tanner et al., (1999) even found some yet unidentified species that were restricted exclusively to the patients. This study showed the limitations of classic microbiology as PCR managed to detect nine species from one patient alone. It has been also speculated (but not proved) that coryneforms could grow in the prostate as a biofilm that would enhance antibiotic resistance (Tanner et al., 1999).

Since coryneform bacteria may be associated with prostatitis, it may be valuable to know about their susceptibility. Domingue and Hellstrom (1998) and Funke et al., (1996) have investigated and reviewed the huge differences in the antibiotic susceptibility patterns of coryneform species. The resistance profiles of corynebacteria have been species-specific but not without their peculiarities. For example, a generally multiresistant species (C. amycolatum) was susceptible to tetracyclin, while a resistance against the same antibiotic characterized a species (C. seminale) that was clearly not multiresistant (Funke et al., 1997). One species, C. resistens, even got its name from its characteristic multi-resistance (Otsuka et al. 2005). As concerns penicillin resistance, it is very unlikely that coryneforms were producing β-lactamase (Martinez-Martinez et al., 1996). Several lines of evidence report high resistance of coryneforms to macrolides and lincosamides (Fernandez et al. 2001; Funke et al. 1997;
Martinez-Martinez et al. 1996; Soriano et al. 1995; Ubaldi et al. 2004). Macrolide and lincosamide resistance occur together in MLSb (Macrolide-Lincosamide-Streptogramin B) resistance pattern, as suggested by Rosato et al. (2001). As concerns nitrofurantoin, low MICs have been reported, so far (Soriano et al., 1995; Riegel et al., 1995). It seems pertinent to fluoroquinolone susceptibility that Corynebacterium sp. lack Topoisomerase IV (Sierra et al., 2005), the enzyme that is the main target of trovafloxacin, levofloxacin, ciprofloxacin and norfloxacin, and a secondary target of gatifloxacin andsparfloxacin (Takei et al., 2001). Theoretically, lack of Topoisomerase IV should grant partial intrinsic resistance to fluoroquinolones.

Hence, possible differences in coryneform composition of patients’ and controls’ microbiota as well as their role in etiopathogenesis of prostatitis are not finally elucidated. Moreover, as their susceptibility patterns are not uniform, the testing of prostatitis-associated strains would provide information for physicians who implement empiric antibacterial therapy.

2.8.2.5. Mycoplasmas in case of prostatitis

Mycoplasmas are the smallest freely living bacteria that are classified into Mollicutes because they have no cell wall. It is believed that Mollicutes probably derived from lactobacilli, bacilli or streptococci by losing cell wall and some biosynthetic pathways until they became the smallest and simplest free-living and self-replicating cells of today (Razin et al., 1998; Woese et al., 1980).

More than third of prostatitis patients harbor some Mollicutes (Corradi et al., 1992). Urologically relevant species have been considered Ureaplasma urealyticum, U. parvum, Mycoplasma genitalium, and M. hominis. These bacteria seem at least mildly harmful because of associations with decreased sperm quality and OxS (Weidner et al., 1978; Berger et al., 1989; Lin et Lu 2007; Sugata et al., 1987; Weidner et Anderson 2008; Potts et al., 2000). Yet, reviewers from last three decades have stated that the relations between prostatitis and mycoplasmas have not been finally elucidated and there are probably significant differences between species (Ludwig et Weidner 1995, Weidner et al., 1978; Taylor-Robinson et al., 2002; Bartoletti et al., 2007).

Although associated with urethritis rather than prostatitis, M. genitalium was more common than C. trachomatis or N. gonorrhoeae in prostatitis patients also (Cao et al., 2003; Taylor-Robinson 2002) yet it has been found from a healthy man as well (Takahashi et al., 2006). M. hominis is quite infrequent in male genital tract (Lin et Lu, 2007; Szőke et al., 1998; Takahashi et al., 2006) and is considered not relevant to prostatitis although it may affect semen parameters adversely (Gdoura et al., 2007).

During the last decade a novel species U. parvum was separated from U. urealyticum that was referred to as Ureaplasma urealyticum biovar 1 or B or parvo before 1999 (Robertson et al., 2002; Kong et al., 1999). This species has been found from 23% of healthy men (Takahashi et al., 2006) and has been re-
searched mostly in association with urethritis. Earlier studies have frequently reported the prevalence of *U. urealyticum* without differentiating its biovars and therefore in these studies it may actually include *U. parvum* as well (Yoshida *et al*., 2007; Martin 2008). No studies of *U. parvum* in prostatitis patients have been available yet.

According to a recent review (Martin, 2008), *U. urealyticum* is considered a pathogen of male urinary tract. The prevalence of *U. urealyticum* among prostatitis patients has ranged wildly from 13.7% to 70.8% (Brunner *et al*., 1983; Yan *et al*., 2003, Corradi *et al*. 1992; Weidner *et al*., 1980) while among healthy men from 3% to 22% (Zeighami *et al*., 2007, Takahashi *et al*., 2006 and Weidner *et al*., 1980, respectively). *U. urealyticum* has been associated with higher levels ROS in semen, while there was no direct association with WBC concentration (Potts *et al*., 2000). It has been reported that ureaplasmas have good protection from host defenses and antibiotics by virtue of forming a biofilm (Garcia-Castillo *et al*., 2008).

Hence, the association of different mycoplasma species with prostatitis needs elucidation in further studies.

### 2.8.3. Anatomic and traumatic

Two major anatomic abnormalities might have initiated and propagated prostatitis: obstruction and reflux. Obstruction of the lower urinary tract caused by either bladder neck hyperplasia, benign prostatic hyperplasia, external sphincter dyssynergia, urethral stricture, meatal stenosis or even phimosis can cause high pressure dysfunctional voiding (reviewed by Nickel, 2002). That may be associated also with calcifications of urethral valves due to the uric acid crystals and a purine-rich diet (Mueller *et Marshall* 1983; Motrich *et al*. 2006) or pathological spasm (Hellstrom *et al*. 1987). The high pressure turbulence caused by such obstruction changes the flow characteristics of urine through the urethra, creating currents and back eddies that can literally drag bacteria from the distal urethra into the area of the prostatic urethra, a potential scenario exists for intraprostatic reflux. Urine with potentially harmful and toxic constituents (potassium, immunogenic proteins, etc.) and/or microorganisms passing through or dragged into the prostatic urethra can reflux into the prostatic ducts or even the acini. Prostatic ductal architecture is such that the peri-urethral area and the peripheral gland would be involved first, which appears to be the case in the pathogenesis of prostatic inflammation (reviewed by Nickel, 2002). In an animal model, urine reflux causes up-regulation of COX-2 in the prostate. COX-2 participates in the synthesis of prostaglandins (including PGF$_{2\alpha}$) from polyunsaturated fatty acids and it is usually upregulated in case of inflammation (Liu *et al*. 2008). This theory for the initiation of prostatic inflammation and subsequent symptoms would explain the benefits of a number of medical (e.g. alpha-blockers, finasteride) and surgical (e.g. incision of the bladder neck) therapies (Nickel, 2002).
Repetitive perineal trauma may result in chronic perineal and pelvic pain as well. This was first described in medical literature with patients experiencing chronic perineal pain associated with horseback riding or riding on hard, wooden seats in poorly suspended buggies. This has been described more recently in long-distance bicycle riders, and clinicians are generally aware of this syndrome occurring in many truck, tractor and heavy equipment drivers. Most probably, this repetitive trauma affects the local perineal muscle and nervous system, perhaps even the vascular system (i.e. local ischemia). It has been even hypothesized that if the perineum is thought of as a limb, repetitive perineal trauma may result in a local reflex sympathetic dystrophy syndrome. This would explain the muscular, neurogenic and perhaps even vascular symptoms and signs associated with this variant of chronic prostatitis/chronic pelvic pain syndrome. It also suggests various avenues of treatment, primarily avoidance of potentially traumatic experiences (reviewed by Nickel, 2002). As concerns the dangers of bicycling, the danger may actually be limited to improper use and not normal use, as mentioned before (Asplund et al. 2007).

2.8.4. Autoimmune

Nickel (2002) has considered autoimmunity an unlikely initiator but a likely propagator of CPPS. There is evidence that lymphocytes from some prostatitis patients proliferate (or secrete IFN-γ) in response to seminal plasma or its antigens, such as PSA (Motrich et al., 2005, Batstone et al., 2002; Ponniah et al. 2000; Alexander et al. 1997). John et al. (2001) has discovered that there were large numbers of intra-acinar T cells in NIH IIB patients and these T cells associated with changes in blood and ejaculate interleukin levels. Rudick et al. (2008) have shown the association between pain and autoimmune prostatitis in mouse model. They investigated the development of antigen-induced CP/CPPS and pinpointed prostate as the source of the pain. This mouse model also suggested the contribution of spinal or supraspinal mechanisms to pain sensation because an analgesic effect was obtainable with gabapentin. Pertinent to CPPS sub-typing, Krieger et al., (2002) brought out that the symptoms of inflammatory CPPS tended to be worse than symptoms of non-inflammatory CPPS.

2.8.5. Neuromuscular and neural

The most unpleasant symptom of prostatitis is the sensation of pain. The routinely used concentration of leukocytes is not an adequate correlate of pain. Hence, in order to find better intervention strategies, it would be useful to identify which processes exactly are responsible for maintaining or exacerbating the chronic pelvic pain. Substance P (Tang et al., 2007, Meyer-Sieglen et Vera 2004; Chen et al., 2005) and calcitonin-gene related peptide (CGRP) (Geppetti et al., 2008) can be the important mediators of pelvic pain. Animal models sug-
gest the presence of spinal component and an important role for substance P secretion resulting in a cascade of MIF↑, NGF↑, COX-2↑ and that of c-fos↑ (a transcription factor and a proto-oncogene) so that the prostate damage will cause consequences in bladder as well (Meyer-Siegler et Vera, 2004; Vera et Meyer-Siegler 2004; Zhang et al., 2007). Preliminary data suggests that pain may be associated with NGF↑, IFN-γ↑, IL-2↑ and IL-10↑ (Miller et al., 2002) although there exists a partial disagreement with other results (Duan et Yang, 2005).

The cross-sensitization in spinal level has been published as an explanation for variability of CPPS symptoms (Malykhina 2007, Chen et al., 2005). Nociceptive pathways can be suppressed by α-blockers, gabapentin, botulinum toxin, capsaicin and resiniferatoxin (Geppetti et al., 2008; Rudick et al., 2008; Tang et al., 2007; Chuang et al., 2006).

Hetrick et al. (2003 and 2006) has analyzed pelvic floor muscles of prostatitis patients and found, among other pelvic muscles, increased tension in levator ani and coccygeus muscle. The muscles of CPPS patients tended to have increased prebaseline resting tonicity but weaker endurance contraction. Peng et al., (2009) have hypothesized that frequent sex activities with ejaculation could cause accumulation of free radicals and lactic acid in prostatic muscles to the point of fatigue, inflammation and dysfunction. Other researchers (Berger et al., 2007; Shoskes et al., 2008) have found increased tenderness also and a neuromuscular spasm in the distal urethra or sphincters has been suggested as a likely cause of prostatic urine reflux (Hellstrom et al., 1987). Hedelin and Jonsson (2007) have pointed out that cold might initiate a process initiating CP/CPPS.

### 2.8.6. Genetic

Arisan et al. (2006) found a genetic risk factor of CPPS – a manganese superoxide dismutase (Mn-SOD) polymorphism at nucleotide number 47. This polymorphism was associated with weaker defenses against OxS, thus linking genetics with OxS. Among other things, defects of this antioxidant enzyme may be responsible for higher prostate cancer risk in smokers and in men with low long-term lycopene status as well as with early-onset prostate cancer (Iguchi et al., 2009; Mikhak et al., 2008; Arsova-Sarafinovska et al., 2008).

The results of a cytokine polymorphism study by Shoskes et al. (2002) indicated existence of two types of patients. One group had decreased IL-10; these were refractory to treatment with quercetin, an anti-inflammatory antioxidant polyphenol compound. Elevated TNF-α discriminated NIH IIIA from NIH IIIB. Others had high levels of IL-10, these were not so responsive to anti-inflammatory treatment. Hence, they concluded that IL-10 and TNF-α could be used to assess the potential effect of anti-inflammatory treatment.

Liu et al., (2008) provided a genetic link between prostatitis and prostate cancer using an animal model where overexpression of Vav3 oncogene caused first chronic prostatitis and then prostate cancer.
2.8.7. Hormonal

Androgens have been long time pertinent to prostatitis research. Although an early study of Yunda et Imshinetskaya (1977) indicated that most of the prostatitis patients had considerable reduction in testosterone excretion, a recent study of Dimitrakov et al. (2008) reported elevated levels of androgens instead, and diminished levels of glucocorticoids and mineralocorticoids in prostatitis patients. Symptoms correlated with 17-hydroxyprogesterone, aldosterone and, inversely, with cortisol levels. The authors associated such situation with dysfunctional enzyme CYP21A1 (P450c21) that partially blocked the synthesis of mineralocorticoids and glucocorticoids, and channeled the production towards the end of androgen synthesis. In addition, according to Mukaratirwa et Chitura (2007) uncastrated dogs have a higher prevalence of prostatitis and BPH. Also, androgen antagonists have been of benefit in an animal model (Seo et al., 2003). Reduction of anti-inflammatory signal in castrated rats has been shown by Quintar et al., (2006). They also indicated that castrated animals had an increased expression of TLR4 in prostate epithelial cell surface – a molecule that recognizes endotoxin of Gram-negative bacteria.

Estrogens have been found relevant, too. Inflammation occurs also after administering estrogens to adult rats (Seethalahkshmi et al., 1996; Harris et al., 2007; Bernoulli et al., 2007). There are reports of laboratory rat prostatitis due to peri- or neonatal exposure to estrogens (Stoker et al., 1999; Naslund et al., 2008). While testosterone seemed beneficial, estrogen antagonists did not seem to do any good to the rats (Naslund et al., 1998). In contrast to rats, humans have benefited from an estrogen antagonist: mepartricin was superior to placebo in a controlled trial (De Rose et al., 2004).

2.8.8. Oxidative stress in prostatitis

Oxidative stress (OxS) is a condition in which the delicate balance that exists between equally necessary pro-oxidants and antioxidants is skewed towards pro-oxidants (Halliwell et Cross 1994). This condition is characterized by an imbalance between increased exposure to free radicals, principally derived from oxygen, and antioxidant defenses, comprised of both small molecular weight antioxidants, such as glutathione (GSH), and antioxidant enzymes, such as superoxide dismutase (SOD). Free radicals can be generated endogenously from various sources (for example, mitochondria and oxidative burst during phagocyte activation) or derived from exogenous sources such as environmental toxins and cigarette smoke. Free radicals cause direct damage to critical biomolecules including DNA, lipids and proteins. OxS is recognized as a prominent feature of many acute and chronic diseases including cancer, cardiovascular disease, neurodegenerative disease, lung disease as well as the normal aging process.
According to recent pain research (primarily animal research), spinal OxS seems to be a major pathogenetic component in chronic states of pain. More precisely, both ROS (Keeble et al., 2009) and lipid peroxidation products (Taylor-Clark et al., 2008a; Taylor-Clark et al., 2008b) might directly evoke pain while pain is suppressed by spinal glutathione (Rossato, 2010) and SOD (Schwartz et al., 2009).

2.8.8.1. Spermatozoa

Maintaining a fine balance between reactive oxygen species (ROS) and antioxidants is essential for sperm maturation and function (Drevet, 2006). Depending upon the nature and the concentration of the ROS, either a beneficial or a detrimental effect on sperm function could be expected (Aitken, 1997). Several studies have shown that peroxidase positive leukocytes in semen (mostly polymorphonuclear leukocytes and macrophages) produce large amounts of ROS. Although immature sperms may also contribute to ROS production at some extent, the leukocytes produce at least 1000 times more ROS than spermatozoa. Excessive ROS levels disrupt human sperm function by peroxidation of unsaturated fatty acids within the sperm plasma membrane, diminishing motility and leading to incompetence for sperm-oocyte fusion. Damage caused by ROS may also be targeted at DNA. ROS can cause chromatin cross-linking, DNA base oxidation, and DNA strand breaks. The latter may accelerate the process of germ cell apoptosis leading to decline in sperm counts associated with male infertility and deterioration of semen quality. While separated from the seminal fluid, the spermatozoa are very vulnerable to OxS because their plasma membranes contain large quantities of polyunsaturated fatty acids and their intracellular defense against ROS is negligible (de Lamirande et Gagnon, 1995; Saleh et al., 2002; Aitken et al., 1991, Twigg et al., 1998, Agarwal et al., 2003). Recently, the effects of microwave radiation emitted by cellular phones have caught scientific attention. Although not directly related to prostatitis, it seems relevant that mobile phone radiation interferes with antioxidant levels and motility of spermatozoa (Agarwal et al., 2008).

2.8.8.2. Seminal fluid

The seminal fluid usually protects spermatozoa from OxS with its huge antioxidative properties because it normally contains high amounts of antioxidants like spermin, thiols, uric acid and vitamin C (Henkel et al., 2005). Nevertheless, leukocytes can breach even great antioxidative defenses if they produce huge amounts of ROS (Saleh et al., 2002; Tremellen, 2008; Agarwal et al., 2003; Lemkecher et al., 2005). Inflammation in case of leukocytospermic prostatitis is a typical situation where excessive production of ROS within the genital tract system is very much elevated and can deprive the anti-oxidative protection sys-
It has been found that OxS may be present in semen of prostatitis patients even if the leukocyte counts are very low (Pasqualotto et al. 2000).

2.8.8.3. Systemic

Investigations of systemic OxS in case of prostatitis are not numerous. In the studies of Lou et al. (2006) and Zhou et al. (2006) occurrence of systemic OxS has been observed in case of NIH II prostatitis. In blood plasma, they observed an increase of blood nitric oxide (NO) and malondialdehyde (MDA) and a decrease of vitamins C and E as well as β-carotene. In erythrocytes, a reduction in levels of antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase was registered. Their regression analysis revealed that during the course of disease the NO and MDA increased while antioxidant levels decreased.

Systemic antioxidants are associated with skeletal muscles. In mice, vitamin E produced intramuscular anti-inflammatory effect (IL-6↓, IL-1β↓) in case of lipopolysaccharide stimulation (Huey et al., 2008). In the same time, at least some exogenic antioxidants (vitamin C, vitamin E, allopurinol) may disrupt the innate adaptation to exercise-related oxidative stress (SOD↑, glutathione peroxidase↑) (Gomez-Cabrera et al., 2005; Ristow et al., 2009). In fact, the changes in OxS parameters resulting from aerobic training (SOD↑, 8-OHdG↓ and 8-EPI↓) might explain why prostatitis patients benefit from exercise (Devries et al., 2008; Gomez-Cabrera et al., 2005; Giubilei et al., 2007).

8-EPI deserves special mention, because it is a relevant metabolite of free-radical oxidation, which is used as a marker of systemic oxidative stress. Elevation of 8-EPI is found in case of atherosclerosis and metabolic syndrome (Kals et al., 2006) as well as in case of immune response directed against periodontitis, which is a polyfactorial biofilm infection (Offenbacher et al., 2009). Systemic 8-EPI is secreted via urinary tract and 8-EPI is both cause and consequence of neuromuscular activity near prostate. In more precise terms, 8-EPI is formed in urinary bladder muscle and mucosa in response to physiologic and pathologic stimuli (stretching of detrusor muscle, injury to urothelium, stimulation of the nerves) and the same 8-EPI causes smooth muscle contractions in relatively low concentrations in human bladder (Jeremy et al., 1987; Maggi, 1992; Tarcan et al., 2000). Hence, 8-EPI may be an important variable in the pathogenesis of prostatitis even if we consider prostatitis a process limited to the prostate and its immediate vicinity.

OxS participates in the pathogenesis of prostatitis but the mechanisms are still poorly understood. Since Shahed et Shoskes (2000, 2001) have shown that OxS in semen is linked to pain susceptibility (more precisely, inhibition of opioidergic antinocicptive mechanisms) it became plausible that OxS may be an element of major importance in the pathogenesis of chronic pelvic pain.
2.9. Diagnostic procedures

The most common (routinely used) diagnostic measures are as follows: validated NIH-CPSI questionnaire for quantifying subjective symptoms, digital rectal examination (DRE), and measuring concentrations of WBC-s and aerobic cultivable bacteria from prostate-specific secretions (semen, EPS or VB3). Some other methods can be used as well – other questionnaires, ultrasound, measuring flow of urine and checking for underlying STDs.

No unified diagnostic scheme has been proposed to date since several studies have shown controversial results. 4-glass test is a ‘gold standard’ in theory but in practice, it does not see much use, because it is just too cumbersome and expensive, and, hence, in practice a simpler 2-glass test is used (Kiyota et al., 2003; Nickel et al., 2006). As concerns detection of inflammation in prostate specific specimens, according to Krieger et al., (2003) measuring inflammation from different materials (semen, VB3, EPS) was not equivalent because the measured concentrations of inflammatory cells did not correlate to each other. At the same time, measuring different materials increased sensitivity and specificity (Krieger et al., 2002; 2003). As concerns detection of causative agents, doctors diagnose bacterial prostatitis according to extant classification: the prostatitis is bacterial if and only if the patient is infected with traditional uropathogens (Gram-negative aerobic rods or enterococci). Other Gram-positive organisms in semen, EPS or VB3 have been usually considered as commensals although Shahed et al. (2000) have suggested that those ‘commensals’ might actually be pathogens, too. Whether and how relevant the Gram-positive organisms are in the etiology and pathogenesis of prostate has been a good question without a good answer (Naber, 2008).

In addition to aforementioned diagnostic measures, there is a multitude of immunological and biochemical markers that have been investigated. A long list of biomolecules has been researched in the context of prostatitis (Table 4). The study by Penna et al. (2007) is of special interest because it compared an impressive list of cytokines and found that IL-8 was the strongest correlate of prostatitis. Seminal IL-6 was another good marker of inflammatory prostatitis (Orhan et al., 2001; Kopa et al., 2005) as shown also by our previous study (Korrovits et al., 2006). Although promising, the cytokines are not introduced into clinical practice yet.

As concerns measuring blood flow of the prostate, the results have been contradictory (Neimark et al., 2000; Cho et al., 2000). According to Shoskes et al., (2007) calcifications in the prostate (detected by ultrasound) associated with bacteria and more WBC-s but were mutually exclusive with pelvic floor spasm and sensitization.
Table 4. Markers of prostatitis under investigation

<table>
<thead>
<tr>
<th>Marker of CPPS</th>
<th>Specimen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA1 upon exacerbation</td>
<td>semen</td>
<td>Kastner and Jakse, 2003; John et al., 2003</td>
</tr>
<tr>
<td>IgG1 upon healing</td>
<td>semen</td>
<td>Kastner and Jakse 2003; Parsons et Albo 2002, Hassan et al., 2007. It was not a good marker according to Yilmaz et al., 2004.</td>
</tr>
<tr>
<td>K+ sensitivity</td>
<td>N/A</td>
<td>Parsons et Albo 2002, Hassan et al., 2007. It was not a good marker according to Yilmaz et al., 2004.</td>
</tr>
<tr>
<td>IL-1β †</td>
<td>EPS</td>
<td>Alexander et al., 1998; Orhan et al., 2001</td>
</tr>
<tr>
<td>IL-2 †</td>
<td>EPS</td>
<td>Li et al., 2006; Duan et Yang 2004</td>
</tr>
<tr>
<td>IL-8 †</td>
<td>EPS</td>
<td>Hochreiter et al., 2000; Orhan et al. 2001; He et al., 2004; Li et al., 2004 and 2006; Penna et al., 2007</td>
</tr>
<tr>
<td>IL-6 †</td>
<td>EPS</td>
<td>Orhan et al., 2001; Kova?chuk et al., 2007; Penna et al. 2007; Stancik et al., 2008</td>
</tr>
<tr>
<td>IL-10 ↓</td>
<td>EPS</td>
<td>Duan et Yang 2004</td>
</tr>
<tr>
<td>IL-10 †</td>
<td>EPS</td>
<td>Li et al., 2004</td>
</tr>
<tr>
<td>IL-4 †</td>
<td>serum</td>
<td>Li et al., 2006</td>
</tr>
<tr>
<td>TNF-α †</td>
<td>EPS</td>
<td>Alexander et al., 1998; Nadler et al., 2000; Orhan et al., 2001; He et al., 2004; Motrich et al., 2006; Li et al., 2006</td>
</tr>
<tr>
<td>IFN-γ †</td>
<td>EPS</td>
<td>Motrich et al., 2006; Ding et al., 2006</td>
</tr>
<tr>
<td>TGF-β †</td>
<td>EPS</td>
<td>Ding et al., 2006</td>
</tr>
<tr>
<td>NO †</td>
<td>semen</td>
<td>Motrich et al., 2006</td>
</tr>
<tr>
<td>CRP †</td>
<td>EPS</td>
<td>Li et al., 2007</td>
</tr>
<tr>
<td>CCL-1 †</td>
<td>semen</td>
<td>Penna et al., 2007</td>
</tr>
<tr>
<td>CCL-2 †</td>
<td>EPS</td>
<td>Desireddi et al., 2008</td>
</tr>
<tr>
<td>CCL-3 †</td>
<td>Semen, EPS</td>
<td>Penna et al., 2007, Desireddi et al., 2008</td>
</tr>
<tr>
<td>CCL-4 †</td>
<td>semen</td>
<td>Penna et al. 2007</td>
</tr>
<tr>
<td>CCL-17 †</td>
<td>semen</td>
<td>Penna et al., 2007</td>
</tr>
<tr>
<td>CCL-22 †</td>
<td>semen</td>
<td>Penna et al., 2007</td>
</tr>
<tr>
<td>CXCL5 †</td>
<td>EPS</td>
<td>Hochreiter et al., 2000 (NIH IIIA)</td>
</tr>
<tr>
<td>Mg ↓</td>
<td>semen</td>
<td>Edorh et al., 2003; Mg was not a good marker according to Colleen et al, 1975.</td>
</tr>
<tr>
<td>Zn ↓</td>
<td>VB3, semen</td>
<td>Canale 1986, Kavanagh et al., 1983; Zn was not a good marker according to Zaichick et al. 1996 or Colleen et al., 1975</td>
</tr>
<tr>
<td>Citrate ↓</td>
<td>EPS</td>
<td>Zdrodowska-Stefanow et al., 2008; Kavanagh et al., 1982; Chen et al., 2007. Total citrate output ↓ – Comhaire et al., 1989.</td>
</tr>
<tr>
<td>Prostatic stones</td>
<td>prostate</td>
<td>Geramoutsos et al., 2004; Shoskes et al., 2007</td>
</tr>
</tbody>
</table>
2.10. Treatment options

There is currently no standard treatment for CP/CPPS, and there are country-specific variations in prostatitis treatment. Usually, prostatitis is treated with fluoroquinolone antibiotics, and this tradition seems to apply for any kind of prostatitis. Of prescription drugs, α-blockers and certain other antibiotics (TMP/SMX and tetracyclines) are common as well. In addition to the above-mentioned drugs, men with CPPS most probably consume over-the-counter anti-inflammatory and herbal supplements. Unlike routinely used ones, the experimental therapies are numerous (Table 5).

2.10.1. Antibacterial agents

Antimicrobial agents and especially fluoroquinolones are the most popular drugs for prostatitis treatment due to their good penetration to prostate tissue. Fluoroquinolones have been suggested for initial treatment of NIH I, NIH II, NIH III and NIH IV, that is any prostatitis category (Nickel, 2000; Murphy et al., 2009). According to both clinical experience and an open-label study by Nickel et al. (2001), CP/CPPS patients have frequently a positive treatment response with fluoroquinolone therapy (reviewed by Murphy et al., 2009). At the same time, in a couple of controlled studies their effect has been comparable to placebo (Nickel et al., 2003; Alexander et al., 2004) and several papers indicate overuse (Ku et al., 2003; Taylor et al., 2008; Duclos et al., 2007). On the other hand, several lines of evidence suggest that fluoroquinolones have immunomodulatory properties as well that could influence therapeutic effect (Zhang et Ward 2007; Lahat et al., 2007, Dalhoff 2005, Williams et al., 2005; Takeyama et al. 2007). When compared to consumption of fluoroquinolones, the turnover of other antibiotics seems moderate. A minority of doctors uses tetracycline as primary or secondary treatment (Kiyota et al. 2003) while TMP/SMX is a first line agent in Canada (Nickel et al., 1998). Szöke et al. (1998) used amoxicillin/clavulanic acid or clindamycin and Magri et al. (2007) used a fluoroquinolone and a macrolides in a combination treatment targeted against unusual or traditional pathogens that caused prostatitis.

Despite unclear etiology of prostatitis and paucity of evidence-based suggestions for antibacterial treatment, susceptibility patterns of potential causative agents must be recorded in order to improve the extant empirical treatment of inflammatory prostatitis.

2.10.2. α-blockers

α-blockers relax smooth muscles and inhibit nociception. Contrary to antibiotics, five out of six controlled studies justify the use of α-blockers (Cheah et al., 2003; Mehik et al., 2003; Sivkov et al., 2005; Nickel et al., 2004; Alexander
et al., 2004; Evliyaoğlu et al., 2002), a class of drugs originally designed for high blood pressure treatment. Yet the results regarding the combinations of fluoroquinolones and a-blockers have produced divergent results (Ye et al., 2008, Kulovac et al., 2007, Jeong et al., 2008; Barbalias et al., 1998).

2.10.3. Anti-inflammatory agents

Concerning treating prostatitis, the researched anti-inflammatory treatments have included glucocorticosteroids, COX inhibitors, herbal products, and even leukotriene inhibitors. Both zafirlukast and prednisolone were clearly not superior to placebo (Goldmeyer et al., 2005; Bates et al., 2007). Pollen extracts and quercetin have been successful in controlled studies (Elist et al., 2006, Shoskes et al., 1999). Pollen extract can suppress pro-inflammatory cytokines and mast cell degranulation (Asakawa et al., 2001; Ishikawa et al., 2008; Nakajima et al., 2009). It is prescribed in Japan for prostatitis about as frequently as antibiotics (Kiyota et al., 2003). Much more is known about quercetin. In addition to before mentioned properties of pollen, the other pertinent properties of quercetin include improving the function of endothelium, being a potent inhibitor of myeloperoxidase while not interfering with adaptive responses to OxS due to exercise (Okoko et Oruambo, 2009; Park et al., 2008; Romero et al., 2009; McAnulty et al., 2008; Gomez-Cabrera et al., 2005). In general, phytotherapy seems as the most promising kind of anti-inflammatory treatment.

2.10.4. Other pharmacological treatment modes

Finasteride, an inhibitor of 5-α-reductase, is a component in a multimodal therapy (or for patients with co-occurring BPH) rather than a primary treatment option (Kaplan et al., 2004; Nickel et al. 2004; Nickel, 2007). Neuronal desensitization therapy is yet highly experimental but deserves attention. It relates to the concept of neurogenic inflammation. Resiniferatoxin only desensitizes afferent neurons while botulinum toxin paralyzes the prostate because it affect efferent neurons, too (Geppetti et al., 2008; Dinis et al., 2005; Cruz et Dinis 2007, Tang et al., 2007; Maria et al., 2005). Pentosan polyphosphate, a heparinoid surfactant, has been shown to relief pain and discomfort related to interstitial cystitis. That drug has been used for treatment of prostatitis also (Wedrén 1989; Nickel et al. 2005). Pentosan polysulphate probably works by avoiding urothelium damage caused by mast cell degranulation (Chiang et al, 2000). Glutathione and anti-oxidant vitamins C, E and Q_{10} have been recommended for curbing OxS in prostatitis patients (Sheweita et al., 2005), although the risks associated with antioxidant supplementation should be weighted against the expected benefits (meta-analysis by Bjelakovic et al., 2008).
Table 5. Treatment options of chronic pelvic pain syndrome

<table>
<thead>
<tr>
<th>Treatment mode*</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibiotics</strong></td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones (0/2)</td>
<td>Nickel 2002; Ku et al., 2005; Luzzi 2002</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Magri et al., 2007; Ku et al., 2005; Luzzi 2002</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Paulson et al., 2006; Luzzi 2002</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>Nickel et al., 1998; Luzzi 2002</td>
</tr>
<tr>
<td><strong>Alpha-blockers</strong></td>
<td></td>
</tr>
<tr>
<td>doxazosin, alfuzosin, tamsulosin,</td>
<td>Mehik et al., 2003; Nickel 2008; Ye et al., 2008;</td>
</tr>
<tr>
<td>terazosin (5/6)</td>
<td>Cheah et al., 2003; Jeong et al., 2008;</td>
</tr>
<tr>
<td><strong>Anti-inflammatory agents and antioxidants</strong></td>
<td></td>
</tr>
<tr>
<td>Lycopene</td>
<td>Han et al., 2008</td>
</tr>
<tr>
<td>NSAID-s, glucocorticoids, leukotriene</td>
<td>Nickel et al., 2003; Goldmeyer et al., 2005;</td>
</tr>
<tr>
<td>antagonists (0/3; rofexocib – minor effect)</td>
<td>Bates et al., 2007</td>
</tr>
<tr>
<td>Quercetin (1/1)</td>
<td>Shoskes et al., 1999</td>
</tr>
<tr>
<td>Pollen extract (2/2)</td>
<td>Elist et al., 2006, Wagenlehner et al., 2009</td>
</tr>
<tr>
<td>(<em>Serenoa repens, Epilobium parviflorum, Chinese herbs</em>)</td>
<td>Capodice et al., 2005; Steenkamp et al., 2006;</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>Chen et al., 2006</td>
</tr>
<tr>
<td>Terpenes</td>
<td>Persson et al., 1996; Ziaee et al., 2006</td>
</tr>
<tr>
<td><strong>Non-pharmacologic</strong></td>
<td></td>
</tr>
<tr>
<td>Prostate Massage</td>
<td>Nickel et al., 1999</td>
</tr>
<tr>
<td>Acupuncture (minor effect)</td>
<td>Lee et al., 2008; Capodice et al., 2005</td>
</tr>
<tr>
<td>TUMT, TURP or TUNA (0/1)</td>
<td>Kastner et al., 2004; Barnes et al., 1982,</td>
</tr>
<tr>
<td>Biofeedback and pelvic muscle training</td>
<td>Leskinen et al., 2002</td>
</tr>
<tr>
<td>Distal urethral web surgery</td>
<td>Cornel et al., 2005; Nadler 2002</td>
</tr>
<tr>
<td>Hot sitz baths</td>
<td>Vega et al., 2002</td>
</tr>
<tr>
<td>Laser</td>
<td>Drach, 1975; Ku et al., 2005</td>
</tr>
<tr>
<td>Shockwave therapy</td>
<td>Capodice et al., 2005; Kozdoba et al., 2007</td>
</tr>
<tr>
<td>Aerobic exercise (1/1)</td>
<td>Zimmermann et al., 2008</td>
</tr>
<tr>
<td>Electromagnetic therapy (1/1)</td>
<td>Giubilei et al., 2007</td>
</tr>
<tr>
<td>Transcutaneous Electrical Nerve Stimulation (TENS)</td>
<td>Rowe et al., 2005</td>
</tr>
<tr>
<td>Myofascial physical therapy</td>
<td>Sikiru et al., 2008</td>
</tr>
<tr>
<td><strong>Other substances</strong></td>
<td></td>
</tr>
<tr>
<td>Botulin</td>
<td>Maria et al., 2005; Cruz et al., 2007</td>
</tr>
<tr>
<td>Finasteride (2/2)</td>
<td>Kaplan et al., 2004, Nickel et al., 2004</td>
</tr>
<tr>
<td>Pentosan polysulphate (minor effect)</td>
<td>Wedrén 1987; Nickel et al., 2005</td>
</tr>
<tr>
<td>Phosphodiesterase inhibitor</td>
<td>Esilevskii et al., 2005</td>
</tr>
<tr>
<td>Mepartricin 1/1</td>
<td>De Rose et al., 2004</td>
</tr>
</tbody>
</table>

* – in brackets the ratio of successful/total of controlled studies have been shown, if any.
2.10.5. Non-pharmacological treatments

There are several traditional or very experimental non-pharmacological treatment modes (Table 5). The most common non-pharmacological treatments are psychotherapy and prostate massage (Yang et al., 2008). A simple experimental treatment is an aerobic training that improved the health of the patients when compared to non-aerobic training used as placebo (Giubilei et al., 2007). Acupuncture is another successful placebo-controlled non-pharmacologic treatment (Lee et al., 2008). Transurethral needle ablation (TUNA) was promising in preliminary study but failed in controlled study (Leskinen et al., 2002). Other experimental options include transurethral resection of the prostate (TURP, Nickel, 2000); transurethral microwave therapy (TUMT, Kastner et al., 2004); surgical resection of distal urethral web (Vega, 2000); shock wave therapy (Zimmermann et al. 2008). Adequate sexual activity has seemed as a factor that protects from prostatitis (Wallner et al., 2009), and Branigan et al. (1994) has suggested that frequent ejaculations in the background of antibiotic treatment can be beneficial. On the other hand, frequent masturbation or having multiple partners have been identified as prostatitis risk factors (Gao et al., 2007; Bartoletti et al., 2007) and Peng et al., (2009) have explained how frequent ejaculations may cause prostatitis. Since the drugs do not selectively help a patient to avoid the extremes of behavior and do not consider individual differences, it seems that counseling and self-help might contribute to patients’ welfare.

***

Hence, in spite of multiple studies on etiopathogenesis of NIH categories III and IV chronic prostatitis that have applied wide variety of methods, the existing data set is controversial and incomplete and thus additional studies are needed to clarify this issue.

Previous research of our workgroup has indicated that inflammatory prostatitis is frequently associated with abundant polymicrobial microbiota in semen (higher frequency, intensity and diversity in terms of colonization) while it is not finally clear whether this colonization is a cause, a consequence or a side effect. Yet it is very likely that inflammatory prostatitis may be associated with dysbalance of genital tract microflora and therefore more focused studies targeted on certain microbial groups of this microbiota are needed. In this thesis, we concentrated upon Corynebacterium sp. and morphologically related bacteria as well as upon Mycoplasmatales in order to determine their etiological role in inflammatory prostatitis.

In addition, previous studies have indicated that OxS participates in the pathogenesis of prostatitis but the mechanisms are still poorly understood. Therefore, additional data are needed in order to see whether and exactly how OxS fits with the extant theories of prostatitis, and how it is linked with inflammation. To meet this challenge, we collected the complex data about OxS in its different forms (antioxidants versus oxidation products and pro-oxidants) and levels – local (seminal plasma and in the spermatozoa) and systemic (urine and blood).
AIMS OF THE RESEARCH

The general aim of these experiments were to elucidate some factors and mechanisms that are associated with chronic inflammatory prostatitis. We therefore attempted to clarify whether *Corynebacterium* sp. and *Mycoplasma* sp. are etiological factors of prostatitis, and to observe possible associations between seminal microbiota, inflammation, and oxidative stress that could explain the pathogenesis of this disease.

The specific aims were as follows:
1) To compare the prevalence and species composition of coryneform bacteria in semen of chronic prostatitis patients and controls.
2) To determine the susceptibility patterns of coryneform bacteria isolated from semen.
3) To compare the prevalence and species composition of mycoplasmas in semen of chronic prostatitis patients and controls by simultaneously comparing culture method with PCR.
4) To assess aspects of oxidative stress (antioxidants, pro-oxidants and oxidation products) in the organism of chronic prostatitis patients in the systemic level as well as in seminal fluid and spermatozoa.
5) To detect possible associations between seminal microbiota, inflammation, oxidative stress, and basic semen parameters.
Table 6. Study subjects, microbial strains and performed investigations

<table>
<thead>
<tr>
<th>Group</th>
<th>No of subjects/strains</th>
<th>Study description*</th>
<th>No of subjects/strains in sub-groups</th>
<th>Presented in Papers:</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Individuals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy Men</td>
<td>99 men</td>
<td>Screening and antimicrobial susceptibility testing of coryneforms</td>
<td>59</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxidative stress in prostatitis</td>
<td>9</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycoplasmas in prostatitis</td>
<td>40</td>
<td>III</td>
</tr>
<tr>
<td>Men with NIH IV prostatitis (&gt;0.2M WBC/ml)</td>
<td>54 men</td>
<td>Screening and antimicrobial susceptibility testing of coryneforms</td>
<td>30</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxidative stress in prostatitis</td>
<td>11</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycoplasmas in prostatitis</td>
<td>24</td>
<td>III</td>
</tr>
<tr>
<td>Men with NIH IIIA prostatitis (&gt;0.2M WBC/ml)</td>
<td>58 men</td>
<td>Screening and antimicrobial susceptibility testing of coryneforms</td>
<td>20</td>
<td>I, II</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxidative stress in prostatitis</td>
<td>10</td>
<td>IV</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mycoplasmas in prostatitis</td>
<td>38</td>
<td>III</td>
</tr>
<tr>
<td>Men with NIH IIIB prostatitis</td>
<td>59 men</td>
<td>Mycoplasmas in prostatitis</td>
<td>59</td>
<td>III</td>
</tr>
<tr>
<td><strong>Microbial Strains</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coryneform strains</td>
<td>148 strains from 109 patients</td>
<td>Screening and identification of coryneforms</td>
<td>148 of 148</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antimicrobial susceptibility testing of coryneforms</td>
<td>62 of 148</td>
<td>II</td>
</tr>
</tbody>
</table>

* – see also Fig. 2.
Fig. 2. A. Formation of study groups. B. Composition of study groups.
3. Subjects and study design

Altogether 270 men participated in the studies (Table 6). The subjects were recruited into the studies according to the NIH Classification of the Prostatitis Syndromes (Table 1, Fig. 2A). The cut-off points for detecting leukocytospermia were $10^6$ WBC/ml according to WHO guidelines (WHO, 1999) and $2 \cdot 10^5$ WBC/ml, according to our previous study results (Punab et al., 2003) where the concentration and the mean number of different microorganisms was compared against WBC concentrations in semen specimens using ROC curve analysis. As a result, we found that an alternative cut-off level of $2 \cdot 10^5$ WBC/ml has the most optimal sensitivity/specificity ratio to differentiate between men with or without significant bacteriospermia. Exclusion criteria for study subjects were stated according to the suggestions of the NIH workshop on chronic prostatitis (National Institutes of Health Summary Statement, 1995). None of the men had received antimicrobial therapy within 3 months, and men of oxidative stress study had abstained from other medicines and vitamins for 2 weeks.

3.1. Men in the study of coryneform bacteria

This study recruited 109 men (Table 6, Fig. 2B) who participated in a prospective study Environment and Reproductive Health (EU 6th FP project QLRT-2001-02911) (72 participants, mean age 18.6 ± 0.2 years old) or the prospective case-control study of chronic prostatitis (37 participants, mean age 32.8 ± 1.3 years old). The mean age of prostatitis patients was 28.5 (SE ±0.62) years; the mean age of controls was 20.0 years (SE ±1.32) years ($p<0.05$).

3.2. Men in the study of oxidative stress

This study included initially 43 men who participated in the prospective study of the etiopathogenesis of chronic prostatitis (Table 6, Fig. 2B). Due to difficulties in finding suitable men whose semen samples were large and concentrated enough (in many cases too few spermatozoa were available for biochemical analyses after Percoll centrifugation) the specimens of 30 men were available for the complex analysis. The controls as well as NIH IV category prostatitis patients consulted a physician due to infertility of the couple or prophylactic purposes. The mean age of prostatitis patients and controls was 32.3±1.4 years and 31.2±2.6 years, respectively ($p>0.05$).
3.3. Men in the study of mycoplasmas

This study involved 161 men who participated in the prospective study of the etiopathogenesis of chronic prostatitis (Table 6, Fig. 2B). The controls as well as NIH IV category prostatitis consulted a physician due to infertility of the couple, for prophylactic purposes or their partner’s chronic gynecological infections. Prostatitis patients were somewhat older than controls, as the mean age of the prostatitis patients was 34.2±0.57 years and that of controls 28.7±0.61 years.

3.4. Ethical considerations

Participation in the study was voluntary. Informed consent was obtained from the participants. All subjects were at least 18 years old. The studies were approved by the Ethics Review Committee on Human Research of the University of Tartu.

4. Bacterial strains tested in vitro

A total of 62 coryneform strains isolated from semen of prostatitis patients (36 strains) and controls (26 strains) were analyzed with E-test antimicrobial susceptibility testing: Corynebacterium seminale (29 strains), Corynebacterium group G (8), C. jeikeium (7), C. striatum (4), Dermabacter hominis (4), Cellulomonas/Microbacterium sp. (4), Corynebacterium group F1 (2), Brevibacterium sp. (1), Turicella otitidis (1), Arthrobacter sp. (1), and C. mucifaciens (1). The set of the bacteria determined for susceptibility testing consisted of as many as possible Corynebacterium group G, Arthrobacter sp. and C. jeikeium strains, because the first two associated with inflammation while the latter was of interest because of possible multiresistance. Not in agreement with that intent, three of the four strains identified as Arthrobacter sp. were unavailable to testing because of storage problems. The rest of the strains were chosen randomly. Groups of Corynebacterium sp. are designations of CDC (Centers for Disease Control and Prevention), while Cellulomonas/Microbacterium is a category of API Coryne identification system.

5. Specimens

5.1. Semen

The samples were obtained by masturbation and were collected in a sterile collection tube. After ejaculation, the semen was incubated at 37°C for 25–45 min for liquefaction. The samples were processed within 1 h (including time spent on liquefaction).
5.2. Urine

5.2.1. Urine samples for microbiological analysis

The first-catch urine was collected into a sterile collection tube by patients in a private room near laboratories after they washed their glans penis with soap and water. The urine samples were microbiologically investigated in 36 randomly selected men (30 with and 6 without leukocytospermia) who participated in the study of oxidative stress. The samples were cultured within 1 h.

5.2.2. Urine samples for biochemical analysis

Urine samples of 30 men who participated in the study of oxidative stress were subjected to biochemical analysis. Prior to the biochemical analyses, urine was frozen for duration less than one year.

5.3. Blood

Blood samples were obtained by venipuncture, serum was obtained by centrifugation at 3000g by 5 min and analyzed immediately (for hsCRP, IL-6, prealbumin, Fe, ferritin, and transferrin receptors using standard methods) or kept frozen at -70°C (for the rest of analyses). The exact methods for specific markers are specified further.

6. Routine analyses of semen

6.1. Cytological analysis of semen for detection of leukocytospermia

Semen smears were made for detecting white blood cells (WBC). The smears were air-dried, Bryan-Leishman stained, and examined with the use of oil immersion microscopy (magnification: x1000) by an experienced microscopist. Polymorphonuclear (PMN) leukocytes were differentiated from spermatids by the presence of segmented nuclei, bridges between lobes of nucleus, and specific granulation of the cytoplasm (Couture et al., 1976). The WBC concentration in semen \((C_{\text{WBC}})\) was calculated by using the known sperm concentration (as \(10^6/\text{mL}\)) according to the following formula:

\[
C_{\text{WBC}} = \frac{\text{number of WBCs counted} \cdot \text{semen sperm concentration}}{\text{number of sperm counted}}
\]
One hundred round cells were counted twice, and their mean value was registered.

The counting of WBC-s was used to divide the patients with prostatitis symptoms between categories of NIH IIIA and IIIB, and the subjects without prostatitis symptoms between category NIH IV and controls, as well as to divide the patients into subgroups with either severe or moderate leukocytospermia (Fig 2A).

6.2. Basic semen parameters

The analysis of semen was performed according to WHO guidelines (WHO, 1999). Semen volume was estimated by weighing the collection tube with the semen sample and subsequently subtracting the predetermined weight of the empty tube assuming 1g=1ml. Motility was assessed in order to report the number of motile spermatozoa (WHO motility classes A+B). Sperm concentration was assessed using the Neubauer haemocytometers. Total sperm count was calculated by multiplying semen volume by sperm concentration. A qualified laboratory technician performed all semen analyses.

6.3. Detection of interleukin-6 (IL-6) in semen

Interleukin-6 levels of seminal plasma (100 µl of specimen was required for the assay) were measured in serum by chemoluminescent immunoassay IMMULITE 2000 Analyzer (Siemens Medical Solutions Diagnostics), according to manufacturer’s instructions (Kit Catalog Number: L2K6P2). Assays were solid-phase, enzyme-labeled sequential chemoluminescent immunometric tests, which were performed automatically on the IMMULITE 2000 automated analyzer with 2 incubation cycles per 30 minutes, analytic sensitivity of 2 pg/ml for IL-6 and calibration range of up to 1000 pg/ml. Granules coated with antibodies directed towards IL-6 were mixed with the samples. After washing, alkaline phosphatase-labeled antibodies were added. Free antibodies were washed away and chemoluminescent reagent was supplied. The reaction between alkaline phosphatase and the chemoluminescent reagent resulted in light production, which was measured in the Immulite 2000 automated analyzer. The antibody used in assay is highly specific to IL-6 and has no cross-reactivity with IL-1α, IL-1β, IL-2, IL-4, IL-8, TNF-α or IFN-γ (IMMULITE 2000 IL-6, 2006).
7. Microbiological methods

7.1. Preparation and cultivation of specimens

Semen and urine samples were cultured quantitatively by “four corners” streak plate method to detect anaerobic, microaerophilic and aerobic bacteria within 1 h from collection. Freshly prepared blood agar and chocolate agar, Wilkins-Chalgren medium (Oxoid) supplemented with 5% horse blood, Wilkins-Chalgren medium supplemented with 5% horse blood and GN supplement (Oxoid), MRS agar (Oxoid) and Gardnerella vaginalis-selective agar (Oxoid) were used. Aerobic (blood agar) and microaerobic (chocolate agar, MRS agar, and G. vaginalis-selective agar in 10% CO₂ atmosphere) cultures were incubated at 37 °C for 1–3 days and anaerobic cultures (Wilkins-Chalgren media in an anaerobic glove box) for 3–5 days. The gaseous environment of anaerobic glove box consisted of 90% molecular nitrogen, 5% of carbon dioxide and 5% molecular hydrogen.

7.2. Isolation and identification of cultivable microorganisms

7.2.1. Coryneform bacteria

Bacterial colonies that occurred on blood agar, chocolate agar, MRS agar, Wilkins-Chalgren medium (with or without GN supplement), and G. vaginalis-selective agar were isolated and subjected to identification. Primary screening for coryneform bacteria was performed by Gram stain and subsequent microscopy as well as the catalase test. The coryneform strains were identified using the API Coryne biochemical identification system (BioMérieux) according to the manufacturer’s instructions with the exception of Corynebacterium semenale strains that were identified on the basis of β-glucuronidase test on blood agar with 4-methylumbelliferyl-β-D-glucuronide (MUG) supplement (Oxoid), which visualizes a positive reaction as a fluorescence near colonies under 254 nm ultraviolet light. In case of the urine strains, only C. semenale was identified to the species level.

7.2.2. Other bacteria

Other microorganisms were identified mostly to genus level (Murray et al., 1999). A latex test (Oxoid) was employed for discriminate Staphylococcus aureus from coagulase negative staphylococci (CONS). Novobiocin disks were used for identification of Staphylococcus saprophyticus. Streptococci and enterococci were identified by the absence of catalase production and differentiated by fermentation of esculine. Group B streptococci were identified using a latex test (Oxoid). Gardnerella vaginalis was identified by its ability to grow on se-
7.3. Susceptibility testing of coryneform bacteria

The E-test susceptibility testing method was chosen since it has shown a good correlation of MICs with both broth microdilution and agar dilution in tests with Corynebacterium sp. (Funke et al. 1997). The E test strips (AB Biodisk) on calcium adjusted Mueller-Hinton agar (Oxoid) were used as described elsewhere (Isenberg 2004) and following manufacturer’s recommendations. Aid of a nephelometer (Becton Dickinson) was used for obtaining the suspensions with the desired turbidity (Mc Farland 0.5). Incubation at 37 °C at normal atmosphere was used for incubation (usually 24h, 48h for slow growers). Minimal inhibitory concentrations (MICs) for 8 antibacterial agents were determined. CLSI (Clinical and Laboratory Standards Institute, formerly NCCLS) interpretive criteria for corynebacteria were used for penicillin G, trimethoprim-sulfamethoxazole (TMP/SMX), doxycycline, erythromycin and clindamycin (Clinical and Laboratory Standards Institute 2005). Because no CLSI interpretive criteria for corynebacteria exist with regards ampicillin-sulbactam, norfloxacin and nitrofurantoin, breakpoints for staphylococci were used for these antibiotics as suggested elsewhere (Clinical and Laboratory Standards Institute 2005; Funke et al. 1996; Otsuka 2005). The strains were considered susceptible (resistant) if their MICs were as follows: penicillin G ≤1 (≥4) µg/mL, ampicillin-sulbactam ≤8/4 (≥32/16) µg/mL, TMP/SMX ≤2/38 (≥4/76) µg/mL, doxycycline ≤4 (≥16) µg/mL, erythromycin ≤0.5 (≥2) µg/mL, clindamycin ≤0.5 (≥4) µg/mL, norfloxacin ≤4 (≥16) µg/mL and nitrofurantoin ≤32 (≥128) µg/mL. The MIC values that were higher than susceptible but less than resistant were termed as intermediately resistant.

7.4. Detection of mycoplasmas

7.4.1. Commercial kit method (Mycoplasma IST test)

The Mycoplasma IST kit was applied to the semen samples of all 161 men to investigate Mycoplasma hominis and Ureaplasma urealyticum according to the manufacturer’s instructions, as described previously (Clegg et al., 1997). The 16 wells of each IST kit strip contained a pH indicator and a lyophilized growth medium. When the inoculates of aforementioned Mollicutes in medium provided by manufacturer were inserted into the wells of the strips, then the changes due to pH change were interpreted as signs of growth as follows: from
pale yellow to amber for U10C broth, and from amber to red for arginine broth. Hence, the strips provided information on the presence or absence of \textit{M. hominis} and \textit{Ureaplasma sp.}, an estimate of the density of each organism (>10^4 CFU) as well as antibacterial agent susceptibility data.

7.4.2. Polymerase chain reaction method (PCR)

PCR was additionally used in the semen samples of 60 randomly selected men to investigate \textit{Mycoplasma genitalium}, \textit{Ureaplasma parvum} and \textit{U. urealyticum}. DNA was extracted from 200 µl of semen using the High Pure PCR Template Preparation Kit (Roche Biochemicals), and 10 µl of extracted DNA was used for PCR. Primers MgPa1 and MgPa3 were used for specific \textit{M. genitalium} genome amplification; they amplify a 281-bp segment of the 140-kDa adhesion protein gene (Jensen \textit{et al.}, 1991). The cycling parameters were as follows: 95 ºC for 2 min; 40 cycles at 95 ºC for 30 s, 65 ºC for 30 s and 72 ºC for 25 s; and 72 ºC for 5 min. Primers UMS-125 and UMA-226 were used for specific \textit{U. parvum} genome amplification; they amplify a 403-bp segment of the multiple-banded antigen gene (Teng \textit{et al.}, 1994). Cycling parameters were as follows: 95°C for 2 min 30 s; 40 cycles at 95°C for 40 s, 60°C for 50 s and 72°C for 40 s; and 72°C for 5 min. PCRs were carried out using the thermal cycler Mastercycler (Eppendorf). Recombinant Taq DNA Polymerase (Fermentas) was used. Primers P6 and U8 were used for specific \textit{U. urealyticum} genome amplification; they amplify a 1300-bp segment of the 16S rRNA gene (Robertson \textit{et al.}, 1992). Cycling parameters were as follows: 95°C for 3 min 30 s; 40 cycles at 95°C for 1 min, 56°C for 1 min 20 s and 72°C for 2 min; and 72°C for 5 min. PCRs were carried out using the thermal cycler RoboCycler Gradient 40 (Stratagene). Recombinant Taq DNA Polymerase (Fermentas) was used. The PCR products were separated by electrophoresis in a 2% agarose gel and visualized under UV light with ethidium bromide.

8. Biochemical methods

8.1. Semen sample preparation for biochemical analyses

A discontinuous Percoll density gradient centrifugation was used to separate spermatozoa from leukocytes (Tucker \textit{et al.} Jansen, 2002; Nakamura \textit{et al.}, 2002; Aitken \textit{et al.}, 1998). Percoll gradient centrifugation yields a highly motile fraction of spermatozoa relative to starting sample and removes seminal plasma and other cells (Mortimer, 1994) which remain in less dense fractions after gradient centrifugation (Makler \textit{et al.}, 1998). Jouan CR3i centrifuge and glass test tubes (dimensions 12x104 mm, volume 8 ml) were used for centrifugation as described in producer's manual. The specimen was centrifuged to separate seminal plasma and spermatozoa at 300g for 10 minutes at +2°C, the pellet was re-
suspended in saline and centrifuged once more. The pellet was resuspended in saline and processed in a discontinuous Percoll density gradient (30%, 50%, 70% and 90% SIP). A Neubauer counting chamber (haemocytometer) was used for measuring the concentration of spermatozoa in the fractions, enabling to select the most spermatozoa-rich fraction for further analyses. The samples were diluted in saline to the density 10⁶ spermatozoa/ml, stored for further analyses in liquid nitrogen and disrupted by quick freezing-thawing for several times.

8.2. Detection of iron (Fe), zinc (Zn) and nickel (Ni)

400 µl of suspended spermatozoa or seminal plasma was pipetted to HDPE plastic vessel and 4 ml of the ultra pure water was added. The concentrations of metal ions in the solution were determined using a Varian (Varian Inc. Scientific Instruments, Mulgrave, Australia) Liberty II axial inductively coupled plasma atomic emission spectrometer (ICP-AES). The detection of metals was performed in accredited laboratory according to EN ISO 11885:1996 (Water quality – Determination of 33 elements by inductively coupled plasma atomic emission spectroscopy).

8.3. Detection oxidative stress

8.3.1. Detection of total antioxidative activity (TAA)

Total antioxidative activity (TAA) of seminal plasma (dilution 1:20) was assessed by using the linolenic acid test (LA-test). This test evaluates the ability of sample to inhibit linolenic acid peroxidation, which indicates ability of a sample to inhibit oxidation in a lipid-soluble environment. The standard of linolenic acid in 96% ethanol (1:100) was diluted in isotonic saline (1:125). 0.01% sodium dodecyl sulphate was added to 0.4 ml linolenic acid, diluted in isotonic saline and the sample. The incubation started by adding 100 µl FeSO₄ (final concentration 200 µM) and the mixture was incubated at 37°C for 60 min. Then the reaction was interrupted by adding 0.035 ml butylated hydroxytoluene and the mixture was treated with 0.5 ml acetate buffer (pH 3.5) consisting of acetic acid glacial and sodium acetate trihydrate and heated with freshly prepared 1% thiobarbituric acid solution (TBA) at 80°C for 40 min. After cooling the mixture was acidified by adding 0.5 ml cold 5 M HCl, extracted with 1.7 ml cold 1-butanol and centrifuged at 3000g for 10 min and the TBA reactivity (as µM of malondialdehyde equivalents) of butanol fraction was measured spectrophotometrically at 534 nm. The TAA of sample was expressed as inhibition by sample of LA-standard peroxidation as follows: \[1 - \left(\frac{A_{534 \text{ (sample)}}}{A_{534 \text{ (LA as control)}}}\right) \times 100\]. The higher numerical value (%) of TAA indicates the higher TAA of sample. Peroxidation of LA-standard in the isotonic saline (without serum) served as a control.
8.3.2. Detection of total antioxidative status (TAS)

TAS indicates the ability of a sample to inhibit the ROS-mediated oxidation in an aqueous environment. To measure total antioxidative status (TAS) of blood serum, seminal plasma and in spermatozoa we used a commercially available kit (Randox Laboratories Ltd.). To measure total antioxidative status (TAS) of blood serum, seminal plasma and in spermatozoa we used a commercially available kit (Randox Laboratories Ltd.). This method is based on the inhibition of the absorbance of the ferrylmyoglobin radicals of 2,2’-azinobis-ethylbenzothiazoline-6-sulfonate (ABTS+) generated by activation of metmyoglobin peroxidase with H$_2$O$_2$. The suppression of the absorbance of ABTS+ radicals by sample depends on TAS of the sample under investigation (Rice-Evans et al., 1994). The assay procedure was as follows. To 0.02 ml of blood serum (blank was ultrapure water) and standard (6-hydroxy-2,5,7,8-tetramethylchroman), 1 ml of chromogen (metmyoglobin) solution was added, mixed well and initial absorbance was read. Then 0.2 ml of substrate (hydrogen peroxide in stabilized form) was added, mixed, incubated at 37°C and absorbance was read exactly after 3 minutes at 600nm. The TAS values are expressed as Trolox units (mmol/L).

8.3.3. Detection of ascorbic acid (AsA)

Ascorbic acid is a major antioxidant of the aqueous environment. Ascorbic acid (AsA) is oxidized by 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy, a stable free radical (TEMPO) to dehydroascorbic acid (DAsA). The latter condenses with o-phenylenediamine (OPDA) to form a quinoxaline derivative that absorbs light at 340 nm. The change in absorbance at 340 nm is proportional to the concentration of AsA in the specimen. Briefly, heparinized plasma or seminal plasma (0.025 mL) and 0.2 mL of TEMPO solution (200 mg/L in phosphate buffer, pH 6.5) were incubated at 37°C for 5 min. Then 0.085 mL of the OPDA solution (500 mg/L in phosphate buffer, pH 6.5) was added and the absorbances at 340 nm were determined every 60 s for 3 min. We used the phosphate buffer as a reagent blank to correct for any non-specific absorbance. We used a 25 mg/L AsA calibrator to calculate the concentration of AsA in the specimens (Ihara et al., 2000). TEMPO, OPDA dihydrochloride and AsA were obtained from Sigma Chemical.

8.3.4. Detection of glutathione and oxidized glutathione

Antioxidative activity of the major antioxidant glutathione (GSH) is mediated by the –SH (sulfhydryl) functional group of the molecule. Total glutathione and oxidized glutathione were measured by using the method described earlier (Griffith, 1980). The samples were deproteinated with 10% solution of meta-
phosphoric acid. The equal volume of the metaphosphoric acid was added to the sample and mixed vigorously. The mixture was allowed to stand at room temperature for 5 min and centrifuged at 3000 g for 5 min. In cases where the assay was not performed immediately, the supernatant was carefully collected and stored at –20ºC. Glutathione content was measured by adding 0.005 ml of triethanolamine 4M solution in water to 0.1 ml of sample and mixed immediately. Thereafter, the sample was divided into two parts. For assay of oxidized glutathione (GSSG), reduced glutathione (GSH) was derivatized by adding 0.1 ml 2-vinylpyridine in 1 mM ethanol to first part of the sample, mixing on a vortex mixer and keeping at room temperature for 1 h. To determine the content of GSSG, 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA, 0.5 U glutathione reductase and 0.3 mM NADPH was added to the 0.1 ml of derivatized sample and mixed immediately. The enzymatic reaction was initiated by addition of 0.1 ml of 1 mM 5,5′-dithio-bis-2-nitrobenzoic acid in 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA (Griffith, 1980). The change in optical density was measured after 10 min at 412 nm spectrophotometrically. The glutathione content was calculated based on a standard curve generated with known concentration of glutathione. Amount of GSH was calculated as a difference between the total glutathione and GSSG (total glutathione − GSSG = GSH). The glutathione content was expressed as µg/ml of sample or as glutathione redox ratio (GSSG/GSH).

8.3.5. Detection of 8-isoprostanes (8-EPI)

8-isoprostanes (8-EPI) are major stable end-products ROS-mediated prostanoid oxidation. We used the method with what we had previously measured the content of 8-EPI in urine of healthy humans (Kullisaar et al., 2003). This assay is a competitive enzyme-linked immunoassay (ELISA) for determining levels of 8-EPI in biological samples (BIOXYTECH 8-Isoprostane Assay, Cat. No. 21019). Briefly, 8-EPI in the samples or standards competes for binding (to the antibody coated on the plate) with 8-EPI conjugated to horseradish peroxidase (HRP). The peroxidase activity results in color development when the substrate is added. The intensity of the color is proportional to the amount of 8-EPI-HRP bound and inversely proportional to the amount of 8-EPI in the samples or standards. The urinary concentrations of isoprostanes were corrected by urinary creatinine concentrations to account for the differences in renal excretory function.

8.3.6. Detection of diene conjugates (DC)

Lipid peroxidation results in the formation of diene conjugates, which are used as lipid peroxidation markers. Diene conjugates were measured according to the method previously described (Recknagel et al., 1984) with minor modifications (Starkopf et al., 1995). Briefly, samples (0.15 ml) + 0.15 ml 0.9% NaCl (reagent blank contains only isotonic saline) were incubated at 37 ºC for
30 min, 0.25% butylated hydroxytoluene (0.015 ml) was added and the lipids were extracted by heptane/isopropanol (1:1, whole volume 1.8 ml). Then the samples were acidified by 5M hydrochloric acid (0.5 ml). After extraction by cold heptane (1.6 ml), samples were centrifuged (for 5 min at 3000 rpm) and absorbance of heptane fraction was measured spectrophotometrically at absorbance maximum at 234 nm.

**8.3.7. Detection of 8-Hydroxy-2′-Deoxyguanosine (8-OHdG)**

8-OHdG shows ROS-mediated damage to DNA. The BIOTECH 8-OHdG Kit is for quantitative measurement of 8-OHdG in tissue, serum, plasma and urine resulting from oxidative damage of DNA. The 8-OHdG monoclonal antibody and the sample or standard were added to a microtiter plate well that has been precoated with 8-OHdG. The 8-OHdG in the sample or standard competes with the 8-OHdG bound on the plate for the 8-OHdG monoclonal antibody binding sites. Therefore, higher concentrations of 8-OHdG in the sample solution leads to a reduced binding of the antibody to the 8-OHdG on the plate.

**9. Statistical analysis**

Statistical analyses were performed with the use of SigmaStat (Jandel Scientific) and Excel (Microsoft Corp, Redmond, WA, United States of America) software programs. In the study of oxidative stress, the study groups were compared with t-test (in case of normal distribution) and Mann-Whitney rank sum test (in case of non-parametric distribution). Spearman rank order correlation was used to find out correlations between different markers. In the study of coryneform bacteria, microbial counts were compared with Mann-Whitney rank sum test. The occurrence of microorganisms in different groups was compared with Fisher exact test. In the study of mycoplasmas, Fisher’s exact test, Chi square test and logistic regression analysis were used to compare the occurrence of mycoplasmas between the different study groups. Cohen’s kappa coefficient \( \kappa \) for diagnostic agreement was used to compare the two methods. \( p \leq 0.05 \) was considered significant in all analyses.
RESULTS AND DISCUSSION

10. Coryneform bacteria in semen of chronic prostatitis patients

10.1. Prevalence of coryneform bacteria in male genital tract

On the level or primary screening, no differences between patients and controls were found as coryneform bacteria were present in the semen of 38 (76%) inflammatory prostatitis patients (both NIH IIIA and NIH IV categories) as well as 49 (83%) controls (p>0.05). The subjects had 0...6 (mean 1.3) different coryneforms present, 0...4 (1.4) in prostatitis patients and 0...6 (1.2) in controls (no statistical differences). Since no difference was found also between NIH IIIa and NIH IV category patients (data not shown), these categories were analysed together.

Substantial differences were revealed between prostatitis patients and controls on the species level. Two coryneform bacteria were significantly more frequently found from prostatitis patients with severe inflammation than controls – Corynebacterium group G (33% vs. 2%, p=0.0003) and genus Arthrobacter (17% vs. 2%, p=0.03). In general, the most frequent species in male genital tract was Corynebacterium seminale, being present in 30 prostatitis patients and 34 controls (Table 7).

We compared the bacteria of semen with that of first-catch urine that enabled us to distinguish between urethral contamination and true microbiota of semen. Half of men (50%) harbored corynebacteria in both semen and urine, 22% of men harbored them in semen only and 3% in urine only. Their total concentration was greater in semen than in urine (median 5000 vs. 100 CFU per ml) yet the difference did not reach the level significance (p=0.053). Of urine strains, only C. seminale was identified to species level (present in 39% of men).

Coryneform bacteria have been previously found from urogenital tract of both healthy men (Willen et al., 1996) and patients with prostatitis (Tanner et al., 1997; Riegel et al., 1999; Domingue et Hellstrom, 1998). It has been stated more than 30 years ago (Drach, 1974) that 12% of studied prostatitis cases were due to coryneform organism in pure culture or with associated bacteria. Culturable coryneform species isolated from semen or expressed prostatic secretions include C. seminale (Riegel et al., 1995), C. singulare (Riegel et al., 1997), C. freneyi (Renaud et al., 2001), C. striatum/amycolatum, C. macginley, C. jeikeium, Dermabacter hominis (Jędrzejczak et al., 2005) and even C. diphtheriae (Machado et al., 1989). Gardnerella vaginalis is a catalase negative coryneform that has been strongly associated with bacterial vaginosis in women. That microorganism occurred less frequently in our study than in some other studies, where G. vaginalis has been found in 19%...26% of semen specimens (Hillier et al., 1990; Virecoulon et al., 2005). Domingue et al. (1997) found Corynebacterium group ANF and C. minutissimum from EPS, while Tanner et al.,
(1999) found several species by PCR from the same material. Of the species found, *C. genitalium* and *C. tuberculostearicum* seemed to have affinity towards prostatitis, and unknown *Corynebacterium* sp. related to *C. coyleae, C. imitans* or *C. seminalis* were found exclusively from prostatitis patients. Hence, earlier studies have associated prostatitis with different sets of *Corynebacterium* species. Our results followed the suit.

As concerns methodological aspects, biochemical identification with API Coryne is less exact than genotype-based methods (Roux *et al.*, 2004). Some API Coryne bacterial groups may be polyphyletic, as *Cellulomonas* sp. and *Microbacterium* sp. are clustered into one category. *Corynebacterium* group G probably consists of several species since according to additional tests that were performed later, most of our *Corynebacterium* group G strains were fructose negative, which does not fit the description of *Corynebacterium* group G provided by von Graevenitz et Bernard (2006). The API identification profiles of our strains of this group were 6000325, 61003025, 6140325 and 1200325.

### 10.2. Quantitative composition of seminal coryneflora

We subsequently set a threshold limit of ≥10⁴ CFU per ml to bacterial concentration in order to reveal possible differences between patients and controls at quantitative level. In control subjects, only four bacterial groups managed to outnumber this threshold: *C. seminalis*, *C. jeikeium*, *Corynebacterium* sp. and catalase-negative coryneforms (Fig. 3). In prostatitis patients also *Arthrobacter* sp., *Brevibacterium* sp., *Cellulomonas*/ *Microbacterium*, *Corynebacterium* group F1 and *Corynebacterium* group G exceeded that threshold.

![Fig. 3. Quantitative differences in seminal coryneflora of inflammatory prostatitis patients and controls. The list of the different isolates that can be found in significant (>10⁴ CFU/ml) quantities from prostatitis patients is longer than the analogous list for controls.](image-url)
<table>
<thead>
<tr>
<th>Coryneform bacteria</th>
<th>Men with severe inflammation (&gt;1 M WBC per ml of semen) n=18</th>
<th>Men with moderate inflammation (0.2–1 M WBC per ml of semen) n=22</th>
<th>Controls n=59</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log10 of CFU/ml Mean (range)</td>
<td>Proportional positive spermogram</td>
<td>log10 of CFU/ml Mean (range)</td>
</tr>
<tr>
<td><em>Arthrobacter</em> sp.</td>
<td>3.8 &lt;2 (&lt;2–5.0) 17% *</td>
<td>&lt;2 &lt;2 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 3%</td>
</tr>
<tr>
<td><em>E. coli</em> sp.</td>
<td>3.4 &lt;2 (&lt;2–4.7) 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 3%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium ovisae</em></td>
<td>&lt;2 &lt;2 0%</td>
<td>1.5 &lt;2 (&lt;2–3.0) 3%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium group A</em></td>
<td>&lt;2 &lt;2 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium group F1</em></td>
<td>3.7 &lt;2 (&lt;2–5.0) 6%</td>
<td>2.8 &lt;2 (&lt;2–4.0) 6%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium group G</em></td>
<td>3.8 &lt;2 (&lt;2–5.0) 33% *</td>
<td>2.5 &lt;2 (&lt;2–4.0) 3%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium jeikeium</em></td>
<td>3.8 &lt;2 (&lt;2–5.0) 11%</td>
<td>1.5 &lt;2 (&lt;2–3.0) 3%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium mucinolyticus</em></td>
<td>&lt;2 &lt;2 0%</td>
<td>2.5 &lt;2 (&lt;2–4.0) 6%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium semenale</em></td>
<td>4.9 &lt;2 (&lt;2–5.7) 61%</td>
<td>4.8 &lt;2 (&lt;2–5.7) 59%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium striatum</em></td>
<td>3.5 &lt;2 (&lt;2–4.7) 11%</td>
<td>3.7 &lt;2 (&lt;2–5.0) 9%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Cellulomonas/Microbacterium</em></td>
<td>0.7 &lt;2 (&lt;2–2.0) 6%</td>
<td>3.8 &lt;2 (&lt;2–5.0) 9%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Corynebacterium sp.</em></td>
<td>5.5 &lt;2 (&lt;2–6.7) 23%</td>
<td>4.0 &lt;2 (&lt;2–5.0) 19%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Dermabacter hominis</em></td>
<td>&lt;2 &lt;2 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>Gardnerella vaginalis</em></td>
<td>2.7 &lt;2 (&lt;2–4.0) 6%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td><em>T. vaginalis</em></td>
<td>&lt;2 &lt;2 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
<tr>
<td>Catalase negative coryneform</td>
<td>4.5 &lt;2 (&lt;2–4.7) 17%</td>
<td>3.5 &lt;2 (&lt;2–5.0) 22%</td>
<td>&lt;2 &lt;2 &lt;2 (&lt;2–3.0) 0%</td>
</tr>
</tbody>
</table>

* p<0.05 (Fisher exact test)
6 — API Coryne category
In our prospective quantitative study with 6 media and 3 different environments, the coryneform species emerged as the most frequent group of microorganisms, in agreement with our previous data (Punab et al., 2003; Kermes et al., 2003; Korrovits et al., 2006) as well as to the data of Hillier et al. (1990) who found them in 86% of semen specimens. According to our previous studies (Punab et al., 2003; Kermes et al., 2003; Korrovits et al., 2006), the seminal microflora of prostatitis patients differs significantly from that of controls since the total concentration and number of different microorganisms are much higher in the semen of prostatitis patients than in the controls. Hence, these data pointed out the idea of the polymicrobial nature of prostatitis. The present study has taken under examination one group of this microbial community and these data indicate that some coryneform species may appear a major component of this microbiota.

10.3. Susceptibility of coryneform bacteria

The minimal inhibitory concentrations and numbers of non-susceptible (resistant plus intermediate) strains are presented in Table 1 at Paper II. All strains were susceptible to ampicillin-sulbactam and only a few were resistant to penicillin G and TMP/SMX while nearly one third of strains were resistant or intermediate to doxycycline (35%) and norfloxacin (29%), and more than half to clindamycin (63%), nitrofurantoin (62%) and erythromycin (53%). Similar susceptibility pattern was characteristic to the most common species, C. seminale, most of its strains were resistant or intermediate to clindamycin, erythromycin, norfloxacin and nitrofurantoin.

The strains showing resistance to at least 3 antimicrobials belonged to Corynebacterium group F1, Corynebacterium seminale and Cellulomonas/Microbacterium sp. One Cellulomonas/Microbacterium sp. strain was resistant to four (erythromycin, clindamycin, penicillin G and nitrofurantoin), one Corynebacterium group F1 strain to 3 (erythromycin, clindamycin, doxycycline) and three C. seminale strains to three antimicrobials (erythromycin and clindamycin combined with norfloxacin, nitrofurantoin or TMP-SMX). In addition, 20 strains (12 C. seminale, four Corynebacterium group G, two D. hominis, one C. striatum, and one Cellulomonas/Microbacterium sp.) were resistant to two antimicrobials. A distinct co-occurring macrolide and lincosamide resistance pattern was common. The susceptibility of the strains originating from prostatitis patients and those of controls were compared (data not shown) and no significant differences were found.
The results of our study were in general agreement with the data of previous reports about frequent resistance among coryneform bacteria to several antimicrobials. The available data describe mostly the invasive nosocomial pathogens and very scarce information exists concerning the mucosal strains yet they may become under certain conditions the source of infection. Since our strains originated from the male genital tract, we have tested their susceptibility mainly to antibiotics commonly used in andrological practice. *Corynebacterium* group G that was associated with prostatitis showed resistance to several antibacterial agents including norfloxacin that is commonly used for treatment of male genital tract infections (Fig. 4). Since treatment of prostatitis usually does not aim for a particular target, susceptibility of possible pathogens may give valuable information for choosing an antibiotic. Caution is advised for interpreting our *in vitro* data for the purpose of *in vivo* application. Relevant factors like prostate tissue penetration and biofilm associated resistance should be taken into account.

Although bimodal spread of susceptibility (MIC either <0.5 or >4 µg/mL) to ciprofloxacin has been described (Fernandez et al. 2001), similar pattern did not appear in our study. *Corynebacterium sp.* did not display any level of universal intrinsic resistance to norfloxacin that could be expected from the absence of Topoisomerase IV in these bacteria.

Resistance to β-lactam antimicrobials among the coryneforms varies, *C. seminale, T. otitidis* and *Arthrobacter* sp. have been more susceptible to peni-

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**Fig. 4.** Susceptibility pattern of *Corynebacterium* group G. White – susceptible; striated – Intermediate; black – Resistant.
cillin G than *C. jeikeium*, *C. striatum*, and *D. hominis* (Funke et al., 1996; Funke et al., 1997; Radtke et al., 2001; Ubaldi et al. 2004). Our strains were highly susceptible, except *Cellulomonas/Microbacterium* group.

Our data corresponds to several studies that have shown a high macrolide and lincosamide resistance of coryneforms (Fernandez et al. 2001; Funke et al. 1997; Martinez-Martinez et al. 1996; Soriano et al. 1995; Ubaldi et al. 2004) and MLSb resistance (a co-occurring resistance to Macrolide, Lincosamide and Streptogramin B) (Rosato et al. 2001). In our study, 16 strains (13 *C. seminale*, 2 *Cellulomonas/Microbacterium* sp. and 1 *Corynebacterium* group F1) showed concurrent resistance to erythromycin and clindamycin.

Unlike earlier studies (Riegel et al. 1995; Soriano et al. 1995), in our study, *C. jeikeium* and *C. seminale* did not have low MICs of nitrofurantoin. Another discord with earlier reports was revealed in case of TMP/SMX that was highly active on the majority of the strains studied by us while weak or missing activity of it against *C. striatum*, *C. jeikeium*, *D. hominis* and *T. otitidis* has been described (Martinez-Martinez et al. 1996; Traub et al. 1998; Troxler et al. 2001). Our strains were susceptible to TMP/SMX, which was declared as a drug of choice in Canada (Nickel et al. 1998).

Resistance to tetracyclines among corynebacteria is controversial – generally multiresistant species as *C. jeikeium* and *C. amycolatum* are relatively susceptible, while *C. seminale* and *C. striatum* quite resistant (Funke et al. 1997; Martinez-Martinez et al. 1996). *D. hominis*, *T. otitidis* and *Cellulomonas* sp. (Troxler et al. 2001; Funke et al. 1997) have been susceptible. In contrast to these studies, our *C. striatum* strains were susceptible to doxycycline.

II. Mycoplasmas in semen of chronic prostatitis patients

II.1. Mycoplasmas detected by Mycoplasma IST test

Mycoplasma IST test gave positive results in all studied groups – three prostatitis categories (NIH IIIA, NIH IIIB and NIH IV) and controls (Table 8, Fig. 2). *M. hominis* was found only in one NIH IIIB patient, in a low count (10^4 CFU /ml). At the same time, ureaplasmas were found in nearly 20% of prostatitis patients and in 12% of controls using the Mycoplasma IST test.

Mycoplasma IST and newer IST 2 tests have been implemented for analyzing mycoplasmas in male patients. Mycoplasma IST test has been reported to be superior to MycoFast All-In test for detecting *M. hominis* (Vázquez et al., 1995). While analyzing the patients with non-gonococcal urethritis using IST test, Kilic et al. (2005) found that 24 of 50 these men harbored *U. urealyticum*, and eight of those 24 harbored *M. hominis*. In a study by Zdrodowska-Stefanow et al., (2006) *U. urealyticum* was found in 8% of prostatitis patients while *M. hominis* was not found using IST 2 test - that corresponds to our data.
11.2. Mycoplasmas detected by PCR method

A quarter of chronic prostatitis patients harbored mycoplasmas confirmed by PCR in their semen (Fig. 5). This proportion was even higher in case of inflammatory CP/CPPS (NIH IIIa) patients of whom one third were colonized by mycoplasmas, at the same time they were present only in 1 out of 25 healthy controls (4/11 vs 1/25; \(p=0.023\)).

Using PCR, most of the ureaplasmas found using the IST test were re-identified as U. parvum, which emerged as the most common species (Table 8). U. parvum was not found from healthy men but it was found from all prostatitis groups (NIH IIIA, NIH IIIB and NIH IV). One patient in NIH category IV had both Ureaplasma species. M. genitalium occurred only in NIH category IIIA patients.

Previous investigators have found numerous mycoplasma species in humans. For certain species such as Ureaplasma sp., M. hominis and M. genitalium, the genital tract is thought to be the main site of colonization (Baseman et Trully 1997, Uusküla et al., 2002). Apparently, U. urealyticum is the most widespread mycoplasma in the genital tract of both sexes, its reported prevalence in human semen varying from 10% to 40% (Keck et al., 1998). U. urealyticum has been related to non-gonococcal urethritis and prostatitis but it also quite frequently colonizes asymptomatic men (Keck et al., 1998, Potts et al., 2000). Unfortunately, in most previous studies, no distinction was made between U. urealyticum and U. parvum. U. parvum (formerly U. urealyticum biovar 1) was distinguished from U. urealyticum (Kong et al., 1999), and it has been shown in some studies that most ureaplasmas in semen may actually be U. parvum (Knox et al., 2003). A similar tendency could also be seen in our study, where U. parvum occurred more frequently among PCR-confirmed mycoplasmas than U. urealyticum and, interestingly, it was present only in prostatitis patients.

M. genitalium is a probable cause of non-gonococcal urethritis (Jensen 2004), and is associated with prostatitis (Krige et Riley 2002). The prevalence rate of M. genitalium in a biopsy study of nonbacterial prostatitis patients was 4% (Krieger et al., 1996) although some contradictory results can be found as well (Taylor-Robinson 2002). M. genitalium can affect fertility as it was shown to adhere to the spermatozoa, which became immotile when many M. genitalium were attached (Svenstrup et al., 2003). In our study, this species was associated with NIH category IIIA prostatitis patients.

11.3. Impact of PCR on interpreting IST test results

We found substantial agreement between the two methods used for the genus Ureaplasma: in 48/60 men both tests were negative and in 7/60 both tests were
positive (κ=0.69, p=0.0007). As the exception, four men were IST positive but PCR negative while one man was IST negative but PCR positive.

A similar situation has been discussed by Stellrecht et al. (2004), who found some culture-positive but PCR-negative semen specimens, although they cultured mycoplasmas on A7 agar instead of using the Mycoplasma IST test. Ras-tawicki et al. (2004) found also some IST test positive but PCR and culture negative specimens and explained it with oversensitivity of IST test. In our study, the false-positive results may have been caused by other urease-positive microorganisms, as the IST test detects ureaplasmas by means of this enzyme.

Despite of agreement between the methods, IST test does not enable differentiation of U. urealyticum and U. parvum. As U. parvum was most common mycoplasma, and present only in prostatitis patients, there is an argument for using the PCR method instead of the Mycoplasma IST test. The IST test does not detect prostatitis-associated M. genitalium as well and it is another argument to suggest PCR method for detecting mycoplasmas in prostatitis patients.

Table 8. Occurrence of mycoplasmas in semen

<table>
<thead>
<tr>
<th>Test Method</th>
<th>Microorganism</th>
<th>NIH IIIA</th>
<th>NIH IIIB</th>
<th>NIH IV</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycoplasma IST</td>
<td>Ureaplasma sp.</td>
<td>8/38 (21%)</td>
<td>10/59 (17%)</td>
<td>6/24 (25%)</td>
<td>5/40 (12%)</td>
</tr>
<tr>
<td></td>
<td>M. hominis</td>
<td>0/38 (0%)</td>
<td>1/59 (2%)</td>
<td>0/24 (0%)</td>
<td>0/40 (0%)</td>
</tr>
<tr>
<td>PCR</td>
<td>U. urealyticum</td>
<td>0/11 (0%)</td>
<td>1/20 (5%)</td>
<td>1/4 (25%)</td>
<td>1/25 (4%)</td>
</tr>
<tr>
<td></td>
<td>U. parvum</td>
<td>2/11 (18%)</td>
<td>3/20 (15%)</td>
<td>1/4 (25%)</td>
<td>0/25 (0%)*</td>
</tr>
<tr>
<td></td>
<td>M. genitalium</td>
<td>2/11 (18%)</td>
<td>0/20 (0%)</td>
<td>0/4 (0%)</td>
<td>0/25 (0%)**</td>
</tr>
</tbody>
</table>

*,**, p=0.087 (Fisher’s exact test); p=0.028 (Chi square test)
***p=0.080 (Fisher’s exact test); p=0.045 (Chi square test)

Fig. 5. Significance of mycoplasmas in relation to prostatitis according to PCR results
* p=0.032 (Fisher’s exact test)
** p=0.026 (Fisher’s exact test), p=0.029 (logistic regression analysis)
12. Oxidative stress in chronic prostatitis patients

We compared the presence and rate of oxidative stress (OxS) in inflammatory prostatitis patients (NIH IIIA and NIH IV categories; Fig. 2) and controls. No significant differences were observed when patients and controls were compared for age, period of sexual abstinence and basic sperm parameters (sperm volume, sperm concentration, total sperm count and motility). In addition to elevated WBC counts, we observed an elevation of IL-6 levels in the patient group that confirmed inflammatoriness of the prostatitis (Table 9).

Table 9. Clinical and basic semen parameters of the prostatitis patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Prostatitis patients (n=21)</th>
<th>Controls (n=9)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>32.3±1.4</td>
<td>31.2±2.6</td>
<td>ns</td>
</tr>
<tr>
<td>Period of abstinence (days)</td>
<td>4.08±0.41</td>
<td>5.11±0.87</td>
<td>ns</td>
</tr>
<tr>
<td>Semen volume (ml)</td>
<td>3.89±0.32</td>
<td>4.77±0.63</td>
<td>ns</td>
</tr>
<tr>
<td>Sperm concentration (million/ml)</td>
<td>56.47±13.73</td>
<td>57.11±13.95</td>
<td>ns</td>
</tr>
<tr>
<td>Total sperm count (million)</td>
<td>213.53±48.78</td>
<td>234.27±44.03</td>
<td>ns</td>
</tr>
<tr>
<td>A+B motility (%)</td>
<td>45.33±3.67</td>
<td>50.79±4.98</td>
<td>ns</td>
</tr>
<tr>
<td>White blood cells in semen (million/ml)</td>
<td>2.48±0.96</td>
<td>0.11±0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-6 in seminal plasma (pg/ml)*</td>
<td>73.74±29.59</td>
<td>11.48±5.14</td>
<td>0.020</td>
</tr>
<tr>
<td>hsCRP in blood serum (mg/L)</td>
<td>0.89±0.14</td>
<td>1.26±0.51</td>
<td>ns</td>
</tr>
<tr>
<td>Prealbumin in blood serum (g/L)</td>
<td>0.41±0.02</td>
<td>0.44±0.03</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns – not significant
* – IL-6 measurements were performed in 8 prostatitis patients and 6 controls

We did find relevant information on various aspects of OxS (antioxidants, pro-oxidants and oxidation products) from local samples (spermatozoa and seminal plasma) as well as from samples of systemic nature (urine and blood).

12.1. Oxidative stress in spermatozoa

The total antioxidative status in water environment (TAS) of spermatozoa decreased in inflammatory prostatitis patients compared with controls, as shown in Table 10. Level of TAS also exhibited a strong negative correlation with WBC (R=-0.64, p<0.001). At the same time, lipid peroxides (diene conjugates, DC) were statistically significantly increased mainly because of the damage of spermatozoon membrane.

This evidence suggests that spermatozoa of the leukocytospermic men have increased OxS. For the interpretation of the OxS parameters in the spermatozoa it should be noted that the spermatozoa need low levels of ROS, especially hyd-
rogen peroxide and superoxide radical for the capacitation (tyrosine phosphorylation, hyperactivation and acrosome reaction) (Aitken 1997, 2004, Rivlin et al., 2004). If the generation of ROS becomes elevated for any reason, spermatozoa possess a limited capacity to protect themselves from OxS (Baker and Aitken, 2005). TAS measurement in spermatozoa has been implemented for purpose of comparing infertile and fertile men, without finding any difference (Verit et al., 2007). In the spermatozoa, the thiols seem much more important contributors to antioxidant defense than vitamins C and E (Lewis et al, 1997).

### 12.2. Oxidative stress in seminal plasma

Oxidative damage of seminal plasma lipids in the inflammatory prostatitis patients appears higher than in the healthy controls, as suggested by the higher DC content in the seminal plasma of leukocytospermic men (Table 10). Also, the level of total antioxidative activity (TAA%) showed lower values in prostatitis patients compared to these of healthy controls. We also observed an analogous but weak trend concerning total antioxidative status (TAS). Not surprisingly, there was also a strong negative correlation between seminal plasma measurements of TAA% and DC (R=−0.54, p=0.002). Our data correspond to previous studies that have shown lower TAS in leukocytospermic men (Omu et al. 1999; Omu et al. 1998; Pasqualotto et al., 2001; Agarwal et al., 2003).

The TAS, indicating total antioxidative status in water-soluble environment and based on water-soluble molecules like vitamin C (Erel, 2004), revealed a good positive correlation with that vitamin, indeed (R=0.69, p<0.0001). The vitamin C levels in seminal plasma had lower values in prostatitis patients with severe inflammation (>1 M WBC/ml) than in control group (28.06±10.47 vs 40.79±10.47, p=0.0002) although the whole group did not reach the level of statistical significance (p=0.09). It has been shown that vitamin C deficiency may reduce sperm characteristics and facilitate DNA damage (Ebesunun et al., 2004, Song et al., 2006); the same applies to TAS (Koca et al., 2003).

We observed very low concentrations of GSH in seminal plasma that correspond with the earlier studies (Yeung et al., 1998; Storey et al., 1998) and there were no significant differences between control and patients group.

In our study, the level of Zn in seminal plasma was lower in prostatitis patients compared to healthy controls yet not reaching the significance level. Zinc levels in seminal plasma have been positively associated with sperm concentration and motility in some studies (Fuse et al., 1999; Chia et al., 2000) but not in others (Lewis-Jones et al., 1996; Lin et al., 2000). Reduced zinc levels in seminal plasma of men with leukocytospermia or Ureaplasma urealyticum infection have been reported (Omu et al. 1999; Han et al., 2003). The exact concentration of Zn in seminal plasma in vivo is unknown since the unbound Zn fraction depends on the post-ejaculatory redistribution of the ion from prostate to high affinity vesicular ligands (Carpino et al., 1998).
12.3. Systemic oxidative stress

We found differences between the inflammatory prostatitis patients and healthy men concerning markers showing systemic OxS, as revealed by 8-EPI in urine and glutathione (GSH) in red blood cells. The levels of 8-EPI in the urine of inflammatory prostatitis patients were significantly elevated when compared to healthy controls (Table 10). This marker correlated well with 8-OHdG, which indicates oxidative damage of DNA \((R=0.45, \ p<0.01; \ Fig. \ 6)\). In addition, 8-OHdG correlated with intraspermatozoal Fe \((R=0.52, \ p<0.004)\) and Ni \((R=0.48, \ p=0.008)\).

Isoprostanes are prostaglandin-like substances that are produced \textit{in vivo} independently from a cyclooxygenase (COX), primary by free radical-induced peroxidation of arachidonic acid (Morrow \textit{et al.}, 1990). They are released in response to cellular activation, circulate as a free form or as esters in phospholipids in plasma and excreted in urine. The measurement of isoprostanes in biological fluids has prompted clinical investigations on the pathophysiological role of lipid peroxidation in human disease.

Elevated production of ROS associated with DNA damage in immature spermatozoa seems to impair spermatogenesis (Ollero \textit{et al.}, 2001; Baker and Aitken, 2005). Elevated level of ROS may cause a release of iron from endogenous iron proteins such as tissue ferritin and transferrin, as well as modulate their expression (Niwa \textit{et al.}, 2003; Polla \textit{et al.}, 2003). Ni may affect genetic material directly or indirectly, via Fenton-like chemistry leading to ROS, causing DNA strand breaks and oxidative modifications of bases (Manini \textit{et al.}, 2003). Induction of DNA single-strand breaks, DNA protein cross-links, sister chromatid exchanges and chromosomal aberrations has been demonstrated with various nickel salts (Doreswamy \textit{et al.}, 2004).

In this study, we found no relation between glutathione and sperm motility although in a placebo-controlled double-blind infertility study it was shown that glutathione supplementation positively affected sperm motility (Lenzi \textit{et al.}, 1993). Unlike the studies regarding NIH II (chronic bacterial) prostatitis by Zhou \textit{et al.} (2006) and Lou \textit{et al.} (2006), our results did not reveal differences in vitamin C levels, which could be expected to contribute to the antioxidative defense as well.
12.4. Seminal microflora in respect to oxidative stress

In an agreement with our earlier studies, no sterile semen samples were found from patients (Table 3 in Paper IV) or controls. 119 isolates were successfully identified and subsequently allocated into 20 microbial groups. 32% of strains were identified to species levels; others were identified to genus level or to a broader category like coagulase-negative staphylo cocci (CNS). The number of different microorganisms in one sample ranged from 1 to 8, the total microbial concentrations ranged from $5 \times 10^3$ to $7 \times 10^5$ CFU/ml. *Corynebacterium* group G showed association with inflammatory prostatitis because its quantities in the semen samples correlated with concentration of seminal white blood cells ($R=0.55, p=0.002$). Another coryniform species, *C. seminale* emerged to show the inverse properties showing a positive correlation with intracellular TAS ($R=0.44, p=0.02$). These two microorganisms were inversely associated as well ($R=-0.38, p=0.04$).

Elevated level of leukocytes in semen has generally been considered an indicator of infection although routine cultures are rarely successful. Our previous studies have revealed a wide profile of microorganisms in the semen of chronic prostatitis patients where extended quantitative microbiological methods were used (Punab *et al.*, 2003; Kermes *et al.*, 2003; Korrovits *et al.*, 2006). Therefore, we applied a similar analysis in this study. As expected, the polymicrobial communities were found in all prostatitis patients containing both aerobic and anaerobic bacteria.

Previous investigators have performed an *in vitro* test where some microorganisms were assessed for their capability to produce ROS and damage the lipid membranes of spermatozoa when coincubated with WBC. As a result, *Bacteroides ureolyticus, Staphylococcus haemolyticus* and *Escherichia coli* were found to cause more damage to sperm membrane lipids than *Streptococcus oralis* and *Ureaplasma urealyticum*; the role of ROS was confirmed by measuring malondialdehyde levels (Fraczek *et al.*, 2007). In spite of relatively modest proclivity for damaging sperm membrane lipids, *U. urealyticum* has been nevertheless associated with increased ROS in the semen of prostatitis patients (Potts *et al.*, 2000). In a study by Shahed and Shoskes (2000), a connection between Gram-positive organisms in semen and isoprostane levels was found. However, their pioneering publication provided no details on the numbers and composition of species, though.
Table 10. Markers of oxidative stress and related metals in semen, urine and blood of inflammatory prostatitis patients and controls

<table>
<thead>
<tr>
<th></th>
<th>Prostatitis patients (n=21) Mean ± SE</th>
<th>Controls (n=9) Mean ± SE</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In spermatozoa:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC (µM)</td>
<td>9.80±0.98</td>
<td>5.02±0.47</td>
<td>0.001</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>0.08±0.01</td>
<td>0.19±0.02</td>
<td>0.001</td>
</tr>
<tr>
<td>Zn (mg/L)</td>
<td>0.10±0.03</td>
<td>0.14±0.05</td>
<td>ns</td>
</tr>
<tr>
<td>Fe (mg/L)</td>
<td>0.55±0.14</td>
<td>0.57±0.15</td>
<td>ns</td>
</tr>
<tr>
<td>Ni (mg/L)</td>
<td>0.17±0.04</td>
<td>0.12±0.03</td>
<td>ns</td>
</tr>
<tr>
<td>Zn/Fe ratio</td>
<td>0.38±0.07</td>
<td>0.40±0.12</td>
<td>ns</td>
</tr>
<tr>
<td><strong>In seminal plasma:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC (µM)</td>
<td>6.16±0.81</td>
<td>2.96±0.63</td>
<td>0.015</td>
</tr>
<tr>
<td>TAA (%)</td>
<td>35.00±1.32</td>
<td>41.11±1.23</td>
<td>0.009</td>
</tr>
<tr>
<td>Vitamin C (mg/L)</td>
<td>30.57±2.60</td>
<td>40.79±6.78</td>
<td>ns</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.56±0.04</td>
<td>1.69±0.11</td>
<td>ns</td>
</tr>
<tr>
<td>GSH (µM)</td>
<td>0.92±0.11</td>
<td>0.85±0.28</td>
<td>ns</td>
</tr>
<tr>
<td>Zn (mg/L)</td>
<td>72.7±53.6</td>
<td>101.4±28.6</td>
<td>ns</td>
</tr>
<tr>
<td>Fe (mg/L)</td>
<td>0.10±0.01</td>
<td>0.27±0.17</td>
<td>ns</td>
</tr>
<tr>
<td>Ni (mg/L)</td>
<td>0.02±0.00</td>
<td>0.02±0.00</td>
<td>ns</td>
</tr>
<tr>
<td>Zn/Fe ratio</td>
<td>739.41±254.95</td>
<td>925.75±236.42</td>
<td>ns</td>
</tr>
<tr>
<td><strong>In urine:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-isoprostanes (ng/mL)</td>
<td>5.79±1.56</td>
<td>2.53±0.92</td>
<td>0.0001</td>
</tr>
<tr>
<td>8-OHdG (ng/mL)</td>
<td>11.66±4.62</td>
<td>8.04±2.47</td>
<td>ns</td>
</tr>
<tr>
<td><strong>In blood:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DC (µM)</td>
<td>45.90±2.28</td>
<td>43.17±2.56</td>
<td>ns</td>
</tr>
<tr>
<td>TAS (mmol/L)</td>
<td>1.13±0.03</td>
<td>1.09±0.04</td>
<td>ns</td>
</tr>
<tr>
<td>Vitamin C (mg/L)</td>
<td>6.85±0.60</td>
<td>6.26±0.90</td>
<td>ns</td>
</tr>
<tr>
<td>GSH (µM)</td>
<td>983.43±57.22</td>
<td>1157.44±72.65</td>
<td>0.037</td>
</tr>
<tr>
<td>GSSG (µM)</td>
<td>38.29±7.01</td>
<td>29.44±11.09</td>
<td>ns</td>
</tr>
<tr>
<td>Glutathione redox ratio</td>
<td>0.04±0.01</td>
<td>0.03±0.01</td>
<td>ns</td>
</tr>
<tr>
<td>Fe (µmol/L)</td>
<td>21.12±1.93</td>
<td>19.78±0.98</td>
<td>ns</td>
</tr>
<tr>
<td>Ferritin (µg/L)</td>
<td>130.77±14.66</td>
<td>152.09±37.05</td>
<td>ns</td>
</tr>
<tr>
<td>Transferrin receptors (mg/L)</td>
<td>1.59±0.07</td>
<td>1.62±0.11</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns – not significant
Fig. 6. The positive association between the levels of (A) 8-isoprostanes and 8-OHdG in urine; (B) Fe in spermatozoa and 8-OHdG in urine; and (C) Ni in spermatozoa and 8-OHdG in urine.
GENERAL DISCUSSION

In the 21st century, scientific community has paid somewhat more attention to prostatitis than in the past century. From the theoretical viewpoint, there have been no paradigm shifts but a gradual accumulation of knowledge about epidemiology, etiology and pathogenesis. In addition to the main etiological theories (infectious, autoimmune and neuromuscular), other theories (urothelial, distal urethral web) have been developed as well (Parsons, 2007; Vega, 2002).

Since the etiopathogenesis of prostatitis syndrome is still largely unknown and thereby the evidence based treatment suggestions are very scarce, additional studies are urgently needed in this field. Therefore, we have taken under investigation some microorganisms that may remain beyond attention or cannot grow during routine cultures. We also tried to clarify the role of oxidative stress (OxS) as one possible pathogenesis mechanism of prostatitis.

13. Role of coryneform bacteria in prostatitis

Coryneform bacteria are Gram-positive rods. While leaving obvious exceptions like toxigenic \textit{C. diphtheriae} aside, the mucosal Gram-positive microorganisms are generally commensals. However, Shahed \textit{et al.} (2000) suggested that these common Gram-positive organisms could actually be associated with prostatitis in some men, and they even classified these organisms as indicators of bacterial prostatitis (NIH II). Our current as well as previous data (Kermes \textit{et al.}, 2003) support the evidence that Gram-positive organisms may sometimes be associated with prostatitis but it depends on their species and counts.

On one side, according to our data, coryneform bacteria in general are more or less equally prevalent among patients and controls. On the other side, their species composition and total counts can be individually variable and significantly health-associated. The latter was shown by differences between prostatitis patients and controls both at qualitative (list of species) and quantitative (predominant species) level. Hence, our data supports the idea that coryneform bacteria are not uniform but a diverse group of organisms containing both commensals and pathogens, and some species are probably more associated with prostatitis than the others are. It is possible that these coryneforms are not as much individual pathogens but indicators of derailed relationships between urinary tract microbiota and mucosal immunity. One of paradigms that explain that kind of situation is that of dysbacteriosis, which is defined as disturbance of balance between immune system and normal microbiota that leads to an abnormal immune response combined with respective aberrations of normal microbiota. Such imbalance between mucosal immune control and bacterial growth could be caused by three likely factors. A combination of urine reflux into the prostate combined with impaired urothelium would facilitate microbial invasion into the prostate together with irritation of periurethral tissues. A neuromuscular spasm could cause an accumulation of ROS and reduction of
antioxidant levels to the point where immune barriers deteriorate and allow bacterial entrance. If the prostatic microbiota and mucosal immunity are altered due to either urine reflux or ischemia due to neuromuscular spasm, then the causative therapies would be probiotics, behavioral training aimed at relaxing the tone of pelvic floor muscles and urothelium-fortifying compounds like pentosan polysulphate. In addition, it might be necessary to count the complex interactions between prostate region and central nervous system. This is necessitated by interactions between sensory input and motor output, and even cross-organ sensitization. The latter could explain both pleomorphic nature of pain and comorbidities like neurological diseases and motor alterations of organs that are innervated by the same region of the spinal cord. As concerns the question whether a particular microbiota is normal or abnormal, perhaps it answered by our observation that there were four species that grew in significant quantities in healthy men while other such species were found only from men with inflammation.

There is electron microscopy and PCR-based evidence, which suggests that prostatitis can be caused by calcificate-associated *E. coli* biofilm infection (Mazzoli, 2010). Similar information about coryneforms is currently not available. Prostatic calcifications would be ideal foothold for the bacteria that manage to enter prostate. Calcificate-related biofilm infection can explain why the inflammation has the nature of having low intensity, being focal, recurrent and antibiotic tolerant. If calcificate-associated biofilms are a frequent cause of prostatitis, then the main problems are that of calcifications and antibiotic tolerance. With a bit of luck, prostate massage, alpha-blockers and perhaps tetracyclin can reduce the quantity of microcalcificates (“nanobacteria”), and contribute towards a critical improvement of the situation.

If bacteria persist in the prostate, then it would be interesting to know why. Blaser and Kirchner (2008) have analyzed the dynamics of bacterial persistence by the use of game theory (pioneered by Neumann et al., 1944). Their formula explains the mechanisms behind bacterial persistence in hosts:

\[ R_0 = \frac{BN}{(\alpha + b + \nu)}, \]

where \( R_0 \) is transmission potential of the microorganism, BN is the transmission rate depending on population size N, \( \alpha \) is the mortality rate of hosts due to microorganism’s virulence, \( b \) is mortality of hosts not due to microorganisms (lifespan), \( \nu \) is the rate by which host’s immune system eradicates the bacteria from the host. If all variables were independent, then a negative \( \alpha \) (symbiosis) would be favorable but if virulence grants better transmissibility, then some virulence would be favorable, instead, especially if there are plenty of interacting hosts that create many opportunities for transmission. Neither urogenital mycoplasmas nor coryneforms are highly virulent but the virulence might increase over time if there is an increase in virulence-dependent transmission opportunities and if unhealthy lifestyle contributes to prostate’s immune systems inability to keep these unwanted guests at bay. While risk factors of
prostatitis have been discussed in detail in previous chapters, it may be of interest that the transmission opportunities (carriage) of nasal coryneforms were reduced in persons who had high titers of antibodies against diphtheria toxoid (Bergamini et al., 2002).

The spectrum and pathogenicity of coryneforms could be conclusively demonstrated by a high-quality metagenomic biopsy study. Confocal-microscopic study of prostatic microcalcifications obtained from EPS stained with a Corynebacterium-specific probe might provide a good proof-of-principle without a need for the unwelcome prostate biopsy. The problems with biopsies include difficulties to avoid contamination (at least in a living patient), and difficulties in recruiting a control group.

14. Role of mycoplasmas in prostatitis

Mycoplasmas are the smallest free-living bacteria that cannot be cultured by routine methods. Therefore, our current knowledge about them is scarce. These organisms have been associated with male genital tract infections during last decades. Moreover, their classification has been changed over time and therefore comparison of published data is complicated. To date, Mycoplasma genitalium is considered a more likely causative agent of urethritis than ureaplasmas. Since prostatitis may be a consequence of an untreated sexually transmitted disease, the role of mycoplasmas needs elucidation in the context of prostatitis as well.

By our data, mycoplasmas in general are more common among the patients than controls. Our study also confirmed that the lack of discrimination between Ureaplasma urealyticum and Ureaplasma parvum has been an important shortcoming in many studies. Before the tests that are more specific became available, Ureaplasma urealyticum was considered as prostatitis pathogen by Ohkawa et al. (1993) and Skerk et al. (2002) although not by Berger et al. (1989). According to our study, it was actually Ureaplasma parvum that associated with prostatitis, not Ureaplasma urealyticum. Hence, this former lack of discrimination between two ureaplasmas probably presented U. urealyticum as a probable cause of prostatitis. Although Mycoplasma genitalium was found from only a few of our study subjects, all of these men had category IIIa prostatitis that supports the possible association of this species with prostatitis.

In the light of current evidence, the associations between prostatitis and mycoplasmas could be explained by the concept of imbalanced microbial communities like in case of coryneform bacteria. Alternatively, the route of sexual transmission might at least partially explain the recurrence of a mycoplasma infection. Without regard to prostatitis symptoms, the adverse effect of some mycoplasmas on the spermatozoa justify using antibacterial agents directed against these microorganisms, anyway. One reason for this is that U. urealyticum is detrimental for motility of the spermatozoa (Zeighami et al., 2009). As is the case with coryneforms, the pathogenicity of different mycoplasma species
could be conclusively demonstrated by a high-quality metagenomic prostate biopsy study.

15. Role of oxidative stress in prostatitis

15.1. Interaction between oxidative stress and fertility

Oxidative stress (OxS) means usually the free radical oxidants that are harming biological systems (a distress) but in some contexts, oxidative stress may refer to a free-radical-facilitated process that is beneficial (an eustress). While infectious etiology has been suspected for several decades, OxS has not been that relevant so far. Oxidative stress may be either beneficial (eustress) or harmful (distress). Breathing is an oxidative process, and even spermatozoa require some ROS in order to become functional (Aitken et al., 2007). Excessive OxS is detrimental, though. OxS might be related to fertility, as suggested by previous studies, especially by mechanism of prostate infection and leukocytospermia (Shahed et Shoskes, 2001; Saleh et al., 2002; Pasqualotto et al. 2000). These and other earlier experiments that showed importance of oxidative mechanisms in prostatitis and fertility gave us incentive to include an analysis of OxS in our prostatitis study.

We have performed a many-sided complex study by measuring markers of both local and systemic OxS (antioxidants, pro-oxidants and oxidation products). Our most important conclusion is that the patients have OxS not only on the level of semen and spermatozoa but on the systemic level as well, which could be a risk factor for many other diseases. Systemic OxS was demonstrated by increased 8-isoprostane (8-EPI) concentrations in urine that was in good correlation with a marker of DNA damage. As concerns semen, seminal plasma is usually well endowed with an array of antioxidant defenses to protect spermatozoa against oxidants. Antioxidants in the seminal plasma usually compensate for the deficiency of cellular enzymes in the spermatozoa. The high-grade OxS will develop during genitourinary infection or inflammation because of reduced antioxidant levels and/or increased production of free radicals.

According to our data, the bodies of prostatitis patients have both more oxidation products and less antioxidants. We found less antioxidants even in the spermatozoa, where the absolute quantities of antioxidants are very low both in physiological as well as pathogenic conditions. Oxidative damage in spermatozoa correlated with major pro-oxidants iron and nickel measured in the same site. The antioxidant system present in seminal plasma in conditions of natural reproduction must exert its action over a relatively short period, ranging from ejaculation to sperm transfer into the female tract, whereas the antioxidant system present in membranes of the spermatozoa must maintain their activity over a prolonged period while spermatozoa must spend their time in the female reproductive tract.
15.2. Interaction between oxidative stress and bacteria

From the viewpoint of host defense, local OxS may be useful, because it has been observed that hydrogen peroxide (product of SOD), even in low concentrations (0.1 mM) decreases uropathogenic E. coli’s adhesion, serum resistance and resistance to phagocytosis as well (Hegde et al., 2008). The old theory that ROS, especially superoxide or hydrochloric acid are the compounds that are directly responsible for killing the microorganisms has been refuted, as it has been shown that proteolytic enzymes are the most important antibacterial compounds while pumping superoxide into phagosome is actually necessary for generating an alkaline environment that enables the action of these proteolytic enzymes (Segal, 2005). Antioxidant defenses of host or exogenous antioxidants can influence bacterial adherence. The proofs of that principle are the observations by Arita et al. (2004) and Lianou et al., (2003), which demonstrate how Mn-SOD inhibits the adherence of P. aeruginosa to airway epithelial cells, and that ascorbic acid reduces the ability of four out of five bacterial species to adhere to buccal cells.

From the viewpoint of pathogen virulence, Fraczek et al. (2007) have associated the harmful effect of bacteria upon spermatozoa with OxS, and demonstrated that this effect is species-specific: Escherichia coli, Staphylococcus haemolyticus and Bacteroides ureolyticus caused more lipid peroxidation in spermatozoa than Streptococcus oralis and Ureaplasma urealyticum did, as measured by malondialdehyde levels. Shahed and Shoskes (2000, 2001) have shown that OxS in semen is linked to Gram-positive bacteria.

Our own observations were rather quirky in nature. Corynebacterium group G associated with inflammation while C. seminale associated with better antioxidative status of spermatozoa and displayed a tendency towards mutual exclusion with Corynebacterium group G. The current evidence does not allow determining causal relationships between microorganisms and OxS. It would be necessary to determine microorganisms’ ROS production, ROS tolerance and capability to inhibit each other’s growth. Preliminary data suggests that C. seminale and C. group G do not inhibit each other (unpublished data). It is certain that microbiota are relevant for fertility, especially in association with adverse effects. The field of possible mutualistic relations is much less investigated, although there is at least one extant proof-of-principle of fertility-related mutualism – between a nematode and intracellular bacteria (Hoerauf et al., 1999).

15.3. Interaction between oxidative stress and cardiovascular system

The simplest explanation of systemic OxS and elevation of 8-EPI is that OxS is a result of inflammation. Elevation of systemic 8-EPI in prostatitis patients reminds an analogous elevation of 8-EPI in vascular disease patients. Prostatitis is associated with increased risk of cardiovascular diseases, indeed
(Pontari et al. 2005). This analogy led to think about a possibility that prostatitis and cardiovascular diseases might share systemic OxS as a pathogenetic component. Because the proportion of prostatitis patients with cardiovascular diseases is only 11%, this association does not lend much explanatory power to the cardiovascular-related explanations of prostatitis. In addition, Franiel et al. (2009) reported that prostatic blood flow is generally increased rather than decreased in case of prostatitis. Another explanation to the apparent lack of cardiovascular diseases among the majority of prostatitis patients is that the cohort of prostatitis patients is relatively young while cardiovascular diseases appear later.

15.4. Interaction between 8-EPI and lower urinary tract symptoms

According to Shoskes et al. (2007), as if two types of prostatitis patients existed: one group had infection, inflammation and prostatic stones but no pelvic spasms, while the others had pelvic muscle sensitization. This makes it plausible to believe that the primary cause of prostatitis is either infection or sensitization. The possibilities of propagation are numerous: autoantibody-driven inflammation, lifestyle choices, ROS-mediated positive feedback in urinary tract or nervous system, mild biofilm infection associated with prostatic calculi, etc. Currently, there are many competing theories that ought not be prematurely welcomed as ‘final’, or discarded altogether. Some of these theories can be viewed as pathogenetic elements that can be linked by positive feedback mediated by lipid peroxidation products. In other words, the current scientific data enables to identify at least two possible vicious circle mechanisms for prostatitis.

Another explanation ties together urinary reflux theory, urothelium dysfunction theory, calcifications, infection and OxS. If bacteria in urine encounter prostatic calcifications, then the consequence is a biofilm infection and immune activation. If these bacteria irritate dysfunctional urothelium, then the result is irritation and immune activation. Local immune activation results in local OxS that becomes systemic as it is being ‘diluted’ by circulation. Systemic OxS results in urinary excretion of 8-EPI and PGF$_{2\alpha}$. The ordinary viewpoint and application of 8-EPI considers it as a marker and as a product of lipid peroxidation. From the viewpoint of this theory, 8-EPI is regarded as a causative component, instead. It has been shown 8-epi-PGF$_{2\alpha}$-epi can contract urethra or bladder of rabbits (Tarcan et al. 2000). For humans, there is data about only about the ability of PGF$_{2\alpha}$ to contract urethra or bladder (Andersson and Persson, 1977). Curiously, it has been shown that most of the PGF$_{2\alpha}$ forms by free-radical pathway like 8-EPI, and, analogously, its generation is not inhibited by NSAIDs (Yin et al., 2007). They managed to determine the ratio of enzymatically derived and free-radical derived PGF$_{2\alpha}$ because enzymatically produced PGF$_{2\alpha}$ is optically pure while free-radical derived is PGF$_{2\alpha}$ racemic. It
is likely that 8-EPI and PGF$_{2\alpha}$ may have overlapping receptor specificity (Morrow, 2006).

Since smoking raises 8-epi-PGF$_{2\alpha}$ levels, it may explain how smoking contributes prostatitis (Patrino et Fitzgerald 1997). Urethral contractions might cause pain, especially if the contractions occur in the context of impaired urothelium or biofilm infection. This suggests a possible vicious circle (Fig. 7).

15.5. Interaction between oxidative stress and pain

Another vicious circle involves a prostatic injury that induces OxS in central nervous system. OxS-induced changes sensitize central nervous system to further stimuli from prostate or other sites that is innervated by that part of central nervous system (Schwartz et al., 2008; Lee et al., 2007; Schwartz et al., 2008). Peripheral injury causes generation of ROS in the mitochondria of neurons in the dorsal horn of the spinal cord (Schwartz et al., 2008). Prostatitis-associated Mn-SOD polymorphisms are relevant to this, because Mn-SOD is a mitochondrial enzyme. SOD dismutates superoxide into hydrogen peroxide. Although hydrogen peroxide can stimulate pain receptor TRPV1 (Keeble et al., 2009), SOD activity actually protects from the pain (Schwartz et al., 2009). That apparent contradiction may be explained by ability of superoxide to react with nitric oxide to form peroxynitrite that can nitrate opiate receptors and

Fig. 7. Possible vicious circle in case of chronic prostatitis
interfere with glutamatergic pain transmission (Salvemini et Neumann, 2009; Ryu et al., 2010).

Another explanation for the relevance of Mn-SOD is that it must dismutate superoxide into hydrogen peroxide in order to prevent the reaction between superoxide and water (\(O_2^- + H_2O = HO_2^- + OH^-\)). Unlike superoxide or hydrogen peroxide, the hydroperoxyl radical (HO\(_2\)-) is a potent initiator of lipid peroxidation (Thomas et al., 1985). Isoprostanes are lipid peroxidation products that are similar to prostaglandins, both chemically and biologically (Morrow, 2006). The possibility that free-radicals and lipid peroxidation products participate in the pathogenesis is compatible with the association between prostatitis and Mn-SOD polymorphisms as well as clinical improvement achieved with antioxidants rather than NSAIDs (Arisan et al., 2006; Shoskes et al., 1999; Nickel et al., 2003). Our observation that chronic prostatitis patients have reduced levels of glutathione in their blood is explainable by reactions that occur between glutathione and lipid peroxidation products that are chemically more reactive than the stable marker 8-EPI (reviewed by Blair, 2006). From the viewpoint of causality, it is important whether OxS precedes pain or is it the other way around. Evidence suggests that the interaction is reciprocal – long-lasting painful stimulus can cause neural OxS and neural OxS can cause long-term sensitization to pain (Schwartz et al., 2009).

In any case, it must be repeated again that the mere activity of ROS, in physiological concentrations, controlled and properly compartmentalized, is a crucial part of our healthy physiology, which probably functions better without unnecessary interference (Gomez-Cabrera et al., 2005; Bjelakovic et al., 2008).

### 16. Some considerations of prostatitis treatment

Considering that CP/CPPS is a chronic pain syndrome that is difficult to treat, it is no wonder that both patients and doctors may be quite frustrated with prostatitis (Nickel, 2000). In fact, initial treatment of prostatitis is quite simple and straightforward – prescription of fluoroquinolones (Nickel 2002; Murphy et al., 2009). As explained by Wood et al., (2007) the doctors have social responsibility and must deal with immediate needs of the patients. Such urgent needs, or perception thereof, may get priority over long-term needs of both patient and doctor. Considering that doctors may have to comply with patients’ expectations quickly as well as justify their treatment decisions later, it seems possible that doctors make decision pertaining treatment before they see the results of microbiological analysis. According to Liu et al. (2008) as well as Ku et al. (2005), culture test results actually did not influence treatment decisions – it seemed that ordering culture test was just a tradition or a means to rationalize previously made treatment decisions. Majority of physicians are willing to prescribe a second course of fluoroquinolones even after they have failed the initial one (Ku et al., 2005). It has been reported that there is a sevenfold overuse of fluoroquinolones (Taylor et al. 2008). The arguments in favor of
common fluoroquinolones include low price, safety (Meropol et al., 2008) good penetrability into prostate (Nickel, 2002), successful treatment experiments (Jeong et al., 2003), and a belief into infectious etiology (without regard to microbiological evidence). The arguments against using fluoroquinolones consist of controlled studies (Nickel et al., 2003; Alexander et al. 2004). This discrepancy in the treatment of prostatitis is of utmost importance concerning health, economy, and research. Therefore, more evidence-based suggestions for treatment are needed.

If it is assumed that the symptoms of prostatitis are a consequence of infection, then one must identify (1) causative agents or at least likely causes of infection; (2) means that can eliminate the causes or at least ameliorate the consequences (universally or case-by-case). There is a reason to believe that in addition to acknowledged urinary tract pathogens (like E. coli and enterococci), prostatitis may be associated with some other bacteria that can be eliminated with antibiotics. Certain coryneform bacteria may be one of the candidates. Their possible association with prostatitis has been proposed in some previous studies (Tanner et al., 1999; Domingue 1998) as well as our present study. Since half of the prostatitis-associated Corynebacterium group G were not susceptible to norfloxacin, it raises questions about the suitability of fluoroquinolone treatment. Traditional and cheap antibiotics like penicillin and TMP/SMX have quite good activity against the urogenital coryneform strains in vitro. Penicillin susceptibility is the common denominator of Corynebacterium group G, as well as β-hemolytic streptococci and peptostreptococci, the latter being associated with prostatitis by our earlier research (Kermes et al., 2003). Therefore, β-lactams that penetrate into the prostate have potential. For examples, ampicillin penetrates into the prostate with a serum-tissue ratio of 2:1 (Jeppesen and Frimodt-Møller, 1984), and piperacillin-tazobactam is superior to ciprofloxacin in preventing infections after transrectal prostate biopsy (Cormio et al., 2002).

Another group of microorganisms that shows association with prostatitis, according to our study, is that of mycoplasmas. Only a short list of antibiotics can be used for the eradication of these facultatively intracellular bacteria: macrolides, tetracyclines and fluoroquinolones. Use of these antibiotics is certainly justified if either M. genitalium or U. parvum is found from a prostatitis patient. Tetracycline is preferable against U. parvum while azithromycin is preferable against M. genitalium (Biernat-Sudolska et al., 2009; Mena et al., 2009).

At the same time, there are several other factors to consider, in addition to antibiogram of supposed causative agent(s). Damage of beneficial microorganisms should be avoided to prevent overgrowth of adverse communities. From that aspect, antibiotics of narrow spectrum are preferred. In addition, in vivo treatment results may differ from in vitro susceptibility results of suspected bacteria since the bacteria might persist in calculi, biofilms or urinary epithelial cells. Therefore, targeting the patient or removing the habitat of bacteria – surgical correction of anatomical deviations and riddance of prostatic stones – may
be a more reasonable strategy in (Shoskes et al. 2007; Domingue et Hellstrom 1998; Vega 2000). Interactions between host and microbiota might be improved by immunomodulation and dietary or supplementary antioxidants. The latter suggestion is in accordance with our study results, which indicated that both local as well as generalized oxidative stress is a major participant in the pathogenesis of inflammatory prostatitis.

If restoration of prostatitis-associated fertility is aimed by means of antibacterial treatment, then the association between *C. seminale* and improved antioxidant defenses of spermatozoa combined with inherent tetracycline resistance of that species might influence antibiotic choice in treating men who are colonized with *C. seminale*. Another beneficial facet of tetracyclines is that these target *Ureaplasma urealyticum*, and *Chlamydia trachomatis* that deteriorate the quality of semen (Zeighami et al., 2009; Mazzoli et al., 2009).

Older tetracyclines, penicillins and fluoroquinolones are all quite inexpensive and safe. Long-term consumption of doxycycline, ciprofloxacin and amoxicillin causes very few hospitalization-requiring severe adverse effects (respectively up to 0.9 and 1.2 and 5.2 cases per 100 000 days of treating a patient) according to Meropol et al. (2008). There is an avenue for limiting healthcare costs by not extending the subsidies for the consumption of newer fluoroquinolones until their worth in prostatitis treatment is backed up by evidence.

Since the etiopathogenesis of prostatitis is still largely unknown and since antibiotic are not always successful, a long list of other treatment modes is in use as well. As concerns evidence of controlled studies, α-blockers have been superior to placebo in five of six studies (Cheah et al., 2003; Mehik et al., 2003; Sivkov et al., 2005; Nickel et al., 2004; Alexander et al, 2004; Evliyağlı et Burgut, 2002). Two of two controlled studies (Elist et al., 2006, Wagenlehner et al., 2009) agree with pollen extracts being useful. There is currently no evidence whether and how these treatments affect interactions between the host and urogenital microbiota, but it would be both theoretically and practically interesting to see whether modifying the host responses could correct the host-bacteria relationships in case of inflammatory prostatitis, too.
CONCLUSIONS

Our study updates the current knowledge of the role of seminal microbiota and oxidative stress in the etiopathogenesis of chronic inflammatory (categories NIH III and IV) prostatitis.

1) Polymicrobial communities are present in the semen of all prostatitis patients containing both aerobic and anaerobic bacteria. Coryneform bacteria form a significant proportion of this microbiota. Localization study suggests that their source in prostatitis patients is not the normal-microflora-containing urethra. In addition, significantly longer list of coryneforms can be found in high concentration in inflammatory prostatitis patients than in controls. *Corynebacterium* group G is associated with inflammatory prostatitis in case of severe leukocytospermia, which suggests that this species might participate in the etiopathogenesis of prostatitis as an initiator of inflammation.

2) Penicillins and TMP/SMX express the highest *in vitro* antimicrobial activity against seminal coryneform bacteria that are frequently non-susceptible to several other antimicrobials. MLSb resistance pattern is common among seminal coryneforms. *Corynebacterium* group G strains frequently resist norfloxacin, nitrofurantoin, clindamycin, and erythromycin. Susceptibility data are useful for empiric therapy of prostatitis patients and clinical drug research.

3) Our findings suggest that some mycoplasma species, *U. parvum* and *M. genitalium* participate in the etiology of chronic prostatitis. Distinction between *Ureaplasma urealyticum* and *M. parvum* is certainly necessary since only the latter has a tendency of being more prevalent among prostatitis patients than controls. *Mycoplasma genitalium* may be linked with NIH IIIa prostatitis.

4) Inflammatory prostatitis patients have both local and systemic oxidative stress, as evident in its various forms (reduced antioxidant levels and increased levels of pro-oxidants and oxidation products). Local oxidative stress comprises increased oxidative stress in both seminal plasma and spermatozoa. As the sperm cells are highly susceptible to oxidative injury, this mechanism may be associated with prostatitis-related decrease of fertility. In addition to local shifts, decreased GSH in erythrocytes and increased 8-EPI in urine (in association with DNA oxidation) are signs of systemic oxidative stress. As 8-EPI can cause smooth muscle contraction in the urinary bladder, this oxidative stress byproduct may therefore facilitate the urinary tract dysfunction in prostatitis patients and it may contribute to the propagation of a vicious circle that upkeeps the prostatitis symptom complex. Moreover, systemic oxidative stress means that inflammatory prostatitis patients may have increased risk for other diseases.

5) The association between *Corynebacterium seminale* and better antioxidant status of spermatozoa as well that of between *Corynebacterium* group G and severe inflammation suggests that these bacteria deserve further attention because of their therapeutic or diagnostic potential, respectively.
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