DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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SILVER TÜRK

Etiopathogenetic aspects of chronic prostatitis: role of mycoplasmas, coryneform bacteria and oxidative stress

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Department of Microbiology, University of Tartu, Estonia

Department of Biochemistry, University of Tartu, Estonia

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Supervisor: Associate professor Reet Mändar, MD, PhD
Department of Microbiology, University of Tartu, Estonia

Co-supervisor: Senior researcher Tiiu Kullisaar, PhD
Department of Biochemistry, University of Tartu, Estonia

Reviewed by: Senior researcher Paul Naaber, MD, PhD
Department of Microbiology, University of Tartu, Estonia

Researcher Jaak Kals, MD, PhD
Department of Biochemistry, University of Tartu, Estonia

Opponent: Professor Sandra Mazzoli, PhD
Professor of Applied Microbiology and Clinical Microbiology, University of Florence, Italy
Chief of Sexually Transmitted Disease Center, S.M. Annunziata Hospital, Health Florence Agency, Florence, Italy

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Paper I: study design, performing all microbiological analyses of the semen and urine, data analysis, writing the paper.

Paper II: study design, performing all susceptibility testing of bacteria, data analysis, writing the paper.

Paper III: participation in data analysis and writing the paper.

Paper IV: study design, performing separation of cellular fractions of semen, participation in performing biochemical analyses, performing all microbiological analyses, data analysis, writing the paper.

ABBREVIATIONS

8-EPI	8-Isoprostanes
8-OHdG	8-Hydroxy -2´- Deoxyguanosine
ABTS+	2,2'-AzinoBis-Ethylbenzothiazoline 6-Sulfonate
ANF	Absolute Nonfermenter
API	Analytical Profile Index
AsA	Ascorbic Acid
BC	Before Christ
BEA	Bile Esculin Agar
BPH	Benign Prostate Hyperplasia
CCL	Chemokine (C-C motif) Ligand
CC	Current Contents
CDC	Centers fod Disease Control and Prevention
CFU	Colony Forming Unit
CGRP	Calcitonin Gene-Related Peptide
CNS	Coagulase Negative Staphylococci
COX	Cyclooxygenase
CP/CPPS	Chronic Prostatitis / Chronic Pelvic Pain Syndrome
CPPS	Chronic Pelvic Pain Syndrome
CRP	C-Reactive Protein
CXCL	Chemokine (C-X-C motif) ligand
DC	Diene Conjugates
DNA	Deoxyribonucleic Acid
DRE	Digital Rectal Examination
EDTA	Ethylenediaminetetraacetic Acid
EN	European Standard
EPS	Expressed Prostatic Secretion
GPSS	Giessen Prostatitis Symptom Score
GPX	Glu
GSH	Glutathione
GSSG	Oxidized Glutathione
HRP	Horseradish Peroxidase
hsCRV	high-sensitivity C-Reactive Protein
IC	Interstitial Cystitis
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectrometer
IFN-γ	Interferon-gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IPSS	International Prostate Symptom Score
iNOS	Inducible Nitrix Oxide Synthethase
ISO	International Organization for Standardization
LA	Linolenic Acid
LOX	Lipooxygenase

LUTS	Lower Urinary Tract Symptoms
MA	Maryland
MIC	Minimal Inhibitory Concentration
MIP	Macrophage Inflammatory Protein
MLSb	Macrolide-Lincosamide-Streptogramin B
MPO	Myeloperoxydase
MRS	de Man-Rogosa-Sharpe
MUG	4-methyl-umbelliferyl 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
$PGF_{2\alpha}$	Prostaglandin F 2 alpha
PMN	Polymorphonuclear
PSA	Prostate-Specific Antigen
Q_{10}	Coenzyme Q_{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	Tributyric 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

Prostatites 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 surficially 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 prostatitis. 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 musculofascial 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. Canalicular 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 prostasomes (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; Vivacqua *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 epididymidis 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 following categories: pain or discomfort, urination disorders, impact of symptoms and quality of life.

Inflammation is measured by default from semen, postmassage urine (VB3) or EPS and if invasive methods are justified, then prostate biopsy is chosen. The older prostatitis classification stands upon on the Meares-Stamey 'four glass test' and 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

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

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 (Gümüs *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 (Kohnen *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-Błaszczyk *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 patiens 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 Zn^{2+} solution was injected directly into the prostates of the rats. The distribution of Zn^{2+} is influenced by stress, adrenergic signals or glucocorticoids in one way (intracellular $Zn^{2+}\uparrow$), and by cholinergic signal in another (intracellular $Zn^{2+}\downarrow$) (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* Ezeanyika 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* Ezeanyika, 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. 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

Prevalence	Study population	Reference
14,2%,	1832 men from Finland, 20–59 years old. Cross-sectional postal survey, 75% response rate.	Mehik *et al.,* 2000
13,8%	2006 men from 28 centers, 25–50 years old. Prospective case-control study of 28 urology clinics.	Bartoletti *et al.,* 2007
12,8%	8503 men from Italy, 16–83 years old. Cross-sectional study by 70 urologists.	Rizzo *et al.,* 2003
12,2%	1507 men from Nsukka, Nigeria, 20–70 years old. Random cross-sectional survey.	Ejike *et* Ezeanyika, 2008

Prevalence	Study population	Reference
9,7% (11,5% in the younger, 8,5% in the older subgroup)	868 men divided into subgroups of younger and older men, 20–50 and 51–74 years old, respectively. Cross-sectional postal survey.	Nickel *et al.,* 2001
4,5%	Computer database research in USA. Prostatitis-patients were compared with age-matched controls.	Clemens *et al.,* 2007
2,7%	1765 men, 20–79 years old	Marszalek *et al.,* 2007
2,7%	6037 men, comparative prevalence study of prostatitis, interstitial cystitis and epididymitis. The patients were seen by randomly recruited urologists. Urology outpatient study.	Nickel *et al.,* 2005

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 3. Epidemiological correlates of CP/CPPS

Risk Factor	Reference
Cigarette smoking	Bartoletti *et al,* 2007
High-calorie diet with low intake of fruits and vegetables	Bartoletti *et al.,* 2007
Sexual relationship with more than one partner	Bartoletti *et al.,* 2007
Coitus interruptus	Bartoletti *et al.,* 2007
Frequent masturbation	Gao *et al.,* 2007
Long-time urine holding	Gao *et al.,* 2007
Sitting or driving	Gao *et al.,* 2007; Chiappino *et* Pisani, 2003 ;
Constipation	Bartoletti *et al.,* 2007
Meteorism	Bartoletti *et al.,* 2007

Risk Factor	Reference
Slow digestion	Bartoletti *et al.,* 2007
Dyspepsia	Clemens *et al.,* 2007
History of UTI-s	Gao *et al.,* 2007 ; Daniels *et al.,* 2007 ; Pontari *et al.* 2005
Cold environment and stress	Gao *et al.,* 2007; Mehik *et al.,* 2000
Cardiovascular diseases	Pontari *et al.* 2005; Collins *et al.,* 2002
Neurological disease	Pontari *et al.* 2005;
Psychiatric conditions (mood, anxiety, other)	Pontari *et al.* 2005;, Clemens *et al.,* 2007; Collins *et al.,* 2002
Hematopoietic, lymphatic, infectious disease	Pontari *et al.* 2005;
Esophageal reflux	Clemens *et al.,* 2007
Being widowed	Mehik *et al.,* 2000
Similar diseases (BPH)	Clemens *et al.,* 2007; Collins *et al.,* 2002
History of STD-s	Collins *et al.,* 2002

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 α_1- or α_{1A}-adrenoblockers (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 impact 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, 'prostatocentric 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).

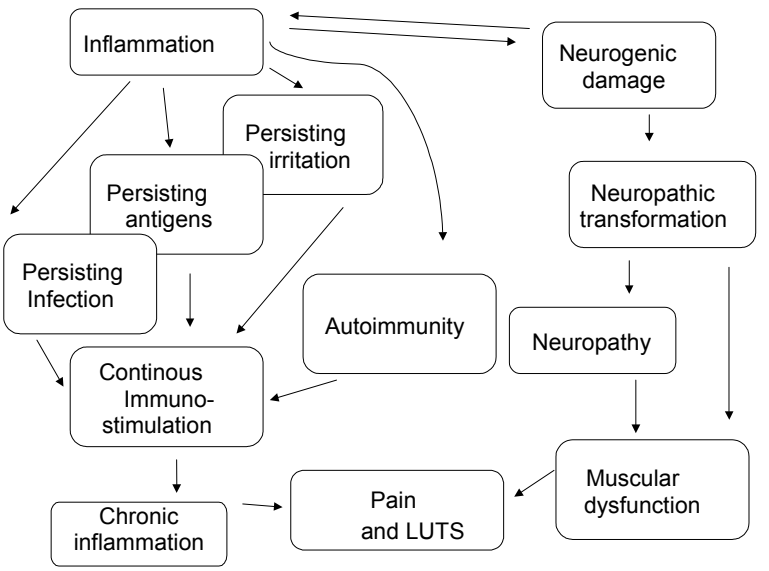

Fig. 1. Scheme of multifactorial cascade in pathogenesis of prostatitis (Modified from Nickel *et al.,* 2002).

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, peptosptreptococci, 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 *Peptoniphilus*, *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.

2.8.2.2.3. 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 *Escherischia*), 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* (Jedrzejczak *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 multiresistance (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 and sparfloxacin (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; Szöke *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 IIIB 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 dysfunctioning 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 molecule 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.

2.8.8.1. Spermatozoa

Maintaining a fine balance between reactive oxygen species (ROS) and anti-oxidants 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 anti-oxidative properties because it normally contains high amounts of anti-oxidants 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 system (de Lamirande *et* Gagnon, 1995). 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 antinociceptive 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* Shoskes (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

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

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-analgesics 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* Burgut, 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

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

* – 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 this study was to elucidate some factors and mechanism that are associated with chronic inflammatory prostatitis. We therefore attempted to clarify whether *Corynebacterium sp.* and *Mycoplasmatales* 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.

MATERIAL AND METHODS

Table 6. Study subjects, microbial strains and performed investigations

Group	No of subjects/ strains	Study description*	No of subjects/ strains in sub-groups	Presented in Papers:
Individuals				
Healthy Men	99 men	Screening and antimicrobial susceptibility testing of coryneforms	59	I, II
		Oxidative stress in prostatitis	9	IV
		Mycoplasmas in prostatitis	40	III
Men with NIH IV prostatitis (>0,2M WBC/ml)	54 men	Screening and antimicrobial susceptibility testing of coryneforms	30	I, II
		Oxidative stress in prostatitis	11	IV
		Mycoplasmas in prostatitis	24	III
Men with NIH IIIA prostatitis (>0,2M WBC/ml)	58 men	Screening and antimicrobial susceptibility testing of coryneforms	20	I, II
		Oxidative stress in prostatitis	10	IV
		Mycoplasmas in prostatitis	38	III
Men with NIH IIIB prostatitis	59 men	Mycoplasmas in prostatitis	59	III
Microbial Strains				
Coryneform strains	148 strains from 109 patients	Screening and identification of coryneforms	148 of 148	I
		Antimicrobial susceptibility testing of coryneforms	62 of 148	II

* – see also Fig. 2.

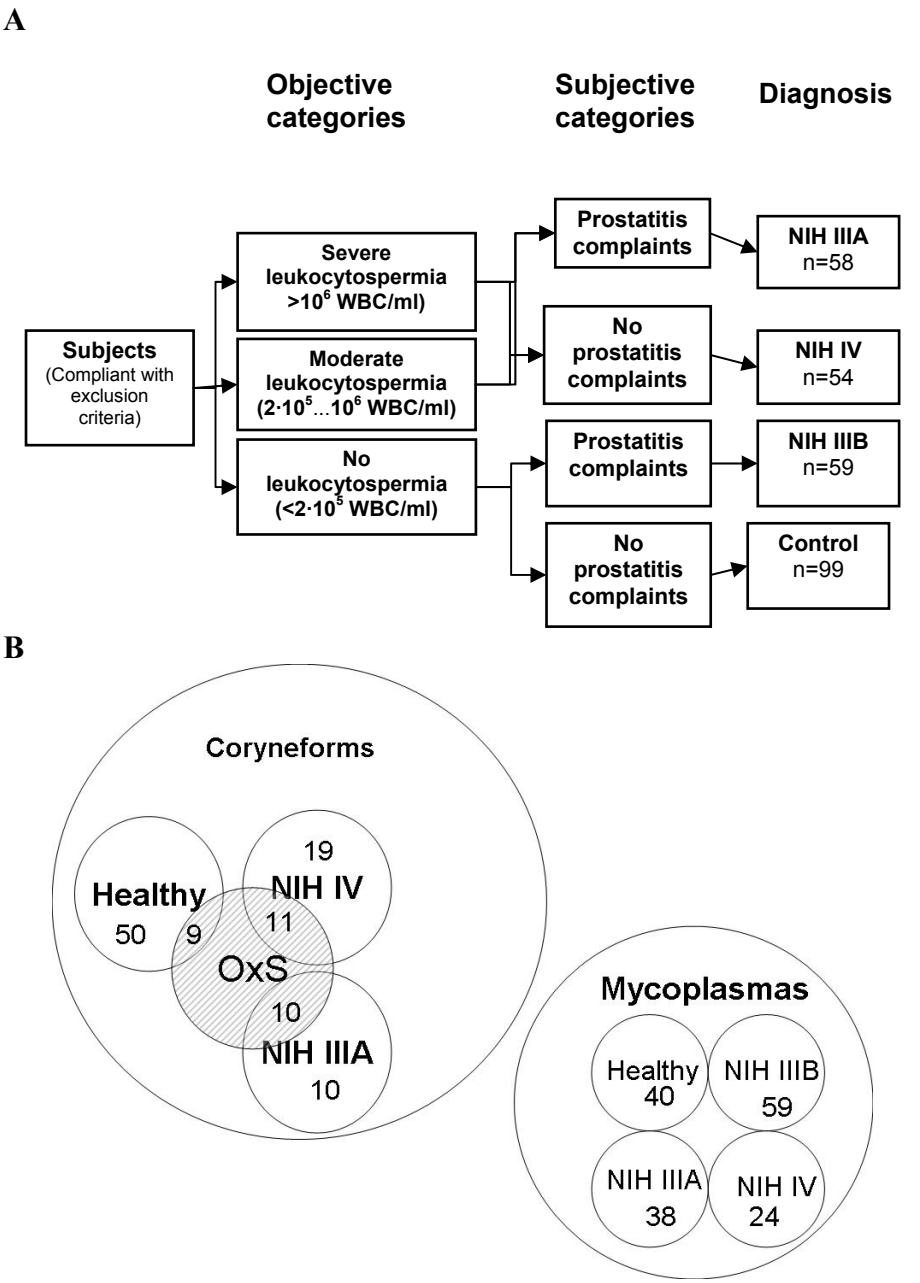

Fig. 2. A. Formation of study groups. B. Composition of study groups.

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_{WBC}) was calculated by using the known sperm concentration (as 10^6/mL) according to the following formula:

$$C_{WBC} = \frac{\text{number of WBCs counted} \cdot \text{semen sperm concentration}}{\text{number of sperm counted}}$$

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% CO_2 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 seminale* 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. seminale* 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-

lective medium, characteristic morphology and negative catalase test. The anaerobes were identified by absence of aerotolerance, growth on selective media, colony and cellular morphology, Gram stain reaction, some diagnostic disks (bile, brilliant green, colistin, vancomycin and kanamycin), rapid tests (indole and potassium hydroxide tests), fluorescence and ability to produce pigment.

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 cation 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 *M. hominis* and *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 *Mycoplasma genitalium*, *Ureaplasma parvum* and *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 *M. genitalium* genome amplification; they amplify a 281-bp segment of the 140-kDa adhesion protein gene (Jensen *et al.,* 1991). The cycling parameters were as follows: 95 °C for 2 min; 40 cycles at 95 °C for 30 s, 65 °C for 30 s and 72 °C for 25 s; and 72 °C for 5 min. Primers UMS-125 and UMA-226 were used for specific *U. parvum* genome amplification; they amplify a 403-bp segment of the multiple-banded antigen gene (Teng *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 *U. urealyticum* genome amplification; they amplify a 1300-bp segment of the 16S rRNA gene (Robertson *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 *et* Jansen, 2002; Nakamura *et al.,* 2002; Aitken *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 *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 10^6 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 $FeSO_4$ (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 – (A534 (sample) / A534 (LA as control)] x 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_2O_2. The suppression of the absorbance of ABTS+ radicals by sample depends on TAS of the sample under investigation (Rice-Evans *et* Miller, 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^{o}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* Glende, 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 κ 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.*, 1999; Riegel *et al.*, 1995; 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. seminale* 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 $\geq 10^4$ 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. seminale, 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 treshold.

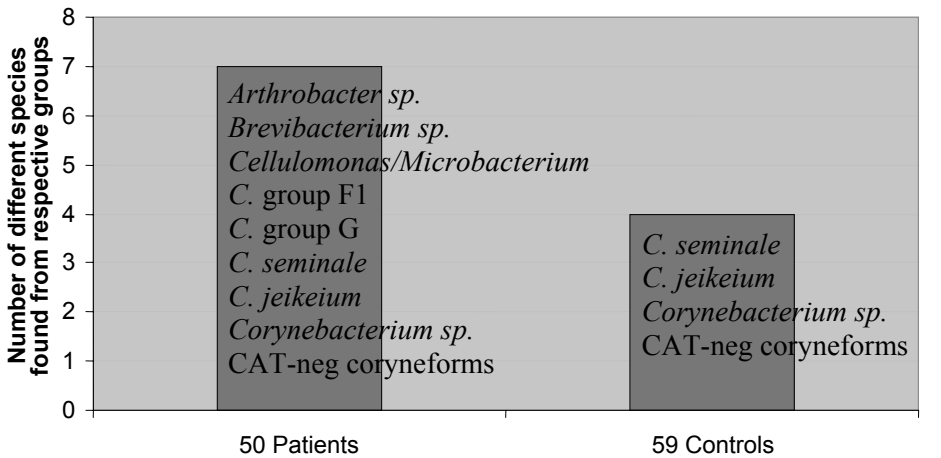

Fig. 3. Quantitative differences in seminal coryneflora of inflammatory prostatitis patients and controls.
The list of the different isolates that can be found in significant ($>10^4$ CFU/ml) quantities from prostatitis patients is longer than the analogous list for controls.

Table 7. Quantities of coryneform bacteria in the semen of inflammatory prostatitis patients (categories NIH IIIA and NIH IV) and controls.

Coryneform bacteria	Men with severe inflammation (>1 M WBC per ml of semen) n=18			Men with moderate inflammation (0.2…1 M WBC per ml of semen) n=32			Controls n=59		
	log10 of CFU/ml		Proportion of coryneform positive specimens	log10 of CFU/ml		Proportion of coryneform positive specimens	log10 of CFU/ml		Proportion of coryneform positive specimens
	Mean	Median (range)		Mean	Median (range)		Mean	Median (range)	
Arthrobacter sp.	3.8	<2 (<2...5.0)	17% *	<2	<2	0%	1.2	<2 (<2...3.0)	2% *
Brevibacterium sp.	3.4	<2 (<2...4.7)	6%	<2	<2	0%	1.9	<2 (<2...3.7)	2%
Corynebacterium coyleae	<2	<2	0%	1.5	<2 (<2...3.0)	3%	<2	<2	0%
Corynebacterium group A	<2	<2	0%	<2	<2	0%	1.2	<2 (<2...3.0)	2%
Corynebacterium group F1	3.7	<2 (<2...5.0)	6%	2.8	<2 (<2...4.0)	6%	<2	<2	0%
Corynebacterium group G	3.8	<2 (<2...5.0)	33%*	2.5	<2 (<2...4.0)	3%	0.2	<2 (<2...2.0)	2% *
Corynebacterium jeikeium	3.8	<2 (<2...5.0)	11%	1.5	<2 (<2...3.0)	3%	4.3	<2 (<2...6.0)	7%
Corynebacterium mucifaciens	<2	<2	0%	2.5	<2 (<2...4.0)	6%	1.9	<2 (<2...3.7)	2%
Corynebacterium seminale	4.9	2 (<2...5.7)	61%	4.8	3 (<2…5,7)	59%	4.5	2 (<2...6.0)	58%
Corynebacterium striatum	3.5	<2 (<2...4.7)	11%	3.7	<2 (<2...5.0)	9%	1.3	<2 (<2...3.0)	3%
Cellulomonas/Microbacterium£	0.7	<2 (<2...2.0)	6%	3.8	<2 (<2...5.0)	9%	1.3	<2 (<2...3.0)	3%
Corynebacterium sp.	5.5	<2 (<2...6.7)	28%	4.0	<2 (<2...5.0)	19%	4.1	<2 (<2...5.7)	29%
Dermabacter hominis	<2	<2	0%	<2	<2	0%	2.9	<2 (<2...4.7)	7%
Gardnerella vaginalis	2.7	<2 (<2...4.0)	6%	<2	<2	0%	4.3	<2 (<2...6.0)	10%
Turicella otitidis	<2	<2	0%	<2	<2	0%	0.2	<2 (<2...2.0)	2%
Catalase negative coryneform	4.5	<2 (<2...4.7)	17%	3.5	<2 (<2...5.0)	22%	4.1	<2 (<2...5.7)	19%

#* $p<0.05$ (Fisher exact test)

£ – 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 an 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.

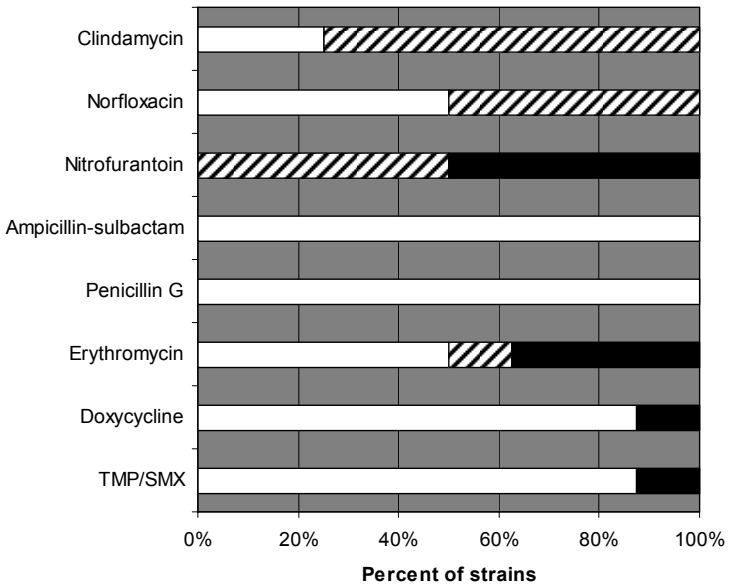

Fig. 4. Susceptibility pattern of *Corynebacterium* group G.
White – susceptible; striated – Intermediate; black – Resistant.

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 penicillin G than *C. jeikeium, C. striatum*, and *D. hominis* (Funke *et al.*, 1996; Fun-

ke *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.

11. Mycoplasmas in semen of chronic prostatitis patients

11.1. 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 (Kriger *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. Rastawicki *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

Test Method		Positive specimens N, %			
	Microorganism	NIH IIIA	NIH IIIB	NIH IV	Controls
Myco-plasma IST	*Ureaplasma sp.*	8/38 (21%)	10/59 (17%)	6/24 (25%)	5/40 (12%)
	M. hominis	0/38 (0%)	1/59 (2%)	0/24 (0%)	0/40 (0%)
PCR	*U. urealyticum*	0/11 (0%)	1/20 (5%)	1/4 (25%)	1/25 (4%)
	U. parvum	2/11 (18%)*	3/20 (15%)***	1/4 (25%)	0/25 (0%)*,***
	M. genitalium	2/11 (18%)**	0/20 (0%)	0/4 (0%)	0/25 (0%)**
*,** 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)					

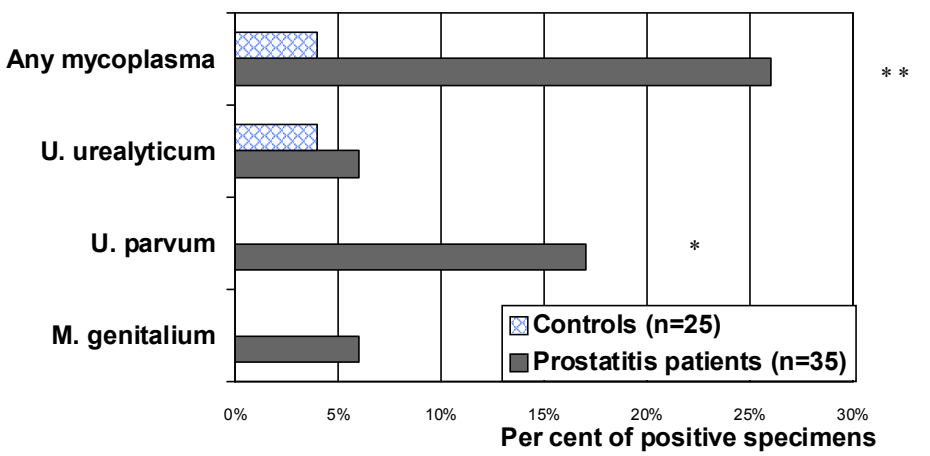

Fig. 5. Significance of mycoplasmas in relation to prostatitis according to PCR results
* p=0.032 (Fisher's exact test)
** p=0.026 (Fisher's exact test), p=0.029 (logistic regression analysis)

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 (semen 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

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

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, prooxidants 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 *in vivo* independently from a cyclooxygenase (COX), primary by free radical-induced peroxidation of arachidonic acid (Morrow *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 *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 *et al.,* 2003; Polla *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 *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 *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 *et al.,* 1993). Unlike the studies regarding NIH II (chronic bacterial) prostatitis by Zhou *et al.* (2006) and Lou *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 staphylococci (CNS). The number of different microorganisms in one sample ranged from 1 to 8, the total microbial concentrations ranged from $5x10^3$ to $7x10^5$ 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 coryneform 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 *Escherischia 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

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

ns – not significant

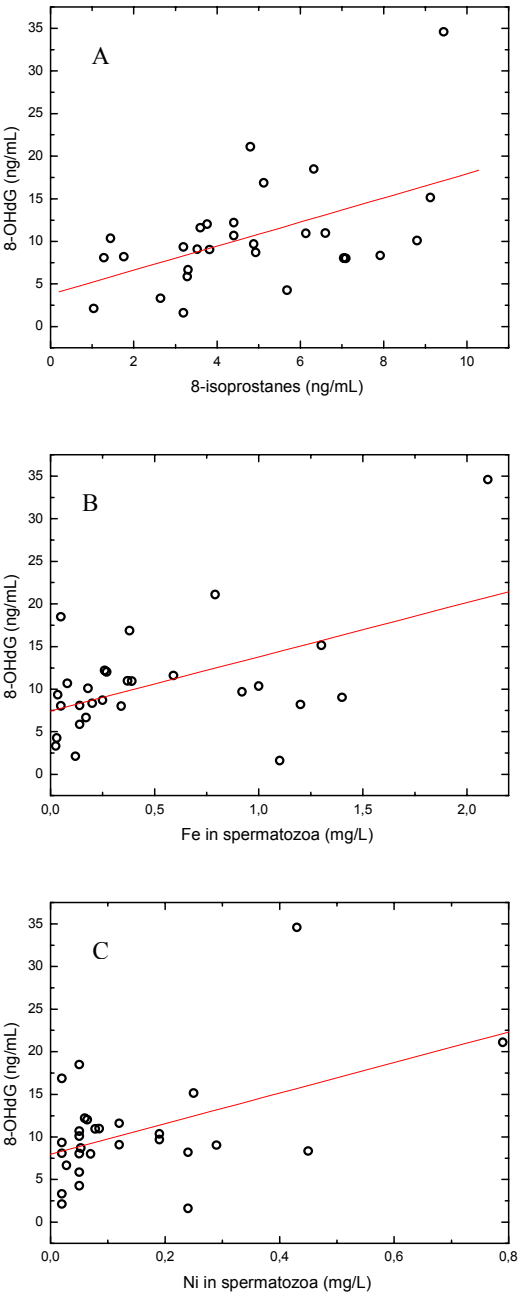

Fig. 6. The positive association between the levels of (A) 8-isoprostanes and 8-OHdG in urine; (B) Fe in spermatozoa and 8-OHdG in urine; and (C) Ni in spermatozoa and 8-OHdG in urine.

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 cannot be revealed or that may remain beyond attention 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 *C. diphtheriae* aside, the mucosal Gram-positive microorganisms are generally considered commensals. However, Shahed *et* Shoskes (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 *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. However, these microorganisms do not probably act as separate pathogens but they may rather fit into multibacterial community existing in male genital tract in case of prostatitis. The large number of unusual species in high quantities might colonize prostatitis patients not as much because of particular virulence of these species but rather because of reduced immunity of the patients. The situation can be explained better by paradigm of dysbacteriosis – a disturbance of balance between immune system and normal microbiota leads to an abnormal immune response combined with respective aberrations of normal microbiota. Alternatively, if only one or two endogenous opportunists from the pool of many potential agents occupy the niches pre-

viously protected by functional immune system, then opportunistic infection would be a better description of the prostatitis. Tentatively, the following three examples could eventually lead to imbalance of microbiota: urinary reflux combined with an impaired urothelium negates desquamation, facilitates invasion and irritates the surrounding tissues; neuromuscular spasm could cause an accumulation of ROS and a depletion of antioxidants, which could lead to deterioration of the immune barriers of the prostate; calcifications in the prostate provide an opportunity for a foreign body biofilm infection for the occasional bacteria that have incidentally migrated into the prostate. In addition, this could explain focal and recurrent nature of infections, as antibacterial treatment can probably suppress but not eradicate a biofilm infection from the prostate. Therefore preventive strategies, probiotics and immunomodulation might be more helpful. The spectrum and pathogenecity of coryneforms could be conclusively demonstrated by a high-quality metagenomic biopsy study. 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* to scientific community as more pathogenic than it actually is. Although *Mycoplasma genitalium* was found in our study from few subjects only, 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

Although definitions of OxS may vary, usually it means that harm to biological systems is being facilitated by free radical oxidants. 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). In one hand, breathing is an oxidative process, and even spermatozoa require some ROS in order to become functional (Aitken *et al.,* 2007). On the other hand, excessive production of free radicals is detrimental for the organism. Studies have suggested causes of OxS: neural sensitization (Schwartz *et al.,* 2008), autoimmunity (Motrich *et al.,* 2008), prostate infection and leukocytospermia (Elkahwaji *et al.,* 2008; Shahed *et* Shoskes, 2001; Saleh *et al.,* 2002), smoking (Kiziler *et al.,* 2007), diet (Holt *et al.,* 2009) and reduced physical activity (Ghosh *et al.,* 2009). Earlier experiences that showed importance of oxidative mechanisms in various diseases 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 OxS (antioxidants, pro-oxidants and oxidation products) from levels of both local (spermatozoa, seminal plasma) and systemic (blood and urine). 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. Since 8-EPI are markers of lipid peroxidation in vascular diseases, hence prostatitis and some cardiovascular diseases share a pathogenetic component that could explain associations between these diseases. In fact, isoprostanes (and even $PGF_{2\alpha}$) can be produced by free radical mechanisms (Proudfoot *et al.,* 1995; Roberts *et al.,* 2004; Yin *et al.,* 2007). 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 in the case of 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 prolonged periods, covering also duration of spermatozoid storage in the female reproductive organs.

From the microbiological point of view, local OxS may be useful, because it has been observed that hydrogen peroxide (product of SOD), even in low concentrations (0.1 mM) decreases abilities of uropathogenic *E. coli* to adhere and resist blood serum and phagocytosis (Hegde *et al.,* 2008). On the other hand, 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 microbiological results from OxS study 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. Hence, our data may indicate that microbiota might be relevant for sperm quality.

16. Possible vicious circles in the pathogenesis of prostatitis

The current scientific results enable to construct possible vicious circle mechanisms for prostatitis. Oxidative stress may be one of the key links in a vicious circle.

One possible vicious circle would tie together urinary reflux theory, urothelium dysfunction theory, calcifications, infection and oxidative stress. 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 oxidative stress that contributes *via* circulation to systemic oxidative stress. Systemic oxidative stress results in urinary excretion of isoprostanes and $PGF_{2\alpha}$, which by irritating the prostate region locally close a positive feedback loop (It has been shown that $PGF_{2\alpha}$ causes urethral contractions in humans while 8-epi-$PGF_{2\alpha}$-epi has exhibited same traits in animal study (Tarcan *et al.* 2000, Andersson *et* Persson*,* 1977)). Since smoking raises 8-epi-$PGF_{2\alpha}$ levels, then we would have an example mechanism how smoking could contribute to prostatitis (Patrono *et* Fitzgerald 1997). Urethral contractions might cause pain in cases of both calcifications and impaired urothelium. This suggests a possible vicious circle (Fig. 7).

Another vicious circle involves a prostatic injury that induces oxidative stress in central nervous system, which in its own turn becomes as if reciprocally sensitized to pain (Schwartz *et al.,* 2008; Lee *et al.,* 2007). Animal study from Schwartz *et al.,* (2008) suggests that ROS generation in spinal cord due to peripheral injury takes place in mitochondria, and that is precisely where Mn-SOD is usually located. Although SOD dismutates superoxide into hydrogen peroxide that is itself capable of causing pain through TRPV1 receptor (Keeble et al., 2009), an observation of SOD levels in spinal cord suggests that SOD activity actually protects from the pain (Schwartz et al., 2009). Perhaps that is because superoxide may react with nitric oxide to form peroxynitrite that seems a truly harmful molecule as it can escalate oxidative stress and exacerbate pain as nitration of SOD and opiate receptors leaves superoxide levels and nociception, respectively, unchecked (Salvemini et Neumann, 2009). Nevetheless, 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).

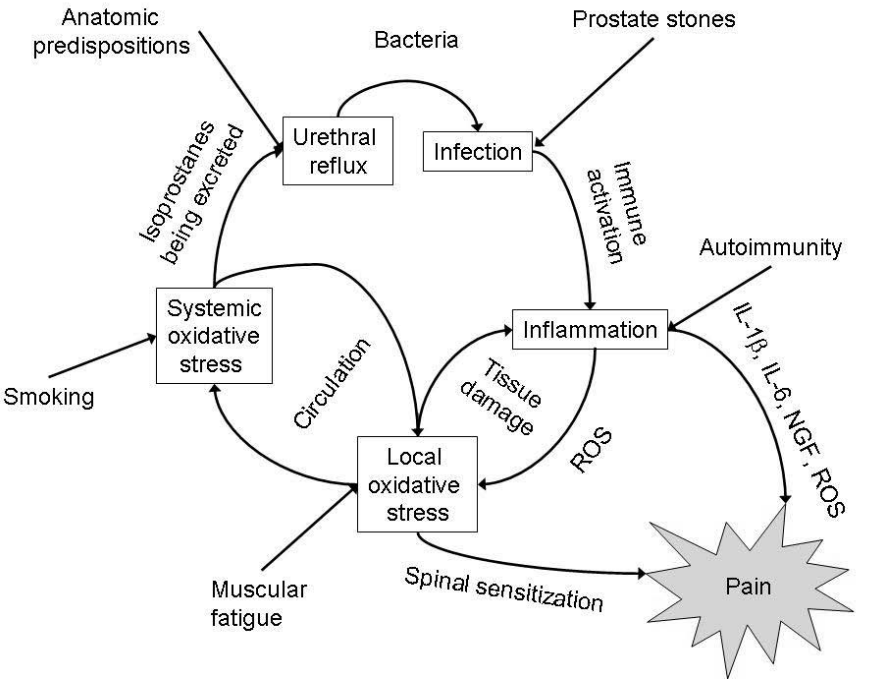

Fig. 7. Possible vicious circle in case of chronic prostatitis

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* Morgenstern, 1944). Their formula explains the mechanisms behind bacterial persistence in hosts:

$$R_0 = BN/(\alpha+b+\nu),$$

where R_0 is transmission potential of the microorganism, BN is the transmission rate depending on population size N, α is the mortality rate of hosts due to microorganism's virulence, *b* is mortality of hosts not due to microorganisms (lifespan), ν is the rate by which host's immune system eradicates the bacteria from the host. If all variables were independent, then a negative α (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).

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 prostatitis begins either by infection or by 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.

While hardly a confounder, it is difficult to estimate whether oxidative stress is an initiator or a propagator of CPPS. Be it one way or the other, or both of them, it seems that oxidative stress is a central pathogenetic mechanism of prostatitis.

17. 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), success stories of treatment (Jeong *et al.,* 2003), and belief into infectious etiology that is not based on 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 is not susceptible to norfloxacin, it raises questions about the feasibility of treating prostatitis with fluoroquinolones. Instead, 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 might have potential, especially those, which penetrate into the prostate. 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; Evliyaoğlu *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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SUMMARY IN ESTONIAN

Kroonilise prostatiidi etiopatogeneetilisi aspekte: mükoplasmad, korüneformsed bakterid ja oksüdatiivne stress

Sissejuhatus

Eesnäärmepõletik ehk prostatiit on lai mõiste, mis hõlmab haiguste gruppi, millest osa on ebaselge tekkepõhjusega. National Institutes of Health (NIH) klassifikatsioonis on kaks selge etioloogiaga prostatiiti – äge bakteriaalne prostatiit (NIH I) ja krooniline bakteriaalne prostatiit (NIH II) (NIH Chronic Prostatitis workshop in Bethesda, MD, 1995). Ülejäänud prostatiidi vormid (NIH III ja NIH IV) on oluliselt sagedasemad, kuid samas ebaselge etioloogiaga. NIH III kategooria ehk kroonilise prostatiidi / kroonilise väikevaagnavalu sündroom diagnoositakse siis, kui mehel on kolme kuu jooksul olnud vaagnapiirkonna valu ja muid kroonilise prostatiidi sümptomeid, kuid patogeene ei leita. Põhjusteks peetakse peamiselt infektsiooni, autoimmuunsust või närvi-lihassüsteemi häiret. Prostatiidi olulisus tuleneb eelkõige massilisest levimusest – ligikaudu kümnendik meestest kannatab kroonilise prostatiidi sümptomite all (Mehik *et al.,* 2000; Bartoletti *et al.,* 2007; Rizzo *et al.,* 2003; Ejike *et* Ezeanyika, 2008; Clemens *et al.,* 2007; Marszalek *et al.,* 2007; Nickel 2005). Samal ajal on haiguse kulg sageli krooniline ning elukvaliteedi langust prostatiidi ja müokardi infarkti puhul peetakse võrreldavateks (Tripp *et al.* 2004). NIH IV kategooria ehk asümptomaatiline põletikuline prostatiit on sündroom, mis sageli avastatakse juhuslikult muude protseduuride käigus (viljatuse ja vähi diagnostika). Sümptomite puudumise tõttu on seda prostatiidivormi suhteliselt vähe uuritud, kuid on täheldatud tema negatiivset mõju viljakusele (Korrovits *et al.,* 2008).

Kuna teadmised NIH III ja NIH IV kategooria prostatiitide tekkepõhjuste ja -mehhanismide kohta on puudulikud, on ka vahendid diagnoosimiseks ja ravimiseks kasinad, mis tingib vajaduse täiendavate uuringute järele. Üheks käesoleva töö uurimisvaldkonnaks olid mükoplasmad, mille olemasolu rutiinse prostatiidi diagnostika raames ei määrata, kuigi neid seostatakse järejst enam uretriidiga. Samuti uurisime korünebaktereid, mille etioloogilist rolli prostatiidi korral on varem kahtlustatud (Tanner *et al.,* 1999), aga rutiinse analüüsi käigus jäävad nad enamasti tähelepanu alt välja. Kompleksuuringu käigus selgitati prostatiidi patogeneesi ühe võtmelüli, oksüdatiivse stressi seost mikrofloora koostise, mikroelementide ja sperma baasparameetritega. Käesoleva dissertatsiooni raames teostatud uurimused teostati TÜ Mikrobioloogia Instituudi, TÜ Biokeemia Instituudi ja SA TÜK Androloogiakeskuse koostööna.

Uurimuse eesmärgid

Uuringu eesmärgiks oli saada uusi teadmisi prostatiidi etiopatogeneesi kohta. Selleks pöörasime põhitähelepanu mükoplasmadele ja korünebakteritele kui võimalikele etioloogilistele faktoritele ja oksüdatiivsele stressile kui võimalikule patogeneesimehhanismile.

Kitsamad ülesanded olid järgmised:

1) Võrrelda korünebakterite levimust ja liigilist koostist prostatiidiga patsientidel ja tervetel.
2) Selgitada spermast isoleeritud korünebakterite antibiootikumtundlikkus.
3) Võrrelda kahe erineva meetodiga mükoplasmade levimust ja liigilist koostist prostatiidiga patsientidel ja tervetel.
4) Määrata kroonilise prostatiidiga patsientide organismis oksüdatiivse stressi erinevaid aspekte (antioksüdandid, pro-oksüdandid ja oksüdatsiooniprodukid) nii lokaalselt (mõõdetuna spermaplasmast ja spermirakkude seest) kui ka süsteemselt (mõõdetuna uriinist ja verest).
5) Leida võimalikud seosed meessuguteede mikrofloora ja oksüdatiivse stressi ning põletikunäitajate vahel.

Materjalid ja meetodid

Korüneformsete bakterite uuringugruppi kuulus 10^9 meest (59 tervet ja 50 põletikulise prostatiidiga patsienti). Põletiku olemasolu hinnati leukotsütospermia alusel – tugev (>10^6 WBC/ml; N=18) või mõõdukas ($2x10^5$ ... 10^6 WBC/ml; N=32). Vastavalt sümptomite olemasolule diagnoositi kas NIH IIIA (N=20) või NIH IV (N=30) kategooria prostatiiti. Patsientide keskmine vanus oli 28,5 aastat, kontrollgrupis osalejate keskmine vanus oli 20,0 aastat. Korüneformide uuringust osa võtnud 30 meest (21 haiget ja 9 tervet) osalesid ühtlasi oksüdatiivse stressi uuringus. Kõikidele patsientidele tehti sperma baasuuring vastavalt WHO juhendile (WHO, 1999). Patsientide spermast ning esmasuriinist tehti ühe tunni jooksul kvantitatiivsed aeroobsed, mikroaerofiilsed ja anaeroobsed algülvid nn „nelja nurga" meetodil järgmistele söötmetele: veriagar, šokolaadagar, MRS agar, *Gardnerella vaginalis*'e agar, Wilkins-Chalgren agar, Wilkins-Chalgren GN agar. Korüneformsed bakterid samastati Gram'i värvumuse, katalaasitesti, MUG-lisandiga söötme (Oxoid) ning API Coryne (BioMérieux) abil. Nende antibiootikumtundlikkust testiti kaheksa antibiootikumi (tetratsükliin, TMP/SMX, penitsilliin, ampitsilliin-sulbaktaam, norfloksatsiin, nitrofurantoiin, erütromütsiin, klindamütsiin) suhtes E-test meetodil (AB Biodisk).

Mükoplasmade uuringus osales 161 meest (38 NIH IIIA, 59 NIH IIIB, 24 NIH IV ja 40 kontroll-uuritavat). Leukotsütospermiana käsitleti WBC kontsentratsiooni üle $2x10^5$ WBC/ml. Patsientide vanus oli 22...50 (keskmiselt 34,2) aastat, kontrollide vanus 21…36 (keskmiselt 28,7) aastat. Kõikide meeste spermast määrati Mycoplasma IST süsteemiga (BioMérieux) *Mycoplasma hominis*

ja *Ureaplasma* sp. Kuuskümmend proovi analüüsiti täiendavalt PCR meetodi abil *M. genitalium, U. parvum* ja *U. urealyticum* esinemise suhtes. DNA eraldamiseks oli kasutusel High Pure PCR Template Preparation Kit (Roche Biochemicals), praimerid ja Taq polümeraas pärinesid firmalt Fermentas. *M. genitalium* avastati praimeritega Pa1 ja MgPa3, *U. parvum* avastati praimeritega UMS-125 ja UMA-226, *U. urealyticum* avastati praimeritega P6 ja U8. PCR produktid eraldati elektroforeesi abil 2% agaroosgeelil ning visualiseeriti etiidiumbromiidi ja UV-kiirguse abil.

Oksüdatiivse stressi uuringus osales 21 prostatiidiga patsienti ning 9 kontroll-uuritavat, keskmised vanused olid vastavalt 32,3 ja 31,2 aastat. Uuritavatel määrati nii oksüdatiivse stressi kui antioksüdantse kaitse näitajaid spermides, spermaplasmas, veres ja uriinis. Spermide ja spermaplasma eraldamine toimus tsentrifuugimisel. Spermide lahutamine põletikurakkudest teostati astmelise Percoll gradient-tsentrifuugimise teel (30%, 50%, 70% ja 90% SIP), seejärel määrati nende puhtuseaste ja kontsentratsioon Neubaueri hemotsütomeetri abil, lahjendati kontsentratsioonile 10^6 spermi/ml, külmutati vedelas lämmastikus ning purustati pehmel meetodil (vahelduv kiire külmutamine ja sulatamine). Spermaproove hoiti transpordi ja tsentrifuugimise ajal jääl või +2°C juures.

Uuringutes osalemine oli vabatahtlik, kõik uuringud said Tartu Ülikooli Inimuuringute Eetika Komitee nõusoleku.

Tulemused ja järeldused

1) Prostatiidi-patsientide spermas esinevad polümikroobsed kooslused, mis sisaldavad lisaks aeroobsetele ka rohkesti anaeroobseid baktereid. Korüneformsed bakterid moodustavad nendes kooslustes märkimisväärse osa. Lokalisatsiooniuuringu põhjal pärinevad prostatiidi-patsientide spermas leiduvad korünebakterid ülemistest suguteedest, mitte ureetrast, millest pärineb normaalne mikrofloora. Lisaks sellele võib patsientidelt võrreldes kontrolluuritavatega leida oluliselt rohkem korüneformseid liike, mis esinevad kõrges kontsentratsioonis. Kuigi *Corynebacterium seminale* on kõige sagedamini esinev korüneformne bakter meessuguteedes, esineb *Corynebacterium* grupp G märksa sagedamini tugeva leukotsütospermiaga prostatiidipatsientidel kui kontrollidel, mis võib viidata, et viimane osaleb prostatiidi etiopatogeneesis häirunud mikrobioodi ühe komponendina.
2) Penitsilliinid ja TMP/SMX on parimad *in vitro* inhibiitorid sperma korünebakteritele, samas kui need bakterid on sageli vähetundlikud mitmetele teistele antibiootikumidele. MLSb resistentsus on sperma korüneformide seas levinud. Prostatiidiga seotud *Corynebacterium* grupp G on resistentne mitmetele antibiootikumidele, mida kasutatakse prostatiidi raviks, sealhulgas on see liik sageli resistentne nitrofurantoiini, klindamütsiini, norfloksatsiini ja erütromütsiini vastu. Prostatiidi polümikroobse olemuse ning sagedase

empiirilise ravi tõttu on potentsiaalsete etioloogiliste faktorite antibiootikumtundlikkuse andmed ravi valikul olulised.

3) Meie andmed viitavad võimalusele, et mõned mükoplasmade liigid võivad olla prostatiidi tekkega seotud. Ureaplasma liikide (*U. urealyticum* ja *U. parvum*) eristamine on oluline, kuna ainult viimane esineb prostatiidiga patsientidel sagedamini kui kontroll-uuritavatel. *Mycoplasma genitalium* võib olla seotud NIH IIIA prostatiidiga, kuna teda leiti ainult selle kategooria patsientidel.
4) Põletikulise prostatiidiga patsientidel esineb oksüdatiivne stress nii lokaalselt (rakusiseselt spermides ja rakuväliselt spermaplasmas) kui ka süsteemselt (veres ja uriinis). Lokaalne oksüdatiivne stress seisneb ühelt poolt oksüdatiivse kaitsevõime vähenemises ning teiselt poolt oksüdatsiooniproduktide ja pro-oksüdantidehulga suurenemises (TAS↓, TAA↓, DC↑ ja Fe↑). Kuna spermid on väga tundlikud oksükahjustustele, võib see mehhanism olla aluseks prostatiidiga kaasnevale viljatusele. Lisaks lokaalsetele muutustele eksisteerib märkimisväärne süsteemne oksüdatiivne stress, mida näitab 8-isoprostaanide sisalduse tõus uriinis ja selle korreleerumine DNA kahjustuse indikaatoriga (8-OHdG). Kuna uriinis leiduvad 8-isoprostaanid on suutelised tekitama põie kontraktsioone ja põletiku ägenemist, siis patsientide kuseteede talitluse häired ning patogeneesiahela muutumine suletud ringiks võib olla seotud just 8-isoprostaanidega. Üldisemas plaanis võib süsteemne oksüdatiivne stress olla paljude haiguste riskifaktoriks.
5) *Corynebacterium seminale* seos spermide parema antioksüdantse kaitsega ning *Corynebacterium* grupp G seos põletikuga viitab vajadusele pöörata nendele bakteritele rohkem tähelepanu nende diagnostilise või terapeutilise potentsiaali tõttu.

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PUBLICATIONS

CURRICULUM VITAE

Date and place of birth: January 13th, 1980, Tallinn
Citizenship: Estonian
Address: Department of Microbiology, Tartu University,
Ravila 19, 50411, Tartu, Estonia
Telephone: (+372) 737 4170
Fax: (+372) 737 4172
E-mail: silver.turk@mail.ee

Education

1987–1992	Sõle Secondary School of Tallinn
1992–1996	Lilleküla Secondary School of Tallinn
1996–1998	Mustamäe Secondary School of Tallinn
1998–2003	University of Tartu, Faculty of Medicine, graduate study of pharmacy
2004–2009	University of Tartu, Faculty of Medicine, postgraduate study of microbiology

Professional Employment

2002	East Tallinn Central Hospital, Central Pharmacy, apprentice
since 2003	University of Tartu, Department of Microbiology, research assistant

Scientific work

Main fields of research:
- Etiology and pathogenesis of prostatitis
- Microbiology, especially that of coryneform bacteria
- Oxidative stress in case of prostatitis

Five scientific publifications (CC and Medline cited) and 12 conference presentations.

ELULOOKIRJELDUS

Sünniaeg ja -koht: 13. jaanuar, 1980, Tallinn
Kodakondsus: Eesti
Aadress: Mikrobioloogia Instituut, Tartu Ülikool,
Ravila 19, 50411, Tartu, Eesti
Telefon: (+372) 737 4170
Faks: (+372) 737 4172
E-post: silver.turk@mail.ee

Haridus

1987–1992	Tallinna Sõle Gümnaasium
1992–1996	Tallinna Lilleküla Gümnaasium
1996–1998	Tallinna Mustamäe Gümnaasium
1998–2003	Tartu Ülikool, Arstiteaduskond, farmaatsia diplomiõpe
2004–2009	Tartu Ülikool, Arstiteaduskond, Mikrobioloogia Instituut, mikrobioloogia doktorantuur

Teenistuskäik

2002	Ida-Tallinna Keskhaigla, Keskapteek, praktikant
alates 2003	Tartu Ülikool, Mikrobioloogia Instituut, vanemlaborant

Teadustöö

Peamised uurimisvaldkonnad:
- Prostatiidi tekkepõhjused ja patogenees
- Mikrobioloogia, eriti korüneformsete bakterite mikrobioloogia
- Oksüdatiivne stress seoses prostatiidiga

Viis avaldatud teaduspublikatsiooni (CC ja Medline rahvusvahelistes andmebaasides) ja 12 konverentsiettekannet.

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