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E tudo isso, que é tanto, é pouco para o que eu quero.

And all of that, which is a lot, is not enough for what I want.

Álvaro de Campos

TABLE OF CONTENTS

LIST OF ABREVIATIONS

18FDG-PET scan	18F-deoxyglucose Positron Emission Tomography Scan
ATP	Adenosine Triphosphate
AUA	American Urology Association
AUC	Area under the Curve
BPH	Benign Prostatic Hypertrophy
CT	Computer Tomography
DAB	3,3′-Diaminobenzidine
DRE	Digital Rectal Examination
EAU	European Association of Urology
EDIM	Epitope Detection in Monocytes
H&E	Hematoxylin and Eosin
IHC	Immunohistochemistry
LHRH	luteinizing-hormone-releasing hormone
MRI	Magnetic Resonance Imaging
NADPH	Reduced form of Nicotinamide Adenine Dinucleotide
PCA	Prostate Cancer
PSA	Prostate-specific Antigen
RCT	Randomized Clinical Trials
ROS	Reactive Oxygen Species
TKT	Transketolase
TKTL1	Transketolase-like 1
TMA	Tissue Microarray
TNM	Tumour Node Metastasis Classification
TURP	Transurethral Resection of the Prostate

1 INTRODUCTION

Prostate cancer is the second most common cancer among men worldwide, so an urgent need exists for new diagnostic, prognostic and predictive tests. The intent of developing new tests would be to reduce the number of unnecessary biopsies (pre-diagnostic tests), identify the aggressiveness of the disease, predict disease progression, predict biochemical recurrence and survival (post-diagnostic tests), and hopefully offer better detection rates and disease monitoring.

Prostate cancer is a heterogenic cancer, with numerous aggression levels and outcomes. In this study we intend to better understand the molecular pathogenesis of prostate cancer, specifically glucose metabolism deregulation. This thesis hypothesizes that glucose metabolism deregulated markers – TKTL1 – may play a role in the tumorigenesis of prostate cancer.

1.1 BACKGROUND: PROSTATE CANCER

1.1.1 EPIDEMIOLOGY

Prostate cancer is the second most common cancer among men worldwide (excluding non-melanoma skin cancer), with nearly 1,1 million new cases in 2012 [1].

In 2012 in Europe, prostate cancer was the most common cancer among men, and lung cancer the second most common. Prostate cancer has an estimated incidence for European men of 92.1 per 100,000 inhabitants, which places it first in incidence, but not in mortality ranking — lung and colorectal cancers occupy the top spots of that chart [2].

Germany's incidence rate of prostate cancer is higher than in the rest of Europe at 114.1 per 100.000 inhabitants, but the mortality rate in Germany is lower (17.8 per 100,000 inhabitants). In 2012, 63,710 new cases of prostate cancer were detected in Germany, being the prognosis for 2016 of 66,900 men. The most common T stage at diagnosis was T1 (27%) and T2 (48%), between ages 70-74 and more than 85 [3].

1.1.2 RISK FACTORS

In comparison to other common cancers, the etiology is still not well understood, and the only three risk factors established are age, ethnicity and family history.

Ageing increases the risk of prostate cancer. Autopsies confirm the prevalence of prostate cancer increases with age [4], and is mainly detected in patients older than 50. Almost half the population older than 70 years of age had prostate cancer [5].

Prostate cancer incidence varies among countries and continents; **ethnic origin** is considered a well-established risk factor. Asians have the lowest incidence while Australians and New Zealanders have higher rates [1].

Disparities between races might be caused by environmental and/or genetic conditions. Observational studies of prostate cancer in migrant inhabitants show Japanese migrants (a country with low incidence) in the United States (high incidence) will show a rise in prostate cancer rates after immigrating [6].

The last well-established risk factor is **hereditary**. A study by Morganti et al. in 1956 described familial aggregation in prostate cancer, in men who had relatives with the disease [7]. The probabilities of having prostate cancer is higher for men with a stronger family history [8].

Definition of hereditary prostate cancer
Three or more affected relatives
or
At least two relatives who developed early-onset disease (before age 55)

Diet, lifestyles and potential exposures may influence the development and incidence of prostate cancer worldwide. Currently there is no strong evidence to suggest or recommend alterations in diet or lifestyles to reduce the risk of prostate cancer [9].

1.1.3 CLASSIFICATION

1.1.3.1 GRADING SYSTEM

When prostate cancer is diagnosed it is graded histologically. The current grading system was created by D.F. Gleason in 1966 [10], and since then has been slightly modified [11,12]. It still remains one of the most powerful prognostic tools in prostate cancer [13].

The Gleason system is an architectural assessment of hematoxylin and eosin stained prostatic tissue sections based on a low-power microscopic (10x–40x) assessment. The architectural changes are classified from 1 to 5 — the higher the number, the less normal the cells are (poorly differentiated).

To assign a Gleason score/sum, the pathologist sums the Gleason grade of the 2 most common types of glandular growth patterns within the tumour, this means the Gleason score ranges from 2 to 10. A sum of 7 can result from 3+4 or 4+3, and Gleason score 3+4=7 does not have the same prognostic outcome as a Gleason score 4+3=7, the latter having a worse prognostic outcome, but both are placed in the same prognostic group (in D'Amico risk classification) [14,15]. Grades 1 and 2 closely resemble normal tissue and are rarely diagnosed [16], so the diagnosis is almost always undergraded. Grades 1 and 2 are often an incidental finding in transition zone carcinomas in specimens

extracted from transurethral resection of the prostate (TURP). In 2000, Epstein mentioned that Grade 1 and 2 on needle biopsies should not be made, leading to a narrowing of possible Gleason sum results, from 6 to 10 [17].

Recently a new alternative grading system for prostate cancer has been validated that overcomes these previous deficiencies (see Table 1) [18].

ISUP Modified Gleason Grading	
Group 1	Gleason score ≤6
Group 2	Gleason score 3 + 4 = 7a
Group 3	Gleason score 4 + 3 = 7b
Group 4	Gleason score 8
Group 5	Gleason score 9-10

Table 1: Modified Gleason score - 2014 International Society of Urological Pathology *(ISUP)*

1.1.3.2 STAGING SYSTEM

The staging system used in prostate cancer is the Tumour Node Metastasis (TNM) Classification, an anatomically based system describing the primary and regional nodal extent of the tumour and the presence or absence of metastases. TNM is a dual staging system that includes a clinical (pretreatment) and a pathological (postsurgical histopathological) classification (see Table 2).

Primary tumour	
TX	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour not palpable or visible by imaging T1a Tumour incidental histological finding in 5% or less of tissue resected T1b Tumour incidental histological finding in more than 5% of tissue resected T1c Tumour identified by needle biopsy (e.g. because of elevated PSA level)
T2	Tumour confined within the prostate T2a Tumour involves one half of one lobe or less T2b Tumour involves more than half of one lobe, but not both lobes T2c Tumour involves both lobes
T3	Tumour extends through the prostatic capsule T3a Extracapsular extension (unilateral or bilateral) including microscopic bladder neck involvement T3b Tumour invades seminal vesicle(s)

T4	Tumour is fixed or invades adjacent structures other than seminal vesicles: external sphincter, rectum, levator muscles, and/or pelvic wall
Regional lymph node	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis	
MX	Distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis M1a Non-regional lymph node(s) M1b Bone(s) M1c Other site(s)

Table 2: TNM Classification – Prostate cancer

1.1.3.3 EAU RISK GROUP CLASSIFICATION

The EAU risk group Classification is essentially based on D'Amico's classification system. The D'Amico classification was created in 1998 to stratify patients with prostate cancer into 3 groups: low, intermediate and high-risk of biochemical recurrence after surgery (see Table 3). The parameters used included the clinical TNM classification, biopsy Gleason score and preoperative PSA-levels [19].

Low risk	Intermediate	High risk	
PSA < 10 ng/mL And GS < 7 And cT1-2a	PSA 10-20 ng/mL or GS 7 or cT2b	PSA >20 ng/mL or GS >7 or cT2c	Any PSA any GS cT3-T4 or cN+
Localized			Locally advanced

Table 3: EAU risk group classification. GS: Gleason Score, PSA: Prostate specific antigen

Since Gleason scores 7a and 7b don't have the same outcome [20], a modification of the EAU risk group classification due to the implementation of the new Gleason grading might be needed in the near future.

1.1.4 DIAGNOSIS

1.1.4.1 PSA BASED SCREENING

PSA is a glycoprotein secreted by the prostatic cells into the glands' lumen. Its function is believed to be liquefy the seminal fluid (proteolysis of semenogelin I, SEMG1 and SEMG2) when activated in the lumen [21]. PSA is produced in normal, in benign prostatic hypertrophy (BPH) and in prostate cancer. When there is a disruption of the basal membrane and a distortion of the normal architectural configuration of the gland happens, PSA leaks (exaggeratedly) into the circulatory system leading to a rise in the serum PSA. This PSA-leakage and rise in serum can occur in normal tissue, during infection, in BPH or in cancer [22]. So using total PSA as a single element to detect prostate cancer is risky and not recommended.

Using the PSA-testing alone for screening is not recommended, but a new concept was introduced: early diagnosis [9]. Early diagnosis/opportunistic screening is still recommended on an individual basis under certain circumstances. The AUA Guidelines do not distinguish screening and early diagnosis, indicating both early detection and screening imply detection of disease at an early, pre-symptomatic stage when a man would otherwise have no reason to seek medical care — an intervention referred to as secondary prevention [23]. In contrast, EAU Guidelines / recommendation differentiate mass screening from early detection distinctly, the latter requiring an informed consent from the patient following a discussion about the pros and cons of PSA based screening [9].

A threshold of total PSA >4ng/ml was proposed to detect prostate cancer, but this has remained controversial from the beginning [24]. PSA works as a biomarker with diagnostic, predictive and prognostic [25] properties, but its use has limitations, so the need to find new biomarkers remains [26]. PSA can be present in blood in a variety of forms, the majority bound to protease inhibitors — complexed PSA. The non-complexed form of PSA is called free PSA. The

free PSA ratio test can be used to help differentiate BPH from prostate cancer, especially when total PSA values vary between 4 and 10 ng/ml, values which are often difficult for the urologist to interpret and act upon (See Table 4.) [27].

Total PSA, ng/ml	Free PSA, %	Probability of cancer, %
4-10	0-10	56
	10-15	28
	15-20	20
	20-25	16
	>25	8

Table 4: Probability of cancer based of free PSA when total PSA lies between 4 and 10ng/ml [28]

1.1.4.2 DIGITAL RECTAL EXAMINATION (DRE)

The zone palpated during DRE is the peripheral zone, where most prostate cancers are located. A clinical suspicion of DRE and/or PSA levels usually lead to a prostate biopsy, although this decision is normally not so straightforward as wished. An abnormal DRE is always an indication for a prostate biopsy [9].

1.1.4.3 BIOPSY

Diagnosis of prostate cancer must be confirmed with a needle prostate biopsy. A transrectal approach is used more frequently, although a perineal approach is also possible. The number of core biopsies recommended is between 10 to 12 under transrectal ultrasound guidance [28]. Other approaches exist, but they will not be mentioned in this thesis.

1.1.5 TREATMENT OPTIONS

Treatment options for prostate cancer are vast and may sometimes elicit contradictory opinions. It is beyond the scope of this thesis to describe all available treatment options in detail.

1.1.5.1 WATCHFUL WAITING

Tumour growth is relatively slow and constant in many patients, so this treatment option should be given to those with localized disease ineligible for local curative treatment, or with a life expectancy less than 10 years [29]. If the

patients develop disease-related symptoms in the future, palliative therapy should be offered to improve quality of life. These measures might include transurethral resection of the prostate (TURP), hormonal therapy, or radiotherapy.

1.1.5.2 ACTIVE SURVEILLANCE

To avoid overtreatment of men with low-risk prostate cancer, active surveillance should be offered. In contrary to watchful waiting, *active surveillance* has a curative intent. The patient should be under a regular follow-up schedule, including PSA-testing and DRE, with eventual biopsies and MRI to determine the optimal time for curative treatment. It appears active surveillance reduces the overtreatment rate, is safe [30], and does not compromise the overall survival compared to patients receiving upfront local curative therapy [31].

1.1.5.3 RADICAL PROSTATECTOMY

This surgery includes removal of the prostate, part of the urethra, seminal vesicles, and surrounding tissue to obtain a negative margin. Bilateral pelvic lymphadenectomy should be performed in patients with an intermediate or high-risk. The surgery can be performed as an open surgery, minimal invasive as either laparoscopic or robotic assisted [32]. The robotic assisted radical prostatectomy is becoming more common, probably due to the lower rate of complications [33], including decreased blood loss, blood transfusions, and hospital stay duration. [34] Most importantly, the oncological and functional outcome is similar to open surgery [35,36].

1.1.5.4 RADIOTHERAPY

Radiotherapy is also commonly used as a curative treatment. It may be offered to patients with low risk prostate cancer without locally advanced disease. It can be given alone, in combination (with hormone therapy), as an salvage agent when PSA increases, or in a multimodalidity setting in patients with locally advanced cancer or in N1 Stage (pelvic external irradiation).

While there are multiple approaches in radiotherapy, two of the most used in prostate cancer are: External-beam radiotherapy, consisting of high-energy radiation beams administered on a daily basis as an outpatient therapy. The recommended modality is the Intensity-modulated radiotherapy (IMRT) [37], Here, the clinician can alter the intensity according to the target volume and proximity to pelvic organs [38]. Brachytherapy is another option, preferentially given to patients with low risk assessments. Here radioactive seeds (around 100 in number) are inserted in the prostate gland working locally [39]. Brachytherapy has been shown to be an effective treatment for low-risk and low-intermediate-risk prostate cancer [40].

1.1.5.5 HORMONE THERAPY

Prostate growth is regulated by androgens (testosterone and dihydrotestosterone [DHT]). Initially, PC usually is dependent on androgens for growth and survival, so androgen suppression in patients with advanced prostate cancer is recommended, a process known as castration [41]. One of the earliest forms of castration performed was the bilateral orchiectomy and the use of oestrogens, including Diethylstilbestrol, but in 1960s the VACURD study revealed the use of oral oestrogen to lower serum testosterone was associated with high cardiovascular morbidity and mortality [42–44].

In the 1960s and 1980s new therapy forms started to develop, involving inhibition of testicular androgen secretions or inhibition of the circulating androgens' action, or a combination of these two pathways.

Currently the main type of hormone therapy is the long-acting luteinizing-hormone-releasing hormone agonists, also known as analogues of LHRH. Another form of hormone therapy is the LHRH antagonist which, in contrast to the previous therapy, will directly block LHRH receptors, achieving an earlier state of castration and avoiding the flare-up phenomenon. The third group includes the anti-androgens, which block the effects of adrenal androgens at the androgen receptor.

Castration is achieved when both PSA and testosterone remain below certain thresholds. If during treatment there is a progression in PSA-levels even though an optimal castrate serum testosterone is present, we are faced with a castration-resistant prostate cancer. Here other therapy options should come into play, including abiraterone acetate (CYP17 inhibitor), enzalutamide (novel anti-androgen) or chemotherapy. Examples of chemotherapeutic agents used include docetaxel and cabazitaxel [45].

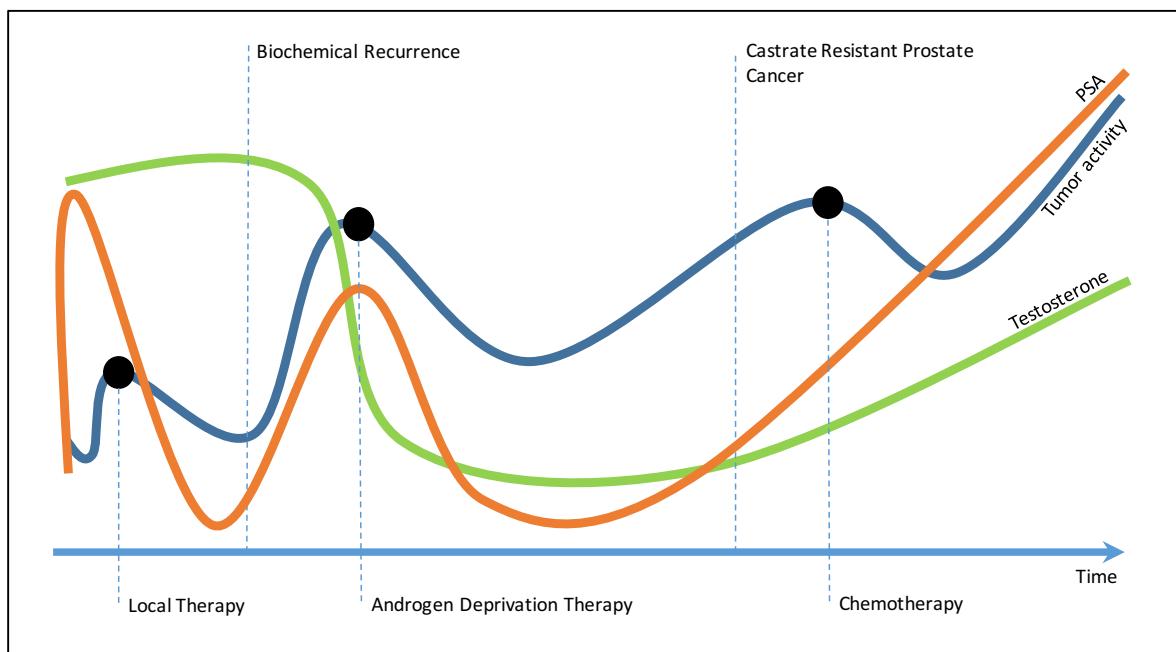## 1.1.6 OVERVIEW OF DISEASE PROGRESSION

Figure 1: Overview of the alterations in testosterone and PSA along prostate cancer progression. An increase in tumour activity after local therapy is indicated by rising in PSA levels, described as biochemical failure or recurrence. The next step will be initiating a medical castration, which will reduce the testosterone levels, diminishing the tumour activity resulting in PSA levels decrease. The period of time which the PSA levels maintain low during hormonal therapy is described as hormone sensitive prostate cancer. If there is an augmentation of the tumour activity, it will be showed a rise in PSA even though testosterone level maintains low, entering in a phase of hormone-resistance prostate cancer.

1.2 BACKGROUND: CANCER AND METABOLISM

In recent decades, carcinogenesis has been intensively studied and great interest has been generated worldwide to discover the cure for cancer. In 2000, an article was published suggesting the existence of 6 traits normal cells must acquire to transform into malignant cells. These traits deregulate certain physiological pathways, leading to cellular instability. The 6 Hallmarks of cancer proposed are: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [46]. In the past 16 years, multiple experimental drugs were developed to target these 6 characteristics. Although there has been intense research in a variety of areas including molecular biology, genetics, metabolism, and pharmacology, an optimal approach to cancer has not been yet found. However, new traits have been discovered, and four traits were proposed in 2011: Emerging Hallmarks include avoiding immune destruction and **deregulating cellular energetics,** and two enabling characteristics including tumour-promoting inflammation and genome instability and mutation [47].

In this thesis the focus is especially on the emerging **Hallmark -- deregulating cellular energetics**.

1.2.1 OVERVIEW OF GLUCOSE METABOLISM

The organism is generally exposed to a constant supply of nutrients, maintaining equilibrium between nutrient availability and cell proliferation. Nutrient uptake and metabolism are highly regulated to prevent abnormal proliferation. An altered or reprogrammed metabolism is considered one of the hallmarks of cancer cells [48], in order to support their rapid proliferation and expansion across the body.

Glucose is a key metabolite in human metabolism; the main purpose of glucose degradation is the generation of energy (Figure 2) — adenosine triphosphate (ATP). There are various pathways to metabolize storage or regenerate

glucose. The first step in glucose degradation is named glycolysis, which the end product is pyruvate and ATP. More ATP can be produced when the glycolysis process continues: in an aerobic condition, pyruvate will be transported to the mitochondrial matrix and converted in Acetyl-Coenzyme A (Acetyl-CoA) in two ways, or suffer a complete degradation/oxidation in the citric acid cycle, or for synthesis of cholesterol and fatty acids. When in anaerobic conditions, pyruvate will be converted in lactate, a process commonly found in red cells by absence of mitochondria [49]. It will be released in the bloodstream and recycled in the presence of oxygen. Another possible pathway for pyruvate is the synthesis of amino acids. When there is excess glucose, the cell will store it in the form of glycogen, to be broken down when needed.

Another pathway, important for pentoses production is the pentose phosphate pathway (PPP). This pathway produces ribose from glucose concomitantly producing 2 reduced nicotinamide adenine dinucleotide (NADPH). The pentoses are needed for formation of DNA and RNA, and also work as cofactors in enzymes. The production of NADPH is important for other pathways, which need NADPH and to avoid or prevent oxidative damage by radicals [50]. PPP neither requires ATP nor produces it. If excess NADPH is generated, it will inhibit the pathway in a feedback process. Pentose in excess can transform back to glucose, a process involving numerous enzymes, including **transketolase** and transaldolase. Special attention will be given to the PPP and transketolase throughout the thesis.
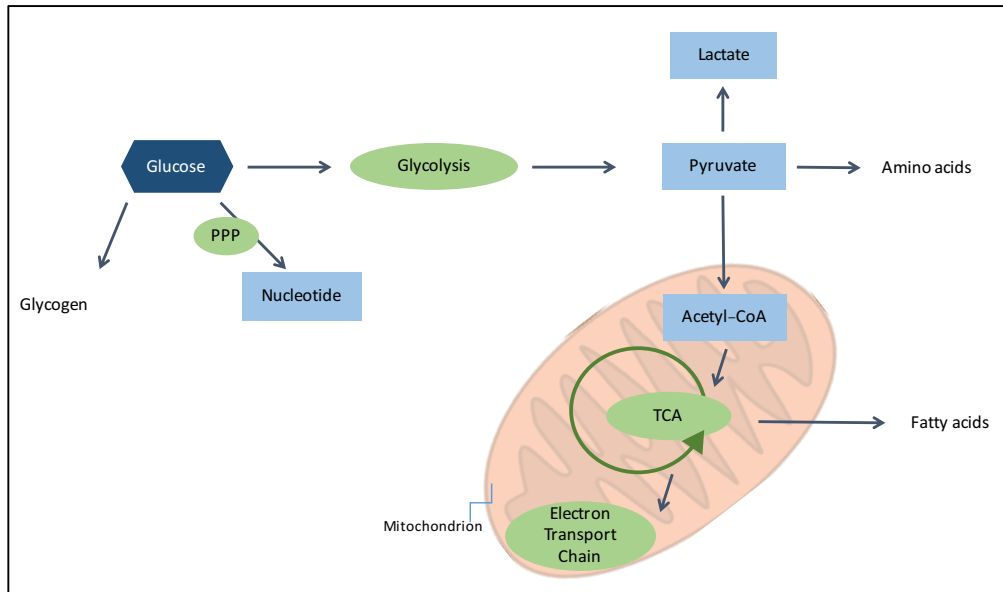
Figure 2: Overview of glucose metabolism in normal cells, adapted from Pérez-Mancera P. et al 2014 [188]

1.2.1.1 Research in Glucose metabolism in malignant cells

Cancer cells exhibit enhanced glycolysis and in aerobic conditions tend to have increased glucose uptake and lactate secretion [51]. This metabolic phenomenon is known as "aerobic glycolysis" (see Figure 3). This finding was first described by Otto Warburg in the 1920s [52] and for historical reasons glucose degradation to lactate even in the presence of oxygen is also named Warburg effect. In spite of the low energy outcome of aerobic glycolysis, it seems to be advantageous for tumour cells to supress mitochondrial metabolism. This may lead to increased cell proliferation (deviation from apoptosis) and a faster growth of cells [53–56].

In 1929, Herbert Crabtree replicated the findings of Warburg and also showed that high concentrations of glucose decreased mitochondrial function, a reversible phenomenon influenced by external/trigger factors, such as environment or genes [57,58].
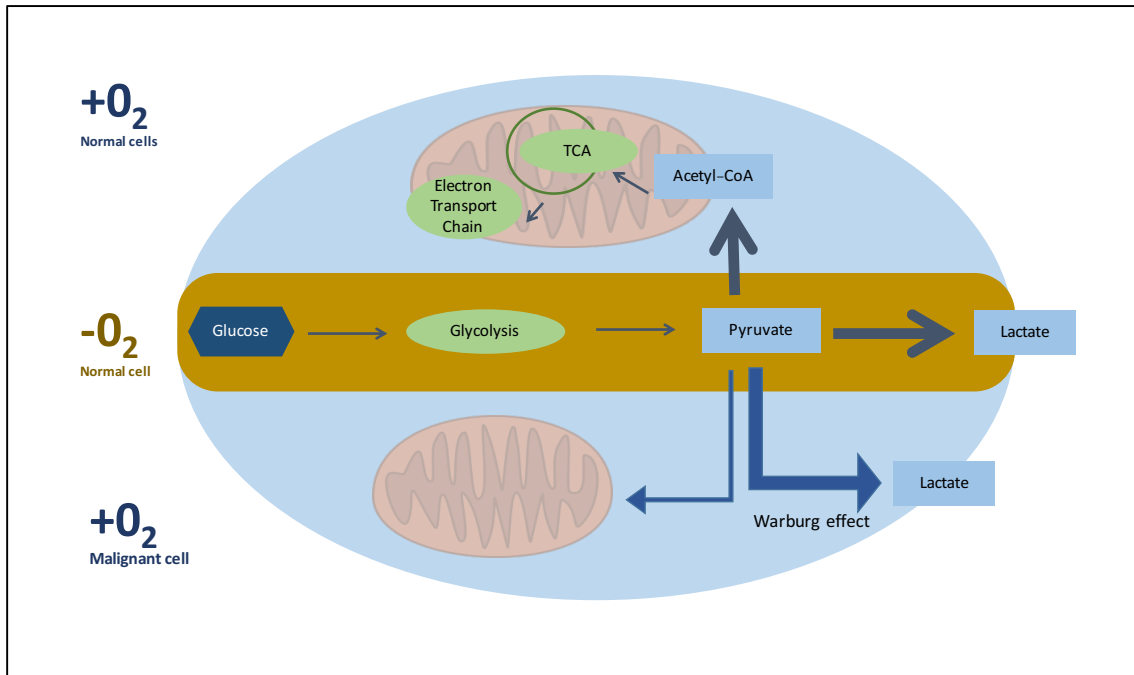
Figure 3: Schematic overview of glycolysis- anaerobic glycolysis and aerobic glycolysis in the presence or absence of oxygen, adapted from Zhang W. Et Al 2015 [189]. $+O_2$ - in presence of oxygen; $-O_2$ - in absence of oxygen.

In the late 1970s, a growing interest in cancer regulation mechanisms and signalling pathways started to arise, and oncogenes emerged as an established field in the early 1980s [59,60]. First, oncogenes were discovered through the study of retroviruses [61]. In the past decades the impact of genetic mutations, tumour suppressors, and oncogenes has been massively investigated. But only in the last 20 years, a link between the regulators of cell division and cell metabolism has been discovered. Here, it was shown that alterations in cellular signalling pathways could lead to aberrant metabolic programming [62–65].

It is becoming clear that many oncogenic signalling pathways (see Table 5), affect tumour cell metabolism in order to support their growth and survival.

Regulatory factors	Reviews
PI3K pathway (PTEN, AKT1)	[66–69]
HIF1 and MYC	[70]
AMP-activated protein kinase.	[71,72]
P53 and OCT1	[73]

Table 5: Example of regulatory factors controlling glycolysis in cancer cells

Increased knowledge of malignant cell metabolism is driving the discovery of new drug targets and new diagnostic tools. One example is the use of 18F-deoxyglucose positron emission tomography scanning (18FDG-PET scan) for imaging of solid tumours. This diagnostic tool has clearly shown us Warburg's findings in a macro scale — an accelerated glucose metabolism is the basis of 18FDG-PET in detecting cancer [74].

1.2.1.2 THE PENTOSE PHOSPHATE PATHWAY

The pentose phosphate pathway is a major pathway for *glucose catabolism* and the main source of NADPH in the body. A series of reactions, called glycolysis, break down glucose. Some of the glucose-6-phosphate (first metabolite of the glycolysis process) is shunted from the glycolysis process and enters the pentose phosphate pathway. Here the glucose-6-phosphate will be broken down, but the end product will not be ATP as expected, but ribose-5-phosphate and NADPH (primary product of PPP) (see Figure 4).

Ribose-5-phosphate - is a precursor to nucleotide, DNA and RNA synthesis.

In cancer cells these are extremely important due to the high rate of proliferation. So a high flux of glucose in the PPP will help the tumour cell to divide rapidly [75].

NADPH - is a reducing agent, needed to control reactive oxygen species (ROS) levels. Higher levels of ROS stimulate apoptosis.

In cancer cells, due to increased metabolism, ROS levels will increase. NADPH helps maintain a better environment to promote cell growth by suppressing apoptosis [76].

As shown in Figure 4, the PPP has 2 arms: an oxidative arm (non-reversible) and a non-oxidative arm (reversible). An important rate-limiting enzyme of the oxidative arm is the *glucose-6-phosphate dehydrogenase*. Its role in oncogenic metabolism and overexpression in multiple tumours has been described in different cancers [77,78], including cervical cancer [79], gastric cancer [80], hepatocellular carcinoma [81], colon cancer [82], clear-cell renal cell carcinoma [83], prostate cancer [84] and urothelial cancer [85].
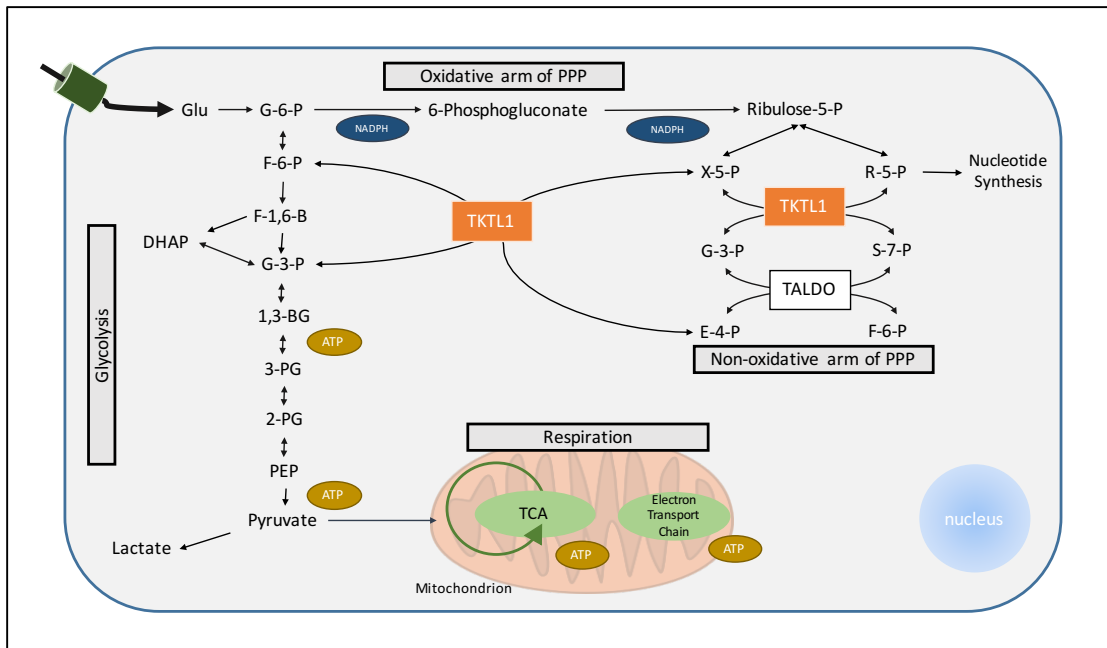
Figure 4: Schematic overview of glycolysis, pentose phosphate pathway and respiration. PPP: pentose phosphate pathway, TCA: tricarboxylic acid, adapted from Fets L. et al 2013 [86]

Other important enzymes from the non-oxidative arm are **Transketolase (TKT)** and transaldolase (TALDO), which have also been associated with oncogenic mechanisms, usually found overexpressed.

1.2.2 TKTL1

Transketolase is an important reversible rate-limiting enzyme that catalyzes several key reactions of the non-oxidative arm of the PPP. This enzyme, together with transaldolase, regulates and sustains the metabolic needs of the cells. Since the non-oxidative arm is reversible, TKT can either switch the flux to produce NADPH or to produce pentoses, depending on metabolic needs. In other words, TKT has a pivotal role as a reversible link between PPP and glycolysis (see Table 6).

Gene	TKTL1 in Xq28
Structure	Homodimer
Molecular mass	74,2 kDa per monomer
Active sites	2 identical, formed at the dimer interface between the PP domain and the Pyr domain
Cofactor	Divalent metal ions (Ca^{2+})
Domains	N-terminal PP Middle-terminal PYR C-terminal
Coenzyme	Thiamine diphosphate (ThDP) - biologically active derivative of *vitamin B1*
Function	Cell metabolism (glycolysis and PPP)

Table 6: Characteristics of TKTL1 [86,87]

Three human genes have so far been discovered: TKT-gene and TKT-like gene 1 and 2 [87]. The TKTL1 protein is commonly expressed in the testis, thymus and retina. Of the three TKT gene family the TKTL1 plays an important role in carcinogenesis and is overexpressed in multiple tumours [88,89], including colonic and urothelial tumours [90,91], uterine cervix cancer [92], breast cancer [93], ocular adnexal tumours [94], non-small cell lung cancer [95,96], and esophageal squamous cell carcinoma [97].

The presence of an overexpression of TKTL1 has also been linked to disease progression with variations in TKTL1 expression being described in different stages of disease. In some cases a correlation with patients outcomes has been found, although this association is not yet understood [57,98–101]. Discussion of this topic is still ongoing without consensus in the scientific community. In a study from Kämmerer U et al. it was shown that the presence of TKTL1 was unrelated to either the rate of glucose consumption or lactic acid production [102]. A consensus regarding TKTL1 and its interactions and function has not been reached yet.

1.2.2.1 TKTL1 IN CARCINOGENESIS

As referred to earlier, TKTL1 expression is overexpressed in multiple tumours, presumably to enable cell survival by maintaining a rapid utilization of large amounts of glucose in the absence of oxidative stress. Cancer cells with a deregulated glucose metabolism tend to have a higher uptake of glucose, because ATP generation via glycolysis is less efficient than by oxidative phosphorylation. In order to have a constant high rate of proliferation, cancer

cells need to maintain a high level of metabolic activity, and counteract against apoptosis and reactive oxygen species.

Although the glycolytic pathway produces lesser ATP (2 vs 36 ATP/mol glucose, in comparison with oxidative phosphorylation) it provides its own advantages, including: production of pentoses needed for synthesis of DNA and RNA as also NADPH redox equivalents, to reduce the reactive oxygen species [89,103]. Studies inhibiting and enhancing TKTL were conducted:

- Inhibiting TKTL activity led to a suppression of tumour growth [104–107]
- Activation of TKTL led to enhanced tumour growth [108]

Most of the prior studies concluded that TKTL1 inhibition may emerge as a future oncological therapeutic target.

1.2.2.2 EDIM-TKTL1 BLOOD TESTS

A new EDIM blood test was developed as a result of prior studies, which showed a correlation between overexpression of TKTL1 and malignancy. This blood test uses the epitope detection in monocytes (EDIM) technology, the concept being that circulating macrophages in peripheral blood, which had contact with cancer cells, will carry present tumour-related material, in these case TKTL1.

In 2012, a study revealed that EDIM-TKLT1 blood test had good agreement with 18FDG-PET/CT [109]. A 2013 presentation to the American Society of Clinical Oncology of EDIM-TKLT1 blood tests in breast cancer patients, showed a good concordance between the EDIM-test and clinical observations. In the same year, a study involving patients with colon carcinoma concluded that EDIM TKTL1 test might indicate metastasis earlier than established tumour markers [110]. However, the EDIM-TKTL1 blood test has not yet been approved for clinical application.

2 SCOPE OF STUDY

The overall aim of this study was to investigate TKTL1 expression in tissue microarrays of human prostate cancer and benign prostatic tissue. More specifically, the aims of the study were as follow:

- To identify expression differences in TKTL1 expression between prostate cancer and normal prostatic tissue, in order to detect if TKTL1 protein is integrated or plays a role in the vast network of pathways in tumorogenesis in prostate cancer.
- To evaluate the expression of TKTL1 in different stages of disease, eventually helping to better understand the tumorogenic process of prostate cancer, questioning if glucose reprogramming is a cornerstone to its development and progression.
- To evaluate the expression of TKTL1 with the clinical profile and pathological data of patients, to help detect if TKTL1 expression could bring more information about the outcome, progression and aggressiveness of the disease, and eventually helping to better manage patients with prostate cancer.

3 MATERIALS AND METHODS

3.1 PATIENTS

The study population consisted of 124 patients who had undergone surgery in the Department of Urology of the University Hospital of Tuebingen, between 2003 and 2014. The median age of patients was 66 (62-70), ages were calculated as of time of operation. Specimens included were from 53 patients with previous histologically verified prostate cancer (through a transrectal ultrasound-guided prostate biopsy) who underwent radical prostatectomies, and 45 specimens from patients with metastatic prostate cancer that underwent palliative TURP. Leading to a total of 98 patients with prostate cancer, in different stages of disease. As negative controls, 26 patients without prostate cancer were also included.

An informed consent was obtained from all patients and the ethic approval was obtained by the local ethics committee (842/2916B02).

53 radical prostatectomy specimens were included in the study. This group of patients were men who had been diagnosed with prostate cancer. The patients did not receive preoperative chemotherapy, nor radiotherapy or any other type of treatment. From these specimens the goal was to retrieve 3 types of tissues, peritumoral tissue far and near the tumour and the tumoral tissue itself.

The 26 benign specimens are from patients with benign prostatic hyperplasia who underwent TURP (n=11), transvesical prostatic adenomectomy (n=8), and also, from patients with muscle-invasive bladder cancer that underwent radical cystectomy (n=7). Usually a transvesical prostatic adenomectomy is chosen instead of TURP for multiple reasons, including high prostatic volume (over 100 ccm) or other concomitant bladder pathologies, following bladder stones or diverticula. In the non-prostatic cancer group, patients with muscle-invasive bladder cancer were included who underwent a radical cystoprostatectomy, which consists of the removal the entire bladder, the prostate, part of the

urethra, seminal vesicles, and part of the vas deferens, which gave an opportunity to retrieve a normal prostatic specimen. In this group of patients, if prostate cancer or an infiltration of the known bladder cancer in the prostate was detected, the prostatic specimen was not included in the study.

Patients who underwent palliative TURP had metastatic prostate cancer. Patients from this group developed obstructive voiding symptoms, which interfered with the quality of life, being a relatively common finding in the clinical practice. Although TURP is commonly performed in patients with BPH, TURP can also be a therapy for the relief of bladder outlet obstruction for patients with advanced or metastatic prostate cancer.

This group of patients is relatively heterogeneous, in terms of different stages of disease concerning the TNM classification as also hormonal sensitivity of the disease, being either hormone sensitive prostate cancer or hormone refractory. Theses are two different stages of disease, the latter meaning a disease progression, marked by a consecutive elevation of PSA-levels under a castration level of testosterone, being conventionally used the level 20 ng/dl.

The clinical information of the patients (26 benign prostatic tissues, 53 prostatectomy specimens and 45 prostatic specimens from palliative TURP) was retrospectively reviewed and inserted in a database in Excel Software Program (2010, v14.0).

3.2 Tissue samples

Healthy normal tissue, prostate cancer and normal adjacent to prostate cancer tissue samples were obtained from formalin-fixed and paraffin-embedded blocks from the Department of Pathology in the University Hospital of Tübingen. All specimens were previously submitted for pathological evaluation in Department of Pathology. All prostatectomy specimens were submitted for routine histological investigation, including measurements (vertical, transverse and sagittal), weight, histological type, location of tumour, extraprostatic extension, seminal vesicle invasion, bladder neck invasion, perineural invasion, Gleason grade, TNM classification, lymphovascular invasion, as also resection margins. Normal prostatic specimens retrieved from TURP, transvesical prostatic adenomectomy and radical cystectomy were also routinely analyzed in the Department of Pathology.

The specimens were conserved in 4% Formalin fixed and paraffin-embedded in room temperature; paraffin embedding offers a good option for long-term preservation of tissue samples.

For the purpose of the study, a pathologist reviewed all hematoxylin and eosin stained slides to confirm the target diagnosis, and marked where the tissue would be punched to construct the tissue microarray block.

3.3 Tissue Microarray (TMA)

3.3.1 Tissue Microarray (TMA) Technology

The tissue microarray technology is a technique to amount multiple biological samples on a single solid support. In 1998 in Nature Medicine, Kononen et al. described the use of this technique, mentioning that as many as 1000 cylindrical tissues biopsies from different tissues could be distributed in a single tissue microarray - in a single slide [111].

Before the TMA is constructed, the histological blocks will be stained with hematoxylin and eosin (H&E) and reviewed by a pathologist to confirm, if the slide is representative of the block. If so, the pathologist will mark the areas of interest, where the needed cores will later on be removed. The cylindrical tissue cores' typical sizes are 0.6 mm, 1.0 mm, 1.5 mm and 2.0 mm and the number of cores varies with size of tumour, size of population and purpose of the study. When building the TMA block (see Figure 5), controls should be placed on each of them, existing a variety of tissues options, to help the examiner to navigate. The pathologist should review the TMA sections (stained with H&E) to confirm if the previous marked areas are present in the TMA final section.

This method reduces time, as well cost, reagent usage, storage place and reduce experimental heterogeneity - all cores are treated identically (the variables, including temperature and preparation method will be the same for all cores). A potential point of criticism of TMA, is that the cores are small in size, and may lead to a misrepresentation of specimens, especially when heterogeneous [112]. The scores given in the tissue microarrays are not always coherent to the scores given in the whole section [113,114]. Studies concluded that when multiple cores from the same area are included, the TMA's representativeness increases [115].
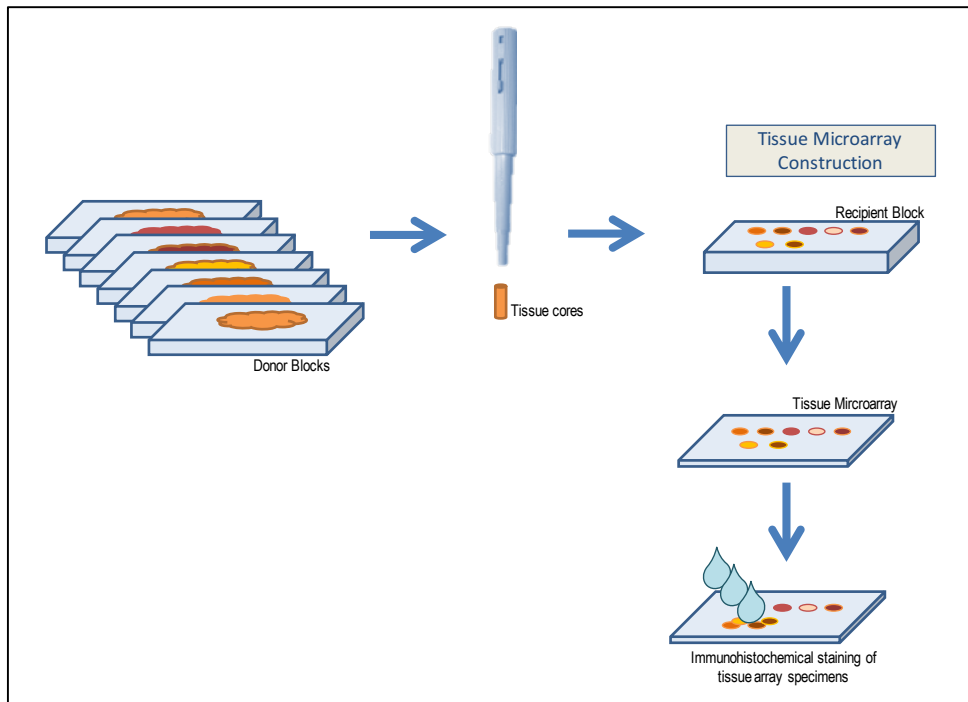
Figure 5: Schematic overview of the process of tissue mircroarray construction and immunohistochemical staining
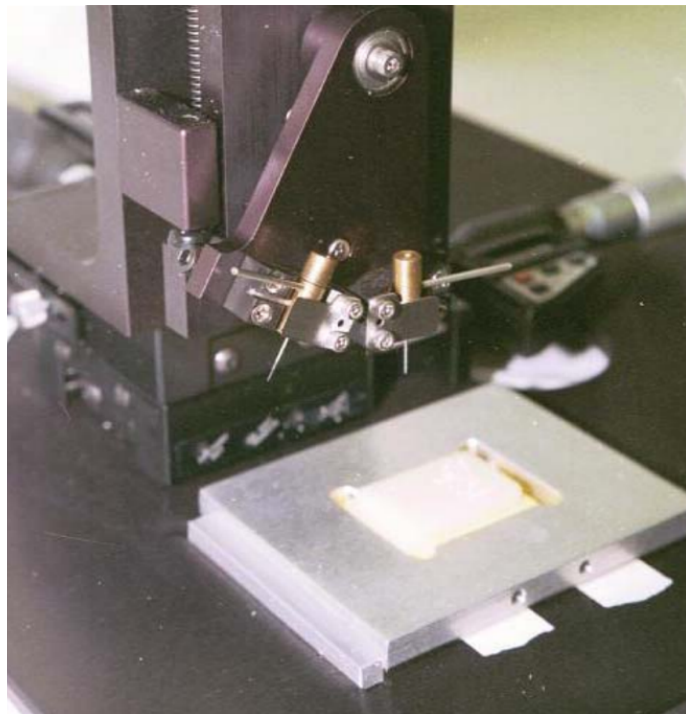

Figure 6: Placement of the cores in the recipient block. Source: Instructional Manual of MTA-1, Beecher Instruments Inc.

3.3.2 DESCRIPTION OF THE PROCESS

Formalin-fixed and paraffin-embedded preparations of radical prostatectomy specimens were evaluated and the pathologist marked the areas of tumour and areas of benign tissue, after which the samples could be arrayed. Benign tissues of the prostatectomies were divided in 2 groups: near and far from tumour. From each case were retrieved at least two to six replicates of each area, to ensure an adequate number for the statistical analysis, knowing that a loss of up to 10% of tissue samples is normal after tissue preparations. The same number of punches was retrieved from normal tissues with BPH as also malignant tissues obtain by palliative TURP cases.

The block from which the histocores were taken is referred as donor block, and the histocores were placed into the recipient block. The tissue microarray instrument (MTA-1, Beecher instruments, Sun Prairie, WI, USA) (see Figure 6), used is designed to produce circular cores that are 1mm in diameter, and the core were placed at a specifically coordinate. TMA maps were recorded using Microsoft Excel.

Using a microtome (Leica RM-2125 RT, Wetzlar, Germany) (see Figure 7), 5-10 μm sections were cut from the microarray blocks' generating the tissue microarray slides for the immunohistochemical analyses. In total of 12 microarrays slides were obtained in this study, each containing around 8x4 to 8x6 cores (see Figure 8).

A pathologist to ensure that the histocores were taken from the wished areas, to detect eventually punches from wrong areas or to detect artefacts, evaluated the final TMA slides.

Figure 7: Microtome (Leica Rm-2125 Rt) Source: Leica Biosystems
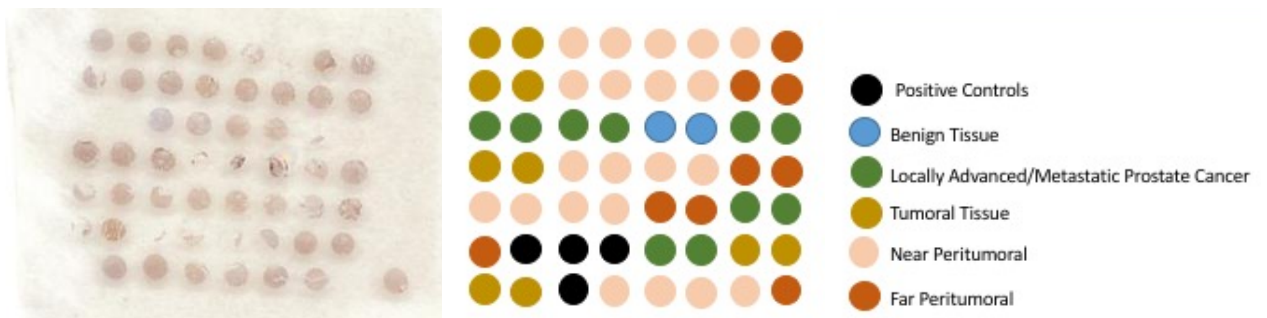

Figure 8: Example of a TMA slide (left) containing 56 tissue samples. The complete TMA comprises 12 sections with a total of 642 samples. Schematic layout of the TMA section (right).

3.4 IMMUNOHISTOCHEMICAL STAINING OF TISSUE ARRAY SPECIMENS

3.4.1 IMMUNOHISTOCHEMISTRY

Commonly, immunohistochemistry is used as a laboratory technic to interpret the TMA material, including in the field of research.

IHC is used routinely in oncology to help distinguish benign from malignant cells, detect prognostic factors, subtyping neoplasia, detect primary site of malignant cells in undifferentiated neoplasia [116,117].

IHC is a method used to detect certain cell types or tissue antigens, inspired in different scientific disciplines, immunology, biochemistry and histology. The concept behind this method is that the binding of an antibody and an antigen in specific tissue sections will be enhanced through a histochemical process or with fluorochromes, being visible under an ultraviolet light. The bind between antibody and antigen is not visible under the microscope, here it will be applied a substrate which will be converted by an enzyme to an insoluble colored product that is deposited at the area of antigen expression – chromogenic detection. Dealing with IHC has two steps: the process of staining and secondly the interpretation and quantification of the expression.

IHC often aids in the diagnosis of prostate cancer. Its diagnosis is based in morphological characteristics, but in some cases the tumour is so undifferentiated or the morphology assembles a benign tissue, which leads to the need of other methods to better interpret the specimens.

It is known that absence of basal cells is one of the major criteria to diagnose prostate cancer [118,119]. Important immunohistochemical stains, specifically basal cell markers (CK HMW, CK 5/6, CK 14) are used to help to detect malignancy or not. Another example of the applicability of IHC in prostate cancer is to distinguishe from other malignant cells, including urothelial cancer which usually express positivity for p63, while prostate cancer does not [120,121] or Prostein which in contrary is expressed in prostate cancer and not in urothelial carcinoma [122]. To distinguish between colorectal cancer, CK20 and CDX2 are used, which CDX2 is not expressed in prostate cancer and is expressed in colorectal cancer [123,124].

Some other important immunohistochemical stains include: AMACR, PSA, PSAP, CK AE1/AE3 [125,126].

As purpose of the study, we used a monoclonal antibody (murine) commercially available, named JFC12T10, which recognizes and binds to the C-terminal

fragment of recombinant TKTL1 protein molecule present in the tissues samples. These antigen-antibody complexes are then only detected after treatment of the tissue samples, with various methods mentioned below. For the chromogenic process, 3,3′-Diaminobenzidine (DAB) was used, which results in the formation of an insoluble color precipitate, brown-red-pink, which can be good distinguished from the blue Mayer's Hematoxylin Counterstained cells under a light microscope. Immunohistochemical stain with JFC12T10 antibody shows a cytoplasmatic staining in certain prostatic cells.

Positive controls should be performed in each test for quality control purposes, it helps to detect any disturbances in the functionality of the anti-TKTL1 antibody. In this study TKTL1- stained testicular tissue specimens were used as positive controls, because it is known that TKTL1 expression is found in high levels in this type of tissue [127]. As negative control, the antibody was omitted on testicular tissue to show absence of staining.

Although IHC has really good properties, being an advantage comparing to other methods, due to its easy availability, low cost, quick method it has also its own disadvantages. Reading/Interpretation method is a subjective interpretation and there is currently no standardization. The expression of certain protein is an indirect detection and it's subject to various potentially preparation bias (dilution, dehydration, staining, fixation, antigen retrieval). The interpretation of the expression will be more detailed during the thesis.

3.4.2 DESCRIPTION OF THE PROCESS

The first section was stained with H&E for histological review. Additional sections served for immunohistochemistry.

Formalin-fixed and paraffin-embedded sections (5-10 µm) of matched normal and cancerous tissue were dewaxed using Xylol (3 times 10 minutes), rehydrated in a series of graded alcohols and rinsed with distilled water, in a sequence of 2 times 5 minutes with 100% Ethanol, 2 times 5 minutes 96% Ethanol and 1time 5 minutes with 70% Ethanol. Pre-treatment with 3%

Hydrogen peroxide, H_2O_2, (for 20 minutes) for inactivation of endogenous peroxidase was performed, and then rinsed with distilled water for 5 minutes. The antigen retrieval step was performed using sodium citrate buffer (pH 6.0) and exposure to high energy in a microwave for 2 x 5 minutes. Slides were allowed to cool down for 30 minutes in room temperature and then washed with TBST (0.05M Tris pH 7.6; 0,15M NaCl; 0.1% Tween 20) 2 x 5minutes. Subsequently, slides were incubated for one hour in room temperature with monoclonal mouse anti-TKTL1 antibody (clone JFC12T10, Monocolonal Mouse IgG2b; Vector Linaris, Darmstadt, Germany) diluted (1:800) in antibody diluent (DAKO Real Antibody Diluent, Glostrup, Denmark). After 3 x 5 minutes washes in TBST, Advance Horseradish Peroxidase HRP Link (Advance kit, Dako, Glostrup, Denmark) was applied to the slides for 30 minutes in room temperature. Sections were washed in TBST 3 x 5 minutes, and then it was incubated with Advance HRP Enzym (Advance kit, Dako, Glostrup, Denmark) for 30 minutes in room temperature. After incubation slides were washed in TBST 3 x 5 minutes and then the complex was visualized by adding ImmPACT DAB (1000µl Buffer + 20µl 3,3′-Diaminobenzidine (DAB), Burlingame, CA, USA) for 4 minutes, being afterward washed in TBST 2 x 5 minutes. The slides were counterstained with Mayer's Hematoxylin Solution followed by a step of blueing where slides were under warm running tap water for 7 minutes. Slides were dehydrated successively in 96% Ethanol 2 x 3minutes, 100% Ethanol 2 x 5 minutes ended with 3 x 5 min with Xylol. To preserve the histochemical stain it was used the Vectamount Mouting medium (Burlingame, CA, USA). Appropriate positive and negative controls were included in each slide, testicular tissue.

The slides were viewed by light microscopy (Axio, Zeiss Lab. A1, Oberkochen, Germany). Cores that were absent, folded, or contained an insufficient number of tumour cells were not scored, and therefore excluded.

3.5 SCORING SYSTEM FOR IMMUNOHISTOCHEMICAL STAINING

There is still no consensus on a protocol for scoring of immunohistochemical staining. Numerous of different IHC evaluation scales are used nowadays. Some pathologists describe the immunoreactivity as negative, weak or strong, other studies use a more complex scoring system, by multiplying the percentage of positive cells (P) by the intensity staining (I) – weak staining (1), moderate staining (2) and strong staining (3).

$$Q = P \times I$$
Minimum= 0; Maximum = 300

A scoring system for TKTL1 was proposed as guide in breast tissues sections (paraffin-embedded) by the R-Biopharm AG, when presenting its product, RIDA®PentoCheck® IHC, which consisted of:

Score 0: 0–20% of tumour cells exhibit TKTL1 staining
Score 1: 21–50% of tumour cells exhibit TKTL1 staining
Score 2: 51–80% of tumour cells exhibit TKTL1 staining
Score 3: > 80% of tumour cells exhibit TKTL1 staining

But since the cytoplasmatic staining with TKTL1 is heterogeneous in prostate tissue, the semiquantitative score should be more complex, and the chosen one for this study was the Histo-score (H-score). The fraction (in percentage) of cells would be multiplied by the stain intensity, negative (0), weakly positive (1), positive (2), strongly positive (3), in two different highly power fields. The product of the percentage of positive cells and staining intensity ranged score between 0 and 300 [128].

The sections were first evaluated at low power magnification. In 100x magnification the majority of strongly positive cases were easily detectable. The fraction of stained tumour cells was verified using a higher magnification of 200x. When difficulty to classify the stained cells between score 1 and 2 increased, a higher power field was used of 400x.

Each area of interest gave rise to more than one core, so the final H-score of the selected area was the average of all H-score of the corresponding area (see Figure 9 and 10).

To score the histocores, each slide was examined twice by the same examiner on a light microscope (see Figure 11). The final score given was the average between the two examinations. Some cores were documented, and photos were taken using a digital camera integrated in the microscope.
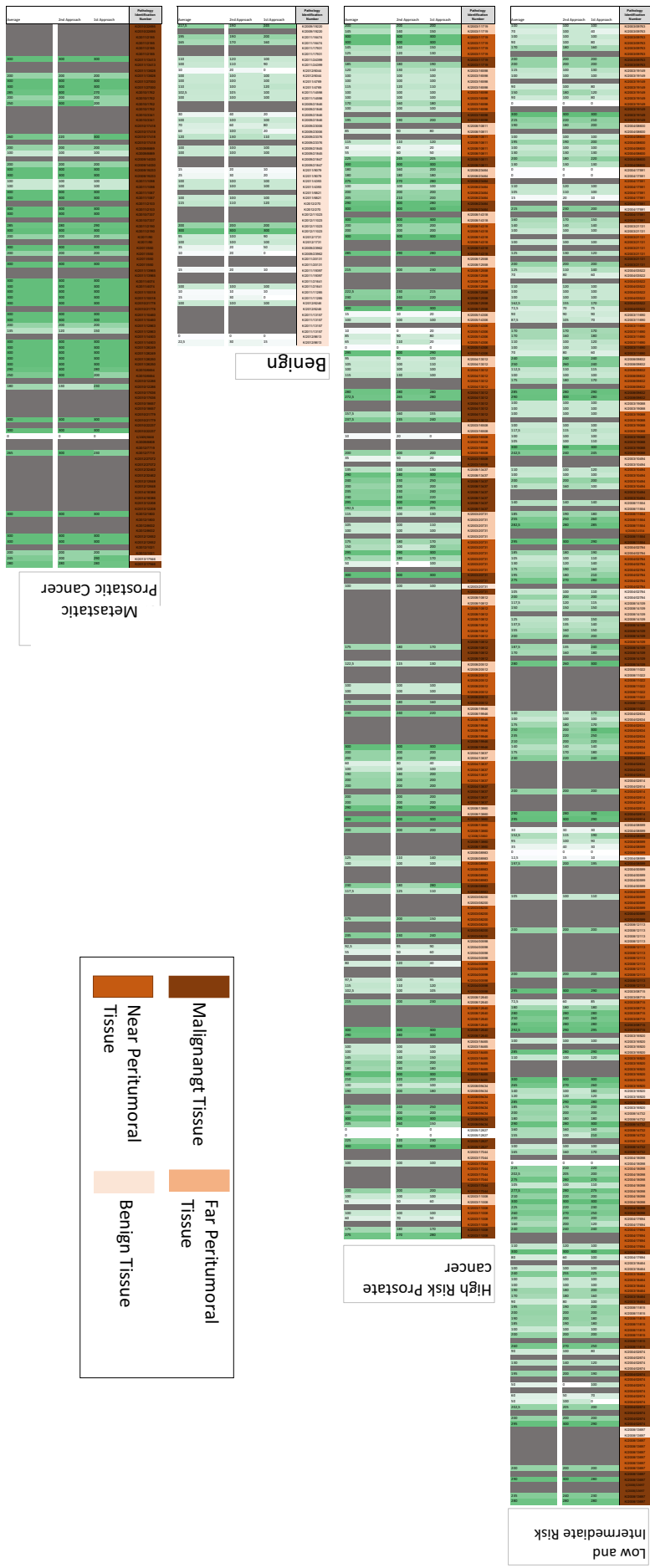
Figure 9: overview of the h-score in a gradient scale of greens within the four group of tissue samples: high risk, intermediate and low risk, benign and locally metastatic prostate cancer. Light to darker equals 0 to 300.
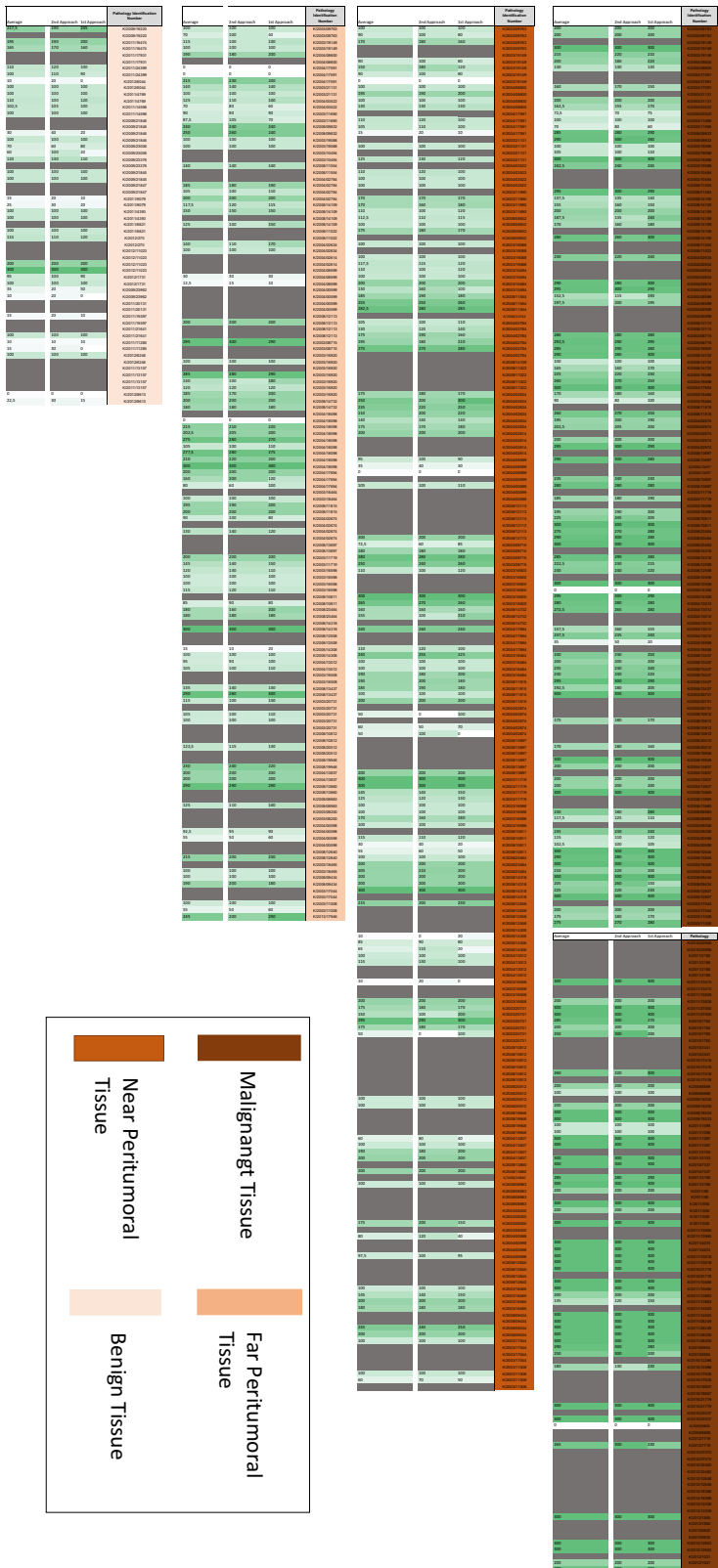
Figure 10: Overview of the h-score in a gradient scale of greens within the four group of tissue samples: tumour, near peritumoral, far peritumoral and benign. Light to darker equals 0 to 300.
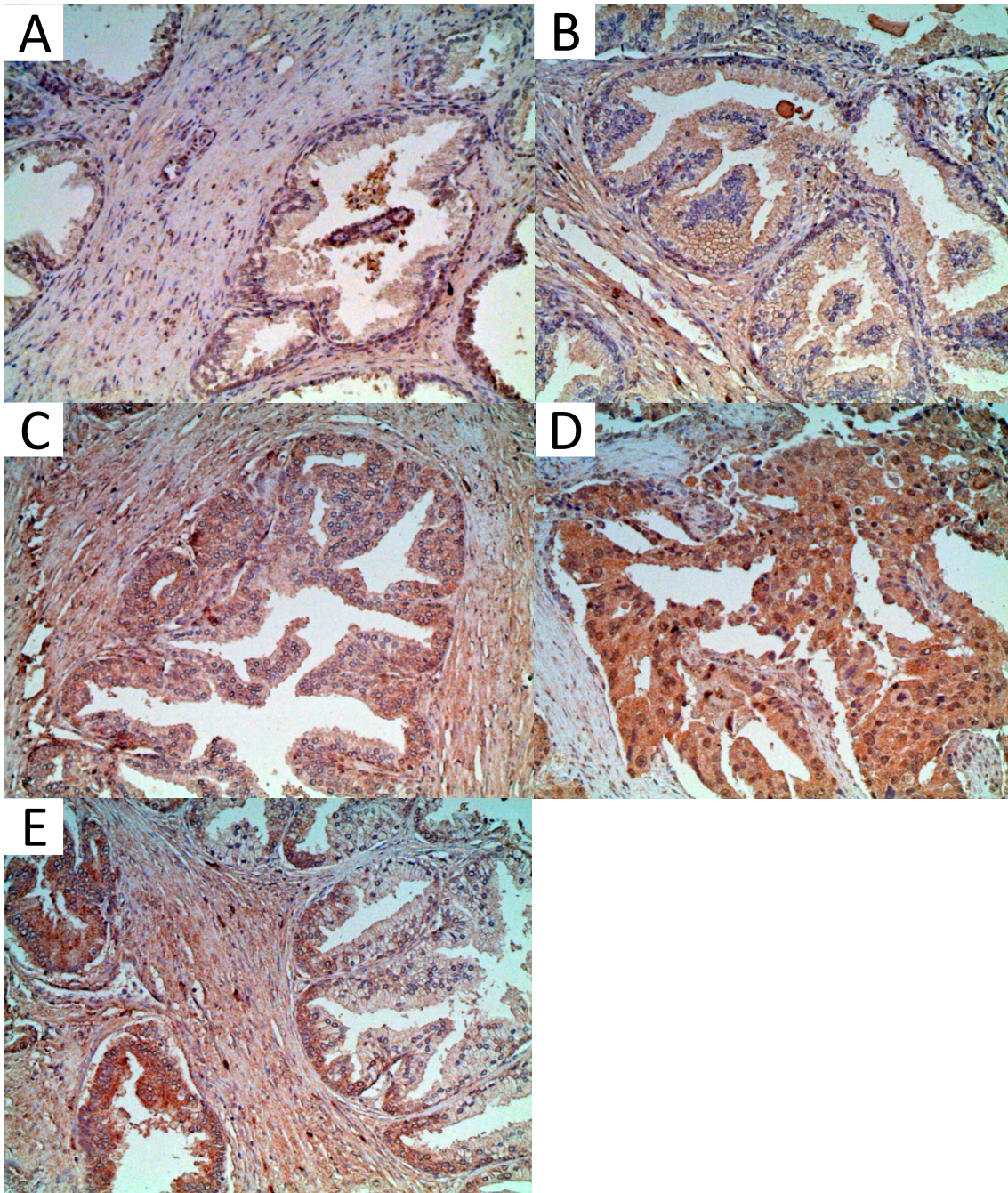
Figure 11: A: TKTL1 score 50. B: TKTL1 score 100. C: TKTL1 score 200. D: TKTL1 score 300
E: example of heterogeneity of TKTL1 expression found in tumoural tissue, on the left TKTL1
expression is higher than on the right side of the image

3.6   STATISTICAL ANALYSIS

Statistical analysis was performed using R (Version 3.3.2.) for Linux. High and low TKTL1 expressing patient groups were compared and the cutoff was determined by the midpoint TKTL1 score between M1-stage and M0-stage.

- Shapiro-Wilk test to determine if data is normally distributed
- Chi-square test for categorical variables
- Mann-Whitney U-test to detect differences among the medians of the continuous variables
- Receiver operating characteristics (ROC) analysis for determination of cutoff between benign and tumour – Area under the curve (AUC), specificity and sensibility
- Kruskal-Wallis analysis to compare means of three or more samples
- Post-hoc comparisons of relevant variables were done using Wilcoxon with Bonferroni Adjustment for multiple comparisons
- Spearman's rank correlation coefficient was used a nonparametric measure of rank *correlation*

A P value less than 0.05 was considered statistically significant. Results are given as median levels (interquartile range).

4 Results

4.1 Clinicopathological findings

From the initial total number of patients (n=124), 24 were excluded, because of insufficient data or tissue artefacts resulting in 100 eligible specimens. The median age of patients with prostate cancer was 65 years (61-70). Clinical and pathologic characteristics of patients included are shown in Table 7.

Tumour tissue of 46 patients who underwent prostatectomy, as well as corresponding adjacent non-neoplastic prostate tissue, were tested for TKTL1 expression by immunohistochemistry. They had a median age of 64 (61-68) and none of them had metastasis at time of surgery. The predominant pathological T-classification was pT2c (63.04%), while pT3a was the second most common (21.74%) and only 2.17% had pathological involvement of lymph nodes. The most common Gleason score were 6 and 9 (23.91%). Histologically, all tumours were conventional acinar adenocarcinomas.

22 patients were included as controls. These had undergone TURP, transvesical prostatic adenomectomy and radical cystectomies. Age range predominance was between 65 and 85 (86.36%), with median age of 71 years (67-77).

A total of 32 patients with metastatic prostate cancer were also included. 90.63% of these patients were older or equal to 65 years, the median age being 73 years (71-73). The TMN classification given to this group is a clinical parameter, in contrast to patients who underwent prostatectomy, which are classified according to pathological criteria. 43.75% had no T-stage information, 40.63% had no clinical N-stage, and 3.12% had no M-stage.

Total patients	100		
Age median (IQR)	66 (62-70)		
i. Benign	22		
Age			
<65	3 (13.64%)		
65-85	19 (86.36%)		
>85	0		
ii. RP-PCa	46	iii. TURP- PCa	32
Age		**Age**	
<65	23 (50%)	<65	3 (9.38%)
65-85	23 (50%)	65-85	28 (87.5%)
>85	0	>85	1 (3.13%)
T stage		**T stage**	
T1	0	T1	0
T2a	4 (8.70%)	T2a	0
T2b	0	T2b	0
T2c	29 (63.04%)	T2c	1 (3.13%)
T3a	10 (21.74%)	T3a	1 (3.13%)
T3b	3 (6.52%)	T3b	4 (12.5%)
T4	0	T4	12 (37.5%)
Unknown	0	Unknown	14 (43.75%)
Metastases		**Metastases**	
M0	46 (100%)	M0	0
M1	0	M1	31 (96,88%)
Unknown	0	Unknown	1 (3.12%)
Lymph node stage		**Lymph node stage**	
N0	44 (95.65%)	N0	2 (6.25%)
N1	1 (2.17%)	N1	17 (53.13%)
Unknown	1 (2.17%)	Unknown	13 (40.63%)
Gleason score		**Gleason score**	
6	11 (23.91%)	6	0
7a	8 (17.39%)	7a	2 (6.25%)
7b	5 (10.87%)	7b	1 (3.13%)
8	10 (21.74%)	8	2 (6.25%)
9	11 (23.91%)	9	21 (65.63%)
10	1 (2.17%)	10	3 (9.38%)
Unknown		Unknown	3 (9.38%)

Table 7: Clinicopathological findings of the study population. Benign group include: 26 benign specimens from patients. RP-PCa group: 46 radical prostatectomy specimens from men with clinically detected PCa and the TUR-P PCa group: 32 TURPs specimens from patients with obstructive urinary disorder with metastatic PCa.

4.2 TKTL1 EXPRESSIONS SCORES

The TKTL1 scores from all tissues were not normally distributed (p<0.001). For these reason non-parameteric tests were used to test for differences between groups.

4.3 TKTL1 EXPRESSION IN BENIGN TISSUE AND PROSTATE CANCER

Figure 12 shows the staining intensity of TKTL1 in normal and tumoral prostatic tissue (from all patients with diagnosed prostate cancer, including those with metastatic disease). In our study the TKTL1 staining was cytoplasmatic. The mean TKTL1 expression score in benign tissue was 100 (57.5 - 105), while the mean TKTL1 expression score in tumour tissue was 243.13 (187.04 - 295). TKTL1 expression was significantly higher in tumour tissue in comparison with normal tissue (*p<0.001*).
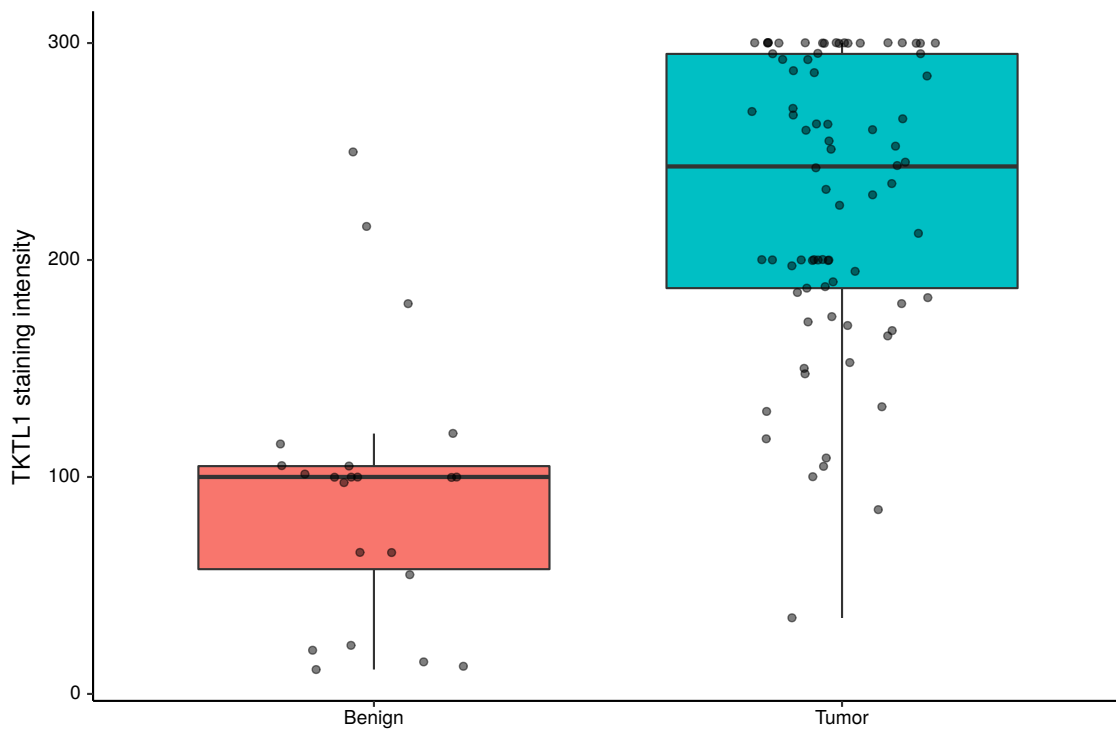
Figure 12: Comparison between TKTL1 expression in benign and tumoral tissue

ROC analysis was used to determine how accurately TKTL1 expression could discriminate between cancer and benign, as well as to calculate a sane value that would serve as a cutoff, separating the two groups. We found 125 to be the best value for this purpose with AUC=92.42%, 95% CI [85.94%–98.9%] with 86.36% specificity and 92.30% sensitivity (see Figures 13 and 14).
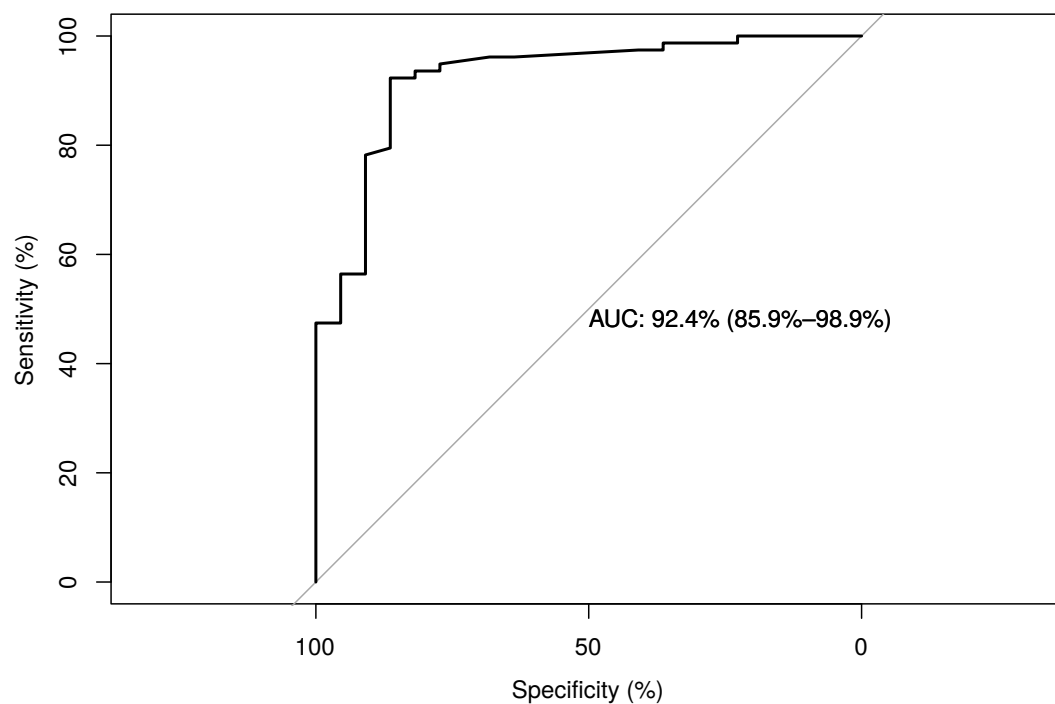
Figure 13: Receiver-operating characteristic curve for TKTL1 as immunohistochemical marker.
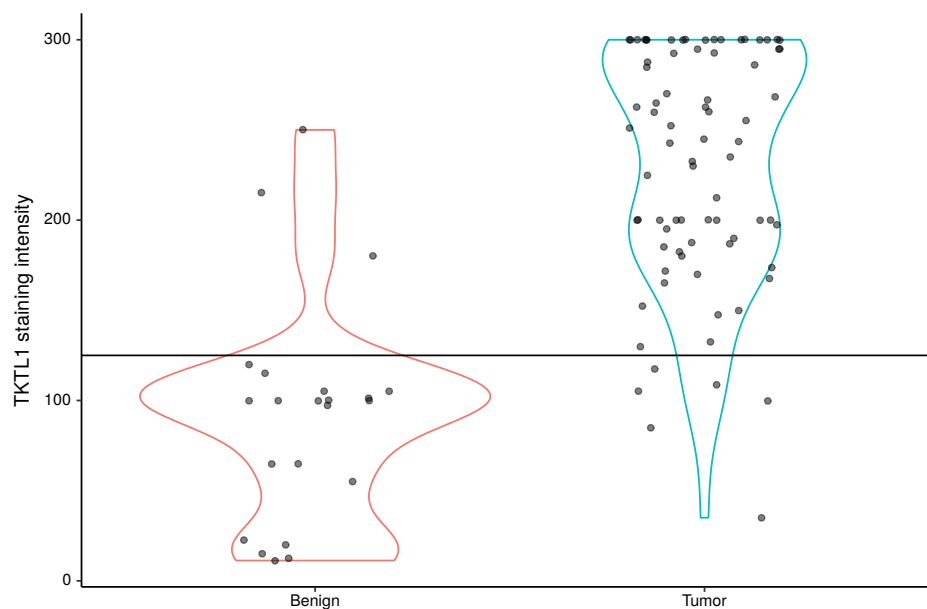
Figure 14: Violin plots of TKTL1 expression in benign tissue samples and tumour samples. Black lines represent cutoff (125) between benign and tumour, coloured lines are density plots of data distribution.

4.3.1 TKTL1 EXPRESSION IN PERITUMORAL NON-NEOPLASTIC TISSUE AND PROSTATE CANCER

Figure 15 shows the staining intensity of TKTL1 in benign tissue, peritumoral non-neoplastic tissue and prostate cancer in patients who underwent prostatectomy. The results show peritumoral non-neoplastic tissue had a lower TKTL1 staining intensity than in tumoral tissue (135.42 (100-195.16) compared with 200 (172.19-254.38), $p<0.0001$). Compared to benign tissue, TKTL1 staining intensity of peritumoral non-neoplastic tissue was higher (135.42 (100-195.16) compared with 100 (57.5, 105), $p=0.0006$). In summary, there is a positive progression of TKTL1 concentration from benign, to peritumoral to tumoral tissue.
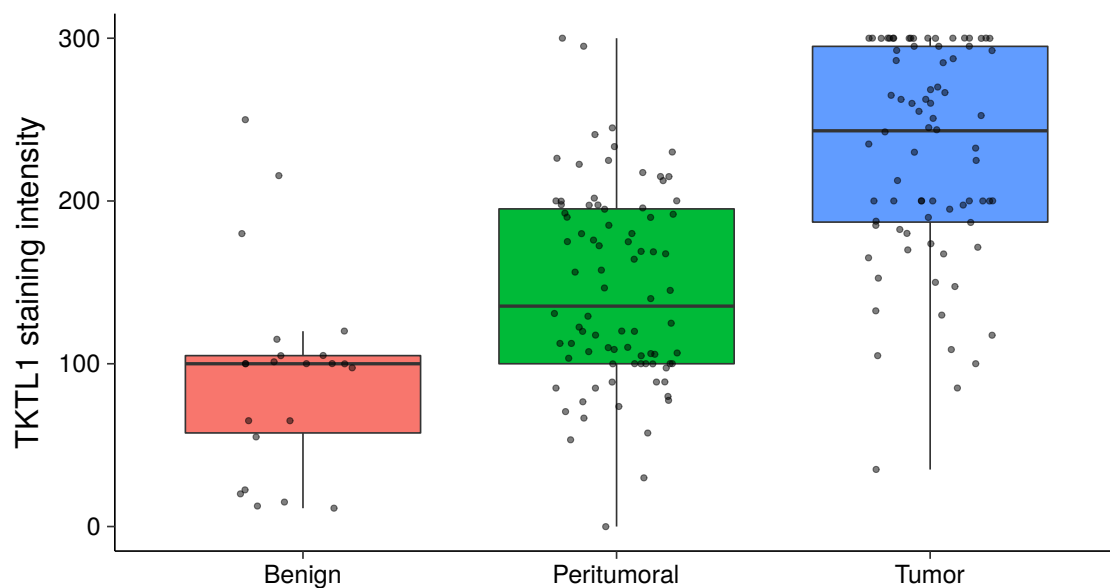

Figure 15: Comparison between TKTL1 expression in benign, peritumoral and tumoral tissue from patients who underwent prostatectomy.

4.3.2 TKTL1 Expression in far, near peritumoral tissue and tumoral tissue

Figure 16 shows the staining intensity of TKTL1 in peritumoral non-neoplastic tissue far, near and prostate cancer in patients who underwent prostatectomy. The results show no difference between *far* peritumoral non-neoplastic tissue TKTL1 staining intensity and *near* peritumoral non-neoplastic tissue (123.75 (100-195.16) compared with 156.88 (100-195.16), *p*=0.4625). In summary, a progression of TKTL1 concentration from far peritumoral to near peritumoral was not detected.

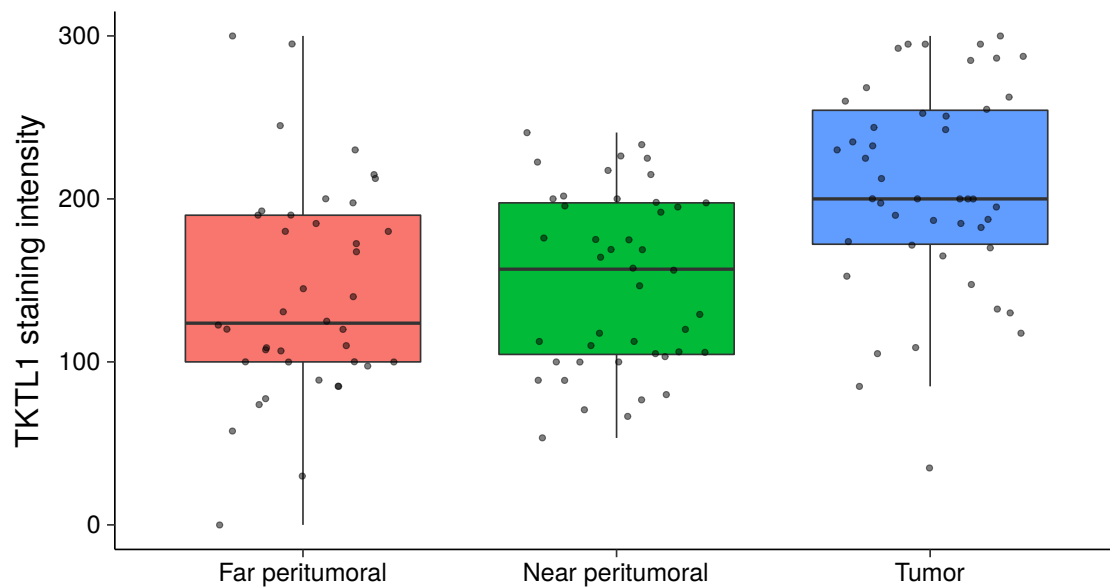

Figure 16: Comparison between TKTL1 expression in far peritumoral, near peritumoral and tumoral tissue, from patients who underwent prostatectomy.

4.4 TKTL1 EXPRESSION IN METASTATIC AND NON-METASTATIC PROSTATE CANCER

Regarding M-stage, patients with metastatic prostate cancer had a significantly higher TKTL1 expression than those with non-metastatic disease (200 (172.19-254.38) compared with 300 (222.50-300), *p*<0.0001, see also Figure 17).
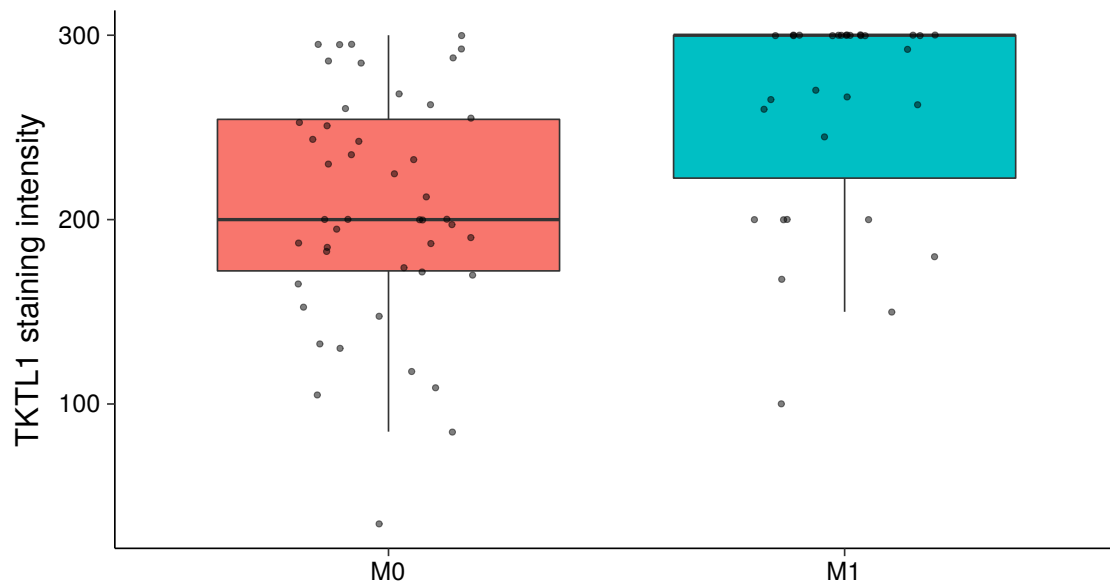

Figure 17: Comparison between TKTL11 expression in non-metastatic and metastatic prostate cancer.

4.5 TKTL1 EXPRESSION AND HORMONE SENSITIVITY

We found no difference in TKTL1 expression between hormone refractory and hormone sensitive prostate cancer in patients with metastatic prostate cancer who underwent palliative TURP (300 (215.63-300) compared with 296.25 (248.75-300), *p*=0.9015) (see Figure 18).
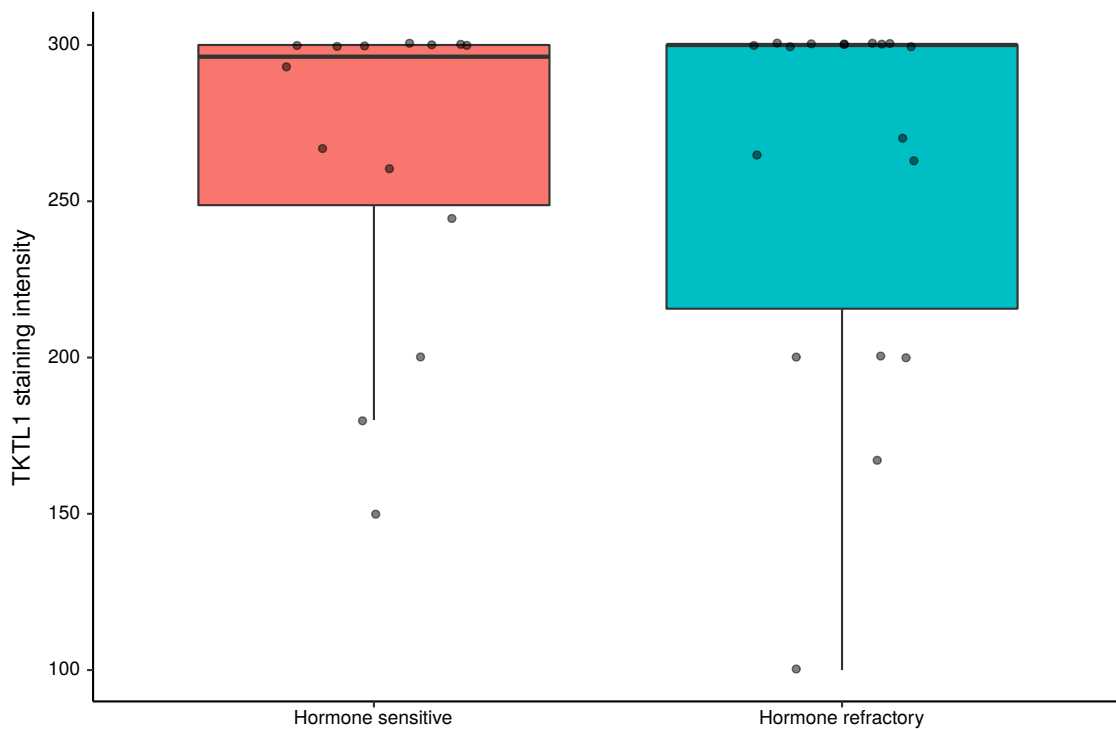

Figure 18: Comparison between TKTL1 expression in hormone sensitive prostate cancer and hormone refractory prostate cancer in patients who underwent TURP.

4.6 TKTL1 EXPRESSION AND GLEASON SCORE

A comparison within the group of *all patients with prostate cancer* regarding the Gleason score was also performed. Here, we decided to compare the tissues from patients with a Gleason score ≥ 8 and < 8 (see Figure 19). We found a significant difference between the two groups, being the expression of TKTL1 found higher within the group of patients with a Gleason score ≥ to 8 (261.25 (200-300) compared with 187.50 (158.75-237.50), *p*<0.001).
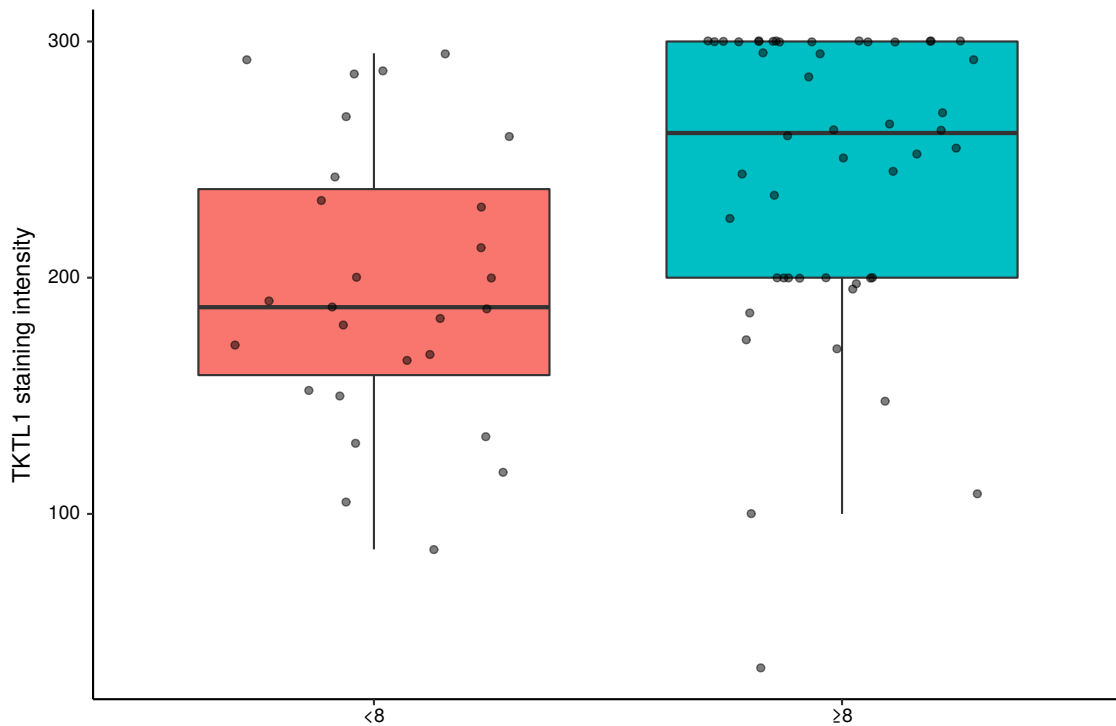

Figure 19: Comparison between TKTL1 expression when Gleason Score ≥ 8 or < 8 in all patients with prostate cancer.

Results obtained using Kruskal-Wallis test indicated some differences in Gleason score and TKTL1 expression in all patients with prostate cancer $\chi^2(5)$ =19.25, p=0.0017 (see Figure 20). Post-hoc comparisons revealed a significant difference between Gleason scores 6 and 9 (p=0.0047), all other pairwise comparisons had p>0.1011.
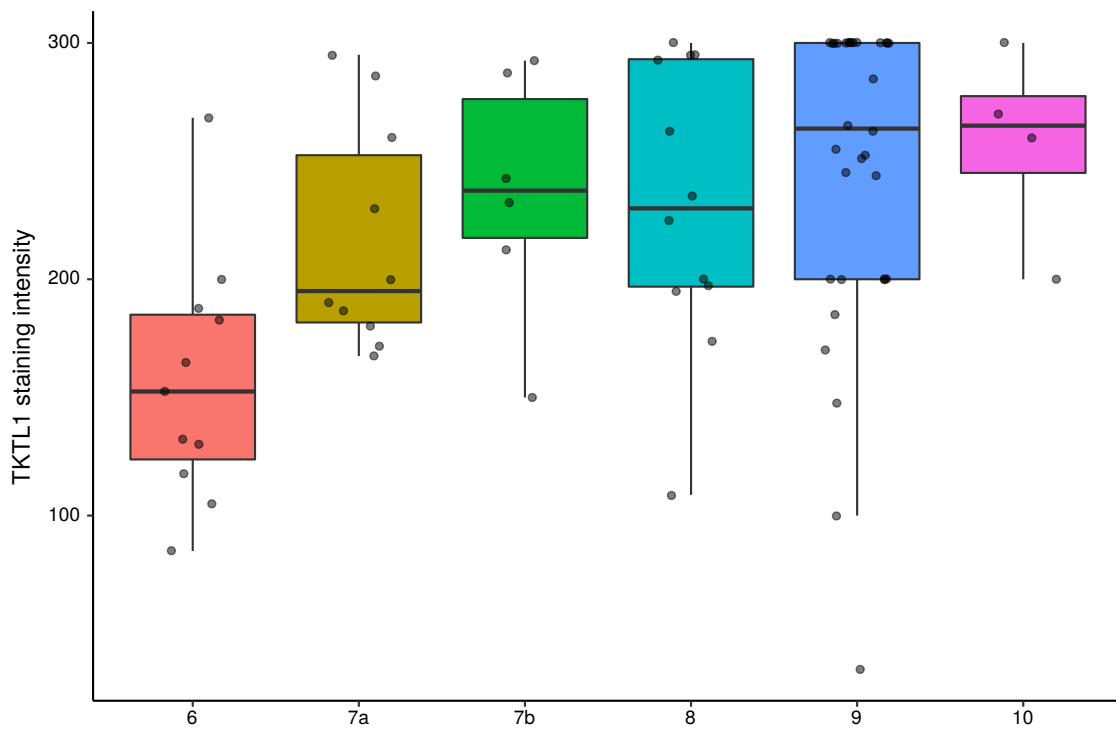

Figure 20: Comparison between TKTL1 expression in different Gleason Scores in all patients with prostate cancer.

We also compared tissues from patients who underwent prostatectomy with a Gleason score ≥ 8 and < 8 (see Figure 21). We found no significant difference between the two groups (212.50 (187.50-254.38) compared to 195.00 (161.88-246.88), p=0.3384).
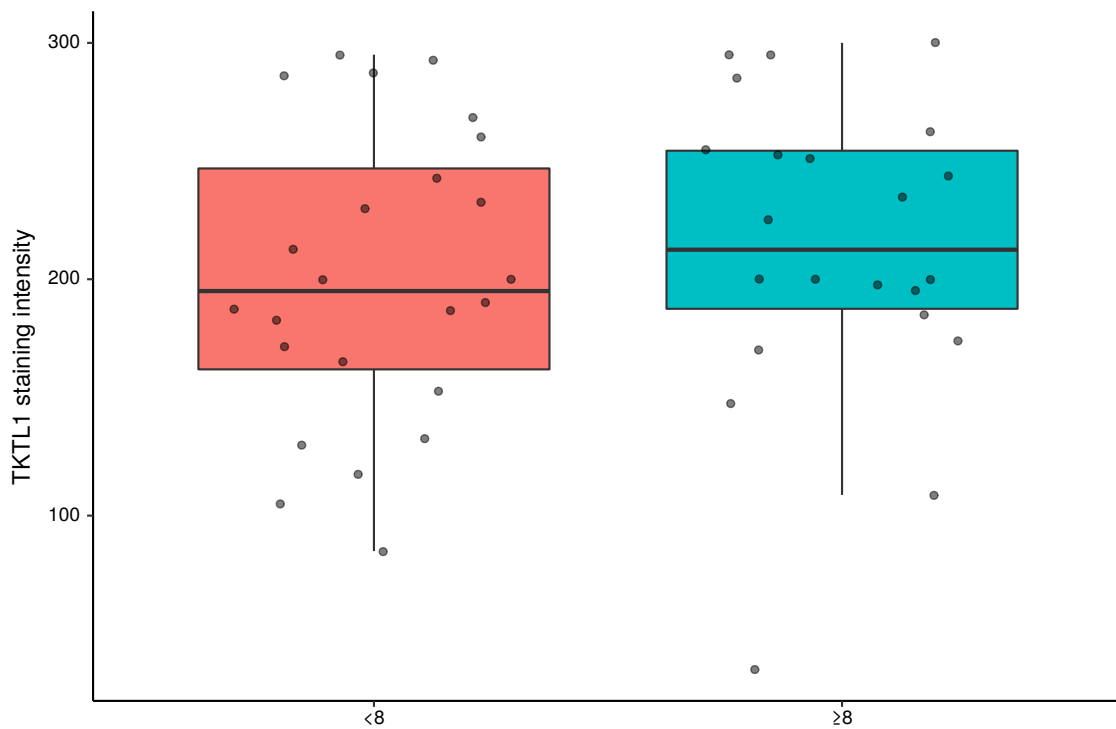
Figure 21: Comparison between TKTL11 expression when Gleason Score ≥ 8 or < 8 in patients who underwent prostatectomy.

4.7 TKTL1 EXPRESSION AND PATHOLOGICAL T-STAGE

A comparison within the group of patients with prostate cancer who underwent radical prostatectomy regarding the pathological T-stage was also performed. Here, we decided to compare the TKTL1 expression from patients with a lower and equal pT2 (organ-confined) and higher than pT2 (locally advanced). We did not find a significant difference between the two groups; the expression of TKTL1 was similar within both groups (200 (165-242.5) compared with 243.75 (197.5-286.5), p=0.0537, Figure 22).
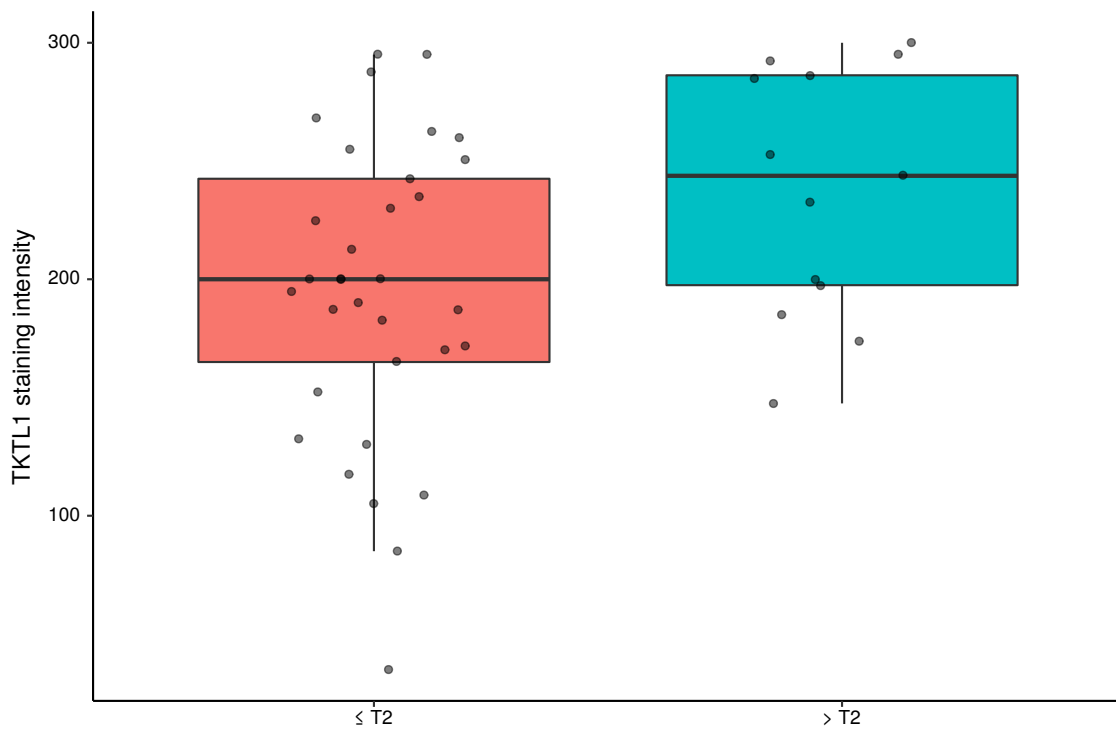
Figure 22: Comparison between TKTL1 expression in T-stage ≤ 2 and T-stage > 2.

Kruskal-Wallis test was performed and pT-stage 2a – 3b failed to predict TKTL1 expression in all patients who underwent radical prostatectomy $\chi^2(3)$ =5.61, p=0.132 (see Figure 23).

Post-hoc comparisons revealed no significant difference between T-stages (all p > 0.23).
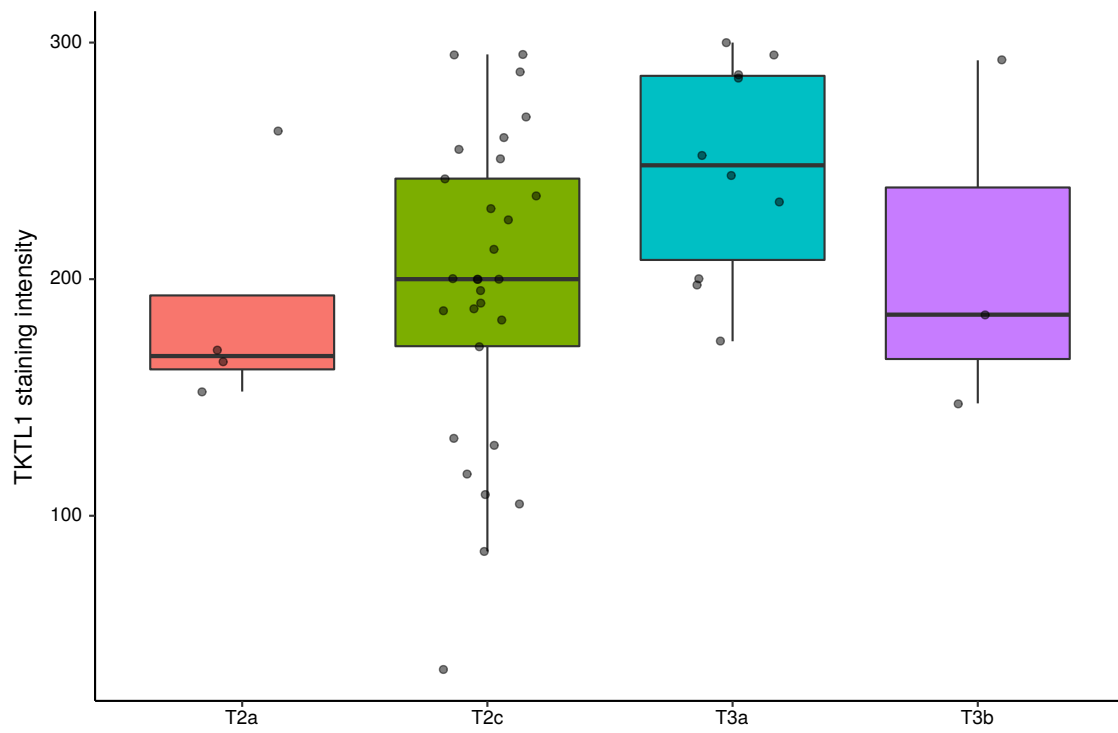
Figure 23: Comparison between TKTL1 expressions in different T-stages. No patient was classified with stage T2b.

4.8 TKTL1 EXPRESSION AND DISEASE PROGRESSION

A significant difference is seen between the following four groups of tissues: benign, peritumoral, non-metastatic tumor tissue and metastatic tumoral tissue (see Figure 24 and Table 9)
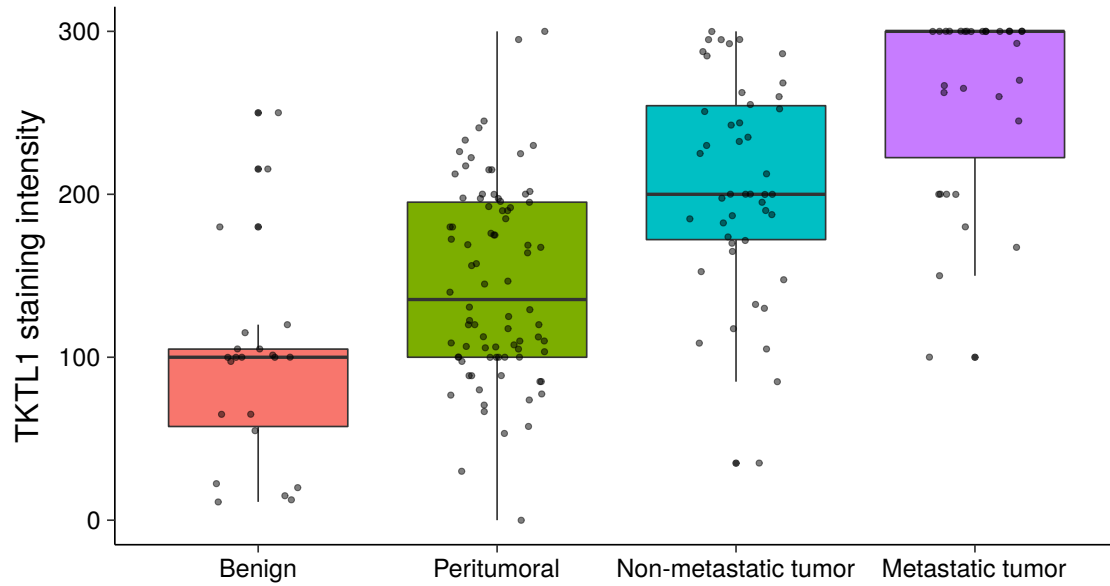
Figure 24: TKTL1 expression in progression of disease, from benign through peritumoral and non-metastatic PCa to metastatic PCa.

4.9 PSA CONCENTRATION AND TKTL1 EXPRESSION

The median PSA concentration of the total number of patients was 7.4 ng/ml. These levels did not significantly correlate with TKTL1 expression (Spearman´s ρ= 0.18, p=0.0847, Figure 25).

Figure 25: PSA concentration plotted against TKTL1 expression in all patients. To aid visualization, PSA values above 300 ng/ml were excluded.

4.10 AGE AND TKTL1 EXPRESSION

The median age of the total number of patients was 66 (62-70). A correlation between TKTL1 expression and age was not found in all patients (Spearman´s ρ= 0.09, p=0.3911, Figure 26).
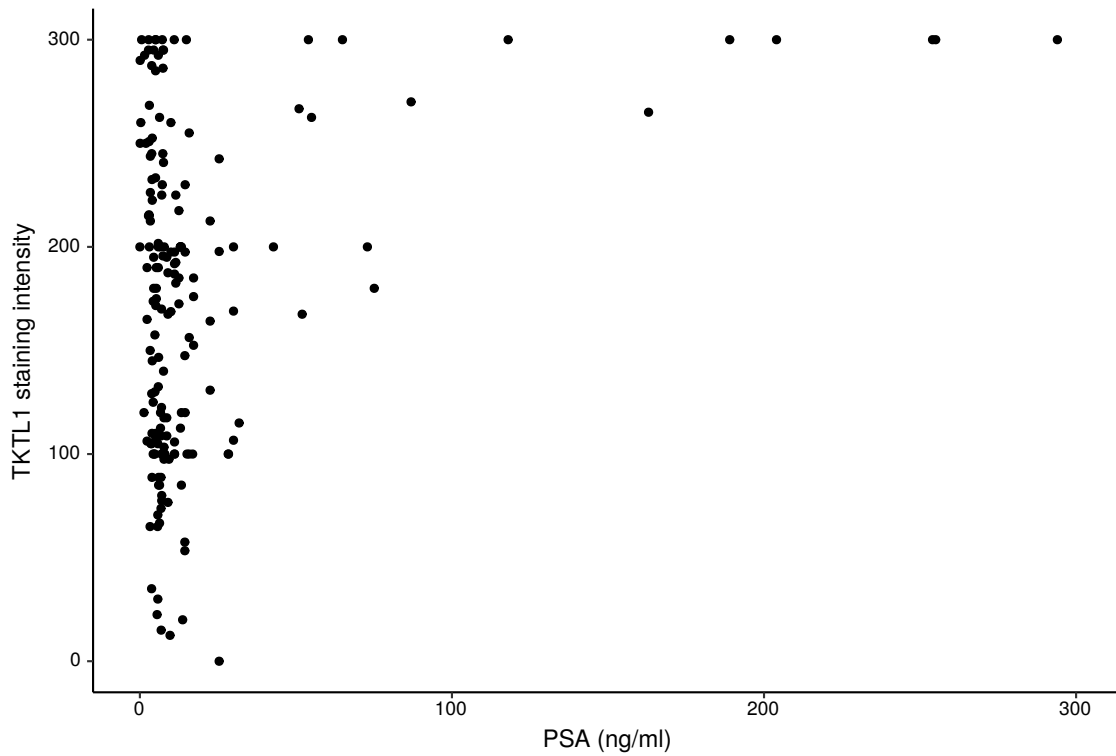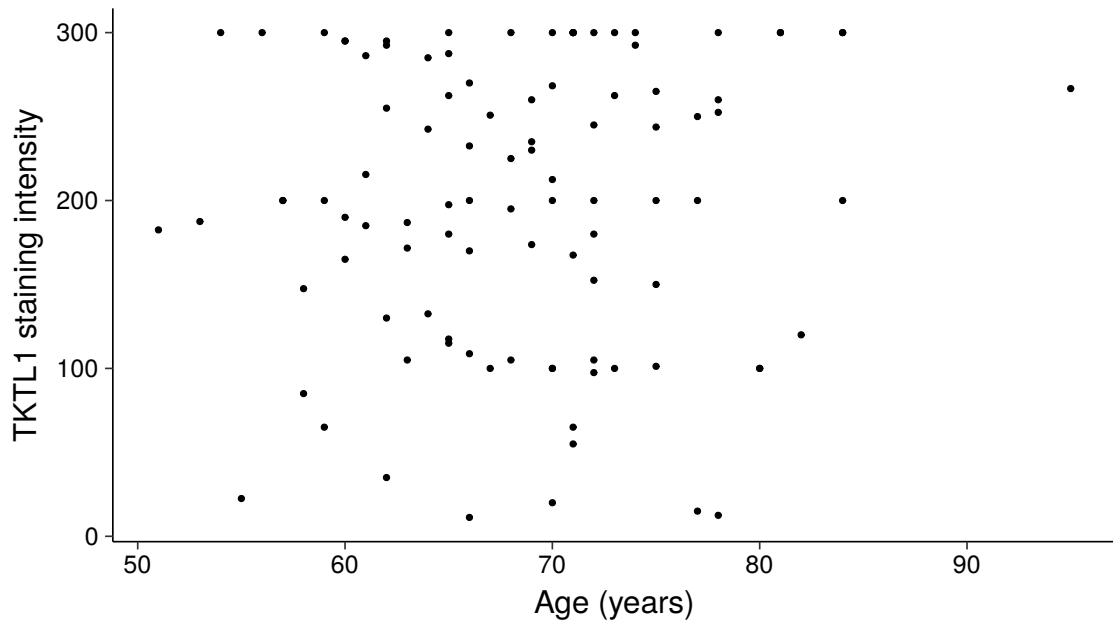

Figure 26: Age plotted against TKTL1 expression in all patients.

4.11 TKTL1 EXPRESSION AND CLINICOPATHOLOGICAL PARAMETERS

All patients with PCa were split into two groups, into low and high, based on whether a patient's TKTL1 expression score was below or above the midpoint between the mean of non-metastatic and metastatic PCa. Patients in both groups were compared across several clinicopathological parameters (see Table 8). We found a significant difference between low and high TKTL1 expressing groups regarding T-stage, M-stage, N-stage and Gleason score. Age groups differences were non-significant in these two expression groups.

In order to simplify all above statistical analysis a summary was compiled in a table (see Table 9).

	Low expression	High expression	p-value
Age			0.1565
<65	16 (20.51 %)	11 (14.10 %)	
≥65	21 (26.92 %)	30 (38.46 %)	
T-stage			**0.0027**
T1+T2	23 (29.49 %)	11 (14.10 %)	
T3+T4	14 (17.95 %)	30 (38.46 %)	
Metastases*			**0.0023**
M0	29 (37.66 %)	17 (22.08 %)	
M1	8 (10.39 %)	23 (29.87 %)	
Lymph-node stage*			**0.0257**
N0	28 (43.75 %)	18 (28.13 %)	
N1	5 (7.81 %)	13 (20.31 %)	
Gleason score*			**0.0075**
6	10 (13.33 %)	1 (1.33 %)	
7a	7 (9.33 %)	3 (4.00 %)	
7b	3 (4.00 %)	3 (4.00 %)	
8	6 (8.00 %)	6 (8.00 %)	
9	10 (13.33 %)	22 (29.33 %)	
10	1 (1.33 %)	3 (4.00 %)	

Table 8: Correlation of TKTL1 expression and clinicopathological features (both clinical and pathological TNM findings) in 78 prostate cancer cases. *One patient has no M-stage, 14 have no N-stage and 3 without Gleason score.

TKTL1 score	Benign	Tumoral tissue	p-value
	100 (57.5-105)	243.13 (187.04-295)	**< 0.001**

TKTL1 score	Benign	Peritumoral*	p-value
	100 (57.5-105)	135.42 (100-195.16)	**0.006**

TKTL1 score	Peritumoral*	Tumoral tissue*	p-value
	135.42 (100-195.16)	200 (172.19-254.38)	**<0.0001**

TKTL1 score	Far peritumoral tissue*	Near peritumoral tissue*	p-value
	123.75 (100-195.16)	156.88 (100-195.16)	0.4625

TKTL1 score	Metastatic	Non-metastatic	p-value
	300 (222.50-300)	200 (172.19-254.38)	**<0.0001**

TKTL1 score	Hormone-Refractory	Hormone-Sensitive	p-value
	300 (215.63-300)	296.25 (248.75-300)	0.9015

TKTL1 score	Gleason score < 8	Gleason score ≥8	p-value
	187.50 (158.75-237.50)	261.25 (200-300)	**<0.001**

TKTL1 score	T-Stage ≤ 2*	T-Stage > 2*	p-value
	200 (165-242.5)	243.75 (197.5-286.5)	0.0537

Table 9: Median TKTL1 levels among all patients, median (interquartile range). P-values from Mann-Whitney U-Tests. *Tumor tissues from specimens obtain only from prostatectomies.

5 DISCUSSION

5.1 OVERVIEW OF DISCUSSION

An estimated 1.1 million men worldwide were diagnosed with prostate cancer in 2012 [1]. The rate of prostate cancer incidence increased greatly in the 1990s, then became stable over the last decade, possibly due to introduction of ready PSA testing.

However, men's life expectancy has generally increased. Since age is a well-known risk factor for prostate cancer, it is presumed that the economic burden will also increase over the years, unless new diagnostic, prognostic and therapeutic tools are discovered. In 2010, the total cost of handling prostate cancer (diagnosis, treatment and 5 year follow-up) was 567.906.109 Euros in Germany – 12,794 euros per patient [129].

Current diagnostic tools for detecting and screening prostate cancer include PSA and digital rectal examination. DRE should always accompany the PSA-test when performing an early diagnosis. A multicenter clinical trial comparing DRE and PSA serum in the early detection of prostate cancer revealed a PCa detection rate of 3.2% for digital rectal examination, 4.6% for PSA and 5.8% for the 2 methods combined, with a positive predictive value of 32% for PSA and 21% for DRE [130].

When prostate cancer is suspected, a prostatic biopsy -- conventionally, a 12-core transrectal ultrasound-guided biopsy – should be performed to diagnose prostate cancer [9]. When the diagnosis is made, we should have the PSA-values, the Gleason score (acquired though prostate biopsy) and the T-staging (through DRE or transrectal ultrasound). The clinical staging may be further extended when a suspicion of extraprostatic, locally advanced, or metastatic disease is present, including a pelvic MRI-staging and bone scan. These and clinical findings are the current preoperative parameters used by

urologists/oncologists for determining treatment options in patients with newly diagnosed prostate cancer.

If a surgical approach is chosen, a radical prostatectomy may be offered, involving complete removal of prostate, seminal vesicles, part of the urethra, and the surrounding lymph nodes. After surgery, the histological findings are analyzed and used as prognostic factors, together with the preoperative findings. These factors are used clinically on a daily basis to better monitor the patient and promptly intervene when recurrence is detected.

However, current diagnostic, predictive and prognostic parameters are still not so accurate, showing a need to explore other molecular biomarkers, to better predict the outcomes and prognoses of treated and untreated patients.

To better understand the progression of prostate cancer, TKTL1 expression was evaluated to determine its usefulness in detecting certain outcomes including disease aggression.

5.2 CURRENT TOOLS IN PROSTATE CANCER – PREOPERATIVE FINDINGS

Cancer screening programs are not universally accepted and there is no worldwide consensus that benefits outweigh the risks. A review from 2013 involving 5 randomized clinical trials (RCTs, including 341,342 men) concluded prostate cancer screening *did not* significantly decrease prostate cancer-specific mortality, though any reduction in prostate cancer-specific mortality may take up to 10 years to accrue. Overdiagnosis and overtreatment are common and are associated with treatment-related harm [131]. In other words, treatment offered to patients who would not have developed clinically relevant prostate cancer leads to unnecessary complications and side effects [132,133].

Later on, various studies attempted to evaluate whether PSA reduces prostate cancer mortality rates and those results remain controversial. The latest large randomized study performed by a European Randomized Study of Screening for Prostate Cancer (ERSPC) in 2014 showed substantial reduction in prostate cancer mortality attributable to PSA testing [134].

PSA-testing was originally approved by the FDA in 1986 to monitor the progression of prostate cancer and is still considered the gold-standard test for that purpose. And years later has been employed as a screening tool also.

As a screening tool, the PSA test has its own limitations and pitfalls, including false positive outcomes where there is an overlap in the serum PSA levels among men with cancer and those with benign disease, so is not disease-specific. In the absence of better tools, PSA is still useful, but lacking, so a great deal of effort is being devoted to improve its performance. Examples are the free PSA and the PSA density, detected by dividing the serum PSA level by the prostate volume detected by TRUS. The higher the density, the more likely it is to find a positive prostate biopsy. Prostate Health Index (PHI) is a recently approved diagnostic blood test, which uses a formula combining total PSA, free PSA and p2PSA (a PSA isoform found in peripheral blood). Patients with high total PSA and p2PSA and low free PSA are more likely to present with prostate cancer at clinically significant levels [135,136]. A meta-analysis from 2014, analyzing 7 studies, showed PHI had a specificity of 0.88 (95% CI, 0.82–0.92) and sensitivity of 0.74 (95% CI, 0.66–0.81) for prostate cancer detection [137].

Additionally, a transrectal ultrasound can be performed and is considered the procedure of first choice for prostate imaging. It should be noted that TRUS has poor accuracy in prostate cancer detection and staging [138].

Multiparametric magnetic resonance imaging, combining the morphological assessment of T2-weighted imaging with diffusion-weighted imaging is a tool used ever more frequently to help detect prostate cancer [139,140].

Despite significant progress in early detection, the majority of prostate cancers detected at early stages come with uncertain prognoses. This explains increasing efforts to discover and develop better clinical tools to monitor and

detect earlier recurrence, predict treatment response, and better understand the course of disease progression.

5.2.1 PROGNOSTIC PARAMETERS IN PROSTATE CANCER

48 radical prostatectomy specimens were obtained from male patients in this study, who underwent a curative surgical approach.

The prognostic tools used in standard practice include the pre- and post-operative PSA levels, [141–143] histological grade of tumour- Gleason score [16,144], histological subtype, TNM [145], performance status, [146,147] age [148,149], serum acid phosphate levels [150,151] and surgical margin [145]. These are the current parameters used to prognosticate the disease outcome following treatment and to predict biochemical recurrence.

The College of American Pathologists published a Consensus Statement in 1999 categorizing prognostic factors into three groups: Category I included proven and useful prognostic factors, Category II included promising factors, which were extensively studied, but not yet proven to be statistically significant, and Category III included factors that have not reached prognostic value. [145]

From the group of patients who underwent radical prostatectomies, we could retrieve all Category I parameters, including PSA-values, pathologic stage, Gleason score, and surgical margin.

The median preoperative PSA-value from patients who underwent radical prostatectomies was 6.93 (4.8-11.1). The most frequent pathological stage found was pT2c. Since radical prostatectomy has a curative intent, it was expected to find in this group of patients a pathological stage corresponding to a confined prostate cancer. All patient cancers were non-metastatic, as metastasis would contraindicate a radical prostatectomy. The most commonly found Gleason score were 6 and 9.

Research into biomarkers has exploded over the last decades. Some examples include prognostic factors/markers, only used in some centers, include Ki-69 (MIB-1), cell cycle markers (p27, p21, p43), DNA ploidy, p53, bcl-2, ELISA to human glandular kalikrein 2, and AMACR [152].

An abundance of other markers, used currently only in the research field, might be applied in clinical practice to better understand prostate cancer, especially its progression.

In the last decade some oncogenic signaling pathways and proteins, classically presented as regulators of cell division, revealed to have a link with glucose metabolism leading to aberrant metabolic programming. One example of a protein, which is associated with glucose reprogramming in oncogenesis is TKTL1. An existence of studies involving prostate cancer and TKTL1 immunostaining has, as of yet, not been found.

5.3   TKTL1 PROTEIN

The TKTL1 protein regulates and sustains the metabolic needs of the cells, playing a pivotal role as a reversible link between PPP and glycolysis, switching flux to meet cellular metabolic needs. Alterations in energy flow help the malignant cell to maintain its characteristics and to improve cell survival, proliferation and progression in a process called metabolic reprogramming. For these reasons, cancer cell metabolism is an area of great current interest to researchers [153].

A 2010 study found a close link between lactate dehydrogenase (LDH) isoenzymes (enzymes which catalyze pyruvate into lactate) and TKTL1 expression. This could indicate that TKTL1 is involved in glucose metabolism reprogramming, as mentioned above [154]. Another interesting study revealed a slowed cell growth, glucose consumption and lactate production in malignant cells (colon carcinoma cells) after TKTL1 suppression, revealing the importance of TKTL1 activity in the tumorigenesis process within some cancer cells [89].

The presence of an overexpression/upregulation of TKTL1 has been found in multiple cancers, including oral squamous cell carcinoma [155], rectal cancer

[156], gastric cancer [157], serous papillary ovarian adenocarcinomas [99], human endometrial cancer [158], renal cell cancer [100] and papillary thyroid carcinoma [159]. And in gene-silencing TKTL1 studies, cancer proliferation was significantly decreased in human hepatoma cell line [106] and human gastric cancer cells in vitro and in vivo [160].

Prior studies suggest TKTL1 may be indispensable in tumorigenesis. Through the PPP two major products are produced, ribose and NADPH, which will help to maintain an oxidative homeostasis; a high TKTL1 activity will also maximize the production of antioxidants and macromolecules, including nucleotides.

5.4 TKTL1 AND PROSTATE CANCER

To our knowledge, few studies have explored the association between variations in TKTL1 expression (in immunohistochemistry) in prostate cancer. Published studies looking into the relationship between TKTL1 and prostate cancer involved only the EDIM-TKTL1 test which presented contradictory results [109,161,162]. Grimm et al. showed EDIM-TKTL1 (with EDIM-Apo10, another marker under study) were highly sensitive and specific for detecting patients with prostate cancer, where 105 of 115 patients with prostate cancer showed positive EDIM-TKTL1 results [155].

A 2016 study involving prostate cancer patients revealed no correlation between serum TKTL1 (using Enzyme-linked immunosorbent assay, ELISA methods) with clinicopathological findings. This conclusion opposes prior studies using EDIM-TKTL1 test blood [162].

5.5 TKTL1 EXPRESSION - BENIGN VS PROSTATE CANCER

TKTL1 expression levels were firstly compared between benign and tumour tissue, revealing a significant difference. Benign tissue was retrieved from patients who underwent TURP, transvesical prostatic adenomectomy and

radical cystectomy. Concomitantly, although an open surgery has its own risk, an advantage compared to TURP is the prevention of TURP syndrome, a life-threatening condition where absorption of irrigation fluid during TURP leads to a hyponatremia.

Since TKTL1 is a relative new marker, a validated threshold has not yet been established, and neither has a scoring system. In the present work, the cutoff for benign vs. cancer was first determined using ROC analysis, a method commonly used in other medical areas and starting to be adopted by studies using IHC [163–165]. This was done as a first step, because we believe the ability to safely differentiate benign and cancer patients to be paramount. Afterwards, we tried to establish an adequate expression cutoff level that would differentiate patients with low and high TKTL1 concentration, in order to evaluate whether any clinicopathological characteristics are related to different degrees of protein expression. Unfortunately, this is not an easy task, because depending on the chosen outcome for the ROC analysis (N-stage, M-stage, Gleason score e.g.), the resulting groups will contain a different amount of patients. Taking into account current practices in published studies, we decided against using a clinical variable such as M- or N-staging as the defining characteristic for the cutoff. Instead, we began by using ROC analysis to find a TKTL1 expression cutoff that would safely separate benign tissue from tumour. This border was found to be a score of 125, with additional visual confirmation from plots. The cutoff from high to low TKTL1 expression was the midpoint TKTL1 score between M1-stage and M0-stage, which was 233. The validity and reproducibility of such approach could be debatable but, as mentioned above, it's the current practice in well regarded studies [166–171].

5.6 TKTL1 EXPRESSION IN RADICAL PROSTATECTOMY SPECIMENS

Among our cases, the median age of men with prostate cancer (who underwent prostatectomy) was 64 —not far from the expected age at diagnosis in patients

fit for surgery. A similar median age was reported in a study from Denmark, which included 1350 patients who underwent radical prostatectomy. The median age at surgery in this study was 63 years [172].

In Germany most tumours are discovered in the early T-stages (T1 and T2) [173]. In our cohort, 71.74% had a T-stage equal or less than T2.

From each radical prostatectomy specimen, we retrieved around 8-12 cores, grouped in 3 classes: far peritumoral, near peritumoral and tumour tissues.

Here, we found a significant difference between peritumoral (non-neoplastic) prostate tissue and prostate cancer, with a higher TKTL1 expression being found in tumoral tissue. The median TKTL1 score in peritumoral tissue was lower than in the tumour, but not lower than 125, the cutoff found between benign and cancer.

Presumably, the pre-tumorigenesis process had already begun in the peritumoral prostatic tissue, even though it was classified as non-malignant due to lack of observable architectural changes, potentially pointing out that molecular changes of cancerogenesis occur prior to the change of histological architecture. Ultimately, we believe the surrounding non-malignant tissue had initiated glucose metabolism alterations, in order to maintain and provide the required needs of a malignant cell, thus in order to maintaining a high metabolic rate and suppressing the potentially prejudicial effects of reactive oxygen species, improving survival against oxidative stress [174].

Interestingly, a significant difference was also found between the peritumoral tissue and benign tissue (with BPH). Revealing a positive progression of TKTL1 concentration from benign, to peritumoral to tumoral tissue. It could then be argued that, in peritumoral tissue, the altered metabolic alterations (present in cancer cells) had already begun, even though no morphological change could be found—it is likely that these cells had not yet accumulated enough changes to be reflected in the cellular architecture / morphology, which the Gleason score classification is based on.

Taking these results together, the significant difference between benign, peritumoral and tumoral tissue, shows a general trend of increased TKTL1 expression with disease progression from benign to malignant tissue.

Tentatively, this could be extrapolated to imply that the glycolytic metabolism alteration, specifically the shift from the oxidative phosphorylation pathway into aerobic glycolysis, is not so much an on/off switch, but a fluid progression (potentially parallel to other oncological pathways and reversible) that follows tumorigenesis.

5.7 TKTL1 EXPRESSION IN METASTATIC PROSTATE CANCER

As one of the aims of the study was to evaluate the progression of expression of TKTL1 in the disease progression of prostate cancer, we also included patients with metastatic PCa to better evaluate the TKTL1 expression in advanced or metastatic prostate cancer, the final stages of disease progression. Since in this group of patients a radical prostatectomy is not recommended, tissue samples were retrieved from patients who underwent palliative TURP.

Comparing the TKTL1 expression between this group of patients and patients who underwent radical prostatectomy showed a significant difference, TKTL1 being expressed higher in the group of patients who underwent palliative TURP.

Further analysis showed a significant difference between TKTL1 expression in patients with metastatic and non-metastatic prostate cancer, being found in lower expression in the latter group. Presumably, TKTL1 expression arises with tumour activity or volume.

Still analyzing the tissues of this group of patients, no difference was found between TKTL1 expressions in tissues from patients with hormone-sensitive or hormone-refractory prostate cancer. Perchance the mechanism of change from hormone-sensitive to refractory does not involve a change in the metabolism of

glucose solely. The growth of tumoral cells that have adapted to a castration environment, in order words, in low levels of androgens, is a complex mechanism, which is thought to be associated with androgen receptor pathway distortion [175–177].

5.8 TKTL1 EXPRESSION AND CLINICOPATHOLOGICAL FINDINGS

The construction for the clinicopathological correlation analysis was not an easy task due to the existence of two heterogeneous group of patients. One, in which the operation (radical prostatectomy) had curative intent, while the other group undergone a TURP, with palliative intent to relieve urinary obstructive symptoms. So the acquired clinical information is different in both groups, including the TMN classification. This means that the patients who underwent prostatectomy where classified based on a pathological report (pTNM), while the palliative patients had a clinical TNM (cTNM), based on clinical findings. Still, we believe both groups must be included in the analysis, since they represent different disease stages.

The results showed a correlation between high and low TKTL1 expression and M-stage, N-stage, T-stage and Gleason score.
When analyzing the data comparing the clinicopathological findings with a continuous TKTL1 levels such findings were not observed. The division in two groups (high and low TKTL1 expression) was determined by using a cutoff of 233.52, which is the midpoint between mean TKTL1 values of M1 and M0.

An association was found between higher Gleason scores and high TKTL1 expression. A phenomenon that is not yet understood, with further studies definitely encouraged; studies with a specific design aiming to understand the interaction of TKTL1 expression and aggressiveness of disease. There are no registered studies evaluating TKTL1 expression and Gleason score.
A progression of TKTL1 expression with Gleason score would be expected, because since morphological changes presumably arise from cytological

alterations, the more architectural distortion, the more intrinsic alterations should be found.

An association was also found between high TKTL1 expression and higher T-stages. When analyzing the TKTL1 expression between ≤ T2-stage (organ-confined disease) and >T2-stages a significant difference was found. The same result was achieved when comparing M1-stage to M0-stage, and N1-stage to N0-stage. Presumably a higher TKTL1 expression may be found to correlate with tumor load or activity, in other words, with higher numbers of cancer cells, increased size of a tumor, or with increased amount of cancer in the body. Higher TKTL1 values are significantly associated with more advanced disease stages. There was no correlation found with age nor PSA levels.

Although interesting results were found from the clinicopathological correlation analysis, it does not allow for definitive conclusions to be drawn. For that purpose, a follow-up study should include more patients in homogeneous groups. A longitudinal study following patients from the first diagnosis onwards, while logistically more complex, would pinpoint when the metabolic alterations start and better chronologically separate the TKTL1 increase and eventually determine outcomes.

This study has shown us that a dysregulation of metabolism may be integrated in the tumorigenesis of prostate cancer. The process of transformation to a more malignant cell, may involve a metabolic shift of flux or an imbalanced energy production not through a mitochondrial oxygen-dependent ATP production instead in aerobic glucose degradation. The overexpression of TKTL1 found in prostatic cancer cells support a finding of increased flux in the PPP, especially in metastatic prostatic cancer cells.

5.9 IMMUNOHISTOCHEMISTRY- PITFALLS

The prostate cancer diagnostic grading system is based on architectural organization and morphological characteristics of H&E stained prostatic tissue. In prostate cancer research numerous potential biomarkers are currently under study, some in the field of immunohistochemistry. Similar to Gleason score, immunohistochemistry has intra-observer and inter-observer variability [178–181]. Although this method has its limitations, including pre-analytical variability (fixation, antigen retrieval, incubation, endogenous peroxidase block, staining, counterstaining, mounting) and subjective interpretation and scoring systems, it still is a technique often used to help discriminate benign from malignant tissue, confirm the origin of poorly-differentiated carcinoma, subtyping carcinomas, and providing prognostic and therapeutic information [178].

Besides taking into account factors such as temperature, incubation time, fixation type, materials and control samples, *interpretation and scoring* will also play an important role when dealing with outcomes from studies using IHC.

Although immunohistochemistry has its limits, it's still a very important technique, already integrated in current diagnostic algorithms for cancers, including HER2 protein in breast cancer and HER2 in gastric cancer [116,182] .

In prostate cancer, immunohistochemistry is also applied to distinguish primary adenocarcinoma of the prostate from secondary tumours, using PSA, PSMA (prostate-specific membrane antigen), AR (androgen receptor), ERG (Ets-related gene product), Prostein (P501s), NKX3.1 (homeobox protein NKX3.1) [122,183,184].

Most studies use a semi-quantitative approach to determine expression, involving a visual scoring system. A major problem, that will aggravate with time due to increase number of IHC studies, are the contradictory results in literature and incompatibility to compare results due to lack of conformity in interpretation of IHC expression. Attempts are being made to find a better solution to interpret the staining, including automated IHC measurements involving software algorithms and whole-slide imaging systems. Studies have

shown that digital image analysis was superior to visual score in detecting differences within tissues [185,186].

TMA slides were reviewed two times. Estimating intensity of stain and extent is a common method used as a visual scoring system. However, estimation of extent in some tissue may not be an ease. The distribution / extension of a protein expression is usually not bimodal, the staining of cancer cells is heterogeneous and it displays a continuous range of staining intensities, being difficult to estimate the exact staining intensity as also the extent of each staining score. Which resulted in multiple cluster of TKTL1 expression H-scores, the impact of these cluster effect in the study was not determined.

Another limitation often found when working with immunohistochemistry is the determination of the cutoff score. In studies using novel markers in immunohistochemistry, a cutoff score is often determined arbitrarily—negative and positive, low and high. The method used to determine cutoff score varies in different studies. The cutoff between prostate cancer and benign was determined by ROC analysis. The cutoff from high to low TKTL1 expression was the midpoint TKTL1 score between M1-stage and M0-stage.

Secondly, in order to minimize pre-analytical variability, the TMA construction as also the staining process was performed by the same person, an expertise in the field of immunohistochemistry. Concerning the method of interpretation, a suggestion of future study would be to review and analyze again the data using an automated IHC measuring system, and compare it with the current data.

5.10 Future Directions

5.10.1 TKTL1 as a Diagnostic biomarker

An interesting result when comparing benign, non-malignant peritumoral and prostate cancer was revealed. TKTL1 expression in non-malignant peritumoral tissue was lower than in tumoral tissue and higher than in benign tissue.

From this finding we could extrapolate that glycolytic metabolism alteration is a fluid process following the tumorigenic transformation. TKTL1 expression reflects an intrinsic process of metabolomic reprogramming and therefore is not detectable through the diagnostic criteria of the Gleason score, since this classification is only based on morphological alterations. This means that, when analyzing these peritumoral tissues, they will be classified as non-tumoral, in spite of a tumorigenesis process that might have already begun.

What can this finding bring to the clinical practice?

The clinical diagnostic tools used to detect prostate cancer are, classically, the PSA-value, digital rectal test and biopsy (as imaging tests, we could include the transrectal ultrasound of the prostate and the multiparametric MRT-Prostate). Elevated PSA is a common indication for prostate biopsy, and due to the pitfalls associated with this marker, many biopsied patients with elevated PSA will yield a negative result for malignancy. This situation creates anxiety for the patient and the urologist, because the follow-up of this group of patients, and also the decision to re-biopsy, is never taken lightly.

Since TKTL1 expression is different in non-malignant peritumoral and in benign tissue, further research should be pursued in order to find if TKTL1 expression could help in patients with elevated PSA and prior negative biopsies to differentiate BPH from potentially pre-malignant process, helping the urologist to offer an adequate follow-up (more rigorous monitoring) and decide if a re-biopsy is needed, and if needed, when.

5.10.2 TKTL1 AS A PREDICTIVE OR PROGNOSTIC BIOMARKER

Prostate cancer is a heterogeneous disease with high variability in regards to therapeutical response and clinical outcome. Due to this variability the process of screening, diagnosing, therapy offers and monitoring can be difficult to the urologist, and achieving a successful outcome still remains a challenge. So a high demand in optimizing each step is crucial, leading to an increased interest in designing novel biomarkers.

Previously, the importance of discovering new screening/diagnostic biomarkers was discussed, but the importance and the valuable contribution in clinical practice of predictive and prognostic biomarkers should not be discarded. The finding of new predictive markers would help divide different populations with respect to the outcome, for example response to determined/target treatment. Differently, a prognostic marker would help to know which population of patients has a risk for a certain outcome, in case standard treatment or no treatment is given. The two terms were carefully described in order to clarify and alert that predictive and prognostic are to different concepts, being frequently misused and wrongly interchangeably used.

Since in the clinicopathological correlation analysis it was found a significant relationship between T-, M- and N-stage as also Gleason score with higher/lower levels of TKTL1, it would be interesting to perform a study with a higher number of patients in order to better clarify the clinical significance of TKTL1 expression in prostate cancer. Additionally, it would be interesting to conduct studies to analyze TKTL1 expression and patient's outcome. A study of this magnitude is difficult to manage and design, but if in a near future TKTL1 protein proves to be a potential biomarker or a potential target therapy, this kind of studies are crucial to perform

5.10.3 TKTL1 INHIBITION AS A CANCER TREATMENT

Another point of relevance for further studies is the impact of TKTL1 inhibition in prostate cancer, reinforcing the idea of a potential therapeutic innovation. Since it has already been published that inhibition of TKTL1 activity resulted in a diminished tumour growth rate, further research efforts should focus on uncovering the mechanism behind this change. Such findings could open the door for new target therapies in different cancer, hopefully in prostate cancer too. This concept has been proposed by Dr. Coy, which in 2005 invented a patent, which related to methods concerning the uses of antagonists or agonist of TKTL1 for treatment, detection and diagnosis of disorders associated with an altered transketolase (European patent EP 1701165 A1).

A recent published study from 2016, showed the potentiality of oxythiamine as an inhibitor of TKTL1 [187]. Further studies should be encouraged in this area.

An interesting study would be to evaluate the impact of TKTL1 inhibition in tumour growth or progression in prostate cancer.

6 CONCLUSION

In our study, TKTL1 showed a high specificity (86.36%) and sensitivity (92.30%) to distinguish benign from malignant prostatic tissue in IHC. The TKTL1 protein immunoexpression pattern ranges from a low level in benign prostatic tissue, to moderate immunoexpression in organ-confined prostate cancer, and high in metastatic prostate cancer. An upregulation of TKTL1 expression in prostate cancer was evident, and multiple clinicopathological parameters correlated with TKTL1: M-stage, N-stage, T-stage and Gleason score. A significant difference in the median of TKTL1 expression was found between peritumoral tissue and benign tissue. Both are considered normal tissue when using the Gleason score grading, but TKTL1 score was found higher in peritumoral tissue comparing to benign. A finding, which raises new questions in the area of pre-malignant lesions and incentives further research in this area.

Although the study revealed an association between TKTL1 expression with prostate cancer progression, the clinical value of TKTL1 is still unclear. Its place in the metabolic chain also makes it a potential therapeutic target, with the possibility of disrupting cancer cell metabolism. The applicability of TKTL1 in the clinical practice as an adjunct test in prostate cancer also warrants further investigation.

7 ABSTRACT

Deregulated cellular energetics has been recently proposed as an emerging hallmark of cancer. Cancer cells, in aerobic conditions, show an increased glucose uptake and lactate secretion in order to support their rapid proliferation. Transketolase like 1 (TKTL1) helps cancer cells meet their energy demands and combat oxidative stress and has been reported to be over-expressed in several human tumours including urothelial carcinomas, ovarial, colon and breast carcinoma. However, there is few data on TKTL1 in prostatic adenocarcinoma (PCa). Prostate cancer is the second most common cancer among men worldwide, and an urgent need exists for new diagnostic, prognostic and predictive tests.

In order to investigate TKTL1 expression in PCa in different stages of progression we included 124 tissue samples. Tumour tissue of 53 patients who underwent prostatectomy, as well as corresponding adjacent non-neoplastic prostate tissue were analyzed. A total of 45 patients with metastatic prostate cancer were also included. 26 patients were included as controls. From the TMA slides, we evaluated the expression of the TKTL1 protein, using the H-score (score range from 0 until 300) by immunohistochemical analysis. Due to artefacts or insufficient data, tissues from 24 patients were excluded from the study, leaving a total of number of 100 eligible patients.

TKTL1 expression was significantly higher in tumour tissue in comparison with normal tissue (243.13 (187.04, 295) compared to 100 (57.5, 105), *p<0.001*). The TKTL1 protein expression pattern ranges from a low level in benign prostatic tissue (100 (57.5, 105) to moderate expression in non-metastatic PCa (200 (172.19-254.38) and metastatic PCa (300 (222.50-300). In the group of patients with metastatic prostate cancer no difference was found between TKTL1 expressions in tissues from hormone-sensitive or hormone-refractory prostate cancer (p=0.9015). An upregulation of TKTL1 in prostate cancer was evident, multiple clinicopathological parameters showed significant relationship with high or low levels of TKTL1: M-stage (p=0.0023), T-stage (p=0.0027), N-stage (p=0.0257) and Gleason score (p=0.0075).

A significant difference was found between peritumoral (non-malignant) prostate tissue and benign tissue, a higher TKTL1 expression being present in peritumoral tissue (135.42 (100-195.16) compared with 100 (57.5-105), p=0.006). TKTL1 expression reflects an intrinsic process of glucose metabolism reprogramming and therefore is not detectable through the diagnostic Gleason score grading system, since this classification is only based on morphological alterations. Both group tissues were classified as non-malignant, in spite of a signs of tumorigenesis process being seen in the non-malignant peritumoral tissue.

TKTL1 protein is still not explored enough and has not yet reached its final potential. This enzyme could be a potential candidate as an adjunct test for prostate cancer or as a targeted inhibitor of tumour growth.

8 ZUSAMMENFASSUNG

Deregulierte zelluläre Energetiken wurden vor kurzem als ein sich neu herausbildendes Kennzeichen von Krebserkrankungen vorgeschlagen. Krebszellen zeigen, unter aeroben Bedingungen, eine erhöhte Glukoseaufnahme und Laktatsekretion um ihre rasche proliferation zu ermöglichen. Transketolase like 1 (TKTL1) hilft Krebszellen dabei ihren Energiebedarf zu decken und gegen oxidativen Stress anzukämpfen. Berichten zufolge ist TKTL1 in mehreren menschlichen Tumoren überexprimiert, darunter das Urothelkarzinom, Ovarialkarzinom, Kolonkarzinom und Mamakarzinom. Jedoch stehen nur wenige Daten über TKTL1 in Prostatakarzinomen zur Verfügung. Prostatakrebs ist die zweithäufigste Krebserkrankung in Männern weltweit. Es besteht ein dringender Bedarf nach neuen diagnostischen und prognostischen Methoden sowie predikativen Tests.

Um die TKTL1 Expression in PCa in unterschiedlichen Progressionsstadien zu untersuchen, bezogen wir in diese Studie 124 Gewebeproben ein. Tumorgewebe und non-malign peritumorales Gewebe aus Prostatektomien von 53 Patienten wurde analysiert. Auch wurden 45 Patienten mit metastasierenden Prostatakarzinomen in dieser Studie untersucht. 26 Patienten wurden als Kontrollen miteinbezogen. Aus den TMA Schnitten evaluierten wir die Expression des TKTL1 Proteins mit Hilfe des H-scores (von 0 bis 300) mit immunohistochemischer Analyse. Aufgrund von Artefakten oder unzureichenden Daten wurde Gewebe von 24 Patienten aus der Studie ausgeschlossen, was zu einer Gesamtzahl von 100 evaluierbaren Patienten führt.

TKTL1 Expression war in Tumorgewebe signifikant erhöht im Vergleich zu normalem Gewebe (243.13 (187.04- 295) vs 100 (57.5- 105), *p<0.001*).

Das Immunoexpressionsmuster des TKTL1 Proteins reicht von einem niedrigen Niveau in benignem Prostatagewebe (100 (57.5-105) über moderate Immunoexpression in nicht-metastasierenden PCa (200 (172.19-254.38) und metastasierendem Prostatakrebs (300 (222.50-300).

In der Patientengruppe mit metastasierendem Prostatakrebs wurde kein Unterschied in TKTL1 Expression in Geweben aus hormon-sensiblem oder hormon-refraktärem Prostatakrebs gefunden (p=0.9015). Die hochregulation von TKTL in Prostatakrebs war eindeutig, und wurden multiplen klinisch-pathologische Korrelationen mit dem M-Stadium (p=0.0023), T-stadium (p=0.0027), N-stadium (p=0.0257) und Gleason score (p=0.0075) gefunden. Ein signifikanter Unterschied zwischen peritumoralem (non-malignem) und benignem Prostatagewebe zeigte eine erhöhte TKTL1 Expression in peritumoralem Gewebe (135.42 (100-195.16) vs. 100 (57.5-105), *p*=0.006). TKTL1 expression reflektiert einen intrinsischen Prozess der Neuprogrammierung des Glukosemetabolismuses, und ist nicht mit dem diagnostischen Gleason score Bewertungssystem bewertbar, da diese Klassifikation nur auf morphologischen Veränderungen basiert. Beide Gewebegruppen wurden als non-malign klassifiziert, trotz Anzeichen   von Tumorgenesisprozessen die in dem non-malignem peritumoralem Gewebe festgestellt wurden.

Das TKTL1 Protein ist noch nicht genug erforscht worden und hat sein Potential bisher noch nicht erreicht. Dieses Enzym könnte künftig ein potentieller Kandidat (als eine Zusatzuntersuchung) für Prostatakrebs oder ein zielgerichteter Inhibitor des Tumorwachstums werden.

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10 Erklärung zum Eigenanteil der Dissertationsschrift

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Ich versichere, das Manuskript selbständig verfasst zu haben und keine weiteren als die von mir angegebenen Quellen verwendet zu haben.

Tübingen, den

Inês Anselmo da Costa

11 LIST OF TABLES

12 LIST OF FIGURES

13 ACKNOWLEDGEMENTS