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## Role of Wnt5a in Prostate Cancer

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# Role of Wnt5a in Prostate Cancer

Azharuddin Sajid Syed Khaja



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Faculty of Medicine

## **Academic Dissertation**

By due permission of the faculty of Medicine, Lund University, Sweden, to be defended at the main lecture hall, Pathology building, entrance 78, Skåne University Hospital Malmö, on Wednesday October 10<sup>th</sup>, 2011, at 9 am for the degree of Doctor of Philosophy, Faculty of Medicine

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Abstract  Wnt5a is a non-canonical secreted glycoprotein of the Wnt family that plays important roles in organ development and tissue orientation. Previous studies have reported that Wnt5a was upregulated at both mRNA and protein levels in prostate cancer, but information regarding its role in predicting clinical outcome in patients after radical prostatectomy is limited. The aim of the present thesis is to define the role of Wnt5a protein expression in prostate cancer.  We started by evaluating Wnt5a protein expression by immunohistochemistry in a large, well-defined and population-based cohort and found Wnt5a protein expression to be upregulated in prostate cancer cells compared to benign epithelium. Interestingly, it predicted a favorable outcome for patients after radical prostatectomy as patients with preserved overexpression of Wnt5a protein in tumor cells had longer biochemical recurrence free time compared to patients with low Wnt5a protein expression. We demonstrated that this effect may be explained by the ability of Wnt5a to impair invasion in prostate cancer cells as recombinant Wnt5a treatment decreased invasion in 22Rv1 and DU145 cells while Wnt5a knockdown resulted in increase in invasion in LNCaP and 22Rv1 cells. In the light of conflicting reports on the role of Wnt5a in prostate cancer outcome, we validated our findings in an external population-based cohort. Again, we showed that Wnt5a protein expression was predictive of recurrence after radical prostatectomy in patients with low-grade prostate cancer and this was further enhanced when Wnt5a was combined with prostate cancer tissue biomarkers of known predictive value. We also demonstrated a positive correlation between Wnt5a and ERG protein expressions and that high Wnt5a protein and presence of ERG expression predicted a more favorable outcome. Despite this we observed that rWnt5a treatment of VCaP cells significantly decreased their ERG protein expression. Therefore, the relation between Wnt5a and ERG clearly need further exploration to better understand their functional interplay.  In conclusion, our study indicates a tumor suppressor function of Wnt5a protein in localized PCa and that it can be used as a predictive tissue biomarker. Further, we suggest a novel therapeutic approach for patients with localized PCa targeting Wnt5a signaling to impair progression of PCa in these patients by using a Wnt5a mimicking peptide (Foxy5).			
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# Role of Wnt5a in Prostate Cancer



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# List of Papers included in this thesis

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- **Azharuddin Sajid Syed Khaja**, Leszek Helczynski, Anders Edsjö, Roy Ehrnström, Anna Lindgren, David Ulmert, Tommy Andersson, Anders Bjartell (2011) *Elevated Level of Wnt5a Protein in Localized Prostate Cancer Tissue Is Associated with Better Outcome*. PLoS ONE 6(10): e26539. doi:10.1371/journal.pone.0026539
- **Azharuddin Sajid Syed Khaja**, Lars Egevad, Leszek Helczynski, Peter Wiklund, Tommy Andersson, Anders Bjartell (2012) *Emphasizing the role of Wnt5a protein expression to predict favorable outcome after radical prostatectomy in patients with low-grade prostate cancer*. Cancer Medicine (2012), 1: 96-104.
- **Azharuddin Sajid Syed Khaja**, Anders Edsjö, Leszek Helczynski, David Ulmert, Tommy Andersson, Anders Bjartell. *TMPRSS2:ERG - Wnt5a interaction in prostate cancer*. Manuscript

## Paper not included in the thesis

Kristofer Ahlqvist, Karunakar Saamathy, **Azharuddin Sajid Syed Khaja**, Anders Bjartell, Ramin Massoumi. *Expression of Id proteins is regulated by the Bcl-3 proto-oncogene in prostate cancer*. Oncogene. 2012 May 14. doi: 10.1038/onc.2012.175. [Epub ahead of print]



# List of abbreviations

5-AR	5- $\alpha$ -reductase
A2M	$\alpha$ 2-macroglobulin
ACT	alpha1-antichymotrypsin
ADT	androgen deprivation therapy
AMACR	alpha-methylacyl CoA racemase
APC	adenomatous polyposis coli
API	$\alpha$ 1-protease inhibitor
AR	androgen receptor
BCR	biochemical recurrence
BPH	benign prostatic hyperplasia
CRPC	castration-resistant prostate cancer
DHT	Dihydroxytestosterone
DRE	digital rectal examination
Dvl	Dishevelled
ECE	Extracapsular extension
ETS	erythroblast transformation specific
fPSA	free PSA
Fz	frizzled
GnRH	gonadotropin hormone-releasing hormone
GS	Gleason Score
GSK-3	
$\alpha/\beta$	glycogen synthase kinase-3 alpha/beta
H&E	Hematoxylin and eosin
hK-3	human kallikrein-3
IHC	immunohistochemistry
JNK	jun N-terminal kinase
LRP	low-density lipoprotein receptor-related protein
LUTS	lower urinary tract symptoms
MSMB	$\beta$ -microseminoprotein
NE	neuroendocrine

ORP	open retro-pubic radical prostatectomy
PAP	prostatic acid phosphatases
PCa	Prostate Cancer
PCP	planar cell polarity
PIA	proliferative inflammatory atrophy
PSA	prostate specific antigen
RALP	robotic-assisted laparoscopic prostatectomy
RP	Radical Prostatectomy
RPLND	retroperitoneal lymph node dissection
RT	radiotherapy
rWnt5a	recombinant Wnt5a
SFM	serum-free medium
SMS	Surgical margin status
SVI	Seminal vesicle incision
T	Testosterone
TMA	Tissue microarray
TMPRSS2	transmembrane protease serine 2
tPSA	total PSA
TRUS	transrectal ultrasound
VEGF	Vascular endothelial growth factor
Wnt	wingless-related MMTV integration site

# Human Prostate Gland

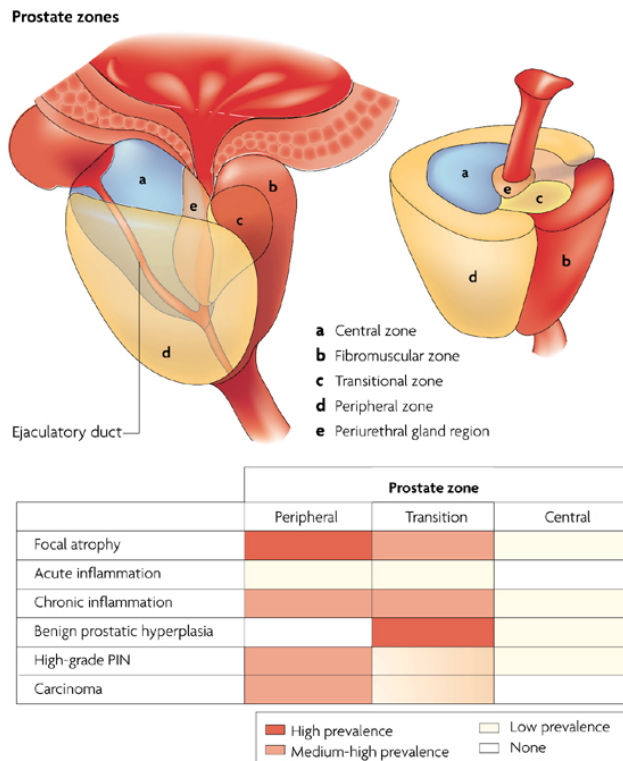
## Anatomy and Physiology

The normal human prostate gland is a wall-nut sized, approximately 3 centimetres long and weighs around 20 grams. It is a part of male reproductive system, located beneath the bladder and in front of rectum, through which it can be felt during a digital rectal examination. It surrounds the beginning of the urethra, a tube running from the bladder through the penis, which carries both urine and semen from the body. The main function of prostate is to store and secrete a milky, slightly alkaline fluid that constitutes 25-30% of the semen volume and is integral for reproductive function. Prostate is also the place where testosterone (T), an androgen predominantly produced by Leydig cells of the testes is converted to the more active androgen dihydroxytestosterone (DHT) by 5- $\alpha$ -reductase (5-AR).

Histologically, the prostate gland can be divided into different zones (**Figure 1**); peripheral zone, central zone, transition zone and anterior zone. **Peripheral Zone** constitutes about 70% of the prostatic volume and **contains** majority of the prostate glands. It is the site of about 70% of prostate cancer (PCa). **Central Zone** surrounds the ejaculatory duct and constitutes about 25% of the total prostate volume. About 5% of PCa cases originate in the central zone. **Transition Zone** is the innermost part of the prostate gland that surrounds urethra and comprises of 5% of the total prostate volume. Nearly 20% of the PCa develop in this zone. When men pass the age of 40, the transition zone enlarges leading to the tightening of the urethra that causes voiding problems. Thus, this zone is the site where benign prostatic hyperplasia (BPH) arises. **Anterior Zone** is located in the front of the prostate gland, close to the abdomen. It comprises mainly non-glandular muscular tissue [1-3].

The prostate is composed of muscular stromal (30%) and glandular epithelium compartments (70%) surrounded by a “prostatic capsule”. The capsule is important in determining the extracapsular extension (ECE) status of tumor growth at radical prostatectomy. The stromal compartment consists of extracellular matrix and variety of cells including smooth muscle cells, fibroblasts, nerves, infiltrating lymphocytes and macrophages and endothelial cells etc. The epithelial compartment in normal prostate consists of stem cells, basal cells, secretory epithelial cells (luminal cells), neuroendocrine cells and intermediate cells [3]. The secretory epithelial cells are tall,

columnar and secrete different proteins such as prostate specific antigen (PSA),  $\beta$ -microseminoprotein (MSMB) and prostatic acid phosphatases (PAP) into the lumen which is connected to urethra through ducts. Basal cells form a single layer at the base of the epithelial cells. These are small and flattened non-secretory cells with low proliferative index. Neuroendocrine cells are terminally differentiated and sparsely scattered among secretory epithelial cells in the normal prostate. The biological functions of these cells are not clear, though it has been suggested that the secretions from these cells play important role in growth and differentiation of surrounding epithelial cells in prostate gland [4, 5].



Nature Reviews | [Cancer](#)

**Figure 1: An illustration of different zones of the prostate gland and predisposition to Prostate Disease.**

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## Prostate Disorders

Prostate disorders are usually connected to aging; as the age increases, the probability of developing prostate problems also increases. The three major, common prostate disorders are;

**Prostatitis** is histological (microscopic) inflammation of the tissue of the prostate gland. It is the most common genitourinary diagnoses in men aged between 18 – 50 years, though it can affect men of any age [6]. Prostatitis can cause symptoms like painful urination and ejaculation, increased urinary frequency and urgency, pain and discomfort in the lower back region and chronic, recurrent symptoms. It might result due to bacterial infections, but evidence and causes of infection are not always found. Bacterial prostatitis (acute and chronic) accounted for less than 10% of cases and is caused by bacteriae of Enterobacteriaceae and enterococci families. But the most common form of the disease, accounting for 90% of the cases, is chronic nonbacterial prostatitis/chronic pelvic pain syndrome, the cause of which is not clearly understood [7, 8].

**Benign Prostatic Hyperplasia** is an extremely common disease in men aged above 60 and is rarely a threat to life. It refers to the enlargement of prostate in the transition zone as a result of benign growth of prostatic stromal and epithelial cells due to increased proliferation and/or impaired apoptosis. Due to enlargement of prostate, the layer of tissue surrounding it stops it from expanding, resulting in the compression of prostatic part of urethra. As a result of this, BPH symptoms comprise frequent urination, especially at night, urgency and leaking or dribbling of urine, urinary tract infections and finally acute urinary retention [3].

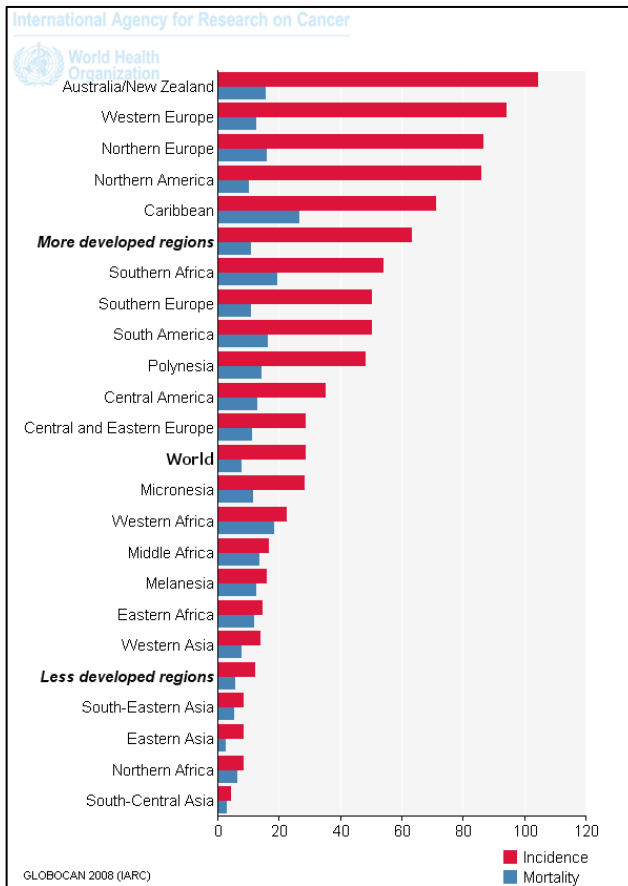
**Prostate cancer** is one of the most common forms of cancer and is the second leading cause of cancer-related death among men in western world [9]. More information about PCa will be described in the following pages as it forms a large part of this thesis.





# Prostate Cancer

In 2008, there were 899 000 new cases diagnosed with PCa with approximately 258 000 deaths worldwide [10]. Incidence and mortality rates vary within different geographical regions worldwide. The highest rates of PCa incidence was observed in Australia/New Zealand, Europe and Northern America (**Figure 2**), whereas PCa mortality rates were higher in Caribbean, followed by Africa, America and Western Europe, lowest incidences being in Southeast Asia and China. These variations in PCa incidence and mortality could be attributed to differences in diet, ethnicity, previous family history, variations in quality of healthcare and also due to the



**Figure 2: Estimated age-standardized Prostate cancer Incidence and Mortality rates (World) per 100,000. Source: GLOBOCAN 2008 (IARC)**

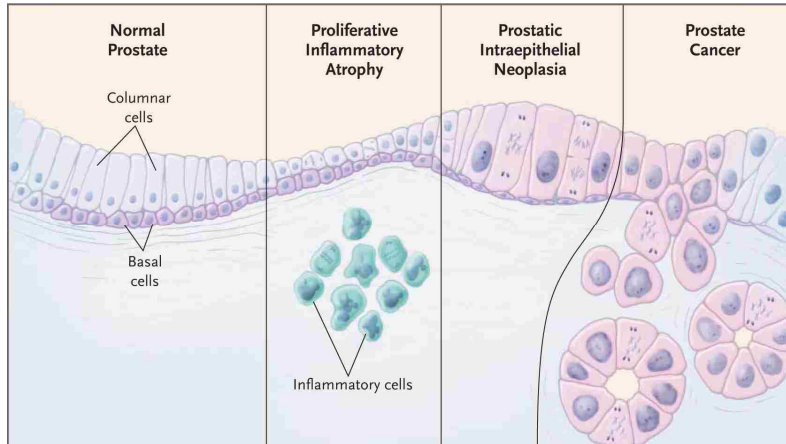
widespread adoption of prostate specific antigen (PSA) screening and the accuracy of cancer registrations in these countries. In Sweden, in 2010, nearly 33% of all diagnosed cancers are prostate cancers, making it the most common malignancy among Swedish men [11]. PCa also accounted for 2398 deaths in 2010. The number of diagnosed cases has increased rapidly in the early 1990s, mainly due to the introduction of screening by PSA blood test and early detection methods.

## Risk Factors

The causes of PCa are poorly understood, but the risk of PCa increases with increase in **age**. Prostate cancer is rarely seen in men aged below 40, but as the age increases risk of developing PCa also increases; more than half of all cases are diagnosed in men aged over 65 [12]. Risk of PCa also increases with a **positive family history**; men with first-degree relative diagnosed with PCa have twice the risk of developing PCa compared to men with no PCa in their family [13]. Though most of the PCa cases are sporadic (i.e., in individuals with no family history), 10-15% of PCa are attributed to familial history and/or hereditary susceptibility. **Race/ethnicity** is also an important risk factor for developing PCa; with African-Americans being more prone to PCa compared to others. **Environment, obesity, diet** and geographic movements also play important roles in etiology of PCa [3, 14-17].

## Pathogenesis of Prostate Cancer

The chain of events leading to prostate carcinogenesis is still unknown, though there are reports suggesting a possible role of inflammation/infection along with other dietary and environmental factors in the initiation of PCa [18-25]. Inflammatory cells secrete numerous microbial oxidants which are necessary for the removal of infectious organisms, but these microbial oxidants might also cause cellular or genomic damage in the normal prostate epithelial cells. As a result of this damage increased proliferation might occur in the prostate epithelial cells that don't differentiate into secretory cells. This morphologic state of proliferation, described as proliferative inflammatory atrophy (PIA) of the prostate by De Marzo et al [18], usually occurs in the periphery of the prostate, a site of PCa. By accumulating more genomic changes, such as down-regulation of glutathione S-transferase pi (*GSTP1*, due to promoter hypermethylation) these lesions later progress to the precursors of PCa known as prostatic intraepithelial neoplasia (PIN) (**Figure 3**).



**Figure 3: A model for prostate cancer progression illustrating proliferative inflammatory atrophy as a precursor to prostatic intraepithelial neoplasia and prostate cancer.**

(Reproduced with permission from [23], Copyright Massachusetts Medical Society).

## Diagnosis of prostate cancer

In the early stages, PCa is asymptomatic; usually there are no or only few signs and symptoms. In locally advanced cases, lower urinary tract symptoms (LUTS) arise because of the blockage of urethra due to enlargement of prostate gland, and include typical symptoms such as urgency to urinate, nocturia, frequency, and hesitancy in urination, weak urine stream, difficulty in emptying the bladder completely, blood in urine or semen and frequent pain in the lower back, hips, or upper thighs [26, 27].

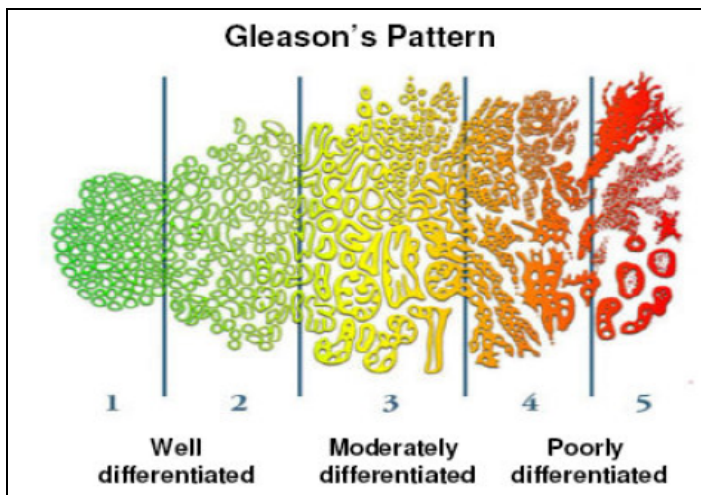
Elevated blood PSA levels and abnormal digital rectal examination (DRE) are the most commonly used diagnostic tools to detect PCa [28]. Although PSA is relatively prostate specific, it is not specific for PCa. Blood PSA levels may also be moderately elevated during BPH or prostatitis [29]. Moreover it is still debatable at what PSA levels should a biopsy be performed. When it was first introduced, PSA levels 0-4.0ng/ml were thought to be normal, and anything above would indicate an abnormality in prostate. Today, patients with serum PSA values >3.0ng/ml are usually recommended for further examination for suspect of PCa [30].

Before the availability of PSA screening, DRE was mainly used for PCa diagnosis; a physical examination during which a urologist inserts a lubricated, gloved finger through the rectal wall to feel for prostate abnormality. But it is also associated with many shortcomings. DRE has low sensitivity and is highly dependent on the examiners. Although PCa screening through PSA and DRE indicate possible

abnormalities in the prostate gland, they do not provide more information about the severity or extent of the disorder. Therefore a definitive diagnosis of PCa is made by transrectal ultrasound (TRUS)–guided needle biopsy. At least eight core biopsies are performed in the event of an elevated PSA test and/or abnormal DRE in order to make a definitive diagnosis of PCa [31, 32]. Biopsies are examined by a pathologist and graded according to the Gleason Grading system if cancer is found [33].

### *Gleason Grade*

Prostate biopsies are graded according to the microscopic description of cancer aggressiveness. Gleason grading is the most commonly used prostate cancer grading system and has a scale of 1 to 5, based on the primary histological features (growth pattern) of the tumor when viewed under a microscope (Figure 4). Grade 1 is not used any longer. Cancer cells that form well differentiated glandular structures are given a grade of 2. Gleason grade 3 relates to cells that are moderately differentiated with a more prominent variation in glandular elements. Gleason grades 4 and 5 indicate more aggressive cancers with poorly defined boundaries. The most common pattern is given a grade of 2 to 5 (primary grade). If there is a second most common pattern, the pathologist again gives it a grade of 2 to 5 (secondary grade), and adds the two most common (primary and secondary) grades together to make the Gleason score (GS) [34]. Thus, GS is the sum of primary and secondary Gleason pattern, ranging from 2 to 10. In case of a tertiary high-grade pattern in a needle biopsy, primary and highest grade patterns are recorded [34, 35].



**Figure 4: Schematic diagram of the Gleason grading system in prostate cancer**

## *Staging*

After PCa is diagnosed using pretreatment parameters (such as PSA, DRE and biopsy results), staging is required to determine whether the cancer has spread outside the prostate and, if so, which parts of the body are affected. Accurate and uniform staging is important in order to plan treatment. Clinical staging is performed by evaluating the size and location of the primary prostate tumor and how far it may have spread beyond the prostate capsule. For this **TNM** classification system is used. It describes the extent of the primary tumor by DRE (cT stage), the absence or presence of dissemination to nearby lymph nodes (N-stage) by retroperitoneal lymph node dissection (RPLND) or imaging procedures and absence or presence of distant metastasis by bone scan (M-stage) [36-38]. According to TNM classification system, a stage T0 means there is no evidence of primary tumor, in T1 and T2 stage, tumors are still confined within the prostate, whereas stages T3 and T4 represent locally advanced tumors which have spread to other organs outside the prostate.

## Treatment

After the cancer has been diagnosed, graded, and staged, appropriate treatment will depend on whether the cancer is confined within the prostate gland (localized), or has spread just outside of the prostate like seminal vesicles or regional lymph nodes (locally advanced), or has spread to other parts of the body like bone, other organs or distant lymph nodes (metastatic). Treatment options also depend upon the patient age, the patient's overall life expectancy and stage and grade of the tumor. Treatment options include active surveillance, surgery, radiotherapy, hormonal therapy and chemotherapy. Based on the risk of recurrence of the disease, PCa patients are stratified into three groups based on PSA, Gleason score and clinical stage [39]; low risk (if PSA < 10ng/ml and GS ≤ 6), intermediate risk (PSA of 10-20ng/ml or Gleason score of 7) and high risk (PSA > 20ng/ml, or GS 8-10).

Active surveillance may be an option in men with low-risk localized PCa, during which the patients are not given any definitive medical treatment but are closely monitored for disease progression with regular PSA blood tests, DRE and re-biopsies [40-44]. The main objective of active surveillance is to minimize overtreatments with possible side-effects [45]. When there are symptoms that indicate tumor progress (e.g. rising PSA levels, abnormal DRE or increased grade at re-biopsy), a definitive treatment is initiated. A radical prostatectomy (RP) which is the surgical removal of the prostate gland, is performed by open retro-pubic radical prostatectomy (ORP), laparoscopic prostatectomy (LRP) or robotic-assisted laparoscopic prostatectomy (RALP), the latter being the predominant procedure today . Though surgery increases

the overall survival and decreases the risks of metastasis and local progression [46], it is associated with erectile dysfunction and various degrees of urinary incontinence. These problems seem to be reduced with improvements in surgical procedures [47-49]. The other alternative to surgery is radiotherapy (RT), which can be external beam radiation therapy (EBRT) [50] or implantation of radioactive seeds directly into the tumor, brachytherapy, though it is also associated with late side effects including impotence [51-53].

In case of locally advanced PCa, hormonal therapy (androgen deprivation therapy, ADT) is also given either before (neo-adjuvant) or after (adjuvant) RT. Hormonal therapy can be in the form of orchiectomy (surgical castration), gonadotropin hormone-releasing hormone (GnRH) agonists or antagonists, anti-androgens or estrogens [54-59]. Hormonal therapies though not curative for PCa, can make tumors grow slowly for various times. Cancer cells initially respond to ADT, as it is evident by a decrease in PSA blood levels and/or symptomatic improvements. But in later stages there is relapse of PCa; cancer cells become insensitive to androgen treatment and fatal castration-resistant prostate cancer (CRPC) develops, as indicated by increase in serum PSA levels. Once the cancer has metastasized to other parts of the body, no curative treatment is available. Hormonal therapy is also associated with side effects such as hot flushes, erectile dysfunction and loss of sex drive, loss of bone mass, weight gain and tiredness. Chemotherapy may also be considered for cancers that have metastasized and don't respond to ADT [60-62].

## Prognostic factors

Since the introduction of PSA blood test, most of the cancers are detected at an early stage. But, it is still not clear which cancers are clinically insignificant and unlikely to grow during a man's lifetime where treatment results in unnecessary side-effects (over treatment), and which cancers need to be treated which if not treated might progress into lethal cancers. Prognostic factors help in selecting any appropriate treatment methods and hence are important in any cancer field. A prognostic factor may be defined as a factor which predicts the probable course and clinical outcome of a disease independent of treatment. In PCa, outcome of the disease is usually based on PSA relapse or biochemical recurrence (BCR) after RP. BCR is usually defined as a rise in blood PSA level of at least 0.2ng/ml, with a subsequent confirmatory value. There are some well-defined clinical and pathological prognostic factors, like GS, pathological T-stage, extracapsular extension (ECE), seminal vesicle invasion (SVI) and surgical margin status (SMS).

Gleason grading of prostate biopsies is one of the strong and established prognostic factors [63-66] and has been shown to predict outcome in several studies [65, 66].

Based on prognosis, three categories of PCa patients have been suggested;  $GS \leq 6$ ,  $GS = 7$ ,  $GS \geq 8$ . Patients with  $GS \geq 8$  have worse outcome compared to patients in other groups. Later reports suggested that prognosis of PCa patients varied within  $GS 7$ . A  $GS 7$  represents either a 3+4 score, where pattern 3 is the primary and prevalent Gleason grade or a 4+3 score, where pattern 4 is the primary and predominant Gleason grade. It was reported that within  $GS 7$ , patients with Gleason 4+3 have three times increased risk of developing lethal prostate cancer compared to patients with Gleason 3+4 [64, 67, 68]. In the present study, we have classified our patient material into two groups based on primary Gleason grade. Tumors of  $GS 3+4$  or lower were classified as “low-grade” cancers, whereas  $GS 4+3$  or higher represented “high-grade” cancers.

Clinical staging (cT) is based on test performed before surgery (DRE, imaging tests, PSA and needle biopsy results), whereas pathological T-stage (pT) is determined after histopathological examination of the prostate gland after RP. Since pathological stage is done after RP, it estimates the extent of the disease more accurately, and hence it is more useful than clinical stage in predicting outcome after RP. Any spread of the tumor beyond the confines of the prostate (ECE, SVI or lymph nodes metastasis) indicates a worse prognosis and an increased risk of recurrence of the disease [69-75]. Similarly a positive surgical margin status, which implies that the tumor has not been completely resected, is associated with adverse outcome after RP [71, 76-78].

## Biomarkers in PCa

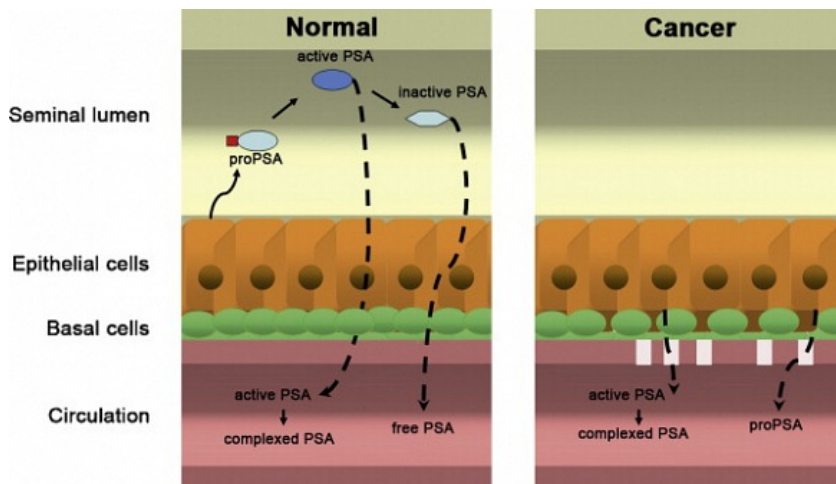
Advances in research led to the discovery of PCa tissue biomarkers that would help in diagnosis and prognosis of the disease. Some of the promising ones are PSA, alpha-methylacyl CoA racemase (AMACR), p63, low molecular weight cytokeratin and Ki-67. AMACR is overexpressed in approx. 95% of prostatic tumors and negative in benign prostatic tissue and a useful diagnostic tissue biomarker [79, 80] in combination with p63, a specific nuclear marker in basal cells, present in benign glands and PIN and absent in invasive PCa [81].

Prostate Specific Antigen, also known as human kallikrein-3 (hK3), is an androgen-regulated serine protease. It is a major protein in semen where its main function is to liquefy the semen by cleaving large gel-forming proteins including semenogelins thus allowing sperms to swim freely. It is produced primarily in prostate gland by secretory epithelial cells and is secreted directly into lumen [82]. PSA can diffuse into blood circulation, where it is present in free or complexed form. In serum, majority of the PSA is bound to protease inhibitors alpha1-antichymotrypsin (ACT),  $\alpha_2$ -macroglobulin (A2M) and  $\alpha_1$ -protease inhibitor (API). In prostate lumen active PSA is also converted to inactive PSA by proteolysis, which enters blood circulation and is



present as free, unbound form (fPSA, 5% to 40% of PSA) (**Figure 5**). Both cPSA and fPSA (total PSA, tPSA) are measurable in blood serum except for the PSA bound to A2M, as it completely masks all PSA epitopes.

Blood PSA levels are elevated during prostate disorders (BPH, prostatitis and PCa), but at moderately elevated levels, it can neither distinguish between PCa and other prostate disorders, nor between aggressive and non-aggressive PCa. Several methods have been evaluated to increase the predictive value of PSA. During PCa the basal cells are lost and normal prostatic architecture is disrupted, resulting in a decrease in proteolytic processing of PSA. As a result of this, there is an increase in the fraction of serum PSA bound to ACT and proPSA and a decrease in fPSA. Hence fPSA levels are lower in PCa compared to BPH [29, 83]. This led to the measurement of percentage of the ratio of free to total PSA (F/T PSA). Age-related reference ranges for PSA, PSA density, PSA kinetics etc. are also used to improve the specificity of PSA measurement [84] but not yet implemented in clinical practice. PSA is used not only in the diagnosis and prognosticate PCa, but also in post-therapy monitoring of the disease. After definitive therapy, remaining and increasing PSA levels indicate disease progression and recurrent disease [85].



**Figure 5: Prostate-specific antigen (PSA) biosynthesis in normal prostate epithelium versus cancer.**

ProPSA is secreted by normal secretory epithelia cells into the lumen where it is converted to active PSA. Some of the active PSA diffuses into the blood circulation, where it is bound to proteases such as ACT. The luminal active PSA undergoes proteolysis to yield inactive PSA, which may also enter the circulation to circulate in the free-state. In PCa, the tissue architecture is lost which results in relative increase in bound PSA and proPSA in serum. Reprinted from Jansen et al (2009), *European urology*, 55:563-574 [86], with permission from Elsevier.

# Gene Fusions in Prostate cancer

Chromosomal rearrangements are exclusively characteristic features of leukemias, lymphomas and sarcomas. For instance, the *BCR:ABL* translocation or the Philadelphia chromosome which results from the t(9;22)(q34;q11) is a distinctive feature of chronic myeloid leukemia. These rearrangements, however, were initially considered to be rare events in epithelial tumors. But this perception was questioned with the discovery of common and recurrent gene rearrangements in prostate cancer between androgen regulated, prostate specific *TMPRSS2* (transmembrane protease serine 2) and erythroblast transformation specific (ETS) family of oncogenic transcription factors by Tomlins et al in 2005 [87]. These findings were later confirmed by other groups [88-90]. The predominant variant of these rearrangements involves *TMPRSS2* fused to *ERG*, a member of the ETS gene family, which is reported to represent more than 90% of all the ETS gene fusions in prostate cancer. In addition to *ERG* and *ETV1*, some studies have reported additional but rare gene fusions (comprising less than 10% of total gene fusions) involving other ETS family members in PCa, such as *ETV4* and *ETV5* [91-93]. Moreover, in addition to *TMPRSS2*, fusions involving other upstream fusion partners have also been reported, like *SLC45A3:ETV5*, *SLC45A3:ETV1*, *SLC45A3:ERG*, *HNRPA2B1:ETV5* and *HNRPA2B1:ETV1* [92, 94-98]. Remarkably, majority of these gene fusion partners donating their promoter elements for fusion with ETS genes are androgen regulated, and hence these ERG rearrangements were found to be specific for PCa [99].

*TMPRSS2* (21q22.3) and *ERG* (21q22.2) genes are located 3Mb apart on chromosome 21. Fusion between promoter and 5' region of *TMPRSS2* and the coding sequence of *ERG* occurs by deletion (in majority of cases) or translocation of the intervening DNA region [87, 100, 101]. As a result of these rearrangements ETS factors are brought under the influence of androgen receptor (AR) signaling, resulting in the overexpression of normal or truncated forms of ERG fusion transcripts. Wang et al showed that there are multiple splice variants of the fusion gene [102, 103]. Though some studies have reported fusion between exon 1/2, 0, 4 or 5 of *TMPRSS2* and *ERG* exon 5, the most common transcript contains exon 1 of *TMPRSS2* fused to exon 4 of *ERG* [87, 89, 90, 104-108]. These gene fusions can be detected on prostate biopsies or RP specimen either by using specific primers of fusion transcripts by RT-PCR, by fluorescence in-situ hybridization (FISH) or by immunohistochemistry (IHC) using ERG antibody as characterized by Park et al [87, 109].

Rearrangement between *TMPRSS2* and *ERG* is by far the most frequent genetic aberration described to date in solid tumors. It is reported to be a common and early event in prostate carcinogenesis and is detected in 40-70% of primary PCa, [87, 101, 104, 105, 110-112]. *TMPRSS2:ERG* fusion is also observed in some benign glands surrounded by cancer and PIN areas (“PCa field effect”) [112, 113]. Studies also revealed the presence of gene fusions in HG-PIN, which is a precursor lesion for PCa, and in low GS; indicating that it is an early event in prostate carcinogenesis [101, 114-117], though other studies observed *TMPRSS2:ERG* fusion gene expression in aggressive and moderate to poorly differentiated tumors [94, 102, 108, 118-120]. Conflicting reports also exist on the role of these genetic rearrangements on outcome after RP for localized PCa. In some studies, this gene fusion was associated with favorable prognosis [121-123], while no significant effect on BCR was reported by others [108, 110, 111, 124]. Other studies have described *TMPRSS2:ERG* gene fusions to be associated with clinically aggressive PCa and disease-specific mortality [119, 120, 125, 126].

Given the high prevalence and specificity of *TMPRSS2:ERG* for PCa, current research is focusing on using these gene fusions in clinical settings to detect cancer by noninvasive methods such as in blood or in urine. Several studies have detected *TMPRSS2:ERG* fusion transcripts in urine samples of PCa patients [93, 127-129], thus making it a useful diagnostic PCa biomarker. A large number of PCa diagnostic markers have been reported, but almost all the biomarkers have shortcomings. No single biomarker can accurately detect the presence of PCa in individuals, but using a biomarker in combination with other markers might increase the accuracy of detecting PCa. Since *TMPRSS2:ERG* fusion is not present in all PCa cases, it could be used in conjunction with other diagnostic biomarkers such as DRE, urine PCA3 and serum PSA to detect cancer before biopsy. Hessels et al used *TMPRSS2* in combination with PCA3 to significantly improve the sensitivity for PCa diagnosis in urine samples after DRE [127]. Similarly, Laxman et al used 4 different biomarkers, *GOLPH2*, *SPINK1*, *PCA3* and *TMPRSS2:ERG* to accurately diagnose PCa with good specificity and sensitivity [128].

# The Wnt proteins

Wnt proteins constitute a family of secreted cysteine-rich glycoproteins, ~ 40 kDa in size, that are highly conserved among species [130]. The name Wnt comes from “wingless-related MMTV integration site” and was originally suggested by Nusse and co-workers in 1991 [131]. As many as 19 mammalian Wnt proteins have been described, which play important roles during development and in cell fate specification, cell proliferation, cell migration, cell polarity and tissue homeostasis [132, 133]. To regulate intracellular signal transduction, Wnt proteins bind to cell surface receptors, members of the Frizzled (Fz) family receptors, in the absence or presence of co-receptors such as LRP 5/6, ROR and RYK [133, 134]. ROR and RYK have also been described as receptors that are activated independent of Wnt ligands. There are 10 known Fz receptors in humans.

## Wnt Signaling pathways

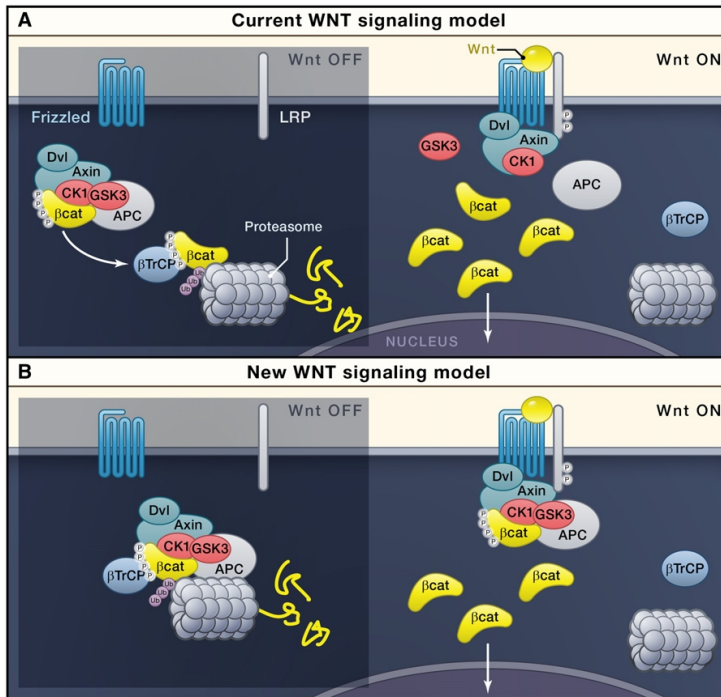
Binding of Wnts to the cell surface receptors initiates many distinct intracellular signaling pathways. These can be grouped into canonical signaling (Wnt/ $\beta$ -catenin) and non-canonical signaling (Wnt/calcium ( $\text{Ca}^{2+}$ ) signaling, planar cell polarity pathway (PCP) etc.) [134, 135].

### Canonical signaling:

Canonical signaling involves  $\beta$ -catenin-mediated transcription, hence also called Wnt/ $\beta$ -catenin signaling [130, 136, 137]. In the absence of a canonical Wnt ligands (e.g. Wnt1, Wnt3a, and Wnt8), the free cytoplasmic  $\beta$ -catenin is bound by the destruction complex, made up of Axin (a scaffold protein), APC (adenomatous polyposis coli), glycogen synthase kinase-3 alpha/beta (GSK-3  $\alpha/\beta$ ) and casein kinase-1 (CKI), and is phosphorylated by the kinases. Subsequently, it is ubiquitinated and is degraded by the proteasome. Thus Wnt/  $\beta$ -catenin target genes are kept inactive. Following the Wnt binding to the Fz receptor and LRP5/6 co-receptor, canonical signaling is activated. The activated ligand-receptor complex, through a cascade of events involving Dishevelled (Dvl), inactivates the destruction

complex. Though the mechanism is unclear, it results in the phosphorylation and activation of LRP5/6. Axin complex then binds to the cytoplasmic tail of the LRP6 receptor. As a result of this,  $\beta$ -catenin is not phosphorylated by the kinases and accumulates in the cytoplasm. The unphosphorylated  $\beta$ -catenin enters the nucleus, binds to LEF-1/TCF and starts the transcription of Wnt responsive genes, like *c-myc*, *cyclin D1*, *MMP7* and *VEGF* [138, 139].

Recently a new model of inhibition of  $\beta$ -catenin degradation is proposed by Li et al (2012) [140]. They show that the binding of Wnt ligand to Fz receptors neither changes the composition of the destruction complex nor affects the kinases' activity, but induces the association of the destruction complex with phosphorylated LRP. The kinases still phosphorylates  $\beta$ -catenin, but the phosphorylated  $\beta$ -catenin is not subjected to ubiquitination and subsequent degradation. Thus it does not leave the destruction complex, but saturates and inactivates it. Newly synthesized non-phosphorylated  $\beta$ -catenin accumulates in the cytoplasm, translocates to the nucleus and starts the transcription of Wnt targeted genes (**Figure 6**) [130, 140]. Canonical Wnt signaling through  $\beta$ -catenin plays important roles in cell proliferation, regulating stem cell maintenance and expansion, embryo development and tissue homeostasis among various other functions [141]. Aberrant activation of this pathway either due to mutations or ligand over-expression is associated with many cancers, e.g. colorectal, gastric, lung cancers etc. [138, 142].



**Figure 6: A schematic representation of Canonical Wnt signaling.**

A) Current model illustrates that in the absence of Wnt (Wnt OFF), cytoplasmic  $\beta$ -catenin is phosphorylated by GSK3 and CKI at the destruction complex. The phosphorylated  $\beta$ -catenin then leaves the complex, and is ubiquitinated by  $\beta$ -TrCP and subsequently degraded by proteasome. In presence of Wnt (Wnt ON), destruction complex is rendered inactive by the activated Wnt-receptor complex, leading to the accumulation of  $\beta$ -catenin in the cytoplasm which translocates to nucleus and starts the transcription of Wnt-related genes. B) The new model (proposed by Li et al., 2012) illustrates that, in absence of Wnt,  $\beta$ -catenin is phosphorylated by GSK3 and CKI within the destruction complex. Phosphorylated  $\beta$ -catenin is then recognized by  $\beta$ -TrCP for ubiquitination within the destruction complex.  $\beta$ -catenin is removed from the destruction complex by proteosomal degradation. Wnt binding to the receptor does not leave the destruction complex inactive; it still phosphorylates  $\beta$ -catenin. But the phosphorylated  $\beta$ -catenin does not leave the destruction complex, and is not ubiquitinated and degraded, but it saturates and thus inactivates the destruction complex. The newly synthesized and unphosphorylated  $\beta$ -catenin then accumulates in the cytoplasm and translocates to the nucleus to start the transcription of the related genes [140].

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## Non-canonical Wnt signaling:

Compared to canonical signaling, the non-canonical Wnt signaling pathways are poorly defined and refer to various Wnt pathways which are independent of  $\beta$ -catenin mediated transcription. Some of these pathways include Wnt/ $\text{Ca}^{2+}$  and planar cell polarity.

Wnt/ $\text{Ca}^{2+}$  pathway involves non-canonical Wnts, such as Wnt5a, Wnt-11 and Fz2 receptor. Binding of non-canonical Wnts to Fz receptors activates phospholipase C (PLC) via G proteins and Dvl, which subsequently triggers intracellular release of calcium ions [143, 144]. Calcium activates protein kinase C [145], which regulates cell adhesion and tissue separation during gastrulation via Cdc42. Intracellular accumulation of  $\text{Ca}^{2+}$  also activates calmodulin-dependent protein kinase II [146], which can antagonize canonical Wnt signaling by activating TAK1 (TGF $\beta$  activated kinase) and NLK (Nemo-like kinase) [147].

Another non-canonical Wnt pathway is the PCP pathway which regulates the cytoskeletal polarization in *Drosophila*. In vertebrates, the Wnt/jun N-terminal kinase (JNK) is similar to the PCP pathway in *Drosophila*. Binding of non-canonical Wnts, such as Wnt-11 to Fz receptor activates Dvl (different domains of the Dvl protein than the canonical Wnt signaling). Dvl mediates cytoskeletal rearrangements through the activation of small GTPases like Rho and Rac and downstream protein kinases such as JNK and rho kinase, leading to the cytoskeletal reorganization [148-150].

Recent advances in research led to the emergence of other pathways mediated by Wnts through Fz receptors, receptor tyrosine kinases and intracellular signaling proteins [151]. Wnt signaling classification into canonical and non-canonical pathways is challenged by the finding that some Wnts can activate both the signaling pathways. The canonical Wnt3a has been shown to activate non-canonical Wnt signaling through PKC during bone formation [152], whereas Wnt11, a non-canonical Wnt, is shown to activate canonical Wnt signaling during axis specification in *Xenopus* [153]. Another example of this is Wnt5a, which seems to involve in almost all Wnt signaling pathways [154, 155]. Signaling through a Wnt molecule, though, is complex and pathways are overlapped. The complexity in signaling depends on the cell or tissue type, receptor it uses for the signaling and presence or absence of co-receptors and other growth factors.

## Wnt5a

Wnt5a is one of the most extensively studied Wnts and is a representative of  $\beta$ -catenin-independent non-canonical Wnt signaling. It is a secreted glycoprotein and plays essential roles in organ development, tissue orientation and cell proliferation and migration. Recent studies have demonstrated an important role of Wnt5a in normal development of organs. Yamaguchi et al showed that Wnt5a<sup>-/-</sup> knockout mice exhibited perinatal lethality and embryos had severe abnormalities such as dwarfism, facial abnormalities and shortened limbs and tails, dysmorphic ribs and vertebrae, absence of the genital tubercle, and abnormalities in distal lung morphogenesis [156]. Wnt5a requirement for adequate morphogenesis of the midbrain and for tooth development has been revealed in recent studies [157, 158]. Wnt5a is also essential for controlling the planar cell polarity in vertebrates [159]. A role of Wnt5a in normal prostate gland development has been demonstrated by Huang et al [160].

In addition to signaling through non-canonical pathways such as PCP and Ca<sup>2+</sup> pathways [154, 155], Wnt5a can also activate or inhibits canonical Wnt signaling depending on presence or absence of certain receptors/coreceptors [161-163]. Mikels et al showed that binding of Wnt5a to Ror2 receptor inhibited  $\beta$ -catenin-dependent canonical pathway by suppressing the transcriptional activity of TCF/LEF, but in presence of Fz 4 receptor with LRP5 as co-receptor Wnt5a activated the  $\beta$ -catenin pathway [162]. Wnt5a has also been shown to inhibit the canonical pathway by inducing the degradation of  $\beta$ -catenin through Siah2 [163]. Given its functional roles in canonical as well as non-canonical pathways, unsurprisingly recent studies point out that any misregulation of Wnt5a signaling results in cancerous growth of certain tissues, but it is debatable whether it has a tumor-suppressor effect or tumor promoting effect. Wnt5a might play various roles in different types of cancer and may act as a tumor promoter gene or as a tumor suppressor gene, depending upon the additional factors/regulators/receptors available [154, 155].

A large number of studies indicated a tumor suppressing effect of Wnt5a, as it is shown to be downregulated in certain malignancies such as colorectal cancer [164], invasive ductal breast carcinomas [165, 166], neuroblastoma [167], hepatocellular carcinoma [168] and leukemias [169]. Loss of Wnt5a expression has been associated with unfavorable outcomes in stage Dukes B colon cancer and in neuroblastoma [164, 167], and with higher tumor stage in hepatocellular carcinoma [168], advanced stage and metastatic disease in breast cancer [166]. Tumor suppressive role of Wnt5a was further supported by inhibition of breast cancer cell invasion *in vitro* and *in vivo* by synthetic peptide (foxy5, as Wnt5a agonist) [170, 171]. Some other studies, though, have pointed out a tumor promoting role of Wnt5a in other cancers, like melanoma



[172], gastric cancer [173], non-small cell lung cancer [174], pancreatic cancer [175] and in breast cancer cells [176].

### *Wnt5a in prostate cancer*

Wnt5a has also been implicated in PCa as studies have shown an upregulation of Wnt5a gene in PCa [177, 178]. Wang et al showed that aberrant upregulation of the Wnt5a gene was due to the hypomethylation, and that this epigenetic regulation may be important for PCa progression [179]. Elevated Wnt5a protein expression in PCa compared to benign tissue has also been discussed before [180, 181]. Yamamoto et al observed that Wnt5a positivity correlated with high GS and malignant stages of prostate cancer and those patients had shorter biochemical relapse-free survival of prostate cancer, indicating Wnt5a to have an oncogenic effect in PCa progression [180]. They also demonstrated *in vitro* that Wnt5a knockdown resulted in reduced invasion in PCa cells, and invasion was stimulated in PC-3 cells with Wnt5a overexpression. Wang et al, however, did not find PC-3 cells to be more invasive upon recombinant Wnt5a treatment [181].

# Aims of the current study

Although reports on Wnt5a mRNA expression in PCa do exist, studies on Wnt5a protein expression are very scant. The role of Wnt5a in PCa is not clarified. Information regarding its effect on clinical outcome is scarce.

The current study was performed to

- ... evaluate a possible role of Wnt5a protein expression to predict outcome after radical prostatectomy in a large population-based cohort of patients with localized PCa
- ... evaluate Wnt5a protein expression in correlation with other tissue biomarkers of PCa
- ... validate the role of Wnt5a protein expression to predict outcome in PCa patients after radical prostatectomy utilizing an independent population-based cohort
- ... study interactions between Wnt5a and TMPRSS2:ERG in PCa outcome prediction



# Materials and Methods

## *Ethics statement*

The studies (paper I & III) were performed after approval from Regional Ethical Review Board in Lund. Study on paper II was approved by ethics committees at the Karolinska University Hospital, Stockholm (2006/4:10) and at IARC, Lyon (06-08).

## *Patients and tissue microarray construct*

For studies on paper I & III (Malmö cohort I), formalin-fixed and paraffin-embedded prostate sections from a consecutive series of 503 patients were used. These patients were operated for RP between 1988 and 2003 at the Department of Urology, Skåne University Hospital, Malmö, Sweden. Hematoxylin and eosin (H&E) stained slides of were used for Gleason grading and histopathological staging of the disease. Selected areas of 1.0 mm diameter of PCa and the corresponding benign areas from each patient in duplicates were mounted in a total of 17 paraffin blocks by using an manual tissue arrayer (Beecher Instruments Microarray Technology, Woodland, MD) for tissue microarray (TMA) construct [182]. Each core was examined for Gleason grade and prostatic intraepithelial neoplasia (PIN) by a senior National Board certified pathologist (LH). Patients receiving neoadjuvant radiation therapy or hormonal therapy (n=39) were ignored, leaving 464 patients for subsequent correlation analyses. Whereas cores that were either lost, or were not properly placed on slides, or were damaged during TMA construction were also discarded. For survival and multivariate statistical analyses, a total of 397 patients remained after excluding patients with no information available on GS (29) and patients where PSA levels were not completely 0 after RP and hence no BCR (n=75).

Study II was performed on consecutive series of 289 patients who underwent RP between May 1998 and November 2002 at the Karolinska University Hospital, Stockholm, Sweden. A total of 14 TMA blocks, each containing up to 24 tumors cores in triplicates were constructed (2 cores from primary Gleason grade and one from secondary Gleason grade). Each TMA had three cores of benign prostatic tissue as controls. None of the patients received androgen deprivation treatment or radiation therapy prior to RP [183]. We had access to tumors from 312 patients, with complete clinical follow-up data available from 262 of them.

In our studies we classified patients into 2 groups based on the GS, as low-grade cancers ( $GS \leq 3+4$ ) and high-grade cancers ( $GS \geq 4+3$ ).

### *Immunohistochemistry*

Consecutive sections of 4 $\mu$ m thicknesses were mounted on Superfrost Plus glass slides (Fisher Scientific, Göteborg, Sweden). The slides were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol solutions. Antigen retrieval step was performed by heating the TMA slides in PT Link from 65°C to 98°C for 40 min. Slides were then processed for IHC staining for Wnt5a (1:100), AR (1:100), Ki67 (1:100), VEGF (1:100) and ERG (1:200) using EnVision™ Flex, High pH reagent (code K8010), in Autostainer Plus (Dako A/S Glostrup, Denmark) according to the manufacturer's protocol.

### *Scoring of stained TMA cores*

Paper I and III: While scoring, staining intensities as well as percentage of positive cells were recorded. Based on staining the cores were scored as 0, if no staining was observed, 1 (weak staining), 2 (moderate staining) or 3 (strong staining). For Wnt5a and VEGF, cytoplasmic staining was recorded, whereas nuclear staining was evaluated for AR, VEGF and ERG. Ki-67 slides were scored as 0 (0-1%), 1 (1-3%), 2 (4-10%) and 3 (11-20%) based on nuclear fraction positivity. Since the cores are present in duplicates, we decided to use the maximum score for subsequent analyses. For statistical analyses, based on protein expression Wnt5a, AR, VEGF and Ki-67 were divided into 2 groups; scores of 0 and 1 as “weak/low” and 2 and 3 as “strong/high”. For ERG expression, staining in vascular endothelial cells and macrophages served as internal positive control, and the grouping was based on presence or absence of the staining in cores; ERG negative if no staining (0) is observed or ERG positive if staining (1, 2 and 3) is observed.

In paper 2, we recorded Wnt5a cytoplasmic staining intensity as well as percentage of positive cells (fraction). For cytoplasmic staining intensities, a scale of 0 to 3 was used as described earlier; 0, if no staining was observed, 1 (weak staining), 2 (moderate staining) or 3 (strong staining). Percentage of positive cells (0, 5, 10, 20, 30 ...100%) was then multiplied by the predominant staining intensity score to get a value (“multiplication score”) for each core, ranging from 0 to 300. Since cancer cores from each patient were prepared in triplicates, we decided to use the average value for further analyses.

### *Source of antibodies*

The following antibodies were used for immunostainings: Wnt5a (rabbit polyclonal): antibody was developed in our laboratory against a Wnt5a sequence with 100% homology between human and mouse; androgen receptor (AR) (code AR 441, mouse monoclonal, Thermo Fisher Scientific Inc., Fremont, CA), Ki-67 (mouse monoclonal, MIB-1 code M7240, Dako Denmark A/S, Glostrup, Denmark); VEGF (A-20, rabbit polyclonal, code sc-152, Santa Cruz Biotechnology, Inc., Santa Cruz, CA); ERG (1:200, rabbit monoclonal, clone EPR3864, Epitomics, Burlingame, CA);  $\beta$ -actin (mouse monoclonal, code C4, MP Biomedicals, Solon, OH),  $\alpha$ -Tubulin (mouse monoclonal, sc-32293, Santa Cruz Biotechnology).

### *Cell lines*

For *in vitro* studies we used one immortalized PNT2 normal human prostate epithelial cells (cat No. 95012613, European Collection of Cell Cultures (ECACC), Sigma-Aldrich, St. Louis, MO) and five human PCa cell lines LNCaP, VCaP, 22Rv1, PC-3, and DU145 (American Type Culture Collection [ATCC], Manassas, VA). Cells were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub>. LNCaP, 22Rv1, DU145 and PNT2 cells lines were cultured in RPMI-1640 medium; whereas VCaP cells and PC-3 were grown in DMEM high glucose medium and Hyclone Ham's F12 medium respectively. Media were supplemented with 10% fetal bovine serum (FBS) and 1% pest (penicillin and streptomycin). Cell confluency was maintained at ~70% for all *in vitro* experiments. All cell lines were regularly tested for the absence of mycoplasma infection.

### *Recombinant Wnt5a treatment*

For LNCaP and VCaP cells, recombinant Wnt5a (rWnt5a, 0.4  $\mu$ g/ml) treatment for protein isolation and mRNA isolation were performed at the same time for 6h, 12h and 24h time points. Cells were initially grown for 12h in respective media with 2% FBS. For 24h time point, media was replaced after 12h of treatment with fresh media with rWnt5a. For control treatment PBS (with 0.2% BSA) was used.

### *Reverse transcription and Real-Time PCR*

After rWnt5a treatment, we isolated RNA from cells RNeasy<sup>®</sup> plus mini kit (#74134, Qiagen) and used 1  $\mu$ g of RNA for reverse transcription into cDNA using

QuantiTect® Reverse Transcription kit (205311, Qiagen) according to manufacturer's protocol. After cDNA synthesis, the samples were diluted 20 times and Real-time PCR was performed with 10µl of diluted samples using 2x Maxima SYBR Green/ROX qPCR master mix (Fermentas) and 0.1 µM of forward and reverse primers in 25 µl of reactions using the manufacturer's recommended thermocycling conditions. Expression level of each target gene was normalized to the expression level of housekeeping gene.

### *Western blot Analysis*

We used western blot analyses as a measure for protein expression. Cells were washed with PBS twice, trypsinized (in trypsin for 3 min) and centrifuged at 1000 rpm for 4 minutes. Cells were then lysed on ice in RIPA buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 1% Triton x-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1mM EDTA, 0.1mg/mL Phenylmethylsulphonyl fluoride with the addition of Complete Mini protease inhibitor cocktail (Roche, Mannheim, Germany) for 30 min. lysed cell pellet was centrifuged at 15,000 rpm for 25 min at +4°C, and protein lysates were collected as supernatants. Protein concentration was measured by Bradford assay and 100µg of each protein sample was loaded on 10% SDS – polyacrylamide gels. Proteins were separated using gel electrophoresis and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Nitrocellulose membrane was blocked for non-specific binding in 5% dry milk for 45 min at room temperature and washed twice in buffer (0.05% Tween in PBS) for 10 min. The membrane was then incubated overnight with primary antibody in 2.5% dry milk (Wnt5a antibody: 1:750; AR: 1:500; β-actin: 1:3,000; α-Tubulin: 1:1000; ERG: 1:1000) at +4°C. After incubation (for 60 min at room temperature) with horseradish peroxidase – conjugated anti-rabbit/anti-mouse secondary antibodies (1:10000 in 5% dry milk, Amersham Life Science, Alesbury, UK), the unbound antibodies were washed away in washing buffer. Membrane-bound antibodies were detected by using Western blotting Luminol Reagent (Santa Cruz) using BioRad® Chemidoc™ XRS imager.

### *Transfection with Wnt5a siRNA*

Two different Silencer® Select Pre-designed (Inventoried) Wnt5a siRNAs (S1 and S2) and Silencer® Select negative control siRNA were purchased from Applied Biosystems (Ambion, CA). A cocktail of two different siRNAs (120nM) in nuclease-free water was transfected into  $1 \times 10^5$  cells in a total volume 250µL of serum free medium (SFM) using 10µL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Media was changed after 5 hours of transfection. After 24 hours of transfection, media was

changed to SFM, and cells were used 24 hours later for analysis of their Wnt5a protein expression and invasive capacities.

### *Invasion Assay*

Cell invasion capacities were measured in a standard commercial invasion assay. In this study, we used BD BioCoat™ Matrigel™ Invasion Chambers (BD Biosciences, Bedford, MA) in accordance with the manufacturer's protocol. Briefly, cells were grown in SFM for 24h, harvested using versene (Invitrogen, Carlsbad, CA), washed in PBS and resuspended at a concentration of 50,000 cells/ml in SFM. To the lower well 0.7 ml serum containing medium (10% FBS) was added. To the invasion chamber 0.5 ml (25,000 cells) of the cell suspension, containing either 0.4µg/ml recombinant Wnt5a (rWnt5a, R&D Systems, Minneapolis, MN) or 100mM Foxy5 (formyl-Met-Asp-Gly-Cys-Glu-Leu peptide, Pepscan Systems, Lelystad, Netherlands) or PBS (with 0.2% BSA) was added, and were incubated for 24 h at 37°C. After 24h, cells that invaded through the Matrigel were fixed in 4% paraformaldehyde and stained with 0.2% crystal violet in 20% methanol (Sigma-Aldrich, Saint Louis, MO, USA). Remains of the Matrigel were removed with a cotton stick moistened in PBS. Membranes from invasion chambers were separated and mounted on glass slide using VectaShield® mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Invaded cells were counted either in an inverted microscope or in Olympus BX51 Fluorescence Microscope (Olympus optical Co. Ltd, Japan).

### *Statistical analysis*

All statistical analyses were performed using SPSS version 17.0 and 20 (SPSS, Chicago, IL) and Microsoft Excel 2010. We used Wilcoxon Signed Ranks test to examine any significant difference in protein expressions between cancer and benign tissues. Spearman's rank-order correlation was performed to know significant correlations between Wnt5a, AR, Ki-67 and VEGF staining. Kaplan-Meier method was used to determine BCR-free survival (outcome) and Log Rank (Mantel-Cox) test was used to compare BCR free survival among different Wnt5a expression groups. In some analyses, expression pattern of two different proteins were grouped together, for example, while performing survival curves and Cox regression analyses Wnt5a and AR staining intensities were grouped together, making four different groups. Patients with low Wnt5a and low AR staining constituted group 1, group 2 had patients with low Wnt5a and high AR staining, patients with high Wnt5a and low AR were kept in group 3, whereas group 4 consisted of patients with high Wnt5a and high AR staining intensities. The same criterion was applied while combining Wnt5a staining



intensities with Ki-67/VEGF scorings. To examine the effect of ERG and Wnt5a protein expressions on outcome of PCa patients after RP, their staining intensities were grouped together into 4 different sub-groups; High Wnt5a and ERG +ve, High Wnt5a and ERG -ve, low Wnt5a and ERG +ve and low Wnt5a and ERG -ve.

For *in vitro* studies, Student t-Tests were performed using Microsoft Excel 2010 to study the significant differences between treated and control groups. P-values of < 0.05 were considered significant.

# Results and Discussion

In **Paper I**, we used cohort I (Malmö TMA) to evaluate Wnt5a protein expression by IHC, and correlated its expression with other PCa tissue biomarkers such as AR, VEGF and Ki-67. We also investigated its possible role in outcome of PCa patients after RP. In **Paper II** we verified our results on the effect of Wnt5a protein expression on outcome of PCa after RP in a different well-defined patients' cohort II (Stockholm TMA). In **Paper III** we studied interactions between Wnt5a protein expression and fusion gene, *TMPRSS2:ERG* in PCa. From these cohorts we excluded patients who received neoadjuvant radiation or hormonal therapies before RP in order to avoid effects of radiation or hormonal treatments on clinical outcome. We classified our patient material into two groups based on Gleason grade; patients with GS up to 3+4 were grouped as “low-grade” cancers, whereas patients with GS as 4+3 or higher were included in “high-grade” cancers. In cohorts I and II, we observed 89% and 76% of patients with low-grade cancers respectively which was to be expected from population-based studies; since most of the cancers are detected in early stages due to PCa screening by PSA testing.

*Elevated Wnt5a protein expression in cancer cores compared to benign and its correlation with other PCa tissue biomarkers such as AR, Ki-67 and VEGF (Paper I)*

We started our study by investigating protein expressions of Wnt5a, AR, VEGF and Ki-67 by IHC in a large, population-based cohort consisting of 503 PCa patients' samples of cancer cores and adjacent benign tissue cores in duplicates (Malmö TMA). These patients had undergone RP between 1988 and 2003 at Department of Urology, Skåne University Hospital, Malmö. We observed Wnt5a protein expression mainly in the cytoplasmic regions of the epithelial cells with some stromal staining in both cancer and benign regions. We found increased Wnt5a protein expression in cancer cores (82%) compared to benign (35%) in this cohort, and this increase was significant ( $p < 0.0001$ ) when Wilcoxon Rank sum test was performed. Recent studies have also pointed out increased Wnt5a protein expression in malignant cores compared to benign prostatic tissue [180, 181].

Our observation that cancer areas have more Wnt5a protein expression compared to benign cores was further supported by *in vitro* studies. We observed that endogenous expression of Wnt5a protein levels were low in PNT2 cell line, an SV40 immortalized cell line derived from normal human prostate epithelium compared to that of the PCa cell lines LNCaP and 22Rv1. We then assessed Wnt5a expression in different grades of cancer. We did not find any significant differences in distribution of Wnt5a protein expression between different grades of cancers ( $p = 0.649$ , Fisher's exact test). In low-grade cancers, 81% of patient cores were strongly stained for Wnt5a, whereas 86% cores had strong Wnt5a staining in high-grade cancers, though *in vitro* we found that Wnt5a expression was less in more aggressive PCa cell lines (PC-3 and DU145) compared to less aggressive LNCaP and 22Rv1 cells.

We also analyzed protein expressions of AR, VEGF and Ki-67 on the subsequent sections of the same TMA. IHC stainings of AR and Ki-67 were predominantly nuclear, whereas VEGF was localized mainly in the cytoplasm of cancer and benign epithelial cells. The staining intensities of these proteins were intense in cancer cores compared to benign, and these differences were statistically significant ( $p < 0.0001$ , Wilcoxon Rank sum tests). A role of AR in normal prostate gland and PCa development has been discussed previously [184, 185], Ki-67, a proliferation marker, has also been extensively studied in PCa outcome [186], and hence increased stainings of AR and Ki-67 in cancer compared to benign were expected. In fact we used Ki-67 staining as an internal control of our clinical material in subsequent outcome analyses.

To better understand a possible role of Wnt5a in PCa, we performed further statistical analyses of potential correlations between Wnt5a protein expression and that of AR, Ki-67 and VEGF. Nearly 60% of patients with strong Wnt5a immunostaining in their cancer cores also exhibited intense AR staining, and 65% of patients had strong Wnt5a and VEGF stainings, indicating an association between Wnt5a and AR and VEGF. Indeed, in this Malmö TMA, Wnt5a expression showed a positive and statistically significant correlation with VEGF expression (Spearman's rho ( $\rho$ ) = 0.396,  $p < 0.0001$ ), weak but still statistically significant correlations with AR and ( $\rho = 0.159$ ,  $p = 0.007$ ) and Ki-67 expressions ( $\rho = 0.233$ ,  $p < 0.0001$ ). The correlation between Wnt5a and AR was further supported by our *in vitro* results. Protein expression analyses by Western blot indicated that cell lines with high Wnt5a protein expression also had high AR expression, and vice versa. On the other hand, Kawano et al showed that Wnt5a inhibited AR transcriptional activity in 22Rv1 cells when these cells were transfected with a Wnt5a plasmid [187].

Wnt5a protein expression also had weak but significant association with Ki-67; indicating a role of Wnt5a in cell proliferation, but our subsequent *in vitro* data suggested that Wnt5a treatment did not affect proliferation in PCa cell lines. Moreover the association between Wnt5a and Ki-67 in clinical samples was in

contrast to the study on hepatocellular carcinoma where Wnt5a had a tumor suppressing effect and loss of Wnt5a strongly correlated with high Ki-67 proliferation index [168]. In non-small-cell lung cancer Huang et al found that intratumoral Wnt5a expression significantly correlated with Ki-67 proliferation index [174]. Taken together, these data suggest that the role of Wnt5a signaling in the regulation of tumor cell proliferation is not the same in different malignancies.

### *Increased Wnt5a expression predicts longer relapse-free time (Paper I)*

We evaluated a possible role of Wnt5a protein expression in predicting PCa outcome after RP by plotting Wnt5a protein expression against time to BCR. Kaplan-Meier curves illustrating Wnt5a protein expression and BCR free time revealed a favorable outcome for patients with high/strong Wnt5a protein expression compared to those with low/weak expression in the whole patient material. This was further enhanced by our *in vitro* results as demonstrated by Western blot analyses; more aggressive PCa cell lines (PC-3 and DU145) had low endogenous Wnt5a protein levels compared to LNCaP and 22Rv1, which are less aggressive PCa cell lines. Since several studies pointed out an antagonistic effect of Wnt5a on  $\beta$ -catenin dependent canonical signaling [155, 162, 163], our results were also consistent with the findings of Chen et al who observed that high expressions of Wnt1 and  $\beta$ -catenin were associated with advanced, hormone-refractory disease and could serve as markers for PCa progression [188].

### *Wnt5a effect on invasion of PCa cells is independent of cell proliferation (Paper I)*

In order to better understand this clinical finding we used four PCa cell lines, LNCaP, 22Rv1, PC-3 and DU145 in subsequent 24 hour invasion assay experiments. We found that addition of rWnt5a decreased invasion in 22Rv1 and DU145 cells, whereas there was no significant effect on invasion of LNCaP and PC-3 cells. The results with PC-3 cells are in line with a recent report by Wang et al, who showed that addition of rWnt5a did not affect PC-3 cell motility in a migration wound scratch assay [181]. LNCaP cells are known to be less invasive, and this might explain why these cells did not respond to rWnt5a treatment. However, LNCaP cells showed a significant increase in invasion when Wnt5a expression was knocked down using si-RNAs. Knocking down of Wnt5a also affected invasion in 22Rv1 cells as these cells were more invasive after Wnt5a knockdown. These results indicated that for PCa cells to invade, Wnt5a must be actively silenced. This hypothesis is in agreement with findings of Yamamoto et al where they did not observe strong Wnt5a staining at the invasive front of the PCa [180], and our *in vitro* results where invasive PC-3 and

DU145 cells had less endogenous levels of Wnt5a protein compared to LNCaP and 22Rv1 cells.

Next, we wanted to know if the decrease in invasion of 22Rv1 and DU145 cells after rWnt5a treatment was associated with a decrease in proliferation of these cell lines. All 4 PCa cell lines were treated with rWnt5a for 24 hours, and then proliferation rate of these cells were evaluated using BrdU assay. We did not find any significant effect of rWnt5a treatment on proliferation in these cell lines during the 24 hours used for the invasion assay.

### *Clinical significance*

Addition of rWnt5a decreased invasion in 22Rv1 and DU145 cells. Hypothetically, Wnt5a can be used in a clinical setting to treat PCa patients. Previously it was shown that Wnt5a treatment decreased motility in breast cancer cells by activating adhesion receptor discoidin domain receptor 1 (DDR1) [189]. Since Wnt5a protein is relatively large (43kDa), Foxy5, a small formylated hexapeptide was derived from the amino acid sequence of Wnt5a protein [170]. Like rWnt5a, it was shown to impair motility of breast cancer cells with low endogenous levels of Wnt5a and to significantly inhibit breast tumor metastasis to lung and liver *in vivo* [171]. We used Foxy5 to investigate its effect invasion of 22Rv1 and DU145 cells. Interestingly, we found that it significantly repressed invasive capabilities of these two PCa cell lines like rWnt5a. Therefore, after further research on this topic, Foxy5 may serve as a hypothetical future therapeutic option in PCa.

### *Conflicting data and Validation study (Paper II)*

There are no contrasting reports about the upregulation of Wnt5a levels in PCa compared to benign tissue. However there are conflicting data on the role of Wnt5a in PCa outcome. Even though Yamamoto et al [180] did not observe strong Wnt5a staining at the invasive front of the PCa, they showed that Wnt5a promoted aggressiveness of PCa and those patients with low/negative Wnt5a protein levels in their tumors had longer BCR free time. They also showed that knockdown of Wnt5a decreased invasion and overexpression of Wnt5a increased invasion in PCa cells. These results were in disagreement with our clinical findings and *in vitro* results, where we demonstrated that patients with high Wnt5a levels had longer relapse free time, and that rWnt5a treatment reduced invasion in 22Rv1 and DU145 cells. These conflicting results urged us to confirm our results about the role of Wnt5a to predict outcome in PCa patients utilizing an external independent patient cohort.

For the validation study we used a different cohort (cohort II) than previously described (cohort I). This cohort II consisted of 312 PCa patients with cancer cores in triplicates; 2 cores from primary Gleason grade and one from the secondary Gleason grade to represent heterogeneity in PCa. After IHC staining with Wnt5a, staining intensity score from 0 to 3 and fraction of positively stained cells were recorded. We multiplied the intensity score with the fraction to get a value which we described as “multiplication score”. Presence of triplicate cores from each patient prompted us to use average value from the three multiplication scores of each patient. We then used classification and regression tree analyses to find optimal cut-offs for dichotomization of the patient material into Wnt5a subgroups of prognostic value in relation to BCR. The optimal cut-off value was identified at multiplication score 195. Patients were then grouped as low expression/weak staining (with score 0 - 195), and high expression/strong staining (196 - 300).

Like the Malmö TMA study (Cohort I, Paper I), we did not find any significant differences in distribution of Wnt5a protein expression between different grades of cancers ( $p = 0.183$ , Fisher’s exact test) in Stockholm TMA (Cohort II, Paper II). Nearly 41% of cancer cores had high Wnt5a protein expression, whereas in our previous Malmö TMA study, we reported high Wnt5a protein expression in 82% of cancer cores. Kaplan-Meier curves plotted between Wnt5a expression and time to BCR showed no significant differences in outcomes between patients with different Wnt5a expression levels. These discrepancies in results might be attributed to; i) cohort II (24%) included a larger proportion of high-grade cancers compared to cohort I (10%), ii) there are more PCa recurrences in cohort II (38%) compared to cohort I (27%), and iii) for cohort II we used average score as it contained tumor cores in triplicate, while we used best score of the two cores in cohort I study. But when patients in cohort II were dichotomized based on GS as low-grade cancers and high-grade cancers, we observed that Wnt5a protein expression could significantly predict outcome in low-grade cancer patients. We found that patients in this group with high Wnt5a protein expression had significantly longer BCR-free time after RP compared to patients with low Wnt5a expression ( $p=0.017$ ). In high-grade cancer patients a predictive value of Wnt5a could not be found and there were no significant differences in outcome between patients with different Wnt5a expressions in this group of PCa patients.

Reasons behind conflicting results between our group and the Japanese group are not known. A number of factors differ between these studies, including but not limited to

- Sample size – we performed our two studies on a large number of patients, whereas Yamamoto et al performed their study on relatively less patient samples (98 patients).
- Classification – we classified our patient group based on primary pathological Gleason grade; tumors with GS 3+4 or lower were classified as low-grade cancers (76%) and tumors with GS 4+3 or higher represented high-grade cancers (24%). The reasons for such classification were described above. On the other hand Yamamoto et al classified tumors as  $GS \leq 7$  (75.5%) and  $GS \geq 8$  (24.5%).
- Antibody – different antibodies were used in these studies. The Wnt5a antibody which we used in our studies was well characterized and documented by peptide antigen and recombinant Wnt5a blocking experiments and IHC and Western blot of PCa cells after silencing Wnt5a expression by siRNA.
- Genetic variations – these studies were performed on patients from different genetic ancestry.
- Source of study samples – we performed our studies on sections from TMAs of RP specimens, while Yamamoto et al performed their study on whole tissue sections from prostatectomies.
- Different scoring methods – The scoring method we used has been described above in detail. Yamamoto et al used only percentage of positively stained cancer cells in tumor region and described tissues as Wnt5a positive when the staining was present in more than 50% of the cancer cells.

The cohort II had 24% high-grade tumors which is similar to 24.5% of PCa patients classified as  $GS \geq 8$  by Yamamoto et al. Despite this similarity in proportion of low- and high-grade cancers, we could not find the same predictive ability of Wnt5a expression as they found in their study. However, we successfully validated our previous finding of a predictive value of Wnt5a protein in PCa patients and showed that high Wnt5a protein expression was significantly associated with longer relapse-free survival time in low-grade PCa.

### *Clinical significance*

Since the introduction of PSA testing, the detection of PCa at early stages and incidence of indolent tumors have increased [190]. However, the PSA test is not good enough to distinguish between clinically significant and insignificant tumors, which resulted in a serious increase in over-detection and overtreatment of less harmful

prostate cancers [191, 192]. Our finding that low-grade PCa patients with high Wnt5a protein expression in their tumors have a longer BCR free time can be used to identify such patients and to avoid overtreatment in this group of patients. However, this needs to be further studied in preoperative biopsies before it can be implemented in a clinical setting.

### *Wnt5a in combination with established PCa tissue biomarkers (Paper I & II)*

In cohort I, we demonstrated that elevated levels of Wnt5a protein expression in localized PCa predicted longer relapse free time after RP. As a control of our clinical material in cohort I, we plotted Kaplan-Meier curves between GS and BCR free time and showed that patients with low-grade cancers had statistically significant better outcome compared to those with high-grade cancers. We also used Ki-67 staining, a validated tissue biomarker in PCa [186] for evaluation of the clinical material. Patients with high Ki-67 expression had reduced relapse free survival time when compared with patients with a low number of Ki-67 expressing tumor cells. Cohort II was evaluated for both GS and SMS. SMS is also an established prognostic factor and patients with negative SMS have a significantly longer relapse-free time after RP compared to patients with positive SMS [193]. In both cohort I and II, there was a statistically significant difference in clinical outcome between patients with negative and positive SMS when Kaplan-Meier curves were plotted.

In cohort I we combined Wnt5a protein expression with AR, Ki-67 and VEGF to examine if we could further increase the predictive power of tissue biomarkers. As mentioned in Materials and Methods, we combined staining scores of two proteins to make four subgroups. The best prediction model was obtained when Wnt5a protein expression was combined with AR and Ki-67; patients with high Wnt5a and low AR or low Ki-67 expression showed better relapse free survival ( $p < 0.0001$ ), whereas patients with low Wnt5a expression and high AR or high Ki-67 expression had the worst outcome after RP. When plotted alone, VEGF expression did not affect the outcome after RP, but when combined with Wnt5a, it could predict outcome, as patients with high Wnt5a and low VEGF protein expressions had better outcome compared to other groups ( $p = 0.003$ ).

In cohort II we found a predictive value of Wnt5a protein in low-grade cancers. We also noticed that when combined with SMS, Wnt5a could have an effect on patients with positive SMS; patients with low-grade cancers, displaying high Wnt5a protein expression and positive SMS have similar relapse-free time after RP compared to patients with low Wnt5a staining and negative SMS. This finding might be of importance from a therapeutic point of view and indicated an opportunity to treat PCa patients with low-grade tumors and positive SMS by targeting Wnt5a signaling.



### *TMPRSS2:ERG gene in PCa (Paper III)*

As described earlier, fusion between androgen regulated *TMPRSS2* and *ERG* genes resulted in androgen-induced overexpression of truncated or normal ERG protein. Using an antibody which has been used in several studies since its characterization by Park et al, we evaluated ERG expression, a surrogate marker for gene fusion by IHC in a previously described, large and well defined population-based cohort (I, Malmö TMA). ERG expression was localized in nuclear region and was found in 58% of PCa patients which was in similar range as reported by recently published studies using the same antibody [110-112]. The staining was present mainly in cancer and PIN areas, though in some rare cases ERG expression was also observed in benign glands surrounded by cancer/PIN, which might be due to “PCa field effect” as reported in earlier studies [112, 113].

An analyses of ERG expression in different GS revealed that ERG expression was more frequent in low-grade cancers (60%) compared to high-grade cancers (40%). This difference in distribution of ERG staining in different grades of cancers was statistically significant ( $p = 0.029$ , Fisher’s Exact Test) and supported the hypothesis that ERG expression is an early event in prostate carcinogenesis. Several previous reports supported this view [116, 117, 123, 124], though Rajput et al [118] reported the presence of gene fusions more in poorly differentiated cancers compared to well-differentiated cancers. ERG expression did not correlate with SMS, SVI and ECE.

Further analyses revealed that ERG significantly correlated with Wnt5a ( $p < 0.001$ ) and AR ( $p = 0.007$ ) protein expressions in this cohort I. In our earlier studies (Paper I) we described Wnt5a and AR immunostainings. ERG correlation with AR was expected since in PCa, AR regulates *TMPRSS2* and thus ERG expression after *TMPRSS2:ERG* fusion. An association between AR and ERG has also been described by Minner et al [111]. ERG protein expression also correlated with Wnt5a protein expression. To our knowledge, this was the first study in PCa describing a correlation between Wnt5a and ERG.

### *ERG expression either alone or in combination with Wnt5a predicted longer relapse free time for PCa patients after RP (Paper III)*

Contradictory findings have been reported regarding the effect of *TMPRSS2:ERG* gene fusion on outcome after RP for localized PCa. To find out if ERG, a gene fusion marker, could predict outcome in PCa patients after RP, Kaplan-Meier curves were plotted between time to BCR and ERG protein expression. We found a positive association between ERG expression and longer relapse free time in cohort I. Cox regression analysis revealed that patients with low ERG expression had nearly double the risk of PCa relapse than patients with high ERG. Our data in this regard

was in strong agreement to reports from Petrovics et al, Saramaki et al and Winnes et al [121-123]. One of the earlier findings from Petrovics et al found ERG expression as determined by Q-PCR to be significantly associated with longer relapse free survival in PCa after prostatectomy [121]. Saramaki et al also found TMPRSS2:ERG rearrangement to be associated with good prognosis in a study of 150 prostatectomy specimen [122]. Other reports though have linked ERG expression to more aggressive PCa [119, 120, 125]. Reasons behind these contrasting reports are not clear, but might depend upon sample sizes, methods of gene fusion detections (e.g., Q-PCR, IHC or FISH), duration of patients' follow-up etc. Further research is required to better understand conflicting results.

We previously showed a positive role of Wnt5a protein expression in predicting longer relapse free time for PCa patients after RP in the same cohort. To find out if these proteins together could predict outcome in these patients, we stratified protein expressions of Wnt5a and ERG into four subgroups as described in Materials and Methods. Kaplan-Meier curves revealed a better prognosis for a subgroup of patients who have high Wnt5a and ERG expressions compared to other subgroups.

### *Wnt5a effect on ERG protein expression and mRNA levels (Paper III)*

To better understand the functional consequences of an association between Wnt5a and ERG, *in vitro* studies were performed. VCaP cells were treated with rWnt5a for 6h, 12h and 24h, ERG levels were then investigated at protein and mRNA levels by western blot and Q-PCR analyses respectively. Though there was a positive correlation between Wnt5a and ERG protein expressions and high Wnt5a protein and positive ERG expression predicted favorable outcome in patient samples, the same was not observed *in vitro*. Instead we found that in VCaP cells rWnt5a treatment for 6h significantly decreased ERG protein expression and the effect was even more significant after 24h of rWnt5a treatment. To find out if rWnt5a treatment also affected ERG mRNA level, we performed Q-PCR analyses using primers specific for TMPRSS2:ERG. ERG mRNA expression decreased after 6 hours of rWnt5a treatment, however, its expression increased following 12 hours of rWnt5a treatment. It is possible that the decrease in ERG protein levels was due to reduced mRNA levels after 6h of rWnt5a treatment, and this effect on protein level remained until 24h. Our results need to be further explored for a better understanding.

ERG fusion has been reported as an early event in PCa and our results also suggested the same, as ERG expression was observed mainly in low-grade cancers, and patients with positive ERG expression had longer survival times. In our earlier studies we highlighted the positive association between high Wnt5a protein expression and longer relapse-free survival for PCa patients after RP. These two factors whose positivity suggest longer BCR free time in PCa patients after RP, but *in vitro* one

factor suppressed the other factor's expression. These seemingly conflicting results might indicate that cancer cell lines are not always true representation of the tumors and different molecular mechanisms might regulate ERG expression in patients than in the cells. The other reason might be that the ERG though associated with longer relapse free survival in PCa patients promoted invasion of the cancer cells.

Earlier we showed that Wnt5a decreased invasion in PCa cells and a recent report also pointed out a role of TMPRSS2:ERG in invasion. Downregulation of ERG fusion attenuated invasion in VCaP cells [194]. In the present study there was a decrease in the invasion of VCaP cells after rWnt5a treatment.

# Conclusions

## *Paper I*

- Wnt5a protein expression is elevated in PCa cells compared to the benign epithelium.
- Preserved overexpression of Wnt5a protein predicts a favorable outcome after RP.
- Combining Wnt5a protein expression with other PCa markers with known predictive value could further improve the performance of Wnt5a to predict recurrence.
- Wnt5a suppresses invasion of PCa cells *in vitro*.
- Our study indicates a tumor suppressor effect of Wnt5a in localized PCa and that it can be used as a predictive tissue biomarker.
- Our results suggest a novel therapeutic approach for patients with localized PCa by targeting Wnt5a signaling to impair progression of PCa in these patients by using Foxy5 (a Wnt5a mimicking peptide).

## *Paper II*

- We have confirmed that a group of patients with preserved overexpression of Wnt5a protein in PCa cells have a lower risk of recurrence after RP.
- Further studies should aim at investigating Wnt5a as a target in new PCa therapies.

### *Paper III*

- *TMPRSS2:ERG* gene fusion is a frequent and early event in prostate carcinogenesis and is associated with low-grade cancers.
- ERG protein expression as a surrogate marker for *TMPRSS2:ERG* gene fusion either alone or in combination with Wnt5a predicts longer relapse free time for PCa patients after RP.
- The association between Wnt5a and ERG, needs to be further explored to better understand the functional consequences.

### *Disclosure of Potential Conflicts of Interest*

Tommy Andersson and Anders Bjartell are shareholders of WntResearch AB.

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