STAT3 in Prostate Cancer

Don-Doncow, Nicholas

2017

Document Version:
Publisher's PDF, also known as Version of record

Link to publication

Citation for published version (APA):
Don-Doncow, N. (2017). STAT3 in Prostate Cancer Lund: Lund University, Faculty of Medicine

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the public portal

Take down policy
If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
STAT3 in Prostate Cancer

Nicholas Don-Doncow

DOCTORAL DISSERTATION
by due permission of the Faculty of Medicine, Department of Translational Medicine, Division of Urological Cancers, Lund University, Sweden
To be defended at Lilla Aulan Jan Waldenströms Gata 5
February 2nd 2017 at 13:00.

Faculty opponent
Hing Leung MD, PhD
Professor of Urology and Surgical Oncology, Institute of Cancer Sciences,
University of Glasgow, United Kingdom
Prostate cancer is the second most common diagnosed cancer among men. Currently there are no reliable biomarkers to distinguish between indolent and aggressive disease. When men progress to advanced stages of the disease treatment options are limited and resistance to treatments available today is a growing problem. Recently the signal transducer and activator of transcription 3 (STAT3) has been suggested as both a potential target for treatment, and a biomarker in advanced prostate cancer.

In this work we explored the potential of activated STAT3 (pSTAT3) as a tissue biomarker in two different cohorts. We also investigated the mechanism of action of the STAT3 inhibitor galiellalactone as well as its effect in combination treatments with chemotherapeutic agent docetaxel.

In a rapid autopsy cohort of patients that have died of metastatic prostate cancer we found that expression of pSTAT3 was present in all metastases, with highest expression in the bone, likely an effect of the tumor microenvironment. A second cohort of hormone naïve patients with localized prostate cancer showed that pSTAT3 expression was higher in benign tissue compared with tumor tissue. Lower pSTAT3 expression in tumor cells was predictive of shorter time to recurrent disease. These two cohorts suggest that targeting pSTAT3 would be valuable at later stages of the disease.

We also investigated galiellalactone, a natural fungal compound, and its mechanism of action in inhibiting STAT3. We found that galiellalactone binds directly to STAT3, thus blocking the ability of STAT3 to bind to DNA. When galiellalactone was used in combination with docetaxel it was able to produce a synergistic inhibitory effect, which was likely due to the observed downregulation of genes involved in docetaxel resistance.

In conclusion, our results suggest that STAT3 is a promising treatment target in late stage prostate cancer and may lead to benefit when used in combination with docetaxel.

Key words: prostate cancer, STAT3, biomarker, immunohistochemistry, drug resistance

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature: __________________________ Date: __________________________
STAT3 in Prostate Cancer

Nicholas Don-Doncow

LUND UNIVERSITY
Coverphoto by Nicholas Don-Doncow

Copyright Nicholas Don-Doncow

Faculty of Medicine, Translational Medicine, Division of Urological Cancers

ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2016
To Catja and Spencer
“Well I guess now is the time for me to say something profound...
Table of Contents

Table of Contents ........................................................................................................... 8
List of Papers .................................................................................................................. 11
Abbreviations .................................................................................................................. 13
I. The Prostate ..................................................................................................................... 17
   Structure ......................................................................................................................... 18
   Cellular Structure .......................................................................................................... 19
   Prostate Cancer and Diagnosis ..................................................................................... 20
   Androgen Receptor ....................................................................................................... 20
   Treatment Options ........................................................................................................ 21
II. Biomarkers ................................................................................................................... 23
   Introduction ................................................................................................................... 24
   Immunohistochemistry ................................................................................................. 24
   Prostate Cancer Biomarkers ......................................................................................... 25
       Gleason Score ............................................................................................................. 25
       PSA ............................................................................................................................. 26
       PCA3 .......................................................................................................................... 27
       TMPRSS2-ERG .......................................................................................................... 27
       AR-V7 ........................................................................................................................ 27
       PTEN .......................................................................................................................... 28
       Other Biomarkers ...................................................................................................... 28
III. STAT3 .......................................................................................................................... 29
   Introduction ..................................................................................................................... 30
   STAT3 activation and signaling pathway .................................................................... 31
   IL-6/STAT3 pathway .................................................................................................... 31
   STAT3 signaling ............................................................................................................. 33
   STAT3 in cancer ............................................................................................................. 33
   STAT3 in PCa ................................................................................................................. 34
   STAT3 and cell proliferation and apoptosis .................................................................. 34
   STAT3 and metastases ................................................................................................. 35
<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAT3 and angiogenesis</td>
</tr>
<tr>
<td>STAT3 and immune suppression</td>
</tr>
<tr>
<td>Targeting STAT3</td>
</tr>
<tr>
<td>Galiellalactone</td>
</tr>
<tr>
<td>IV. Drug resistance</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>ADT</td>
</tr>
<tr>
<td>Docetaxel resistance</td>
</tr>
<tr>
<td>STAT3 and drug resistance</td>
</tr>
<tr>
<td>Combination Therapies</td>
</tr>
<tr>
<td>V. The Current Investigation</td>
</tr>
<tr>
<td>Paper I</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Results and Discussion</td>
</tr>
<tr>
<td>Paper II</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Results and Discussion</td>
</tr>
<tr>
<td>Paper III</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Results and Discussion</td>
</tr>
<tr>
<td>Paper IV</td>
</tr>
<tr>
<td>Introduction</td>
</tr>
<tr>
<td>Results and Discussion</td>
</tr>
<tr>
<td>Concluding Remarks</td>
</tr>
<tr>
<td>Popular Science Summary</td>
</tr>
<tr>
<td>Acknowledgements</td>
</tr>
<tr>
<td>References</td>
</tr>
<tr>
<td>VI. Papers</td>
</tr>
</tbody>
</table>
List of Papers

The thesis is based on the following papers:


II. Immunohistochemical expression patterns of activated STAT3 and androgen receptor in hormone-naïve prostate cancer. Don-Doncow N*, Krzyzanowska A*, Marginean F, Hellsten R, Bjartell A. Preliminary Manuscript


IV. The STAT3 inhibitor galiellalactone down-regulates genes involved in drug resistance and enhances the anti-proliferative effects of docetaxel in prostate cancer cells. Don-Doncow N, Bjartell A and Hellsten R. Preliminary Manuscript.

* = Authors have equally contributed
Abbreviations

AA anti-androgen
ABC adenosine triphosphate binding cassette
ADT androgen deprivation therapy
ALDH1 aldehyde dehydrogenase 1
ALK anaplastic lymphoma kinase
AR androgen receptor
ARE androgen-responsive elements
AR-V7 androgen receptor splice variant 7
AS active surveillance
ASTRO American Society of Therapeutic and Radiology Oncology
BAD Bcl-2-associated death promoter
BCR biochemical recurrence
BCL-2 B-cell lymphoma 2
BCL-XL B-cell lymphoma-extra large
BCR-ABL breakpoint cluster region – abelson
BCRP breast cancer resistance protein
BPH benign hyperplasia of the prostate
c-PARP cleaved poly(ADP-ribose) polymerase
CDK cyclin dependent kinase
CML chronic myeloid leukemia
CRPC castration-resistant prostate cancer
cT clinical tumor stage
CZ central zone
DHT dihydrotestosterone
DNA deoxyribonucleic acid
DRE digital rectal examination
EBR external beam radiation
EGF epidermal growth factor
EGFR epidermal growth factor receptor
EMA European Medicines Agency
ERG erythroblast transformation-specific-related gene
ERSPC European Randomized Screening Study for Prostate Cancer
FAS apoptosis antigen 1
FDA Food and Drug Administration
gp130 glycoprotein 130
GS Gleason score
H&E hematoxylin and eosin
HER2 human epidermal growth factor receptor 2
HIF1α hypoxia-inducible factor 1α
HSP heat shock protein
IGF1R insulin-like growth factor 1 receptor
IHC immunohistochemistry
IL interleukin
IL-6R interleukin 6 receptor
JAK Janus kinase
LHRH Luteinizing Hormone-Releasing Hormone
LIF leukemia inhibitory factor
MCL-1 BCL2 family apoptosis regulator
MMP matrix metalloproteinase
MRP1 multidrug resistance-associated protein 1
NFκB Nuclear factor kappa-light-chain-enhancer of activated B cells
nRTK non-receptor tyrosine kinases
P-gp P-glycoprotein
PCa prostate cancer
PCA3 prostate cancer antigen 3
PIM1 proto-oncogene serine/threonine-protein kinase
PLCO Prostate, Lung, Colorectal and Ovarian Cancer Screening
PSA prostate-specific antigen
pT pathological tumor stage
PTEN phosphatase and tensin homolog
PZ peripheral zone
RAC1 Ras-related C3 botulinum toxin substrate 1
RP radical prostatectomy
RT radiation therapy
S727 serine 727
SH2 Src homology 2
SOCS3 suppressor of cytokine signaling 3
STAT3 signal transducer and activator of transcription 3
T testosterone
TAB total androgen blockade
TGF1β transforming growth factor-1 beta
Th17 T helper 17 cell
TMA tissue microarray
TMPRSS2 transmembrane protease serine 2
TRUS trans-rectal ultrasound
TZ transition zone
VEGF vascular endothelial growth factor
VEGFR vascular endothelial growth factor receptor
Y705 705 tyrosine
I. The Prostate

“The good thing about science is that it's true whether or not you believe in it.”

Neil DeGrasse Tyson
Structure

In males, located below the bladder, there exists a walnut sized exocrine gland: the prostate [1]. The prostate develops in men and reaches maturity at puberty. The prostate’s main function is to secrete about 30% of the fluid that makes up semen. This fluid is alkaline and rich in enzymes and simple sugars that allow the sperm to be maintained and flourish. The prostate is situated around the urethra and during ejaculation it squeezes the seminal fluid into the urethra. The fully formed prostate is made up of 3 different zones; the peripheral zone (PZ), the central zone (CZ) and the transition zone (TZ) [2] (Figure 1). The largest zone, the PZ, occupies around 70% of the total prostate and is the most common location for tumors. The CZ makes up about 25% of the prostate and is the location of around 20% of tumors. [3, 4]. Finally, the smallest area of the prostate, the TZ makes up the remaining 5% of the prostate and it is the main source of benign prostate hyperplasia (BPH), an enlargement of the prostate. The TZ position around the urethra makes it troublesome since its enlargement may lead to a blockage of the urethra, causing problems urinating [5].

Figure 1. This is a depiction of the prostate showing three zones; peripheral zone (PZ), central zone (CZ) and transition zone (TZ) as well as the urethra and the connections to the penis, bladder and seminal vesicles.
Cellular Structure

The prostate is composed of glandular structures of epithelial cells at the microscopic level. These glands contain a lumen that allows for secretion through ducts into the urethra. A single layer of basal cells surrounds the epithelial cells. These basal cells are potentially stem cell-like cells that can differentiate into epithelial cells [6]. A striking difference between the two cells at the microscopic level is their different structure and shape and at the molecular level their reliance on the androgen receptor (AR) [7]. In a normal gland, the epithelial cells are tall and columnar in shape and basal cells are flat or cuboidal [8]. During cancer development a loss of the basal cell layer can be observed as well as a change in shape and size of epithelial cells and their nuclei. An example staining with hematoxylin and eosin (H&E) of both benign and cancerous prostate tissue is shown in Figure 2. H&E staining is a common method used in diagnosing cancer by the appearance of the glandular structure.

![Figure 2. Progression from normal glandular structure (left) to a very aggressive tumor (right) with sample H&E stainings below.](image-url)
Prostate Cancer and Diagnosis

Cancer of the prostate is the most commonly diagnosed cancer in men in the United States [9]. Most diagnoses of prostate cancer (PCa) are not life threatening, with most men dying of unrelated causes/diseases [10]. Risk for PCa increases with age and the majority of PCa diagnoses are in men over the age of 65, with an average age of diagnosis of 66 [9]. Other risk factors include; genetics, ethnicity and diet [11]. PCa is diagnosed via a digital rectal examination (DRE), where the physician examines the prostate with their finger, a rise in prostate specific antigen (PSA) levels, detected via a blood test, and a trans-rectal ultrasound (TRUS)-guided biopsy [12]. PCa is driven by the expression of androgen receptor (AR), which is also responsible for the expression of PSA. PSA testing was introduced in the 90s and it relies on a blood test that determines the level of total PSA in the blood, with increased levels of PSA suggesting a possibility of PCa [13, 14]. Its introduction has vastly changed the landscape of PCa diagnosis and led to an increase in diagnosis of PCa. Multiplex tests measuring different forms of PSA are commercially available today, including prostate health index (PHI), four kallikrein panel (4K score) and STHLM3 and they all have been shown to increase the predictive accuracy in PCa diagnosis [15-17].

Androgen Receptor

The AR is a 110-kDa receptor protein found in the cytoplasm. It is held in an inactive state by a heat-shock protein complex (HSP) [18, 19]. The AR is made up of 3 important functional domains comprising a transcription regulation domain, found at the N-terminal region, a DNA binding domain, and a ligand binding domain [20]. Activation occurs when either testosterone (T) or dihydrotestosterone (DHT) bind to the AR, resulting in a conformational change that sheds the HSP chaperone protein complex [21] (Figure 3). Testosterone is produced in the testes and adrenal glands with cytochrome P450 and 5 α-reductase converting testosterone to DHT. Active AR then homo-dimerizes and translocates to the nucleus where it binds to androgen-responsive elements (ARE) and stimulates the transcription of a number of downstream targets including PSA [22]. AR is an important driver of normal prostatic growth and development but it is also a driver of carcinogenesis in the prostate. AR expression has been shown to be present at all stages of the disease and importantly overexpressed in castration-resistant PCa (CRPC) [23, 24].
Figure 3: Simplified androgen receptor (AR) signaling. Testosterone (T) is converted to dihydrotestosterone (DHT), which binds AR and releases it from its heat shock protein (HSP) chaperone complex. AR then dimerizes and goes to the nucleus where it binds to androgen-responsive elements (ARE) to induce transcription of specific genes.

Treatment Options

Treatment for PCa is dependent on the stage and progression of the disease. At diagnosis, patients can be stratified into the following groups based on outcome: low, intermediate or high risk. The first risk classification system was described by D’Amico and based on PSA level, clinical tumor stage (cT) (by DRE) and Gleason score (GS) [25]. Today, different classification systems have been suggested but they are usually based on the same three parameters as D’Amico’s
system, with additional parameters taken into account [26, 27]. The majority of patients have localized PCa with low GS (explored further in Biomarkers chapter), low cT, and PSA level <10 ng/ml)[12]. These patients are considered low risk and treatment can be either active surveillance (AS) where a patient is monitored for PSA levels over time and with repeat biopsy, or they can have local treatment with curative intent. Local treatment for PCa is usually a radical prostatectomy (RP), with a complete removal of the prostate or radiation therapy (RT). RP is performed either via robotic, laparoscopic or open surgery but all of them have a similar rate of oncological outcome [28]. RPs are good at mitigating further risk for patients with local low-grade disease but the side effects incurred from this treatment are non-negligible, including impotence and incontinence. Today low risk patients are often recommended AS until disease progression is observed [29, 30]. Radiation therapy can be either full-dose external beam radiation (EBR), interstitial radiation by brachytherapy or a combination [12].

Once a primary tumor is treated, patients are monitored for any raise in PSA. In around 20-40% patients who have undergone RP and in about 30-50% of patients who have had adjuvant or salvage RT, biochemical recurrence (BCR) occurs within 10 years [31]. BCR for RP is defined as a “patient who has a measured PSA level of 0.2 ng/ml after RP, which is confirmed again after a few weeks”. With patients who have undergone primary RT the definition is a bit more complex; according to the American society of therapeutic and radiology oncology (ASTRO), the BCR is defined as “PSA nadir and the first of 3 consecutive rises in PSA” [32]. After a patient has been diagnosed with BCR, the most common form of treatment is androgen deprivation therapy (ADT). Currently the standard of care is to use a Luteinizing Hormone-releasing Hormone (LHRH) agonist, which includes but is not limited to leuprorelin or goserelin. LHRH acts by indirectly inhibiting the secretion of hormones such as DHT and T. LHRH antagonists, like degarelix, which has a more rapid effect, are also available but not used to the same extent as agonists [33]. Orally administered anti-androgens (AA) like bicalutamide and flutamide may be given as monotherapy in the case of limited metastatic spread or in combination with LHRH agonists for total androgen blockade (TAB).

The treatment options for men who have failed ADT and moved onto CRPC are mainly novel AR signaling inhibitors, like abiraterone and enzalutamide, chemotherapeutic drugs such as docetaxel and cabazitaxel or radium-233, a radiotherapeutic drug [34-38]. A need exists to identify new targets for treatment of aggressive PCa as well as better diagnostic tools to at an early stage identify those patients who may develop more aggressive forms of cancer. A large number of new drugs and combinations, including immunotherapy, are currently tested in clinical studies (www.clinicaltrials.gov).
II. Biomarkers

“You want to know how I did it? This is how I did it, Anton: I never saved anything for the swim back.”

Vincent, Gattaca
Introduction

Biomarkers are molecules, genes or other characteristics that are specific to a given disease and can help in its identification. Prognostic, diagnostic and predictive are the most common types of biomarkers. Prognostic biomarkers are intended to evaluate a patients overall outcome, for example in PCa the likelihood of BCR or developing metastases after localized treatment. Meanwhile predictive markers determine a possible benefit that a treatment or combination will have [39]. Diagnostic markers help a physician determine the presence of a disease; for example increased PSA values. Some biomarkers are also considered as potential drug targets. Biomarkers in cancer have been hotly pursued for over 50 years, the first genetic biomarker in cancer was discovered in the 1960’s in Chronic myelogenous leukemia, called the Philadelphia chromosome [40]. Since then a number of different biomarkers have been discovered. Biomarkers can be present in a number of samples including blood, tissue, urine and saliva. The methodology for identifying biomarkers is broad and includes analysis of aberrations in germ-line and somatic DNA, epigenetics, transcriptome, proteome, metabolome and lipidome. In this summary, I will primarily focus on immunohistochemistry (IHC).

Immunohistochemistry

IHC is a method that is used on fixed tissue to stain for proteins of interest using antibodies specific to the target protein. IHC is one of the main current methods in trying to identify biomarkers. Biomarkers are easily quantifiable but the problem with IHC is that relies on qualitative scoring that can be subject to interpretation. Currently there are very few IHC tests that are approved for diagnostic and prognostic tests in cancer. The HER2 test in breast cancer, and detection of ALK protein in non-small-cell lung cancer are two of IHC tests currently used for diagnostic detection [41, 42]. The largest problem with IHC that exist today is the lack of standardization in tissue collection, preparation and staining. Samples are taken from patients and can be fixed in a number of different solutions, something that has been shown to affect the phosphorylation of proteins [43]. Another factor to consider is if tissue is affected during the process of removal from the patients. In prostate, contradictory results were found: one study claimed that tissue ischemia lead to changes in expression of 41 genes within 1 hour after the removal of the prostate [44], whilst another found that the ischemia did not affect the tissue for up to 2 hours [45]. Many attempts have been made to standardize the IHC procedure but to date no international standardization requirements exists. Most institutions have their own way of handling samples and staining. A study from 75
institutions in Canada in 2011 tested the most common IHC techniques in use at the time; pan-cytokeratin and low molecular weight cytokeratin. Their findings suggested that more than half of the participating institutions used incorrect methods [46]. Another problem in IHC is the need for positive and negative controls. There are multiple methods suggested for determining antibody specificity but most methods cannot give clear evidence that an antibody is specific for a given molecule [47]. A very useful method for determining antibody specificity is Western blot but even then it must be run in very similar conditions to the tissue that will be stained otherwise the information gleaned from it may not be valid due to antibodies reacting in different ways in different tissue/cell types [48]. Even with these problems IHC presents a useful tool for exploratory work into target proteins as biomarkers.

Prostate Cancer Biomarkers

One of the largest problems in PCa is the inability to distinguish between aggressive and indolent disease. Still, we are lacking a reliable biomarker of this kind. Below is a discussion of some of the current biomarkers used as well as those that are being researched for potential benefit. Recently, genetic tests came on the market (Oncotype DX, Prolaris, and others) but they all need further validation before they can be generally recommended [49].

Gleason Score

Currently the best prognostic marker in PCa continues to be GS[50]. GS was first introduced in the 1960s by pathologist Donald F Gleason [51]. It is based on the pattern that cells express when stained with H&E staining. The system has been amended a few times, most notably in 2005, but the basic method involved five different growth patterns (grades) (Figure 4). The tumor sample is graded on an addition of what the primary Gleason grade (most common) and secondary Gleason grade (second most common), this produces a final GS between 2-10. Originally if the secondary grade was less than 3% of the sample it was ignored and the primary grade was used for both [50]. At an international consensus meeting in 2005, the Gleason scoring system was upgraded with a removal of the score 1 and some modifications on how diagnosis worked for 3 and 4 [52]. More recently in 2014 a smaller modification has been suggested and approved involving the different patterns that may be apparent when looking at PCa, such as cribriform and glomeruloid glands and intraductal carcinoma [53]. With the current GS system the lowest grade given is a 6 (out of a 2-10 system) resulting in
an implication of intermediate cancer, therefore a new scoring system to be used in parallel with GS was devised giving a score from 1-5 instead [53].

**Figure 4.** The classical representation of the Gleason Grades 1-5 as drawn by Donald Gleason. Cells progress from an ordered and uniform structure to sheets of undifferentiated cancer. Image has been adapted from a public domain image available from the NIH [54].

**PSA**

PSA is used for diagnosis of PCa but since it can also be found in patients that have enlarged prostates due to benign disease, its use has been questioned. In 2012 the United States Preventive Services Task Force recommended against using PSA screening based on two large studies of PSA effectiveness [55-57].

Controversy has skirted the use of PSA, with opponents pointing to an over-diagnosis of non life threatening diseases that leads to overtreatment while proponents point to a decrease in mortality from PCa. There have been multiple studies conducted to check the effectiveness of screening PSA. The European Randomized Screening Study for Prostate Cancer (ERSPC) found that mortality was decreased with screening with reduction of 1.28 deaths per 1000 men [58]. The Prostate, Lung, Colorectal and Ovarian cancer screening trial (PLCO) found no significant difference in mortality between the screened and unscreened group.
after 7-10 years of follow up, but it has been heavily criticized for contamination of PSA testing in the control group [55].

Due to the continuing controversy with PSA a need to find more robust markers for diagnosis is currently underway.

**PCA3**

Prostate cancer antigen 3 (PCA3) is a non-coding RNA that can be detected in the urine. PCA3 is highly expressed by PCa cells and has been correlated with higher chance of a positive biopsy if tested for directly after a DRE [59, 60]. PCA3 has also been suggested as a useful prognostic tool to determine outcome of treatment [61]. Although the Food and Drug Administration (FDA) have approved it, it is not widely used today.

**TMPRSS2-ERG**

Trans membrane protease serine 2-ETS-related gene (TMPRSS2-ERG) first described in 2005 by Tomlins et al. is a fusion gene between TMPRSS2 and ERG, which occurs via genetic rearrangement. Since the TMPRSS2 gene has androgen response elements, its fusion with ERG leads to an increased expression of the oncoprotein ERG directly driven by the AR [62]. Transfecting cells with the fusion gene has shown an increase in invasion-associated gene expression suggesting that TMPRSS2-ERG may play a role in metastases [63]. Studies in patient cohorts have not suggested a predictive role for TMPRSS2-ERG on its own but there have been suggestions that its combination with other markers may have clinical value [64-66]. There has been a controversy about the role of TMPRSS2-ERG as a prognostic biomarker in prostate cancer, but most studies have not been able to show a significant prognostic value [67].

**AR-V7**

AR mutants have been theorized to help PCa become castrate resistant [68]. More recently AR mutations have been explored as potential biomarkers for prognostic value. AR splice variant 7 (AR-V7) has recently been discussed due to its active transcriptional function without a functional ligand binding domain suggesting its constitutive activation. AR-V7 has recently been found to be associated with resistance to enzalutamide and abiraterone treatment, but confirmatory studies are needed [69-71].
PTEN

Phosphatase and tensin homolog (PTEN) is a well characterized tumor suppressing gene that acts by dephosphorylating PI3K, therefore deactivating it [72]. PTEN mutation or loss is a very common occurrence and an early event in a number of different cancers [73, 74]. In PCa PTEN loss has been correlated with worse GS and tumor stage but has not been found to be as predictive as GS [75, 76].

Other Biomarkers

Examples of other markers that have had more diverging results are interleukin 6 (IL-6) and transforming growth factor-1 beta (TGF1β). Studying IL-6 in patient tissue using IHC has shown that PCa has significantly higher expression of IL-6 and its receptor, IL-6R [77]. In a study that evaluated circulating IL-6 and sIL-6R in the plasma of patients, elevated levels were found in patients with bone metastases [78]. Similarly to IL-6, TGF1β has been shown to have increased expression on an IHC level in cancer tissue compared to normal tissue [79]. Also TGF1β has been reported to be increased in plasma [80, 81] but another study suggested that plasma levels of TGF1β were similar in patients who had cancer vs. those who did not [82]. Interestingly the same study found that urinary levels of TGF1β were more predictive of cancer [82]. Downstream target of IL-6, the signal transducer and activator of transcription 3 (STAT3) has been suggested as a possible biomarker in PCa and will be the focus of papers I and II.
"It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts."

[Sherlock Holmes] Sir Arthur Conan Doyle *A Scandal in Bohemia*
Introduction

Signal transducer and activator of transcription 3 (STAT3) is a transcription factor that belongs to the STAT protein family. STAT3 consists of an N-terminal domain, a DNA binding domain, an SH2 domain involved in protein-protein interactions and a C-terminal domain that contains an area for transactivation [83, 84]. STAT3 can be activated by phosphorylation on two residues: the 705 tyrosine (Y705) and the 727 serine (S727) [83, 85]. The Y705 phosphorylation is essential for activation; the S727 is not necessary for activation but has been shown to enhance activation [86].

Figure 5. Overview of STAT3 signaling. Activation can occur via a number of different receptors: cytokine receptors (left), receptor tyrosine kinases (middle) and non-receptor tyrosine kinases (nRTK) (right). Activation of STAT3 occurs via phosphorylation and is followed by a homodimerization step before translocating to the nucleus and acting on transcription of a number of genes.
STAT3 activation and signaling pathway

STAT3 is a tightly regulated transcription factor with many upstream activators including different cytokine and growth factors. Most commonly STAT3 is activated by the Janus kinases (JAKs) by interaction with different cytokine receptors (IL-6R and IL-10R). It can also be activated by a number of receptor tyrosine kinases (e.g. EGFR, VEGFR, IGF-1R) and non receptor tyrosine kinases (e.g. Src, BCR-ABL) [87] (Figure 5).

IL-6/STAT3 pathway

The most common activation of STAT3 is via IL-6 [88, 89]. IL-6 is a small cell signaling protein, known as a cytokine, and has both pro- and anti-inflammatory effects [90]. IL-6 binds to its receptor (IL-6R). The IL-6R is made up of two different molecules; the IL-6Ra (also known as gp80) and gp130 (also known as IL-6Rβ) [91, 92]. The IL-6Ra binds directly to the cytokine IL-6 but it has a relatively short cytoplasmic domain (82 kDa) [93], with very limited activation activity [89]. The gp130 receptor on the other hand has a larger cytoplasmic domain with multiple motifs for intracellular signaling, which allows binding to JAK [94]. Upon IL-6 binding to the IL-6Ra, the complex binds to gp130. This three-molecule complex then homodimerizes to create a hexamer with the resulting complex constituting its active state [95]. The hexamer does not directly activate STAT3 but instead activates JAKs via a JAK binding domain present on the gp130 subunits [96]. The JAKs and IL-6R interact and trans activate each other [88]. The activated JAK can then in turn phosphorylate and activate STAT3 [97]. Following this phosphorylation, STAT3 dimerizes and goes into the nucleus to activate downstream targets. It is important to note that there is also a soluble IL-6R (sIL-6R), which lacks the cytoplasmic component and is created via cleavage or alternative splicing [98]. This sIL-6R can also bind IL-6 and form the same hexameric structure with IL-6 and gp130 as the membrane-bound IL-6Ra [88] (Figure 6). The gp130 protein is not specific for IL-6 signaling and is also found to complex with a number of other receptors including but not limited to; IL-27, LIF and IL-11 [99]. STAT3 activation is tightly regulated by a number of its own downstream targets leading to a negative feedback loop. Part of the common IL-6 pathway is its activation of transcription of the suppressor of cytokine signaling 3 (SOCS3), which inactivates the IL-6R and allows for tight control of the IL-6 signaling axis [100].
Figure 6. IL-6/JAK/STAT3 pathway. An illustration of the IL-6 pathway showing the binding of IL-6 to IL-6Rα (1) and the formation of the hexameric structure with gp130 (2) that goes on to cross phosphorylate with the JAK (3). JAK then phosphorylates STAT3 (4) which homodimerizes (5) and goes to the nucleus where it binds to DNA and is involved in transcription of target genes (6). One of these targets the SOCS3 protein goes on to bind gp130 (7) and block its phosphorylation of JAK (8), which leads to a shut down of the STAT3 pathway. Illustrated to the left is the sIL-6Rα which undergoes the same signaling process but without a cytoplasmic domain on the IL-6Rα.
STAT3 signaling

STAT3 has been linked to a number of processes in normal cell function including embryogenesis, innate and adaptive immune function, regulation of cell differentiation, growth, and apoptosis. Through knockout of STAT3 in mouse embryos, it was determined that STAT3 is required for the embryo to come to term [101]. It has been shown that STAT3 is required in stem cell renewal and determination of stem cell phenotype [102, 103]. STAT3 is involved in immune response with both an inhibitory and promoting role. It has been shown that STAT3 expression can lead to T-cell tolerance, while loss of STAT3 expression was shown to lead to T-cell priming [104]. STAT3 is also needed to prevent the apoptosis of IL-6 stimulated T cells, via activation of BCL-2 [105].

STAT3 in cancer

Normal STAT3 signaling can become aberrantly activated, leading to an oncogenic state. The constitutive activation of STAT3 has been postulated to occur through a number of different abnormalities in upstream receptors of STAT3. The first evidence of constitutively activated STAT3 was published in 1995 and connected with the proto-oncogene tyrosine protein kinase Src [106]. This groundbreaking paper showed that infection of different cell lines with v-Src, a tyrosine kinase known to induce cancer in chickens, was able to activate STAT3. This association was later confirmed in a number of studies showing that v-Src transformation of cells was mediated by STAT3 and in the absence STAT3 the transformation would not occur [107, 108]. Studies of human samples in different cancer types found high expression of activated STAT3 [109-112]. Different methods of constitutive activation have been suggested in different cancer types. In lung carcinoma a mutant epidermal growth factor receptor (EGFR) was shown to be responsible for constitutive STAT3 activation [113]. Meanwhile in breast cancer the overexpression of different cytokines related to inflammation, including IL-6 have been linked to constitutive STAT3 activation [114]. Constitutive activation of STAT3 can also occur through STAT3 mutation, a common occurrence in cancers associated with the blood [115, 116] Constitutive STAT3 activation has been linked to oncogenesis due to its ability to activate a number of downstream targets that are involved in cell proliferation and apoptosis, metastatic potential, angiogenesis and immune evasion [117].
STAT3 in PCa

In PCa, STAT3 has been found to be more highly expressed in benign and tumor areas of PCa patients compared to healthy patients and STAT3 activation has also been observed in a number of cell lines [118, 119]. It has been found that activated STAT3 correlated with worse prognosis and higher GS in some studies [120-122]. Metastatic potential of DU145 and PC3 cells transfected with STAT3 was increased, with high expression of STAT3 observed in metastatic samples from patients [123]. STAT3 has been linked with direct interaction to the AR, one of the main drivers of prostate cell growth [124]. Meanwhile downregulation of AR was shown to induce a stem cell like phenotype that was driven by IL-6/STAT3[125]. There is evidence that anti-androgen treatment may lead to a more stem cell-like cell that is more likely to metastasize [126]. Profiling of PCa stem cells has revealed over 500 changes in gene expression with many of them linked to the JAK/STAT3 pathway [127].

STAT3 and cell proliferation and apoptosis

Cell proliferation and apoptotic escape are a hallmark of tumor cells, which are able to continuously proliferate without ever reaching senescence and escape programmed cell death. STAT3 has been shown to influence cell proliferation in a number of different cancer types [128, 129]. Cyclin D1 functions by regulating cyclin dependent kinases (CDK), CDK4 and CDK6 and allowing cells to progress through the G1 phase of the cell cycle. In cancer it has been linked to stimulating anchorage independent growth and cell proliferation [130]. Specifically in PCa cyclin D1 has been linked to proliferation via IL-6/STAT3 [131]. Another method of cell proliferation increase in PCa happens via upregulation of Wnt3a via the interference of TGF-β signaling promotes cell proliferation in a STAT3 dependent manner [132]. STAT3 increased cell proliferation has also been linked to presence of HSP90, which is specifically linked to the activation of both AR and STAT3 [133]. IL-6, in a STAT3 dependent manner, is involved in the expression of AR, the main driver of cell proliferation in the prostate [134]. Interestingly STAT3 was also shown to activate MMP-3 with suggested involvement in both metastases and cell proliferation [135].

Meanwhile apoptosis, a method of programmed cell death first discovered in the Caenorhabditis elegans model system [136] has been linked to STAT3 tumorigenic function. One of the most studied families of apoptosis is the BCL-2 family of proteins [137]. STAT3 has been linked to the upregulation of BCL-XL, MCL-1 and BCL-2, all anti-apoptotic members of the BCL-2 family [138-140].
Not only does STAT3 activate these anti-apoptotic genes but it is also known to downregulate p53, one of the main drivers of pro-apoptotic expression, indirectly leading to the downregulation of pro-apoptotic genes such as BAX, Fas and caspase 6 [141, 142].

**STAT3 and metastases**

Metastasis is an important process in a tumor's progression to an aggressive form. Matrix metalloproteinase (MMPs) have been linked to cellular invasion in a number of different cancer types [143, 144]. STAT3 has been shown to regulate the expression of numerous MMPs in different cancer types: MMP7 in pancreatic cancer, MMP2 in melanoma, MMP9 in breast, and MMP1 in bladder cancer [145-148]. STAT3 has also been linked to cellular migration through regulation of a number of proteins including Ras-related C3 botulinum toxin substrate 1 (RAC1) [149] and stathmin [150]. In squamous cell carcinoma, overexpression of STAT3 was shown to lead to higher invasion [151]. Specifically, in PCA, it has been shown that STAT3 enhances migration via integrin β6 and MMP3 [152].

**STAT3 and angiogenesis**

Angiogenesis is the process of attracting and forming new blood vessels. In tumors angiogenesis is important for the uptake of nutrients, for continued growth, and for metastatic potential. One of the most important proteins involved in angiogenesis is the vascular endothelial growth factor (VEGF). It has been shown that VEGF is downstream of STAT3 and hyperactivation of STAT3 can lead to increased expression of VEGF [153]. Not only is STAT3 involved directly in VEGF expression, there is evidence that AR can also be involved in VEGF expression through hypoxia inducible factor 1α (HIF1α) [154]. Interestingly STAT3 has been shown to induce HIF1α expression [155]. This complex overlap, though well controlled in normal cells, is strongly affected when STAT3 becomes constitutively active and leads to angiogenic activation in tumors.

**STAT3 and immune suppression**

Immune evasion is key to a tumor's success locally, in the blood stream and at metastatic sites. STAT3 is linked to immune evasion in a number of ways: with
involvement in the tumor cells, the microenvironment and the immune cells themselves. Evidence exists that STAT3 expression in the tumor cells can induce IL-6 and IL-10 that activate STAT3 in myeloid cells [156]. Upregulation of IL-10 has also been linked to a decrease in proliferation to a number of different immune cells [157, 158]. STAT3 has also been linked to the differentiation of T cells into Th17 cells via the expression of IL-6 and TGFβ [159, 160]. STAT3’s role in immune response is complex but many studies have pointed to STAT3 activation as key to the tumor cells ability to escape the immune system via it functions in different immune cells [161].

Targeting STAT3

The tumorigenic potential of the STAT3 pathway has made it an attractive target for potential therapeutic purposes. Multiple targeting methods have been explored that include targeting upstream, at the receptor or kinase level, as well as directly to STAT3.

Multiple cell surface receptors that are upstream of STAT3 have been studied as potential targets, among them EGFR, VEGFR and IL-6R. IL-6R and its binding cytokine have both been targeted with different drugs. In 2012 the results of a phase 2 study with siltuximab, an IL-6 chimeric monoclonal antibody, suggested treatment did not yield outcome benefit in CRPC [162]. Tocilizumab, an antibody targeting the IL-6R instead has also shown promise in vitro and in vivo but has not been clinically approved for any cancer treatments [163, 164]. Success has been seen in vivo and in vitro in oral squamous cell carcinoma and breast carcinoma in targeting the EGFR receptor, which led to a decrease in STAT3 phosphorylation [165, 166]. The most successful receptor target so far has been VEGFR with a number of drugs currently approved to be used in the clinic, including sorafenib, sunitinib and axitinib, all of which have been shown to affect STAT3 [167-172].

By targeting cell surface receptors upstream of STAT3 there is a clear reduction in STAT3 activity but there is a need for more directed therapies in order to reduce side effects. Therefore, there is more focus on the more direct approaches to target either the JAKs or STAT3 directly. Currently a large number of JAK inhibitors exist but only two, ruxolitinib and tofacitinib, have been approved for treatment in rheumatoid arthritis, but not in cancer. JAK inhibitors still pose a large amount of side effects with tofacitinib only being approved by the Food and Drug Administration (FDA) but not by the European Medicines Agency (EMA) due to its side effects.
Directly targeting the STAT3 protein has a high potential for therapeutic effect with fewer side effects. A large number of compounds are currently being tested with many targeting different parts of STAT3: disrupting translation, disrupting phosphorylation, disrupting protein-protein interaction, nuclear translocation or the DNA-binding of STAT3 [173, 174]. As previously mentioned, the SH2 domain of STAT3 is involved in protein-protein interaction, specifically its association with the JAKs for activation as well as its homodimerization and nuclear translocation. Targeting the SH2 domain of STAT3 was one of the first direct STAT3 targeting techniques and has proven to shutdown constitutive activation of STAT3 [175, 176]. One of the most successful SH2 targeting compounds is Stattic, which specifically targets the dimerization of STAT3 [177]. Due to STAT3s transcriptional activity another big target on STAT3 is its DNA binding domain. The DNA binding domain of STAT3 consists of three loops that interact with the DNA double helix at specific STAT3 consensus sequences [83]. By binding to the DNA binding domain on the STAT3 protein you can, in theory, disrupt the ability of STAT3 binding to DNA and therefore inhibit its transcriptional abilities. This direct effect on the DNA binding domain would likely not interfere with the phosphorylation and dimerization of STAT3. A number of options exist to target the DNA binding domain of STAT3. A common method is to introduce decoy oligonucleotides which mimic the response element of the target transcription factor [178]. Major problems exist in stabilizing the decoy oligonucleotide but a recent clinical trial was successful in modulating STAT3 expression with a decoy oligonucleotide in patients with head and neck cancer [179]. Another method for targeting the DNA binding domain is to use a small molecule inhibitor such as inS354A18 [180].

Galiellalactone

Small molecules present an attractive treatment option due to their many advantages such as; easy to get into the cell, stable, can bind directly to the target and can target specific interactions of the target [181].

We used a small molecule STAT3 inhibitor named galiellalactone in papers III and IV of the current investigation. Galiellalactone is a small molecule that can be found in the Galiella Rufa mushroom [182]. Galiellalactone has been successfully synthesized by Johansson and Sterner [182, 183]. First suggested as an inhibitor of the IL-6 pathway in 2000 by Weidler et al. it has since become an interest for study for its potential clinical benefits [184]. Our group has shown that galiellalactone is an effective treatment in castrate resistant cell lines that express constitutively active STAT3 [185]. Galiellalactone has also been shown to have a
potent effect on ALDH1+ cells, which are considered stem cell-like cells, and are present in small subpopulations in PCa cell lines [186]. Our group has also shown that galiellalactone inhibited metastases in a mouse model [187]. The current study focuses on the mechanism of action of galiellalactone in paper III and the use of galiellalactone in combination with docetaxel in paper IV.
IV. Drug resistance

“Quoth the Raven ‘Nevermore.’”

Excerpt from *The Raven* by Edgar Allan Poe
Introduction

The largest problem facing current cancer treatments is the tumor's ability to overcome the effects of treatment and become resistant [188]. Resistance to drugs can fall into two categories: intrinsic and acquired [189]. Intrinsic resistance occurs when the resistance mechanism is already present in some or all cells in the tumor. Due to tumor heterogeneity it is unlikely that all cells are resistant meaning that treatment will still lead to a reduction in tumor size, but eventually the resistant cells are able to propagate and recurrence is observed. Acquired resistance occurs when cells initially respond to the treatment but due to mutation, differential expression of the drug target, or activation of an alternative pathway, become resistant.

ADT

As discussed in Chapter 1, when local therapy fails, a rise in PSA levels represents BCR. The most common treatment after the observation of BCR is ADT. ADT benefits last for up to 2-3 years before patients become resistant, progressing to CRPC [190, 191]. ADT focuses on blocking the AR signaling pathway. AR is the main target of ADT and resistance to ADT stems from modifications to the AR pathway. After ADT treatment, AR levels are commonly very low in patients but tumor cells are able to develop high sensitivity to the low levels of AR via amplification [192] or mutations that allow activation without native ligand, or via alternative ligands [193]. Some examples of these mutations that lead to higher AR activity are a mutation at the 877 alanine or the substitution of histidine 874 for tyrosine [194, 195]. AR can also be activated by a number of other signaling pathways such as IL-6, IGF-1 and EGF [134, 196].

Once ADT options have been exhausted, one of the main treatments relies on the chemotherapeutical agents, like taxanes. Docetaxel was approved in 2004 while cabazitaxel was developed later and approved in 2010 [197, 198]. Currently there is ongoing research to determine which taxane is more effective, with early evidence suggesting that in PCa, cabazitaxel is more effective than docetaxel but that this is not true in all cancer types [199, 200]. There have been a few clinical studies suggesting that patients that fail docetaxel are still responsive to cabazitaxel [201, 202]. Progression to resistance to both cabazitaxel and docetaxel is a major challenge today [203, 204]. Paper IV is about docetaxel resistance mechanisms and therefore the rest of this chapter will focus on docetaxel.
Docetaxel resistance

Docetaxel is a taxane that binds the β subunit of tubulin in microtubules; this causes a stabilization of microtubules. The stabilized microtubules are unable to depolymerize, which does not allow the cell to go through mitosis [205]. The resistance methods to docetaxel are not well elucidated but there are a number of possible mechanisms that are currently being studied. The most studied form of docetaxel resistance is through the multidrug transporters belonging to the adenosine triphosphate binding cassette (ABC) family. There are 49 members but the most studied across all cancers are P-glycoprotein (P-gp), MDR protein 1 (MRP1) and breast cancer resistance protein (BCRP). In PCa P-Gp has been shown to be associated with resistance to chemotherapy [206] and worse clinical outcome [207]. MRP1 has been shown to be expressed in DU145 and PC3 PCa cell lines [208], and correlated with advanced disease and GS [209-211].

Docetaxel treatment has also been shown to increase the expression of a number of cytokines such as IL-6 and IL-10 possibly leading to inflammation or apoptotic escape [212, 213]. Patients with high serum levels of the cytokine IL-6 had worse outcome with docetaxel treatment [214, 215]. These increases in IL-6 expression may lead to higher activation of STAT3 and indeed a number of the mechanisms discussed further in this chapter are downstream targets of STAT3. Docetaxel is also known to induce apoptosis by indirectly leading to phosphorylation of the anti-apoptotic B-cell lymphoma 2 (BCL-2) causing BCL-2 to become inactive [216, 217]. Apoptotic escape has been a growing topic of interest in docetaxel resistance with multiple proteins involved being implicated in resistance including: BCL-2 and Proto-oncogene serine/threonine-protein kinase (PIM1).

The BCL-2 family of proteins has been extensively studied in PCa and many of its members have been shown to be highly expressed in aggressive PCas [218]. Recent focus for docetaxel resistance has focused on BCL-2 due to its indirect targeting by docetaxel, making its mutation or overexpression a possible resistance mechanism [219]. In PCa cells it has been shown that directly targeting BCL-2 resulted in higher sensitivity to docetaxel [220]. Upregulation of both BCL-2 and BCL-XL has been suggested as a possible resistance mechanism via activation of the glucocorticoid receptor [221]. Other BCL-2 family members such as MCL-1 and survivin have also been implicated in docetaxel resistance [222, 223]. Furthermore, upstream modulators of the BCL-2 family have also been suggested as possible docetaxel resistance mechanisms. One of these is PIM1; its main mechanism of action is phosphorylation of the BAD protein, another member of the BCL-2 family [224, 225]. It has been shown that PIM1 can be activated by docetaxel and possibly leads to docetaxel resistance via anti-apoptosis by
activation of NFκB [226]. It has not only been linked to docetaxel resistance but to drug resistance in general [227].

Another suggested form of resistance is the chaperone protein clusterin. Clusterin exists in a number of different isoforms and interestingly the amount of specific isoforms of the proteins have different effects with higher nuclear clusterin associated with apoptosis and cell death while secreted clusterin has an opposing effect [228, 229]. In PCa cell lines, secreted clusterin is activated through AKT and can lead to a docetaxel resistant phenotype, with implications that the Src kinase is involved in the expression of secreted clusterin [230].

STAT3 and drug resistance

STAT3 has been implicated in multiple cancers as a resistance mechanism to a wide variety of drugs. In lung cancer, in cells that were addicted to EGFR, increased activation of STAT3 was observed in response to erlotinib treatment, an EGFR inhibitor, and conferred resistance [231]. Moreover in chronic myeloid leukemia (CML), treatment with an inhibitor of the tyrosine kinase, BCR-ABL, increased not only pSTAT3 expression but also its downstream targets MCL-1 and BCL-XL [232].

An increase of pSTAT3 and BCL-2 coupled with a reduction of p53 expression was observed in cisplatin-resistant gastric cancer cell lines compared to sensitive parental cell lines. When these resistant cells were treated with STAT3 inhibition their sensitivity to cisplatin increased [233]. In renal carcinoma, non-Hodgkin lymphoma and multiple myeloma, resistance to chemotherapy was linked to IL-6 and IL-10, two cytokines known to activate STAT3 [140, 234, 235].

In PCa STAT3 has been linked to resistance of both chemotherapy and anti-androgens. Activated STAT3 expression was shown to be associated with an enzalutamide-resistance in cell lines, with STAT3 inhibition leading to a re-sensitization to enzalutamide [236]. It has also been suggested that the PIM1 kinase involvement in docetaxel resistance is mediated in a STAT3 dependent manner and leads to the expression of NFκB [226].

Combination Therapies

As resistance mechanisms have become more studied, the idea of combination therapies has arisen as a possible solution. Combination therapies focus on treatment of two different targets that would make adaptation for the cancer cells
harder than a single target. Due to its high expression in a number of cancers and the fact that many proteins implicated in resistance are downstream targets, STAT3 seems like a viable option for combination treatment with ADT and chemotherapy. Specifically combination of STAT3 inhibition with docetaxel seems promising with a number of the resistance mechanisms previously discussed in this chapter being linked to STAT3.
V. The Current Investigation

The aim of this work was to investigate the expression of STAT3 in tumor tissue from PCa patients from different stages of the disease and to investigate the potential therapeutic benefit that targeting STAT3 holds. STAT3 targeting was specifically investigated with the use of galiellalactone.

Specific Aims

- Determine the expression of STAT3 and pSTAT3 in metastases from CRPC patients and if the metastases have differential expression of STAT3 within and across patients
- Determine the expression of pSTAT3 in hormone naïve patients and if it correlates to disease progression and AR expression
- Study the molecular mechanism of galiellalactone’s inhibitory effect on STAT3
- Investigate the feasibility of using galiellalactone in combination with the chemotherapeutic agent docetaxel
Paper I

Introduction

There is a need in PCa to find new treatment targets and new markers that can help understand PCa progression. Since STAT3 has been shown to be constitutively activated in a number of malignancies, and has been linked to metastases, we wanted to examine STAT3 and its main upstream receptor IL-6R in patients with metastatic CRPC [152, 237]. We collaborated with researchers from Washington University who provided us with metastatic samples in tissue microarray (TMA) format from multiple sites taken from patients who had died from CRPC (samples were taken at autopsy). We used IHC staining to determine the level of activated STAT3 (phosphorylated at the Y705) and IL-6R followed by an analysis of the mRNA expression of STAT3 and IL-6R. We were interested if the levels of expression varied between metastatic sites because a recent study has shown that visceral metastases have much worse prognosis than bone and lymph node metastases [238].

Results and Discussion

We found that all patients expressed both IL-6R and pSTAT3 in at least one of their tissue samples. The IL-6R expression was higher in bone compared with lymph node but not visceral metastases but this was not seen at the mRNA level. We then examined the expression of pSTAT3 where we found that bone metastases had higher expression than both lymph node and visceral metastases. In order to get a better understanding of the expression in a single patient we narrowed down our analysis to only patients with all three metastases available. When looking at only matched patients we still found significant differences between bone and visceral metastases for both pSTAT3 and IL-6R, showing that the metastases were highly heterogeneous. Our results suggest that pSTAT3 activation is a common event in PCa metastases. The high pSTAT3 expression may explain why downstream STAT3 targets have been shown to be more highly expressed in bone [239]. Low correlation between IL-6R and pSTAT3 suggests
that multiple pathways maybe involved in the activation of STAT3 in metastases. The high expression of pSTAT3 specifically in the bone may be due to presence of IL-6 in the microenvironment [240]. Our results suggest that pSTAT3 may be a viable target in CRPC metastatic patients. The role of STAT3 in metastases and progression needs to be further explored in patient samples with larger cohorts where samples from different stages of the disease are available.
Paper II

Introduction

To date, there is a lack of good biomarkers to indicate whether PCa is indolent or aggressive [241]. Since we observed in Paper I that metastases had high expression of pSTAT3 we were interested in examining the expression of pSTAT3 (phosphorylated at Y705) and AR in hormone-naïve patients to determine if they could add clinical value to diagnosis. Previously, smaller cohorts were shown to have some correlations between pSTAT3 and GS or between pSTAT3 and BCR [118, 122, 242]. More recently Pencik et al. have suggested the opposite, that loss of STAT3 expression is a more tell-tale sign for more aggressive disease, but these patients were also negative for PTEN [243].

Results and Discussion

We examined TMAs from 300 patients that had undergone RP but not been subjected to any pretreatment (ADT or RT). The intensity of the pSTAT3 IHC staining was scored using h-index, where both percent and intensity were taken into account. Most patients included in the study were still alive at time of analysis and therefore BCR was used as a surrogate marker for survival. Surprisingly, we found that pSTAT3 expression was higher in the benign regions compared to the tumor. We also found that lower pSTAT3 expression significantly correlated to higher risk of BCR but no correlation with GS was observed, unlike previous reports from other groups. This discrepancy most likely stems from a difference of methodology and cohort size. Previous publications did not use an h-index, instead opting to only score intensity or percentage stained. Our scoring methodology was stringent and only took into account epithelial cells ignoring any stromal staining and inflammatory staining, something not explored or mentioned in earlier studies. Some of the earlier studies included whole cores/tissue sections for analysis, without separating by structure nor taking into account whether the area was tumor or benign. Given the importance of stroma and inflammation in tumor progression, it would be crucial to investigate the expression of pSTAT3 in those areas in
future studies of our cohort. In order to better understand the finding of pSTAT3 levels significantly predicting BCR, we compared the concordance index of pSTAT3, GS and pathological tumor stage (pT) in predicting BCR. The expression of pSTAT3 was significantly predictive but was not as robust as GS and pT, it also did not add predictive power to either GS or pT.

Similar to previous studies, we found increased expression of AR in the cancerous tissue compared to benign, but AR did not provide any predictive benefit for BCR. Slight correlation was observed between AR and pSTAT3 in benign areas but not cancerous areas [244]. In conclusion we found that hormone-naïve patients had lower expression of pSTAT3 in their tumors compared with benign areas and lower pSTAT3 in the tumor tissue correlated with worse outcome in terms of BCR. This data suggests that STAT3 targeting therapy would not likely benefit hormone-naïve patients. Similar to Paper I more studies need to be done with larger cohorts.
Paper III

Introduction

STAT3 is known to be constitutively activated in prostate tumor samples from patients and PCa cell lines [119]. Previous studies have indicated that targeting the STAT3 pathway may be beneficial in PCa [174].

Galiellalactone is an inhibitor of STAT3 and has previously been shown to affect PCa cell growth both in vivo and in vitro in cells that have high expression of pSTAT3 [185, 186]. We were interested in exploring the exact mechanism of action of galiellalactone’s STAT3 inhibitory effect. In order to study galiellalactone’s interaction with STAT3, we used a biotinylated galiellalactone compound for pull down of STAT3 bound to galiellalactone and for confocal microscopy to visualize co-localization of STAT3 and galiellalactone. Mass spectrometry with recombinant STAT3 was used to determine the binding sites of galiellalactone on STAT3.

Results and Discussion

We used a biotinylated galiellalactone that had a similar effect on cell proliferation as galiellalactone. With the aid of mass spectrometry the binding areas of galiellalactone on STAT3 were determined to be three cysteines of which two (c367 and c468) were located in the DNA binding domain and one was located in the linker domain (c542). The cysteine residues that were modified by galiellalactone in STAT3 were not present in STAT1 or STAT5, indicating specificity for STAT3. Competition assays with galiellalactone and biotinylated galiellalactone and wash-out experiments indicated irreversible binding. Pull-down in LNCaP cells, which express an inactive STAT3, confirmed that galiellalactone binding was irrespective of STAT3 activation. No effect on phosphorylation was observed in DU145 cells treated with galiellalactone for 24 h. Confocal microscopy showed co-localization of biotinylated galiellalactone and STAT3 in the nucleus. We also confirmed that galiellalactone decreased the ability of STAT3 to bind its specific DNA response elements by using an electric motility
shift assay. In short, our paper showed that galiellalactone binds to cysteines in the STAT3 DNA binding region affecting its ability to bind to DNA. The covalent interaction with STAT3 and the inability of competition assays to displace the compound, coupled with effects seen even after removal of galiellalactone suggests that this targeting method can be useful in inhibiting STAT3 until it is degraded. Since STAT3 has been shown to be a vital protein in cancer metastases, cell proliferation and apoptotic escape targeting STAT3 with galiellalactone presents a potential treatment option with the added benefit of binding regardless of the activating method.
Paper IV

Introduction

Docetaxel is a common second-line treatment in CRPC but unfortunately many patients display inherent or acquired resistance [245]. Docetaxel is also highly toxic with a large amount of detrimental side effects reported. Due to high rates of resistance, combination therapies have been tested in a number of clinical trials, mostly with disappointing outcome. As discussed in the drug resistance chapter, cancer cells are very adept at overcoming single agent treatments; therefore targeting multiple targets at one time may stem resistance or increase the time to resistance with the added potential of lowering effective doses. Docetaxel treatment has been implicated in an upregulation of a number of downstream STAT3 targets, which have been linked to eventual docetaxel resistance. Therefore targeting STAT3 concurrently with docetaxel may in theory block certain resistance mechanisms. We used the PCa cell line LNCaP-IL6, a model of aggressive PCa that has constitutively activated STAT3, to carry out experiments of combination treatments between docetaxel and the STAT3 inhibitor galiellalactone.

Results and Discussion

Galiellalactone and docetaxel were administered to cells in varying concentrations as single agents and in combination to determine their IC$_{50}$ values and their combination index. Combination index was determined with the aid of CompuSyn that uses a previously published algorithm developed by Chou to determine synergy between treatments [246]. We found that galiellalactone and docetaxel showed synergy at a number of different concentrations. The gene expression of genes involved in docetaxel resistance (PIM1, BCL-2, IL-6 and clusterin) were investigated by qPCR analysis. Three of these genes PIM1, BCL-2 and IL-6 are known to be regulated by STAT3. We found that the gene expression of BCL-2, PIM1 and IL-6 were downregulated by galiellalactone alone and in combination with docetaxel compared to control while all three gene expressions were
upregulated under docetaxel treatment. Clusterin was unaffected by galiellalactone treatment but was upregulated by docetaxel. A slight downregulation of clusterin was observed in docetaxel and galiellalactone treated cells compared with docetaxel alone. Western blot analysis indicated an increase in cleaved poly(ADP-ribose) polymerase(s) (c-PARP) in both galiellalactone and combination treated samples. This data suggests that galiellalactone and docetaxel can be used in combination to synergistically inhibit cell proliferation and increase apoptosis by c-PARP. The synergistic effect likely stems via STAT3 targeting leading to a downregulation of BCL-2, PIM1 and IL-6. Combination therapy between STAT3 inhibitors and docetaxel may lead to an increase in effect while potentially decreasing the docetaxel dose resulting in reduced side effects.
Concluding Remarks

PCa is a common disease in the western world affecting a large number of men. Current detection methods are decent and lead to better treatment for many men but there is a need for more robust methods to identify the patients with aggressive disease. A need for new biomarkers has spawned a number of possible candidates but their validation has been spurred with controversy. Treatment options have heavily centered on the AR pathway and chemotherapy, with the latter causing detrimental side effects for short survival benefit. New targets and biomarkers must be found to increase survival and decrease unnecessary treatment.

STAT3 is here presented as both a potential biomarker and target for treatment. We found that in early stages of PCa STAT3 did not add predictive value to outcome as measured by BCR but we did find that in metastases, STAT3 was almost ubiquitously expressed suggesting its importance in later stages of the disease. We explored the STAT3 inhibitor galiellalactone and found that it bound directly to STAT3, blocking its DNA binding ability. It was also found that galiellalactone treatment in combination with chemotherapeutic docetaxel reduced expression of genes involved in docetaxel resistance. These results coupled with earlier publications on STAT3 targeting suggest that direct STAT3 targeting is a viable treatment in more aggressive PCas.

It is imperative that we continue to study STAT3 and other potential biomarkers in larger cohorts with as much clinical information as possible to validate or exclude their potential uses for predictive, diagnostic or prognostic benefit.
Prostate cancer is the most diagnosed non-skin cancer in the world among men, it is also the second leading cause of death (due to cancer). Two problems face current patients diagnosed with prostate cancer: over diagnosis with the inability to distinguish between aggressive and non-aggressive cancer and the cancer's ability to gain resistance to current treatment options. This thesis is split into two parts consisting of four papers that deal with these two issues.

Patients being overdiagnosed with prostate cancer is directly linked to a shortage of valuable markers that can identify more aggressive tumors. Currently the only markers used for diagnosis are the assessment of Gleason Score of biopsy samples and measuring PSA levels in the blood. The screening of blood for PSA can detect whether a man has an increased androgen activity but it cannot distinguish between changes associated with cancer or benign hyperplasia. This results in the overtreatment of men that have non-life threatening disease. It is of great importance to find new markers that can identify which patients are in need of treatment and what treatment is beneficial. STAT3 is a protein involved in controlling the levels of a number of proteins, many of which have been linked to a cancer's ability to metastasize, escape programmed cell death and escape the immune system. We explore the presence of STAT3 in different groups of patients in papers I and II. In paper I the levels of STAT3 were studied in various metastatic samples from patients who had died from prostate cancer. STAT3 expression was observed to be present in at least one sample of each patient, with bone metastases having the highest levels. Levels of a cell receptor, IL-6R, which can activate STAT3 were also studied and similar results to STAT3 levels were observed. Meanwhile paper II focused on a group of patients that had undergone a surgical removal of the prostate but had not had any other treatment. In this early stage of the disease it was found that STAT3 levels were lower in the tumor tissue compared to the normal tissue and the lower STAT3 levels in the tumor suggested a more severe disease.

Removal of the prostate is an effective treatment option in prostate cancer but has a number of high risk side effects including impotence and incontinence. Even when patients have their prostates removed there is a high likelihood that the cancer will come back. When the cancer comes back, treatment options are limited to chemotherapy, radiotherapy and targeted therapy focused on the androgen...
receptor and its activating hormones. Many patients receiving these therapies gain resistance after a few years. There is a need to find new drug targets in prostate cancer. As mentioned earlier STAT3 has been shown to be involved in a number of cancer related processes and is therefore a good candidate for treatment.

Paper III focused on determining the mechanism of action of the STAT3 inhibitor galiellalactone. Galiellalactone was found to interact with STAT3, limiting its ability to bind to DNA. Since STAT3 controls the levels of a number of proteins linked to resistance to chemotherapy, paper IV explored the ability of galiellalactone to enhance the effects of docetaxel, a common chemotherapeutic agent. Galiellalactone treatment was shown to have synergistic effects when used in combination with docetaxel. The synergy may in part be due to galiellalactones ability to decrease production of genes that had increased levels when treated by docetaxel treatment alone.

The four papers in this thesis help to establish what patients are suitable for treatment with a drug that blocks STAT3 and how this drug interacts with the STAT3 protein.
Acknowledgements

“Always take a banana to a party, bananas are good!”

Tenth Doctor, Doctor Who

First and foremost I would like to thank the funders, this work was supported by the Swedish Cancer Foundation, Swedish Scientific Council, Gunnar Nilsson Cancer Foundation, the BioCare program at Lund University, the Skåne University Hospital Research Foundations, Government Funding of Clinical Research with-in the National Health Services and Lund University (ALF) to Anders Bjartell. The funding for paper I was also supported by the Pacific Northwest Prostate Cancer SPORE program (P50CA97186), a PO1 NIH grant (PO1CA085859), R01 CA165573 and the Richard M. Lucas Foundation.

A big thanks goes out to my supervisor Anders, you challenged me with interesting projects and gave me the opportunity to pursue a PhD. I look up to you and your ability to handle so many different things at once. You always seem to be doing something but always have time for questions and discussions, thank you.

To my co-supervisor, Rebecka, thank you for always having an open door to discuss any problems with experiments. You also have given be invaluable insight into drug development research and I appreciate all of the help you have given me, whether it be with analysis or experimental design.

Agi, thank you for everything!!! You have helped me so much with so many different aspects, analysis, writing, etc., basically figuring out which direction is up. I know that at this point, especially as I sit writing this section you just cannot wait until this book is printed. I apologize to you for all the times I have sent you work texts late or on weekends. You have been a valuable mentor and I could not have done it without you.

Giacomo, I teased you that you wouldn’t get an acknowledgment but you deserve huge thanks from me. You’ve taught me so many things, I still can’t believe I can
find a mouse prostate. I have probably talked your ear off but thank you for being there to teach and listen.

**Giuseppe**, you helped me in the impossible sea that is statistical analysis, thank you.

To the rest of the group, **Aseem**, thanks for your suggestions, **Felicia**, thanks for your help with IHC, **Annica**, for putting the TMA database together, **Susan**, for teaching me some animal work and for being the other American, **Anna Stiehm** thank you for all of the assistance and **Anna Holst** thank you for all the work you’ve done behind the scenes.

**Kiki** and **Elise**, without your IHC expertise we would not have had the great stainings that have given us so much information, thank you.

I would like to thank all of the patients who made this work possible.

I am grateful to investigators **Drs. Robert Vessella, Celestia Higano, Bruce Montgomery, Evan Yu, Paul Lange, Martine Roudier**, and **Lawrence True** for their contributions to the University of Washington Medical Center Prostate Cancer Donor Rapid Autopsy Program.

To all my coauthors, thank you; **Ilsa Coleman, Peter S. Nelson, Roy Ehrnström, Colm Morrissey, Zilma Escobar, Martin Johansson, Sven Kjellstrom, Victor Garcia, Eduardo Munoz** and **Olov Sterner**.

To my office mates past and present **Krzysztof** (I still don’t know how to spell your name without looking), **Roni, Lisa, Melly, Carro** and **Greta** thank you for the strange conversations, the useful conversations and most importantly with all the help you were able to give whether it be for a poster or a method.

To the rest of the CMP, **Karin, Martin, Maite, Tamae, Johan, Totte, Ben, Camilla, Helen** and **Elisabet** thank you for all the help.

**Mom** and **Dad**, you were always very supportive of me and helped me whenever I needed it. You gave me support and were always there in my time of need even across an ocean. You didn’t even blink an eye when I said I was going to Sweden even though I know that you both have missed me as I have missed you. You always wanted what was best for me. I love you.

**Tomas**, what can I say? You are that person who is always there, we have spent many hours (I checked and you don’t want to know) playing destiny (and other games of course). Its nice how we “hang-out” online its as if we are in the room together, its been tough not being home sometimes but I know that I can always reach you and the best part, our sleeping schedules are so insane that we end up having conversations at the oddest hours.
To my friends in Sweden, Leandro and Johan the game nights we had were a welcome distraction and all those times throwing the football around, Pavle always there to grab a beer and just talk, Mike always with some new idea did you ever think that when you met me in 2009, I would still be in Sweden in 2017?

To my friends in the US, Bev and AJ, I know at this point you are asking, “When is this guy coming home?” and no one knows the answer to that question, thanks for being there, the random texts we send each other never get old. To Stephen, its crazy to think that we spent high school and university together and now I’m halfway across the world, thanks for all the fun we’ve had.

To George Cachianes and Maureen Boland, the two teachers who set me on the path to molecular biology, thank you.

To Catjas family, Daina, Louise, Mats, Torbjörn, Anne, Calle and Max thank you for making me feel at home.

Catja, 6 years, did you ever think that you’d be together with that weird American for that long? What can I say, there is so much? You have helped me through some tough times but mainly we’ve had a lot of fun. I don’t know how I would have done it without you. I love you. (I know you hate batman, but do you realize that without the joker, we wouldn’t be here, now?)

Finally, one last acknowledgement remains, to Spencer, you wont be able to read this but maybe you can chew on one of the books. As a rabbit your life is simple; dig, sleep, bite Nick, rinse and repeat. Thank you for being cuddly (sometimes).

...Nothing comes to mind”
Colonel Jack O’Neill Stargate SG-1
References


VI. Papers

“All of life in its complexity and beauty is forever minted in the gold of words.”

[D-503] Yevgeny Zamyatin, We