Image analysis of prostate cancer tissue biomarkers

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2015

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LUND
UNIVERSITY

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 Lecture Hall, Pathology building, Jan Waldenströms gata 59, Malmö

Thursday 28th of May 13:00.

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Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
Department of Translational Medicine, Division of Urological Cancers	Date of issue
Author(s) Giuseppe Lippolis	Sponsoring organization

Title and subtitle Image analysis of prostate cancer tissue biomarkers

Abstract

Prostate cancer is the second most common cancer in men. In order to improve diagnosis and prognosis, new sensitive and specific biomarkers are needed. Tissue biomarkers carry expression and morphological information of the tissue where they are expressed. However their use is still limited by technological problems, lack of standardized procedures and inadequate interpretation.

In this work we investigated a group of tissue biomarkers as well as new technologies and computerized approaches for consistent and reproducible analyses. We also tested an automated approach for performing Gleason grading.

In order to validate previous *in silico* studies, we investigated the expression of ERG (as a surrogate marker of *TMPRSS2:ERG* gene fusion status) and TATI (encoded by *SPINK1*) proteins in a large TMA of localized prostate cancer patients. We observed a mutually exclusive expression pattern, further supporting the idea of tailored treatment for genotypically different cancers. In the second and third studies we introduce the use of image analysis for an integrated approach that uses Time Resolved Fluorescence Imaging on PSA and AR, immunofluorescence on cytokeratin as well as brightfield microscopy on H&E and p63/AMACR. The workflow includes the following automated steps: multi-modality image registration, identification of regions of interest, recognition of benign *versus* cancer areas and protein quantification. PSA seemed to decrease in cancer while AR increased in AMACR+ and decreased in AMACR- cancer tissue compared to benign. Finally, we developed a system based on SIFT features and BoW approach to automatically perform Gleason grading. The system was able to distinguish between grades with very high accuracy.

Key words prostate cancer, image analysis, Time Resolved Fluorescence, automated Gleason, PSA, AR, fusion gene, TMAs

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title 1652-8220	ISBN 978-91-7619-144-6
Recipient's notes	Number of pages 152 / Price / Security classification

Image analysis of prostate cancer tissue biomarkers

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Faculty of Medicine, Translational Medicine, Division of Urological Cancers
ISBN 978-91-7619-144-6
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2015


Alla mia famiglia

Contents

List of papers

Included papers

The thesis is based on the following papers:

I. A high-density tissue microarray from patients with clinically localized prostate cancer reveals ERG and TATI exclusivity in tumour cells. **Lippolis G**, Edsjö A, Stenman U-H, Bjartell A. *Prostate Cancer and Prostatic Diseases*. 2013;16(2):145-150. doi:10.1038/pcan.2013.7.

II. Automatic registration of multi-modal microscopy images for integrative analysis of prostate tissue sections. **Lippolis G**, Edsjö A, Helczynski L, Bjartell A, Overgaard NC. *BMC Cancer*. 2013;13:408. doi:10.1186/1471-2407-13-408.

III. Quantitative Time Resolved Fluorescence Imaging of Androgen Receptor and Prostate Specific Antigen in Prostate Tissue Sections. Krzyzanowska A*, **Lippolis G*,** Peltola M, Pettersson K, Helczynski L, Anand A, Lilja H, Bjartell A. *Manuscript*

IV. Automated Gleason grading of prostate cancer using SIFT and BoW. **Lippolis G**, McCarthy N, Hamilton P, Bjartell A and Overgaard NC. *Manuscript*

* = authors have equally contributed

Papers not included

I. miQ--a novel microRNA based diagnostic and prognostic tool for prostate cancer. Larne O, Martens-Uzunova E, Hagman Z, Edsjö A, **Lippolis G**, den Berg MS, Bjartell A, Jenster G, Ceder Y. *Int J Cance*r. 2013 Jun 15;132(12):2867-75.

II. Tractable and Reliable Registration of 2D Point Sets. Ask E, Enqvist O, Svärm L, Kahl F, **Lippolis G**. *Computer Vision – ECCV 2014 Lecture Notes in Computer Science Volume* 8689, 2014, pp 393-406

III. Serum and tissue levels of prostate specific antigen are dependent of androgen receptor CAG repeat number. Nenonen H, Skjærpe P*; **Lippolis G***; Sajid A; Björk C; Bjartell A; Svartberg J; Giwercman A; Giwercman Y, *Manuscript*

* = authors have equally contributed

Abbreviations

ADT Androgen Deprivation Therapy

AJCC American Joint Committee on Cancer

AMACR alpha-methylacyl-CoA racemase

AR androgen receptor

AUA American Urological Association

BCR biochemical recurrence

BoW Bag of Words

BPH benign prostatic hyperplasia

COPA Cancer Outlier Profile Analysis

CRPC castration resistant prostate cancer

CZ central zone

DAB diaminobenzide

DHT dihydrotestosterone

DoG difference of gaussians

DRE digital rectal examination

ERSPC European randomized screening prostate cancer

FISH fluorescence in situ hybridization

GnRH gonadotropin-releasing hormone

H&E Haematoxylin and Eosin

HMWCK high molecular weight cytokeratins

HOG Histogram of oriented Gradient

IHC immunohistochemistry

LUTS lower urinary tract symptoms

MSER Maximally Stable Extremal Regions

PCa prostate cancer

PHOW Pyramidal Histogram of Visual words

PLCO Prostate, Lung, Colorectal and Ovarian

PSA Prostate Specific Antigen

PZ Peripheral zone

RANSAC RANdom Sample Consensus

ROIs regions of interest

SIFT scale invariant feature transform

SURF Speeded Up Robust Features

SVM Support vector machine

TATI tumour associated trypsin inhibitor

TMA Tissue Microarray

TRFI Time resolved fluorescence imaging

TRUS transrectal ultrasound

TURP transurethral resection of the prostate

TZ transition zone

UICC Union for International Cancer Control

USPSTF United States Preventive Service Task Force

Prostate cancer

This chapter gives an introduction to prostate cancer (PCa). It touches upon biological and medical aspects of the disease, starting with a description of the anatomy and physiology of the normal prostate. In addition, causes of PCa, related statistics, current diagnostic procedures, tools to assess disease extension and aggressiveness and treatment options will also be discussed.

By the end of the chapter, the reader will know that:

- ✓ The prostate gland is part of the male genitourinary system. It is hormonally controlled and it produces substances that favour the mobility and viability of sperm.
- ✓ Many pathological conditions affecting the prostate are not life threatening while the risk of PCa should be adequately assessed.
- ✓ With approximately 1 million cases in the world, PCa is one of the most common cancers in men. PCa affects mainly men above 60 years old. Even if the causes are not yet clear, some factors have been identified that increase the risk of occurrence.
- ✓ We have a set of clinical tests including DRE and PSA that allow for diagnosis but that unfortunately still have low specificity with consequent risk of overdiagnosis and overtreatment
- ✓ Clinical data allow for staging of the disease which is the estimation of the actual extension and aggressiveness. Data collected from resected prostates, following curative treatment, help to predict the future development of the patient's disease. Gleason grading is an assessment of the microscopic structure of the cancerous prostate and is still the most powerful prognostic tool.
- ✓ Not all PCas are lethal; the majority of the cases are actually not life threatening or detected at an early stage when successful treatments can be offered to the patient. Advanced PCa is not curable, yet palliative treatments are available.

The human prostate

The prostate is an exocrine organ located below the bladder and surrounding the urethra. In its normal state, the prostate is similar in shape and size to a walnut [1]. Throughout life the prostate grows until puberty and it remains in that state unless other conditions arise.

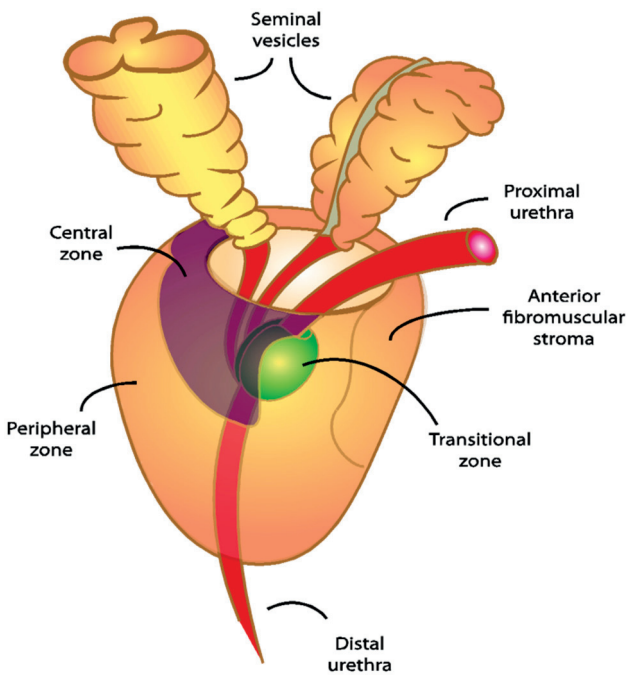

Figure 1 - prostate anatomy adapted from *Campbell-Walsh Urology 9th edn (Saunders Elsevier, Philadelphia, 2007)*.
The central, peripheral and transition zones, together with the anterior fibromuscular stroma are visible. The urethra and the ejaculatory ducts of the seminal vesicle pass through the prostate

Anatomically (Figure 1) the prostate is constituted of an anterior fibro-muscular stroma and three glandular zones: peripheral zone (PZ), transition zone (TZ) and central zone (CZ) [2]. This distinction is important because the different zones are subject to different pathological conditions. The PZ is the largest with 70% of the total prostate volume and harbours the majority of the prostate tumours. The TZ occupies only 5% of the prostate volume, but its critical position, right adjacent to

the urethra, can make it a cause of distress and problems for men when benign prostatic hyperplasia (BPH) occurs. BPH is a non-malignant enlargement of the prostate which is a common occurrence in elderly men [3]. The CZ constitutes around 25% of the organ and, although less frequent than the PZ, it can harbour PCa with highly aggressive features [4].

The name prostate is derived from Greek and means ‘protection’, which seems to suggest that the prostate position at the ‘entrance’ of the urinary system my play the role of a barrier to exogenous agents towards the upper urinary ways.

As part of the male genitourinary system, the function of the prostate is to produce the fluid component (about 30%) of the semen which is rich in simple sugars, enzymes and alkaline substances to maintain and nourish the sperm. The gland cells secrete proteolytic enzymes that, by breaking down proteins, favour the sperm’s motility and viability [1].

Microanatomy of prostatic tissue

The normal prostatic tissue (Figure 2) consists of glandular structures, tubules surrounded by stroma, the two of which by which are separated through a basement layer.

The glands consist of epithelial cells which surround an empty lumen where the secretory proteins are released to then reach the urethra. There are three types of epithelial cells: the basal cells, close to the basement layer the luminal cells opening to the lumen and the neuroendocrine cells (less conspicuous). These cells have distinct function and express proteins that are differently involved in various stages of PCa. The luminal cells require androgens for survival and without them they undergo apoptosis; the basal cells are highly proliferative, androgen independent cells that might contain a stem-like cell population responsible for development of all epithelial cells [5, 6]. The most common PCa phenotype (ca 90% of the cases) has epithelial origin and is called adenocarcinoma [7]. The stroma compartment is formed of extracellular matrix and smooth muscle cells as well as fibroblast. Stromal cells produce paracrine factors that influence the glandular cells. The stroma-epithelium (micro-environment) interaction is central both in normal and in pathological conditions, as is shown in several studies [8, 9].

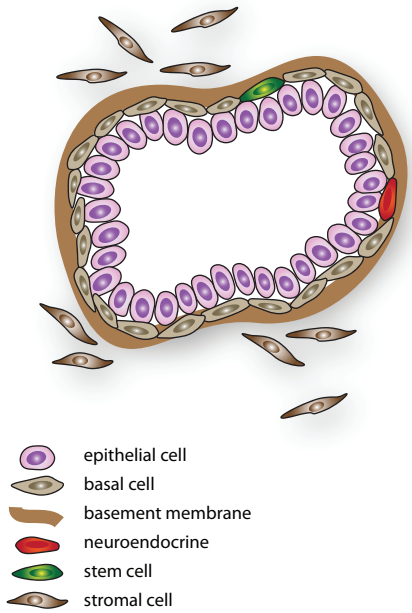

Figure 2 - normal prostatic glandular structure
One layer of epithelial cells is surrounded by one layer of basal cells and a basement membrane. Neuroendocrine cells are rare and stem cells originate in the basal cell compartment. The gland is separated from the stroma by a basement membrane.

A normal gland is formed by a single layer of luminal cells, surrounded by a layer of basal cells. The absence of basal cells is a hallmark of cancer. The shape of a normal gland is typically irregular, with branches and infoldings. The cells have normal abundant cytoplasm with polar shapes toward the lumen. The nuclei are regular in size and shape. When cancer arises, a loss of basal cells is observed and the tissue undergo some changes that affect cells and gland architecture (Figure 3)

The Haematoxylin and Eosin (H&E) staining is the traditional way to make the microstructure visible through light microscopy. The architectural structure of the glands and the appearance of the glandular cells is analysed to make a diagnosis and for grading of PCa. The Gleason grading (see Gleason grading section) is in fact an assessment of the gradual transformation of the normal gland into malignant, undifferentiated tissue (Figure 4).

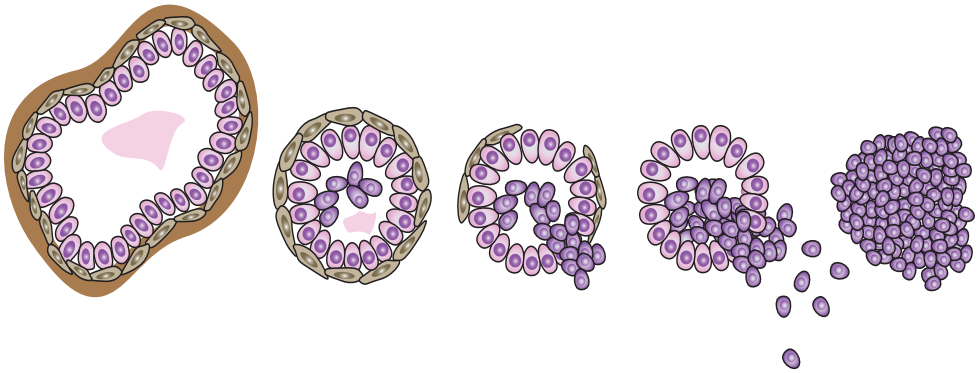

Figure 3 - tumour progression
From left to right, the schematic shows the gradual transformation of a normal gland into a more undifferentiated structure. Basal cells tend to disappear, the glands become smaller, lumina are not well defined and eventually there are only cancer cells, scattered or forming solid sheets.

Androgen signalling

The normal growth and physiology of the prostate depends on androgen supply. Among the androgens, testosterone is produced by the testes while dehydroepiandrosterone is synthetized in the adrenal glands. Testosterone circulates in the blood and when it enters the prostate epithelial cells, it interacts with 5-α-reductase and gets converted to dihydrotestosterone (DHT), which is the most potent ligand to the androgen receptor (AR) [9, 10].

The AR belongs to the nuclear steroid receptor family and when inactive is bound to heat shock proteins. Upon DHT binding with AR, there is consequent dissociation of AR from the heat shock proteins. At this point the AR enters the nucleus and binds to the androgen responsive elements within promoter regions of AR target genes, and initiates transcription. In order to perform this process, the newly formed AR-DNA complex recruits a multitude of co-transcription factors [11].

Prostate Specific Antigen (PSA), which will recur in the thesis, is one of the targets of the AR pathway.

The AR and androgen signalling [12, 13] are involved not only in the correct functioning of the prostate but also in PCa development and lethal castration resistant prostate cancer (CRPC).

Aetiology of PCa

PCa is one of the most common cancers in the world with 1.1 million new diagnoses in 2012 which accounted for 15% of all cancers in men. Interestingly almost 70% of the cases occur in the developed countries. The incidence of PCa varies significantly across countries, with the highest peaks occurring in Australia/New Zeeland, north America and western and northern Europe and the lowest rates in Asian populations [14]. Even if this seems to suggest a geographical factor for PCa, the result could also be explained with the introduction of PSA screening in the late 1980's in the most developed countries resulting in an increase in the detection of PCa in those areas [15].

The causes of PCa are still not completely understood, although a number of risk factors have been identified.

Age is one of the most important risk factors for PCa as the disease is rarely found in patients aged below 40. Other risk factors are related to familiar history, ethnicity, diet, and certain sporadic genetic events.

Men with a brother or father who developed PCa are twice as likely to develop the disease [16]. African American men have among the highest incidence rate and a 2.5-fold higher risk of developing advanced PCa compared to the white Caucasians [17]. The ethnical/geographical influence has been investigated further in several studies. Shimizu et al. [18] studied incidence of PCa for Spanish-surnamed whites, other whites and Japanese populations in Los Angeles County. The incidence was much higher than in the 'homeland' populations and similar to those of US-born patients. The incidence of PCa in Asian immigrants in the United States and their descendants was studied also by Cook et al. [19] who observed an increase compared to homelands, however still lower than white men born in the US. These kinds of studies seem to suggest that life style such as dietary habits, may play a role in modulating the risk of developing PCa. Some studies have shown that men that carry mutations in the genes BRCA1 and BRCA2 seem to have a higher risk for developing PCa [20].

Loeb et al [21] analysed the influence of environmental factors on genetic events that are related to PCa risk (like single nucleotide polymorphisms). They showed that selenium supplements may reduce genetic risk of advanced PCa; aspirin, ibuprofen and vegetables may reduce risk of non-advanced PCa.

In a recently published work [22], a set of risk factors was analysed to predict the 10-year risk of 11 common cancers: age, Body Mass Index, Townsend deprivation score, ethnicity, smoking status, family history, manic depression, type 1 diabetes and type 2 diabetes. Again, significant differences were observed between ethnic groups with south Asian and Chinese having the lowest and black African and

Caribbean men having the highest risk. They also implemented a risk calculator, the Qcancer, accessible online (http://qcancer.org/10yr/).

Diagnosis

The current diagnostic tools to detect PCa are PSA blood test, digital rectal examination (DRE) and transrectal ultrasound (TRUS)-guided biopsy.

The PSA test measures the level of the antigen in the blood. Elevated levels may suggest PCa and advocate for more tests. The routine was introduced in the early 1990's [23] and has changed the PCa scenario, resulting in a surge in disease detection.

During a DRE the urologist looks for hard lumps in the prostate that might be indicative of tumours. Adding the PSA test increases the accuracy of the diagnosis but it is still far from being adequately specific for PCa. In fact, abnormalities in DRE and PSA can be due to other conditions such as BPH or prostatitis (prostate inflammation). Symptoms that might alarm a man, the so called lower urinary tract symptoms (LUTS), are related to problems with storage and voiding of urine, however these can be attributed to several other causes affecting bladder, urethra, prostate or sphincter tissue and generally are common in aging men. These symptoms are not related specifically to PCa and should be addressed appropriately [24].

The single conclusive way to diagnose cancer is to biopsy the prostatic tissue [25].

A diagnostic procedure is conceptually different from screening. Screening means to perform clinical tests on patients without clinical symptoms. The aim is to detect the disease at an early stage in order to increase the chances to have a curative treatment.

The question of whether it is really beneficial for increasing survival is contradictory. The two biggest studies trying to reach a conclusion were the European randomized screening prostate cancer (ERSPC) and Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening.

The ERSPC [26] observed a 20% decrease in mortality, however 1055 men needed to be screened to prevent one death from PCa during a median follow-up duration of 11 years. The PLCO [27] showed that after 7-10 years the rate of death from PCa in screening *versus* non screening did not differ significantly. In a 2009 review [28] and then in a combined meta-analysis taking in consideration even other studies [29], the authors concluded that PCa screening does not significantly decrease PCa-specific mortality. Interestingly, just one study (ERSPC) observed a reduction of

mortality. Reduction in PCa specific mortality can take up to 10 years to be evident, so if life expectancy is shorter than that, screening should not be suggested.

Moreover, overdiagnosis and overtreatment that derives from this, can have harming consequences (infections, pain deriving from biopsies and bleeding) for the patients. Quality of life needs to be taken in high consideration.

For these reasons the United States Preventive Service Task Force (USPSTF) did not stand in favour of large-scale screening for PCa, stating that the harms would outweigh the benefits (2012). The American Urological Association (AUA) was also critical about the screening approach [30].

PSA screening has certainly resulted in a decrease in stage (see Staging and grading section) of detected PCa at the time of diagnosis and 80% of these are localized to the prostate [31].

It is clear that the main problem is not only to detect PCa with minimally invasive techniques and limited harmful consequences for the patient but also to identify clinically significant PCa. PLCO and ERSPC have increased our awareness about diseases, pointing out that, although detected through the screening, some PCas would have never become symptomatic or led to cancer specific death. This case is called indolent PCa, or sometimes insignificant which must be distinguished from the significant PCa [32]. The histopathological definition of insignificant PCa is based on the analysis of the whole prostate and requires Epstein criteria of a volume $<0.5cm^3$, Gleason score ≤ 6, no evidence of Gleason grade 4 and pathological stage pT2 [33] (see Staging and grading section). A clinical translation of the aforementioned is needed but challenging. Clinically insignificant PCa fulfils clinical and biopsy criteria, including clinical Epstein criteria [32].

Staging and grading

Two types of staging, clinical and pathological, are currently in use. The first is the doctor's estimation based on DRE and lab test conducted on the patient. The second is performed on the actual removed tissue after prostate excision. This is generally more accurate than the clinical staging because the doctor can have a view of the whole prostate and avoid the risk of underestimation.

The American Joint Committee on Cancer (AJCC) and the Union for International Control (UICC) have adopted the TNM staging [34].

The TNM system is comprised of: T, the extent of the primary tumour, N, the state of nearby lymph nodes and M, the presence of distant metastasis. T0 and TX define the absence of a primary tumour or the impossibility to give a clear assessment, respectively. T1 indicates a primary tumour that is not palpable but that was found

incidentally. This could occur following a transurethral resection of the prostate (TURP) for relieving the symptoms of BPH or following a needle biopsy performed upon an elevated level of PSA. T2 describes a tumour that is palpable or visible through imaging but still confined to the prostate: a T2a tumour is present in one half or less of one lobe, a T2b is still limited to a single lobe but larger than a half, a T2c is present on both lobes. T3 stage is given when the primary tumour has grown outside the prostate: a T3a has exceeded the prostatic capsule while a T3b has spread to the seminal vesicles. Finally T4 stage defines a tumour that has spread to adjacent tissues and organs such as the bladder, rectum or pelvis. As for the lymph nodes state, NX means the lack of an assessment, N0 and N1 indicate absence or presence of tumour in the lymph nodes, respectively. M describes the state of distant metastases: M1a indicates the spread to distant lymph nodes, M1b the spread to the bones and M1c the spread to other organs such as liver, lungs, brain or bones. Bone is the most common site of metastasis for PCa.

TNM system is used along with blood PSA value and biopsy Gleason score to form the stage-grouping, a scale from I to IV from the least to the most advanced disease. While patients with low risk might be candidates for active surveillance, the high-risk patients are offered a number of possible treatments depending on stage and clinical data. This would support the doctor in deciding the best treatment for the patient.

Tools called nomograms can help predict the actual extension of the disease from the clinical tests. The so called “Partin tables” in their updated version [35] use serum PSA, biopsy Gleason score and clinical stage to predict the pathological stage (whether or not the tumour is confined or not to the prostate) at radical prostatectomy.

Other staging tools used less frequently include the Whitmore-Jewett system that identifies 4 categories and the D’Amico that also uses PSA level, Gleason score and T stage to stratify patients in 3 risk groups [36].

Gleason grading

Gleason grading was introduced by Dr D. Gleason in 1966 [37–39] and revised recently in the 2005 consensus [40]. The Gleason scoring is an assessment of the architectural structure of the prostatic tissue. It identifies five patterns or grades, from 1 to 5, indicating tumours ranging from resembling a benign gland to the least differentiated, more invasive ones. This scale represents the gradual loss of glandular structure that takes place in the most aggressive form of the disease. The final Gleason score is the sum of the two most common grades found within the tumour, therefore ranging from 2 to 10.However importance has also been given to the tertiary pattern, if this is particularly high.

The original Gleason grading system was conceived before the PSA testing era and the majority of patients, diagnosed with the aid of DRE, presented with advanced disease and often metastasis at the time of diagnosis. Only 8% were diagnosed with a palpable localized tumour [38]. Since then, advancements in biopsy collection (with the aid of 18-gauge thin needle biopsies introduced in the late 1980s [41]) and immunohistochemistry (IHC) have shown that PCa is heterogeneous and multifocal. Conditions such as adenosis or prostatic intraepithelial neoplasia (PIN) would have likely been misinterpreted as very low Gleason grades in the past. Also, newly diagnosed forms of PCa such as mucinous carcinoma or foamy gland carcinoma need to be taken into account. For these reasons the 2005 USCAP meeting strived to reach a consensus over the new issues related to Gleason grading in the PSA era. The new reformulation (Figure 4) is defined as [42]:

- Pattern 1 = a circumscribed nodule of medium sized acini, uniform and still separate.
- Pattern 2 = still circumscribed nodule with glands that are arranged in a more loose fashion and less uniform than the first grade.
- Pattern 3 = the glands are generally smaller than previous grades with more variation in size and shape; they are still discrete but the can start to infiltrate normal areas.
- Pattern 4 = there is a fusion of glands that start to lose their normal structure with an ill-defined lumen. A cribriform pattern is also observed.
- Pattern 5 = it is not possible to distinguish discrete glands anymore and they are replaced by solid sheets or single scattered cancer cells.

It should be noted that reporting Gleason scoring for biopsies and for whole prostatectomies may differ and different considerations are generally made. Since biopsies only show small samples of the whole organ, pathologists are generally more careful in dealing with this. As multiple biopsies are taken from different locations in the prostate, the samples may display different patterns. One way of reporting this would be to identify the most common patterns, however, several studies have shown that if some cores show the presence of a highest degree pattern, a score reporting the most common and the highest grade (even if not the second most common) correlates better with pathological stage at radical prostatectomy [43, 44]. This is a different approach from the traditional Gleason grading.

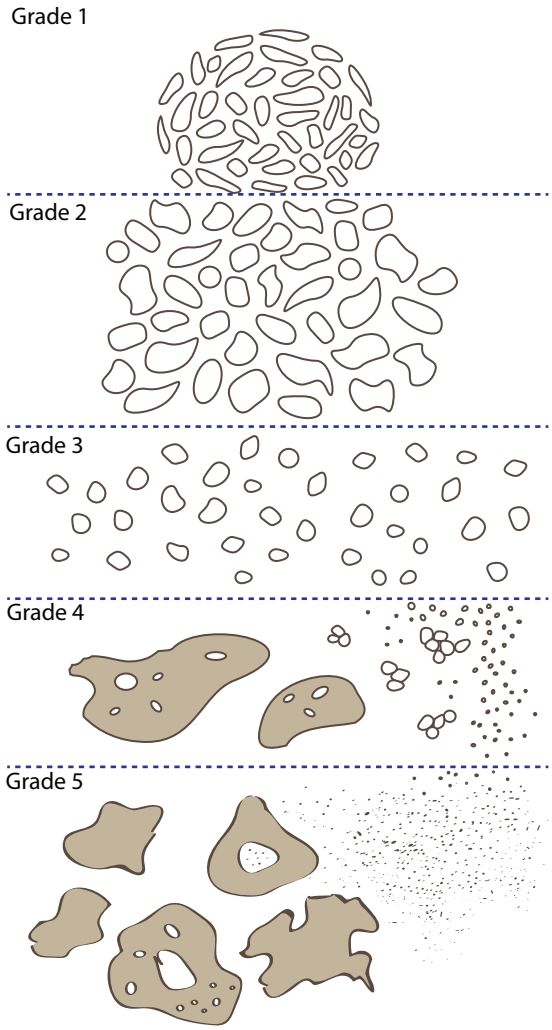

Figure 4 - Gleason Grading schematic adapted from *An update of the Gleason Grading System. Epstein J. The journal of Urology 2010*
The system describes the gradual change in the glandular architecture. With increasing grades, the glands tend to become smaller, fuse together and eventually disappear into undifferentiated, scattered, highly invasive cancer cells.

The new modified grading system has resulted in an increase in the concordance between biopsy Gleason score and radical prostatectomy score from 58% to 72% [45].

There are, of course, still incongruences and areas of lacking consensus in analysing and reporting Gleason score of needle biopsies and radical prostatectomies [46].

One other issue related to Gleason scoring is the difference between pattern 3 and pattern 4, as pattern 4 marks a more defined change of prostatic glandular structure

with increased tumour invasive capability [47]. This makes the capability if distinguishing between grade 3 and grade 4 particularly important.

Finally it is clear that Gleason grading is highly dependent on the pathologist experience and a certain inter and intra observer variability has been shown in some studies [48].

Treatment

Choosing the adequate treatment must take into consideration the stage of the disease, the general health status of the patient and their life expectancy. Patients with tumours that are not expected to grow for several years may be put on a regime of active surveillance. This means that regular DRE and PSA tests are conducted every three or six months, yet other treatment is deferred until the tumour displays signs of progression.

Localized PCas that are not thought to have spread beyond the organ are candidates for curative treatments including surgery or radiotherapy. The surgical operation is called radical prostatectomy and consists in removing the whole prostate. While retropubic (frontal opening from the belly to the pubic bone) and perineal (incision between the anus and the scrotum) are the traditional techniques, nowadays laparoscopy is becoming more and more common. It consists of performing several small incisions in the abdomen, through which a camera and the operation tools are inserted. The robotic-assisted laparoscopy is a an evolution which further reduces the bleeding and other side effects (incontinence, impotence) with comparable outcome to retropubic prostatectomy [49]. Radiotherapy can be recommended for tumours that have started to grow outside the prostate, advanced disease in order to shrink the tumour as well as following prostatectomy in case of biochemical recurrence (BCR).

Advanced PCa is not yet curable. Patients that present an advanced stage cancer at diagnosis and are not operable as well as those where the tumour has recurred after prostatectomy are given palliative treatments. The first treatment is generally Androgen Deprivation Therapy (ADT) since, at least in the beginning, PCa cells are dependent on the AR-androgen interaction for proliferation [12]. The most used ADT therapy involves gonadotropin-releasing hormone (GnRH) agonists and antagonists that block production of testosterone from the testes. While the GnRH have an effect on testosterone produced from testes, they cannot stop other cells from producing the hormone (PCa cells themselves can do that). The drug Abiraterone (Zytiga®) is able to stop this process by blocking the enzyme CYP17 in tumour cells. Anti-androgens can also be used for their antagonist activity: they bind competitively to the AR impeding its activation. Unfortunately the ADT therapy only has an initial temporary effect. The cancer inevitably stops responding,

becoming castration-resistant (CRPC) and eventually metastasizing to distant areas. At this stage chemotherapy can be provided using drugs such as Docetaxel (Taxotere®) that, for example, target cell division. New interesting techniques include cancer vaccines that make the immune system attack PCa cells, however even this is not a curative treatment.

Histopathology

Histopathology is the microscopic assessment of tissue specimens, processed and stained, to highlight cellular and tissue architecture for both clinical and research purposes.

The chapter will highlight the following points:

- ✓ The importance and need for biomarkers for diagnostic and prognostic purposes
- ✓ Tissue biomarkers have great potential because they carry both expression and morphological information in contrast to serum markers. However, their use is still hampered by lack of standardization in material processing, analysis tools and interpretation.
- ✓ There are a number of biomarkers that are currently being investigated. Some, such as alpha-methylacyl-CoA racemase (AMACR) and p63, help in diagnostic settings while others are under investigation for their prognostic role.
- ✓ The introduction of tissue microarrays (TMA) has boosted the research in high-throughput analysis of tissue biomarkers.
- ✓ There are different staining techniques: the main methods are chromogenic and fluorescence. They both have pros and cons. Chromogenic staining is more widely used while immunofluorescence is limited mostly to research setting.
- ✓ We investigate a novel immunofluorescence technique, the Time Resolved Fluorescence Imaging (TRFI), which seems to be particularly suitable for biomarker quantification on formalin-fixed paraffin embedded tissue sections.

Tissue biomarkers

Broadly speaking, a biomarker is a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [50]. Talking about

biological processes can be difficult so it is fundamental to define relevant clinical endpoints against which to test a specific biomarker [51]. Clinical endpoints in PCa include BCR, defined as serum PSA rising after curative treatment, metastasis onset and survival.

A biomarker should not only be objective, quantifiable and reproducible but easily accessible.

During the ‘80s and ‘90s biomarker research in solid tumours mainly focused on immunohistochemical markers. However, the majority of these markers were never introduced in the clinical setting which led to decreased interest in immunohistochemical biomarkers and instead an increased focus on blood/serum markers [52].

Traditionally, analytes in the blood/serum have been the main biomarkers, probably for their easy access. However markers expressed within tissue have the advantage that they also carry spatial information, that is, information regarding where a specific event is taking place. In fact, in contrast to circulating biomarkers, in tissue there are intermingled populations of cancer and normal cells. This allows for a differential expression analysis with the possibility to look also at field effects.

An example of a tissue biomarker that has been successfully implemented in the clinical setting is the human epidermal growth factor 2 (HER2), whose expression in the membrane of breast epithelial cells is used in breast cancer patients. Guidelines for the correct use and interpretation of the biomarker have been issued by ASCO/CAP [53] and the test has gone a long way with approval from the American Food and Drug Administration (FDA).

Generally, immunohistochemical analysis of tissue biomarkers has been limited to a ‘binary’ assessment (presence/absence of a biomarker) because a quantitative or semi-quantitative assessment poses some problems.

Factors that have been impairing the widespread use of tissue biomarkers include:

- Limited datasets and lack of validation sets.
- Lack of appropriate cohort to test tissue biomarkers.
- Wrong choice of the adequate endpoints (is BCR a good surrogate of failure or specific death?).
- Datasets that are not well described,
- Inadequate statistical analysis,
- Heterogeneity of PCa. A problem that especially occurs with biopsies where the sampling might miss the most significant foci.
- Absence of standardized protocols for staining, data assessment and interpretation.

The reproducibility of many studies has been a problem and therefore we lack validation studies.

Nevertheless tissue biomarkers are still needed for improving diagnosis, better staging, stratifying patients and predicting patient outcome with or without a treatment.

It is improbable that a single marker can give a complete description of an aspect of the disease; hence the latest studies try to look at a group of biomarkers.

Also a new biomarker must be powerful enough to significantly improve the current prediction model (based on serum PSA) in order to be taken in consideration [54]. At the time being, due to the heterogeneity and lack of concordance of the studies [55], blood PSA is the only biomarker routinely used in nomograms for PCa.

The new advances in the 'omics' field together with new technologies, high-throughput schemes and computerized image analysis can aid the search for new, reliable biomarkers.

Diagnostic markers

Definitive diagnosis of PCa is performed on biopsies that have been stained with H&E. Using a microscope, the pathologist analyses the tissue architecture and the cellular atypia. However, sometimes this is not sufficient to rule out the presence of cancer, possibly due to focus being too small to detect the atypia as it happens in at least 5% of biopsies [56]. For this reason other immunohistochemical staining can be used to help the diagnosis. The most used ones include the following:

AMACR

AMACR [57, 58] is generally expressed in the cytoplasm of cancer epithelial cells and is normally negative in benign tissue. It can however be expressed in PIN as well as occasionally in benign lesions such as atrophy and adenosis [54].

AMACR is reported to be expressed in about 80% of cancer cells, although some variability has been observed [59]. Some forms, such as foamy gland PCa, are less often positive [60]. Moreover, although quite specific for cancer cells, AMACR expression may decrease in more advanced cancers as was suggested by Rubin et al. [61].

P63

AMACR is generally utilized in combination with p63 and/or high molecular weight cytokeratins (HMWCK). p63 is a basal cell marker [62, 63] and therefore a marker of benign tissue. However false negative p63 stainings can occur and sometimes basal cells could be absent in small foci due to the cutting procedure. p63 can be used alone or in combination with HMWCK because, differently from epithelial cells, they are expressed by basal cells. The problems might arise for those benign conditions such as atrophy that are occasionally negative for HMWC or for aberrant expression in some adenocarcinomas [64].

A double immunohistochemical staining for p63 and AMACR therefore has a very important diagnostic utility [25, 65] which is why we employed it in our studies. In particular the possibility to simultaneously navigate images of the same tissue section stained sequentially for H&E and p63/AMACR can be a valuable investigative tool as demonstrated by Helin et al. [66].

Prognostic markers

Prognostic markers are molecules that predict the possible outcome of a disease in an untreated individual whereas *predictive* markers can predict the response to a specific treatment for a specific disease [67]. PCa is a very heterogeneous disease: it can be very aggressive from its onset or it can present with clusters of tumour cells with different phenotypes [54] and different aggressive features.

There are a number of prognostic markers that have been described and investigated.

Ki-67

Ki-67 is a cell proliferation marker and is expressed in G1, S, G2 and M phase of the cell cycle. A number of studies have shown its association to aggressive features of PCa and its predictive capability after treatment.

Berney et al. [68] showed that Ki-67 expression in TURP samples was correlated to overall survival in conservatively treated patients and added an independent prognostic power to Gleason score and serum PSA. Ki-67 seems to correlate with Gleason score in diagnostic biopsies [69] as well as in subsequent prostatectomies [70]. Rubio et al. [71]observed that Ki-67 in needle-biopsies predicted disease-free survival after prostatectomy.

To summarize Ki-67 expression is related to unfavourable clinicopathological characteristics of PCa as well as with cancer specific death.

Fusion gene *TMPRSS2:ERG*

In 2005 Tomlins et al. [72] used a bioinformatics approach, Cancer Outlier Profile Analysis (COPA), to discover oncogenic chromosomal abnormalities. They found that ERG and ETV1, two members of the ETS family of protein, were overexpressed in a subset of PCas. In addition, they observed recurrent fusion of these proteins to the 5' untranslated region of the androgen dependent *TMPRSS2* gene. In particular the *TMPRSS2:ERG* fusion occurs in approximately 50% of PCas [73].

Several groups have tried to study the association of this fusion gene with clinicopathological features and outcome of the disease, however the results have so far been inconclusive.

Rajput et al. found the fusion to happen more frequently in moderately to poorly differentiated tumours [74]. Perner et al. [75] also observed association of the fusion gene (through deletion) with higher tumour stage and metastatic onset. However a study by Barros-Silva et al. displayed that the gene fusion detected in biopsies was associated with low Gleason score and serum PSA [76]. A similar association with low-grade morphological features was observed by Fine et al. [77]. Furthermore, no association with clinicopathological parameters was found by Rubio-Briones et al. [78] and when checked against outcome in patients treated by prostatectomy Gopalan et al. [79] did not find any significant predictive power as we also observed in a large cohort of localized PCas, in paper I [80].

With regards to needle biopsies, immunohistochemical staining for ERG is sometimes used to rule out PCa mimickers, although the use of p63-AMACR is generally preferred.

It is likely that the fusion gene is more of an early event in PCa rather than a predictor of tumour progression.

SPINK1

SPINK1 which encodes the tumour associated trypsin inhibitor (TATI) is expressed in various tissues, both normal and cancerous and its prognostic role was investigated by Paju et al. [81]. The use of COPA unveiled *SPINK1* as another outlier in PCa, being highly expressed in a subset of cancer cases that did not harbour any ETS rearrangements [82]. These cases (approx. 10% of all cases) seemed to have an increased risk for BCR.

Subsequent studies further investigated the association of TATI with the features of the disease. TATI expression seems to increase in high grade tumours and to be associated with invasiveness in the PCa cell line 22RV1 where it is regulated by androgens [81]. Leinonen et al. [83] analysed the status of the *TMPRSS2:ERG*

fusion gene and *SPINK1* in a set of endocrine-treated PCas. They observed that the fusion gene was not associated with patient outcome after treatment, while the *SPINK1* expressing cases had more aggressive disease and a shorter progression-free survival. However there was no association with clinicopathological parameters. In our work in paper I, we did not observe any association between TATI in localized PCa and BCR.

In order to understand more about *SPINK1* biology, some groups have studied the association of TATI with other potential biomarkers in castration-resistant PCa. TATI overexpression was observed in tumours characterized by absence of AR amplification and PTEN deletion [84].

AR

The AR is a nuclear receptor that becomes active and translocates from cytoplasm to nucleus when bound by androgens. In the nucleus, the activated AR binds to DNA and acts as a transcription factor regulating expression of several target genes.

The AR is involved in the maintenance of the normal prostate [85] however it also plays a role in prostate tumorigenesis [86], development and CRPC [87]. The AR pathway is also the target of modern hormonal treatment with abiraterone and enzalutamide although more evidence is being brought forward suggesting that the resistance pattern in advanced disease are related to ligand independent AR activation, intra-tumoral androgen production, increased mRNA, AR mutation and AR splice variants [88].

The prognostic role of AR has been debated for some time and some studies have concluded a limited prognostic power [89]. An increase in the AR gene expression [90] in tumour areas compared to normal tissue has been reported to correlate with worse prognosis after radical prostatectomy. In addition, an increase in AR protein seems to be associated with shorter BCR free survival and more aggressive clinicopathological features [91, 92]. High level of AR was also associated with Ki-67 and therefore with proliferative features [91].

In patients with advanced disease like CRPC, the increased AR expression upon biopsy as well as in radical prostatectomy was associated with shorter cancer-specific survival [93].

PSA

The PSA is the protein encoded by the KLK3 gene which belongs to the kallikrein gene locus consisting of 15 genes on chromosome 19q13-4 [94].

KLK3 is a known target of the AR and the PSA is expressed by prostatic epithelial cells in both normal tissue, BPH and most tumours [95]. This makes the PSA a tissue specific but not a tumour specific marker. For this reason the diagnostic specificity is far from optimal, as changes in serum PSA can be due to several conditions. While its diagnostic specificity is not very high, its predictive role after treatment is still important.

Only few studies have been conducted on PSA expression in tissue. Some of these have observed a decrease of PSA at both mRNA and protein level in cancer compared to benign tissue [95, 96] and an adverse association with adverse clinicopathological features [97]. However tissue PSA has not proven to be an independent predictor of BCR [97].

The role of TMAs

The analysis of immunohistochemical markers has received a great boost by the introduction of TMAs.

TMAs have represented a decisive step forward in translational research in the attempt to bring the discoveries of important genes and proteins from the basic research to the large-scale clinical studies with direct implications for patients' health.

TMAs, along with automated image analysis applications, contributed to the creation of high-throughput systems for the simultaneous analysis of hundreds of samples, considerably reducing costs [52].

The high density TMAs were introduced in a study of breast cancer by Kononen et al. [98] and since then the technology has become automated [99] and well described [100]. The technique is based on collecting core biopsies (generally 0.6 mm diameter) from paraffin-embedded tissue samples of different patients and arraying them in another paraffin block resulting in a 'matrix' of potentially thousands of samples. At this point the new block can be sliced into a large number of sections that can be utilized for the study of several biomarkers by means of any method including IHC, immunofluorescence or *in situ* hybridization.

The major advantage of TMAs is the possibility to analyse a large quantity of samples at once with reduced amount of resources. In addition, TMAs provide certainty that all samples are processed in the same way during the staining

procedure, reducing in this way the sources of error and variability. Any following analysis can be done in an easier and faster way.

A drawback, that could be relevant especially for PCa, is that a single core of such limited size is not likely to represent and capture the heterogeneity of the disease. Rubin et al. [101] tried to address the issue in a TMA study of Ki-67. They concluded that the optimal number of cores coming from single donor was 3 and that these would represent accurately the intrinsic variability of PCa.

The potential of TMA format can be significantly and successfully exploited when it is coupled with a well-organized dataset reporting pathological and clinical information of each core.

There are different types of TMAs according to the nature of the samples represented. The main interest is in constructing TMAs that show the transition from normal to precancerous (HPIN) and cancer lesions or from normal to cancer and metastatic disease [102]. In the first case, being able to identify markers that change expression at the normal/HPIN interface, might give the basis for preventive studies. In the second case, biomarkers differently expressed in primary and metastatic tumours could represent potential targets for treatment studies.

In the case of PCa, the construction of TMAs that can unveil the association of certain markers with the disease outcome (cancer-specific death) is made difficult by the long natural history of the disease. For a biomarker to be significant it is necessary to collect outcome data for at least 10 to 20 years.

Despite the increasing number of TMA studies and the identification of candidate biomarkers, very few tissue biomarkers have been implemented in the clinical setting.

Chromogenic staining

Chromogenic staining is based on enzyme-based precipitations of chromogens that produce a colour allowing the detection of a certain substrate. They are still the most used staining methods in IHC. In this procedure an antibody generally carries an enzyme that reacts with the chromogen. This means that any substrate that attaches to the antibody becomes detectable through traditional bright-field light microscopy

The most common chromogen is diaminobenzide (DAB). This molecule is highly thermochemically stable and produces a brown staining. Other chromogens are available such as Vina Green and Warp Red. The imaging process is based on the absorption of light. The more substrate is present, the more light would be blocked, resulting in a more intense staining. The chromogenic staining is generally used with a counterstain like Haematoxylin in order to create a contrast that can be easily

read. However the simultaneous use of multiple chromogens is limited by the ability to distinguish colours on normal light microscopy especially in co-localization studies. Moreover the qualitative quantification of colour by eye is a hard task, as it has been shown to suffer of inconsistencies within and across different observers and labs [103].

H&E staining

H&E is a routine staining that has been used since the beginning of the 20th century and it is the most common technique in pathology laboratories for cancer diagnosis and evaluating tissue morphology. Haematoxylin is a basic dye formed by hematein and aluminium ions that bind basophilic structures, such as nucleic acids and colours them blue. Eosin is an acidic dye and therefore binds to acidophilic structures like proteins and other components in the cytoplasm, colouring them pink [104].

A good H&E staining should allow for visualization of nuclei and nuclear structures (like the presence of nucleoli), cytoplasmic compartments, stromal cells and other structures.

In the PCa setting, H&E staining of the biopsy is the way to establish cancer diagnosis (sometimes with other immunohistochemical staining like p63/AMACR) [25] and to perform Gleason grading.

Even if H&E is an old, quite standardized technique, there are still some issues related to inter-patient variation and preparation inconsistencies that should be taken into account [105].

Fluorescent staining

Fluorophores are molecules that absorb and emit light at different wavelengths. The light absorption causes a change of the molecule's electronic state (excitation) that is followed by relaxation with a consequent emission of photons at a longer wavelength. Optical detection needs a proper excitation light source such as xenon-arc or mercury-vapour lamps, and a system of mirrors and filters to acquire the specific signal emitted by the fluorophore of interest. Fluorescent microscopy has proven to be quite sensitive and especially suitable for multistaining, providing that the fluorophores used have non-overlapping emission-spectra. Because of this, we can have generally up to 4-5 fluorophores simultaneously. Fluorescence has been extensively used in *in vitro* and *in vivo* applications. However even fluorescence microscopy suffers from some drawbacks including photobleaching, short life-times, pH sensitivity as well as unspecific autofluorescent phenomena that especially limit its use in paraffin-embedded tissues.

In the last years, in addition to traditional organic molecules such as Alexa dyes, DAPI, cyanines, some inorganic molecules have with useful features have been developed. Nanoparticles have been recently introduced in bio-assays for their ability to overcome some of the problems of traditional fluorophores. For instance, Quantum dots [106] are nanocrystals of semiconductors with high quantum yield and narrow emission wavelengths. In addition, they show a good resistance to phtobleaching and degradation.

Some methods have been proposed to increase the number of used fluorophores by applying sequential staining, signal acquisition and alkaline quenching which would allow for detection of more than 60 targets [107].

Other proposed methodologies include the use of lanthanide chelates as fluorophores with desirable features to improve fluorescent microscopy (see TRFI section).

To summarize, fluorescence staining are more suitable for for co-localization studies as well as signal quantification on digital images, however, absence of standardized quantitative methods, analytical tools and physical drawbacks still hamper their use in clinical setting.

TRFI

TRFI is an evolution of traditional immunofluorescence. As described above immunofluorescence is not particularly suitable for staining of paraffin embedded tissue sections because they produce a large, unspecific autofluorescence signal making immunofluorescence less sensitive. TRFI overcomes some of the limitations of immunofluorescence. First of all, it makes use of a special family of fluorophores, the lanthanide chelates such as europium, terbium and samarium. These fluorophores were used in time-resolved fluoroimmunoassays [108] and they have then been tested in cytochemistry [109], in histochemistry and *in-situ* hybridization [110–116]. Lanthanide chelates have also been used in high performance RT-PCR [117].

The production of these fluorophores involves a development step, Delfia principle [118] which makes the chelates fluorescent.

These lanthanide chelates have:

- long stokes shifts (wavelength distance between excitation and emission peaks) (figure 5),
- long decay times (Figure 6)
- high stability

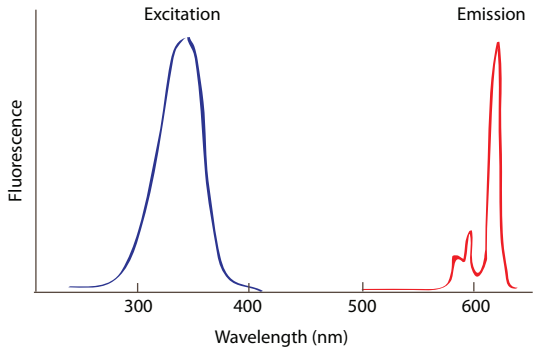

Figure 5 - absorption and emission spectra for europium
The schematic shows the absorption/emission spectrum of europium and its long Stokes shift (maximum absorption peak at 340nm and maximum emission at 615nm).

Autofluorescence is a fluorescence signal emitted by substances other than the fluorophores of interest. Both assay buffers and biological samples can contain substances that autofluoresce, in response to the same excitation light used for the specific fluorophore. The autofluorescence intensity, which increases the background signal may depend on the excitation wavelength and in general tends to be lower at wavelength above 650nm.

Autofluorescence of a biological sample has a short decay time (100ns) [110]. As a result, if the fluorophores of interest emit light with significantly longer decay time, part of the emitted signal can be free of autofluorescence. Lanthanide chelates have a decay time from 10 up to 1000μs which is more than 4 fold larger than normal background duration.

When applied to tissue sections, TRF has been used to investigate PSA, alpha-1-antichymotripsin [114, 115] and kallikrein 2 in prostatic tissue sections [119] where the use of two lanthanide chelates, europium and terbium, to label the antibodies of interest was shown.

In paper II we used anti-PSA (clone 2E9) and anti-AR (clone AR-441) monoclonal antibodies conjugated respectively to terbium and europium chelates [120]. A direct staining of prostatic tissue using only primary antibodies can make the signal intensity more linear with antigen concentration.

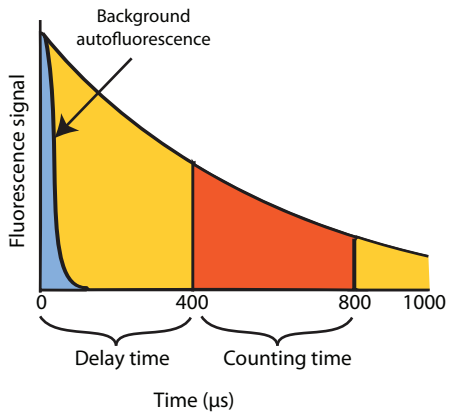

Figure 6 - Emission time of lanthanides
The lanthanide emission (1000µs) lasts much longer than the autofluorescence (100ns). This property allows for gated acquisition of the signal that can start when the autofluorescence decays.

A TRFI workstation [110], like the one used in our work is described in Figure 7 and it consists of a Nikon Eclipse E600 light microscope with ET630/50m and ET560/40m as well as standard FITC filters (Chroma technologies). The system is equipped with a Xenon flash lamp (60 W, Perkin-Elmer Life Sciences, Boston, MA, USA) which has suitable spectral and temporal characteristics for the detection of europium and terbium. The Xenon lamp produces a pulsed excitation of circa 1µs and energy of 0.4 J/pulse at 340 nm wavelength which is the wavelength of maximum absorption for the lanthanides. The acquisition of the lanthanide signal is gated after a lapse of time longer than the decay time of the autofluorescent compounds. A rotating chopper is placed in the emission light path to block the light for 300µs. The chopper is electronically controlled in order to be synchronized with the excitation pulse. A CCD camera, Apogee Alta U32 (Apogee Imaging Systems) is used to capture the image.

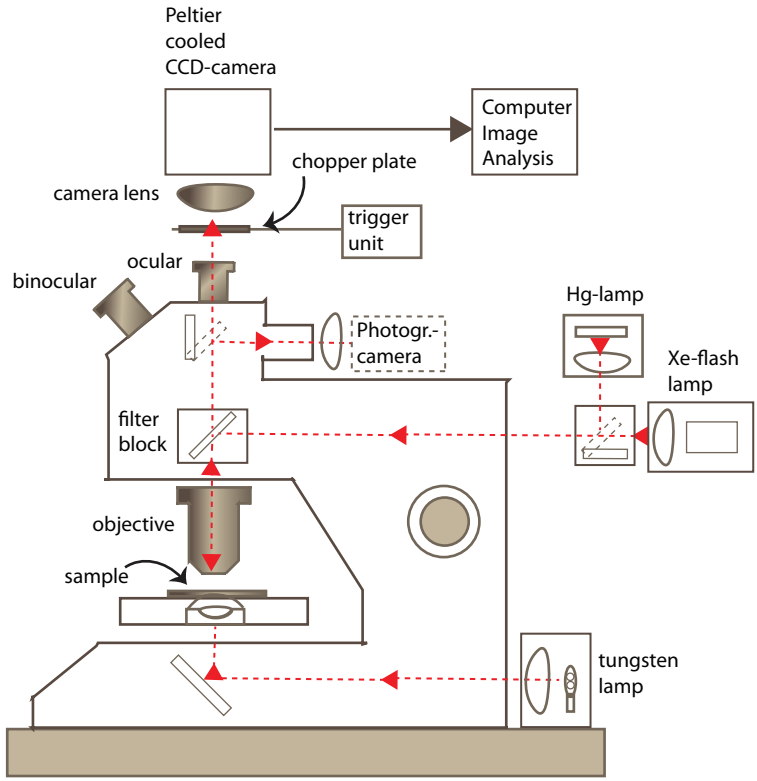

Figure 7 - schematic of TRFI prototype microscope
A normal fluorescent microscope is equipped with a Xenon flash lamp, suitable filters for europium and terbium spectra and an electronically controlled chopper, which gates the signal acquisition after the autofluorescence has decayed.

Image Analysis

This chapter gives an overview of the increasing use of image analysis in histopathology. The following points will be highlighted:

- ✓ New imaging techniques, scanning devices, increased storing possibility and computational power have extended the use of image analysis in biomedical and histopathological research
- ✓ The main aim of using image analysis in biomedical and histopathological research is to make assessment more consistent and independent of observer ability and momentary condition.
- ✓ Image analysis tools aim at automatically extract quantifiable characteristics that can be used to segment objects of interest (i.e. cells, glands), quantify marker expression, classify and align multi-modality images
- ✓ Digital imaging allows for visualization at any time and for easy and fast sharing of interesting diagnostic cases across different institutions.
- ✓ Some image analysis tools applied to different tasks will be presented.

Needs and aims

The application of image analysis to microscopy imagery and histopathology is not new but only in the last years it has acquired an important place both in research and in the clinical setting. This is due to a great improvement in hardware and software that have made possible an easier access to fast scanning devices, to larger storing facilities for digital images and to increased computational power to run complex algorithms.

The use of image analysis serves several purposes:

- To assess images of cytological and histological samples in a fast, consistent, and reproducible way and in a high-throughput manner.
- To provide a quantitative rather than qualitative analysis of biomarkers expression, cell and tissue details, morphological features

- To make easier the user's (pathologist/researcher) work by providing assistive tools, like Computerized-Assisted Diagnosis (CAD) systems [121]
- To analyse the samples in an unsupervised way, in order to find characteristics that are not immediately clear to a human observer but that can be related to important aspects of the disease.

The digitization that is taking place in almost any lab has also another advantage which is the possibility to easily share images with web-based applications [122] between researchers and pathologist across the world, allowing for multisite collaborations, teaching [123], and second opinion in difficult diagnostic cases.

Need for biomarker quantification

Quantification of tissue biomarkers through image analysis is often based on segmentation techniques that identify the regions of interest (ROI) where the biomarker is expressed. There is a vast literature on nuclear, cytoplasmic and membrane algorithms for both chromogenic and fluorescent stainings [124] and using these tools there have been attempts to improve for example current prediction of BCR [125]. All these algorithms return quantitative values like number of cells positive for certain biomarker and signal intensity and they can be applied on large TMAs for high-throughput biomarker discovery.

Need for morphology quantification

Heterogeneity of tissue architecture and tumour features need to be closely analysed to evaluate the aggressiveness of a tumour. Given the variability in human assessment [48], researchers have tried to implement algorithms for automated Gleason grading, using different approaches and sets of features. The ability to accurately recognize and distinguish grades can have huge consequences on patient prognosis and treatment planning [47].

In the rest of the chapter we will go through some technical aspects concerning image analysis in tissue investigation.

Digital images

A digital image is produced by a device which collects the light hitting a sensor and transforms it into a quantized value. The resulting digital image is therefore an array of elements (pixels) whose values (uni- or multi-dimensional) are limited to a specific range.

The size of a pixel defines the resolution of the image which is the smallest detail that can be distinguished.

In our applications, the value of a pixel can be either mono-dimensional (in the case of grey-scale images produced by CCD camera in immunofluorescence application) or 3-dimensional, (in the case of brightfield microscopy). In the latter case, the 3 channels, red (R), green (G) and blue (B) represent the most common system to describe the colour of an image. In most cases the values of the pixels are quantized using 8-bit format which produces a maximum of 256 intensity values (in our brightfield images).

Colour deconvolution

Typically histological images contain two or three different stains. The purpose of Colour Deconvolution is to separate a digital image into channels corresponding to the specific stains in order to analyse selectively each of those (Figure 8). Ruifrok et al. [126] suggested a deconvolution method based on the Lambert-Beer's law. According to this law, the intensity of I_c, the detected light in channel C, transmitted by the sample, is given by:

$$I_c = I_o e^{-\alpha C}$$

Where I_o is the incident light, α is the absorbance of the pure stain and C is the concentration of the stain. From this the optical density (OD) can be derived as

$$OD = -\ln \frac{I_c}{I_o} = \alpha \, \mathrm{C}$$

The OD has the property of being linearly related to the stain concentration. If we had three stains and considering that the camera captures three colour channels (red, green and blue) then we would have three characteristic wavelength absorbance for each stain. The OD can then be expressed as

$$\overrightarrow{OD} = \begin{bmatrix} \alpha_{r1} & \alpha_{r2} & \alpha_{r3} \\ \alpha_{g1} & \alpha_{g2} & \alpha_{g3} \\ \alpha_{b1} & \alpha_{b2} & \alpha_{b3} \end{bmatrix} \begin{bmatrix} C_1 \\ C_2 \\ C_3 \end{bmatrix}$$

So the stain concentration can be expressed as

$$\vec{C} = M^{-1}\,\overrightarrow{OD}$$

With $M = \begin{bmatrix} \alpha_{r1} & \alpha_{r2} & \alpha_{r3} \\ \alpha_{g1} & \alpha_{g2} & \alpha_{g3} \\ \alpha_{b1} & \alpha_{b2} & \alpha_{b3} \end{bmatrix}$ where the elements of the matrix *M* could be found by measuring the optical density of single pure stains.

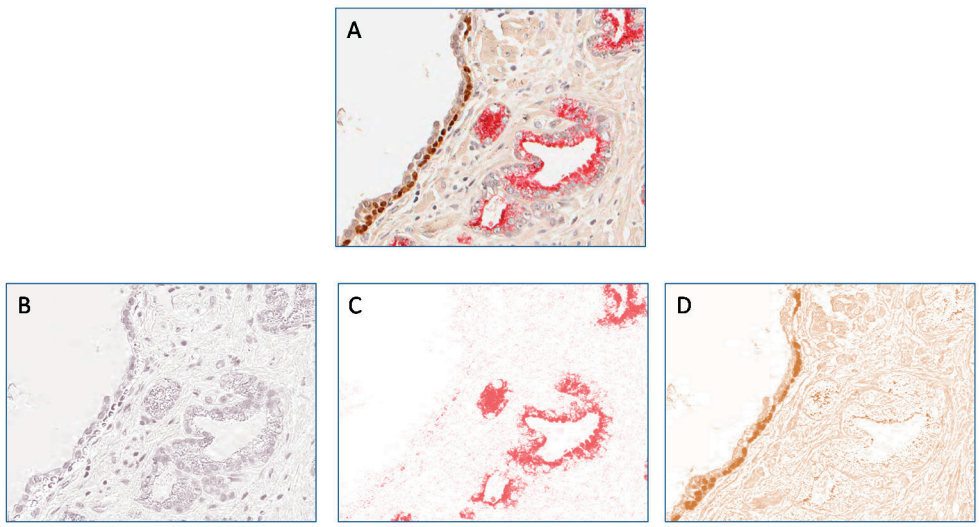

Figure 8 - colour deconvolution result
A, original image of a p63/AMACR stained sample. p63 (D) is expressed in the nuclei of basal cells and it is stained brown; AMACR (C) is expressed in the cytoplasm of cancer cells and it is stained red. A blue counterstain is obtained by applying Haematoxylin (B). Colour deconvolution separated the three stains in three images.

Stain Normalization

Stain/colour variation is an intrinsic problem in histopathology.

Theoretically it would be desirable that two specimens that contain the same amount of antigen would produce the same staining appearance.

However, the staining procedure involves many steps and each one of these affects the quality of the final result. Factors that play a role in the variability are: origin of the biological sample (large specimens like whole prostates or small biopsies pose different levels of difficulty on handling), the pre-processing steps which include the quality of the fixation and the appropriate storing method, the differences in the staining procedures across different labs but also the variability at a single institution depending on the technician's experience, the differences in the manufactures or batches of stains and, in the case of digitized images, the difference in the scanning systems.

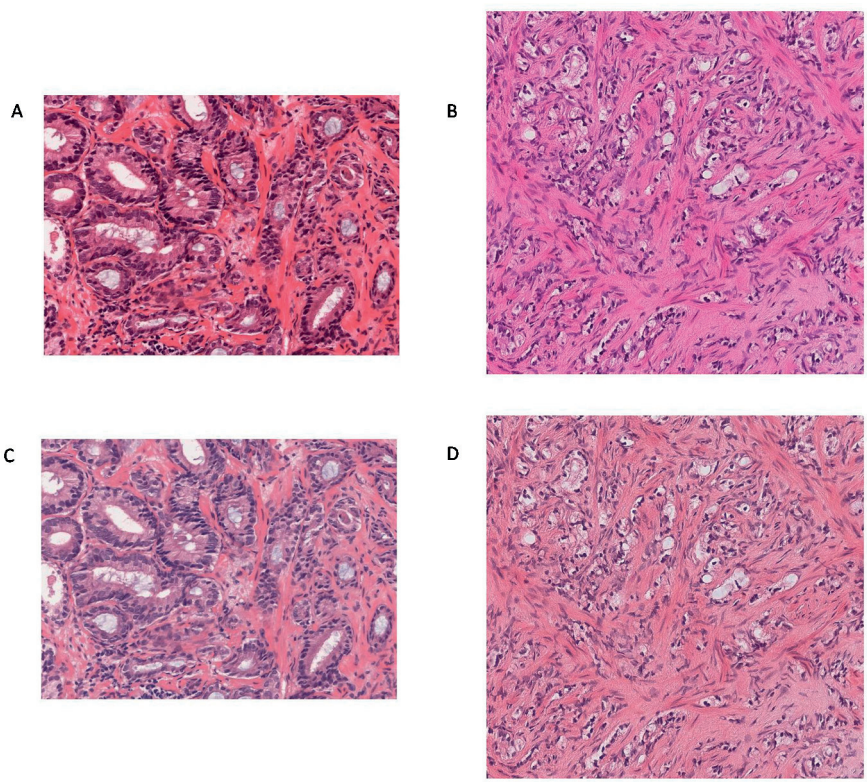

Figure 9 - stain normalization
Original images A and B coming from samples stained for H&E produced in two different laboratories. The colour appearance is different. By applying Macenko normalization, the two images are brought into the same colour space and now appear more similar (C and D).

The quality of the staining can be problematic for a correct assessment by a human eye through a microscope but such variation can even be problematic for computerized systems. In fact even such automated approaches often analyse digital histological images, based on some expected colour parameters.

For this reasons several methods have been suggested to normalize the colour distribution of an acquired image to the one of a reference (or target) image in order to make them comparable [127, 128]. Some studies propose histogram equalization [129], some other have used more articulated methods to first detect nuclei and stroma and then estimate the stain information [128]. Another approach is to estimate the stain concentrations through Single Value Decomposition (SVD) as done by Macenko et al. [130] (Figure 9).

Registration

Image registration is the process of aligning two images, generally called reference and sensed one. Under the last decades many new image acquisition devices have been developed and there is an unprecedented amount of data of different modalities that requires registration procedures. Registration is required when the images to be compared/integrated are either captured from different viewpoints or in different moments in time or from different sensors (multimodality) or when an image is compared to a model one (for example actual patients imaging and anatomical atlases).

The applications in medical imaging include for example the combination of different imaging modalities like ultrasound, CT, PET, MRI or histological images [131–136] that can be useful for examining tumour growth or treatment progress or for image guided radiotherapy. Registration of 2D images for 3D reconstruction is important not only in radiology but also in histology to see tumour invasion fronts [137, 138]. Microscopic image registration [139] and fluorescence [140] are important for localization studies. Registration is often a necessary step before further image segmentation and analysis [141].

In order to perform alignment, corresponding points on the reference and sensed images need to be identified.

In general there is no best registration procedure in absolute terms but every application might benefit from a specific algorithm. However any registration procedure includes 4 steps as described by Zitova et al. [142]: 1)feature detection, 2)feature matching, 3)transformation estimation, 4)transformation application and resampling.

Feature detection

This is the process of searching the images for finding corresponding features. They should be, frequent and robust to image degradation and noise. The features should also be invariant to the kind of transformation that reference and sensed images are linked through. This step defines two families of registration procedures: the area (or intensity) based ones and the features based ones. In the first case, there is no real feature detection. Instead this methodology is based on direct intensity correlations between the two images. Therefore here we speak of direct matching. Among the area based algorithms there are the normalized correlation-like methods that directly use the intensity of the reference and sensed images and find the translation (in its original formulation) that maximize the correlation of the images. The problem here is that this approach is sensitive to illumination changes, noise and it is computationally expensive. Fourier methods use the representation of the image in the frequency domain. They are faster and less sensitive to disturbance. When translation, rotation and scale change are present, other formulations [143] are needed. Another algorithm belonging to this family is the Mutual Information which is based on the maximization of the statistical dependence of two datasets. The method is well described by Viola and Wells [144].

On the other hand the feature-based approach requires the detection of some salient objects which can be closed-boundary regions (with their centre of gravity), lines (like object contours) or points (line intersections, corners, local extrema). Region-like features rely on an initial segmentation step, the accuracy of which can influence the accuracy of the registration. Goshtasby et al. [145] suggested an iterative technique where registration and segmentation were done in parallel with iterative refinement. More recent approach include affinely invariant neighbourhoods and Maximally Stable Extremal Regions (MSER) [146]. Line features are based for example on edges like Canny detector [147] and point features include for example corners like Harris-corners [148] or extrema of Laplacian of Gaussians [149–152]. The feature based approach is preferable in situations where the images contain many distinctive objects with some structure.

Area-based approaches are used when the main information is contained in the pixel intensity of the images rather than in an underlying geometrical structure. This implies that the images must have similar intensities or at least statistically dependent [142]. The feature based methods instead are based on higher level features and can therefore be used successfully for the registration of images with different modalities. Moreover they are generally faster than the area-based ones but they can fail if the images do not contain clearly distinctive characteristics.

Some medical images might be difficult to handle because they sometimes lack enough distinctive structures. This situation requires the user to interact by setting corresponding control points on both images.

Feature matching

This step aims at finding the pair-wise correspondences between the detected features on both images. While some methods use spatial relationships between points, another approach is to identify a descriptor for each feature, that is, a mathematical characterization of the local neighbourhood of a feature. These descriptors should be stable (robust to small deformations and noise), invariant (they should be the same regardless of geometric transformations that can be applied to them), and unique (two different features should have different descriptors) [142]. The matching is done by looking for the pairs of most similar descriptors. The most popular features and corresponding descriptors include Scale Invariant Feature Transform (SIFT) [152] (see SIFT section), the Speeded Up Robust Features (SURF)[153], Histogram of oriented Gradient (HOG) [154], MSER [146], Pyramidal Histogram of Visual words (PHOW) [155].

Sometimes, the set of preliminary matching points can contain many false matches. In this case, the RANdom SAmple Consensus (RANSAC) algorithm [156] can be used to overcome the problem.

Transformation estimation

Once the corresponding points on both images have been identified, then the following step is to estimate the transformation that maps one image onto the other so that the corresponding keypoints are as close as possible. This implies to decide the model of the transformation and the parameters of the corresponding function. The choice of the function depends on the specific application, on the prior knowledge about the two images and on the required accuracy. There exist global and local, rigid and elastic methods. Often the detected points used to estimate the transformation are more than the minimum number required; the transformation is then estimated using least-square fitting.

Transformation application and resampling

The mapping function is used to transform each pixel of the sensed image into the new coordinate system of the reference image. In doing so, an interpolation procedure is needed to reconstruct the values of the sensed image on the new grid. Most common interpolation procedure are nearest neighbour, quadratic splines [157], cubic B-splines [158].

In tissue biomarker research field, there is an increasingly compelling need to analyse simultaneously multiple markers as they could describe more thoroughly some biological processes and to integrate the expression date with morphological information related to the spatial localization of the marker (Figure 10). The ability to selectively analyse biomarker expression in benign as opposed to cancer areas and moreover in region of a specific grade of disease aggressiveness can produce relevant knowledge.

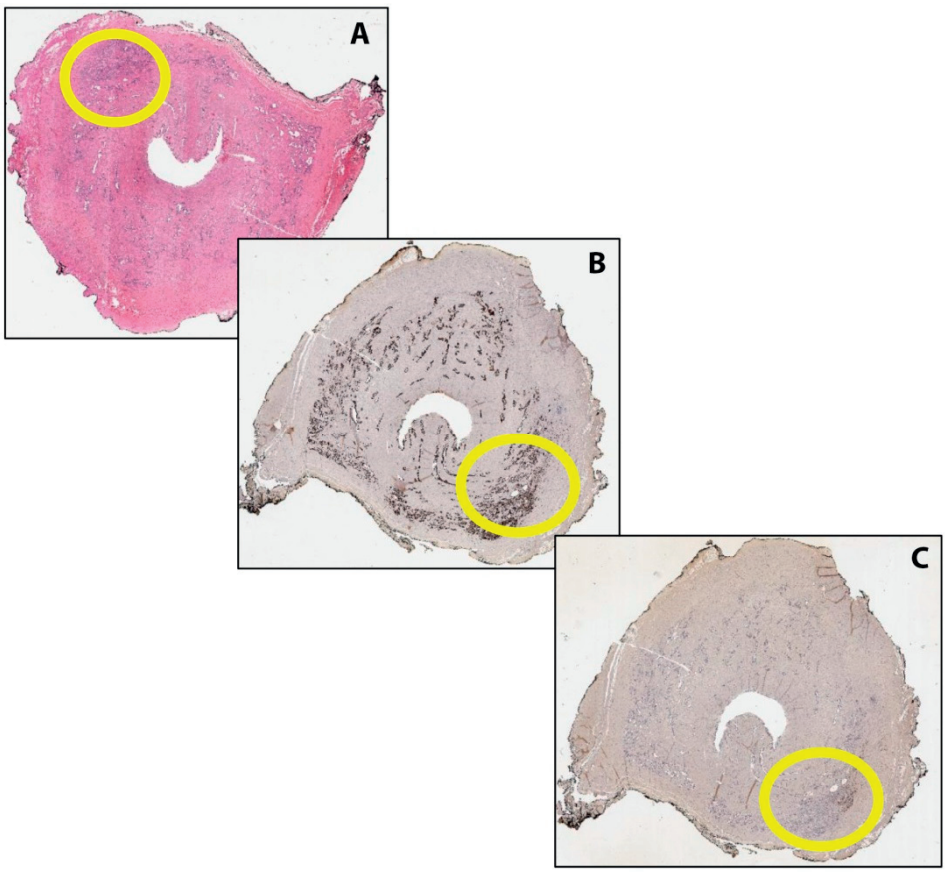

Figure 10 - multi staining analysis
Three consecutive sections from prostatectomy have been stained for H&E for morphological analysis (A) and for two other markers through ICH (B and C). The yellow circle indicates a ROI, the same corresponding area on the three images that should be analysed. In order to perform the analysis the images have to be aligned. However, due to morphological deformations, colour differences and scanning process, registration is not a trivial problem.

SIFT features

As mentioned before, SIFT [152] are some of the most popular features. They are used for registration purposes (as in paper II [159]) as well as for object detection (as in paper IV).

A SIFT feature is a region of an image (keypoint) with an associated descriptor. Keypoints are defined as the extrema of difference of Gaussians (DoG) in scale and

space. The Gaussian scale space can be thought as a pyramid formed by progressively smoothed (and resampled) version of the input image. This is basically equivalent to reducing the resolution of the image. The smoothing level is then referred to as scale of the image. The keypoints represent blob-like structures that keep stable at different scales.

Each keypoint is given an orientation which is determined by the local properties of the image. The local descriptor is then expressed relative to this principal orientation, in this way acquiring rotation invariance.

Some keypoints are then filtered out based on their stability. Those that are located on image edges or those with low contrast are excluded.

The SIFT descriptor is a 3-D spatial histograms of the image gradients in a square patch around the keypoint. The gradient calculated at each pixel location in the patch is regarded as a sample of feature vector formed by gradient orientation and pixel location. The sample is then weighed by gradient norm and stored in the 3D histogram. This is then normalized to form the SIFT descriptor. The original patch is constituted by a 4 X 4 bins square cantered in the keypoint. The size of the bin (in pixels) is a certain multiple of the keypoint scale. In each bin the gradients are accumulated in 8 orientations so that at the end the SIFT descriptor results in a 128 dimension feature vector (Figure 11).

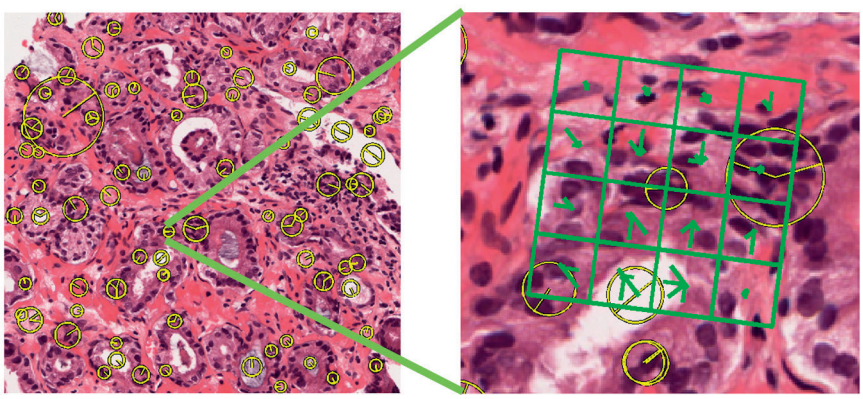

Figure 11 - SIFT keypoints and zoom of local SIFT descriptor.
Left: SIFT detector applied to an H&E image. The yellow circles represent the keypoints, extrema of DoG in scale and space, with their main orientation. Right: the local patch (4x4 bin) where the image gradients are accumulated in a histogram to form a descriptor.

Performance of several descriptors confirming the efficacy of SIFT was investigated by Mikolajczyk et al. [160].

Segmentation

Segmentation is the process of assigning a label to each pixel of a digital image in order to partition the image in 'segments' which share certain characteristics that make them more meaningful to a human user. Segmentation is often the basis of following analysis including feature extraction and classification.

In histopathology this implies to segment the image in order to distinguish for example background from tissue, or specific objects like glandular structures, or identify cells and sub-cellular compartments like nuclei and cytoplasm (Figure 12). Segmentation techniques can be categorized in several ways. One is based on image properties and mainly divides the methods in two families: discontinuity and similarity detection based [161].

The first are based on discontinuities in certain image properties: examples are represented by edge-based algorithms like Canny, corner detectors or borders and line detectors based on the Hough transform [162]. Once the borders of an object are found a postprocessing method is used to create closed-boundary regions.

The second family on the other hand is based on the uniformity of certain properties, like intensity, texture and first and second order statistics in a certain image region. The most popular examples are: thresholding like the traditional Otsu method [163], watershed [164], region growing [165]. There exists several ways of selecting a proper threshold which can be unique (global) over the whole image or adaptive which means that the image is tiled and each tile has an optimal threshold. Some methods estimate the intensity distribution of background and foreground objects and choose a safe threshold above the background intensity as in the case of robust background thresholding used in paper III. Segmentation of fluorescent cytological images [166] is generally easier than histological ones because the latter are more complex and contain more structures [167].

Other segmentation techniques are based on assumed model of neighbourhood pixel dependency like in Markov Random Fields or on a priori models of the objects to be segmented like in active contours [168].

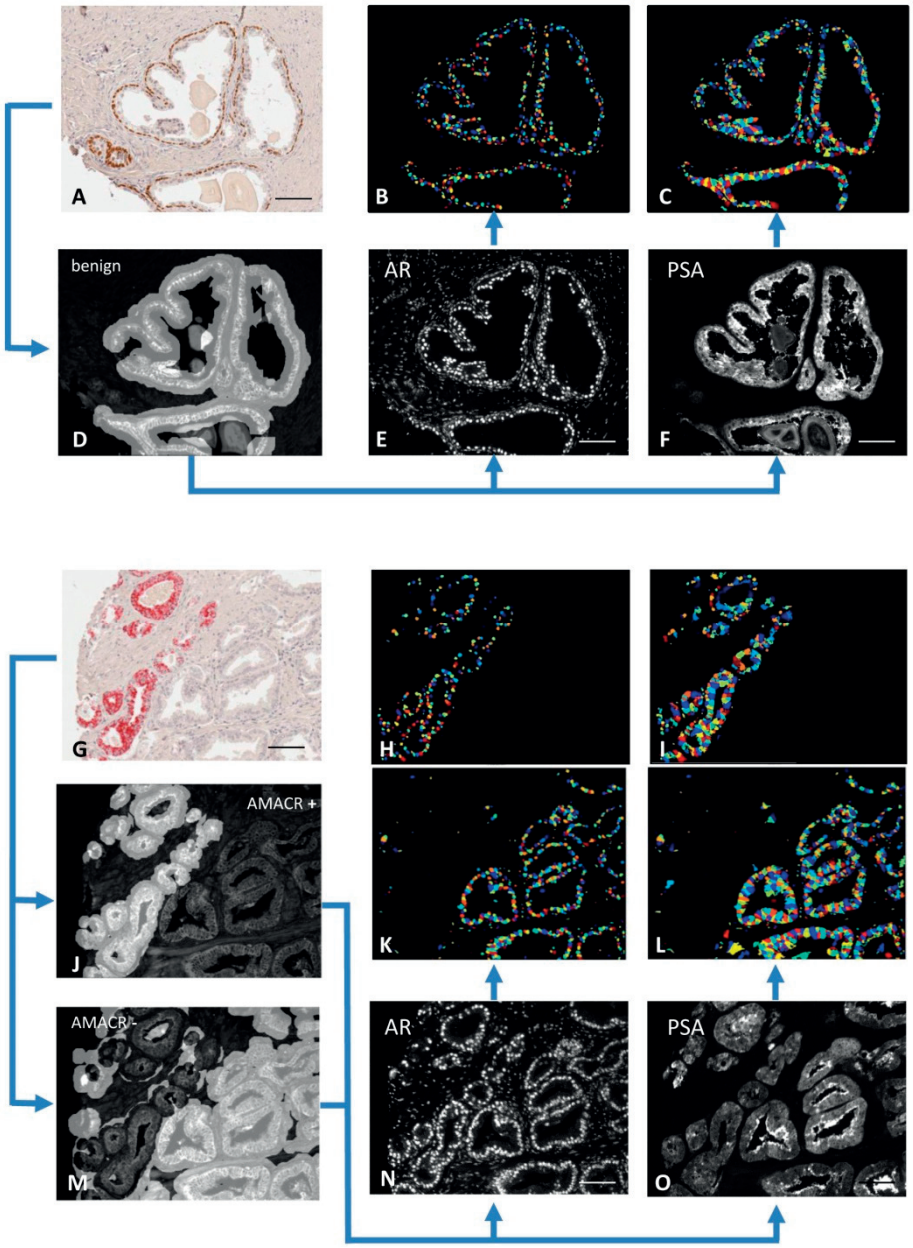

Figure 12 - segmentation of multimodality images.
The figure shows an automated pipeline that includes:1) segmentation of ROIs, that is, benign and cancer (AMACR+ and AMACR-) epithelial cells using pancytokeratin (immunofluorescence) and p63/AMACR (IHC) (A, D, G, J, M); 2) segmentation of cells and nuclei in the ROIs and quantification of AR and PSA on a per-cell base (see paper III for more details).

Segmentation results can be improved by preprocessing steps such as image denoising, spatial and frequency filtering, which tend to enhance certain properties of the image. Another class of algorithms that are used in object identification are the ones based on image saliency such as the spectral residual saliency filter [169] which we used in paper III to segment glandular structures.

Superpixels

Superpixel approach was introduced by Ren and Malik [170] and consists in oversegmenting an image into smaller homogenous regions that adhere to object boundaries. These regions are perceptually more meaningful to a human eye and can replace the individual pixels. They are often used as an initial step to facilitate other segmentation techniques or feature extraction. There exist several implementations of superpixels; an adaptation of the Simple Linear Iterative Clustering (SLIC) [171] was used in paper III to perform segmentation of glandular structures.

Classification

Classification is the process of assigning a certain object to a specific category, or class. In order to perform the classification, an object has to be described adequately. In computer vision terms an object is also called *instance*, the object characteristics *features* and the specific classification procedure is called *classifier*. A classifier is a mathematical function that categorizes new instances.

There are two types of classifications: supervised and unsupervised. Supervised methods build a classifier based on a training dataset, where the class of each instance is known. A good classifier is a method that not only well describes the training data but also generalizes well to a new, independent, dataset. On the other hand, unsupervised classification, which is also called clustering, does not use any pre-labelled training data. The classification in this case is based on some similarity measurement on the features; objects that are close together in the feature space belong to the same class.

Since in many cases the size of available dataset is limited or there is no access to several independent sources, *cross-validation* techniques can be used to estimate the accuracy of the classifier. An example is k-fold cross-validation technique which splits the dataset in k groups, trains the classifier on $k-1$ groups, tests it on the k_{th} group left out and repeats the process k times with each of the k groups used exactly once as testing set. The k accuracies are then averaged to give an estimated accuracy of the model.

In histopathology, objects to be classified can be for instance whole images, ROIs, in case one wants to distinguish between benign and malignant areas or single cells.

Features selection

A feature can be any numerical or categorical descriptor of an object. In histopathology, depending on the specific task, many different features can be extracted from the images. Some features can be related to physical entities directly understandable by a human user, i.e. size of a gland, some others can be the result of mathematical operations like filtering, that a human eye could miss.

Low-level features directly based on pixel values are colour statistics and textural features. Haralick [172] features and Gabor filters [173] are some of the oldest techniques to describe image texture and have been massively used. More modern approach include local binary patterns (LPB) [174, 175] fractal measurements [176] and SIFT-like features based on local gradient histograms (strategy used in paper IV).

Some approaches are based on regular tiling of an image. Multiple features are then extracted from each tile which is then classified independently from neighbouring tiles. Colour and texture features can be used to distinguish for example between stroma and epithelial compartment [177] or between benign and cancer [178].

Other approaches aim at segment objects and then extract object-level features; gland segmentation for example can be a first step before tumour identification and grading [179, 180].

Spatially related features represent another valuable set of topological features that can be described in a mathematically efficient way using graph theory. Voronoi, Delaunay, minimum spanning trees features have been used to describe the cells arrangement in tissue [181].

Attempts to perform Gleason grading have often used large sets of features [176, 182–186] even with multiscale approach to capture significant tissue characteristics [187].

The high dimensionality of the feature space can pose a problem for classification. The so called ‘curse of dimensionality’ describes this phenomenon when adding more variables (features), increases exponentially the complexity of the problem, the computational and storing resources required and the sparsity of the data with consequent detriment for classification. For this reasons dimensionality-reduction methods are applied to try to get rid of redundant or insignificant features before building a classifier. An example is principal component analysis (PCA).

Bag of Words (BoW) approach

BoW model was first introduced in Information Theory as a tool for information retrieval and has then been adopted in computer vision to classify/retrieve images or part of those [188–191].
Given a training dataset of images, the approach consists in the following steps:

- Extract features from every single image
- Encode these features into 'words' and learn a vocabulary of such words
- Create a histogram of occurrences of such words for each image
- The resulting histogram is used as an image descriptor for classification purposes.

The feature extraction techniques are mainly based on two approaches: regular grid (i.e. dense SIFT) and interest point detector [188].
Features encoding is done through vector quantization using for instance k-means or Gaussian mixture models. The histogram can be built over the whole image or as a concatenation of histograms from tiles of an image to retain more spatial information [192].

Support vector machine (SVM)

SVM is a linear classifier in a high-dimensional feature space.

Let's consider a binary (two classes) classification problem where we have a training set of *N* labelled instances $(x_1, y_1), \dots, (x_N, y_N)$, where x_i is called a positive instance if $y_i = +1$ and a negative example if $y_i = -1$. Let's consider that an instance is described by a *d-dimensional* feature vector.

In this R^d feature space, the SVM-predictor is the *(d-1)*-dimensional hyperplane $w^T x + b = 0$ that can separate the training examples in the two positive and negative classes (figure 13).

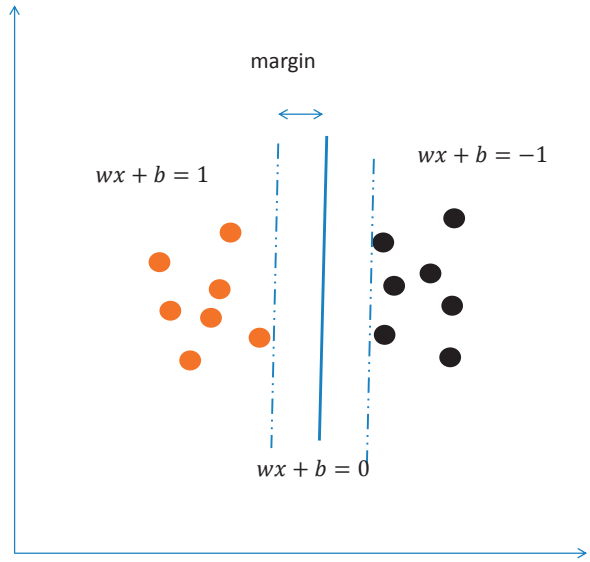

Figure 13 - SVM in a simplified 2D case
The SVM finds the hyperplane $w^T x + b = 0$ the best separates the two classes

The coefficients $w \in R^d$ and $b \in R$ are calculated based on maximization of the margins between the classes and the problem can be solved with a quadratic programming optimization. In many cases the instances of two classes are not perfectly separable; therefore the soft margin approach [193] is used, where the optimization is a trade-off between maximal margins and number of misclassified samples. Once the SVM has been trained, that is, after the coefficients have been trained, any new image will be classified based on the value $y = sign(w^T x + b)$.

The value $s = w^T x + b$, called score can be used for multiclass classification.

SVM can also be used for non-linear classification by using kernel methods [194] which map the feature space to a higher dimensional one, making still possible to use a linear classifier. Linear, polynomial, Gaussian, Chi square kernels (used in paper IV) are examples of this method.

The present investigation

Aims

The overall objective of the present work was to investigate a defined group of PCa tissue markers by applying a suit of novel technologies and Image Analysis tools.

The work can be seen as an itinerary that started with the application of traditional methods for the assessment of tissue biomarkers and developed further towards an automated and more consistent approach.

The specific aims were:

- To confirm the mutually exclusive expression of ERG and TATI protein in PCa tissue.
- To investigate the potential of TRFI as a tool for tissue biomarker quantification.
- To investigate the feasibility of a multimodal, automated system for analysis of tissue sections.
- To apply the developed procedure for analysis of AR and PSA in prostate tissue sections.
- To develop a system for automated Gleason grading.

Paper I: ERG and TATI are expressed in a mutually exclusive way in PCa cells

The multifocality and heterogeneity of PCa poses many problems with regards to determining an accurate prognosis [195]. As in other malignancies, genetic mutations affecting oncogenes and tumour suppressors genes [196], occur in PCa as well.

In 2005, by using COPA bioinformatics tool, Tomlins et al. [72] identified the transcription factors ERG and ETV1, belonging to the ETS family, as outliers in PCa. By using fluorescence in situ hybridization (FISH), a recurrent fusion between

the 5'-untranslated region of *TRMPRSS2* and *ERG* or *ETV1* in those outliers PCas was observed [72]. *ERG* is the most common fusion partner and this rearrangement occurs in at least 50% of PCas [73]. While this seems to be an early event in PCa onset, association with disease stage and the prognostic or predictive role of the fusion gene is still debated [74, 75, 77–79].

In 2008, using the same *in silico* methodology, the *SPINK1* gene was identified as an outlier in PCas that did not harbour the fusion gene event [82]. This subset of *SPINK1* expressing tumours seems to be associated with higher risk of BCR. Moreover *SPINK1* positive cases seem to have worse prognosis following endocrine therapy [83].

Results and discussion

While these findings were based on bioinformatics analysis, there had not been any study that showed mutually exclusive expression at the protein level of *TMPRSS2:ERG* and *SPINK1* in prostatic tumours. Confirming this would support the research in targeting and treating differently patients with genotypically different PCas differently.

We used ERG staining as a surrogate marker of *TMPRSS2:ERG* fusion marker, as described previously [197, 198] and a TATI (the protein encoded by *SPINK1*) monoclonal antibody to perform a double staining of a TMA built from a cohort of 4177 patients who underwent radical prostatectomy with no hormonal adjuvant therapy. Clinicopathological information was collected and TMAs were built as described in previous studies taking one core per patient [199].

Scoring of the TMA was performed manually using a bright field microscope.

We observed ERG positivity in 41.7% and TATI positivity in 5.7% of the cases, in accordance with previous studies. ERG was not expressed by benign epithelial cells. Interestingly we observed ERG positivity also in some PIN, as already described by Furusato et al. [200], which could support the theory that the gene fusion is an early event in PCa onset.

Our data did not show any association between ERG and BCR nor metastatic onset. ERG was positively associated with pathological stage, Gleason score and surgical margin status but not with lymph node status or pre-operative PSA. TATI was moderately associated with pathological stage but not with other parameters, nor was it a predictor of BCR or metastasis.

Our study confirmed, for the first time by immunohistochemical staining, that ERG and TATI are mutually exclusive in PCa cells and therefore provide a basis for stratification and selectively targeting patients with different PCas in order to strive toward personalized treatment.

Paper II and III: integrated image analysis based approach for assessment of PSA and AR in prostatic tissue

The assessment of histological sections is a labour-intensive procedure when one considers the increasing amount of available material. The analysis of tissue biomarkers is now rarely conducted on a single marker, as this is often not enough to understand underlying biological processes. Multi-staining procedures of several markers are therefore necessary. However there is a limit in the number of biomarkers that can be studied at the same time, due to overlapping problems. Various methods have been suggested to analyse multiple markers [107, 201] alternatively registration techniques can be used to analyse consecutive sections.

The use of tissue biomarkers in clinical routine [202] is limited by:

- Pre-processing steps
- Staining procedures
- Objective and reproducible analysis

Even if immunohistochemical staining is still the standard, manual scoring of IHC is rarely reproducible and it has been shown to suffer from inter and intra-observer variability [203–205].

Moreover, there is an unmet need to integrate the biomarker expression data to its spatial location within the tissue. The expression of a protein in specific areas (cellular compartments, benign epithelium, malignant epithelium, stroma, tumour areas with specific aggressive features), can carry important information. The use of a consecutive section stained for H&E is therefore necessary for pointing out ROIs where the biomarkers can be differently expressed.

Such an integrated analysis would rely on an accurate alignment of images from consecutive tissue sections stained for different markers.

Results and discussion paper II

In paper II, we aimed at investigating the feasibility of an automated registration of triplets of images coming from tissue sections stained respectively for H&E, p63-AMACR (standard IHC) and AR (TRFI). As earlier explained, H&E is the primary technique for diagnostic purposes, however it can be assisted by p63-AMACR in particularly complicated cases [25, 65]. We tested a feature based approach using SIFT [152] and Lowe's criterion to detect initial corresponding points on images, RANSAC [156] to reduce the number of points, excluding false correspondences and Procrustes fit to estimate the alignment transformation. We assessed the performance qualitatively and quantitatively and reported the results for different

Gleason grades to see how much the disruption of the normal tissue architecture can affect the accuracy of registration.

We tested the accuracy of the registration for pairs of images: in the first experiment, we performed alignment of H&E and p63-AMACR, in the second, H&E and AR were aligned and in the third, registration of H&E progressively further away from each other was evaluated.

Correct registration was achieved as follows: 96.6% of images in the experiment 1 and 97.2% in experiment 2. Experiment 3 showed a dropped registration success rate in more distant sections. Moreover, as we expected, the performance of the algorithm slightly decreased for the highest Gleason scores cases.

We demonstrated the reliability and speed of the automated registration approach which could therefore be used in paper III.

Results and discussion paper III

In paper III we investigated the expression of PSA and AR, in a TMA from primary PCa samples, as potential biomarkers for the disease. PSA is one of the targets of AR pathway [206]. AR is involved in the normal maintenance of the prostate as well as in the development of PCa and in CRPC [13, 85].

Immunofluorescence allows for multistaining, however it is affected by bleaching problems and it is generally not suitable for paraffin embedded tissues sections due to the high autofluorescence signal from the background.

We suggest the use of TRFI as a method for biomarker quantification at the tissue level. This technique makes use of lanthanide chelates as fluorophores which have high emission peaks, long stokes shifts and long decay times [110]. Thanks to these features we can gate the acquisition of the fluorescent signal after a lapse of time sufficient to almost eliminate the autofluorescence and thereby increasing the SNR. We have optimized a protocol for direct immunostaining of AR and PSA with europium and terbium labelled antibodies which guarantees a better linearity of the signal compared to indirect immunostaining.

Our approach included several staining modalities and techniques: H&E, p63/AMACR - IHC, AR and PSA - TRFI and cytokeratin - immunofluorescence.

The workflow included:

- Automated registration of IHC, H&E and TRFI/immunofluorescence as explained in paper II.
- Identification of ROIs
    - Automated segmentation of IHC image: colour deconvolution of the p63-AMACR images to create a binary mask for benign areas

(those containing p63 positive basal cells) and p63 negative areas containing AMACR (potentially cancer)

    - Segmentation of the immunofluorescence image: pancytoketatin channel is segmented based on the textural features of the glands. We used saliency filter, entropy filter and SLIC superpixel segmentation. The resulting segmented epithelium is then combined with benign and AMACR+ region masks. This results in the final benign, AMACR+ and AMCR- areas.

- Cell segmentation: nuclei are segmented on the europium channel using robust background thresholding and morphological smoothing. We then applied a propagation algorithm to the cytokeratin image using nuclei as seeds to identify corresponding cytoplasm component.
- Extraction of AR and PSA intensity statistics in the ROIs.

We demonstrated differential expressions not only between benign and cancerous tissue but also between AMACR+ and AMACR- cancer areas. We observed a decrease of PSA in cancer tissue *versus* benign and a negative correlation with high Gleason score as reported in other previous works. PSA was higher in AMACR+ than in AMACR- regions. AR expression did not significantly change between benign and cancer areas overall but when analysed separately in AMACR+ and AMACR-, there was a significant increase in the AMACR+ compared to benign and a decrease in AMACR- compared to benign regions. We did not observe any significant correlation between AR and PSA which could be explained with the fact that our data were reported as an average measurement of ROIs and not on a cellular level. Moreover PSA is a secretory protein and this dynamic could be part of the explanation.

Paper IV: automated Gleason grading

Among the current prognostic tools for PCa, the single most important attribute is Gleason grading. Introduced in the 1960's and partially revised in 2005 [40], the system describes the architecture of the prostatic tissue classifying the gradual disruption of the normal glandular structures in more aggressive diseases. The Gleason grade ranges from 1 (most resembling a benign gland) to 5 (loss of glandular structure and scattered cancer cells) and the 2 most common grades in a sample are summed up to form the Gleason score.

Since it has been shown that the scoring is highly dependent on the pathologist's experience and therefore prone to inter as well as intra observer variability [48], in this paper we investigated the possibility to create a computerized Gleason grading system.

Results and discussion paper IV

Our approach involves the use of SIFT features that describe the local texture of patches of an image, the BoW approach to create the image descriptor and the SVM for model building and testing.

In contrast to other studies we used a heterogeneous dataset derived from both prostatectomy and biopsy material and collected and processed at two different institutions. Since each lab has its own protocols and procedures, the resulting H&E staining can be slightly different in appearance. For this reason we considered the use of stain normalization, an algorithmic procedure to make the colours of the images uniform. Using the Macenko formulation [130] we estimated the stain concentration vectors (through SVD) and replaced them with pre-calculated prototype vectors.

The classification framework is based on an appropriate choice of features. Since the Gleason grading is essentially a description of the architecture of prostatic tissue, we decided to focus our analysis on the sole use of texture features. The SIFT [152] finds significant keypoint in an image and appends a mathematical description of their local neighbourhood. SIFT descriptors are rotationally and scale invariant.

After extracting the SIFT feature vectors we then used a BoW approach to cluster the features in visual words and accumulate them in a histogram which is the final image descriptor. This descriptor is then used in the subsequent classification. We used SVM to perform automated classification of different histological cases (benign, Gleason grade 3, Gleason grade 4, and Gleason grade 5) in a binary fashion and reported cross-validation accuracies.

The suggested framework was able to distinguish benign from tumour (images of grade 3, 4 and 5) with 98.1% accuracy. Grade 3 was distinguished from grade 4 with 93.3% accuracy and Grade 3 vs grade 5 yielded 98.2% accuracy. Grade 4 vs grade 5 reached the lowest accuracy, being 87.2%. When we tested the system for low grade (Gleason grade 3) vs high grade (Gleason grade 4 and 5 put together), we observed 93.8% accuracy. Benign tissue seemed to be highly separable from each grade, reaching 98.1%, 97.1% and 100% when compared to grade 3, grade 4 and grade 5, respectively. In a multiclass scheme (one-vs-all), the system performed with 87.3% accuracy.

The use of SIFT was shown to be a valid choice for its scale and rotation invariance and maybe better than dense SIFT, as some of our preliminary experiments suggest. The possible advantage of SIFT is that it identifies the most important landmarks. Our preliminary data seem to show that stain normalization is generally a good method to be applied when working with heterogeneous images. Even if we do not use colour features, the stain normalization is a non-trivial process that changes the appearance of the image and therefore can contribute to producing ‘better’ descriptors.

When compared to other studies[184, 186], the current work has a focus on only one class of features (texture), as they are the ones most closely related to the Gleason system. The proposed framework performed comparably or better than other systems previously described.

Popular science summary

Prostate cancer is one of the most common cancers in the world and the second most common in men. The western world has the highest incidence rates. The causes of prostate cancer are not yet clear, however a number of risk factors have been identified such as familial history, ethnicity, diet and genetic events. Prostate cancer affects primarily elderly men with the majority of the cases happening above 65 years of age. If caught at an early stage, prostate cancer is curable by removal of the whole prostate whereas advanced or recurrent disease is lethal and only palliative methods are available for patients.

Nowadays the tools to diagnose the disease include PSA blood test and a rectal examination conducted by a pathologist to detect suspicious lumps. PSA is a protein produced by the prostate; when its amount goes up beyond a certain level, it may indicate cancer or other pathological conditions that are not life threatening. The only way to be sure that a patient harbours a tumour in the prostate, is to perform a biopsy (generally from multiple areas at once) and analyse it using a microscope. The problem with blood PSA test is that it unfortunately detects many false positives. This can expose the patient to unnecessary treatment and side effects.

The biopsy is used not only to diagnose, but also to assess the potential aggressiveness of the disease by looking at the architecture of the tumour lesions and assigning the so-called “Gleason grade”. The Gleason grade is a prognostic tool, meaning that it is able to predict, to a certain extent, the development of the disease and the response to treatments.

In order to improve both diagnosis and prognosis, we need more reliable markers. A class of such markers is represented by proteins present in the prostatic tissue. Traditionally the way to look at them is by using a normal light microscope, however, this technique is slow and prone to errors and inconsistencies.

In this thesis we investigated the role of ERG, TATI, PSA and AR proteins in prostate cancer by using novel methodologies based on Time Resolved Fluorescence Imaging, digital imaging and automated image analysis.

In paper I we analysed the expression of ERG and TATI in prostate cancer from 4177 patients with a localized disease. We observed that the two proteins were mutually exclusive, as cancer cells that expressed one, did not express the other. This finding is very important because confirms the heterogeneity of prostate cancer

and identifies different families of cancer cells. As a result, the research could focus on targeted therapies and personalized treatments.

In paper II, III and IV we introduced the use of image analysis to study tissue biomarkers. In paper II and III we develop a system for automatic analysis of PSA and AR in tissue sections employing mathematical algorithms for alignment of images, recognition of specific areas of interest within the tissue, and quantification of the markers in those areas. To quantify the markers, we used a novel fluorescence technique that has several advantages over other existing methods. Moreover the use of computerized image analysis allows for consistent and reproducible assessment of tissue sections. Our methods allowed us to observe some interesting expression patterns of the proteins in different clusters of tumour cells and in normal tissue. This kind of differential expression would need to be analysed further to uncover some aspects of the disease. Finally in paper IV we developed an algorithm for automated Gleason grading, which is a system that resembles the pathologist analysis. The system was able to recognize with high accuracy the different Gleason grades and it represents a promising supporting tool for aiding pathologists' work and possibly increasing the accuracy of prognosis.

Acknowledgments

This work was financially supported by European Union 7 Framework/Marie Curie Initial Training Networks (ITN) PRONEST/FP7-PEOPLE Contract no. 238278, and FAST-PATH/FP7-PEOPLE Contract no. 285910, the Swedish Cancer Foundation, the Swedish Research Council, Government Funding of Clinical Research within the National Health Services, Lund University (ALF), the Cancer Foundation at Skåne University Hospital in Malmö, and the Gunnar Nilsson's Cancer Foundation.

Anders, tanks for taking me on board in this long 'trip'. I really respect and admire you as a scientist and as a person. To me it is still a mystery how you can follow so many things, jump in so many projects, operate someone in the morning, have a meeting in Stockholm in the afternoon and talk at a conference in Japan in the evening…what is next? Walking on water? Your energy and dedication are second to none! I want to thank you for the help and the trust you have had in me even when I would talk gibberish (maths, I mean). I am proud of the opportunity you gave me.

Niels-Christian, it was almost half way through my PhD when you joined and since then everything radically changed. Your help has been invaluable. It would have not been possible to achieve my goals if you had not been there with your calm and rationality. Thanks for giving those inputs in my research that I was looking for, for all the fruitful discussions, the *tour de force*s, and all the lunches in Lund!

Kris Cristoforo, my Canadian brother, any word in this case would not be enough. So, actually, I will not say anything...I know you would like me to end the joke there but I can't avoid to thank you for all the support and the incredible moments we have lived during these years from that Bocelli-Xmas-dinner. Fratello. Priceless. Carro, you have been one of the first people I got to know at the lab. You are such a smart and kind person and often wiser than many of us. Thanks for your advices and for being there any time I would be need help. Sofia, giraffe, thanks for the rollercoaster! You showed me the real Sweden and helped me to feel more at home here. You are a valuable person and I have so many nice memories from these years. I hope you will always be happy.

Thanks to all co-authors and collaborators: Anders Edsjö, Ulf-Håkan Stenman, Leszek Helczynski, Agi Krzyzanowska, Mari Peltola, Kim Pettersson, Assem Anand, Hans Lilja, Nick McCarthy, Peter Hamilton, Olivia Larne, Elena Martens-Uzunova, Zandra Hagman, Mirella den Berg, Guido Jenster, Yvonne Ceder, Erik

Ask, Olof Enqvist, Linus Svärm, Fredrik Kahl, Hannah Nenonen, Paal Skjærpe; Azharuddin Sajid; Christel Björk; Johan Svartberg; Alexander Giwercman; Yvonne Giwercman and Ari Kuusisto.

Thanks to past and present members of the group: Azharuddin, Anna D, Susan, Rebecka, Nick, Anna S. Giacomo, and partuclarly Agi for the fruitful collaboration and the great help in this last part of my PhD. Thanks to Anna Holst as well for her constant help. You certainly helped me to grow; I am happy we shared this time together and I am sure there will be other chances in the future.

Thanks to all past and present colleagues of CMP, those who stayed and those who moved to Lund, particularly: Martin, Håkan, Maite, Tamae, Totte, Karin, Marica, Greta, Arash, Roni, Louise, Sophie, David, Jennifer, Susan and the youngest ones. Despite our research has been quite different, I truly enjoyed working together.

Thanks to Christina, Elisabet, Siv and Elise for their competence and help with everything at the lab.

Thanks to all PRONEST fellows, particularly Janine and Ines; you are brilliant and so much fun!

Special thanks to PathXL Ltd, Peter, Jim, David, and all my 'northern Irish mates', particularly Mike, Nick, Catarina, Cathrine, Lourdes, Manuel, Olivier and Ivan for the great adventure I lived in Belfast. You welcomed me as if I had always been part of the group and working there has been a hugely formative experience for me.

To my friends in Sweden, my gratitude is infinite. Ciccio, fratello acquisito (a volte minore, a volte maggiore) di una vita, folle e visionario, come i migliori; Ricky-fru, your mental strength has always impressed me, despite your Alzheimer; Xello il nostro ragazzo d'oro, punto; Juanito you are such a great and loyal friend, during those breaks from your silly training; David S el mas sueco de nosotros, you are our jolly; Tibodó, so positive and optimist despite the 'frenchness'; Daniel, surprising what a great guy you can be when you don't sleep; Tobbe and Samme, unbeatable wine and beer connoisseur; David W one of my first Swedish friends and a true one. You guys have become a real family! You live life to the fullest, I am proud of what you are achieving and I am so lucky to have you.

Un grazie ai miei amici in Italia, specialmente a Francesco, Flaviano, Valerio perché la nostra amicizia non é mai cambiata nonostante la mia lontananza.

Last, but not least, grazie alla mia famiglia, Frá, frater, che, sotto quei modi burberi, covi un gran cuore da terrone, mamma e papa, le persone piú importanti, i miei modelli di vita, esempi di tenerezza, intelligenza e apertura mentale. Siete stati il mio trampolino. Vi voglio bene.

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