<table>
<thead>
<tr>
<th>Title</th>
<th>Authors</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foreword of the editor</td>
<td>Gábor L. Kovács</td>
<td>3</td>
</tr>
<tr>
<td>Focusing on men’s health</td>
<td>Maria Pasic, Vathany Kulasingam and Eleftherios P. Diamandis</td>
<td>4-8</td>
</tr>
<tr>
<td>An emerging role of TEX101 protein as a male infertility biomarker</td>
<td>Christina G. Schiza, Keith Jarvi, Eleftherios P. Diamandis, and Andrei P. Drabovich</td>
<td>9-26</td>
</tr>
<tr>
<td>Genetic and epigenetic changes after spermatogonial stem cell culture and transplantation</td>
<td>Mary K. Samplaski, Marie Deault-Bonin, Kirk C. Lo</td>
<td>27-41</td>
</tr>
<tr>
<td>Mechanisms of androgen-independent prostate cancer</td>
<td>Punit Saraon, Andrei P. Drabovich, Keith A. Jarvi, Eleftherios P. Diamandis</td>
<td>42-54</td>
</tr>
<tr>
<td>Prostate-specific antigen (PSA) screening and new biomarkers for prostate cancer (PCa)</td>
<td>Carsten Stephan, Harry Rittenhouse, Xinhai Hu, Henning Cammann, Klaus Jung</td>
<td>55-78</td>
</tr>
<tr>
<td>miRNA in prostate cancer: new prospects for old challenges</td>
<td>Samy M Mekhail, Peter G Yousef, Stephen W Jackinsky, Maria Pasic, George M Yousef</td>
<td>79-98</td>
</tr>
<tr>
<td>Urothelial bladder cancer urinary biomarkers</td>
<td>Aidan P Noon &amp; Alexandre R Zlotta</td>
<td>99-114</td>
</tr>
<tr>
<td>Estimation of alert and change limits of haematological quantities and its application in the plausibility control</td>
<td>Lourdes Sánchez-Navarro, María José Castro-Castro, Dolors Dot-Bach, Xavier Fuentes-Arderiu</td>
<td>115-127</td>
</tr>
</tbody>
</table>
Editor: Gábor L. Kovács

Dr. Maria Pasic completed her undergraduate studies at the University of Toronto, Toronto, Ontario, Canada, and then attended McMaster University, Hamilton, Ontario Canada, where she completed a PhD on the rare hematologic disorder Quebec Platelet Disorder. She graduated from the Clinical Biochemistry Post-Doctoral Diploma Training Program at the University of Toronto in 2011, and then pursued additional post-doctoral training in Molecular Diagnostics. She is now a Clinical Chemist at St. Joseph’s Health Centre, Department of Laboratory Medicine, Toronto, Ontario Canada. She is also an Assistant Professor in The Department of Laboratory Medicine and Pathobiology, University of Toronto. Dr. Pasic’s current research is focused on Molecular Diagnostic’s in Personalized Medicine.

Dr. Vathany Kulasingam completed her undergraduate and graduate studies at the University of Toronto. She did her PhD in the laboratory of Dr. Eleftherios P. Diamandis on a subject related to identification of breast cancer biomarkers by using mass spectrometry. She then completed the Clinical Biochemistry Post-Doctoral Diploma Training Program at the University of Toronto and became a Certified Clinical Chemist by the Canadian Academy of Clinical Biochemistry in 2011. She is currently a Clinical Biochemist at Toronto’s University Health Network. She is also an Assistant Professor at the Department of Laboratory Medicine and Pathobiology, University of Toronto. The research interests of Dr. Kulasingam evolve around discovery of novel biomarkers for ovarian cancer by using genomics, proteomics and otheromics’ technologies. Dr. Kulasingam has published extensively on strategies for identifying novel biomarkers, and especially for ovarian cancer, by using proteomics.

Dr. Eleftherios P. Diamandis completed his undergraduate studies, PhD and Medical Degree at the University of Athens, Greece. He also completed the Clinical Biochemistry Post-Doctoral Diploma Training Program at the University of Toronto. Dr. Diamandis immigrated to Canada in 1986 and he is currently serving as Division Head of Clinical Biochemistry, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto. He is also Biochemist-in-Chief, University Health Network, Toronto, and is serving as Division Head of Clinical Biochemistry at The Department of Laboratory Medicine and Pathobiology, University of Toronto. Dr. Diamandis has published extensively over the years on the development of new analytical techniques, molecular diagnostics, molecular epidemiology, and on a group of enzymes known as Kallikreins. More recently, he is using proteomics and genomics for discovery of novel biomarkers. Dr. Diamandis has been honored by many organizations for his contributions to Laboratory Medicine and Clinical Chemistry and he is a Fellow of the American Association for the Advancement of Science, a Corresponding Member of the Academy of Athens and a member of the Royal Society of Canada.
Men and women are genetically extremely similar, with women having a 46XX and men a 46XY chromosomal composition. Sex differentiation is driven by a single gene on chromosome Y known as sex-determining region Y (SRY). Despite these similarities, women suffer from some unique disorders, such as breast cancer, while men suffer from others (e.g. prostate cancer). These two cancers represent the most frequently diagnosed and two of the most common causes of death in females and males, respectively. A few years ago, we reviewed the many common features between breast cancer in females and prostate cancer in males (1).

Women’s health has attracted more attention and more funding than men’s health (e.g. the January 2014 special issue of the journal “Clinic of Chemistry” is focusing on women’s health) (2). In this issue of eIFCC we focus on men’s health with special emphasis on prostate cancer. We also include some other health issues exclusive to men such as androgen replacement therapy and male infertility. We have also included one chapter on bladder cancer which is common in both men and women.

A lot has been written already about prostate cancer screening by using prostate specific antigen (3-4). Despite the fact that the long-awaited prospective clinical trials on the usefulness of prostate cancer screening have already been published (5,6), the issue is still widely controversial. The US Preventive Services Task Force issued recommendations which do not support widespread prostate cancer screening. Dr. Carsten Stephan and colleagues review the issue of prostate cancer screening and examine novel biomarkers which may increase its effectiveness. The same topic is examined by Dr. Ulf-Hakan Stenman and colleagues but from a different angle.

MicroRNAs have emerged as major players of gene regulation and a Nobel Prize has been awarded for their discovery (7). More recently, the family of non-coding RNAs has been greatly expanded (8). In addition to mechanistic aspects related to transcription and translation, an emerging field is to use microRNAs as cancer biomarkers (9). The application of these markers to the clinic is still remote but efforts are continuing with a fast pace. Yousef and colleagues review the use of microRNAs as candidate biomarkers of prostate cancer.

The majority of prostate cancers do not kill the patient and many do not even need treatment, but a new form of therapy coined “active surveillance” (10). Prostate cancer is not lethal, unless it becomes
metastatic. Why do the vast majority of patients respond initially very well to various forms of therapies but relapse within 2-3 years with distant metastasis? This is usually due to the establishment of androgen independence. Although some mechanisms of androgen independence are known (11) we still do not understand the fine details or how to reverse it. Keith Jarvi and colleagues review the currently known mechanisms of androgen independence and describe the clinical importance for finding new therapies.

Mass spectrometry is a powerful technique not only for measuring various types of analysts such as hormones, metabolites, proteins and nucleic acids, but also as a discovery tool for novel biomarkers (12, 13). While most researchers focus on identifying genomic or proteomic biomarkers for cancer and other diseases, it is also possible to investigate small metabolites as potential biomarkers, which can be found in either serum or urine. Recently, some highly promising metabolomic biomarkers for prostate cancer (such as sarcosine) have been described (14, 15). Robert Wolfert and colleagues examine how mass spectrometry can be used to study the metabolon and on how to use this information to derive clinically relevant prostate cancer biomarkers.

Kallikreins are a group of serine proteases that are encoded by 15 genes located in tandem on human chromosome 19q13.4 (16). Two members of the kallikrein family, prostate specific antigen (PSA; KLK3) and KLK2 are expressed only in the prostate and are used widely as biomarkers for prostate cancer screening, diagnosis and monitoring (17). KLK3 and KLK2 are not the only prostatic specific kallikreins; at least another two (KLK4 and KLK15) are also almost exclusively expressed in prostate (18). It is likely that these prostate specific kallikreins, which have been found to participate in semen liquefaction (19), could find important applications as individual or combined biomarkers. Judith Clements and colleagues review the status of kallikreins in prostate cancer and suggest on how this group of enzymes can be used in clinical practice.

The genetics of prostate cancer are relatively obscure (20). While there is a familial form of the disease (21), the majority of prostate cancers have not been associated with specific cancer predisposition genes. Recently, whole exome sequencing revealed candidate genes associated with the disease (22). Also, previous genome-wide scans established genetic loci that are associated with prostate cancer predisposition such as those on chromosome 8q (23). Dr. Robert Nam and colleagues review the genetics of prostate cancer and outlines the known genetic components of this disease.

With increased life expectancy, more men live longer while remaining sexually active. There is currently a debate as to the existence of male andropause (24). However, it is also widely accepted that a proportion of aging men lose potency and have other symptoms of possible testosterone deficiency such as fatigue, weakness, loss of motivation and mood swings. Testosterone replacement
therapy is an effective way to reverse these symptoms. However, this therapy may have important side effects (25). Dr. Ethan Grober and colleagues bring us up to date with the status of testosterone replacement therapy and outline advantages and disadvantages.

Stem cells represent one of the most rapidly growing areas of research as they promises to revolutionize regenerative therapies for many diseases (26). Inducible stem cell technologies enjoyed dramatic improvements over the last two years and they are highly promising. Dr. Kirk Lo and colleagues review the status of testicular stem cells and propose important applications in male infertility and other testicular diseases.

Infertility affects approximately 5-10% of all couples and is due to disorders of both partners (50% each). While female infertility has received much attention, male infertility is a less researched area. In men with azoospermia, the question is whether the infertility is due to obstruction of the vas deferens (this is equivalent to vasectomy) or to a non-obstructive cause. Non-obstructive azoospermia is further divided into three major categories (maturation arrest, hypospermatogenesis or Sertoli cell-only syndrome). The most viable current way of establishing if a non-obstructive azoospermic man may be able to have children, is to retrieve sperm from his testes and proceed to artificial insemination. Retrieving sperm from the testes requires testicular biopsy followed by a surgical procedure, testicular semen extraction (TESE). This procedure is invasive, painful, has serious side effects and on many cases does not lead to sperm extraction. Dr. Keith Jarvi and colleagues recently described a new biomarker, TEX101, which may have the potential to discriminate between obstructive and non-obstructive azoospermia, as well as, of various forms of non-obstructive azoospermia (27). Their review is an update on TEX101 and other seminal plasma biomarkers for male infertility.

Last but not least, Dr. Alex Zlotta and colleagues address a cancer that is found in both males and females, bladder cancer. There is an urgent clinical need to identify biomarkers not only to diagnose bladder cancer early, but also to discriminate between low-grade and high-grade bladder cancer since these two forms need different types of treatment.

All-in-all, this compilation covers a wide spectrum of clinical questions and aims to bring the readers up-to-date with new developments in prostate cancer and other areas related to men’s health. We thank all authors for their efforts but the Co-Editors assume responsibility if this special issue falls short of expectations.
References


An emerging role of TEX101 protein as a male infertility biomarker

Christina G. Schiza¹, Keith Jarvi², Eleftherios P. Diamandis¹, 3-5* and Andrei P. Drabovich³*

¹Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada.
²Department of Surgery (Division of Urology), Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada.
³Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada.
⁴Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, University of Toronto, Toronto, ON, Canada.
⁵Department of Clinical Biochemistry, University Health Network, Toronto, ON, Canada.

ARTICLE INFO

*Correspondence to:
A.P. Drabovich, Ph.D.
Lunenfeld-Tanenbaum Research Institute,
Mount Sinai Hospital, Joseph & Wolf Lebovic Ctr., Mount Sinai Hospital,
Joseph & Wolf Lebovic,
60 Murray St [Box 32]; Flr 6 - Rm L6-201
Toronto, ON, M5T 3L9, Canada
Tel: 416-586-4800 ext. 8805;
e-mail: adrabovich@mtsinai.on.ca

and

E.P. Diamandis, M.D., Ph.D.
Mount Sinai Hospital, Joseph & Wolf Lebovic Ctr.,
60 Murray St [Box 32]; Flr 6 - Rm L6-201
Toronto, ON, M5T 3L9, Canada
Tel: 416-586-8443; Fax: 416-619-5521;
e-mail: ediamandis@mtsinai.on.ca

Keywords:
Male infertility
Azoospermia
Biomarkers
Sperm
TEX101
Spermatogenesis
Acrosome reaction

ABSTRACT

Infertility is an important aspect of human reproduction. It affects up to 15% of couples, with the male factor contributing to approximately 50% of all cases. Azoospermia is one of the most severe forms of male infertility, which is characterized by the absence of sperm in semen. The mechanisms underlying male infertility remain unknown. Currently, clinicians rely on semen analysis to predict the reproductive potential of a male, and testicular biopsy is the only reliable method to diagnose different subtypes of azoospermia. Recently, advances in proteomics encouraged the search for novel male infertility biomarkers in seminal plasma. In this review, we focus on TEX101, a testicular germ cell-specific protein, one of the most promising male infertility biomarkers. We discuss its role in spermatogenesis and fertilization and summarize our current knowledge about this new potential biomarker.
Introduction

Infertility, the inability to conceive a child within one year of regular unprotected intercourse, shows a high prevalence (up to 15% of couples) and is associated with both men and women (1). Male factor (exclusive or combined with female abnormalities) contributes to approximately 50% of infertility cases (2). In attempts to overcome this problem, infertile couples seek medical advice and pursue fertility treatments such as hormonal and drug therapy or assisted reproductive technologies (ART) (3).

The impact of male factor infertility was traditionally overlooked despite its significance for men’s health. However, over the last decade, this topic has received the required attention and there has been a notable progress in the field of male infertility. Cellular and molecular mechanisms of reproduction and their impact on the clinical outcome are still not fully understood due to the complexity of the male reproductive system. With 3-4% of couples remaining childless (4), male infertility remains an important clinical and societal issue.

Forms and subtypes of male infertility

Clinical forms of male infertility result in reduced sperm counts (oligospermia), reduced sperm motility (asthenospermia), abnormal sperm morphology (teratospermia) and in most severe cases, a complete lack of spermatozoa in semen, known as azoospermia. Azoospermia accounts for 5-20% of infertile men and could be further categorized as pre-testicular or non-obstructive (NOA), and obstructive azoospermia (OA) (5). In most situations pre-testicular azoospermia is linked to hypothalamic or pituitary dysfunction, resulting in low serum levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). This can cause low testosterone levels and failure of the testes to produce sperm. This group of infertile men can be easily diagnosed utilizing current clinical methods (blood test to determine reproductive hormones levels) (6). NOA may be sub-classified as maturation arrest (MA), Sertoli cell-only syndrome (SCO), and hypospermatogenesis (HS) (7). OA results from physical obstruction in the male genital tract. It resembles vasectomy, a surgical procedure in which vas deferens is severed and sealed, used for male sterilization (8).

Development of sperm antibodies in semen or blood can be an additional cause of male infertility, affecting 5-6% of infertile men. Their presence results in reduction of sperm motility and prevention of sperm-egg binding during fertilization. (9)

Challenges in male infertility diagnosis

If male factor infertility is suspected, a variety of diagnostic approaches are used to identify infertility forms and subtypes and for considering treatment options. Initially, physical examination followed by semen analysis and endocrine profiling are performed. semen analysis, the most common way to
An emerging role of TEX101 protein as a male infertility biomarker

diagnose male infertility, includes examination of macroscopic (coagulation, color, viscosity, pH, and volume) and microscopic (sperm count, concentration, motility, morphology and viability) parameters of seminal fluid (10). Since the composition of seminal plasma can be affected by environmental factors, infections and other pathologies, the results of semen analysis are frequently either normal or ambiguous, leading to inconclusive diagnosis (11). In these cases, patients are diagnosed with idiopathic infertility and specific treatment is not provided (12).

In an attempt to diagnose forms and subtypes of azoospermia, a diagnostic testicular biopsy is often used as the only reliable method to distinguish between OA and NOA (7;13). Testicular biopsy is an invasive surgical procedure with possible complications such as tissue damage, bleeding and development of chronic pain. Thus, there is an urgent need for alternative, non-invasive approaches for identification of categories of male infertility. Recent developments in the –omics technologies should aid in discovering novel infertility treatments (14) and for developing non-invasive tests to eliminate diagnostic testicular biopsies, classify infertility forms and suggest options for treatment.

Search for male infertility biomarkers

Different types of molecules, such as genes, messenger RNA, microRNA, proteins, metabolites or combination of these, can serve as biomarkers (15). Although approaches for biomarker discovery and development are rapidly maturing and the number of biomarker discovery projects are steadily increasing, translation of biomarkers from bench to bedside is still slow. In a recent review, Kovac et al.(16) summarized the most significant genomic, proteomic and metabolomics approaches to identify male infertility biomarkers (Table 1).

Proteins are the most promising molecules to develop disease biomarkers. Alternations in protein abundance and activity in different physiological states reflect dynamic alternations which may hardly be predicted at the genome level (16). Proteomic analysis of seminal plasma (SP) or spermatozoa can provide information regarding the presence of a protein, its abundance, and post-translational modifications. Several studies conducted in the 1980’s resulted in discovering roles of transferrin, heparin-binding proteins (HBPs), prolactin inducible protein (PIP), and human cationic antimicrobial protein (hCAP18) in spermatogenesis and fertilization (16). Recently, by comparing the SP proteome of healthy fertile men before and after vasectomy, Batruch et al. identified a list of testis and epididymis-expressed proteins. Among these proteins, TEX101, PGK2, HIST1H2BA, SLC2A14, SPACA3, GAPDHS, and AKAP4 were the top candidates for developing biomarkers of vasectomy success (17). In the follow-up study, 30 of those biomarker candidates were verified in pre- and post-vasectomy SP samples as well as SP from patients with NOA. Several testis-specific proteins, such as TEX101, LDHC and ECM1, were identified as key male infertility biomarkers (18). Recently, TEX101 and ECM1 were used to develop an algorithm for non-invasive differential diagnosis of azoospermia forms (OA versus NOA) (19). SP levels of TEX101 could also distinguish different subtypes of NOA. TEX101 levels of
120ng/mL or higher denote normal spermatogenesis, while levels of 5-120ng/mL are associated with HS or MA, and levels below 5ng/ml (theoretically zero) indicate SCO syndrome (Figure 1). Apart from azoospermia diagnosis, TEX101 levels in SP may predict the outcome of sperm retrieval procedures used for assisted reproduction. Men diagnosed with OA, NOA-HS, and NOA-MA are recommended for testicular sperm extraction (TESE), while for men with NOA-SCO, the success rate of TESE is negligible. In that case, TESE, a full-scale several hour surgery under general anaesthesia, could be avoided. In this article, we will focus on TEX101 protein, discuss its role in spermatogenesis and fertilization and summarize our knowledge about this new potential infertility biomarker.

**TEX101, a novel testicular germ cell-specific protein**

Mouse TEX101 protein (encoded by testis expressed 101 gene, 
Tex101) was originally identified in mice by Kurita et al. (20). Mouse TEX101 is a testicular germ cell-specific protein predominantly located on the plasma membrane of germ cells during gametogenesis. According to The Human Protein Atlas (www.proteinatlas.org), human TEX101 is not expressed in any other human tissue or cell type, including Sertoli and Leydig cells of the testicular tissue.

The TEX101 gene is located on the long arm of chromosome 19 at position 19q13.31 in humans and chromosome 7 in mouse, with homology of sequence approximately 55% (21). It should be mentioned that most of our knowledge on TEX101 derives from studies conducted using mouse models. However, taking into consideration that the positions of the cysteine residues and the potential glycosylphosphatidylinositol (GPI)-anchoring site within the sequence are highly conserved in mammalian species, we can assume that most of the data generated with mouse models may be valid for human TEX101.

Studying TEX101 at a genomic level, Tsukamoto et al. (22) found that mouse TEX101 gene is regulated by two distinct promoters and forms three major transcripts. Based on nucleotide and amino acid sequence, it is predicted that the human ortholog exists in two isoforms; the cytosolic form and the extracellular GPI-anchored form, under the regulation of two promoters (Figure 2A). However, by performing immunohistochemistry (IHC) experiments Kurita et al. (20) demonstrated that TEX101 is located mainly on the plasma membrane of germ cells during the whole process of gametogenesis, but it is only weakly present in the cytoplasm of the cells. Jin et al. (23) showed that TEX101 is highly glycosylated at all potential sites and that it is expressed as a GPI-anchored protein on the cell surface, as it was speculated by Kurita et al. (20) (Figure 2B). Furthermore, they presented evidence that TEX101 is present in the lipid rafts on sperm surface, as it was also demonstrated by Sleight et al. (24).

Given that TEX101 is present on the cell surface during all stages of spermatogenesis, Takayama et al. (25) intended to examine the fate of mouse TEX101 during sperm transport through the male reproductive tract. They reported that mouse TEX101 is eventually cleaved and released from the cell surface of epididymal sperm while it passes through the caput epididymis.
One of the missing parts regarding TEX101 is its 3-dimensional structure. Considering that it possesses a Ly-6/uPAR (Lymphocyte Antigen 6/ urokinase Plasminogen Activator Receptor) domain defined by 8-10 distinct cystein residues, it can be predicted that the structure of TEX101 might resemble that of uPAR. A number of proteins, comprising the Ly-6 family, share this single Ly-6/uPAR domain, and they are mapped to chromosome 8. However, the uPAR locus, PLAUR, encoding three tandem Ly-6/uPAR domains is found at 19q13.3 (26). Surprisingly, a few members of the Ly-6 family are mapped at the same locus, among which is TEX101 (19q13.31) and SAMP14 (19q.31.33). Based on this fact, we can speculate that these proteins are more closely related to uPAR, and may share the same or similar functions (27).

**Functional roles of membrane-bound and soluble forms of TEX101**

The information that is already gained regarding TEX101 function stems from studies that were based on mouse models (28;29). Nevertheless, the ultimate goal is to translate this knowledge into the human male reproductive system.

Spermatozoa are, inarguably, the most highly differentiated cell type of the human body. Numerous distinct processes need to be completed for generating mature and functional spermatozoa that have the ability to fertilize the oocyte. In the following section we describe step-by-step the events that occur, having as starting point the spermatogenesis in testes, and as terminal point the sperm-egg interaction in the female reproductive tract. Based on the fact that TEX101 accompanies sperm, either anchored to its membrane, or shed into seminal plasma, we are going to highlight all the events in which TEX101 is involved.

**Spermatogenesis**

Spermatogenesis is a highly ordered process that occurs in the male testis and is responsible for the production of a large number of spermatozoa, controlled by a complex system of paracrine and endocrine activity within the seminiferous tubules of the testis. The spermatogonia (the stem cells of the testis) are located in the basal compartment of the seminiferous tubule, between the basement membrane and the Sertoli cells.

Serial cross-sections of a seminiferous tubule show that sperm cells differentiate in spermatogenic cycles. LH-regulated release of testosterone from Leydig cells marks the initiation of spermatogenesis and development of germ cells into spermatozoa (30). Spermatogonial mitosis leads to a sufficient population of cells that will become spermatozoa. Following cell proliferation, diploid spermatogonial cells differentiate into spermatocytes, which in turn undergo meiosis and produce haploid spermatids (31). Finally, during the last stage of spermatogenesis (spermiogenesis), round haploid spermatids transform into mature spermatozoa undergoing a series of morphological and biochemical changes.
During spermiogenesis, the mature spermatozoa acquire all the essential and unique regions: formation of the acrosome, development of flagellum (tail), elimination of cytoplasm and condensation of the nucleus (32). It is estimated that one spermatogonium is able to differentiate into approximately 250 spermatozoa in ~35 days, depending on the species (33). By the end of spermatogenesis, morphologically complete spermatozoa detach from the Sertoli cell microenvironment and they are delivered into the lumen of the seminiferous tubule (34). Released spermatozoa passively migrate to the epididymis for further maturation.

TEX101, as previously mentioned, is a testicular germ cell-specific protein and is located on plasma membrane of spermatocytes, round and elongated spermatids, and testicular spermatozoa. The question that arises is whether TEX101 affects the outcome of spermatogenesis, or it remains on the cells to play its role later in the forthcoming processes.

Recently, two different groups (28;29) independently generated Tex101−/− mice in an attempt to

[Diagram: Figure 1. Differential diagnosis of azoospermia and prediction of subtypes of non-obstructive azoospermia with seminal plasma proteins ECM1 and TEX101.]

When azoospermia is diagnosed by semen analysis, low SP levels of ECM1 (<2.3 µg/mL) and TEX101 (<5 ng/mL) proteins suggest obstructive azoospermia, while high SP level of ECM1 (>2.3 µg/mL) suggests non-obstructive azoospermia. SP concentration of TEX101 protein may also discriminate between non-obstructive azoospermia subtypes of Sertoli cell-only (< 5 ng/mL) and hypospermatogenesis or maturation arrest (5-120 ng/mL). Men with obstructive azoospermia have good chances of sperm retrieval by testicular sperm extraction (TESE), while for men with Sertoli cell-only, sperm retrieval is unlikely and TESE can be avoided.

investigate in vivo the effects of TEX101 knock-out on the production of functional spermatozoa. In both studies, heterozygous (Tex101+/−) and homozygous (Tex101−/−) mutant mice were generated, and disruption of TEX101 caused no deleterious effect. Although Tex101−/− mice had normal mating ability, they were not able to produce offsprings, confirming the infertile phenotype. Interestingly, there was no significant difference in the weight and the histology of the testes between Tex101−/− and Tex101 wild type mice. Furthermore, sperm count, acrosome reaction efficiency, and sperm motility and viability parameters were examined and no significant differences were found. To conclude, although TEX101 is found on the sperm cell membrane during spermatogenesis, there is still no evidence supporting its essential role for normal spermatogenesis.

**Epididymal maturation**

Development of germ cells into testicular spermatozoa is followed by their transit to epididymis for post-testicular maturation. Although the morphology of testicular spermatozoa is similar to sperm cells, they are not fully mature and lack both the motility and the ability required to bind to zona pellucida (ZP) and interact with egg cells (35).

By the time testicular spermatozoa enter the epididymal tubule, post-testicular maturation starts, accompanied by transcriptional and translational silencing (36). Given this translational dormancy, it seems clear that epididymal maturation of sperm is driven mainly by post-translational modifications (PTMs) (37;38). Modifications to sperm surface occur while spermatozoa reside in the epididymal fluid (secreted by epithelial cells) (39). The milieu surrounding spermatozoa is changing along the three regions of the epididymis (caput, corpus, cauda), in terms of protein content and chemical composition. Due to these changes, there is a progressive modification of most of the testicular proteins that are bound to the sperm membrane. These modifications include: (i) sperm surface protein removal by proteolysis, (ii) sperm surface protein processing and maturation, (iii) redistribution of proteins on the surface of the membrane, and finally (iv) transient or permanent integration of epididymal proteins into sperm membrane (40).

It is well-known that GPI-anchored proteins are a major component of the specialized membrane microdomains, called lipid rafts (41). Proteins that are found to be localized in lipid rafts are implicated in the recognition of ZP, the outer layer of the oocyte, by spermatozoa (42). Shedding of some proteins from the sperm surface is crucial for the sperm binding ability, and is attributed to two possible scenarios: (i) GPI-anchored proteins are activated after they are released from the sperm membrane; (ii) GPI-anchored proteins are cleaved to facilitate the sperm-ZP binding (43).

TEX101 is one of these GPI-anchored proteins and is cleaved from the sperm surface and released into the seminal fluid. Although it was proven that TEX101 is enzymatically shed from the surface of epididymal sperm (25), the enzyme involved in that process has not been known until recently.
Interestingly, Kondoh et al. (43) demonstrated that another GPI-anchored protein, testicular angiotensin-converting enzyme (tACE), is also expressed on the cell surface of testicular sperm and is shed from the sperm membrane during epididymal maturation (44). Furthermore, tACE, catalyzed shedding of GPI-anchored proteins from the sperm surface and affected the egg-sperm binding ability during fertilization. Combination of these two findings led to the assumption that tACE was responsible for the release of TEX101 during the epididymal transit. Kondoh’s discovery regarding the novel GPI-ase activity of tACE came along with a number of ‘debate articles’ (45-48), that questioned the significance of the GPI-ase activity in reproduction, arguing that the well-known dipeptidase activity of ACE is crucial for sperm maturation and fertilization. A few years later, the same group (49) concluded that tACE had both GPI-ase and dipeptidase activities (43;47) which are required for sperm-ZP interaction. The dipeptidase activity takes place in the epididymis (50), and angiotensin II, the product of dipeptidase action, maintains pH homeostasis of the epididymal fluid (51). All in all, the dipeptidase activity of ACE indirectly supports its GPI-ase activity by maintaining an optimal pH (6.5) in the epididymis (49). All remaining doubts were unraveled recently by Fujihara et al. (28),

who proved with Ace−/− mouse model that TEX101 is indeed cleaved from epididymal sperm by ACE. Furthermore, the effect of ACE (both isoforms, somatic and testicular) on TEX101 was also examined in vitro, and a good correlation between in vitro and in vivo data was found. Surprisingly, the removal of TEX101 by ACE occurred in a substrate-specific manner, while other testis-specific GPI-anchored proteins, like SPACA4 (27), remained unaffected both in vivo and in vitro. Failure in epididymal maturation of TEX101 is associated with infertility, as it was demonstrated by in vivo experiments with Ace−/− and TEX101−/− mouse models (28;29).

**Role of soluble TEX101 in sperm-egg interaction**

Following the first stage of maturation in the epididymis, sperm undergoes additional remodeling events by the accessory fluids secreted before and at the time of ejaculation. When sperm enters the female genital tract, it has to pass the cervix and the uterus, and eventually reach the oviduct. Three major regions comprise the oviduct: the uterotubular junction (UTJ), the isthmus, and the ampulla. Each one of these regions seems to play a distinct role, and all together they contribute to a successful fertilization. During this transit, sperm interacts with a number of components of the uterine and oviduct secretions, and in this way complete maturation is achieved (52). Although a large amount of ejaculated spermatozoa is initially released in the female tract, only a very low percentage will manage to migrate up to the UTJ, and enter the oviduct, while most sperm is eliminated from the female tract by other mechanisms (53). The significance of sperm migration through the UTJ during sperm transport has been recently demonstrated by Tokuhiro et al. (54).

The UTJ connects the uterus with the oviduct and forms a natural barrier through which only
spermatozoa can pass (54,55). UTJ migration defect was always co-identified with failure of fertilization, associated with male infertility (55). There is evidence that sperm migration through the UTJ is under hormonal regulation, although the exact molecular basis of this transit is not well defined. Sperm from eight different knockout mice was analysed and showed UTJ transport deficiency. Null mice models for Ace, Adam1a, Adam2, Adam3, Calr3, Clgn, Tpst2, and Pdilt (56) revealed infertile phenotypes associated with sperm transport deficiencies. Surprisingly, proteomic analysis of all the mutant mouse lines mentioned, demonstrated that ADAM3, a sperm surface protein, is always absent or located incorrectly in the detergent-rich membrane domains (57). Although, initially, Shamsadin et al (58) reported that Adam3−/− mice had no effect in sperm migration, a subsequent study by Yamaguchi et al (59), revealed the importance of ADAM3 for the oviductal migration. ADAM3 is now recognized as an essential factor for sperm transport through the UTJ.

TEX101 was recently added to the list of molecules which regulate ADAM3 function. Fujihara et al (28), proved that lack of TEX101 was associated with infertile phenotype. They moved one step further and confirmed that infertile phenotype resulted from the defect of sperm migration into the oviduct. Interestingly, spermatozoa directly delivered into the oviduct were capable to fertilize the oocyte. Tex101−/− phenotype closely resembled the Adam3−/− phenotype (59). By investigating ADAM3 in Tex101−/− mice, it was shown that TEX101 interacts with ADAM3 on the surface of the testicular germ cells. Unlike the other proteins associated with ADAM3, TEX101 did not mediate the delivery of ADAM3 on sperm surface, since their interaction took place on sperm surface rather than in the endoplasmic reticulum (ER). During epididymal maturation of Tex101−/− spermatozoa, ADAM3 was processed normally, but it was not resistant to degradation, suggesting that TEX101 was responsible to protect ADAM3 from proteases present in the epididymal fluid. In the cases of Ace deficiency, both proteins remained on sperm surface, but they were dissociated, as it was indicated by their distribution. All these findings were independently confirmed by Li et al. (29), who also found that the absence of TEX101 during maturation in the epididymis affected ADAM4, ADAM5, and ADAM6 proteins. ADAM proteins, known for their adhesion ability (60), accumulated on the testicular sperm, but failed to mature in Tex101−/− sperm, leading to UTJ migration defect. All these facts allow us to conclude that cell adhesion properties of spermatozoa are as essential for sperm migration as their motility.

After passing through the UTJ, spermatozoa are binding to the epithelial cells of the isthmus, the second segment of the oviduct. It was proposed that such interaction mediated by the lectin-like proteins present on the sperm head and carbohydrate residues present on the oviductal epithelial cells (61), formed a reservoir of spermatozoa. Release of sperm occurs gradually (to allow only few spermatozoa to reach the oocyte), and is associated with sperm membrane remodeling, loss of binding molecules (capacitation), and hyperactivation of sperm motility (56).
The sperm release from the isthmus and its passage to the ampulla, the last region of the oviduct where fertilization takes place, is modulated by signaling between the cumulus-oocyte complex (COC), that is already located in the ampulla, and the epithelial cells of the oviduct (62). The cumulus is a layer of cells that surrounds and protects the oocyte. It is very important for the oocyte maturation, being the mediator of various signals. These cells interact not only with one another, but also with the oocyte (63). The ZP is a glycoprotein-enriched membrane surrounding the plasma membrane of the oocyte. It hosts a number of membrane glycoproteins responsible for sperm-binding upon fusion. It is of essential importance for successful fertilization, since failure of sperm-ZP binding is directly associated with infertility (64).

Gradient of temperature and chemoattractants such as progesterone guide the sperm towards the oocyte. Once spermatozoa are delivered, they have to cross the cumulus cells surrounding the oocyte (55;56). It was previously assumed that the acrosome reaction (AR), the release of hydrolytic enzymes and ZP penetration, is induced when sperm meets with ZP. However, it was recently demonstrated by Yin et al. (65;66), that the sperm-cumulus cross-talk modulates the AR while sperm is passing through the cumulus layer. Sun et al. (67), summarized recent data on the acrosome reaction, and the essential role of cumulus cells. Two potential models of AR with regard to its initiation were proposed. The first model suggests that AR occurs in the cumulus layer, and the hydrolytic enzymes released from spermatozoa facilitate the cumulus mass dispersion. This model, however, cannot explain the presence of acrosome-intact spermatozoa close to ZP. According to the alternative model, AR is initiated at the surface of ZP, and the “false-reacted” spermatozoa in the cumulus layer never reach it. Combining the information of all recent reports, it seems that ZP is not the only site of AR, but cumulus cells may also induce this process. A reasonable explanation could be that the acrosome reacted sperm may disperse the cumulus layer, so the intact sperm can pass through and bind to ZP. This means that a large number of sperm have to be sacrificed to facilitate the passage of a small population, which will participate in the fertilization process.

(Fig. 2 - cont’d) Figure 2. Genomic, transcriptomic and proteomic organization of TEX101.


(B) Structure of human TEX101 gene and its splice variants. Exons are represented with gray boxes.

(C) TEX101 protein structure. Signal peptide, post-translational modification sites and uPAR domain are highlighted in red, green and blue, respectively. GPI-anchored signal peptide is highlighted in yellow. SNV variants are marked with orange color.
TEX101 has been recently identified in the cumulus-sperm interaction during the AR (65). The authors support that TEX101 was detected on mature spermatozoa, in contrast to most of the reports demonstrating that it disappears after epididymal maturation (25;28;29). To our understanding, this implication of TEX101 in sperm-cumulus cross-talk, could occur by the involvement either of soluble TEX101 that is present in the seminal plasma, and accompanies sperm while they travel in the female tract, or by a low level of protein that may remain bound to spermatozoa. In a study by Yin et al. (65), TEX101 role in sperm-cumulus interaction was suggested. TEX101 cleavage and binding to the surface of cumulus cells leads to Ca\(^{2+}\) mobilization and progesterone production by the cumulus, events that are known to induce AR and facilitate the penetration of the cumulus layer. In an attempt to study the role of cumulus cells in AR, the same group focused on identifying candidate molecules that mediated TEX101 binding on cumulus cells and induced Ca\(^{2+}\) mobilization (68). Based on their previous study of cancer cells (69), they have proved that TEX101 could bind to uPA/uPAR complex and interfere with uPA activity. The uPA system is known to be involved in signal transduction, apart from its serine protease activity, and is associated with Ca\(^{2+}\) mobilization (68). Cumulus cells express uPA/uPAR (70) and it is possible that TEX101 could interact with this complex on cumulus surface, triggering Ca\(^{2+}\) mobilization and progesterone release required for AR.

**Concluding remarks**

During the past decade there have been intense efforts not only to characterize novel germ cell specific proteins, such as TEX101, but also to elucidate their functions. Certain observations related to TEX101 role and its interactions with other molecules both in male and female reproductive tract still need to be confirmed and validated. For example, reliable and accurate translation of mouse data into humans should be made. Since TEX101 may emerge as a powerful male infertility biomarker, understanding its functional role will be essential.

Accumulating evidence based on animal models indicates that TEX101 interacts with various molecules during post-testicular maturation of spermatozoa, and interaction with oocytes. Some members of the ADAM family, ACE, and the components of uPA system are potential interacting partners of TEX101, but there is still much research to be done for shedding light in all these processes, and their contribution in fertilization. Since testicular and epididymal ADAMs play a crucial role in fertilization (71), further studies on their interaction with TEX101 should provide information about relevant molecular mechanisms. Phylogenetic analysis of ADAM proteins shows their crucial roles in reproductive biology (72). Identification of the human ADAM orthologs would be the next step in this field of research. The interaction of TEX101 with the uPA/uPAR complex is a completely independent field of research that needs further investigation. It seems that TEX101 and the uPA system components are not only co-expressed by sperm cells, but also co-exist in the oviduct and...
are present during sperm-cumulus and sperm-ZP interaction. It has been reviewed (73) that uPA system components are secreted by Sertoli cells, and are involved in tissue remodeling during the last stages of spermatogenesis, facilitating the passage and release of spermatozoa from the Sertoli cell microenvironment within the seminiferous tubules. Different components of the uPA system are also present on the oocyte, cumulus cells, spermatozoa, and in the oviductal fluid where all the aforementioned meet and interact. Recently, Mondejar et al. (74), reviewed the regulation of fertilization by the uPA system components. They report that plasminogen/plasmin system is involved in sperm motility and AR, and its dysfunction leads to infertility in humans. It is also activated upon gamete interaction and it seems to regulate initially, the degradation and penetration of cumulus layer and ZP, through its localized proteolytic activity, and subsequently, the sperm entry in the oocyte, preventing polyspermy. If TEX101 indeed interacts with uPA, this would lead us to the conclusion that TEX101 is implicated in all the processes mentioned, and could mediate their regulation.

Bioinformatic analysis could be an alternative pathway to study TEX101 function and regulation. Apart from the proteomic level, modulation of TEX101 expression and regulation could be studied at the genomic, epigenetic, or transcriptomic level, using publically available databases of gene expression, such as COSMIC. This approach could lead to the identification of Single Nucleotide Variants (SNVs) that affect the expression and activity of TEX101. Additionally, the existence of testicular tissue-specific transcription factors (TF), or microRNAs would be of great importance in the regulation of TEX101 expression at the genomic and transcriptomic levels, respectively. All this information could eventually be useful in developing approaches for male infertility treatments, and development of male contraceptives.

Reference List


**Review criteria:**

The PubMed database was searched for full-text English-language articles published from 1942 to 2013 using the search terms “TEX101”, “NYD-SP8”, “ADAMs”, “male infertility and biomarkers”, “spermatogenesis”, “epididymal maturation”, “acrosome reaction”, “cumulus-oocyte complex”
“fertilization”. The majority of articles included were published after 2005. The reference lists of selected articles were searched for further relevant publications.

**Funding:**
This work was supported by the Canadian Institutes of Health Research (CIHR) and Physician’s Services Incorporated grants. **Author contributions:** C. G. S. wrote the manuscript, and all authors contributed to the design and revision of the manuscript. **Competing interests:** The other authors declare that they have no competing interests.

**Table 1. Potential male infertility biomarkers. OA, obstructive azoospermia; NOA, non-obstructive azoospermia**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Protein name</th>
<th>Clinical application</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECM1</td>
<td>ECM1_HUMAN</td>
<td>Extracellular matrix protein 1</td>
<td>OA vs. NOA</td>
<td>Drabovich et al.(19)</td>
</tr>
<tr>
<td>TKTL1</td>
<td>TKTL1_HUMAN</td>
<td>Transketolase-like protein 1</td>
<td>Azoospermia</td>
<td>Rolland et al. (75)</td>
</tr>
<tr>
<td>LDHC</td>
<td>LDHC_HUMAN</td>
<td>L-Lactate dehydrogenase C chain</td>
<td>OA/NOA vs. NS</td>
<td>Drabovich et al.(19)</td>
</tr>
<tr>
<td>PTGDS</td>
<td>PTGDS_HUMAN</td>
<td>Prostaglandin-H2 D-isomerase</td>
<td>OA diagnosis</td>
<td>Heshmat et al. (76)</td>
</tr>
<tr>
<td>PIP</td>
<td>PIP_HUMAN</td>
<td>Prolactin-inducible protein</td>
<td>Azoospermia</td>
<td>Davalieva et al. (77)</td>
</tr>
<tr>
<td>SPACA1</td>
<td>SPACA1_HUMAN</td>
<td>Sperm acrosome membrane-associated protein 1</td>
<td>Globozoospermia</td>
<td>Fujihara et al. (78)</td>
</tr>
<tr>
<td>SPAG11B</td>
<td>SG11B_HUMAN</td>
<td>Sperm associated antigen 11B</td>
<td>OA vs. NS</td>
<td>Drabovich et al.(19)</td>
</tr>
<tr>
<td>TEX101</td>
<td>TEX101_HUMAN</td>
<td>Testis-expressed sequence 101 protein</td>
<td>OA vs. NOA NOA subtypes</td>
<td>Drabovich et al.(19)</td>
</tr>
<tr>
<td>CAMP</td>
<td>CAMP_HUMAN</td>
<td>Cathelicidin antimicrobial peptide</td>
<td>OA vs. NOA</td>
<td>Drabovich et al.(19)</td>
</tr>
<tr>
<td>AZU1</td>
<td>CAP7_HUMAN</td>
<td>Azurocidin</td>
<td>Infertility</td>
<td>Kumar et al.(79)</td>
</tr>
</tbody>
</table>
Men with testicular failure, either primary or secondary, have been shown to be interested in fertility preservation. Spermatogonial stem cell (SSC) transplantation is currently being investigated as a treatment for this. Currently this experimental technique consists of cryopreservation of a testicular biopsy prior to cancer treatment, followed by optional in vitro expansion of SSCs and auto transplantation after cancer treatment. This technique may restore the pool of SSCs resulting in restoration of spermatogenesis. While this technique has not been applied to humans due to its highly experimental nature and concerns of malignant contamination, animal studies have been successful. While the offspring obtained from SSCs appear to be healthy in rodent models, there is relatively little data on any genetic and epigenetic changes that occur in either the transplanted SSCs or offspring. In humans, male germ cells undergo unique and extensive chromatin and epigenetic remodeling soon after their destiny as a spermatocyte has been secured. Errors in this remodeling may cause altered genetic information to be transmitted to offspring,
resulting in abnormalities. This is particularly pertinent for cancer patients as SSCs obtained from these men may have a predisposition for genetic instability even prior to starting gonadotoxic therapies. In this article, landmarks in the evolution of SSC transplantation are reviewed, along with presently known genetic, epigenetic, and imprinting abnormalities that may occur after in vitro propagation and transplantation.

**Background**

With the use of contemporary oncologic treatment protocols, survival is oftentimes a realistic outcome and the importance of fertility preservation has become more prominent as the majority of such men have been shown to desire children in the future. Treatment modalities such as chemotherapy and radiotherapy can have a profound and irreversible effect on fertility, and most patients will have transient or permanent loss of sperm production following therapy, with only 20-50% recovering spermatogenesis after therapy. While cryopreservation of sperm is a well-established option for post-pubertal men, options are limited for pre-pubertal boys in whom spermatogenesis has not yet started. Similarly, men with conditions resulting in primary testicular failure are in need of novel options for fertility preservation or restoration. Men with severe cases of sickle cell disease or beta-thalassemia major, which may be treated with chemotherapy for the eradication of bone marrow cells, followed by hematopoietic stem cell transplantation, may also end in a state of testicular failure. Clearly these men, in addition to oncologic patients, are a group for which there are currently very few fertility options, and in whom novel options are needed.

Spermatogonial stem cells provide the foundation for spermatogenesis in male. Men have a small number of spermatogonial stem cells (SSCs), also known as male germline stem cells. These cells reside at the base of the seminiferous tubules of the testes, and undergo self-renewing division, proliferation, and differentiation to produce sperm. In mice it is estimated that they constitute approximately 0.03% of the spermatogonia in the testis. In pre-pubertal testes, the absence of differentiating germ cells creates a relatively higher proportion of SSCs compared with adult testes.

These SSCs are responsible for continual sperm production and the transmission of genetic information from males to their progeny. SSCs are derived from gonocytes and divide into two populations. One is constantly active to maintain continuous spermatogenesis, while the other is quiescent under normal conditions but becomes active at the time of gonadotoxic injury. The regenerative potential of SSCs logically leads clinicians to consider options for fertility restoration, in both oncologic and non-oncologic men with testicular failure. As a result, there has been interest in identifying novel options for SSC preservation (Figure 1).
**Figure 1:** With the use of contemporary oncologic treatment protocols, survival is oftentimes a realistic outcome and the importance of fertility preservation has become more prominent. Spermatogonial stem cell (SSC) transplantation is currently being investigated as a treatment for this. Currently this experimental technique consists of cryopreservation of a testicular biopsy prior to cancer treatment, followed by optional in vitro expansion of SSCs and auto transplantation after cancer treatment. These SSCs may then be used with assisted reproductive technologies for fertility options in these patients. While the offspring obtained from SSCs appear to be healthy in rodent models, there is relatively little data on any genetic and epigenetic changes that occurs in either the transplanted SSCs or offspring.

**Historical aspects**

SSC transplantation was first performed by Brinster et al. in 1994. Spermatogonia from fertile mice were transplanted into the testes of infertile mice. Donor spermatogonia were able to colonize the seminiferous tubules of the recipients and initiate spermatogenesis in >70% of recipients, and up to 80% of progeny were sired by donor-derived spermatozoa. This group then applied this technique to cryopreserved donor murine testis cells, which resulted in restoration of spermatogenesis in the recipient seminiferous tubules. Schlatt et al. applied this technique to primates, with similar spermatogenic recovery in gonadotoxin-induced azoospermia treated with autologous testicular germ cells transplantation. These encouraging results suggest that these methods may be successfully applied to humans.
At present, re-establishing spermatogenesis after SSC transplantation is fairly well established in murine models. In mice, transplanted males are able to spontaneously mate and produce offspring and these offspring have been shown to be fertile \(^{13,14}\). However, compared with fertile controls, spermatozoa from SSC transplants have been shown to have a diminished fertilization capacity when used for in vivo conception or in vitro fertilization (IVF), but not intracytoplasmic sperm injection (ICSI), as these sperm have been shown to have a lowered motility \(^{15}\).

The ultimate goal of SSC transplantation is to yield healthy offspring. In this respect, studies have been conflicting but seem to overall suggest favorable outcomes. Early studies demonstrated that IVF conception (but not ICSI) with transplanted mouse SSCs resulted in reduced fertilization rates, delayed blastocyst developmental rates, and smaller litter sizes compared with controls \(^{15}\). Follow-up studies from this group on fetus preimplantation development demonstrated that blastocysts obtained after IVF with sperm from transplanted male mice again showed lower fertilization and developmental rates, as well as reduced numbers of inner cell mass cells and lower inner cell mass to trophectoderm ratios, implicating lower implantation potential. These differences were not seen after ICSI conception; both fertilization and development were normal when comparing controls with ICSI conceptions \(^{16}\). However, these results should be interpreted with caution due to technical differences in ICSI in mice versus humans. Finally, this group evaluated post-implantation development and by mating (spontaneous pregnancy) female mice with male mice after testicular stem cell transplantation. Litter sizes after testicular stem cell transplantation were decreased compared with controls and on the 17th gestational day fetuses demonstrated developmental retardation of a quarter of a day, but no major external abnormalities were observed. The live born pups were able to produce normal litter sizes, with developmentally normal pups, until the 3rd generation \(^{13}\). Live born pups were developmentally normal in this study, which has also been shown in other studies.

Short-term cryopreserved immature mouse or rabbit testicular tissue transplanted into mouse testes, allowed to mature, and then used for ICSI has been shown to result in grossly normal offspring \(^{17}\). Likewise, long-term (>14 years) cryopreserved testis cells from mouse used for ICSI or natural mating have been shown to result in grossly normal offspring \(^{18}\).

Of note, some studies seem to demonstrate reduced litter sizes, which may be due to lower sperm concentrations and poor motility, which have been demonstrated after SSC transplantation \(^{16}\). In addition, the work of Wu et al. \(^{18}\) demonstrates that there is some variability in the number of pups obtained per litter, regardless of if ICSI or natural mating is used. It is likely that offspring conceived by testicular stem cell transplantation have higher rates of spontaneous abortion, a form of natural selection against developmentally abnormal animals, which has never been assessed in the literature. Because small testicular biopsies do not contain sufficient SSCs to fully repopulate the testis after
transplantation, *in vitro* propagation of human spermatogonial stem cells will likely be necessary to obtain an adequate amount of cells for successful transplantation. In 2009, Sadri-Ardekani et al. reported their impressive results on human SSC culture and xenografting \(^{19}\). SSCs were cultured and propagated from testicular tissue from men undergoing orchidectomy as part of prostate cancer treatment, and then transplanted into the testes of immunodeficient mice. SSC numbers increased 53-fold within 19 days in the testicular cell culture and increased 18,450-fold within 64 days in the germline stem cell subculture. In 4 of 6 men, xenotransplantation demonstrated the presence of functional SSCs, even after prolonged *in vitro* culture. Similar experiments were then performed using testis tissue from 2 pre-pubertal boys being treated for Hodgkin’s lymphoma \(^{20}\). Xenotransplantation of cultured cells from these patients showed a 9.6-fold increase in the number of SSCs after 11 days of culture. Eight weeks after xenotransplantation, human SSCs were detected on the basal membrane of seminiferous tubules of recipient mouse testes. As it has been estimated that a 1300-fold increase in SSC number would be adequate to repopulate the adult human testis, based on these results, a 1 month period of culture will likely be sufficient. However, the effect of short versus long-term culture is not currently known.

The effects of *in vitro* culture and transplantation on the genetic and epigenetic characteristics of SSCs are still under investigation. Studies have demonstrated that cells with a high replicative potential often exhibit many abnormalities when they are maintained *in vitro*, in part due to chromosomal abnormalities and also from degenerative cellular changes that culminate in apoptosis \(^{21}\). While stem cells are considered to have special machinery to maintain their replicative potential without accumulating genetic abnormalities \(^{22}\), embryonic stem cells are sensitive to stresses and often exhibit abnormalities in chromosome structure and genomic imprinting patterns after culture \(^{23}\). It is possible that SSCs grown *in vitro* will have a higher risk of being genetically modified by exposure to growth factors and the maturation processes \(^{24}\). Embryonic stem cells have been shown to have a spontaneous mutation frequency that is approximately 100 fold below that of somatic cells \(^{25}\). However, *in vitro* culture of SSCs may induce genetic and epigenetic changes. Therefore, special attention will need to be paid to the genetic and epigenetic status of cells cultured or matured *in vitro* and after transplantation.

While the offspring obtained from SSCs appear to be grossly normal in rodent models \(^{17,18}\), there is relatively little data on any genetic and epigenetic changes that occurs in humans (Figure 1). In humans, male germ cells undergo unique and extensive chromatin and epigenetic remodeling soon after their destiny as a spermatocyte has been secured and during the differentiation process to become a mature spermatozoon \(^{26}\). Errors in this remodeling may cause altered genetic information to be transmitted to offspring, causing abnormalities.
Interestingly, the composition of the culture medium can influence the epigenetic imprinting and gene expression pattern on stem cells. However, there are limited studies of the effects of culture medium on SSCs. One group found that after culture of 2-cell mouse embryos to blastocysts, the imprinted H19 gene exhibited biallelic expression after embryo culture, and this loss of imprinting correlated with the loss of DNA methylation in the differentially methylated region implicated in H19 expression. Another group looking at the effect of different culture media on the behavior of offspring from 2-cell mouse embryos found behavioral differences (anxiety, locomotor activity, and spatial memory) in culture-derived mice, which could not be ascribed to differences in genotype. Other studies have found that the culture of mouse embryonic stem cells may give rise to fetal and offspring abnormalities, which may be linked to alterations in imprinted genes. However, all of these studies were on either 2-cell embryos or embryonic stem cells, not SSCs, and it is possible that embryonic stem cells are more sensitive than SSCs. One of the rare studies on SSCs found that SSCs can change their phenotype according to their microenvironment. Specifically, SSCs cultured on laminin demonstrated increased c-kit tyrosine kinase expression, which correlated with a distinct phenotype and increased renewal pattern. However, even in this study, the genetic and epigenetic fingerprint after culture was not examined. The susceptibilities of embryonic stem cells are likely reflective of their innate susceptibility to subtle changes in the maternal environment. However, it does seem that these stem cells have adapted advanced repair mechanisms, as well as the ability to proceed down an apoptotic route, to prevent the transmission of genetic or epigenetic damage to progenitors, which are generally similar to those found in postnatal stem cells in other self-renewing tissues.

**Genetic Changes**

The first group to look at genetic abnormalities after SSC transplantation was Goossens et al. In 2010, this group examined the karyotype of donor-derived spermatozoa using an array comparative genomic hybridization analysis. Numerical chromosomal aberrations could not be detected in spermatozoa from transplanted males. The karyotypes of first- and second-generation offspring were then evaluated, and all of these karyotypes demonstrated normal chromosome number. The few amplifications or deletions observed in chromosomes 1, 3, 4, 7, 12, 14 and 17 however, were also detected in the mother and therefore confirmed to be polymorphisms. While only 3 primary grafts were examined, the absence of abnormalities in the offspring is reassuring. Although this study is limited by the testing methodology, in that it fails to identify structural chromosome aberrations such as balanced reciprocal translocations of inversions (as these are not genomic losses or gains) and also ploidy variation, these results are nonetheless suggestive of genetic stability after SSC transplantation. In addition, in this study culture of the SSCs was not performed, and as such, the effect of culture on the genetic fingerprint could not be evaluated. Further studies are required to confirm these initial findings.
Embryonic stem cells seem to be sensitive to genetic alterations after long-term culture. Longo et al. showed that more than 70% of embryonic stem cells became aneuploid after only 25 cell passages, and these cells could no longer contribute to the germline by blastocyst injection. Interestingly, these abnormalities seemed to occur at specific chromosomal loci, which differ between species, with human embryonic stem cells susceptible to developing trisomy 17q and 12. However, long-term cultures (greater than 2 years, 139 passages) have demonstrated that SSCs maintain a euploid karyotype and androgenic imprint, even after ~10^8-fold expansion. After long-term culture, these SSCs were transplanted and the resultant spermatozoa used for ICSI to produce fertile offspring. The only genetic difference identified during culture was a gradual shortening of the telomeres, suggesting that these cells are not truly immortal. This shortening occurred despite the presence of telomerase activity, suggesting that SSCs may have different mechanisms for the regulation of telomere length as compared with embryonic stem cells. Nevertheless they do demonstrate remarkable stability, suggesting that SSCs have unique mechanisms to prevent the transmission of genetic alterations to offspring. In addition, the fact that even after prolonged culture they can develop into functionally intact spermatozoa with a relatively normal fertilization potential, with normal appearing offspring, would seem to suggest that no gross genetic alterations are happening. These findings suggest that SSCs seem to be slightly more stable than other mammalian somatic cells, which eventually undergo senescence after a limited number of cell divisions.

**Epigenetic Changes**

We now know that execution of the genetic code is not simply limited to the nucleotide base sequence of DNA but also includes epigenetic programming, heritable changes that affect gene expression. The sperm epigenome is unique because of the requirements for successful fertilization. Notably, there is the need for chromatin to be tightly packaged into the sperm head to facilitate motility and protect the sperm from the hostile environment of the female reproductive tract. During this process, most of the histones are replaced with protamines, and the remaining histones can have a unique pattern of chemical modifications to either facilitate or repress gene transcription. This unique “fingerprint” maintains the sperm in a state in which the key genes are “poised” for possible activation in embryogenesis. Sperm epigenetic abnormalities have been linked with multiple diseases including male factor infertility and poor embryogenesis.

Embryonic stem cells have been shown to have widespread variability their epigenetic state, and after nuclear transfer, variation in imprinted gene expression is observed in most cloned mice, even those derived from the same subclone. This suggests that the variability of gene expression reflects epigenetic changes that occurred during in vitro culture among sister cells derived from a single cell, demonstrating the instability of the epigenetic state of embryonic stem cells. However, in spite of
this epigenetic instability, many of these cloned animals survive to adulthood, and appear normal. However, whether the same can be applied to SSCs is still being determined.

In the study by Kanatsu-Shinohara, where SSCs maintained a euploid karyotype and androgenic imprint, even after ~10^8-fold expansion, altered methylation patterns were found. Specifically, the methylation patterns of the differentially methylated regions of three paternally methylated regions (H19, Meg3 IG and Rasgrf1) and two maternally methylated regions (Igf2r and Peg10) were examined in SSCs after 3, 12, 18 and 24 months of continuous culture. The authors found that the androgenetic pattern was not altered in the two cultures at 24 months, indicating that cells were epigenetically stable. By contrast, a study of multipotent germline cells by the same group after 3 months of culture after a freeze-thaw treatment had a different methylation pattern, with the Meg3 IG region being slightly undermethylated compared with those in the SSCs. These results seem to indicate that, similar to multipotent germline cells and embryonic stem cells, methylation patterns are somewhat variable in SSCs after culture and transplantation.

In a study by Goossens et al. SSCs in testicular cell suspensions from 5-7 day old mice were transplanted into the testes of genetically similar recipients and then allowed to mature for 4 months. Immunohistochemistry was used to look at a specific panel of epigenetic modifications known to be important for the fertilization potential of spermatozoa. The authors found that, in general, the epigenetic modifications were not different after grafting compared with data from adult control mice. Specifically, DNMT1 and DNMT3A expression (the enzymes catalyzing DNA methylation), the general methylation status and the stage-specific histone modifications H3K4me3, H3K9ac, H4K12ac and H4K16ac were not different from fertile adult controls. The only difference identified was in the stage-dependent expression of H4K5ac and H4K8ac in elongated spermatids, which was altered after SSC transplantation. This difference may be a true difference in expression, but may also be due to an inability to detect these marks due to the highly condensed chromatin in these relatively mature gametes. However, the full implications of this difference are still unclear as the specific function of these histone modifications is yet unknown.

Genomic imprinting is a unique epigenetic process by which certain genes can be expressed in a parent-of-origin specific manner. The effect of SSC transplantation on imprinting is still being determined, with some studies suggesting that there transplantation does result in imprinting differences, and others suggesting that it does not. One study has demonstrated findings suggestive of altered imprinting after SSC transplantation in rodents. First generation fetuses obtained after SSC transplantation lower in size and weight compared with controls, and demonstrated developmental retardation. However, these pups were able produce normal litter sizes and weight offspring for the next two subsequent generations. Since subsequent generations did not demonstrate these
abnormalities, the authors postulated that the developmental retardation was due to an imprinting disorder, however it is noteworthy that no genetic testing on these pups was performed and this speculation is based on the gross findings of litter size, weight, and development.

Studies on imprinting status after SSC transplantation are limited, but would suggest that imprinting is not altered. In 2009 Goossens et al. examined the DNA methylation pattern in a paternally methylated gene (Insulin-like Growth Factor-2 \((Igf2)\)), a maternally methylated gene (Paternally Expressed Gene-1 \((Peg1)\)) and a non-imprinted gene \((\alpha\text{-Actin})\) \(^{39}\). For the three genes studied, no alterations in the DNA methylation patterns of spermatozoa obtained after SSC transplantation, nor in first and second generation offspring were observed. Likewise, first and second generation offspring developed normally, having similar length and weights as compared with controls. While this group only looked at 3 genes, it is impossible to know how generalizable these results are.

In a study in which embryonic male germ cells were expanded into SSCs, the resultant cells repopulated seminiferous tubules and produced spermatozoa \(^{40}\). However, the offspring showed growth abnormalities and were defective in genomic imprinting. The imprinting defect persisted in both the male and female germlines for at least four generations. Moreover, germ cells in the offspring showed abnormal histone modifications and DNA methylation patterns, suggesting that fetal germ cells expanded into SSCs lose the ability to undergo epigenetic reprogramming by \textit{in vitro} culture.

Interestingly, it does seem that that the genetic background of the donor cells may have an influence on the incidence of methylation errors \(^{39}\). This is of concern when contemplating the use of SSC transplantation in human cancer survivors; SSCs obtained from cancer patients, even prior to starting gonadotoxic therapies, may have a predisposition for genetic instability. These patients are, by definition, more genetically unstable than non-cancer patients, and there is evidence to suggest that relaxation or loss of imprinting could represent a new epigenetic mutational mechanism in carcinogenesis \(^{41}\). This instability may translate into more genetic and epigenetic abnormalities after transplantation, which may, in turn, be passed on to offspring. This is particularly concerning given some evidence that alteration of SSCs to induce self-renewal machinery can induce the development of seminomatous tumors \(^{42}\). While currently there is no evidence to either support or negate this, it is nonetheless an important consideration.

In addition, the effect of cryopreservation on genetic and epigenetic alterations will need to be elucidated. It is possible that the freezing process may alter the functional epigenetic machinery, and studies should be undertaken to investigate this. One recent study in zebrafish found that cryopreservation produced a decrease in most of the studied transcripts \((cxcr4b, pou5f1, vasa\text{ and } sox2)\) and upregulation of heat shock proteins \((hsp70, hsp90)\), results which were corroborated in
human spermatozoa. These data suggest that genetic alterations caused by cryopreservation should be studied in detail in order to ensure the total safety of the technique 43.

Controlled slow-freezing of testicular tissue is currently offered to pre-pubertal boys when fertility is threatened by gonadotoxic therapies 44, as it has been shown to allow for survival of spermatogonia 45,46.. Cryopreservation conserves tissues by suspending the metabolic activity of the cell, but during cooling and warming, cells are exposed to different forces (thermal, chemical and mechanical), which may interfere with their normal functioning 47. At present, controlled slow-freezing with the cryoprotectant, dimethyl sulfoxide (DMSO) is the method most commonly used to cryopreserve immature testicular tissue 48. The major advantages to controlled slow-freezing are that protocols are well-established, and relatively large samples can be frozen, up to 2 x 4 x 12 mm³ 49. However, it does result in ice crystal formation, which may be damaging to SSCs, and it requires expensive computerized equipment and the process is time-consuming 48.

Vitrification has recently been explored as an alternative cryopreservation option. Samples are cooled at ultrafast rates in liquid nitrogen using high concentrations of cryoprotectants in order to remove a high proportion of cellular water and avoid ice crystal formation, minimizing cellular damage 49. Early studies have shown that vitrification is faster and less expensive than slow-freezing (only a relatively inexpensive -80°C freezer is required), as well has the potential to preserve the integrity of seminiferous tubules and maintain the long-term organotypic survival and proliferation of SSCs due to the absence of ice crystal formation 50. Vitrification has been applied to testicular tissue in immature mice 51, immature non-human primates 52, and immature humans 51,53. One potential disadvantage is that currently vitrification can only be performed successfully on very small sample sizes, up to 5 x 1 x 1 mm³ 49. One of the primary concerns regarding vitrification is biological safety and sterilization, as samples are placed directly in contact with liquid nitrogen, which may mediate the transfer of pathogenic agents 54.

So far, data comparing slow-freezing cryopreservation with vitrification are few, and most seem to suggest that the methods have quite similar outcomes with respect to maintaining pre-pubertal testicular tissue cell ultrastructure, tubular morphology, and tissue function 51,53,55-57. However, at this point, controlled slow-freezing with the cryoprotectant DMSO should still be considered the standard, with vitrification considered a promising technology.

With respect to alterations in genetic and epigenetic fingerprints after cryopreservation, there is little available data. Cryopreservation has been shown to cause DNA fragmentation in spermatozoa 58,59, an effect which seems to be more pronounced in infertile men as compared with fertile men 58,60. This may be because oligozoospermia, teratospermia, and asthenospermia have all been associated
with abnormal methylation of several imprinted genes \[^{61-63}\]. Recent data looking at the short- and mid-term impact of cryopreservation on DNA methylation of different spermatozoal genes showed that 3 maternally imprinted genes (LIT1, SNRPN, MEST), 2 paternally imprinted genes (MEG3, H19), 2 repetitive elements (ALU, LINE1), 1 spermatogenesis-specific gene (VASA) and 1 gene associated with male infertility (MTHFR) in semen samples demonstrated no alteration in methylation pattern regardless of duration of cryopreservation \[^{64}\]. To our knowledge there are no studies of the effect of vitrification on human sperm, likely due to its somewhat experimental nature at this point.

Finally, the choice of intratesticular tissue grafting versus \textit{in vitro} culture and the resultant genetic and epigenetic effects, warrants investigation. While \textit{in vitro} culture is obviously more convenient, intratesticular tissue grafting might be the better choice for fertility restoration because restoration of the stem cell niche might influence epigenetic patterns.

**Conclusions**

While other germline cells often acquire genetic and epigenetic changes \textit{in vitro}, SSCs appear to maintain a state of relative genetic stability. These cells have been shown to retain a constant and stable growth rate after 2 years in culture \[^{21}\], and subsequently maintain functional stability and were able to produce fertile offspring and these offspring displayed normal karyotypes and unmodified methylation levels in three investigated genes \[^{32,39}\]. Of note, some studies have demonstrated that offspring obtained from grafted SSCs have been shown to result in reduced litter size \[^{15}\], altered preimplantation development \[^{16}\], be smaller in size and lower in weight compared with control fetuses, and also have developmental retardation \[^{13}\]. However, others have demonstrated offspring that are grossly normal \[^{17,18}\]. In our opinion it seems likely that fetuses obtained from SSCs will have a slightly higher rate of spontaneous abortion, but that live birth progeny will likely be developmentally normal and have a normal reproductive potential. Current animal studies are limited by the small number of animals and offspring studied. In addition, it should be noted that analysis of gene expression and DNA methylation patterns is currently limited to only a selection of imprinted genes and comparative genomic hybridization is not able to detect small genetic changes. In addition, DNA methylation has not been investigated in human cultured SSCs. More research on the epigenetic level is certainly warranted before these techniques are safe for human application. In addition the optimal testicular tissue cryopreservation conditions need to be further investigated, as the technique itself may induce genetic and epigenetic changes. This is particularly true for cancer patients as SSCs obtained from cancer patients, even prior to starting gonadotoxic therapies, may have a predisposition for genetic instability, which may translate into more genetic and epigenetic abnormalities after transplantation, which may in turn be passed on to offspring.
References


18. Wu, X., et al. Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than


38. Goossens, E., Bilgec, T., Van Saen, D. & Tournaye, H. Mouse germ cells go through typical epigenetic


Mechanisms of androgen-independent prostate cancer
Punit Saraon1,2, Andrei P. Drabovich1,2, Keith A. Jarvi3, Eleftherios P. Diamandis1,2,4*

1 Samuel Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada
2 Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada
3 Department of Surgery (Division of Urology), Mount Sinai Hospital, Toronto, ON, Canada
4 Department of Clinical Biochemistry, University Health Network, Toronto, ON, Canada

ARTICLE INFO

*Author for correspondence
E.P. Diamandis, M.D. PhD
Mount Sinai Hospital, Joseph & Wolf Lebovic Ctr.,
60 Murray St [Box 32]; Flr 6 – Rm 6-201
Toronto, ON, M5T 3L9, Canada
Tel: 416-586-8443; Fax: 416-619-5521;
e-mail: ediamandis@mtsinai.on.ca

Running Title:
Androgen-independent prostate cancer

Keywords:
Androgen-independent prostate cancer,
Castration-resistant prostate cancer,
Prostate cancer progression,
Prostate cancer,
Androgen receptor,
Androgen-independence

Conflicts of Interest: None

ABSTRACT

Prostate cancer is the second leading cause of cancer-related deaths among men in North America. Almost all prostate cancers begin in an androgen-dependent state, so androgen deprivation therapy is administered and results in improved clinical outcomes. However, over time, some cancerous cells are able to survive and grow during this treatment, resulting in androgen-independent prostate cancer. At this point, the disease is fatal, as there are no effective targeted therapies available. Most prostate cancer tumors require androgen receptor (AR) signalling for survival. During the progression to androgen-independence, this signalling cascade has been found to be altered at many levels within prostate cancers. Mechanisms that enhance AR signalling during androgen deprivation include: AR gene amplifications, AR gene mutations, changes in expression of AR co-regulatory proteins, changes in expression of steroid-generating enzymes, ligand-independent activation of AR via ‘outlaw’ pathways, and AR-independent pathways that
become activated, termed ‘bypass’ pathways. One or more of these aforementioned changes can lead to prostate cancer cells to gain androgen-independent properties. Understanding the molecular alterations that occur during this process will allow for improved therapeutic strategies to target key molecules and pathways important for this progression.

Introduction

Prostate cancer is the most commonly diagnosed and second leading cause of cancer-related deaths among men in North America [1]. Statistically, one in six men will develop some form of prostate cancer in their lifetime, and interestingly, almost 50% of men have tumors within their prostate upon autopsy. This indicates that prostate cancer is a slow growing cancer that may not directly lead to morbidity. However, there are aggressive forms of the disease that ultimately lead to fatal outcomes. Prostate cancer is initially diagnosed with a physical digital rectal examination followed by a serum prostate-specific antigen (PSA) test [2, 3]. PSA is one of the best known cancer biomarkers available, however, has its own limitations as well. PSA is also elevated in other pathological conditions of the prostate including benign prostate hyperplasia and prostatitis. In addition, PSA does not provide powerful prognostic potential, as it is unable to discriminate between indolent and aggressive forms of prostate cancer [2, 3]. Patients presenting with positive PSA tests undergo a prostatic biopsy, where histological assessment of prostatic tissue is analyzed to determine whether cancer is present or not [2, 3]. Not surprisingly, 75% of positive PSA cases do not present with cancer, indicating the lack of specificity of the marker. It is for these reasons, that active research is being pursued to identify additional biomarkers that either complement serum PSA and/or discriminate between indolent and aggressive forms of the disease. One of the best prognostic indicators for prostate cancer is Gleason score (GS), which characterizes the glandular architecture of the prostate based on a histological score that represents the level of ‘de-differentiation’ of the cancer [4, 5]. Briefly, GS is comprised of two numbers that represent the common Gleason patterns ranging from 1 to 5, where 1 represents well differentiated cellular architecture and 5 represents an aggressive un-differentiated one. It is now well accepted that the transition from a pattern 3 to 4 represents the development of aggressive prostate cancer [6].

Androgen receptor AR signalling

The AR is a protein that is able to bind to androgens and act a transcription factor to regulate a diverse array of genes. Most endogenous androgens are generated via the hypothalamus-pituitary-Leydig cell axis [17]. There is also a very small amount of androgens generated by the adrenal glands. The hypothalamus releases LHRH which is in turn promotes the pituitary gland to release LH, which is able to bind to Leydig cells of the testes and promote testosterone production (the most common androgen) [17]. Once generated, testosterone is able to enter the bloodstream and localize to
effector tissues including the prostate. Free circulating testosterone is able to enter prostate cells, where it is converted to its more active metabolite, dihydrotestosterone (DHT), by the 5-alpha reductase enzyme [17]. DHT within the prostate cell is then able to bind to cytosolic AR, which then undergoes a conformational change and translocates into the nucleus [18]. In the nucleus, AR acts as a transcription factor, binding to specific DNA sequences known as androgen responsive elements (ARE), leading to the expression of a variety of genes [19]. The AR protein consists of three major domains: ligand-binding domain, DNA-binding domain, and the N-terminal domain. The ligand binding domain is integral for the binding of DHT and testosterone to AR. The DNA-binding domain, as its name suggests, is responsible for the interaction of AR with specific ARE within the DNA in the nucleus. The N-terminal domain has also shown to be very important for AR signalling, as inhibition of this domain results in decreased AR transcriptional activity [20]. Many genes including PSA, are regulated by AR signalling.

AR signalling is absolutely critical for normal prostate cell function, so it’s not surprising that prostate cancer cells also require its signalling for survival. Almost all prostate cancers begin in an androgen-dependent state, where AR signalling is predominant for cancerous growth and proliferation. When ADT is administered, many of the cancerous as well as normal cells undergo cell death due to the reduction of a crucial signalling cascade [21]. However, over time, some cancerous cells are able to manifest specific molecular and cellular changes in order to activate AR signalling, irrespective to whether there is a blockade of androgens. Many mechanisms as to how this is achieved has been studied, including amplification and mutation of the AR gene, changes in expression of co-regulatory proteins, alterations in steroidogenic producing pathways, and activation of the AR via ligand-independent manners known as ‘outlaw’ pathways [14, 22-25]. In addition, recent interest has shifted outside of focusing particularly on the AR pathway, where the much active research is looking at identifying novel ‘bypass’ pathways (AR-independent pathways) for the development of AIPC (Figure 1). Many of these ‘outlaw’ and ‘bypass’ pathways will be further discussed.

AR gene amplifications:

A common way for cells to compensate for the loss of a key cellular pathway is the over-activation or expression of an integral protein within that pathway. In the case of AR signalling and prostate cancer, cancerous cells have been shown to over-express AR at both the mRNA and protein level in vitro and in vivo models [26-28]. Studies have shown that almost 25-30% of AIPC contain AR genetic amplifications [28]. Such genetic amplifications have not been observed in cases where ADT was not administered, providing further evidence that AR gene amplification is a common by-product of hormone therapy. Elevated AR expression at both the mRNA and protein expression has been shown to sensitize cancer cells to lower-than-normal concentrations of androgens [29]. Although ADT is
efficient at reducing endogenous androgen production, it does not completely stop its production; so theoretically, any minimal amount of androgens still present can activate the AR. With excessive AR expression via genetic amplification, even small amounts of androgens can activate the protein resulting in downstream signalling. Interestingly, AR overexpression at the protein and mRNA level has also been observed in the absence of AR gene amplifications, indicating there could be other modes of regulation of AR including epigenetic factors and miRNAs [19]. It is clearly evident based on clinical studies that AR overexpression is a common event that occurs during the development of AIPC, and therefore therapies able to focus on particularly blocking its expression or signalling cascade could potentially be utilized for clinical use.

Figure 1. Prostate cancer diagnosis and treatment.

After an initial physical digital rectal examination followed by a positive PSA test, a prostatic biopsy is examined. Based on histology, the biopsy will either confirm no cancer or cancer, and based on the Gleason scoring system, prostatic cancerous cells will be assessed a Gleason score. Gleason score 6 and less cancers do not require any curative treatments and undergo active surveillance, whereas Gleason 7 or higher cancers are normally treated with radical prostatectomy and androgen deprivation therapy. Patients often regress to androgen-independent prostate cancer, where there are no effective targeted therapies available.
AR gene mutations:

Along with genetic amplifications, another mode of aberrant AR signalling could result due to genetic mutations of the AR gene itself. As previously mentioned, AR consists of three major domains, and specific mutations in each of these domains could have a large impact on the function of the protein. The AR is a gene located on the X chromosome, and loss of function results in a condition known as androgen-insensitivity. Over the years, many novel mutations have been identified within the AR gene. The McGill androgen receptor database (http://androgendb.mcgill.ca), has compiled a list of all the AR mutations identified to date, as well as the specific domains they occur within. We will only focus on the most frequent mutations found in AIPC patients.

The frequency of AR mutations are very low (up to 4%) in patients with early stage tumors [30]. However, in late stage/aggressive tumors, the frequency is elevated to 10-20% in cases of AIPC [31]. This further supports the notion that AR mutations are a common mechanism that prostate cells may utilize to gain androgen-independent properties. The first reported AR gene mutation was in the hormone-dependent LNCaP human prostate cancer cell line derived from a lymph node metastasis [32]. The LNCaP cell line contains a unique missense mutation at codon 877, resulting in the amino acid threonine being substituted to an alanine [32]. Interestingly, this mutation occurs within the ligand-binding domain of the AR protein, and has been shown to reduce the ligand specificity of the protein, whereby other molecular such as progesterone, estrogens and many antiandrogens can also activate the protein. Such a mutation would be highly beneficial for a cancerous cell, as during ADT, they no longer require androgens, but instead, can utilize other common circulating hormones or molecules to activate the AR protein and its downstream signalling cascade. Studies have shown that this specific T877A is very common during AIPC [33]. Localized androgen-dependent cancers have been shown to have less AR gene mutations, whereas tumors that have metastasized and become more aggressive harbour greater number of mutations [30, 31, 34]. An interesting study by Marcelli et al., showed that mutations were found in 8 of 38 patients with lymph node metastasis who were treated with ADT, whereas no such mutations were observed in patients that did not undergo therapy [35]. Other common AR mutations include H874Y, V715M, L701H+T877A and Y741C [31, 34, 36, 37]. All these mutations are also within the ligand-binding domain of the protein, resulting in either broadened ligand specificity or constitutive protein activity.

In addition to AR gene mutations, recent interest in AR splice variants has also been observed in AIPC. In a study by Guo et al., three novel AR splice variants were identified in AIPC, all lacking the ligand-binding domain [38]. Subsequent studies assessing the exact role of these splice variants and their activity need to be further addressed, however, they present another interesting mechanism which prostate cancer cells can potentially utilize to gain androgen-independent properties. Potential drugs that could inhibit such splice variants could represent a novel area of therapeutic intervention.
Altered AR co-regulators:

As mentioned, the AR is a transcription factor, capable of binding to specific DNA sequences (AREs) to induce or inhibit the transcription of a variety of genes. As a result, there are many co-regulatory proteins that are able to bind to AR and either activate (co-activators) or suppress (co-repressors) gene expression of downstream target genes. Alterations in the expression of any of these co-regulatory enzymes could have an impact on AR signalling, and be a possible mechanism for cells to gain androgen-independence. There have been over 170 documented proteins that have been shown to act as coregulators with AR [39]. Any shift in the balance of these proteins can have a drastic effect on the overall expression of AR regulated genes. Some of the more well studied coactivators of AR signalling include TIF2, GRIP1, SRC1, and a broad group known as AR-associated (ARA) proteins [40, 41]. Gregory et al., found that levels of TIF2 and SCR1 were elevated in AIPC samples that also had increased AR expression [42]. On the other hand, two of the most common AR co-repressors include NCoR and SMRT [43]. Both of these proteins are able to recruit histone deacetylases, resulting in chromatin condensing and reduced transcriptional activity [43]. During the development of AIPC, both these co-repressors have been shown to be down-regulated, resulting in increased AR-mediated transcriptional activity [44].

Aberrant androgen-generating enzyme pathways:

The main purpose of ADT is to block/reduce endogenous androgen activity. This can either be achieved via blocking the androgen production pathways or by directly inhibiting androgen affinity towards the AR. Many AIPC patients have aberrant signalling in the precursor pathways that generate androgens, usually in the form of over-production to compensate during ADT [24]. Many of the current androgen-blocking agents are directed towards inhibiting the hypothalamus/pituitary/Leydig axis, and are very efficient as this is the major androgen generating mechanism of the body. However, the adrenal glands are also capable of generating low concentrations of androgens, and blockade of this pathway may also be required for efficient androgen deprivation [45]. In addition, recent studies have shown that tumor cells themselves are capable of generating their own androgens via de novo synthesis [46]. Such a mechanism is very intriguing, as cells that are undergoing ADT can activate certain cellular enzymes and pathways to produce their own endogenous testosterone to active AR. In particular, many enzymes within the cholesterol biogenesis pathway, a precursor to androgen production, have been shown to be elevated in tumor cells [47]. Essentially, prostate cancer cells may be utilizing various alternate pathways to produce endogenous androgens to activate the AR signalling cascade, during times of androgen deprivation.
Outlaw pathways:

The AR protein is preferentially activated by endogenous androgen ligands. However, like other steroid hormone receptors, AR has also been shown to be activated in ligand-independent mechanisms referred to as outlaw pathways. Cytosolic AR has been shown to interact with many molecules in a nongenomic role, and activate various pathways. Various growth factors, cytokines, kinases and other proteins have been shown to interact with and activate AR in a ligand-independent manner.

Some of the most common growth factor proteins that interact with AR include IGF1 and EGF. IGF1 has been extensively studied with respect to AR signalling, as it has been shown to prolong it, even in the absence of androgens [36]. Interestingly, in the presence of antiandrogens, AR signalling is abrogated, indicating that IGF1 and AR have a direct interaction with one another [36]. IGF1 has also been shown to induce the expression of AR co-activator TIF2, indicating another indirect way to potentiate AR signalling [48]. EGF is another growth factor able to induce AR signalling in a ligand-independent way [36]. The EGF-regulated gene, SPINK1, has been shown to be elevated in cases of aggressive prostate cancer, indicating the importance of this growth factor with respect to prostate cancer pathogenesis [49, 50].

In addition to growth factors, various cytokines have also been shown to interact with AR. Specifically, (NF)-kB signalling, which activates the cytokines IL-6 and IL-8, has been found to be elevated in many cases of AIPC [51]. Increased (NF)-kB signalling was shown to increase AR signalling in the LNCaP prostate cancer line, and this activation was halted after inhibition of (NF)-kB signalling [51]. In addition, both IL-6 and IL-8, like IGF1, were shown to directly bind and activate AR, as inhibition via antiandrogen treatment abolished this activation [51].

Receptor tyrosine kinases (RTK) are important signalling molecules that have been shown to be altered in various pathological conditions, especially cancers. One highly studied RTK that has been found elevated in AIPC is HER2/ERBB2 [52, 53]. This protein is overexpressed in many AIPC cell lines in vitro, as well as in many xenograft models of androgen-independence. The overexpression of HER2 in prostate cancer cells can directly activate AR signalling, and unlike IGF1, IL6 and IL8, in the presence of antiandrogens, this signalling is not disrupted [52]. This potentially indicates that activation of AR signalling via HER2 may be independent of the ligand binding domain. Other RTKs that have been implicated to the development of AIPC are the IGF and EGF receptors [10]. These receptors activate essential downstream survival pathways including AKT, MAPK, and STAT, many of which are also dysregulated in AIPC [10].
Bypass pathways:

Thus far we have discussed mechanisms of AIPC progression through AR signalling. Although alterations to various aspects of AR signalling are integral during the progression to androgen-independence, it is also important to mention other pathways, the AR-independent ones, which also become altered during prostate cancer progression. Such pathways are referred to as bypass pathways. Many of the outlaw pathways mentioned earlier can also be classified as bypass pathways, as signalling through various RTKs and receptors are able to activate a diverse and unique signalling cascade that is independent of AR signalling. For example, the IGF1 ligand once bound to its receptor, IGF receptor, is able to transduce a signalling cascade that can activate the expression of genes that are able to promote cellular growth and proliferation, allowing cancer cells another mechanism for enhanced survival. Many bypass pathways act through RTKs that activate a diverse range of kinases including MAPK/Ras/Raf, which in turn can activate various transcription factors such as (NF)-kB and c-MYC, resulting in changes that influence cell cycle regulation and cellular proliferative properties [54, 55].

One major signalling cascade that is altered during AIPC is the Akt pathway [56]. Akt signalling can act both in an outlaw mechanism via activation of AR, or independently through other intermediate proteins that affects major cellular processes including apoptosis and proliferation in prostate cancer cells [56]. Another highly studied molecule in prostate cancer pathogenesis, PTEN, which is a proapoptotic protein that inhibits Akt signalling, has been found to be decreased in expression in many cases of AIPC, further indicating the importance of Akt signalling [57].

Apoptosis is an important mechanism that cells utilize to undergo cell death in order to ensure stability. During androgen deprivation, many prostate cancer cells undergo apoptosis, so a mechanism a cancerous cell could utilize to ensure survival is the activation of proteins that inhibit this process, known as anti-apoptotic proteins. Once such protein, Bcl-2, has been found elevated in many cases of aggressive AIPC [58, 59]. A study conducted by Liu et al., demonstrated that when Bcl-2 expression was blocked in AIPC xenografts, the resulting tumors were smaller than ones that did not have Bcl-2 expression inhibited [59].

Recent progress:

Extensive research has been conducted on the development of AIPC with regards to aberrations in various signaling pathways, most notably AR signalling and others already mentioned (Table 1). In addition to abnormal signalling pathways, other factors including epigenetic alterations and miRNA regulation are also being studied to understand the progression of AIPC.

Epigenetics is an important mode of regulation that cells use to ensure proper gene expression. Changes in cellular epigenetic signatures are common developments during cancer development.
Some common epigenetic alterations during AIPC development include changes in genes involved in cell cycle control, cell invasion, cellular architecture, DNA damage repair, tumor-suppressors and oncogenes. The most notable epigenetic alteration in AIPC is GSTP1 promoter methylation, with a frequency of 70-100% in prostate cancer DNA samples [60]. Recently, RGS2 promoter hypermethylation was also observed in AIPC, as it allows cells to gain a more aggressive androgen-independent phenotype [61].

<table>
<thead>
<tr>
<th>Signalling Pathway</th>
<th>Receptors Involved</th>
<th>Consequence of Pathway</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt Pathway</td>
<td>Various Receptor Tyrosine Kinases</td>
<td>Decreased Apoptosis and Increased Survival</td>
<td>10, 54</td>
</tr>
<tr>
<td>IGF Pathway</td>
<td>IGF Receptor</td>
<td>Increased Cell Growth and Proliferation</td>
<td>10, 36, 48</td>
</tr>
<tr>
<td>EGF Pathway</td>
<td>EGF Receptor</td>
<td>Increased Cell Growth and Proliferation</td>
<td>10, 36</td>
</tr>
<tr>
<td>AR Pathway</td>
<td>Androgen Receptor</td>
<td>Increased Survival and Growth</td>
<td>54</td>
</tr>
<tr>
<td>JAK/STAT Pathway</td>
<td>IL6 Receptor</td>
<td>Increased Survival and Growth</td>
<td>10, 51, 54</td>
</tr>
<tr>
<td>MAPK Pathway</td>
<td>Various Receptor Tyrosine Kinases</td>
<td>Increased Proliferation and Decreased Apoptosis</td>
<td>10, 54</td>
</tr>
<tr>
<td>PKC Pathway</td>
<td>TGFβ Receptor</td>
<td>Increased Proliferation and Decreased Apoptosis</td>
<td>54</td>
</tr>
</tbody>
</table>

Another mode of regulation that has recently been studied for the progression of AIPC is via miRNAs. Various miRNAs have been shown to promote this transition, most notably miR-221, miR-222, mir-125b and miR-146 [62]. Interestingly, miR-221, miR-222 and miR-125b have been found to be over-expressed in AIPC, whereas miR146 has been shown to be down-regulated [63-66].

Further investigation is currently being pursued in several of these fields to identify aberrantly expressed genes that are involved in AIPC progression, in the hopes of generating potential useful clinical biomarkers and treatments.

Conclusion:
Prostate cancer is a curable disease if detected early in an indolent form (ie. radical prostatectomy); however, aggressive forms require ADT which ultimately results in the development of AIPC. Once at
this stage, there are no targeted therapies, and cells will likely have metastasized to distal sites and eventually results in fatal outcomes. Understanding the molecular alterations during the progression of prostate cancer to an androgen-independent state is of utmost importance in order to first understand the disease, and second, to generate effective targeted treatments to enhance patient care. Much research has heavily focused on AR signalling, a definite key player in the process, however, further work identifying other novel molecules and pathways are currently being pursued. Of the aforementioned mechanisms of androgen-independence, AR gene amplification and mutations still remains one of the better accepted modes for this transition. For this reason, many more sensitive inhibitors of AR are being developed and tested in patients with the hopes of alleviating symptoms. In addition, recent interest in blocking circulating adrenal androgens has also provided an interesting avenue of therapeutic intervention for this disease. However, further studies are being conducted to assess the potential of such therapies. Once we are able to fully understand the molecular pathogenesis of this disease, the next steps will be to target key proteins such as AR and other important pathways in order to provide specific therapeutic intervention that can result in decreased morbidity.

References:


32. Wilding, G., M. Chen, and E.P. Gelmann, Aberrant response in vitro of hormone-responsive prostate cancer


Prostate-specific antigen (PSA) screening and new biomarkers for prostate cancer (PCa)

Carsten Stephan\textsuperscript{a,b}, Harry Rittenhouse\textsuperscript{c}, Xinhai Hu\textsuperscript{a}, Henning Cammann\textsuperscript{d}, Klaus Jung\textsuperscript{a,b}

\textsuperscript{a}Department of Urology, Charité - Universitätsmedizin Berlin, Germany
\textsuperscript{b}Berlin Institute for Urologic Research, Berlin, Germany
\textsuperscript{c}IR2Dx, Los Osos, CA, USA
\textsuperscript{d}Institute of Medical Informatics, Charité - Universitätsmedizin Berlin, Germany

\textbf{A R T I C L E  I N F O}

* Correspondence to: PD Dr. Carsten Stephan
  Department of Urology
  Charité - Universitätsmedizin Berlin, CCM, Charitéplatz 1
  D-10117 Berlin, Germany
  Tel.: +49-30-450 515041
  Fax: +49-30 450 515904
  E-Mail: carsten.stephan@charite.de

\textbf{Short title:}
PSA screening and PCa biomarkers

Word count of abstract: 250
Word count of text without abstract: 4868
Number of figures and tables: 3 and 3
Number of references: 103

\textbf{Key words:}
PSA
Screening
Prostate cancer
Prostate health index
PCA3
MPRSS2
Biomarkers

\textbf{A B S T R A C T}

PSA screening reduces PCa-mortality but the disadvantages overdiagnosis and overtreatment require multivariable risk-prediction tools to select appropriate treatment or active surveillance. This review explains the differences between the two largest screening trials and discusses the drawbacks of screening and its meta-analysis. The current American and European screening strategies are described.

Nonetheless, PSA is one of the most widely used tumor markers and strongly correlates with the risk of harboring PCa. However, while PSA has limitations for PCa detection with its low specificity there are several potential biomarkers presented in this review with utility for PCa currently being studied. There is an urgent need for new biomarkers especially to detect clinically significant and aggressive PCa. From all PSA-based markers, the FDA-approved prostate health index (phi) shows improved specificity over percent free and total PSA. Another kallikrein panel, 4K, which includes KLK2 has recently shown promise in clinical
research studies but has not yet undergone formal validation studies. In urine, prostate cancer gene 3 (PCA3) has also been validated and approved by the FDA for its utility to detect PCa. The potential correlation of PCA3 with cancer aggressiveness requires more clinical studies. The detection of the fusion of androgen-regulated genes with genes of the regulatory transcription factors in tissue of ~50% of all PCa-patients is a milestone in PCa research. A combination of the urinary assays for TMPRSS2:ERG gene fusion and PCA3 shows an improved accuracy for PCa detection. Overall, the field of PCa biomarker discovery is very exciting and prospective.

1. Prostate-specific antigen (PSA) and prostate cancer (PCa) screening

**PSA-screening reduces PCa-specific mortality**

The widespread and increasing use of PSA within the last 25 years has revealed PCa to be the most frequent malignancy in the Western world accounts for ~25% of all cancer cases in men [1, 2]. Since 2009, PSA-based screening for prostate cancer (PCa) has been heavily debated with clearly contrasting results of the two largest randomized screening studies. On one hand, the “European Randomized Study of Screening for Prostate Cancer“ (ERSPC) with data on more than 162,000 men from 7 European countries found a PCa-specific mortality reduction of 20% [3] in the PSA screened group, which increased to 21% after a median follow-up of 11 years [4]. When the data is adjusted for nonattendance and PSA-contamination, the mortality risk reduction rises to 29-31% [5].

In marked contrast, the “prostate, lung, colorectal, and ovarian (PLCO) screening trial“ with data from 76,693 American men, found no difference in the PCa-specific mortality after 7 years and also after 10 years of follow-up [6].

The reasons for these large differences and the drawbacks of general screening and several meta-analysis as well as the current screening strategies will be discussed in this review. The second key aspect in addition to screening in this review article is the evaluation of PSA and all PSA-based tumor markers and all currently available serum and urine biomarkers.

**Differences between ERSPC and PLCO trial**

First, the wide distribution of the PSA test in the U.S. resulted in a significant so-called PSA-contamination of the control group in the PLCO trial as at least 52% of the control group underwent at least one PSA test during the six years of screening. With a compliance rate of 85% in the screening group, the real difference in PSA testing was only 33%. However, it is more likely that within the PLCO screening trial actually only 15% of men in the control group never had a PSA test [7]. Thus, when 85% of men in the control group had a PSA test at least once in their life including 44% already before enter-
ing the study, the difference to the 85% of PSA-screened men is actual zero [8]. Thus, the PLCO trial became a comparison of frequent screening versus (somewhat) less frequent screening. Therefore, a mortality difference is very unlikely between PSA-screened and officially non-screened men in the PLCO trial. In the ERSPC study, the PSA contamination rate was much lower with 15% at the most [9]. The highest reported contamination from a single ERSPC center was 24% while other center specific contamination rates were below 10%. With 82% of all screen group participants screened at least once, the difference between screening and no screening in the ERSPC was 67% or at least 58%. This difference is ~2-fold in comparison to the PLCO (33%).

Second, for those screened men in the PLCO trial with positive tests (abnormal digital rectal examination (DRE) and/or PSA ≥4ng/ml) only 40.2% and 30.1% respectively, were biopsied [9]. The low biopsy rate indicates that two-thirds of men suspicious for PCa were not subsequently diagnosed. In the ERSPC, 85.8% of screening participants with positive tests (abnormal DRE and/or PSA ≥4ng/ml, changed 1996–1997 to PSA cutoff ≥3ng/ml without DRE) were in fact biopsied [3]. This rate is 2 to 3-fold higher as compared with the PLCO trial.

Third, there was no difference in stage distribution for all organ confined stages I and II between the screening arm (95.9%) and the control arm (94.4%) in the PLCO trial [6]. Also, the Gleason scores of ≤6 were not different between the screening (65.7%) and the control arm (60.3%). Since a PCa in such early stages normally does not show any symptoms, it is possible that the PSA-contamination in the control group was much higher than 52%. The stage distribution in the screening group in the ERSPC was 80.9% for stages I and II, while the control group had a significantly lower rate of the early stages with 58.9% [3]. Further, the proportions of men who had a less aggressive PCa with Gleason scores of ≤6 were 72.2% in the screening group and only 54.8% in the control group while a Gleason score ≥7 was detected in 27.8% in the screening group and in 45.2% in the control group [3]. These differences were predicted. Additionally, there was a relative reduction of 30% of detected metastatic PCa in the screening group [10].

Beside these above mentioned three important differences between both trials, the shorter follow-up of the PLCO trial as compared with the ERSPC [9] and the insufficient statistical power of the PLCO with its high PSA-contamination do also influence the PCa-specific mortality [8]. A reduced overall mortality should not be expected because the overall risk for men to die of PCa is reduced from 3% to 2.4% with PSA screening as shown from the ERSPC data [11].

All these characteristics of the PLCO trial with a narrow window of only 33% difference in screening between the two arms [9], the low biopsy rate, and the resulting identical stage distribution of the detected PCa in the screen and control arms [9], results in features that make the occurrence of a
difference in PCa-mortality unlikely even with a longer follow-up [9].

**Drawbacks of screening**

On the other hand, the 21% reduced PCa-specific mortality in the ERSPC that increased in single centers to 32% [12], 44% [13] or 51% with correction for nonattendance and contamination [14] has drawbacks with a significant overdiagnosis and detection of insignificant cancers. Overdiagnosis is a major problem for regular PSA screening and the risk to suffer from any PCa is 1.5-fold. The risk of a stage I PCa is almost 2-fold when summarizing data from 6 screening trials with almost 400,000 men [15]. While overdiagnosis itself may harm the patient in the way of a negative psychological effect, subsequent overtreatment can lead to incontinence, impotence and other clinical side effects. Data from the ERSPC show that 32-43% of low risk PCa may have avoided treatment [16]. In those cases, the active surveillance strategy is accepted as a non-treatment option for all low risk PCa-patients (reviewed in [17, 18]). Data from 439 patients initially screened and positively identified with PCa, showed no tumor progression in 86% of individuals after a 10 year follow-up [19].

While active surveillance becomes an increasingly popular management option it should be mentioned that the definition of those early disease stages only relies on biopsy results. An insignificant tumor on biopsy may become clinically significant in the final pathology of the prostatectomy specimen. Two studies on more than 12,000 men treated with radical prostatectomy showed that only 1/4 to 1/3 of tumors were still defined as insignificant on the final prostate pathology [20, 21]. Additionally, 1/5 to 1/3 also showed an upgrading from the biopsy to the final pathological result and ~10% had already extracapsular extension of the disease [20, 21]. This demonstrates that the proportion of men with an apparently insignificant PCa who actually have a clinical relevant tumor is not negligible [20, 21]. The topic of insignificant tumors has been already discussed elsewhere [22]. Finally, at long term follow-up after radical prostatectomy a biochemical recurrence occurs in up to 40% [23], indicating that these tumors were not insignificant but already in an advanced stage.

**Problems of meta-analysis on PSA-screening**

The differences between these two screening studies are substantial and it is questionable if data from the PLCO can be compared with the ERSPC data or used in meta-analysis [11]. Thus, it is not surprising that most meta-analysis incorporating the PLCO study and other screening studies with different clinical designs (reviewed in [24]) could not prove a lower PCa-specific mortality with PSA screening. Since 2010, at least 5 meta-analysis (including updates) have been published [15, 25-27], with almost all concluding no evidence of a PCa-specific mortality reduction. Only one meta-analysis found a 24% PCa-specific mortality reduction with PSA-screening using as exclusion criteria insufficient follow-up length, unacceptably high PSA-contamination in the control group or insufficient
participation in the screening group [27]. All other meta-analysis did not consider those important aspects. Exemplarily, the meta-analysis of Djulbegovic et al. [15] showed an inconsistency grade of 55% and should be therefore valued as questionable [24, 28]. It should be emphasized that a meta-analysis mostly based on studies with severe limitations cannot correctly answer the question of PSA-screening utility [29].

However, other screening studies without randomization (Tyrol study), with low numbers of patients and no PSA in the first two screening rounds (Norköping study), with a too short follow-up (French ERSPC) or with several methodological limitations (Quebec study) have been extensively reviewed elsewhere [15, 30] and will not be discussed here.

Current screening strategies

Despite strong discrepancies, the authors of the ERSPC and PLCO trial found shared conclusions for the future use of PSA in PCa screening [31]. PSA is able to predict PCa up to 30 years in advance [31]. Based on an initial PSA test (without age specification, but 40-45 years seems useful, at least before 60 years [32]) the frequency of follow-up PSA tests should be estimated depending on the individual PCa risk considering age, comorbidities, prostate volume, race and PCa family history [31]. With known PSA values, risk calculators can be used for biopsy indications [31].

Here, the current recommendation of the American Urological Association (AUA) and the European Urological Association (EAU) on PCa screening from 2013 should be mentioned [33, 34]. According to the American guidelines, PSA is not recommended for individuals below the age of 40 years or higher than 70 years. Regular biannual screening after careful counseling should be performed in men aged 55-69 years [33]. In Europe, a baseline PSA is recommended for men 40-45 years to initiate a risk-adapted follow-up approach with the purpose of reducing PCa-mortality and the incidence of advanced and metastatic PCa [34]. To prevent overdiagnosis and overtreatment, multivariable risk-prediction tools will be necessary [34]. This strategy seems to be a reasonably balanced approach so far.

The economically emphasized and widely distributed recommendation of the “US Preventive Services Task Force” completely abandoned PSA as screening tool [35] and has already been critically discussed elsewhere [11, 36].

Considering the above-mentioned points, we view the ERSPC results as reliable. A reduced PCa-specific mortality by more than 20% can be achieved. However, the likelihood of overdiagnosis is about 2-fold. PSA needs to be used in a more rational, strategic way and active surveillance should be included as a serious management option in appropriate patients.

2. Biology of PSA and its correlation with PCa

After the development of the first immunoassay for the PSA antigen in serum, the PSA test replaced
the PAP test and revolutionized the management of PCa. Biologically, PSA is responsible for semen liquefaction and secreted into the seminal plasma but a retrograde release of PSA into the bloodstream is a rare event in healthy men (reviewed in [37]). An excessive escape of PSA into the blood circulation only occurs in cases of destruction of the basement membrane of prostate epithelial cells. Although an increased PSA can also be caused by benign prostate diseases, such as benign prostate hyperplasia (BPH) or prostatitis, there is a strong correlation of serum PSA with the incidence of PCa [37]. Thus, increased PSA levels indicate pathologies of the prostate gland including PCa, but PSA is not cancer-specific. In addition to the relationship of an elevated PSA with a higher PCa risk, PSA can predict the occurrence of PCa several years in advance as already mentioned [37]. Furthermore, PSA can predict death from PCa with up to 25 years in advance [38, 39]. The risk to die from metastatic PCa is as high as 44% for men aged 45 to 55 years when their PSA is within the 10th percentile as compared with those men with a PSA below the median with a risk of <0.3% [39].

3. Efforts to overcome PSA limitations

While PSA is the key parameter for the management of a known PCa, there are decisive limitations for diagnosing PCa. As mentioned, benign prostate diseases as well as prostate manipulations such as bicycling, digital rectal exam (DRE), biopsy, catheterization or ejaculation can also cause at least temporary elevated PSA serum concentrations [40]. This leads to low specificity if a single PSA measurement is used to predict PCa, especially in the PSA “grey zone” of 2-10ng/ml [40]. Avoiding factors such as bicycling, DRE or ejaculation a few days before a PSA blood draw may facilitate interpretation of results. In addition, a biological variation of the PSA value up to 20-30% [41] should be considered. A simple repeat measurement of PSA can significantly reduce the number of prostate biopsies [42] but 60-80% of all biopsies are still unnecessary. The traditional PSA cutoff of 4ng/ml is no longer valid because the PCa detection rate at the 2-4ng/ml range [43] is comparable to the 4-10ng/ml range in the PSA screening environment today [44]. Further, differences between PSA assays additionally with or without WHO calibration may complicate the interpretation of results [45, 46].

To increase the specificity of PSA, different parameters have been developed like PSA density (ratio of PSA to prostate volume), PSA velocity (change of PSA over a time period) or age-/race-specific reference ranges [40]. All these PSA based parameters have been only partially successful. PSA density is perhaps the single most specific parameter but requires an ultrasound procedure to obtain an accurate assessment of prostate size.

4. PSA based serum markers

PSA complexes with proteinase inhibitors

In the early 1990s two independent groups found PSA to exist in different molecular forms [47, 48]. Ap-
approximately 65-95% of PSA is bound to alpha-1-antichymotrypsin (PSA-ACT) while the remaining PSA circulates as free PSA (fPSA). PSA-ACT is higher in PCa-patients compared with non-PCa-patients [48]. The enzymatically-active form of PSA is rapidly and irreversibly complexed with prostate inhibitors while inactive PSA (free PSA) is not complexed [49]. PSA also complexes with alpha2-macroglobulin (A2M), which is not measurable with the current assays. The measurement of PSA-A2M needs rather complicated methods [50]. A very small amount of PSA is also complexed with the protease inhibitor alpha1-protease inhibitor (API). The very small amounts of API to total PSA (tPSA) are analytically challenging [51] so that both the A2M-PSA complex and API-PSA complex assays have never become commercially available. Current PSA immunoassays measure free and complexed PSA which is sometimes referred to as total PSA.

Using a blocking antibody against fPSA, all complexed PSA (cPSA) can be also measured. The cPSA only reaches comparable results to the ratio of fPSA to tPSA (f/tPSA ratio or percent free PSA, %fPSA) when also used as ratio to tPSA, but not as a single parameter [52]. Since the tPSA is the sum of ACT-PSA and fPSA the ratios of cPSA to tPSA should be equal to fPSA to tPSA in a clinical correlation. The fPSA to tPSA ratio was used earlier than cPSA to tPSA ratio. Therefore the vast majority of clinical utility studies on molecular forms of PSA have been published on %fPSA.

Clinical relevance of %fPSA

Since the middle of the 1990s the %fPSA has become a clinically relevant parameter to improve specificity of PSA alone [53]. This has been confirmed (reviewed in [54]). A meta-analysis on %fPSA found an area under the ROC curve (AUC) of 0.68 for more than 2800 patients within the tPSA “grey zone” of 4-10ng/ml [55]. But the authors concluded that %fPSA can only be a useful adjunct to PSA-based screening when reaching extreme values such as <7% [55]. When using high %fPSA cut-offs, the number of unnecessary biopsies could be reduced by ~10-20%. However, for a more accurate interpretation, factors such as prostate volume, prostatitis, or prostatic intraepithelial neoplasia should be considered (reviewed in [52]). Currently, %fPSA is used within multivariable models such as artificial neural networks (ANN) or logistic regression (LR) based nomograms to predict the PCa risk in a subsequent prostate biopsy (reviewed in [56]). Table 1 (modified from ref. [56]) shows the improvement of %fPSA and ANN or LR models compared with tPSA. Regardless of the different assays for tPSA and fPSA [57] and the different PSA ranges investigated, enhanced specificities by using %fPSA within ANN or LR models were observed. However, only some models are freely online available [57, 58] as illustrated in Figures 1 and 2.

Subforms of free PSA

Beginning in 2000, researchers focused to define subforms of fPSA in search for ways to further enhance the specificity of %fPSA. The free PSA became more complex [59]. Figure 3 indicates the different molecular forms of PSA.
The so-called “benign” PSA (bPSA) is a clipped subform of free PSA that is highly associated with the transition zone of the prostate, containing BPH nodules. The bPSA could potentially be used as a marker for BPH, but was unable to distinguish between BPH and PCa [60, 61]. But within a multivariable model, bPSA improved specificity of %fPSA by ~15% [61].

Another fPSA subform was detected by using anti-PSA antibodies that do not recognize internally cleaved PSA at Lys145-Lys146. This special PSA subform was termed “intact”, unclipped PSA (iPSA) [62]. Although iPSA could distinguish between PCa and BPH, its further use has been limited since a commercial assay is lacking. A lab-based test may now be available as a panel termed 4K. This panel combines tPSA, fPSA, iPSA and the human glandular kallikrein 2 (KLK2) and showed a high predictive accuracy [63, 64].

Another subform, proPSA is termed [-7]proPSA and contains a seven amino acid N-terminal pro-leader peptide in this native form, which is rapidly truncated by proteolytic cleavage to [-4]proPSA, or [-2] proPSA. The proPSA derivative [-2]proPSA cannot be cleaved to form enzymatically-active PSA and accumulates in the prostate cancer regions of the prostate. A research assay measuring the [-7, -5] proPSA was of limited usefulness and has subsequently not been commercialized (reviewed in [52]). Only the [-2]proPSA [65] and especially the commercial and FDA-approved [-2]proPSA [66, 67] showed the expected further improvement in specificity over %fPSA. Since 2010 the Prostate health index (phi) (calculated as: [-2]proPSA / fPSA * √PSA) has been used to discriminate between PCa and non-PCa [68]. These data on phi have been confirmed in large multicenter cohorts and it further seems that phi may preferentially detect aggressive PCa [66, 67, 69, 70].

**Clinical importance of prostate health index phi**

In 2012, [-2]proPSA was approved by the FDA to be used for initial biopsy decision in men with PSA in the range of 4-10ng/ml and negative DRE. A comprehensive review summarizes all aspects on different proPSA forms as well as the cost-effectiveness of phi [71]. The addition of phi to the common screening strategy with PSA alone slightly increases the costs of the blood tests but could reduce the number of required office visits, laboratory tests and biopsies [71].

A recent meta-analysis for phi and the percentage of [-2]proPSA to fPSA (%[-2]proPSA) analyzed data from more than 5000 biopsied men within the tPSA range of 2-10ng/ml [72]. At 90% sensitivity a pooled specificity of ~32% for phi and %[-2]proPSA was found; both parameters were superior to tPSA and %fPSA. Table 2 provides data on all available studies using phi with at least 200 biopsy proven patients.

Increasing phi values were associated with an increased probability of detecting Gleason ≥7 PCa [66, 67]. Two studies with more than 2,200 men independently found that the relative risk of any PCa is
3.6-fold [67] to 4.7-fold [66] higher in those men with phi values in the highest as compared with the lowest quartile. The risk of a Gleason ≥7 PCa increases 1.6-fold with phi values the highest quartile [66]. Phi had also significantly higher median values in aggressive PCa and the proportion of Gleason ≥7 PCa increased with the phi score [67].

However, when using phi within multivariable models, the AUC-gain was very modest or not visible [67, 73]. As reviewed [74], the inclusion of new biomarkers such as urinary prostate cancer antigen 3 (PCA3) and [-2]proPSA in risk calculators amounted only to a marginal improvement in the accuracy of these prediction tools. Despite this, phi shows overall promising data, especially when focused to detect aggressive PCa.

5. Other prostate cancer serum marker

The kallikreins

Beside the pancreatic/renal kallikrein KLK1, KLK2 and KLK3 which is widely known as PSA, 12 new members of the human kallikrein family have been characterized [75]. The human kallikrein genes are named KLK1 to KLK15 and they encode for the proteins KLK1 to KLK15.

KLK2 can convert proPSA to active PSA (reviewed in [52]) and has been investigated extensively [75]. However, early promising data could not be confirmed (reviewed in [52] and [54]) and KLK2 has not been transferred into a commercial assay.

Beside KLK2 and PSA, at least 6 other kallikreins (KLK4, KLK10-13 and KLK15) are also expressed in relatively high amounts in prostate tissue [75] but again, no commercial immunoassay is available. Only KLK11 showed promising values but data have not been confirmed independently (reviewed in [52] and [54]). Reviews on kallikreins have been published elsewhere [75, 76].

Other serum markers

Details on several markers like caveolin, IGF, PSP94, macrophage inhibitory cytokine 1, cytokine macrophage migration inhibitory factor, the calcium-binding proteins S100A8 and S100A9 that have never reached clinical significance or at least assay commercialization have been already reviewed [77]. The extracellular matrix protein Spondin-2 [78], and Galectin-3, a tumor-associated protein [79], have been published in 2013. Spondin-2 showed an extremely high AUC of 0.95 as compared with %fPSA (0.81), sarcosine (0.67) and tPSA (0.56) [78]. The galectin-3 levels were in contrast only compared in the sera of metastatic PCa-patients with non-cancer patients [79].

Sarcosine in serum

In the above mentioned study on Spondin-2 [78], sarcosine showed limited success. Others found an increased PCa risk and a further increased risk for aggressive PCa (odds ratio 1.44) with increasing
sarcosine levels [80]. In contrast, another large study found high sarcosine and glycine concentrations to be associated with a reduced PCa risk of borderline significance (odds ratio 0.86) [81]. Other studies on sarcosine in serum and plasma with smaller numbers of patients also showed inherent data [82-84]. Interestingly, first data on sarcosine have been published in urine.

6. Urine markers

Sarcosine

Sreekumar et al. [85] found sarcosine to be significantly higher in urine sediments and supernatants in PCa as compared with men without PCa [85]. In 53 men, the AUC for sarcosine (0.69) was significantly higher than the AUC of PSA (0.53) at PSA levels of 2-10ng/ml [85]. In contrast, another study in 139 men found significantly lower sarcosine values in PCa-patients compared with non-PCa-patients and no difference between healthy men and PCa-patients [86]. Also, %fPSA (AUC: 0.81) had a significantly larger AUC than sarcosine (0.63), and PSA (0.64) was equal to sarcosine [86]. Sarcosine was measured with a commercial amino acid assay and values were normalized to urine creatinine [86]. There was a strong correlation ($r_s = 0.86$) between the sarcosine and creatinine showing that the occurrence of sarcosine in urine is due to renal excretion [86]. Sarcosine is not specific to prostate tissue nor is it related to tumor aggressiveness or recurrence, which is in contrast to PCA3, which is prostate-specific and not found elsewhere. Therefore it is unlikely that sarcosine is suitable as a marker for PCa detection. Further details on sarcosine have been reviewed recently [87].

PCA3

PCA3 is a noncoding messenger RNA (mRNA) and is 66-fold overexpressed in PCa tissue. A molecular assay for PCA3 was introduced in 2006 [88]. In 2012, this assay was FDA-approved to aid in the decision for repeat biopsy in men ≥50 years (reviewed in [89]).

Two independent multicenter studies found excellent clinical value of the PCA3 assay in men with previous negative biopsies [90] and with first and repeat biopsies [91]. Haese et al. [90] found PCA3 to be better than %fPSA and that PCA3 was independent of prostate volume, age and tPSA. The PCa likelihood increased with the PCA3 score. However, the PCa detection rate was only 47% in those patients with PCA3 scores >100 [90]. This problem has been reported in several other studies (reviewed in [89]) proving a low sensitivity with a PCA3 cutoff of 100.

Nonetheless, several studies have proven the clinical value of PCA3 to improve specificity over PSA and %fPSA (reviewed in [89]). Table 3 provides data on studies with at least 200 patients. With exception of two studies (AUC 0.59 and 0.83), the AUCs for PCA3 are ~0.7.

However, PCA3 is not capable to replace PSA as a first-line test in clinical practice due to the lack of an appropriate cut-off level with acceptable performance characteristics. But addition of PCA3 to risk
assessing tools leads to an increase of 4.5-7.1% in predictive capability [92, 93].

In contrast to PSA, PCA3 is not influenced by prostate volume, prostatitis or medication with 5-alpha-reductase inhibitors (reviewed in [89]). Regardless of its complicated measurement procedure, relative high costs (~300 Euros), and lower sensitivity than PSA PCA3 has clearly shown its clinical value. The potential correlation of PCA3 with tumor volume and cancer aggressiveness has shown conflicting results in several studies and needs to be clarified.

**TMPRSS-2**

The detection of gene fusions involving the androgen regulated TMPRSS2 and ETS transcription factor genes in PCa was a research-milestone [94]. Approximately 50% of all PCa-patients do have the TMPRSS2 fusion with the ETS family member that is regarded as a key PCa oncogene [94, 95]. Based on these important findings in PCa tissue, a urinary assay using the same format as PCA3 has been developed [96]. TMPRSS2:ERG in PCa tissue and in urine showed a strong correlation demonstrating a high tumor specificity of this marker. In 2011 a high AUC of 0.77 was reported for the TMPRSS2:ERG urinary assay in a small cohort with only 15 PCa-patients, which was higher than the AUC for PCA3 with 0.65 [97]. So far, only one study reported separate data on the TMPRSS2:ERG urine assay in a larger (n=246) and more balanced cohort [98]. ROC data showed a significant lower AUC for TMPRSS2:ERG (0.63) than for PCA3 (0.74) but both had no difference to phi (AUC 0.68) [98]. All other studies only reported AUCs on PCA3 and TMPRSS2:ERG together without separate evaluation of TMPRSS2:ERG [99, 100].

The use of TMPRSS2:ERG and PCA3 in PCa risk calculators has been published [99, 100]. The TMPRSS2:ERG had independent additional predictive value to PCA3 and to the ERSPC risk calculator parameters for predicting PCa [99]. TMPRSS2:ERG had prognostic value, whereas PCA3 did not [99]. Urinary TMPRSS2:ERG was further associated with clinically significant PCa at biopsy and prostatectomy [100].

It was postulated, that there is a rational basis for the need to combine PCA3 and TMPRSS2:ERG gene fusion for PCa diagnosis [101]. Based on tissue expression, it was visible that most false-negative results of PCA3 were corrected by TMPRSS2:ERG and that the combination of both markers would be capable to improve sensitivity [101].

**Conclusion on urine markers**

Detecting PCa in urine is technically feasible, as demonstrated by numerous studies, but few markers have been validated in multiple large sample sets [102]. There are several new markers like zinc alpha2-glycoprotein, thiosulfate or combinations of markers measured in multiplex models or gene panels that are only reported by one group so far. However, preanalytical conditions in urine are more difficult than in serum and the process of urine collection is subject to variability, which may result in conflicting clinical results [102].
Details on further urine marker have been published elsewhere [102, 103]. However, advanced clinical studies have identified only PCA3 and TMPRSS2:ERG fusion transcripts as promising RNA markers for cancer detection and possibly prognosis [102].

Summary

PSA screening reduces PCa-mortality as shown by the largest screening trial so far, the ERSPC. Other screening trials and meta-analysis from these trials with severe drawbacks should be interpreted cautiously. However, disadvantages of regular screening, namely overdiagnosis and overtreatment can be diminished with selective strategies including active surveillance. The most balanced screening guideline, the European EAU screening guideline, recommends a baseline PSA for men with 40-45 years to initiate a risk-adapted follow-up approach to reduce PCa-mortality and the incidence of advanced and metastatic PCa.

PSA as one of the most widely used tumor markers strongly correlates with the risk of harboring from PCa. This risk is already visible up to 20-30 years in advance but PSA has severe limitations for PCa detection with its low specificity. The FDA-approved and currently best serum parameter phi shows improved specificity over %fPSA and PSA. The best parameter in urine, the FDA-approved PCA3 has also been proven its utility in the PCa detection but correlation with aggressiveness and low sensitivity at high values have to be re-examined. While the detection of TMPRSS2:ERG gene fusion was one research milestone, the urinary assay for TMPRSS2:ERG only shows the expected improved accuracy for PCa detection in combination with PCA3.

Taken together, risk-adapted PSA screening and diagnosing as well as appropriate use of the FDA-approved biomarkers is the most likely scenario in the near future. New techniques such as genomics, proteomics or metabolomics as well as improved imaging devices (multiparametric-MRI) and the simultaneous use of all parameters preferentially within multivariable models may further enhance the accuracy of PCa diagnosis within the next years.

References


42. Singh R, Cahill D, Popert R, O’Brien TS. Repeating the measurement of prostate-specific antigen in symptomatic men can avoid unnecessary prostatic biopsy. BJU Int 2003;92:932-5.


Shin SS, Cruz AB, Chan DW, Sokoll LJ, Roberts WL, van Schaik RH, Mizrahi IA. A multicenter study of [-2] pro-prostate specific antigen combined with prostate specific antigen and free prostate specific antigen for prostate cancer detection in the 2.0 to 10.0 ng/ml prostate specific antigen range. J Urol 2011;185:1650-5.


**Table 1. Examples for multivariate models using %fPSA for diagnosis of PCa (1998-2004)**

<table>
<thead>
<tr>
<th>First Author [Ref.] (n of pts.; % of PCa)</th>
<th>Year</th>
<th>Scree- ning</th>
<th>Model (ranking)</th>
<th>PSA assays (company)</th>
<th>tPSA range (ng/ml)</th>
<th>contributing factors (if numbered, by value)</th>
<th>AUC</th>
<th>Specificity at 95% sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Carlson</strong> (n=3773; 33% PCa)</td>
<td>1998</td>
<td>no</td>
<td>LR</td>
<td>Tosoh (Dianon)</td>
<td>4-20</td>
<td>1.%fPSA, 2.age 3.tPSA</td>
<td>n.a.</td>
<td>34 (LR) 23 (%fPSA)</td>
</tr>
<tr>
<td><strong>Virtanen</strong> (n=212; 25% PCa)</td>
<td>1999</td>
<td>yes</td>
<td>1. LR</td>
<td>ProStatus (Wallac)</td>
<td>3-10 (3-45)</td>
<td>1.%fPSA 2.DRE 3.heredity</td>
<td>0.81</td>
<td>(LR for tPSA 3-45) %fPSA n.a.</td>
</tr>
</tbody>
</table>

*(Table 1) continued on next page*
Table 1 cont’d. Examples for multivariate models using %fPSA for diagnosis of PCa (1998-2004)

<table>
<thead>
<tr>
<th>First Author [Ref.]</th>
<th>Year</th>
<th>Scree-ning</th>
<th>Model (ranking)</th>
<th>PSA assays (company)</th>
<th>tPSA range (ng/ml)</th>
<th>contributing factors (if numbered, by value)</th>
<th>AUC</th>
<th>Specificity at 95% sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Finne [58] (n=656; 23% PCa)</td>
<td>2000</td>
<td>yes</td>
<td>1. ANN 2. LR</td>
<td>ProStatus (Wallac)</td>
<td>4-10</td>
<td>1.%fPSA 2.volume 3.DRE 4.tPSA</td>
<td>n.a.</td>
<td>33 (ANN) 24 (LR) 19 (%fPSA)</td>
</tr>
<tr>
<td>Babaian (n=151; 25% PCa)</td>
<td>2000</td>
<td>yes</td>
<td>ANN</td>
<td>Tandem R (Beckman Coulter)</td>
<td>2.5-4</td>
<td>%fPSA, tPSA, age, PAP, CK</td>
<td>0.74 ANN (0.64 %fPSA)</td>
<td>51 (ANN) 39 (PSAD) 10 (%fPSA)</td>
</tr>
<tr>
<td>Horninger (n=3474; n.a.)</td>
<td>2001</td>
<td>yes</td>
<td>ANN LR</td>
<td>Abbot IMX (Abbott)</td>
<td>n.a.</td>
<td>PSA&gt;4 or DRE+ age, tPSA, %fPSA, DRE, volume, PSAD, PSAD-TZ, TZ-volume</td>
<td>n.a.</td>
<td>~27 (ANN) ~13 (%fPSA) ~13 (tPSA)</td>
</tr>
<tr>
<td>Stephan (n=1188; 61% PCa)</td>
<td>2002</td>
<td>no</td>
<td>ANN LR</td>
<td>IMMULITE (Bayer)</td>
<td>2-20</td>
<td>1.DRE 2.%fPSA 3.volume 4.tPSA 5.age</td>
<td>0.86 (ANN) 0.75 (%fPSA)</td>
<td>43 (ANN) 26 (%fPSA)</td>
</tr>
<tr>
<td>Remzi (n=820; 10% PCa)</td>
<td>2003</td>
<td>no</td>
<td>ANN, LR</td>
<td>AxSYM (Abbott)</td>
<td>4-10</td>
<td>tPSA, %fPSA, volume, PSAD, PSAD-TZ, TZ-volume</td>
<td>0.83 (ANN) 0.79 (LR) 0.745 (%fPSA)</td>
<td>68 (ANN) 54 (LR) 33.5 (%fPSA)</td>
</tr>
<tr>
<td>Finne (n=1775; 22% PCa)</td>
<td>2004</td>
<td>yes</td>
<td>1. LR 2. ANN</td>
<td>ProStatus (Wallac)</td>
<td>4-10</td>
<td>1.DRE 2.%fPSA 3.volume 4.tPSA</td>
<td>0.764 (LR) 0.760 (ANN) 0.718 (%fPSA)</td>
<td>22 (LR) 19 (ANN) 17 (%fPSA)</td>
</tr>
<tr>
<td>Sokoll [70] (n=566; 43% PCa)</td>
<td>2010</td>
<td>not available</td>
<td>0.79 (LR model)</td>
<td>80</td>
<td>45</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Abbreviations:** AUC: area under the (ROC) curve; n.a.: not available; LR: logistic regression; ANN: artificial neural network, PAP: prostate alkaline phosphatase, CK: creatinkinase; PSAD: PSA density, PSAD-TZ: transition zone density; DRE: digital rectal examination
### Table 2. Selected studies with more than 200 subjects on Phi (2010-2013)

<table>
<thead>
<tr>
<th>First author [Ref.] (n of pts.; % of PCa)</th>
<th>Year</th>
<th>Phi cutoff</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sokoll [70]</strong> (n=566; 43% PCa)</td>
<td>2010</td>
<td>not available</td>
<td>0.79 (LR model)</td>
<td>80</td>
<td>45</td>
</tr>
<tr>
<td><strong>Jansen [68]</strong> (n=756; 50% PCa)</td>
<td>2010</td>
<td>2 cohorts</td>
<td>not available</td>
<td>0.75 (0.71)</td>
<td>90</td>
</tr>
<tr>
<td><strong>Liang</strong> (n=250+250; 50% PCa, matched)</td>
<td>2011</td>
<td>36.45 (at 90% spec.)</td>
<td>0.73</td>
<td>42</td>
<td>90</td>
</tr>
<tr>
<td><strong>Guazzoni</strong> (n=268; 40% PCa)</td>
<td>2011</td>
<td>48.5 (at 90% spec.)</td>
<td>Hybr. calibr.</td>
<td>0.76</td>
<td>43</td>
</tr>
<tr>
<td><strong>Catalona [66]</strong> (n=721; 17% PCa)</td>
<td>2011</td>
<td>21.3 (24.1)</td>
<td>Hybr. calibr.</td>
<td>0.70</td>
<td>95 (90)</td>
</tr>
<tr>
<td><strong>Loeb (see also [66])</strong> (n=721; 17% PCa)</td>
<td>2011</td>
<td>24.3 (27.9)</td>
<td>WHO calibr.</td>
<td>0.70</td>
<td>95 (90)</td>
</tr>
<tr>
<td><strong>Lazzeri</strong> (n=222; 32% PCa)</td>
<td>2012</td>
<td>28.8</td>
<td>Hybr. calibr.</td>
<td>0.67</td>
<td>90</td>
</tr>
<tr>
<td><strong>Stephan [67]</strong> (n=1362; 49% PCa)</td>
<td>2013</td>
<td>31 (24)</td>
<td>Hybr. calibr.</td>
<td>0.74</td>
<td>95 (90)</td>
</tr>
<tr>
<td><strong>Ferro</strong> (n=300; 36% PCa)</td>
<td>2013</td>
<td>27.5</td>
<td></td>
<td>0.68</td>
<td>90</td>
</tr>
<tr>
<td><strong>Ito</strong> (n=239; 22% PCa)</td>
<td>2013</td>
<td>23.9 (24.9)</td>
<td>Hybr. calibr.</td>
<td>0.72</td>
<td>95 (90)</td>
</tr>
<tr>
<td><strong>Lazzeri [69]</strong> (n=646; 40% PCa)</td>
<td>2013</td>
<td>27.6</td>
<td>41.5</td>
<td>61.7</td>
<td>0.67</td>
</tr>
<tr>
<td><strong>Scattoni</strong> (n=211; 33% PCa)</td>
<td>2013</td>
<td>28.3 (30.6)</td>
<td>24.1 (35.5)</td>
<td>0.70 all</td>
<td>0.69 1st bx</td>
</tr>
<tr>
<td><strong>Ng</strong> (n=230; 9% PCa)</td>
<td>2013</td>
<td>26.5</td>
<td>Hybr. calibr.</td>
<td>0.78</td>
<td>90</td>
</tr>
</tbody>
</table>

*also PCA3 values available

Abbreviations: AUC: area under the (ROC) curve; bx: biopsy; Hybr. calibr.: Hybritech calibration (for PSA & fPSA); n.a.: not available; WHO calibr.: calculated (not measured) as WHO calibrated
Table 3. Selected studies with more than 200 subjects on PCA3 (2007-2013)

<table>
<thead>
<tr>
<th>First author [Ref.]</th>
<th>Year</th>
<th>PCA3 cutoff</th>
<th>AUC</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marks (n=226; 27% \text{ PCa})</td>
<td>2007</td>
<td>35</td>
<td>0.68</td>
<td>58</td>
<td>72</td>
</tr>
<tr>
<td>Haese [90] (n=463; 28% \text{ PCa})</td>
<td>2008</td>
<td>35</td>
<td>0.66</td>
<td>47</td>
<td>72</td>
</tr>
<tr>
<td>Deras [91] (n=570; 36% \text{ PCa})</td>
<td>2008</td>
<td>35</td>
<td>0.69</td>
<td>54</td>
<td>74</td>
</tr>
<tr>
<td>Ankerst (n=443; 28% \text{ PCa})</td>
<td>2008</td>
<td>25</td>
<td>0.665</td>
<td>63</td>
<td>60</td>
</tr>
<tr>
<td>Chun [92] (n=809; 39% \text{ PCa})</td>
<td>2009</td>
<td>17</td>
<td>0.68</td>
<td>81</td>
<td>45</td>
</tr>
<tr>
<td>Hessels (n=336; 40% \text{ PCa})</td>
<td>2010</td>
<td>35</td>
<td>0.72</td>
<td>61</td>
<td>74</td>
</tr>
<tr>
<td>Auprich (n=621; 41% \text{ PCa})</td>
<td>2010</td>
<td>17 ((24, 35))</td>
<td>0.73-0.75</td>
<td>88</td>
<td>45</td>
</tr>
<tr>
<td>Roobol (n=721; 17% \text{ PCa})</td>
<td>2010</td>
<td>35</td>
<td>0.635</td>
<td>68</td>
<td>56</td>
</tr>
<tr>
<td>Ploussard (n=301; 24% \text{ PCa})</td>
<td>2010</td>
<td>35 ((25, 30))</td>
<td>0.69</td>
<td>44-59</td>
<td>67-79</td>
</tr>
<tr>
<td>Aubin (n=1072; 18% \text{ PCa})</td>
<td>2010</td>
<td>35</td>
<td>0.69</td>
<td>48</td>
<td>79</td>
</tr>
<tr>
<td>De la Taille (n=516; 40% \text{ PCa})</td>
<td>2011</td>
<td>35</td>
<td>0.76</td>
<td>64</td>
<td>76</td>
</tr>
<tr>
<td>Perdona (n=218; 33.5% \text{ PCa})</td>
<td>2011</td>
<td>51</td>
<td>0.83</td>
<td>70</td>
<td>81</td>
</tr>
<tr>
<td>Bollito (n=1237; 26% \text{ PCa})</td>
<td>2012</td>
<td>35 ((39, 50))</td>
<td>0.68</td>
<td>73 ((n=949 \text{ at PSA 4-10}))</td>
<td>49 ((n=949 \text{ at PSA 4-10}))</td>
</tr>
<tr>
<td>Crawford (n=1913; 42% \text{ PCa})</td>
<td>2012</td>
<td>10 ((25, 35))</td>
<td>0.71</td>
<td>86.5</td>
<td>37</td>
</tr>
<tr>
<td>Stephan [98] (n=246; 45% \text{ PCa})</td>
<td>2013</td>
<td>28</td>
<td>0.74</td>
<td>73</td>
<td>64</td>
</tr>
<tr>
<td>Hansen [93] (n=692; 46% \text{ PCa})</td>
<td>2013</td>
<td>21</td>
<td>0.74</td>
<td>79</td>
<td>59</td>
</tr>
<tr>
<td>Scattoni (n=211; 33% \text{ PCa})</td>
<td>2013</td>
<td>16.5 ((13.5, 23.5))</td>
<td>0.59</td>
<td>80 ((90))</td>
<td>16-34</td>
</tr>
<tr>
<td>Tombal (n=1024; 18% \text{ PCa})</td>
<td>2013</td>
<td>20</td>
<td>n.a.</td>
<td>87</td>
<td>55</td>
</tr>
<tr>
<td>Gittelmann (n=466; 22% \text{ PCa})</td>
<td>2013</td>
<td>25</td>
<td>0.71</td>
<td>77.5</td>
<td>57</td>
</tr>
<tr>
<td>Ferro (n=300; 36% \text{ PCa})</td>
<td>2013</td>
<td>22</td>
<td>0.73</td>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>Goode (n=456; 19% \text{ PCa})</td>
<td>2013</td>
<td>35</td>
<td>0.73</td>
<td>62</td>
<td>75</td>
</tr>
<tr>
<td>Ruffion (n=601; 46% \text{ PCa})</td>
<td>2013</td>
<td>35</td>
<td>0.74</td>
<td>63</td>
<td>72</td>
</tr>
</tbody>
</table>
Fig. 1. The program at www.finne.info to estimate the risk of PCa based on ANN and LR at the 95% sensitivity level.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calculating the risk of finding prostate cancer in sextant biopsy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate specific antigen, µg/l</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of free PSA</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prostate volume, ml</td>
<td>25</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digital rectal examination</td>
<td></td>
<td>Normal</td>
<td></td>
<td>Suspicious</td>
<td></td>
</tr>
<tr>
<td>Output value of neural network</td>
<td>0.047</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Output value of logistic regression</td>
<td>0.064</td>
<td>0.039</td>
<td>0.104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(=risk of detecting prostate cancer)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Predict the diagnosis

Select sensitivity level | 95 |

Output cutoff

<table>
<thead>
<tr>
<th>Neural network</th>
<th>Logistic regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.072</td>
<td>0.061</td>
</tr>
</tbody>
</table>

Predicted diagnosis

<table>
<thead>
<tr>
<th>Neural network</th>
<th>Logistic regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>No cancer in biopsy</td>
<td>Cancer in biopsy</td>
</tr>
</tbody>
</table>
Fig. 2. Program “ProstataClass” version 2008 for 5 different PSA assays at http://urologie.charite.de and the link: “ProstataClass”. Provided example of the ANN output (only available in German) indicating “Risiko” (risk)” at the 95% sensitivity level.

Fig. 3. Molecular forms of PSA and the prostate health index phi including the respective times of detection.
miRNA in prostate cancer: new prospects for old challenges

Samy M Mekhail1, 2, Peter G Yousef3, Stephen W Jackinsky3, Maria Pasic1,4, George M Yousef1,2,*

1Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada
2Department of Laboratory Medicine, and the Keenan Research Centre for Biomedical Science at the Li KaShing Knowledge Institute, St. Michael’s Hospital, Toronto, Canada
3American International College of Arts and Sciences, Antigua
4Department of Laboratory Medicine, St. Joseph’s Health Centre, Toronto, Canada
*To whom correspondence should be addressed

A B S T R A C T

Prostate cancer (PCa) is one of the most commonly diagnosed cancers among men but has limited prognostic biomarkers available for follow up. MicroRNAs (miRNAs) are small non-coding RNAs that regulate expression of their target genes. Accumulating experimental evidence reports differential miRNA expression in PCa, and that miRNAs are actively involved in the pathogenesis and progression of PCa. miRNA and androgen receptor signaling cross-talk is an established factor in PCa pathogenesis. Differential miRNA expression was found between patients with high versus low Gleason scores, and was also observed in patients with biochemical failure, hormone-resistant cancer and in metastasis. Metastasis requires epithelial-mesenchymal transition which shares many cancer stem cell biological characteristics and both are associated with miRNA dysregulation. In the era of personalized medicine, there is a broad spectrum...
of potential clinical applications of miRNAs. These applications can significantly improve PCa management including their use as diagnostic and/or prognostic markers, or as predictive markers for treatment efficiency. Preliminary evidence demonstrates that miRNAs can also be used for risk stratification. Circulatory miRNAs can serve as non-invasive biomarkers in urine and/or serum of PCa patients. More recently, analysis of miRNAs and circulating tumor cells are gaining significant attention. Moreover, miRNAs represent an attractive new class of therapeutic targets for PCa. Here, we summarize the current knowledge and the future prospects of miRNAs in PCa, their advantages, and potential challenges as tissue and circulating biomarkers.

Prostate cancer (PCa) is the most commonly diagnosed cancer among men in western populations. The American Cancer Society estimated 239,590 new cases and 29,720 expected deaths in the USA in 2013. One in every six men are at risk of developing PCa during their lifetime (1).

Currently, the standard biomarker for PCa diagnosis is prostate-specific antigen (PSA), which has its limitations, leading to the risks of PCa over diagnosis and harmful overtreatment. The prognostic value of PSA is also questionable (2). Stepping into the new epoch of personalized medicine, molecular markers are urgently needed to improve the different aspects of PCa management (3). miRNAs represent an attractive class of emerging biomarkers that can help in this regard (4;5).

1. Regulation of the biogenesis and function of miRNAs

miRNAs are small single stranded RNA sequences which do not encode for proteins but rather function by controlling the expression of their target genes. The biogenesis of miRNAs starts in the nucleus by a primary transcript (pri-miRNA) which is then processed by an RNase enzyme, Drosha, with the help of a microprocessor complex, to a 60 to 90 nucleotide precursor miRNA (pre-miRNA) that is then exported by Exporin-5 to the cytoplasm. In the cytoplasm, RNase III enzyme, Dicer, cleaves the double-stranded RNA (dsRNA) hairpin structure to form short double stranded 20-25 nucleotide fragments, which are then unwound into two single-stranded (ss) RNAs, namely the passenger strand and the guide strand. The passenger strand is degraded, and the guide strand is incorporated into the RNA-induced silencing complex (RISC) (6). A miRNA identifies its RNA target mainly through a 6-8 nucleotide seeding sequence. Annealing of mature miRNA to its target mRNA 3’-UTR, and the formation of RISC inhibits protein translation in the case of partial sequence complementarity, or triggers target mRNA degradation if their sequence is perfectly complementary (6;7).

miRNA production and processing is under various regulators at the different levels of biogenesis which have been elaborately reviewed (8). For example, the oncogene epidermal growth factor receptor (EGFR) suppresses miRNA maturation in response to hypoxia (9). miRNAs can function as a cross-talk between epigenetic machinery and modulators (10). Recent evidence has demonstrated
complex interaction between the expression of tumor suppressor miR-31 and AR signaling, and that miR-31 and AR could mutually repress each other (11). miR-31 up-regulation was found to suppress AR expression through epigenetic modulation, and inhibit tumour growth in vivo (11). This epigenetic-miRNA interaction is new paradigm in cancer biogenesis gene regulation.

miRNAs are best described as fine tuning modulators of gene expression (6). They have essential roles in many vital processes like cell cycle, survival, differentiation, growth and apoptosis (12). miRNA function can be also tissue-specific. For example, in PCa, miR-125b acts as an oncomiR (a tumor promoter) but as a tumor suppressor in ovarian and breast cancers (7).

2. miRNAs are involved in prostate cancer pathogenesis

The link between miRNA and PCa pathogenesis is well-established in the literature, and linked to more 50 miRNAs (13-16). miRNAs were shown to contribute to PCa tumorigenesis and progression, as reviewed in (5,17) (Figure 1). Some of these miRNAs e.g. miR-125, 145 and 221 were found to be dysregulated in many cancer-related processes such as cell proliferation, differentiation and progression (5). Table 1 summarizes these miRNAs expression pattern as reported in 8 studies (15,18-24). As observed, let-7c, miR-125 and miR-145 were down-regulated in tumors versus normal tissue (15,19,20). Tong et al., found down-regulation of miR-23, miR-100, miR-145, miR-221 and miR-22, tumors versus normal tissues (21), which is keeping consistency with Schaefer et al. who found down-regulation of miR-125b, miR-145, miR-221 and miR-222 (22). Obviously, variation among study results could be attributed to sampling issues, tumor heterogeneity or technical variability (25). However, this differential miRNAs expression could be used as diagnostic biomarkers.

Depending on targeted gene, miRNAs can function as tumor promoters (oncomiRs) or tumor suppressors. Tumor suppressor miR34 was down-regulated in PCa, controls tumour proliferation, apoptosis and invasiveness in PCa. miR-34 is target of tumor suppressor p53 and was reported to be frequently silenced in PCa. Overexpression of miR-34 induced G1 cell cycle arrest (26), and negatively regulated oncogenes E2F3 and BCL-2 (27) (Figure 2). Transition from high grade prostate intraepithelial neoplasia (HGPIN) to localized adenocarcinoma was associated with down-regulation of miRNAs including miR-16 and 146a which repress oncogenic genes such as anti-apoptotic BCL2 and ROCK1 which increase cell growth and invasion (14). Moreover, down-regulation of tumour suppressors miR-16 and miR-15a, in tumor epithelium and its surrounding fibroblasts, promoted tumor growth and invasion via a simultaneous effect on fibroblast growth factor 2 (FGF-2) (28). Ru et al. found down-regulation of anti-metastatic miR-29b in PCa tissues as compared with non-tumor tissues. The authors succeeded to inhibit the metastatic lesion but overexpressing miR-29b in vivo (29). Transition from localized to metastatic adenocarcinoma was associated with down-regulation of tumour suppressors
miR-let7 and 143 (14), which are repressing oncogene RAS (14;15;20;27). miR-21 was also found to be down-regulated in metastatic adenocarcinoma. miR-21 induces tumour suppressor PTEN (14). Thus, tumour proliferation and invasion are correlated with loss of the suppressor miRNAs which repress certain oncogenes. Other miRNAs such as miR-221/222, 125b, miR148 and 145 promote PCa tumourgenesis via hormonal regulation and stemness. Please see Sections 2.1 and 2.4 for more information. Overall, PCa pathogenesis is under the balance between the effect of the tumour suppressor and promoter miRNAs, which could be a therapeutic potential.

### 2.1 Androgen dependence-associated miRNAs

Several miRNAs were found to be androgen-dependent such as miR-125b, miR-101, miR-148, 221/222 and miR-146a. These miRNAs were reported to control tumour proliferation, invasion and metastasis by regulating several genes as BAK1, EZH2, CAND1 and ROCK1 (17). Jalava et al. identified miR-221 and miR-148a as androgen-regulated miRNAs that expressed in CRPC versus BPH (30). In CRPC, miR-221/-222 were also related to PCa relapse and metastasis (13;19;30;31). Table 2 summarizes examples of androgen-dependent miRNAs expression.

AR inhibition is important for PCa therapy. In androgen-independent tumor, AR signaling was found to be susceptible to miRNA regulation (13;17). miRNAs were identified to influence AR expression level in PCa. For example, miR-34a and miR-34c were found to target the AR 3’UTR and decrease its expression, and thus, could affect PCa progression (32). Moreover, miR-124 was found to be significantly down-regulated in malignant compared with benign prostatic cells, and directly target AR, which induces up-regulation of the p53 apoptotic activity (33). Notably, in androgen refractory, hormonal resistant PCa, AR expression and signaling could remain intact (34). Accordingly, the androgen-dependent state of PCa is regulated not only by androgen and AR, but also by miRNAs which are important for tumourgenesis and hormonal therapy.

### 2.2 miRNAs are involved in acquiring an aggressive behavior in prostate cancer

Current experimental evidence suggests that a group of miRNAs; “metastamirs”, are involved in PCa aggressive behavior and metastasis (16). miRNAs expression was significantly different among cases with early PSA recurrence after surgery, and non-aggressive tumours with long remission (>1 year but <5 years) (20). miR-145 and 125b regulate tumor cell cycle progression, apoptosis and cellular transformation (27). miRNAs expression in PCa tumour compared with benign peripheral zone tissues showed miR-125b down-regulation. miR-125b targets candidate genes such as BAK1 and ELF4EBP1 combined with the AKT/mTOR pathway, which could be responsible for the aggressive phenotype characteristics including high Gleason score, stage, and biochemical failure (20;22). miR-145 and miR-143 were found to regulate tumor progression, EMT and cancer stem cells (CSCs) through targeting
Oct4, c-Myc, and Klf4 (35). They were also implicated in PCa cell acquiring invasive behavior, in addition to let-7c and miR-218 (14).

Figure 1. miRNAs involvement in various steps of prostate cancer pathogenesis.

miRNAs ( ) show dysregulation upon transformation of normal glands to high grade prostate intraepithelial neoplasia (HGPIN), and then to invasive PCa. They are also involved in the acquisition of an aggressive behavior including castration-resistant prostate cancer (CRPC), biochemical failure and disease relapse. Tumor spread and metastasis is associated with a number of changes including epithelial to mesenchymal transition (EMT) and gaining cancer stem cell (CSC) characteristics that results in cell detachment and metastasis to distal organs, possibly by circulating tumor cells (CTC). Recent literature showed that miRNA deregulation is associated with many of these processes, as described in detail in the text.
Evidence of aggressive tumor behavior, such as biochemical recurrence, as well as local and distant metastasis, was found to be associated with altered expression of the metastamir miR-32 and miR-21 (30). This miRNA targets tumor-suppressor genes including TPM1 and PDCD4 and decreases BTG2 levels which induce the acquisition of epithelial-mesenchymal transition (EMT) in PCa (16). Moreover, alteration of Dicer expression is documented to be related to tumor growth and progression (19).

Figure 2. Illustration of dysregulated miRNAs in prostate cancer.

Recent literature has shown that certain miRNAs are associated with specific steps in PCa pathogenesis, including androgen receptor (AR) signaling, biochemical failure, metastasis, cancer stem cell (CSC) formation, Gleason score, epithelial to mesenchymal transition (EMT). Other miRNAs were shown to be associated with SNPs that can be useful in screening for cancer risk. miRNAs which are identified in more than two studies are shown in bold.
2.3 miRNAs in epithelial to mesenchymal transition (EMT)

Cancer progression is linked to EMT (Figure 1&2). Cells undergoing EMT share many biological characteristics with CSCs, and literature suggest that the two processes are interrelated. A recent study showed that PCa cells with EMT phenotype displayed stem-like cell features which were associated with decreased expression of miR-200 and the let-7 family (36). Loss of epithelial markers is associated with transcription suppressors such as zinc-finger E-box binding homeobox (ZEB) 1 and 2. In PC3 cells, miR-200b-c overexpression were found to be inversely associated ZEB 1 expression, and acquiring an EMT phenotype (36).

miR-182 and miR-203 are found to be down-regulated during EMT. These miRNAs regulate SNAI2 and P-cadherin (37). Presence of miR-205 is an essential factor for the inhibitory effects of p63, a metastasis suppressor, on EMT markers, ZEB1 and vimentin in PCa cells (38).

2.4 miRNAs and prostate cancer stem cells

CSCs are gaining considerable attention due to their involvement in tumor initiation, progression, therapy resistance, relapse and metastasis (39). The potential effects of miRNAs on cancer stem/progenitor cells are being explored in PCa. Liu et al. found that miR-34a, let-7b, miR-106a and miR-141 are down-regulated in CSCs, whereas miR-301 and miR-452 were up-regulated (26). A recent study demonstrated miR-143 and miR-145 significant role in metastasis by repressing CSC and stemness markers, and cellular viability (35).

Hypoxia regulates CSC through hypoxia-inducible factor (HIF), which activates pluripotent stem cell inducers, including miR-302. HIFs also induces glycolysis- and EMT-associated molecules, miR-181 and let7a (40). Anti-metastatic miRNAs, including miR-34a, and let-7 (27), function by inhibiting certain CSC properties. miR-34a induces G1 cell-cycle arrest and senescence, and let-7 induces G2-M phase arrest without senescence (26).

2.5 SNPs and miRNAs in prostate cancer

Single nucleotide polymorphisms (SNPs) can alter miRNA expression through varied mechanisms. Their presence in miRNA promoter sites can alter immature and mature miRNA transcription. SNPs located at the miRNA-binding sites of target genes could modify the efficiency of miRNA binding to the 3’UTR, leading to gene dysregulation. Oncogenic or tumor suppressor miRNAs function could be modified by miRNA-SNPs site resulting in alteration in protein levels (41).

Genetic mutations contributing to PCa risk groups have been recently investigated. Emerging genome wide-associations studies (GWAS) identified a number of SNPs associated with PCa risks factors such as age at diagnosis, pathological aggressiveness, and family history of cancer (42). PCa
aggressiveness was found to be associated with pairs of SNP-SNP interactions. These SNP network converge on the epidermal growth factor receptor (EGFR) pathway, and could affect PCa oncogenesis and proliferation (43). Genetic variance in miRNA regions could influence PCa development. Risk of disease development is associated with increased with the SNP of miR-146a (rs2910164), miR-196a (rs11614913), miR-499 (rs3746444), and miR-612 (44-47). These SNPs have potential as predictors of PCa risk in high risk groups. It needs to be investigated if these associations are of functional significance.

3. The clinical utility of miRNA as prostate cancer biomarkers

Due to their involvement in cancer pathogenesis, miRNAs have a wide range of potential applications as diagnostic, prognostic, or predictive markers, or as potential therapeutic targets and pharmacogenomic markers for both primary and metastatic cancers (7;18;24;48). miRNAs possess many properties that make them attractive biomarkers, including the ability to detect them in small volume samples, and from formalin-fixed tissues. Furthermore, they can be detected in different body fluids, such as serum and urine, using specific and sensitive quantitative real-time PCR (qRT-PCR) (7).

3.1 miRNAs as diagnostic markers in prostate cancer

Clinical stage, Gleason score and PSA level provide the current parameters for PCa diagnosis. miRNAs provide useful information beyond these parameters, and by incorporating them into these parameters, miRNAs will improve these clinicopathological parameters diagnostic and prognostic effectiveness (Figure 3). For example, miRNAs were found to be associated with clinicopathological state (22), and dysregulated in premalignant prostate lesions before progression to cancerous and then metastatic disease (14). miRNAs were also found to be dysregulated in biochemical failure high risk group (13). The ability to quantify these miRNAs expression from archival formalin-fixed tissues and body fluids makes this approach potentially useful in determining high risk patients. Oncologist can use the archival tissues with full clinical, survival and therapeutic information (16;48;49). However, further studies are needed to examine the implication of tumour heterogeneity, stages and grades on miRNA expression. Experimental studies demonstrated the potential use of miRNAs as PCa diagnostic markers (Table 1). miRNA differential expression can also be used to identify the tissue of origin in undifferentiated tumors, which is an important problem in surgical pathology practice. Tumors from the same embryonic origin were found to share the same miRNA clusters (18;50).

3.2 The prognostic utility of miRNAs in prostate cancer

Assessment of PCa prognosis is a challenge, and now relies on histopathological parameters (like Gleason score) together with PSA levels (Figure 3), which do not always correctly reflect disease status. Many studies have been reported the potential utility of specific miRNA expression profiles
to assist in linking PCa with its aggressive behavior (21;24;31;48;51), either alone or combined with current prognostic tools (52). Schaefer et al. reported high miR-96 expression as a prognostic tissue biomarker associated with decreased recurrence free interval. High miR-96 was found to be associated with high Gleason score, tumour stage and biochemical failure (defined as elevation of PSA after surgery to ≥ 0.2 ng/ml in two successive measurements) remains the only available marker, and Gleason score (22). Differential miRNA tissue expression has also been observed between high grade (Gleason score ≥ 8) and low grade tumors (24).

Conventional clinical parameters have limited value for assessment of clinical outcome after diagnosis, and are not efficient for personalizing the treatment plan for individual patients. miRNAs, alone or in combination with clinical parameters, can be used to enhance patient management plans and this can lead to a significant improvement of outcome.

Statistical survival analysis identified down-regulation of two miRNAs with prognostic importance expressed in prostate tissue, miR-221 and miR-96, which were found to be associated with clinical outcome and biochemical relapse (22;31). Table 2 summarizes the potential utility of miRNA expression to predict aggressive behavior, including extra-prostatic disease, biochemical failure, and CRPC. Recent studies have shown the ability of miRNAs to predict relapse and biochemical failure in PCa (13;48). Differential miRNA expression also correlated with metastases and stem cell formation (40).
A recent study identified 25 differentially expressed miRNAs between patients with high versus low risk of biochemical failure, including miR-331-3p, miR-193a, and miR-125a-b. miR-152 function through targeting ERBB signaling pathways, transforming growth factor-β (TGF-β) signaling, focal adhesion, and extracellular matrix (ECM)-receptor interaction. ERBB signaling is a major steroid-independent activator of AR, which makes miR-152 a biomarker with therapeutic importance (48). miR-10b and miR-222/miR-10b ratio were good predictors of PCa biochemical failure (13).

### 3.3 miRNAs as predictive markers for prostate cancer

Markers that can predict response to therapy allow physician to restrict treatment only to the subgroup of patients who are likely to respond, thus avoiding unnecessary cost and side effects of administering treatment to patients who will not experience a benefit. PCa is a hormone-dependent malignancy. A recent study showed that serum miR-21 levels are elevated in CRPC patients, especially in those resistant to docetaxel-based chemotherapy. This study suggested that miR-21 can be a marker to indicate the transformation to hormone refractory disease, and a potential predictor for the efficacy of docetaxel-based chemotherapy (53). Another study reported that SNPs inside miRNAs and miRNA target sites have potential value to improve outcome prediction in PCa patients receiving androgen deprivation therapy (54). A third study investigated miR-141 as a potential biomarker of therapeutic response in PCa. Serum miR-141 could be a new predictive biomarker to PCa progression, when compared to validated biomarkers such as PSA and CTC. However, it was less specific than PSA (55). Therefore, miRNAs should be combined with other validated biomarkers to increase their effectiveness. Directional changes in PSA, CTC, and miR-141 had sensitivity in predicting clinical outcome in 79% of cases. Logistic regression modeling of the probability of clinical progression demonstrates that miR-141 levels predicted clinical outcomes with an odds ratio of at least 8.3 (55). More research studies are needed to assess the utility of miRNAs as predictive markers for radiotherapy, chemotherapy and androgen suppression therapy (56).

### 4. miRNAs as non-invasive biomarkers

Cellular miRNAs may be released to body fluids such as serum, plasma, urine or saliva. These miRNAs are carried and protected from degradation in complexes with Argonaute proteins (catalytic components of RISC), high-density lipoprotein and microvesicles (57). Current studies are looking to use these cell-free, circulating miRNAs as noninvasive biomarkers for PCa (58). Extracellular miRNAs could be the product of dead cancer cells, circulating tumor cells (CTCs), as well as nonmalignant cells, such as platelets, or the product of nonmalignant cells tissue damage (59).
4.1 Circulating miRNAs in blood

miRNAs originating from prostate cancer can be released into the circulation and can be readily measured in plasma and serum from PCa patients (60). An earlier study showed that miRNAs are present in a remarkably stable form in blood and that they are protected from endogenous RNase activity (58). This study showed that miRNAs originating from human PCa xenografts are readily measured in plasma, and can robustly distinguish xenografted mice from controls. In addition, it was determined that serum levels of miR-141 can distinguish patients with PCa from healthy controls (58).

Table 3 summarizes circulatory miRNAs with diagnostic, prognostic and predictive importance. Recent studies have observed a correlation between circulating miRNA expression and risk assessment models. miR-20a was significantly overexpressed in plasma from patients with stage 3 tumors compared to stage 2 or below, and significant increases in miR-21 and miR-145 expression were also observed with intermediate or high risk D'Amico scores (Table 3) compared to low risk scores (51). Combining miRNAs with the current prognostic tools for risk assessment can improve the accuracy of these models (52).

Serum from patients with metastatic PCa showed up-regulation of five miRNAs; miR-375, miR-9*, miR-141, miR-200b, and miR-516a-3p. Also, miR-141 and miR-375 are associated with metastatic disease in other studies (61-63). Up-regulation of serum miR-93, miR-106a and downregulation of miR-24 are also linked to metastatic PCa (60). Combining circulating miRNAs associated with biochemical failure, such as miR-141, miR-146b-3p and miR-194, with the current prognostic tools can predict disease progression (52). Measuring tumor-derived miRNAs in blood was essential diagnostic step, but endogenous miRNAs baseline, tumour heterogeneity and other possible miRNAs sources should be considered.

4.2 miRNAs in urine

There is growing body of evidence supporting the clinical utility of urinary miRNAs as PCa biomarkers (4). miRNAs are reported to be stable in body fluids which contains RNases. They resist nuclease activity, as well as methylation, adenylation, or uridylation (64). Urine of PCa patients was found to have a higher concentration of miR-150 and -328, whereas miR-107, miR-574-3, miR-196b, miR-200b, miR-100, and miR-106a showed decreased concentrations (4,64). The diagnostic value of these miRNAs has been shown to outperform that of prostate cancer antigen3 (PCA3), a biomarker for PCa that is measured in urine samples. It is vital to realize that miRNAs released into body fluids do not necessarily reflect miRNAs abundance in the cell of origin. A recent study suggested the existence of cellular selection mechanisms for miRNA release, which should be an important consideration in the identification of circulating miRNA biomarkers (57). Extracellular miRNAs are feasible diagnostic
and prognostic biomarkers. However, caution should be taken with study methodology and miRNA normalization reference to achieve concordant data and outcomes.

4.3 miRNAs in circulating tumor cells

Circulating tumor cells (CTCs) have been proven to be of significance as cancer biomarkers, especially as prognostic indicators and therapy-monitoring biomarkers (65). In PCa, CTC enumeration has been extensively studied and validated as a prognostic tool with FDA clearance for use in monitoring advanced disease (66). In addition to quantification of CTC in blood, recent evidence suggests the usefulness of CTCs as sources for DNA analysis. Molecular characterization of captured cells can serve as a “liquid biopsy” of the tumor, reflecting molecular changes in an individual’s malignancy over time.

Current evidence shows that EMT could occur in CTCs in PCa. Consequently, research groups are currently focusing on the development of new markers to detect CTCs with an EMT phenotype. Cells undergoing EMT produce mesenchymal proteins such as N-cadherin, vimentin, tenascin C, laminin_1, type VI collagen and numerous proteinases, and lose epithelial E-cadherin, which protect cancer cells from anoikis. Additionally, expression of EMT transcription factors, Twist, Slug, Snail and SIP was found to protect CTCs from anoikis (65). Tumor-derived circulating miRNAs were studied in the plasma of PCa patients using centrifugation and filtration to exclude CTCs (58). Cell-free miRNAs were detected in the supernatant or filtrate. However, the authors could not exclude the possibility of cellular miRNAs release during blood processing steps (58).

Recent studies in breast cancer documented the capacity of circulating miRNAs to indicate the CTC status and their potential as prognostic markers. CTC is a rapidly developing important biomarker in cancer (59). CTC-associated miRNAs could have higher specificity than the free circulating miRNAs. However, most of the CTC-associated miRNA study findings are preliminary that awaits further validation.

5. miRNAs as potential therapeutic targets

Recently, miRNAs are gaining attention as potential therapies for a wide array of diseases including hepatitis, hypercholesterolemia and cancer (67,68). Some miRNA-based therapies have already successfully passed phase II clinical trials. miRNA therapy has many advantages, as recently outlined (49). A major advantage of miRNAs is that their gene-silencing effects occur in the cytoplasm without disturbing nuclear molecules. Because of their small size, they are much easier to transfect without many side effects. Moreover, miRNAs regulate multiple gene networks, thus offering the advantage of simultaneous down-regulation of multiple cancer-promoting signaling pathways (69).

A number of studies highlighted the potential therapeutic applications of miRNAs in PCa (70). Inhibition
of cancer cell growth and migration with genistein, a small biologically active flavanoid, has been found to act by inhibiting oncogenic miRNAs such as miR-21, 151, 221 and 222, which inactivate Notch signaling, RAC1/VEGF mediating angiogenesis and increase expression of tumour suppression gene ARHI, respectively (71). On contrary, genistein inhibited cell growth by tumor suppressor miR-574-3p up-regulation (71). A natural agent, isoflavone, was found to alter methylation sites of miR-29a and miR-1256, increasing their levels and decreasing expression of TRIM68 and PGK-1, which inhibits PCa cell growth and invasion (72). Moreover, vitamin D was found to up-regulate tumour suppressor miR-98, which suppressed tumour growth by inducing G2/M arrest (73). The aim of these therapeutic maneuvers is manipulating oncogenic and tumour suppressor miRNAs to control tumorigenesis.

6. Future prospects

The current efforts to define PCa diagnostic and prognostic miRNAs are still evolving (Figure 3). The consensus on high value miRNAs as specific biomarkers has not yet been established due to multiple factors. Observed discrepancies among studies could be due to differences in case series examined, specimen type (formalin-fixed vs. frozen tissue and blood), tumor heterogeneity and sampling issues, RNA isolation protocols and method of detection (microarray vs. qRT-PCR, etc). Large scale high quality studies with patients’ clinical and pathological information is an important step in this regard. Further validation of the potential miRNA biomarkers should be conducted as well (3).

The accumulating evidence shows that miRNAs are actively involved in PCa pathogenesis, tumor progression and metastasis (17), and can be used as potential biomarkers for patient management (5). Among promising miRNAs which could be used in future (Figure 2) as biomarkers for tumour proliferation miRs-15, 16, 20a, 21, 23, 32, invasion miRs-143, 145, 205, 221/222 and androgen-independent growth miRs-125b, 146, 205, 221/222 (13;14;21;22;28;30;51).

In the era of personalized medicine, a great hope relies upon the integration of multiple clinical and molecular parameters to establish a patient-specific risk profile useful for clinical decision making (Figure 3) (74). Multiparametric approaches utilizing different types of molecules hold the promise of enhancing the sensitivity and specificity of molecular markers as diagnostic tests. In addition to their value as disease and therapeutic biomarkers, miRNAs have great potential as therapeutic targets.

Reference List

Samy M Mekhail, Peter G Yousef, Stephen W Jackinsky, Maria Pasic, George M Yousef

iRNA in prostate cancer: new prospects for old challenges


Table 1. Differentially expressed miRNAs in prostate cancer.

<table>
<thead>
<tr>
<th>Up-regulated miRNAs</th>
<th>Down-regulated miRNAs</th>
<th>Methods</th>
<th>Number of clinical tissue samples/PCa vs. normal</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>202, 210, 296, 320, 370, 498, 503, 373*</td>
<td>let7a-d, let7g, 16, 23a, 26a, 92, 99a, 103, 125a-b, 143, 145, 195, 199a, 221, 222, 497</td>
<td>• MA</td>
<td>• 5 primary PCa4 hormone - refractory PCa(±hormonal treatment) 4 BPH</td>
<td>(15)</td>
</tr>
<tr>
<td>7d, 195, 203, 34a, 20a, 29a, 25, 95, 197, 1352, 187, 1961, 148, 191, 21, 7i, 198, 199a-2, 30c, 17-5p, 92-2, 146, 181b1, 32, 206, 184prec, 29a prec, 29b-2, 181b, 196prec, 93, 223, 16, 101, 124a, 26a, 214, 27a, 106a, 199a</td>
<td>128a, let7a-2, 218-2, 29a, 149, 24-1</td>
<td>• MA</td>
<td>• 56 primary PCa7 normal prostate tissues</td>
<td>(18)</td>
</tr>
<tr>
<td>32, 182, 31, 26a, 200c, 375, 196a, 370, 425, 194, 181a, 34b, 7i, 188, 25, 106b, 449, 99b, 93, 92, 125</td>
<td>520h, 494, 490, 133a, 1, 218, 220, 128a, 221, 499, 329, 340, 345, 410, 126, 205, 7, 145, 34a, 487, let7b</td>
<td>• MA RT-qPCR</td>
<td>• 60 primary PCa(no hormonalthrapy)16 Normal</td>
<td>(19)</td>
</tr>
<tr>
<td>Let family, 34a, 29a, 16</td>
<td>145, let-7 (7b–g, 7i), 26a-b, 29a-c, 30a-e, 99a-b, 125a-b, 200a-b</td>
<td>• MA RT-qPCR</td>
<td>• 16 PCa30 PCa with relapse10 Normal tissue</td>
<td>(20)</td>
</tr>
<tr>
<td>141, 20a</td>
<td>23b, 100, 145, 221, 222, 143</td>
<td>• mirMA-SART-qPCR</td>
<td>• 40 PCaNormal adjacent tissue</td>
<td>(21)</td>
</tr>
<tr>
<td>524*, 182*, 183, 634, 96, 182, 130b, 375</td>
<td>205, 222, 221, 368, 181b, 149, 31, 16184.145, 125b</td>
<td>• MA RT-qPCR</td>
<td>• 76 PCaNormal adjacent tissue</td>
<td>(22)</td>
</tr>
<tr>
<td>let7a, 17, 21, 93, 101, 141, 182, 375, 720, 1826, 12745, 106a, 106b, 200b, 200c, 20a, 20b, 768-3p</td>
<td>136*, 145, 214, 221, 222, 302d*, 375*</td>
<td>• MA RT-qPCR</td>
<td>• 20 PCaNormal adjacent tissue</td>
<td>(23)</td>
</tr>
<tr>
<td>let7, 1, 98, 126, 132, 142, 143, 144, 205, 210</td>
<td>34c, 29b, 212, 10b</td>
<td>• RT-qPCR</td>
<td>• 37 PCaNormal adjacent tissue</td>
<td>(24)</td>
</tr>
</tbody>
</table>
### Table 2. Prognostic miRNA biomarkers and their applications in prostate cancer

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>miRNAs expression</th>
<th>Methods</th>
<th>Patients numbers</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biochemical failure risk Low vs. high risk</td>
<td>23a, 449a, 449b, 200a, 1233, 10b, 1825, 186, 1275, 532-5p, 193b, 886-3p, 664, 196b, 1274b, 720, 146b5p, 222, 31, 127-5p</td>
<td>RT-qPCR</td>
<td>40 PCa, 12 normal adjacent tissue</td>
<td>(13)</td>
</tr>
<tr>
<td>Hormone refractory tumors</td>
<td><strong>Up-regulated</strong></td>
<td>MA</td>
<td>5 primary PCa, 4 PCa hormone-refractory PCa, (±hormonal treatment), 4 BPH</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td>184, 198, 302c**, 345, 491, 513</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7f, 19b, 22, 26b, 27a, 27b, 29a, 29b, 30a, 30b, 30c, 100, 148a, 205</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra-prostatic disease</td>
<td>101, 200a, 200b, 196a, 30c, 484, 99b, 186, 195, 7f, 34c, 371, 373, 410, 491</td>
<td>MA, RT-qPCR</td>
<td>60 primary PCa, 16 Normal</td>
<td>(19)</td>
</tr>
<tr>
<td>Androgen-regulated tumors</td>
<td>338, 126, 146b, 181b, c (cluster), 219, 221(cluster)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biochemical failure</td>
<td><strong>Up-regulated</strong></td>
<td>mir-MASA, SART-qPCR</td>
<td>40 PCa, 40 normal adjacent tissue</td>
<td>(21)</td>
</tr>
<tr>
<td></td>
<td>135b, 23a, 34c, 194, 218, 96, 16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>342, 154, 140, 298, 129, 126, 122a, 213, 300</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gleason score High vs. low grade</td>
<td><strong>Up-regulated</strong></td>
<td>RT-qPCR</td>
<td>37 PCa, Normal adjacent tissue</td>
<td>(24)</td>
</tr>
<tr>
<td></td>
<td>122, 335, 184, 193, 34, 138, 373, 9, 198, 144, 215</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>96, 222, 148, 92, 27, 125, 12627</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PrognosticAndrogen-regulated</td>
<td><strong>Up-regulated</strong></td>
<td>MA</td>
<td>28 PCa, 14 CRPC12 BPH</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td>21, 32, 148a, 590-5p</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>Down-regulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>99a, 99b, 221</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biochemical failure riskLow vs. high risk</td>
<td>148a, 141, 135a, 19a, 19b, 26b, 29c, 174b, 196b, 26a, 3313p, 193a, 365, 12a, 125b</td>
<td>RT-qPCR</td>
<td>27 PCa with biochemical failure</td>
<td>(48)</td>
</tr>
</tbody>
</table>

mirMASA, microRNA multianalyte suspension array
Table 3. Circulating miRNA biomarker applications

<table>
<thead>
<tr>
<th>miRNA applications (Samples)</th>
<th>Up-regulated miRNAs</th>
<th>Down-regulated iRNAs</th>
<th>Methods</th>
<th>Patients samples</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prognostic for D’Amico scores (^1) High vs. low risk (Plasma)</td>
<td>20a, 21, 145, 221</td>
<td>----------</td>
<td>(\cdot) RT-qPCR</td>
<td>82 PCa</td>
<td>(51)</td>
</tr>
<tr>
<td>Prognostic Biochemical failure (Serum)</td>
<td>141, 146b-3p, 194</td>
<td>----------</td>
<td>(\cdot) MART-qPCR</td>
<td>8PCa8 patients with recurrence</td>
<td>(52)</td>
</tr>
<tr>
<td>Prognostic for metastatic PCa (Serum)</td>
<td>100, 125b, 141, 143, 296</td>
<td>----------</td>
<td>(\cdot) RT-qPCR</td>
<td>25 patients with metastasis 25 healthy volunteers</td>
<td>(58)</td>
</tr>
<tr>
<td>Prognostic for PCa risk factor index (^1,2) (Serum)</td>
<td>20b, 874, 1274a, 1207-5p, 93, 106a</td>
<td>223, 26b, 30c, 24</td>
<td>(\cdot) RT-qPCR</td>
<td>36 patients with metastasis 12 healthy volunteers</td>
<td>(60)</td>
</tr>
<tr>
<td>Prognostic for metastatic PCa (^3) (Serum)</td>
<td>375, 9*, 141, 516a3p, 629, 203, 429, 618, 212, 21, 545, 218, 422, 656, 655, 29c, 200b, 200c, 502-5p</td>
<td></td>
<td>(\cdot) RT-qPCR</td>
<td>14 PCa7 patients with metastasis</td>
<td>(61)</td>
</tr>
<tr>
<td>Prognostic for metastatic castration-resistant PCa and diagnostic (^3) (Serum)</td>
<td>141, 298, 375, 346(^1)</td>
<td></td>
<td>(\cdot) RT-qPCR</td>
<td>25 patients with metastasis 25 healthy volunteers</td>
<td>(62)</td>
</tr>
<tr>
<td>Prognostic for metastatic PCa (Plasma)</td>
<td>125b, 136, 1513p, 200a, 744a*, 9, 8*, 99a, 7d, 126, 142-5p, 15b, 27a, 27b, 30a*</td>
<td>205, 106b, 16, 363</td>
<td>(\cdot) RT-qPCR</td>
<td>25 PCa25 patients with metastasis</td>
<td>(63)</td>
</tr>
</tbody>
</table>

1. D’Amico scores: risk assessment: PSA level, Gleason and T stage. Low-risk: PSA less than or equal to 10, Gleason score less than or equal to 6, and clinical stage T1-2a; Intermediate risk: PSA between 10 and 20, Gleason score 7, or clinical stage T2b. High-risk: PSA more than 20, Gleason score equal or larger than 8, or clinical stage T2c-3a.
2. miRs-141,298,375 are diagnostic and miRs-141 and 375 are prognostic (relapse)
3. Clinicopathology index: age, PSA level and Gleason score
Urothelial bladder cancer urinary biomarkers
Aidan P Noon1,2 & Alexandre R Zlotta1, 2,3

1The University of Toronto, Department of Surgery, Division of Urology, Toronto, Ontario
2Princess Margaret Cancer Hospital, Department of Surgical Oncology, Division of Urology, Toronto, Ontario
3Mount Sinai Hospital, Department of Urology, Department of Surgery, Division of Urology, Toronto, Ontario

ARTICLE INFO

Corresponding author:
Alexandre R Zlotta, MD
Murray Koffler Urologic Wellness Centre
Mount Sinai Hospital
6th Floor, Rm 6004, Box 19
Toronto, ON, M5T 3L9
e-mail: AZlotta@mtsinai.on.ca

Running Title:
Urothelial Bladder Cancer Urinary Biomarkers

Keywords:
Urothelial
Carcinoma
Bladder,
Biomarker
Urinary

Non-standard abbreviations
PCa; prostate cancer, miRNA; microRNA, CSCs; cancer stem cells, AR, androgen receptor, CTC; circulating tumor cells, CRPC; castration-resistant prostate cancer

ABSTRACT

Urothelial bladder cancer is the fourth most prevalent male malignancy in the United States and also one of the ten most lethal. Superficial or non-muscle-invasive bladder cancer has a high rate of recurrence and can progress to muscle invasive disease. Conventional surveillance requires regular cystoscopy and is used often with urinary cytology. Unfortunately, the cystoscopy procedure is invasive for patients and costly for health care providers. Urinary biomarkers have the potential to improve bladder cancer diagnosis, the efficiency and also the cost-effectiveness of follow up. It may also be possible for urinary biomarkers to help prognosticate particularly for patients with high-grade bladder cancer who may want enhanced assessment of their risk of disease progression. In this review the important historical urinary biomarkers and the newly emerging biomarkers are discussed. As will be presented, although many of the tests have good performance characteristics, unfortunately no single test can fulfill all the roles currently provided by cystoscopy and cytology. It is likely that in the future, urinary biomarker testing will be used selectively in a personalized manner to try and improve prognostication or reduce the necessity for invasive cystoscopy in patients understanding the limits of the test.
Introduction

Urothelial Bladder Cancer (UBC) is the 4th most common malignancy affecting American males and the 8th most lethal(1). Urinary biomarkers have been the subject of great interest in the field of UBC as urinary sediment may contain exfoliated (intact) or fragments of UBC cells, potentially allowing clinicians to screen, diagnose, prognosticate and follow up patients with this disease. In order to understand how biomarkers may fulfill these different roles it is necessary to understand the natural history of this disease. UBC presents as two clinically distinct groups as assessed by stage. the first group (70% of new cases) is non-muscle-invasive bladder cancer (NMIBC) where the disease is limited to the urothelium and submucosa but has not invaded the muscularis propria (detrusor muscle) and the second group (30% of new cases) is muscle invasive bladder cancer (MIBC) where the disease has invaded into the detrusor muscle. Approximately 50% of patients with MIBC will harbour occult metastatic disease and therefore despite radical treatments (cystectomy or radiotherapy), will die of their disease(2). Early detection (screening of asymptomatic) is desirable especially for individuals at risk (smokers and workers in industry with carcinogenic exposure(3)), and this is one role that urinary biomarkers may fulfill.

Patients that present with symptoms or signs suggestive of UBC (macro or microscopic hematuria), the standard evaluation involves an office cystoscopy and cytological examination of the urine for malignant exfoliated cells in addition to upper tract imaging (as urothelial carcinoma can be found in the renal pelvis or ureter). There is interest in using urinary biomarkers to enhance the detection of UBC in this setting.

For patients treated for NMIBC approximately 50% may have a recurrence of their disease and 10 – 15% of patients may progress to MIBC. The majority of cases are of low grade with very low potential of progression to life threatening MIBC. Here the goal is to detect disease recurrence and this is currently being performed by using a combination of cystoscopy and urinary cytology(4). The cost of performing this follow up protocol is high and is responsible for UBC being labelled as the most expensive cancer for health care providers to treat (4). As urine is in contact with the entire urothelium, including the upper-tract, which cannot be reviewed by a cystoscope, a urine-based biomarker for detecting recurrence would be desirable, especially if it could obviate the need for cytology and / or cystoscopy in a cost effective manner.

Patients with “high risk” NMIBC (stage pT1, carcinoma-in-situ, or high grade disease) currently provide the greatest challenge, as clinicians need to follow them to ensure that their disease has not progressed or recurred. Unlike in patients with low-risk NMIBC, where the risk of progression is very low, a urinary biomarker in this setting must have excellent sensitivity, as this disease can be lethal if missed. Within the high grade NMIBC group there is also the option for utilizing a urinary biomarker
that can help define which patients may progress (and therefore be recommended to undergo early
cystectomy or more vigorous follow up) from those that will not. The ability of urinary biomarkers to
prognosticate disease has been evaluated and will be discussed.

Urinary UBC biomarkers have been reviewed previously (5, 6). The focus of this review is to highlight
laboratory based urinary biomarker tests that can help clinicians to screen, diagnose, survey and
prognosticate UBCs. Some tests described have been approved by the FDA and are established in
clinical practice. In addition to established tests the surge in genomic evaluation in UBC has identified
a number of candidate genes and gene regulatory networks that may have a role in UBC biology.
Some of the more promising genes will also be discussed.

**Protein Assays**

**Bladder tumour antigen (BTA)**

Bladder tumour antigen (BTA) assays aim to detect the human complement factor H-related
protein (enables UBC to evade host immune responses) in urine (7). There are two commercially
available assays for BTA; BTA STAT™ which is a qualitative point of care test and BTA TRAK™ which
is a quantitative sandwich immunoassay reference laboratory test (POLYMEDCO, Washington, US)
(8). BTA STAT™ has a reported sensitivity of 57% to 83% and specificity of 60% – 92% (9-13). Thomas
et al, used ROC analysis and calculated that the optimum BTA TRAK™ level was 14 kilounits/L (14),
they used this cut off level and calculated a sensitivity of 66% for diagnosing UBC in a population
suspected of harbouring UBC. Including the paper by Thomas et al, the reported overall sensitivity
ranges from 62% to 91% (14-21). Recent publications have further demonstrated that the presence
of hematuria can lead to false positive results with the BTA STAT™ and BTA TRAK™ assays(22-24), given
these limitations the FDA have only approved BTA assays in combination with cystoscopy.

**Nuclear matrix protein 22**

There are two commercially available detection methods for identifying the presence of urinary
nuclear mitotic apparatus protein 22 (NMP22), which is more abundant in the urine of patients
harbouring UBC. The first test is NMP22® (Alere™, Scarborough, Maine, USA) a laboratory-based,
quantitative, sandwich-type, enzyme immunoassay and the second is a qualitative point-of-care test
called BladderChek® (Alere™, Scarborough, Maine, USA). Historically the NMP22® has been shown to
have a variable sensitivity (47% - 100%) and specificity (60% – 90%) depending on the value assigned
for a positive result(9-11, 20, 25-29). False positive results can be seen in any urinary condition that
can cause cell death and release of NMP22 such as benign inflammatory conditions, infection or
urolithiasis(10) and also in concentrated urine(30). NMP22® and NMP22 BladderChek® have been
compared in 100 urine samples collected from patients prior to Trans-Urethral Resection of Bladder
Tumour (TURBT) and 100 normal controls, using the manufacturer ELISA cut-off of 10U/ml. The NMP22® had a sensitivity of 40% and a specificity of 99% where as the BladderChek® was found to have a superior sensitivity of 59% and a specificity of 93%(31). NMP22® has been used to screen an “at risk” population that have had industrial exposure to aromatic amines but the authors did not feel that this test could be used alone for screening this specific population(32). NMP22® has been shown to be a cost effective alternative to urine cytology in the diagnosis of UBC using cystoscopy(33), and it is possible that in certain patients, the NMP22® could be used as an alternative to cystoscopy(34) although the FDA has only approved the test to be used along with cystoscopy.

**Urinary UBC test**

Fragments of cytokeratins 8 and 18 are the markers targeted by three commercially available tests; two quantitative assays - UBC® ELISA, UBC® IRMA (Immunoradiometric Assay) and UBC® Rapid (one stop qualitative point of care assay) all manufactured by IDL Biotech, Borlange, Sweden (35, 36). Hakenberg et al, evaluated the UBC® Elisa (cut off of 12µg/L), UBC® Rapid and cytology prospectively in 181 patients (117 pre TURBT, 43 post TURBT & 47 controls) and found the respective sensitivity and specificity to be 64.4% & 63.6% for UBC® rapid, 46.6% & 86.3% for UBC® ELISA and 70.5% & 79.5% for cytology(37). The UBC® Rapid test was also used to evaluate 180 urine samples from patients with symptoms suggestive of UBC or being followed up post TURBT. In this study 53 patients were found to harbour UBC and the sensitivity of the UBC Rapid test was 66% and specificity was 90%, which outperformed the BTA Stat™ test (sensitivity 53% and specificity 90%)(36). Babjuk et al, evaluated cytology, BTA TRAK™ and UBC IRMA® in the urine of patients with a history of low grade NMIBC undergoing surveillance cystoscopy, the reported sensitivities were 19.8%, 53.8% & 12.1% respectively with specificities of 99%, 83.9% & 97.2%(16). UBC does not have the desirable performance characteristics to replace cystoscopy or cytology.

**BLCA-1 and BLCA-4**

BLCA-1 and BLCA-4 are nuclear matrix proteins. BLCA-1 is not expressed in normal urothelium, while BLCA-4 is expressed in both the tumour and adjacent benign areas of the bladder but not in normal (no history of UBC) bladders(38). BLCA-1 has been evaluated (ELISA) in the urine of patients with UBC and found to have 80% sensitivity and 87% specificity(39). Similarly BCLA-4 has been measured (ELISA cut off value 13 units) in the urine of pre TURBT patients (N=54) and normal controls (N=51) and found to have a sensitivity of 96.4% and a specificity of 100%(40). In the same paper 38 of 202 urine samples from the spinally injured were positive at the BLCA-4 ELISA cut-off. These two markers require validation by another group (in fact a later publication, by the same group evaluating BLCA-4 functionality has recently been retracted(41)) and the lack of published work on these markers in the
last few years, suggests that by themselves, these are unlikely to become established diagnostic tests in their own right.

**Cell Based**

*uCyt+™ / ImmunoCyt™*

Urinary cytology is an established adjunct to cystoscopy, which involves examination of exfoliated bladder cancer cells by a trained cytopathologist. Cytology has been shown to have a high sensitivity for detecting high grade UBC, especially carcinoma-in-situ. To try and increase detection of low grade UBC the immunoCyt™ test was developed (42). This commercially available laboratory test, uCyt+™ (formerly ImmunoCyt™) ((Scimedx Corp., Denville, NJ) combines standard urine cytology with immunofluorescence detection of three monoclonal antibodies (M344, LDQ10, and 19A211) which target carcinoembryonic antigen and two tumour associated mucins (43). The test requires the urine to contain a minimum number of exfoliated cells and is dependent on a trained cytopathologist analysing the sample and is therefore expensive. Comploj et al (42), reported the results of 7,422 consecutive urine cytology and uCyt+™/ImmunoCyt™ tests, they found a sensitivity of 34.5%, 68.1% & 72.8% for cytology alone, uCyt+™/ImmunoCyt™ and both test combined respectively. The specificity was 97.9%, 72.3% & 71.9% for the same test combinations. It is known that false positive results can be seen in men with benign prostatic hyperplasia or cystitis(44). The sensitivity of uCyt+™/ImmunoCyt™ is currently too low for this test to replace cystoscopy, however it may have a role for equivocal standard cytology tests(45).

**UroVysion**

UroVysion® (Abbott Molecular, Inc., Des Plaines, IL) employs fluorescence in-situ hybridization to detect gains in copy number of chromosomes 3,7, and 17 as well as loss of the 9p21, which contains the P16 gene(46). UroVysion® is designed to enhance the normal morphological assessment provided by cytology by assessing molecular changes. A 2008 published meta-analysis comparing UroVysion® to cytology found the sensitivity and specificity of all studies evaluating UroVysion® were 72% (69%-75%) and 83% (82%-85%) respectively, and for cytology the overall sensitivity and specificity was 42% (38%-45%) and 96% (95%-97%)(47). The meta-analysis demonstrated that the superiority of UroVysion® to urine cytology was based on the former tests superior sensitivity for superficial low grade UBC. The cost of UroVysion® is greater than that of urine cytology and requires specialised laboratory testing, it is therefore unlikely to be a cost effective alternative for units that employ cytology. UroVysion® may be able to help in the cases of equivocal urine cytology(48, 49), however what to do in the event of a positive UroVysion® in the absence of cystoscopic or radiological validation poses other clinical dilemmas(50-52).
Genes / DNA tests

TERT

TERT or hTERT is the abbreviated form of human Telomerase reverse transcriptase and is a catalytic subunit of the telomerase complex. Mutations in the promoter of TERT can lead to increased expression of telomerase enabling malignant cells to continue to renew telomeres and avoid end replication problems. It has been reported that TERT promoter mutations are the most common genetic lesion reported to date in NMIBC, seen in 65%, 68% & 86% (pTA LG, pTa HG & C.I.S respectively) of cases(53). Alloy et al, screened two different tumour cohorts and found the TERT promoter mutations present in 70% of tumours of all stages and in a second cohort 80% in NMIBC and 79% in MIBC, using a SNapShot® assay (Applied Biosystems®)(54). From the same paper, the sensitivity of urine samples analysed by SNapShot® assay had a sensitivity of 62% for detecting new tumours and 42% for recurrent samples. It also been shown that the TERT promoter mutations can be detected from urine using PCR amplification and miSEQ. In fact using this technique, Kindle et al(53), found that 8/15 patients with TERT promoter mutations, detected in urine following TURBT (at time of follow up cystoscopy), all 8 (100%) had subsequent recurrence discovered. Using a SNapShot®, Hurst et al, also demonstrated detection of 51/53 positive samples from the urine of patients prior to TURBT(55).

FGFR3 mutations

Fibroblast growth factor receptor 3 mutation is a frequent genetic event, particularly in low-grade tumours (56). Van Oers et al, developed and tested a SNapShot® assay targeting nine FGFR-3 mutations. In this study 64 urine samples were analysed (29 from bladder harbouring a FGFR3 mutant tumour and 35 FGFR3 wildtype tumours), and the calculated sensitivity was 62% and specificity 89%(56). This SNapShot® assay panel was evaluated in the follow up setting of 200 patients known to have low grade NMIBC (67% of tumours were mutant FGFR3), the sensitivity of the assay was found to be 58% for concomitant disease. An FGFR3 positive urine sample was found to have a 3.8-fold higher risk of recurrence versus a negative sample(57). As FGFR3 is associated with low grade / stage tumours it has been combined with other markers to try and improve diagnostic performance, and featured as one of the markers for a bladder cancer screening study(58). FGFR3 in combination with TERT promoter mutations has been shown to have a sensitivity of 70% and a specificity of 71% in detecting new UBC tumours(54). Kandimalla et al (59), combined FGFR3 with a DNA hypermethylation assay and found a sensitivity of 52% for FGFR3 alone but 80% when in combination with the methylation assay. It is very unlikely that FGFR3 will have utility as a urinary biomarker in its own right as tumours with FGFR3 mutation can still progress to MIBC(57) and so cystoscopy could not be obviated in cases of urine positive for FGFR3 mutation.
**STAG2**

STAG2 ("stromal antigen" gene 2) is located on the X chromosome and encodes for a subunit of the cohesion molecule that is important for regulating sister chromatid cohesion during cell division and also regulates gene expression through DNA looping and interactions with transcription factors(60). There has been recent interest in this gene as three papers, performing genomic analysis of UBC tumours, all found STAG2 to be frequently mutated (inactivated) or deleted(60-62). The significance of STAG2 mutations has yet to be truly elucidated, with the published papers not agreeing on the prognostic implications or the exact mechanism of action of STAG2(63). To date no researcher as provided details of urinary evaluation of STAG2 mutation, but the frequency of mutation may make it an attractive future marker.

**AURKA**

The Aurora kinase A (AURKA) gene encodes a serine/threonine kinase that has a role in chromosomal segregation and centromere separation and overexpression of AURKA has been shown to drive oncogenesis(64). Park et al, used a FISH test to assess the urine from 100 patients with bladder cancer and 148 controls patients; they reported a sensitivity of 87.0% and a specificity of 96.6% (64). The AURKA has not been the subject of any other published study since 2008, however the recent development of an AURKA inhibitor, may renew interest in this urinary marker(65).

**Survivin**

Survivin is an important protein involved in the inhibition of apoptosis and tumour cell invasiveness, survivin mRNA has been identified in urine using an immunoassay (66). Smith et al, evaluated urinary survivin in UBC samples, survivin protein and mRNA were detected in all of 46 patients with bladder cancer, but in only 3 of 35 patients with negative cystoscopic evaluation (67). Shariat et al, (using a Bio-Dot microfiltration detection system (Bio-Rad, Hercules, California)) evaluated urinary survivin in 117 UBC cases and 92 controls and described a sensitivity of 64% and specificity of 93% (68). Horstmann et al, used PCR measurement of urinary survivin mRNA in 50 patients with suspicion of new or recurrent bladder cancer prior to transurethral resection (69) to yield sensitivity of 83% & 35% (for HG and LG UBC respectively) and specificity of 88%. The UroScreen study group performed a large prospective screening study on 1,540 chemical workers and analysed 5,716 samples for Survivin mRNA using rt-PCR(70). The study was limited by a very low number of tumours being detected (18) however Survivin demonstrated a sensitivity of 21.1% for all tumours and 36.4% for high grade tumours, surviving had a very low false positive rate and the authors concluded the test may be useful in a multi-marker panel.
RNA species

miRNA

MicroRNAs (miRNA) are small (less than 20bp) noncoding RNAs that post-transcriptionally regulate gene expression (71). Hanke et al, screened urinary sediment from UBC patients and controls for 157 different miRNAs using PCR. In this study miR-126, 182 and 199a were significantly increased in UBC patients urine compared to controls and the ratio of miR126:152 had a sensitivity of 72% for detecting UBC at a set specificity of 82% (72). Puerta-Gil et al, evaluated three miRs (143, 222, & 452 (in the urine of patients harbouring UBC using PCR), they found the diagnostic accuracy of miR-222 to be 77% and for miR-452 to be 85% (73). Miah et al, tested 121 urine samples (68 from UBC patients and 53 symptomatic controls attending cystoscopy clinic) for the presence of 15 miRs(using qPCR) known to be differentially expressed or associated with epigenetic hotspots in UBC. As a result of this analysis they found that a combination of miRs-135b/15b/1224-3p could detect bladder cancer with 94.1% sensitivity and 51% specificity (74). Recently, a study by Snowden et al, found urinary miR-125b to have an average 10.42-fold decrease (p<0.01) and miR-126 showing an average 2.70-fold increase (p=0.30) in UBC samples compared to controls (75). Shimizu et al, evaluated four methylated miRNAs (miR-137, miR-124-2, miR-124-3, and miR-9-3) in the urine of patients harbouring UBC. In this study the panel of four miRNAs was able to detect all UBC with an 81% sensitivity and 89% specificity and stage pTa and low – grade tumours (sensitivity 0.68, specificity 0.89), unlike conventional cytology.

mRNA markers

Messenger RNA (mRNA) based multi-gene commercial assays uRNA® (mRNAs = CDC2, HOXA13, MDK, and IGFBP5) and its derivative (has the additional marker CXCR2) Cxbladder™ (Pacific Edge Ltd, New Zealand) have demonstrated increased sensitivity in detection of UBC in comparison to NMP22 assay and cytology in patients with hematuria (76). In this 485 patient study, uRNA® had a sensitivity of 62.1% compared to NMP22 50% with a set specificity of 85% for the investigational assays. Cxbladder™ assay distinguished low-grade Ta tumours with a sensitivity of 91% and specificity of 90%. Mengual et al(77), have constructed a 12+2 gene expression signature for BC diagnosis and prediction of tumour aggressiveness on urine samples using qPCR assays. The twelve genes comprise: ANXA10, AHNAK2, CTSE, CRH, IGF2, KLF9, KRT20, MAGEA3, POSTN, PPP1R14D, SLC1A6, and TERT. The additional two genes, ASAM and MCM10, can help differentiate between LG and HG tumors. Overall, this gene set panel had 98% sensitivity and 99% specificity in discriminating between cases and controls samples and 79% sensitivity and 92% specificity in predicting tumour aggressiveness (high grade). They have tested the efficacy of this 12+2 gene set in voided urine and observed sensitivities and specificities of 89% and 95%, respectively and of 79% and 91%, respectively for predicting tumour aggressiveness.
<table>
<thead>
<tr>
<th>BIOMARKER</th>
<th>COMMERCIAL NAME</th>
<th>ASSAY TYPE</th>
<th>PERFORMANCE</th>
<th>REFERENCES</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PROTEIN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human complement factor H-related protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BTA STAT™</td>
<td>Qualitative point of care</td>
<td>Sensitivity 57% – 83%</td>
<td>9 - 13</td>
<td>False Positive results with haematuria</td>
</tr>
<tr>
<td></td>
<td>BTA TRAK™</td>
<td>ELISA</td>
<td>Sensitivity 62% – 91%</td>
<td>14 - 21</td>
<td>FDA approved only in combination with cystoscopy</td>
</tr>
<tr>
<td></td>
<td>NMP22®</td>
<td>ELISA</td>
<td>Sensitivity 47 - 100%</td>
<td>9 - 11, 20, 25 - 29</td>
<td>False positives with any cause of cell death e.g. benign inflammatory conditions, infection or urolithias</td>
</tr>
<tr>
<td></td>
<td>BladderChek®</td>
<td>Qualitative point of care</td>
<td>Sensitivity 59%</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 93%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytokeratins 8 &amp; 18</td>
<td>UBC® ELISA</td>
<td>ELISA</td>
<td>Sensitivity 64.4%</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 63.6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UBC® Rapid</td>
<td>Qualitative point of care</td>
<td>Sensitivity 64.4 - 66%</td>
<td>36, 37</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 63.6% - 90%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UBC® IRMA</td>
<td>Immunoradiometric Assay</td>
<td>Sensitivity 12.1%</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 97.2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLCA-1</td>
<td>- ELISA</td>
<td>Sensitivity 80%</td>
<td>39</td>
<td>Results require validation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 87%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BLCA-4</td>
<td>- ELISA</td>
<td>Sensitivity 96.4%</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>CELL BASED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEA &amp; Tumour Mucins</td>
<td>uCyt+™</td>
<td>Immunofluorescence</td>
<td>Sensitivity 72.8%</td>
<td>42</td>
<td>Requires minimum number of exfoliated cells and trained cytopathologist</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specificity 71.9%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(Table 1 cont’d) Table 1. Summary of urinary biomarkers described in the text. ELISA = Enzyme linked immunosorbent assay, FISH = Fluorescence in situ hybridization.

<table>
<thead>
<tr>
<th>BIOMARKER</th>
<th>COMMERCIAL NAME</th>
<th>ASSAY TYPE</th>
<th>PERFORMANCE</th>
<th>REFERENCES</th>
<th>COMMENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromosomes 3, 7, 17 &amp; 9p21</td>
<td>UroVysion®</td>
<td>FISH</td>
<td>Sensitivity = 72% Specificity = 83%</td>
<td>47</td>
<td>More expensive than cytology</td>
</tr>
<tr>
<td>mRNA / DNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TERT promoter mutations</td>
<td>-</td>
<td>SNapShot</td>
<td>Sensitivity = 42% - 62% Specificity = 73% - 90%</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>FGFR3 mutation</td>
<td>-</td>
<td>SNapShot</td>
<td>Sensitivity = 58 - 62% Specificity = 89%</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>AURKA</td>
<td>FISH</td>
<td></td>
<td>Sensitivity = 87% Specificity = 96%</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Survivin</td>
<td>Bio-Dot</td>
<td></td>
<td>Sensitivity = 35% - 83% Specificity = 88% - 93%</td>
<td>68, 69</td>
<td></td>
</tr>
</tbody>
</table>

Epigenetic urinary markers

Analysis of gene methylation has been performed on voided urine (78, 79). Friedrich et al, analysed the methylation status of different markers in urine samples of patients with UBC and found that methylation of DAPK, BCL2, and TERT (see earlier) was detected in the majority of samples (78%), whereas they were unmethylated in the urine sediment from age-matched cancer-free individuals(79). Renard et al, identified TWIST1 and NID2 to be frequently methylated in urine samples collected from UBC patients and reported a sensitivity and specificity for this two-gene panel >90% (80). Scher et al, developed a small urine volume nested methylation-specific PCR assay for the detection of UBC based on methylation of BCL2, CDKN2A, and NID2 with a sensitivity of 80.9% and 86.4% (81). Chung et al used methylation markers (MYO3A, CA10, SOX11, NXX6-2, PENK, and DBC1) to screen urine for UBC with a 81-85% sensitivity and 95-97% specificity (82). Zuiverloon et al (83), using a methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) assay for 4 genes (APC, TERT_A, TERT_B & EDNRB), and found a sensitivity of 72.3% and specificity of 55.2% for detecting NMIBC recurrence.

Conclusion

UBC is a challenging disease for clinicians to manage and demands new methods of performing diagnosis and disease surveillance. Urinary biomarkers have the potential to enhance current diagnostic strategies and perhaps in the future, even replace existing techniques. As UBC is a disease
of the entire urothelium (field change effect), urinary biomarkers may have the potential to inform clinicians of the pathogenicity of the urothelium in the absence of visible carcinoma. As more information emerges about the different genetic pathways in low and high grade BC, it is likely that a much more genetic level based surveillance will be used to guide the success of the bladder sparing treatment protocols (BCG, Chemotherapy, Radiation).

As described, urinary biomarkers from the chromosome down to specific gene mutations have all been evaluated, not only for diagnosis of symptomatic patients but also for screening at risk populations and for predicting and detecting disease recurrence. Although many markers have shown superior test performance to urine cytology, there is a paucity of well-designed prospective trials (with cost-effectiveness) that will be needed to justify a new markers use. It seems more likely that markers will need to be used in combination to enhance the effectiveness of the test. Clinicians may also use these new biomarker tests selectively for example in patients with high grade disease that are pursuing bladder sparing treatments, or possibly for patients with very low risk of disease progression that do not want to undergo routine cystoscopy. Urinary biomarkers will undoubtedly find a role in the future of UBC management but at present, which test or test strategy has yet to be elucidated. Clinicians and patients desire a rapid bedside test particularly as in many health care scenarios UBC is assessed in “one stop” clinics. However the complexity and possibly the number of tests that will be required to be performed will still necessitate in the first instance, laboratory analysis, possibly in designated specialized centers.

References


Estimation of alert and change limits of haematological quantities and its application in the plausibility control

Lourdes Sánchez-Navarro¹, María José Castro-Castro¹, Dolors Dot-Bach¹, Xavier Fuentes-Arderiu²

¹Laboratori Clínic, Hospital Universitari de Bellvitge, L’Hospitalet de Llobregat, Catalonia, Spain
²Clinical Laboratory Sciences Consulting, Barcelona, Catalonia, Spain

ABSTRACT

Introduction: In the process of quality assurance of the measured values of the clinical laboratory, one of the purposes is to perform the validation of patients’ measured values in the most objective way. This validation process is called plausibility control which may be defined as the set of procedures used to decide if a patient’s measured value is valid according to established clinical and biological criteria.

The aim of this study is to propose a model to estimate alert and change limits of measured values of the blood cell count, to be applied to detect doubtful patients’ measured values.

Methods: Some alert and change limits were estimated from the emergency laboratory database of the year 2010 using different percentiles. A verification of the suitability of the proposed model was also performed.

Results: Most of the fractions of the measured values excluded by the alert and change limits were according to the theoretical expected. The overall fraction of the number of doubtful clinical laboratory reports ranged between 0.6 and 47.6 %.

Conclusions: The proposed model helps, improves and standardizes the process of detection of doubtful measured values since they are produced objectively. These limits can also be configured in a laboratory information system letting the clinical laboratory professional staff to save time and efforts.
1. Introduction

In the clinical laboratory, different processes are involved to assure the quality of a clinical laboratory haematology report before releasing it to the requester: the visual inspection of the samples detects possible defects that make them unsuitable for the requested measurements in the pre-analytical phase; the internal quality control, the alarms of the measurement systems and the microscopic examination of stained blood film ensure the suitability in the analytical phase and the so-called “validation process” of measured values decides whether each clinical laboratory report may be issued (validated) or it should be retained for a more detailed final inspection (doubtful or non validated) in the post-analytical phase.

The visual inspection of the samples and the internal quality control are two well defined processes which have been standardized over time. In the same way, some scientific organizations have devoted their efforts to provide consensus rules for microscopic examination [1]. However, there are not guides or recommendations from a scientific or professional organisations providing information on how to conduct the validation process of patients’ measured values. Furthermore, despite all technological improvements, in many clinical laboratories this process is still performed in “manual” way by clinical laboratory specialised staff. So, every clinical haematology laboratory adapts this process in a particular way: some of them do not perform it because of its time consumption and its high costs of clinical laboratory specialised staff and some other, when performed, they do in a not standardized way. The lack of standardization of the validation process of patients’ measured values, performed by clinical laboratory specialised staff, has some disadvantages such as applying many subjective criteria and, consequently, having great interindividual variation, which degree depends on the experience of the mentioned professionals and the criteria used.

The plausibility control can be defined as the set of procedures used to decide if a patient’s measured value is valid or not according to clinical and biological criteria previously established [2]. This is a useful tool to detect doubtful measured values, which, despite of belonging to a series of measurements accepted by the internal quality control, can be erroneous and might be not detected by sample inspection, by the automated analysis or by the microscopic examination of stained blood film. The factors that may generate erroneous measured value are diverse: factors related to the sample (e.g. sample pertaining to another patient, sample collected from intravenous route), factors related to the measurement process (e.g. obstruction of the analyser, errors in manual transcription of measured values), and so on.

Probably, the most efficient way to eliminate the interindividual variation of the plausibility control of patients’ measured values is its computerization which, in addition, makes the process more efficient. In order to automate this process, some tools can be used to detect doubtful measured values: (i)
The plausibility control of measured values should detect previously unnoticed errors produced in any process of the clinical laboratory [2] and should ensure the consistency of these values with the available clinical and biological information.

The aim of this article is to propose a model to estimate alert and change limits of the number concentration of erythrocytes, leukocytes and thrombocytes; number fraction of neutrophils, lymphocytes, monocytes, eosinophils and basophils; mass concentration of haemoglobin; volume fraction and entitic volume of erythrocytes (also known as PRC and MCV, respectively), to detect doubtful measured values in the plausibility control in an objective way.

2. Material and methods

In order to estimate the alert and change limits, measured values of each haematological quantity, from the year 2010 were taken from the database maintained in our emergency laboratory information system Omega 3000 (Roche Diagnostics España S.L., Sant Cugat del Vallès, Catalonia, Spain). The number of measured values obtained ranged between 89231 and 89786, depending on the quantity. In order to verify the suitability of the proposed model, another patients’ measured values of each quantity, from the year 2011 were taken from the same data source. The number of measured values exceeding some alert limits, which define an interval where a large proportion of such a values can be expected to be found; (ii) detection of measured values which are not in agreement with the corresponding preceding one using a change limit (commonly called deltaxcheck); (iii) detection of patients’ measured values which are not in agreement with measured values of other quantities obtained in the same sample [3]; and (iv) detection of measured values which are not consistent with the diagnosis, if known, or the origin of the request [2].
obtained ranged between 38912 to 46003, depending on the quantity.

All quantities were measured in the emergency laboratory in an ABX Pentra 120 DX analyzer (Horiba Medical, Montpellier, France). Number concentrations of erythrocytes, thrombocytes and leukocytes in blood, volume fraction and entitic volume of erythrocytes in blood were measured using impedance whereas haemoglobin mass concentration was measured by the cyanmethemoglobin method, and differential leukocyte count was carried out using a flow cytochemistry method.

The differential leukocyte count was also carried out using microscopic examination of stained blood film when some suspect flags from automated analysis, specified by the manufacturer, appeared or when the consensus guidelines of the International Society for Laboratory Hematology indicated [1]. The smear, the stain and the microscopic examination of 100 leukocytes were carried out by some experienced technicians according to the standardized procedure in use in the laboratory [9].

The metrological characteristics of the measuring system were stable during the years 2010 and 2011 (no changes in the measurement system and in the measurement errors for each quantity, observed in external quality assessment schemes).

The selected alert limits were the percentiles that exclude 10 % or 1 % or 0.1 % or 0.01% of the patients’ measured values and the change limits were the percentiles that exclude 10 % or 1 % or 0.1 % of the relative differences of patients’ measured values. These percentiles were chosen in an arbitrary way but using professional consensus among the authors.

In order to define the alert limits, percentiles 5.00 and 95.00, 0.50 and 99.50, 0.05 and 99.95, and 0.005 and 99.995 of original data from the year 2010 were estimated. The percentiles estimated to define the change limits were 90.00, 99.00 and 99.90 from the same database and year.

In a manual 100 leukocytes differential counting, the number fraction of each type of leukocyte usually is expressed rounding to an integer. To estimate alert and change limits, these values were transformed by adding 0.5 to avoid giving biologically impossible measured values of 0 %.

All data were processed with the software SPSS v.17 (SPSS, Chicago, USA).

For each quantity, a change is the relative difference of a measured value with regard its preceding one. This relative difference \( D \), expressed in percent (%), is calculated taking into account the highest \((x_h)\) and lowest \((x_l)\) values [10]:

\[
D = \frac{(x_h - x_l)}{x_l} \times 100 \%
\]

In this way, from the year 2010, pairs of measured values of each haematological quantity from the same patient, were obtained. The pairs of measured values obtained ranged between 30917 and 31365, depending on the quantity.
To estimate change limits, measured values lower than the corresponding limit of detection have been transformed into the immediately preceding numerical measured value taking into account the number of digits used.

To estimate change limits, the selected time of searching back was one day.

For each haematological quantity, in order to verify the suitability of the estimated alert limits, the percent (%) of measured values from the year 2011 excluded by these limits was calculated. The same procedure was using the change limits.

The criteria applied to decide when a measured value is doubtful were the following: (i) for those measured values not having a preceding one, only the comparison with alert limits may produce doubtful measured values, obviously; (ii) for those measured values having a preceding one, only change limits are accepted to produce doubtful measured values.

For each quantity, the fractions (in %) of the number of measured values excluded by alert or change limits (doubtful measured values) were calculated applying together these limits to measured values from the year 2011. Since four alert limits and three different change limits have been estimated, twelve combinations giving twelve different possibilities were obtained.

In order to assess the real impact of the implementation of alert and change limits in the daily plausibility control, the overall fractions (in %) of the number of doubtful clinical laboratory reports, regardless of the quantity responsible of its exclusion, were also calculated.

The criteria applied to consider the whole patients’ clinical laboratory report as doubtful were, at least, one measured value of any quantity of the blood cell count excluded by alert or change limits.

3. Results

In all tables, haematological quantities are described using the traditional English acronyms but also according to the IUPAC-IFCC recommended syntax in which B means blood; Lkcs means leukocytes, num. means number, vol. means volume; c. means concentration and fr. means fraction [11]. Table 1 shows alert limits for all quantities estimated from original data of the year 2010, corresponding to percentiles 0.005, 0.05, 0.50, 5.00, 95.00, 99.50, 99.95 and 99.995, whereas Table 2 shows, for the same quantities and for the same year, the change limits of relative differences corresponding to percentiles 90.0, 99.0 and 99.9.
Table 1. Alert limits for each haematological quantity estimated with measured values from the year 2010.

<table>
<thead>
<tr>
<th>Percentile</th>
<th>RBC (x10E12/L)</th>
<th>Hb (g/L)</th>
<th>PRC (1)</th>
<th>MCV (fL)</th>
<th>Plt (x10E9/L)</th>
<th>WBC (x10E9/L)</th>
<th>Neu (%)</th>
<th>Lym (%)</th>
<th>Mono (%)</th>
<th>Eos (%)</th>
<th>Baso (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p_{0.005} )</td>
<td>0.9</td>
<td>25</td>
<td>0.088</td>
<td>52</td>
<td>&lt;3</td>
<td>&lt;0.2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>( p_{0.05} )</td>
<td>1.3</td>
<td>38</td>
<td>0.121</td>
<td>59</td>
<td>&lt;3</td>
<td>&lt;0.2</td>
<td>2.2</td>
<td>1.5</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>( p_{0.50} )</td>
<td>1.9</td>
<td>58</td>
<td>0.179</td>
<td>68</td>
<td>13</td>
<td>0.4</td>
<td>13.5</td>
<td>2.5</td>
<td>1.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>( p_{5.00} )</td>
<td>2.5</td>
<td>78</td>
<td>0.239</td>
<td>81</td>
<td>69</td>
<td>3.7</td>
<td>49.6</td>
<td>4.5</td>
<td>2.8</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td>( p_{95.00} )</td>
<td>5.1</td>
<td>155</td>
<td>0.470</td>
<td>103</td>
<td>448</td>
<td>19.5</td>
<td>90.3</td>
<td>38.5</td>
<td>12.5</td>
<td>4.9</td>
<td>1.5</td>
</tr>
<tr>
<td>( p_{99.50} )</td>
<td>5.8</td>
<td>172</td>
<td>0.522</td>
<td>114</td>
<td>746</td>
<td>38.6</td>
<td>94.4</td>
<td>68.5</td>
<td>31.1</td>
<td>10.6</td>
<td>4.2</td>
</tr>
<tr>
<td>( p_{99.95} )</td>
<td>6.6</td>
<td>188</td>
<td>0.572</td>
<td>126</td>
<td>1057</td>
<td>109.6</td>
<td>96.8</td>
<td>91.5</td>
<td>57.4</td>
<td>22.5</td>
<td>12.6</td>
</tr>
<tr>
<td>( p_{99.995} )</td>
<td>7.6</td>
<td>213</td>
<td>0.628</td>
<td>134</td>
<td>2027</td>
<td>275.1</td>
<td>98.5</td>
<td>98.5</td>
<td>82.1</td>
<td>48.7</td>
<td>34.1</td>
</tr>
</tbody>
</table>

RBC: B─Erythrocytes; num.c.; Hb: B─Haemoglobin; mass.c.; PRC: B─Erythrocytes; vol.fr.; MCV: B─Erythrocytes; entitic vol.; Plt: B─Thrombocytes; num.c.; WBC: B─Leukocytes; num.c.; Neu: Lkcs(B)─Neutrophils; num.fr.; Lym: Lkcs(B)─Lymphocytes; num.fr.; Mono: Lkcs(B)─Monocytes; num.fr.; Eos: Lkcs(B)─Eosinophils; num.fr.; Baso: Lkcs(B)─Basophilis; num.fr.; in parentheses: units of measure; \( p \): percentile having the order indicated by the subindex.
Table 2. Change limits for each haematological quantity estimated with measured values from the year 2010.

<table>
<thead>
<tr>
<th>Percentile</th>
<th>DRBC (%)</th>
<th>DHb (%)</th>
<th>DPRC (%)</th>
<th>DMCV (%)</th>
<th>DPlt (%)</th>
<th>DWBC (%)</th>
<th>DNeu (%)</th>
<th>DLYm (%)</th>
<th>DMono (%)</th>
<th>DEos (%)</th>
<th>DBaso (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p90.0</td>
<td>25.9</td>
<td>25.9</td>
<td>25.8</td>
<td>2.9</td>
<td>45.7</td>
<td>67.6</td>
<td>22.3</td>
<td>133.3</td>
<td>100.0</td>
<td>162.5</td>
<td>300.0</td>
</tr>
<tr>
<td>p99.0</td>
<td>68.4</td>
<td>70.7</td>
<td>68.3</td>
<td>6.7</td>
<td>209.8</td>
<td>194.6</td>
<td>75.2</td>
<td>488.9</td>
<td>536.5</td>
<td>516.7</td>
<td>850.0</td>
</tr>
<tr>
<td>p99.9</td>
<td>129.1</td>
<td>174.8</td>
<td>143.8</td>
<td>19.4</td>
<td>1269.0</td>
<td>606.3</td>
<td>473.1</td>
<td>1533.3</td>
<td>2085.5</td>
<td>1377.0</td>
<td>2150.0</td>
</tr>
</tbody>
</table>

Relative differences (D), in fraction (%), of: DRBC: B─Erythrocytes; num.c.; DHb: B─Haemoglobin; mass c.; DPRC: B─Erythrocytes; vol.fr.; DMCV: B─Erythrocytes; entitic vol.; DPlt B─Thrombocytes; num.c.; DWBC: B─Leukocytes; num.c.; DNeu: Lkcs(B)─Neutrophils; num.fr.; DLYm: Lkcs(B)─Lymphocytes; num.fr.; DMono: Lkcs(B)─Monocytes; num.fr.; DEos: Lkcs(B)─Eosinophils; num.fr.; DBaso: Lkcs(B)─Basophils; num.fr.; p: percentile having the order indicated by the subindex.

Table 3 and Table 4 show, respectively, the fraction (in %) of data of the year 2011 excluded by alert limits and change limits.

Table 3. Fraction (in %) of the number of measured values for each haematological quantity from the year 2011 excluded by alert limits.

<table>
<thead>
<tr>
<th></th>
<th>RBC (%)</th>
<th>Hb (%)</th>
<th>PRC (%)</th>
<th>MCV (%)</th>
<th>Plt (%)</th>
<th>WBC (%)</th>
<th>Neu (%)</th>
<th>Lym (%)</th>
<th>Mono (%)</th>
<th>Eos (%)</th>
<th>Baso (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Excluded by</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p5.00 and p95.00</strong></td>
<td>8.09</td>
<td>9.94</td>
<td>10.01</td>
<td>8.11</td>
<td>9.97</td>
<td>10.57</td>
<td>9.70</td>
<td>7.47</td>
<td>10.42</td>
<td>5.96</td>
<td>5.79</td>
</tr>
<tr>
<td><strong>Excluded by</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p0.50 and p99.50</strong></td>
<td>0.86</td>
<td>1.06</td>
<td>1.12</td>
<td>0.61</td>
<td>0.93</td>
<td>1.02</td>
<td>1.13</td>
<td>0.85</td>
<td>1.01</td>
<td>0.73</td>
<td>0.73</td>
</tr>
<tr>
<td><strong>Excluded by</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p0.05 and p99.95</strong></td>
<td>0.08</td>
<td>0.09</td>
<td>0.11</td>
<td>0.07</td>
<td>0.14</td>
<td>0.38</td>
<td>0.17</td>
<td>0.11</td>
<td>0.05</td>
<td>0.03</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Excluded by</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>p0.005 and p99.95</strong></td>
<td>0.01</td>
<td>0.01</td>
<td>0.04</td>
<td>0.02</td>
<td>0.05</td>
<td>0.28</td>
<td>0.01</td>
<td>0.003</td>
<td>0.002</td>
<td>0.003</td>
<td>0.01</td>
</tr>
</tbody>
</table>

RBC: B─Erythrocytes; num.c.; Hb: B─Haemoglobin; mass c.; PRC: B─Erythrocytes; vol.fr.; MCV:B─Erythrocytes; entitic vol.; Plt: B─Thrombocytes; num.c.; WBC: B─Leukocytes; num.c.; Neu: Lkcs(B)─Neutrophils; num.fr.; Lym: Lkcs(B)─Lymphocytes; num.fr.; Mono: Lkcs(B)─Monocytes; num.fr.; Eos: Lkcs(B)─Eosinophils; num.fr.; Baso: Lkcs(B)─Basophils; num.fr.; p: percentile having the order indicated by the subindex.
Table 4. Fraction (in %) of the number of measured values for each haematological quantity from the year 2011 excluded by change limits.

<table>
<thead>
<tr>
<th></th>
<th>DRBC (%)</th>
<th>DHb (%)</th>
<th>DPRC (%)</th>
<th>DMCV (%)</th>
<th>DPlt (%)</th>
<th>DWBC (%)</th>
<th>DNeu (%)</th>
<th>Dlym (%)</th>
<th>DMono (%)</th>
<th>DEos (%)</th>
<th>DBaso (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excluded by $p_{90.0}$</td>
<td>9.83</td>
<td>9.68</td>
<td>9.93</td>
<td>5.55</td>
<td>9.22</td>
<td>10.05</td>
<td>10.52</td>
<td>10.03</td>
<td>11.63</td>
<td>9.95</td>
<td>10.23</td>
</tr>
<tr>
<td>Excluded by $p_{99.0}$</td>
<td>0.63</td>
<td>0.66</td>
<td>0.73</td>
<td>0.80</td>
<td>0.85</td>
<td>1.14</td>
<td>0.95</td>
<td>0.82</td>
<td>1.03</td>
<td>1.06</td>
<td>1.70</td>
</tr>
<tr>
<td>Excluded by $p_{99.9}$</td>
<td>0.07</td>
<td>0.03</td>
<td>0.12</td>
<td>0.03</td>
<td>0.02</td>
<td>0.12</td>
<td>0.09</td>
<td>0.07</td>
<td>0.13</td>
<td>0.09</td>
<td>0.28</td>
</tr>
</tbody>
</table>

Relative differences (D) of: DRBC: B─Erythrocytes; num.c.; DHb: B─Haemoglobin; mass c.; DPRC: B─Erythrocytes; vol.fr.; DMCV: B─Erythrocytes; entitic vol.; DPlt: B─Thrombocytes; num.c.; DWBC: B─Leukocytes; num.c.; DNeu: Lkcs(B)─Neutrophils; num.fr.; Dlym: Lkcs(B)─Lymphocytes; num.fr.; DMono: Lkcs(B)─Monocytes; num.fr.; DEos: Lkcs(B)─Eosinophils; num.fr.; DBaso: Lkcs(B)─Basophils; num.fr.; $p$: percentile having the order indicated by the subindex.

Table 5 shows, for all the quantities studied, the fraction (in %) of the number of doubtful measured values of the year 2011 detected applying both alert and change limits. All fractions (in %) are expressed regarding to the total number of measured values studied.

Table 5. Fraction (in %) of the number of doubtful measured values for each haematological quantity found applying alert and change limits to measured values, having or not a preceding one, from the year 2011.

<table>
<thead>
<tr>
<th>Alert and change limits applied</th>
<th>RBC (%)</th>
<th>Hb (%)</th>
<th>PRC (%)</th>
<th>MCV (%)</th>
<th>Plt (%)</th>
<th>WBC (%)</th>
<th>Neu (%)</th>
<th>Lym (%)</th>
<th>Mono (%)</th>
<th>Eos (%)</th>
<th>Baso (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL1 or CL1</td>
<td>9.14</td>
<td>10.60</td>
<td>10.39</td>
<td>7.54</td>
<td>9.32</td>
<td>9.73</td>
<td>10.07</td>
<td>10.08</td>
<td>10.89</td>
<td>7.61</td>
<td>7.40</td>
</tr>
<tr>
<td>AL1 or CL2</td>
<td>5.95</td>
<td>7.22</td>
<td>7.16</td>
<td>5.82</td>
<td>6.43</td>
<td>6.65</td>
<td>6.77</td>
<td>6.92</td>
<td>7.21</td>
<td>4.56</td>
<td>4.47</td>
</tr>
<tr>
<td>AL1 or CL3</td>
<td>5.76</td>
<td>7.01</td>
<td>6.95</td>
<td>5.55</td>
<td>6.15</td>
<td>6.29</td>
<td>6.47</td>
<td>6.66</td>
<td>6.90</td>
<td>4.23</td>
<td>3.99</td>
</tr>
<tr>
<td>AL2 or CL1</td>
<td>4.07</td>
<td>4.23</td>
<td>4.34</td>
<td>2.45</td>
<td>3.83</td>
<td>4.12</td>
<td>4.43</td>
<td>4.42</td>
<td>4.75</td>
<td>3.98</td>
<td>4.05</td>
</tr>
<tr>
<td>AL2 or CL2</td>
<td>0.89</td>
<td>1.09</td>
<td>1.11</td>
<td>0.73</td>
<td>0.94</td>
<td>1.02</td>
<td>1.13</td>
<td>1.26</td>
<td>1.08</td>
<td>0.93</td>
<td>1.12</td>
</tr>
<tr>
<td>AL2 or CL3</td>
<td>0.69</td>
<td>0.88</td>
<td>0.90</td>
<td>0.46</td>
<td>0.66</td>
<td>0.67</td>
<td>0.83</td>
<td>1.00</td>
<td>0.77</td>
<td>0.59</td>
<td>0.63</td>
</tr>
<tr>
<td>AL3 or CL1</td>
<td>3.47</td>
<td>3.43</td>
<td>3.55</td>
<td>2.05</td>
<td>3.28</td>
<td>3.74</td>
<td>3.71</td>
<td>3.64</td>
<td>4.06</td>
<td>3.45</td>
<td>3.58</td>
</tr>
<tr>
<td>AL3 or CL2</td>
<td>0.28</td>
<td>0.30</td>
<td>0.33</td>
<td>0.33</td>
<td>0.39</td>
<td>0.65</td>
<td>0.41</td>
<td>0.48</td>
<td>0.39</td>
<td>0.40</td>
<td>0.65</td>
</tr>
<tr>
<td>AL3 or CL3</td>
<td>0.09</td>
<td>0.08</td>
<td>0.11</td>
<td>0.07</td>
<td>0.11</td>
<td>0.30</td>
<td>0.11</td>
<td>0.22</td>
<td>0.08</td>
<td>0.06</td>
<td>0.16</td>
</tr>
<tr>
<td>AL4 or CL1</td>
<td>3.42</td>
<td>3.37</td>
<td>3.50</td>
<td>2.01</td>
<td>3.21</td>
<td>3.69</td>
<td>3.63</td>
<td>3.46</td>
<td>4.03</td>
<td>3.42</td>
<td>3.51</td>
</tr>
<tr>
<td>AL4 or CL2</td>
<td>0.23</td>
<td>0.23</td>
<td>0.27</td>
<td>0.29</td>
<td>0.32</td>
<td>0.60</td>
<td>0.33</td>
<td>0.30</td>
<td>0.36</td>
<td>0.37</td>
<td>0.58</td>
</tr>
<tr>
<td>AL4 or CL3</td>
<td>0.03</td>
<td>0.02</td>
<td>0.06</td>
<td>0.03</td>
<td>0.03</td>
<td>0.24</td>
<td>0.03</td>
<td>0.04</td>
<td>0.05</td>
<td>0.03</td>
<td>0.1</td>
</tr>
</tbody>
</table>

(Table 5 continued on next page)
Table 6 shows, for all the quantities studied, the fraction (in %) of the number of doubtful clinical laboratory reports of the year 2011 detected applying both alert and change limits.

Table 6. Fraction (in %) of the number of doubtful clinical laboratory reports found applying alert and change limits.

<table>
<thead>
<tr>
<th>Alert limits (AL)</th>
<th>Change limits (CL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL1 or CL1</td>
<td>AL1 or CL2</td>
</tr>
<tr>
<td>Excluded (%)</td>
<td>AL1 or CL3</td>
</tr>
<tr>
<td></td>
<td>AL1 or CL2</td>
</tr>
<tr>
<td>47.6</td>
<td>32.8</td>
</tr>
<tr>
<td>22.1</td>
<td>7.3</td>
</tr>
<tr>
<td>18.4</td>
<td>3.5</td>
</tr>
<tr>
<td>17.9</td>
<td>3.1</td>
</tr>
</tbody>
</table>

4. Discussion

There are many clinical laboratories around the world producing daily by thousands of patients’ measured values of haematological quantities which are subject to a validation process performed by clinical laboratory specialised staff. This process is difficult and time consuming, so many laboratories cannot afford to carry it out. On the other hand, its subjectivity and interindividual variation make this process less efficient than it could be. As an alternative, computerization of the plausibility control could conduce to a final inspection of those haematological laboratory reports containing doubtful patients’ measured values, and to deliver automatically to the requester those considered acceptable according to unambiguous defined rules. For these haematology laboratories, the computerized plausibility control allows laboratory professional staff a more objective review, saving time and increasing their effectiveness in detecting doubtful patients’ measured values, as has been previously demonstrated for some specialized and computerized plausibility control systems (e.g. VALAB system) [12, 13, 14]. Even though partially computerized plausibility control systems have existed for more than 20 years and they are available in the most of laboratory information systems, published data on the use of them are limited. Some surveys report that about 64% of clinical laboratories use computerized plausibility control systems [15]. Most of them are clinical laboratories with high workload in which plausibility control are applied mainly to biochemical quantities, with a lack of standardization in the algorithms used and criteria applied.
To perform the plausibility control applying the alert and change limits is necessary to develop a procedure for establishing these limits [4-7, 16-18]. In the proposed model, the percentiles of the population distribution of the differences were chosen for estimating change limits, rather than the change limits based on biological variability. The main reason has been that change limits based on biological variability only include physiological variability whereas change limits based on percentiles also include pathological and iatrogenic variability. These change limits are particularly relevant in the plausibility control of measured values from hospital population. In this way, an unmanageable number of doubtful clinical laboratory reports that finally they will be issued after a detailed inspection (false positive) are avoided [4, 6].

The proposed model provide different fractions (in %) of the number of doubtful patients’ measured values of some haematological quantities, excluded by the alert or change limits, depending on the percentiles applied; these clinical laboratory reports which contain doubtful patients’ measured values would not be automatically validated. Since a wide range of combinations are provided, each laboratory may choose the appropriate alert or change limits, that is the appropriate percentiles to obtain a fraction (in %) of doubtful patients’ measured values which the clinical laboratory professionals would decide whether each clinical laboratory report could be issued (validated) or it should be retained for a more detailed inspection. The selection of the appropriate combination is established according to clinical laboratory needs, mainly the time or the clinical laboratory staff available to be dedicated to plausibility control, bearing in mind that the frequency of clinical laboratory errors reported in the literature range from 0.05% to 2% [19-22].

Despite all above, it should be remarked that, as stated in the introduction section, the aim of this study is to propose a model for establishing alert and change limits, but the clinical relevance of the doubtful patients’ measured values is not under the scope of this article.

The fractions (in %) of patients’ measured values excluded by the alert or change limits of some quantities were far from the expected theoretical. Some reasons could explain this fact: those quantities with different percentiles with coincident values do not behave as expected, as in the case of leukocytes number concentration (Table 1, WBC) in which percentiles 0.005 and 0.05 of estimated alert limits were coincident with the corresponding detection limit of the measuring system; or in the case of estimated alert limits of eosinophils (Table 1, Eos) and basophils (Table 1, Baso) number fractions, in which there were low percentiles with coincident values probably due to the narrow range of the measured values; in the case of entitic volume of erythrocytes, only the percentile 90.0 of the relative differences of pairs of measured values was far from the expected theoretical (Table 4, DMCV). The rest of the percentiles were according to the expected.
Despite of estimating all the alert limits for each quantity, there are some cases that the lower limit should not to be included in the plausibility control, as in the case of percentiles 0.005, 0.05, 0.5 and 5.0 of blood monocytes, eosinophils and basophils number fractions due to the low prevalence of these types of leukocytes (lower limits of reference ranges very close to 0 %).

This study shows how to set alert and change limits to apply the plausibility control of a large number of haematological quantities in an objective and standardized way. Despite the estimation of these limits has been focused on a particular sort of clinical laboratory (emergency laboratory from a university hospital), the proposed way to set these limits can be applied to any kind of clinical laboratory without any limitation.

This model has some advantages against other systems. Since these limits are configurable in a simple middleware or an information system that allows the configuration of logic rules, the plausibility control can be carried out without the need of using specialized systems [12, 13, 14]. Thus, the objective and standardized plausibility control remains available to all sorts of clinical laboratories, regardless of their size. Furthermore, the estimation of change limits based on percentiles, which include physiological, pathological and iatrogenic biological variability, is suitable to be used in clinical laboratories working with samples from hospital patients as well as in clinical laboratories working with samples from non-hospital source. Since these limits are estimated in a relatively simple way and taking into account that and the results of the different percentiles may vary depending on the origin of the population (e.g. laboratories serving population from primary health care centres or laboratories of a tertiary hospital with Haematology and Oncology departments among others), the plausibility control can be adapted to workflow and needs from each clinical laboratory by calculating its own limits and selecting the most appropriate combination for each quantity. If interested, these limits can also be estimated by dividing the measured values in some subpopulations according to the origin (inpatients versus outpatients, emergency laboratory versus routine laboratory), requesting department, diagnosis and so on.

The selection of the appropriate combinations, together with an appropriate software, should allow computerizing the plausibility control to help, improve and standardize the process of detection of doubtful measured values which have gone unnoticed in the previous phases (sample inspection, internal quality control and microscopic examination of blood film) and leaving time for other tasks.

As said before, this study is focused on two tools used in the plausibility control: alert and change limits. Nevertheless, the use of another tool applicable to the plausibility control —detection of patients’ measured values which are not in agreement with patients’ measured values of other patho-physiologically related quantities obtained in same sample has been published for biochemical quantities by some of the present authors [3]. These series of studies will be finished (as a doctoral thesis) applying together all these tools to the computerization of the plausibility control.
5. References


Editor-in-chief

Gábor L. Kovács
Institute of Laboratory Medicine, Faculty of Medicine,
University of Pécs, Hungary

Editorial Board

Khosrow Adeli, The Hospital for Sick Children, University of Toronto, Canada
Borut Božič, University Medical Center, Ljubljana, Slovenia
Rajiv Erasmus, Dept. of Chemical Pathology, Tygerberg, South Africa
Nilda E. Fink, Universidad Nacional de La Plata, La Plata, Argentina
Ellis Jacobs, New York University School of Medicine, New York, USA
Bruce Jordan, Roche Diagnostics, Rotkreuz, Switzerland
Evelyn Koay, National University, Singapore
Maria D. Pasic, Laboratory Medicine and Pathobiology, University of Toronto, Canada
Oliver Racz, University of Kosice, Slovakia
Rosa Sierra Amor, Laboratorio Laquims, Veracruz, Mexico
Sanja Stankovic, Institute of Medical Biochemistry, Clinical Center of Serbia, Belgrade, Serbia
Danyal Syed, Ryan Center, New York, USA
Grazyna Sypniewska, Collegium Medicum, NC University, Bydgoszcz, Poland
Istvan Vermes, University of Twente, The Netherlands
Stacy E. Walz, Arkansas State University, USA
Publisher: IFCC Communications and Publications Division (IFCC-CPD)

Copyright © 2014 IFCC. All rights reserved.

The eJIFCC is a member of the Committee on Publication Ethics (COPE).

The eJIFCC (Journal of the International Federation of Clinical Chemistry) is an electronic journal with frequent updates on its home page. Our articles, debates, reviews and editorials are addressed to clinical laboratorians. Besides offering original scientific thought in our featured columns, we provide pointers to quality resources on the World Wide Web.

Contents may not be reproduced without the prior permission of the Communications and Publications Division (CPD) of the IFCC.