Prostate Cancer Diagnosis
Experimental and Clinical Studies With HRMAS NMR Spectroscopy

Katarina Stenman
Dedicated to all men ... 
... and the women who care about them.
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Abstract

A few abnormal cells found in a small piece of prostate tissue are most consequential for a man’s future.

The prevalence of prostate cancer (PCa) is increasing globally. The main instigating factor for this cancer is not yet known, but it appears to be the consequence of many variables such as an increasingly older population, more frequent PSA-testing, and factors involving lifestyle.

Prostate cancer screening, as an equivalent for breast cancer screening, has been suggested but unfortunately there are no accurate diagnostic tools available for this type of screening. The reason for this is simply that the prostate is one of the most difficult organs to diagnose and, consequently, PCa screening would generate far too many false-positive and false-negative results. The prostate is not easily accessible as it is deeply-seated in the male pelvic area, wrapped around the urethra and surrounded by sensitive vital organs. Furthermore, PCa is frequently multi-focal, and the cancer cells have a tendency of assimilating among normal cells and, thus, do not always form solid lumps. Therefore, prostate tumors are often not felt by digital rectal examination (DRE) or identified by imaging. The PSA-test is not reliable as it is more prostate-specific than cancer-specific. Due to increasing prostate awareness, more early-stage and locally confined PCas are being detected. This is saving lives, although there is a high risk of over treatment and unnecessary side-effects. The increased detection of PCa requires sophisticated diagnostic methods and highly skilled clinicians who can discern between indolent and aggressive cancers. The current “gold-standard” for PCa diagnosis is biopsy grading by pathologists using the Gleason score system, which is a difficult task. Therefore, innovative methods to improve the precision of prostate diagnosis, by increased biopsy sensitivity and tumor localization, are of essence.

In light of these difficulties, the metabolomic approach using 1D and 2D high-resolution magic angle spinning (HRMAS) NMR spectroscopy combined with histopathology on intact prostatectomy specimens was evaluated in this research project. The non-destructive nature of HRMAS NMR enables spectroscopic analysis of intact tissue samples with consecutive histological examinations under light microscope. Metabolomics aids in the unraveling and the discovery of organ-specific endogenous metabolites that have the potential to be reliable indicators of organ function and viability, extrinsic and intrinsic perturbations, as well as valuable markers for treatment response. The results may, therefore, be applied clinically to characterize an organ by utilizing bio-markers that have the capacity to distinguish between disease and health.
The aim was to characterize the human and the rat prostate in terms of its intermediary metabolism, which is shown here to differ between species and anatomical regions. Furthermore, the aim is to seek the verification of HRMAS NMR derived metabolites which are known to be a part of the prostate metabolome such as, citrate, choline, and the polyamines which were performed, but also the identification of metabolites not previously identified as part of the local prostate metabolism, such as Omega-6, which was detected in tumors. The extended aim was to elucidate novel bio-markers with clinical potential. In this study, the common phyto-nutrient, inositol, which appears to possess protective properties, was identified as being a potentially important PCa bio-marker for the distinction between the more indolent Gleason score 6 and the more aggressive Gleason score 7 in non-malignant prostate tissues with tumors elsewhere in the organ. Further studies in this area of PCa research are therefore warranted.
Objective

The main objective of this thesis was to investigate and evaluate the application of HRMAS NMR on intact prostate tissues. During the course of the study, it became clear that nothing about the prostate is easy, supporting the validity of the statement below:

“Despite the major progress that has occurred in the biological sciences during the last 50 years, it is rather remarkable that we are about to enter the twenty-first century, and still the specific function of the prostate gland is unknown. Indeed the prostate is the largest organ of unknown specific function in the human body.”

Dr. John Isaacs
John Hopkins School of Medicine

Therefore, as a result, the initial aims and goals have been revised several times and finally resulted in a more comprehensive overview of the complexity of the prostate and a presentation of HRMAS NMR spectroscopy, used in conjunction with histopathology, as a powerful tool to gain a deeper understanding of the intracellular local metabolism of the prostate. This method has proved to be useful in providing valuable pieces to the, far-from-finished, puzzle of the prostate and its diseases.
Outline

This thesis consists of three parts and a total of 10 chapters. The aim is to guide the reader through the complexities that involves the prostate gland in general, throughout the theory behind NMR spectroscopy and its utility and, finally, towards the results and its interpretation and suggestions for future studies.

Part I

Chapter 1 presents a brief, but comprehensive, introduction about the research topic in general; prostate cancer.

Chapter 2 provides information about the prostate such as anatomy, function, and metabolism. It also includes an overview on the subject of experimental rodent prostate cancer models.

Chapter 3 reviews the different diagnostic options available for prostate diagnosis.

Part II

Chapter 4 and 5 presents the method used to generate the results in this thesis, namely NMR, with a strong emphasis on HRMAS NMR and metabolomics.

Part III

Chapters 6-10 comprise the design of experiments, methods, results, and discussions followed by research papers.
Original Papers and My Contributions

The following peer-reviewed papers are included in this doctoral dissertation and referred to by their Roman numerals.

My contributions are shown in Table 1.


Table 1: Contributions

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Note: All three papers were reprinted with kind permissions from the publishers.
Acknowledgements

“Haba na haba, hujaza kibaba”*

Swahili kanga writing

This study has been collaboration with Professor Anders Bergh, Pathology, the Department of Medical Biosciences, Umeå University Hospital; Professor Gerhard Gröbner, Physical Chemistry, the Department of Chemistry, Umeå University; and Professor Katrine Åhlström Riklund, Diagnostic Radiology, the Department of Radiation Sciences, Umeå University Hospital. This work has been funded at large by the Lion’s Cancer Research Foundation and also by generous grants from the Swedish Cancer Foundation, the JC Kempe Memorial Foundation Academic Funds, and the Wallenberg Foundation. Thanks are due to many people and as a precaution not to unintentionally exclude anyone, only the people in close collaboration are therefore mentioned: Dr. Jón Haukson, Professor Mikael Karlsson, Anna Wernblom, Christina Ericsson, Linda Kocsis, Dept. of Radiation Sciences; Professor Pär Stattin, Dept. of Surgical and Perioperative Sciences; Dr. Hans Stenlund, Dept. of Public Health and Clinical Medicine; Professor Dan Johnels, Ingmar Sethson, Professor Jurgen Schleucher, Dr. Izabella Surowiec, Dr. Henrik Antti, Dept. of Chemistry; Birgitta Ekblom, Elisabeth Dahlberg, Pernilla Andersson, Dept. of Medical Biosciences; Kerstin Almroth and Britt-Inger Dahlin, Dept. of Surgical and Perioperative Sciences. Lisbeth, Erica, Eivor, Anna, Ann-Kristin, Margaret, and Ronald, – thank you very much!! Again, special thanks to: Anders Bergh who performed all of the, very difficult and time consuming, histopathological evaluations of the prostate tissues, prior and subsequent HRMAS NMR analysis, and also contributed significantly in the writing of manuscripts; Gerhard Gröbner, who joined the group in a late stage and with his ambitious and efficient ways proved to be the “missing piece” for the completion of this dissertation; and my supervisor, Katrine Riklund, who administered this project. Last but definitely not least, thanks to the patients who generously donated their prostates for this study and thereby contributed to the highest degree to this research and this dissertation. We wish all of you the best of health!

*“Small things, when combined together make up big things”.

As this Swahili proverb says, all things are possible if you just take it little by little, step by step.
Abbreviations

AA  Arachidonic Acid  
Acetyl-CoA  Acetyl-Coenzyme A  
ATP  Adenosine Triphosphate  
AUC  Area Under the ROC Curve  
BPH  Benign Prostatic Hyperplasia  
COX  Cyclooxygenase  
CPMG  Carr-Purcell-Meibom-Gill  
CT  Computerized Tomography  
D₂O  Deuterium Oxide  
DLP  Dorso-Lateral Prostate  
DRE  Digital Rectal Examination  
EFA  Essential Fatty Acid  
EPA  Eicosapentaenoic Acid  
EtOH  Ethanol/Ethyl alcohol  
FID  Free Induction Decay  
GC-MS  Gas Chromatography-Mass Spectrometry  
GS  Gleason Scores  
HRMAS  High Resolution Magic Angle Spinning  
HSQC  Hetero-nuclear Single Quantum Coherence  
Hz  Hertz  
MRSI  Magnetic Resonance Spectroscopic Imaging  
MS  Mass Spectrometry  
NMR  Nuclear Magnetic Resonance  
NSAID  Non-Steroidal Anti-Inflammatory Drug  
OR  Odds Ratios  
PCa  Prostate Cancer  
PG  Prostaglandin  
PPM  Parts Per Million  
PSA  Prostate Specific Antigen  
PUFA  Polyunsaturated Fatty Acid  
RF  Radio Frequent  
ROC  Receiver Operating Characteristic  
TNM  Tumor, Nodes (lymph), and Metastasis  
TOCSY  Total Correlation Spectroscopy  
TRUS  Trans Rectal Ultra Sonography  
VP  Ventral Prostate
Part I
Chapter 1: Introduction

Globally, prostate cancer (PCa) is rapidly becoming the most common form of male specific cancer [Thun et al., 2010]. Consequently, a large international research community is actively engaged in trying to learn more about this deep-seated gland in the reproductive tract of the male body. The prostate, with its heterogeneity and anomalous metabolism, [Costello et al., 1997, 1999, 2000] is one of the most difficult human organs to diagnose. The PCa pathology poses a great challenge for clinicians and scientists [Fleshner et al., 2002; Swindle et al., 2008] and in order to understand this disorder, it is of absolute necessity to build up a thorough comprehension of this organ and learn to interpret its biochemical signals. The majority of PCa are slow-growing cancers and, in fact, more men die from this disease than not. The main goal in the fight against PCa is to find a reliable method that can differentiate between the indolent and the aggressive types [Madu et al., 2010]. There are ongoing discussions about diverse theories relevant to the cause and instigation of PCa. Although not exclusively, PCa and other ailments of the prostate are by far more common in older men [Sharma et al., 2010]. In addition to age, factors, such as, genetic susceptibility, diet and lifestyle, infectious agents, inflammation, etc. are all possible instigators for the development of PCa [Coffey et al., 2001; Gonzales et al., 2010; American Cancer Society, 2010]. The incidence of PCa and other diseases related to the prostate are expected to increase due to an ever increasing aging male population [Thun et al., 2010].

One of the main obstacles involved in the diagnosis of the prostate is its inaccessibility in the body. Furthermore, the prostate, especially a malignant one, is a heterogeneous structure with different cell types and pathologies, and cancer cells often infiltrate among normal cells [Zakian et al., 2005]. Contrary to other solid tumors, prostate tumors do not normally develop into solid lumps. Therefore, they are not always detectable by digital rectal examination (DRE), which, jointly with the Prostate-Specific Antigen (PSA) test, is part of the clinical routine examination when suspecting PCa [Kelloff et al., 2009]. Indirect diagnosis of PCa is achieved through a simple blood test, using the PSA-test. However, PSA is not cancer particular, but more so prostate specific even though PSA is also expressed in other tissues and in women, and thus not a reliable indicator. The value of PCa screening using the PSA-test is not determined given that it generates frequent false-positive results, which involves the increased risk of unnecessary biopsies and treatments [Wolf et al, 2010; Vickers et al., 2011]. PCa is multifocal and the cancer foci can be small, i.e., only millimeters in size, thus making DRE and biopsy sampling a challenge for the clinician. Furthermore, there is currently no imaging method available that can be used with high accuracy to guide biopsies towards the tumors and particularly
towards the most aggressive ones. Multiple biopsies are therefore taken from different parts of the prostate and as biopsies are small, many tumors are often missed [Andriole, 2009]. Biopsy sampling is generally performed in conjunction with Trans Rectal Ultra Sonography (TRUS) which provides an image of the internal organs, i.e. the prostate, and thus aids in guiding of the sampling towards the prostate although many tumors are often not seen. Requiring a skilled pathologist, biopsy specimens are examined and rated through the Gleason grading system; this diagnosis will be the basis for the treatment plan [Berney et al., 2007]. A high Gleason score indicates a poor prognosis; most men are detected with low or intermediate grade tumors and for them prognosis is highly variable and unpredictable. The diagnosis and prognostication of PCa are therefore complicated but research and technological advancements of clinical diagnostic methods and instruments are hopefully changing the situation for the better [Kelloff et al., 2009].

The prostate is often enlarged due to Benign Prostatic Hyperplasia (BPH) in men past the age of 50, sometimes even younger [Kirby, 2000]. BPH and its consequences such as inflammations [De Marzo et al., 2007], does not make PCa diagnosis easier. In fact, it is difficult to know what a completely healthy prostate looks like in a middle-aged man [McLaughlin et al., 2005] and as the senescence factor is a major part of PCa [Sharma et al., 2010] it is, consequently, virtually impossible to find completely healthy, older human controls for prostate cancer research studies. A wide variety of experimental rodent PCa models are available for pre-clinical studies. Many of these models are successfully utilized since it is often not possible to use human subjects due to obvious ethical, practical and economic reasons. However, the anatomy and the metabolisms of rodents differ from that of man. This is a major drawback [Lamb et al., 2005; Kamb, 2005].

There is currently no standardized method that can accurately diagnose, stage and predict the outcome for PCa that affects such a large proportion of the male population [Kelloff et al., 2009; Wolf et al., 2010]. Histological evaluation of prostate biopsies by skilled pathologists is presently the gold standard, used in conjunction with the PSA-test [Rajinikanth et al., 2008; Chun et al., 2010]. Novel diagnostic methods that can detect and accurately stage non-palpable and aggressive tumors at an early stage are urgently needed [Kelloff et al., 2009]. In-vivo Magnetic Resonance Spectroscopic Imaging (MRSI) [Giusti et al., 2010; Klijn et al., 2011; Scheenen et al., 2011], especially when used in conjunction with ex-vivo High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (HRMAS NMR) spectroscopy, has shown to be a promising alternative for the future [Kurhanewicz et al., 2002; Swanson et al., 2003; Santos et al., 2010]. Spectroscopy generates a fingerprint of the metabolism including a visualization of its perturbations due to disease, i.e., loss of homeostasis, which often occurs before morphological changes [van der Greef et
al., 2005; Batthen et al., 2010]. HRMAS NMR spectroscopy is a non-destructive method that allows the analyzed specimen to be examined under light microscopy post NMR [Beckonert et al., 2010; Moestue et al., 2011]. This feature makes it possible to correlate the spectrum with the specific pathology that can be verified with advanced immunohistochemistry.

All of the factors involved in PCa and discussed above affect the metabolism one way or another. Therefore, the focus of this study was to learn more about the local metabolism of the prostate, malignant as well as non-malignant/healthy in both man and rat. HRMAS NMR spectroscopy with subsequent histopathological analysis was used to obtain a correlated spectroscopic and morphologic “fingerprint” of the metabolism.
Chapter 2: The Prostate

2.1 What is a Prostate? Anatomy and Function

The name prostate means protector in Greek. It is believed that its main function is to protect the sensitive reproductive area and prevent urinary tract infections. The prostate is an exocrine gland slightly larger than a walnut that fits snugly, wrapped around the urethra, in the male pelvis. Its strategic location is right below the bladder and in front of the rectum (Figure 1) [McNeal, 1981].

![Figure 1: The male reproductive region.](image)

The terminology of anatomy or functional anatomy of the human prostate is complicated. This is due to the result of the anatomical changes that normally occur with age due to hypertrophy. Thus, the prostate of a young man is not comparable to the prostate of an older man [McLaughlin et al., 2005]. The location of the prostate is not ideal, (Figure 1), when it comes to clinical examinations and treatments as it is deeply seated and located close to vulnerable organs such as the bladder, the rectum, the sphincter muscles contracting the urethra, and the nerves regulating erection. Consequently treatments for prostate cancer may cause problems with the nearby organs with side-effects such as incontinence and impotence.

Four distinct regions or zones make up the prostate: the peripheral, the transitional, the central and the anterior fibro-muscular (Figure 2). The largest and most essential, adjacent to the rectum, the peripheral zone, contains most glands; it is also the site where most cancers arise. The second most important
one, including the periurethral gland region, is the transitional zone which directly surrounds the urethra. This is the zone where BPH originates and explains why BPH may result in urinary obstruction. The central zone surrounds the ejaculatory ducts and is often free of disease. The anterior fibro-muscular zone consists mainly of connective tissues and smooth muscles. The prostate is, at least in part, enveloped by a fibro muscular layer that is referred to as the capsule. [McNeal, 1981; Villers et al., 1991; McLaughlin et al., 2005]

![Diagram of the zones of the prostate](image)

**Figure 2: The zones of the prostate.**
*Image adapted from “De Marzo et al., 2007”.*

The prostate contains two different compartments: the glands and the fibro muscular cells. The glands contain luminal/secretory epithelial cells and basal epithelial cells as well as a few neuroendocrine cells [Sherwood et al., 1991; Roy Burman et al., 2004]. The stroma is contractile but it also generates many growth factors regulating the glands. Besides PSA, the prostate glands secrete a clear and slightly alkaline (pH 7.3) fluid [Mann, 1974], that contains compounds such as: citrate, zinc, spermine, potassium, acid phosphates, and calcium, many of which have protective and antibacterial effects. Including the slightly alkaline fluid produced in the Cowper’s glands, the fluid is drained via the prostatic ductal system into the urethra where it is combined with spermatozoa and fluid generated in the seminal vesicle. Collectively, these compounds make up the semen, expelled during ejaculation. The sperm is produced in seminiferous
tubules that are located in each testis and connected to the epididymal duct which in turn is connected to the vas deferens (Figure 1).

2.2 The Remarkable Metabolism of the Prostate

The human body is equipped with a highly sophisticated metabolic system committed to keeping us alive and healthy. The core of this system is the cellular respiration which is defined as the metabolic reactions and processes that take place in the cells in order to convert biochemical energy from nutrients into adenosine triphosphate (ATP), with the concomitant release of waste products. There are three main steps in this process: the Glycolysis which takes place in the cytosol, and this is where glucose is converted into pyruvate. Pyruvate is converted into Acetyl-Coenzyme A (acetyl-CoA) which enters the mitochondria and the Krebs cycle if oxygen is present. The oxidative phosphorylation step in the mitochondrial cristae comprises the electron transport chain that establishes a chemiosmotic potential across the inner membrane. This step drives the phosphorylation of ADP to produce ATP – the “transporter of chemical energy” (Figure 3) [Mathews et al., 1999].

![Figure 3: The cellular respiration system.](image)

The Krebs cycle is also known as the citric acid cycle or the tricarboxylic acid cycle (TCA cycle). It also goes by the name the Szent-Györgyi-Krebs cycle [Krebs, 1940], named after its two discoverers: Albert Szent-Györgyi (1893 – 1986) and Hans Adolf Krebs (1900 – 1981). Krebs cycle is probably the most important metabolic pathway in aerobic cells and organisms as it provides information
about the chemical intracellular conversions of carbohydrates, fats and proteins into carbon dioxide and water to generate energy. This dynamic and amphibolic process involves a series of enzyme catalyzed chemical reactions that occurs in the mitochondria of the cell. The citric acid cycle begins with the high energy compound acetyl-CoA which condenses with oxaloacetate, and catalyzed by citric acid synthetase, to generate citrate. Citrate is oxidized further in this cycle through a series of isomerisation-, oxidation-, and decarboxylation-steps that finally regenerate oxaloacetate in the final step in the Krebs cycle before the cycle is repeated (Figure 3).

The unusual, truncated Krebs cycle of the prostate (Figure 4) has been, and is still, largely overlooked by a large section of the research community although this is exceptionally significant in order to gain a complete understanding of this complex organ. Contrary to the average mammalian cell, normal and BPH prostate glandular cells accumulate high levels of both zinc and citrate [Mawson et al., 1952; Cooper et al., 1963, Costello et al., 1997, 1998, 2005; Singh et al., 2006].

![Image of the intermediary metabolism of the prostate](Image by permission from Costello LC, Franklin RB. Mol Cancer. 2006;5:17.)

The reason for this, besides the protective effects, is not fully understood and this is not associated with other cells in the human body. High quantities of both zinc and citrate are secreted to the prostatic fluid (Figure 4) where they are...
believed to function as antibacterial agents while the fluid is entering and flushing the many ductal systems in the gland [Fair et al., 1976, 1981; Kavanagh, 1985]. One of the functions of mitochondrial zinc is to inhibit mitochondrial aconitase (m-aconitase) which in turn prevents the oxidation of citrate in the Krebs cycle (Figure 4). Conversely, malignant epithelial cells are metabolically transformed to citrate-oxidizing, energy producing cells that lose the ability to accumulate zinc (Figure 4). This feature, unique for the prostate epithelial cells, is exploited in MRS where the spectroscopic levels of citrate are detectable (Figure 5). [Liney et al., 1996; Kurhanewicz et al., 2002; Swanson et al., 2003; Gillies et al., 2005]. The reduction of intracellular zinc is characteristic of PCa and is not associated with prostatitides or BPH [Zaichick et al., 1996; Costello et al 2009]

![HRMAS NMR spectrum](image)

Figure 5: The characteristic double doublet of citrate seen in a HRMAS NMR spectrum obtained from a healthy prostate tissue.

The reduction of mitochondrial zinc is, however, not due to a reduction of circulatory zinc in the body. Instead, there seems to be a hindrance in the intracellular function that regulates the uptake of zinc which is in agreement with the down-regulation of the ZIP1 transporter in cancer cells [Franklin et al., 2005]. Malignant prostate cells, just as with other malignant cells, have a higher need for energy (ATP) to handle the rapid proliferation involved. Since Otto Warburg’s pioneering studies in the 1920’s, it has been well known that most tumors have an increased glucose metabolism which has been termed the
Warburg effect [Warburg, 1956; Kim et al., 2006; Hsu et al., 2008]. However, the healthy prostate epithelial cell has a very slow metabolism, i.e., it is an energy inefficient cell with high aerobic glycolysis and low aerobic oxidation and its overall reaction is:

$$\text{Glucose} + 2 \text{Aspartate} + 2 \text{O}_2 \rightarrow 2 \text{Citrate} + 2 \text{CO}_2 + 14 \text{ATP}$$

The malignant prostate epithelial cells transforms into energy producing cells with a fully operational Krebs cycle with complete oxidation of glucose and with a low glycolysis:

$$\text{Glucose} + 6 \text{O}_2 \rightarrow 6 \text{CO}_2 + 38 \text{ATP}$$

Thus, the malignant prostate epithelial cells resemble typical, energy efficient non-dividing normal mammalian cells. As previously mentioned, citrate is produced in the mitochondria and is either oxidized by the Krebs cycle or exported to the cytosol for the conversion to acetyl-CoA. Cytoplasmic acetyl-CoA is essential for the production of fatty acids and sterols. In healthy prostate cells most of the citrate is exported to the cytosol and secreted into the prostatic fluid. In malignant prostate cells, there is an increased export of citrate from the mitochondria into the cytosol where it is cleaved by ATP-citrate lyase into the two compounds acetyl-CoA and OAA. Thereafter, the prostate cancer cells most likely uses the acetyl-CoA (after carboxylation) mainly derived from citrate, and some from acetate, for the increased requirement of the de novo fatty acid biosynthesis [Costello et al., 2006]. This complex pathway is suggested in Figure 6.

![Figure 6: Prostate cancer cell metabolism. Image by permission from Costello LC, Franklin RB. Mol Cancer. 2006;5:17.](image)
Prostate tumors (selectively) over express fatty acid synthase (FAS) which is the enzyme responsible for the de novo synthesis of fatty acids [Swinnen et al., 2000; Baron et al., 2004]. However, an alternative pathway to provide OAA and acetyl-CoA for the citrate initiated lipogenesis/cholesterogenesis pathway in PCa is suggested; namely the glutamate pathway where the two major products are citrate and alanine. Another option is the cytosolic direct synthesis of acetyl-Coa [Costello et al., 2006]. As acetate takes part in the cytoplasmic lipogenesis, the PET tracer 11C-Acetate is used and studied in PCa diagnosis; however, its role as a tracer is not determined [Jadvar, 2011]. Choline compounds are increased in prostate tumors and this feature is exploited by MRS [Swanson et al., 2003; McLean et al., 2010] and 11C-Choline PET [Jadvar, 2011]. As citrate alone is not sufficient for the discrimination between PCa and BPH its resonance is usually combined with the choline compounds, which are increased in PCa vs. BPH, in the (total-choline + creatine)/citrate (tCho+Cre/Cit) ratio [Gillies et al., 2005]. However, increases of choline compounds may not to be correlated with proliferation [Gillies et al., 2005]. As a matter of fact, the increased de novo lipogenesis in tumor cells may have a more complex role than earlier believed, possible even as a cellular protector from endogenous and exogenous insults by promoting membrane lipid saturation [Rysman et al., 2010].

Again, this points to the complexity of the human body and, consequently, further studies to elucidate the prostate cancer pathways are warranted in order to assess innovative bio-markers that can be used clinically with accurate results.

2.3 Man vs. Rat Prostate

For decades, researchers have tried to find experimental models that mimic the human prostate and its diseases; however, the perfect model has yet to be discovered [Kamb, 2005]. The underlying reason for these difficulties is, simply, the fact that the prostate is a very complex organ with large species differences. Factors such as heterogeneity, anatomical, zonal and cellular differences, and unpredictable metabolic pathways etc. needs to be taken into considerations when choosing an appropriate model. One important aspect that, unfortunately, is often set aside is age [Badawai et al., 2004; Jara et al., 2004] since it is often practically difficult to age match subjects in a research study. There is an economic factor involved in this [Hahn, 2004], namely the cost and time it requires to breed experimental animals to equal the age of an aging man which is, after all, the main category that acquires problems with the prostate. Thus, there is, unfortunately, not a universal tumor metabolism that is applicable to all tumors, species and anatomical zones. Extrapolations of experimental PCa results into clinical studies should therefore only be made under strict caution [Costello et al., 2005].
Rodent models are widely used in PCa studies, with variable success rate. Each model possesses strengths and weaknesses and is more appropriate for some disorders than others [Lucia et al., 1998]. The rat prostate (which is fairly similar to that in mice) is a large gland surrounding the urethra and is, contrary to the human prostate, composed of distinctly different lobes, the ventral, dorsal, lateral, and anterior lobes. The anterior lobe is also called the coagulating gland. The ventral prostate lobe has been in the focus of several studies although it has no equivalent in the human prostate [Roy-Burman et al., 2004], one reason is that it is easily accessible and comprises roughly half of the entire prostatic complex. The dorsal and the lateral lobes are often combined as the dorsolateral lobe (DLP) as the share the same ductal system and it is also practically difficult to separate the two. The LP is the lobe of the rat prostate that is the most similar to the human peripheral zone. The lateral lobe actually comprises two different ductal systems: the lateral type 1, and the lateral type 2 [Hayasi et al., 1991]. It is interesting to note that the lateral lobe of the rat prostate demonstrates similar cellular citrate and zinc accumulating characteristics as with the human glandular peripheral zone. The rat ventral lobe shares the same characteristics but not the dorsal lobe [Costello et al., 1998].
Chapter 3: Diagnostic Methods of the Prostate

The main clinical challenges in PCa treatment and management rely on accurate diagnosis and initial staging. As the PSA-test has become readily available [Wever et al., 2010] more clinically localized PCa are detected by consecutive histopathological diagnosis and Gleason grading on biopsies. Regrettably, the treatment for early stage disease is poorly defined [Berney et al., 2007]. Early detection of PCa has caused a rapid increase in terms of incidence [Wever et al., 2010]. Early detection and treatment of prostate cancer saves lives [Schröder et al., 2009]. Unfortunately however, early detection of cancer inevitably also leads to an increased risk of overtreatment due to the difficulties involved in determining disease aggressiveness and the accomplishment of accurate staging [Berney et al., 2007; Reese et al., 2011].

3.1 General Diagnostic Procedures

The general procedure for a clinical prostate examination includes: Digital Rectal Examination (DRE) and the Prostate Specific Antigen (PSA) test. Trans Rectal Ultra Sonography (TRUS) imaging if often used to guide biopsies [Cupp et al., 1993]. This examination provides basic, initial information.

3.1.1 Digital Rectal Examination – DRE

This is a routine examination that allows the doctor’s finger to feel the back wall of the prostate through the rectum. Any abnormalities detected will warrant for additional exams and tests. The front and the middle of the prostate are not felt during this exam. This is a subjective test and an urologist most likely has an advantage over a general practitioner.

3.1.2 Prostate Specific Antigen – PSA

PSA is a kallekrein (an enzyme that cleaves peptide bonds in proteins), which is produced by the prostate epithelial cells and it is a major component of the semen, where its function is to break down coagulated semen post-ejaculation. PSA is secreted via the prostatic ductal system and leaks into the bloodstream where it can be detected by a simple blood test. An elevation of PSA may be a sign of PCa since prostate cancer do not have a functioning ductal system that drains the fluid into the urethra. The lack of free passage forces the PSA into the
prostate where it eventually leaks into the blood stream. However, increased values can also be due to BPH, irritations of the prostate such as inflammation and infection. The PSA-test is prostate specific and not cancer specific. Therefore the optimal way to interpret a PSA-test and use it as an indicator for PCa may be to include factors such as: age, race, free vs. bound PSA, PSA velocity (when monitoring yearly), volume of prostate (especially the transition zone where BPH arises), and decide individual cut-off levels based on its baseline value [Oesterling, 1991]. However, a total PSA cutoff value of 3.0 ng/ml is often used as an indication for biopsy although some countries prefer 2.5 ng/ml while others prefer 4.0 ng/ml. The value of prostate cancer screening using the PSA-test is unclear [Vickers et al., 2011], although there seems to be a reduction in mortality albeit with a risk of overtreatment as high as 50% [Schröder et al., 2009]. PSA expression has also been reported in other tissues and also in females [Kraus et al., 2010]

3.1.3 Trans Rectal Ultra Sonography – TRUS

TRUS provides an ultrasound (harmless sound waves) generated image of the prostate and surrounding tissue and allows the physician to access prostate volume and examine the gland for abnormalities. Although this technique is able to detect many of the palpable tumors, it is of less value for early stage diagnostics where the tumors are non-palpable and, furthermore, some tumors are iso-echogenic and are therefore not visualized by TRUS. TRUS is valuable to guide biopsy sampling and to monitor treatments. Technical advancements in this field include contrast-enhanced (microbubble contrast) agent techniques [Aigner et al., 2010], and image fusion with TRUS and MRI [Turkbey et al., 2011].

3.2 Imaging of the Prostate

Abnormal DRE and/or PSA-test calls for a more advanced evaluation using image based diagnostic procedures including, anatomical, functional and molecular imaging techniques. Imaging techniques, such as Computerized Tomography (CT), Magnetic Resonance Imaging (MRI) (Figure 7), and Positron Emission Tomography (PET), facilitates the evaluation of internal organs without the need for surgery. Imaging of the prostate holds an important role in the integrative approach for a patient with PCa where diagnosis, staging, and monitoring in watchful waiting or the response of treatment are part of the regimen. The field of imaging has expanded from the characterization of locally advanced or metastatic disease to include intra and extra prostatic tumor delineation, including morphology and zonal anatomy. Current clinical imaging
techniques cannot detect early disease and consequently generate limited information to be used for accurate staging; therefore, new techniques that can provide anatomical, functional and molecular imaging information are urgently needed [Kelloff et al., 2009].

![Figure 7: CT and MR examinations of prostate with benign hyperplasia and PCa in the same patient. Black arrow (CT) and white arrow (MR) indicate regions containing malignant tissue in hyperplastic prostate gland.](image)

### 3.2.1 Computerized Tomography - CT

CT allows for a cross-sectional visualization of the internal organs, with or without an intravenous contrast material (Figure 7, left). CT does not offer high diagnostic accuracy in PCa and are, therefore, often excluded from the battery of diagnostic tools available for the evaluation of the prostate. It is, however, valuable for the planning of radiation therapy. CT is not indicated in early PCa but can be used to diagnose distant metastases and sometimes lymph node metastases.

### 3.2.2 Positron Emission Tomography - PET

PET is a nuclear medicine imaging technique which produces a 3D image of functional processes in the body. PET is actually more correctly a 4D modality where the 4th dimension is time. The system uses small amounts of radioactive tracers that indirectly emit pairs of gamma rays which are detected on by the PET scanner. The radiotracers are fused on with biologically active molecules, such as glucose, and introduced into the body. The location and amount of radiotracer absorbed is visualized on images offering details on both the structure (from CT) and function (from PET) of organs and tissues. To get
optimal anatomical, functional and molecular information PET combined with CT is used as; PET/CT [Bouchelouche et al., 2010].

\(^{18}\)F-18-fluorodeoxyglucose is the most common PET tracer for tumors; however, it is not useful in local PCa detection [Avril et al., 2010]. The roles of \(^{11}\)C/\(^{18}\)F-acetate or \(^{11}\)C/\(^{18}\)F-choline as PCa PET tracers for primary diagnosis are not clear [Jambor et al., 2010].

### 3.2.3 Magnetic Resonance Imaging - MRI

MRI, preferably with an endorectal coil, is used to improve prostate tumor detection. MRI offers high-quality images of the internal organs (Figure 7, right). The method is based on the magnetic behavior of hydrogen atoms as part of the body’s inherent biomolecules and water in an outer magnetic field and their manipulation by radiofrequency waves; an approach which generates images with valuable information about the human anatomy and morphology.

Normally, a T\(_2\)-weighted MRI obtained by with standard 1.5 Tesla scanners equipped with endorectal coils, is used to detect and localize PCa. Tumors are characterized by decreased signal intensity compared to the normal peripheral zone. 3.0 Tesla scanners provides increased spatial resolution and thus improved localization and staging. However, this MRI method does not provide the sensitivity and specificity needed for accurate results of early stage PCa as the cancer cell clusters often are minuscule. Additionally, there are problems detecting tumors in zones other than the peripheral one [McMahon et al., 2009]. Multiparametric MRI combines T\(_2\)-weighted imaging with one or more of following approaches: Diffusion Weighted Imaging (DWI), Magnetic Resonance Spectroscopy (MRS), and Dynamic Contrast Enhanced (DCE) functional techniques. This is performed in one single study combining the different imaging protocols [De Visschere et al., 2010; Weidner et al., 2011].

Contrast agents such as Ultra Small Supermagnetic Iron Oxide (USPIO) particles add additional, valuable information to an MRI scan by increasing the signal of PCa cells in lymph nodes [Thoeny et al., 2009]. However, lymph node staging is still limited, also with MR. Currently MRI is of great value in difficult cases, although it is not part of the clinical routine.

### 3.2.4 Magnetic Resonance Spectroscopic Imaging – MRSI

MRSI is a highly promising, non-invasive method, useful in the field of PCa diagnosis. This cross-sectional technique offers an excellent overview of the
organ in all dimensions in-vivo, and possibilities for longitudinal assessments of therapy, short and long-term changes related to disease and healing, volume and density changes etc.

MRSI, preferably with an endorectal coil, provides visual anatomical and morphological information simultaneous with spectral information about the local metabolism and provides assessment of metabolic changes during a disease process or treatment responses. This is of special interest since metabolic perturbations often occur before the concomitant morphological changes. The most commonly studied bio-markers for PCa found in the prostate metabolism include citrate, choline compounds, and creatine. The ratio of choline compounds (t-choline) and citrate are by far the most utilized biomarker for the discrimination of malignant and non-malignant tissue, however, it may not be the most optimal one [Swindle et al., 2003].

MRSI is not used routinely for prostate evaluations since it is more in the experimental stage with room for improvements and furthermore, it is both costly and time consuming. The present drawbacks of MRSI include difficulties in obtaining optimal spectral resolution, poor spatial resolution, artifacts that are, for example, caused by movements, in homogeneous external magnetic field, metals, radiofrequent “noise” etc., but also the requirements for specialized software and experienced personnel to acquire and interpret the data since this technique is very much operator dependent and thus, as with most other diagnostic tools, highly subjective [Kreis, 2004]. Furthermore, the prostate is, as mentioned earlier, a heterogeneous organ and the tumor foci are often infiltrated among normal tissue and, thus, will not appear clearly with MRSI. Moreover, a more precise definition and understanding of functional anatomy of the prostate is needed [McLaughlin et al., 2005]. However, MRSI is of immense use in difficult cases. Technological advancements, such as functional, 2D and 3D techniques and higher field strength, will improve tumor localization and render MRSI a highly promising technique for the future [Thomas et al., 2008; Kelloff et al., 2009; Larson et al., 2010].

3.2.5 Small Animal MRI/MRSI

Small animal MRI/MRSI scanners are rapidly emerging in modern research facilities around the world. Animal models, particularly rodents, and the use of MRI/MRSI have greatly advanced researchers’ understanding of various pathologies and the effects of treatments etc. Since this technique is non-invasive it has drastically reduced the number of animals needed since each animal can be used more than once and thus followed longitudinally, i.e. each animal can be used as its own control [Nastiuk et al., 2007]. However, given the
small size of rodents and the associated technical challenges, however, prostate imaging studies in live animals only recently have become feasible, thanks to technological improvements.

3.3 Prostate Biopsy

Taking and evaluating prostate biopsies are a team effort between the urologist and the pathologist, and this is the most significant factor for the final diagnostic verdict which strongly influences decisions regarding options for therapy. TRUS guided biopsy sampling is most commonly used where the needle is inserted through the thin rectal membrane into the prostate. Tissue samples are extracted, with the biopsy needle, from different areas of the prostate. As tumors often cannot be detected by imaging the prostates are systematically sampled with 10-12 or more specimens along the sides of the peripheral zone and it is recommended to sample a few laterally. Prostate tumors usually grow like thin sheets laterally and consequently it is easy to misguide the biopsy needle past the target. A larger prostate requires a more extensive sampling than a smaller size. The biopsies in total however sample less than 1% of the prostate volume. This means that there can be a significant amount of aggressive cancer present that will be undetected by the biopsies. Again, imaging methods such as TRUS, CT or MRI do not have the diagnostic accuracy to detect microscopic disease. Thus, image guided biopsy sampling is not straight forward. PCa awareness, in the era of PSA-testing, has increased the number of men diagnosed with low-grade/low-risk cancers. This has led to treatment options such as focal therapy and active surveillance to avoid unnecessary radical therapy with its many negative side-effects. Since PCa often is multi-focal, both treatment options require improved diagnostic methods in order to rule out aggressive tumor foci elsewhere in the gland [Lazzeri et al., 2010; Ukimura et al., 2011]. Biopsy is an invasive procedure with risks for complications such as: infection, bleeding, and in some cases sepsis. Therefore, it is highly desirable to avoid unnecessary biopsies and to discover bio-markers that possess the ability to detect microscopically small amounts of aggressive cancer cells in the specimen and perhaps indicate that a cancer could be present in the nearby tissue.

3.4 Histopathological Interpretation of Tissue Specimens

Prostate tissue is exceptionally heterogeneous by nature which complicates classification and grading. Tumor grading and staging of prostate cancer is fundamental in characterization and prognosis. The Gleason score system provides information regarding the type of cancer cells while the TNM staging system provides additional information concerning the extent of the cancer.
3.4.1 The Gleason Score – GS

The most widespread method of PCa tissue grading used today is the GS system which is based on microscopic tumor patterns and is the single most important prognostic factor [Rajinikanth et al., 2008]. The tissue specimen is examined under light microscope where cells or groups of cells that are markedly different from healthy prostate tissue are categorized. The greater the disparity between the healthy cells and the malignant cells the higher the grade and the more likely the tumor is aggressive and will metastasize. The GS diagram exhibit five distinctly different tissue patterns that are technically referred to as tumor “grades” (Figure 8). The microscopic determination of the loss of normal glandular structure caused by the cancer is represented by a grade number ranging from 1 to 5, with 5 being the worst grade possible (Figure 8).

![Figure 8: The Gleason score system.](image)

The final Gleason score is a sum, ranging from 2-10, of the primary grade which represents the most common type of tumor cells (>50 %), and a secondary grade which represents the second most common type of tumor cells (<50% but >5%), that are found in the specimen [Gleason et al., 1974]. However, this determination is highly subjective and thus requires a skilled pathologist. The GS from a biopsy frequently differ from the GS of radical prostatectomy (RP). It has been reported that the difference may be as much as 60-70 % and that under grading is often the case in biopsy although the precision of GS has improved over the last years [Rajinikanth et al., 2008]. A modified Gleason score is also
suggested, a score based on the most common and the worst grades present [Epstein et al., 2010].

### 3.4.2 The TNM Table

TNM stands for Tumor, Nodes (lymph nodes), and Metastasis. This widely used staging system gives information about the extent of the primary tumor (T stage), the absence or presence of spread to nearby lymph nodes (N stage) and the absence or presence of distant spread, or metastasis (M stage) (Figure 9).

![TNM Table](image)

*Figure 9: The TNM Table.*

TNM staging, just as with the Gleason score system, requires skilled interpreters. Under and over staging is common, i.e., the clinical staging (pre-surgery) results might often not correspond with the pathological staging (post-surgery or ex-vivo evaluation). This is, however, not surprising since different information for the staging are used. Correct staging is critical because treatment is directly* related to disease stage [Hoedemaeker et al., 2000].

### 3.4.3 Immunohistochemistry

Interpretation of prostate tissue specimens is a challenge. The reason for this is the heterogeneous nature of prostate tissues in general but also the new
demands that comes with earlier detection in the era of PSA-tests/screening [Paner et al., 2008; Chun et al., 2010]. Differentiation of minimal cancer (<5 % of biopsy specimen), atypical small acinar proliferation (ASAP), and high-grade prostatic intraepithelial neoplasia (HGPIN) have increased the use of immunohistochemical methods detection; α-methyl-acyl-coenzyme A racemase (AMACR), nuclear p63, and the basal cell cytokeratin (34βE12). With the use of such markers cancer can generally be distinguished from other conditions, but as there are several mimickers of prostatic adenocarcinoma these immunohistochemical markers are not universally applicable [Epstein, 2004].
Part II
Chapter 4: Nuclear Magnetic Resonance - NMR

4.1 General Introduction

In the recent decades, NMR spectroscopy [Pople et al., 1959; Ernst et al., 1987] emerged as a powerful method to provide structural and dynamical information of molecules at atomic level as well as unique diagnostic information in medicine. Therefore, not surprisingly, a range of Nobel Prizes was awarded in connection with NMR; Otto Stern (USA, 1943), Isidor I. Rabi (USA, 1944), Felix Bloch, and Edward M. Purcell (USA, 1952), Richard R. Ernst (Switzerland, 1991), Kurt Wüthrich (Switzerland, 2002), [The Nobelprize., 2010]. Chemist Paul C. Lauterbur, USA and physicist Peter Mansfield, United Kingdom shared the Nobel Prize in Medicine 2003 for the development of magnetic resonance imaging (MRI). MRI, which has become an invaluable diagnostic method in medicine, is directly derived from NMR, a fact that is often neglected in the medicinal community [Fry et al., 2004].

The technique relies on the property of various nuclei, to possess a nuclear spin, whose size is defined by the quantum number I. The nuclear spin is connected with specific nuclear magnetic properties whose discovery by Rabi was awarded the Nobel Prize in 1944. These magnetic properties, mainly the magnetic nuclear moment, enabled researchers to study these nuclei in magnetic fields for obtaining unique structural, chemical, physical and electronic information about molecules since 1958.

4.2 The NMR Signal

The most common nuclei used in NMR in life science are $^1$H, $^{13}$C, $^{19}$F and $^{31}$P. In general, these NMR active nuclei possess a property called spin with a value of $\frac{1}{2}$. Insertion of a sample into a permanent outer magnetic field ($B_o$) causes the nuclear magnetic moment of these nuclei to adopt different positions with respect to this magnetic field, accompanied by a splitting up of their energy into discrete energy levels. A nucleus with a spin of $\frac{1}{2}$ adopts two possible magnetic quantum states; $m=1/2$ which is the lower energy state also called $\alpha$ state or $E_1$, and $m=-1/2$ which is the higher energy state also called the $\beta$ state or $E_2$ (Figure 10).
This process is accompanied by a precession of the magnetic moments around the $B_0$ like spinning tops. The frequency of this precession is called Larmor frequency and corresponds to:

$$\Delta E = \nu h$$

$\nu$ = Larmor frequency  
$h$ = Planck’s constant: $6.63 \times 10^{-34}$ J s

The energy difference (between two adjacent energy levels) of the nuclear spins in the outer magnetic field and it is dependent on the gyromagnetic ratio $\gamma$ of the nucleus, and the outer magnetic field strength, $B_0$.

$$\nu/2\pi = -\gamma B_0$$

$\gamma$ = gyromagnetic ratio  
$B_0$ = magnetic field strength

However, due to the energy difference between both states (in the case of $I=1/2$), there will be a small excess of spins aligned in the direction of $B_0$, at the lower energy state $E_1$. This population difference will result in a macroscopic magnetization of the sample parallel to the outer magnetic field, represented as
equilibrium magnetization vector, \( M_0 \). This resulting vector \( M_0 \) equals \( M_Z \) since it is aligned along the \( Z \)-axes. \( M_Z \) is referred to as the longitudinal magnetization (Figure 11).

![Figure 11: NMR coordinate system.](image)

The size of \( M_Z \) is dependent on the Boltzmann distribution, as it is determined by the following equation;

\[
\frac{N_{E_2}}{N_{E_1}} = e^{-\Delta E/kT}
\]

\( N_{E_2} = \) higher energy state  
\( N_{E_1} = \) lower energy state  
\( \Delta E = \) energy difference between the spin states  
\( k = \) Boltzmann’s constant: \( 1.3805 \times 10^{-23} \) J/Kelvin  
\( T = \) temperature in Kelvin.

**4.3 The NMR Experiment**

By applying in an NMR experiment radio frequency pulses at a frequency \( \nu \) which corresponds to the energy difference \( \Delta E \) between both states, it is possible to flip the nuclear spins from their respective \( \alpha \) to the \( \beta \) states via absorption of this energy. An absorption of energy occurs when the energy \( E \) of the radio frequency pulse meets the resonance conditions, namely the Larmor frequency corresponding to the energy difference \( \Delta E \) between the two spin states \( E_2 \) and \( E_1 \), which is described in the formula;

\[
E = \Delta E = h\nu B_0
\]
The radio frequent (RF) pulse applied along the X-axis creates a secondary magnetic field that disturbs the alignment of the magnetic moments and causes them to flip away from the equilibrium state. The required frequency is dependent upon the static magnetic field, \( B_0 \), and the nuclei observed. A perpendicular flip of the initial magnetization \( M_z \) from the Z-axis to the XY-plane where the transverse magnetization \( M_Y \) can be detected, requires a 90° pulse along the X-axes. This net magnetization may be described as an oscillating magnetic field in excess that induces an alternating voltage in the detection coil. This is the NMR signal also called free induction decay (FID). In general, this signal diminishes over time due to the decay of the transverse magnetization \( M_{XY} \) in the XY-plane, characterized by the spin-spin relaxation time \( T_2 \) according to:

\[
M_{XY} = M_{XY0} e^{-t/T_2}
\]

In addition, there is a process, called spin lattice relaxation, which describes the return of the longitudinal \( M_Z \) magnetization towards its equilibrium (as prior to the application of the NMR pulse). This process is characterized by a sample specific spin lattice relaxation time, \( T_1 \), and is described in the equation:

\[
M_Z = M_o (1 - e^{-t/T_1})
\]

In an NMR experiment, the FID is time-dependent and can be converted into the frequency domain by Fourier transformation [Jacobsen, 2007]. This will provide the typical NMR spectrum, where frequency differences (magnetic field differences) are plotted as a function versus intensity on a graph (spectrum). This allows the visual observation of specific quantum mechanical magnetic properties of the selected atomic nuclei. The most common type of nuclei frequently analyzed by NMR is hydrogen (\(^1\)H) which is, besides being the lightest, by far the most abundant atom in the universe. NMR is nowadays frequently utilized in the medical fields in the form of MRSI.

### 4.4 Chemical Shift

The chemical shift is a function of the nucleus and its local chemical environment, thereby giving detailed information about the part of the molecule where the nucleus is residing. By understanding different chemical environments it is possible to obtain structural information about the molecule in a sample [Chamberlain, 1959]. This is because the electronic environment, that shields the nucleus from the magnetic field, is distinctive of the particular nucleus and, thus, provides the exact resonance frequency of the nucleus. The difference between the applied magnetic field and the field at the nucleus is
termed the nuclear shielding. The differences in the resonance frequency are referred to as the chemical shift and it provides the information needed to identify the compounds visible by NMR spectroscopy. The frequency shifts are extremely small in comparison to the fundamental NMR frequency. In order to detect the minuscule frequency differences the applied magnetic field must be kept constant throughout the whole sample volume. This is done by using shim coils. Since $B_0$ differs to some extent between spectrometers the frequency measurement in Hertz (Hz) was converted to a standardized scale that measures in parts per million (ppm). This was done in a way that the NMR chemical shift can be universally compared in ppm regardless of magnetic field strength. For $^1$H-NMR the chemical shift range is just over 10 ppm. The chemical shift is calibrated using an internal standard as a reference; such as the commonly used (tetramethylsilane) TMS [Van Dyke Tiers, 1958]. The chemical shift is obtained by using the following calculations:

$$\delta = \frac{(v - v_0)}{v_0}$$

$\delta$ = chemical shift  
$v$ = resonant frequency  
$v_0$ = standard frequency

Frequently, a secondary reference of known chemical shift can be used instead of the common standards such as TMS. A chemical reference standard of known concentration is added to the sample and the concentration of the solute can then be determined by comparing the integrals of the signals. The secondary references are often the residual solvent signals or the unsuppressed H$_2$O signal [Cheng et al., 1998].

The secondary references are often used in quantitative NMR. However, there are challenges involved in this, especially when quantitating HRMAS spectral data. The standard to be used must be compatible with the sample, be chemically inert, have a longitudinal relaxation time ($T_1$) that, preferably, is shorter than that of the sample, and furthermore, not generate resonances that cause overlaps [Akoka et al., 1999]. A semi-quantitative approach is to compare spectral ratios within each sample which does not require the need for suitable internal standards. However, this method does not allow for separate resonances to be studied independent from each other [Swanson et al., 2006; Sitter et al., 2009]. Technological advancements have contributed to the development of new types of internal standards such as the Electronic REference To access In vivo Concentrations (ERETIC). As the ERETIC signal is generated by an electronic device it is therefore not dependent on the sample. This electronic reference signal aids in the determination of absolute concentrations [Akoka et al., 1999;
Modification and improvements of the ERETIC reference include, as an example, the Amplitude-corrected Referencing Through Signal Injection or ARTSI [Mehr et al., 2008].

4.5 Spin-Spin Coupling

Spin-spin coupling is an essential attribute of NMR as it provides detailed structural and conformational information of the molecule. This spin-coupling describes the phenomena that one nucleus feels the presence and spin state of another nucleus nearby via connecting bonds (transferred via the magnetic properties of the bond electrons). Spin-spin coupling can cause splitting of the signal for each type of nucleus into two or more lines depending on the number of chemically bonded neighboring nuclei. Thus, coupling to \( n \) equivalent (spin \( \frac{1}{2} \)) nuclei will split the signal into an \( n+1 \) multiplet with intensity ratios according to Pascal’s triangle, as can be seen in Figure 12. Pascal's triangle [Lash et al., 1987], named after the French mathematician Blaise Pascal, entails a set of numbers aligned in the shape of a pyramid. These numbers represent the binomial coefficients. Nuclei that are chemically equivalent (isochronous) do not exhibit spin-splitting between each other and thus have no effect on the NMR spectra.

<table>
<thead>
<tr>
<th>Neighboring Nuclei</th>
<th>Splitting Pattern</th>
<th>Relative Intensities of Split Peaks</th>
<th>Multiplet</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>L</td>
<td>1</td>
<td>Singlet</td>
</tr>
<tr>
<td>1</td>
<td>L L</td>
<td>1 1</td>
<td>Doublet</td>
</tr>
<tr>
<td>2</td>
<td>L L L</td>
<td>1 2 1</td>
<td>Triplet</td>
</tr>
<tr>
<td>3</td>
<td>L L L L</td>
<td>1 3 3 1</td>
<td>Quartet</td>
</tr>
<tr>
<td>4</td>
<td>L L L L L</td>
<td>1 4 6 4 1</td>
<td>Quintet</td>
</tr>
</tbody>
</table>

*Figure 12: Splitting patterns.*

The size of the splitting; i.e., the coupling constant which is annotated \( J \), is independent of the magnetic field and is therefore measured as an absolute frequency, usually in Hertz.
4.6 High-resolution Magic Angle Spinning (HRMAS) NMR

The line width of an NMR resonance depends on orientation-dependent factors, such as chemical shift, chemical shielding anisotropy, and magnetic susceptibility of the sample and susceptibility differences within the sample. The rapid isotropic motion of the molecules in liquid state NMR averages the anisotropic interactions and reduces broadenings due to dipolar couplings resulting in narrow line widths. Furthermore, by choosing the correct sample geometry, the broadenings, due to magnetic susceptibilities, are minimized [Brown et al., 2001].

Solid or semi-solid samples are more difficult to analyze by NMR due to the lack of molecular mobility and therefore the anisotropic nature of the corresponding NMR spectra, which are usually wideline spectra without any significant resolution. Due to this anisotropic nature, the NMR signal depends on the orientation of the individual molecules with respect to the outer magnetic field; an orientation dependence which for most NMR relevant interactions can be described (in the case of spin I=1/2 systems) by the $2^{nd}$ Legendre Polynom $P_2(\cos \theta)$, with $P_2(\cos \theta)=(3\cos^2\theta-1)/2$. This term becomes zero when:

$$(3\cos^2\theta-1)/2 = 0$$

$$\cos^2\theta = 1/3$$

$$\theta = 54.74^{\circ}$$

This $54.74^{\circ}$ angle is called the “Magic Angle” [Andrew et al., 1959]. By spinning the sample rapidly at the magic angle, $54.74^{\circ}$ with respect to $B_0$ the orientation dependent $P_2$ term will be time averaged to zero. This will reduce anisotropic interactions to zero, or in the case of chemical shift anisotropy to their isotropic value and will break up the wideline NMR spectra into a set of individual narrow lines which can be assigned as in the case of liquid NMR spectroscopy (Figure 13), [Maas et al., 1996].
High Resolution Magic Angle Spinning (HRMAS) NMR thus enables the analysis of intact tissue samples, where the MAS approach even minimizes the susceptibility broadening of the NMR lines due to the heterogeneous nature of the samples (Figure 13). The non-destructive nature of HRMAS NMR allows for consecutive histological examinations under light microscope [Moestue et al., 2011].

### 4.7 2-Dimensional NMR

Contrary to 1D NMR, 2D NMR will plot intensity as a function of two frequencies; F1 and F2 [Martin et al., 1988]. This type of spectrum is often made as a topographical map where each peak is plotted as a contour with the co-ordinates corresponding to frequencies at F1 and F2. F2 is the equivalent to the x-axes in a 1D spectrum. 2D NMR is necessary when trying to elucidate and identify resonances, i.e. confirming the compounds found in the sample.

#### 4.7.1 Total Correlation Spectroscopy - TOCSY

2D TOCSY gives information about spin systems within a molecule. This is especially useful when assigning compounds with similar chemical shifts and multiplet overlap. TOCSY yields through bond correlation. The TOCSY experiment correlates all protons of a spin system via spin-spin couplings [Braunschweiler et al., 1983]. A typical TOCSY spectrum has a mixing period during which only scalar coupling is acting while chemical shift is suppressed. The basic process is transfer of magnetization between pairs of coupled spins.
4.7.2 Hetero-nuclear Single Quantum Coherence – HSQC

HSQC has been used since its invention in 1980 [Bodenhausen et al., 1980]. This type of 2D spectrum displays the chemical shift of $^1$H on the x-axes and the indirect chemical shift of a hetero-nucleus such as $^{13}$C or $^{15}$N on the y-axes. The spectrum show the cross peaks for each unique proton attached to the hetero-nucleus. This provides the chemical shift of both the proton and the hetero-nucleus coupled to the same proton, and thus, important qualitative information for the identification of a compound [Soubias et al., 2003].
Chapter 5: Metabolomics Using HRMAS NMR

5.1 Concept of Metabolomics

Metabolomics is part of the omics sciences, such as genomics, proteomics, transcriptomics, in systems biology. Although the term “metabolomics” is rather novel this type of approach to elucidate metabolic perturbation in biological systems due to disease is not new. The notion that changes in tissues and biological fluids, such as urine, are indicative of disease were explored in the ancient Greece and China [van der Greef et al., 2005; Nicholson et al., 2008], and Dalgliesh et al investigated metabolic patterns in urine and tissue by using GC-MS in 1966 [Nordström et al., 2010].

There has been some confusion regarding the definitions of the terms “metabolomics” and “metabonomics”. Nicholson et al introduced the expression “metabonomics” in 1999 [Nicholson et al., 1999], while Fiehn et al launched the term “metabolomics” just a year later [Fiehn et al., 2000]. Although metabolomics is the more common term in use today, there are differences between the two slightly similar terms: “metabolomics” is usually referred to as the more comprehensive method covering a global range of metabolites typically analyzed by mass spectrometry (MS). “Metabonomics” is often referred to as being based on the use of NMR and thus not as comprehensive as MS. This is due to the fact that MS detects metabolites that are present at concentrations orders of magnitude lower than what can be detected by NMR [German et al., 2006]. The term metabolomics, though NMR based, will be used in this thesis.

The metabolome is defined as the complete set of small molecule metabolites, endogenous as well as exogenous, found in a biological sample, i.e., specific cell, organ or organism [Wishart et al., 2007]. The exploration of the metabolome aids in the search for unique fingerprints responsible for different phenotypes [Koulman et al., 2008]. The distinction between phenotype and genotype was proposed in 1911 by Wilhelm Johannsen with the aim of clarifying the difference between an organism’s heredity and what that heredity produces [Johannsen, 1911]. Phenotypes are the outcome of genetic expressions as well as the influence of environmental factors, and the interactions between the two; and this may be detected in the metabolome.

Both extrinsic and intrinsic factors have input on the metabolome. Extrinsic factors may include lifestyle choices, nutrients and non-nutrients, physical activity, stress, drugs, and colonic micro flora (micro biome). Intrinsic factors may comprise genotype, age, body composition, tissue turnover, reproductive
status, and diurnal cycle [Koulman et al., 2008, Goodacre, 2007]. The metabolites present in a metabolome include metabolic intermediates, amino acids, fatty acids, etc. Metabolomics aids in the unraveling and the discovery of organ-specific endogenous metabolites that has the potential to be reliable indicators of organ function and viability as well as valuable markers for treatment response [Conti et al., 2006]. The metabolites may be viewed individually but in a more holistic context or as a group in, what is termed, metabolic profiling [Beckonert et al., 2007]. Metabolomics is an excellent tool in translational research as it provides a reflection of, for example, the unique metabolism of experimental models where the results ultimately are to be applicable clinically. This is of special interest in the emerging field of molecular imaging where diagnostic technologies such as MRSI or PET are utilized [Spratlin et al., 2009]. The comprehensive investigation of the metabolome will provide a more holistic picture of the biochemical perturbations which are expressed subsequent gene expressions and protein activities [Bathen et al., 2010]. The results may therefore be applied clinically in-vivo to characterize an organ by utilizing bio-markers that have the capacity to discriminate between disease and health. A powerful application of metabolomics is in the differentiation of malignancy vs. non-malignancy. However, despite this, the approach is not commonly used in the clinics for oncology [Spratling et al., 2009].

5.2 Metabolomics in the Characterization of the Prostate

Successful therapy for PCa depends on early diagnosis and accurate evaluation of progression and outcome. Bio-markers aimed for the characterization of the prostate are especially difficult due to the heterogeneity of the prostate, especially when malignant, and the fact that it is difficult to differentiate between the aggressive type vs. the indolent type of PCa [Madu et al., 2010], and in addition, there is a variability between patients which presents a challenge using the diagnostic tools available [Abate-Shen et al., 2009]. Thus, clinical indicators in the form of robust bio-markers are important in modern medicine. The human prostate metabolome, visible by HRMAS NMR spectroscopy, consists of small, freely rotating organic molecules [Swanson et al., 2003, 2006; Tessem et al., 2008]. The Krebs cycle intermediate citrate is one of the most important metabolites found in the prostate metabolism and has been researched thoroughly by different means. By assessing the biochemistry of, for example, the Krebs cycle intermediates using the metabolomic approach where extrinsic and intrinsic factors are included, a more comprehensive observation will be provided [Griffin et al., 2004].
5.3 Metabolomics, Nutrition and Prostate Cancer

The prevention of the development of disease and prostate cancer is even more important than the search for bio-markers [Silberstein et al., 2010]. There is a growing body of evidence suggesting a relationship between nutrition, health, and PCa risk, and where there seems to be a particularly strong link between obesity and PCa [Coffey, 2001; Ornish et al., 2009; Gonzales et al., 2010; Kahn et al., 2010; Thun et al., 2010]. Furthermore, the roles of certain food components as protectors against disease are documented [Davis et al., 2004; Afman et al., 2006]. By defining an individual’s healthy metabolome at baseline under specific nutritional conditions and lifestyle factors may ultimately lead to personalized medicine. The baseline metabolome would aid in the interpretation of disease and a comprehension of optimal nutritional intake and the maintaining of homeostasis on an individual basis [van der Greef 2004; German et al., 2005; Gibney et al., 2005; Trujillo et al., 2006; Goodacre, 2007; Holmes et al 2008]. Essential nutrients are especially interesting since they must be obtained by dietary means. These nutrients: vitamins, dietary minerals, essential fatty acids, and essential amino acids, are required for normal body functioning. The essential polyunsaturated fatty acids (PUFAs) omega-6 and omega-3 has received much attention. Omega-3 is believed to possess protective properties while omega-6 has been known as an instigator for PCa. These PUFAs, along with numerous other nutrients, are detectable by HRMAS NMR.
Part III
Chapter 6: Materials and Methods

“Mti hawendi ila kwa nyenzo”*

Swahili kanga writing

6.1 Experimental Setup

6.1.1 Procedure of HRMAS NMR Spectroscopy

All spectroscopic data were acquired at 11.7 T on a Bruker AMX2 NMR spectrometer (Bruker Biospin GMBH, Karlsruhe, Germany), operating at a $^1$H NMR frequency of 500.13 MHz and equipped with a 4 mm dual band, $^1$H/$^{13}$C, HRMAS probe head from Bruker. NMR spectra were acquired at room temperature (20°C). The magic angle spinning rate was set to 5 kHz for all experiments except for the 2D rotor-synchronized TOCSY experiments were a 41.8 ms mixing time at 2.25 kHz sampling was used. The 2D $^1$H-$^{13}$C GE-HSQC (phase-sensitive) was performed at 5 kHz. The MAS spinning rate suppresses chemical shift anisotropy and the line broadening effects due to dipolar couplings.

Prostate tissues were cut with a surgical steel blade into smaller specimens weighing ~15±5 mg so as to fit the MAS rotor with insert. 4 mm cylindrical ZrO$_2$ rotors with spherical inserts were utilized. Kel-F caps were used to secure the rotors. D$_2$O were used for $^2$H field locking and ten micro liters of D$_2$O were added to each specimen and allowed to soak in for a few minutes. Prostate tissue specimens were packed in rotors and either immediately placed in the HRMAS probe to be analyzed or temporarily stored at -20°C. In order to avoid possible errors all samples were treated as identically as possible.

6.1.2 1D $^1$H HRMAS NMR

For the 1D HRMAS NMR spectroscopy a rotor-synchronized Carr-Purcell-Meibom-Gill (CPMG) [Meiboom et al., 1958], pulse sequence (90°-(τ-180°- τ)n-acquisition) with a 5 kHz spinning rate was used. A T$_2$ filter of 20 ms duration

*You need to have proper tools to carry out any task.
was used to suppress broad signal contributions from macromolecular components. The $T_2=20$ ms was chosen based on $T_2$ time studies performed in-house. The excitation band width was set to 10309 Hz and each spectrum was recorded with 256 transients, using 16K complex time domain data points and with a repetition time of 5.0 seconds. To suppress the resonances arising from H_2O a presaturation pulse during the relaxation delay prior to the CPMG pulse sequence. All 1D spectra were generated, from start to finish, at a maximum time of 30 minutes.

Spectral 1D data were analyzed off-line using Mestre-C 4.9.9.6 and/or MNova 6.0.4. (Mestrelab Research, A Coruña, Spain). The free induction decays (FID’s) of the CPMG edited $^1$H HRMAS spectra were subjected to a 1.5 Hz exponential multiplication, zero-filled to 60k complex points preceding Fourier transformation. The resulting spectra were manually phased and baseline corrected using a multi-point polynomial function. The spectra were referenced to the residual water peak by setting the chemical shift of the lactate doublet equal to exactly 1.33 ppm. A selection of metabolites, that were relatively clear of spectral overlaps, was subjected to a line-fitting procedure/deconvolution so as to obtain metabolite peak areas.

### 6.2 Prostate Tissue Samples

Excised tissues are no longer under enzymatic control outside of its natural environment, which is the body. Therefore, all biological specimens: tissues, and fluids, require strict handling to avoid unnecessary oxidation and degradation [Sitter et al., 2009]. It is not practically feasible to analyze fresh tissues, therefore all excised tissue samples were immediately snap-frozen in N_2(l) and thereafter stored in a freezer with a temperature of -70°C. Thawing does have an effect on the specimens [Middleton et al., 1998; Wu et al., 2003] which needs to be taken into consideration when interpreting the results.

### 6.2.1 Patients

Informed consent was obtained from the patients and the studies were approved by the Research Ethics Committee of Umeå University Hospital, Umeå, Sweden.

Forty patients with PCa were included in this study. All of them underwent prostatectomy and agreed to donate the surgical specimen by a form of written consent. Study participants, aged between 41 and 71 years, were drawn from the northern part of Sweden. The tumor stage was in the range of 1 to 3, and the Gleason scores were GS 6 (3+3) and GS 7 (3+4). Serum PSA value was 12.41 ±
13.02 (range 3.42 – 50.03). None of the patients had undergone any form of treatment before surgery.

Specimens were chilled immediately after surgery. Prostate surfaces were inked for anatomical identification purposes and thereafter the gland was cut into horizontal 1 cm thick sections. Between 2 and 20 tissue punches, approximately 0.5 cm in diameter, were obtained from the peripheral zone of each section. All specimens were snap-frozen in N₂(l) within 30 minutes after surgery and thereafter stored in -70° C freezers. In order to identify the punches sampled each prostate slice were fixed using formalin and embedded in paraffin before being cut into 5-µm thick sections. Each section was stained with haematoxylin and eosin, thus enabling tumor foci identification using microscopy. The foci were thoroughly marked and the frozen punches were preliminary classified as malignant or non-malignant by studying the tissue surrounding the holes from where the punches originated from. The frozen tissue punches were randomly cut into smaller specimen and analyzed by HRMAS NMR.

6.2.2 Rat

The study protocol for the animal experiments was approved by the Animal Research Ethics Committee of Umeå, Umeå, Sweden.

Nine adult male Sprague-Dawley rats (B&K, Stockholm, Sweden) were included in this study. The Sprague Dawley strain is an outbred multipurpose model. The rats were anaesthetized according to the previously described method [Lissbrant et al., 2004], and their ventral prostate (VP) and dorso-lateral (DLP) lobes were removed and immediately frozen in liquid nitrogen.

Six adult male Copenhagen x Fisher F₁ rats (ALAB, Uppsala, Sweden) were transplanted subcutaneously with pieces of highly differentiated Dunning R3327 PAP rat prostatic adenocarcinoma in their right flank as earlier described [Häggström et al., 1998]. Upon growth to 1-2 cm in diameter each tumor was removed and immediately frozen in liquid nitrogen.

6.3 Interpretation of Data

The interpretation of NMR data requires, besides obligatory spectroscopic skills, a thorough comprehension of the particular species analyzed, and general biochemical knowledge. Assignments of the spectral resonances in this study were made by consulting published data, and by 2D TOCSY, and 2D GE-HSQC experiments. The 2D NMR experiments are valuable when assigning overlapping
resonances which are common in HRMAS spectra. It is not uncomplicated to quantify this type of spectral data due to the overlapping resonances, as already mentioned, furthermore, there are challenges involved in weighing the samples correctly due to the natural variation in tissue composition of intact biological specimens. In this study the basic deconvolution method to quantify spectral data was used: linefitting of resonances and thereafter using the spectral ratios within each sample [Inouye et al., 2010].

6.3.1 Pathologic Analysis

Formalin fixed, paraffin embedded specimens were cut in 5 micron thick sections and stained with hematoxylin-eosin (H&E). Frozen samples were cut unfixed and then stained either with H&E or immunostained for high molecular weight cytokeratin (cytokeratin-hmw, DAKO, Stockholm, Sweden) detecting basal epithelial, or the cell proliferation marker Ki67 (DAKO).

The slides were reviewed by a highly experienced uro-pathologist who determined the percentage of non-malignant glandular, non-malignant stroma, and the percentage and grade of prostate cancer. Cancer-containing prostate tissues (glands lacking cytokeratin-hmw positive basal epithelial cells) were compared to non-malignant tissues (glands with an intact basal epithelial cell layer) regardless of relative glandular and stromal composition. The fraction of malignant vs. non-malignant prostate tissue was established using a light microscope with a square-lattice mounted in the eye-piece to count the number of grid-intersections falling on each tissue compartment. The distance between the remaining holes from the punches and the closest tumour was measured on the whole mount sections. As “completely healthy,” i.e. benign, prostate tissues were lacking non-malignant were used as such.

6.3.2 Statistical Analysis

Upon the completion of the NMR measurements and post NMR processing, the results, in the form of integral areas, were brought into Excel (Microsoft Office Excel 2003) for the generation of a matrix. The results from the histopathological analysis and pathological staging were added.

The data matrix was calculated with: SPSS (v. 16.0, SPSS, Chicago, IL, USA) where basic descriptive statistics such as means and SD was generated, and STATA (v. 10, StataCorp LP, College Station, TX, USA), where the data was standard error-adjusted in order to take clustering (dependencies between samples obtained from the same patient) into considerations. The following
statistical methods was used; Multivariate Linear Regression, Binary Logistic Regression, Receiver Operating Characteristic (ROC) and Area Under the ROC Curve (AUC). Odds Ratios (ORs) and 95% confidence intervals (CIs) were calculated. P-values $\leq 0.05$ were considered to be statistically significant.
Chapter 7: Results and Comments

Various types of intact prostate tissues, obtained from both human and rats have been studied using HRMAS NMR spectroscopy. The results from these studies are reported below.

Paper I

The aim of this study was, firstly, to verify metabolites known to be part of the local prostate metabolism according to previous publications. Secondly, our extended aim was to assess and elucidate additional compounds, associated with the prostate but not previously published.

The result from the 1D $^1$H HRMAS NMR analysis confirmed the compounds related to the prostate metabolism but also revealed a spectral resonance at 2.8 ppm that had previously not been documented in spectroscopic studies of the prostate. The 2.8 ppm resonance in the 1D spectrum, which was found in prostate cancer specimens only, was indicative of a polyunsaturated fatty acid (PUFA). There are two PUFAs that are considered essential, namely the omega-3: α-linolenic acid (18:3 ω3) and the omega-6: linoleic acid (18:2 ω6), which are structurally similar as both are 18 carbon fatty acids; however, omega-3 has three double bonds while omega-6 has two (Figure 14). The fact that omega-3 has its first double bond at the third carbon counting from the methyl end while omega-6 first double bond is connected to the sixth carbon from the methyl end, provides the information needed to distinguish these two compounds from each other (Figure 14) by using 2D $^1$H,${^{13}}$C-HSQC NMR. The PUFA in our study was identified as an omega-6 specie. This finding was not surprising as linoleic-acid omega-6 is by far the most common PUFA in western diet [Simopoulos, 2010].

![Alpha-linolenic acid (ALA, C18:3, omega-3)](image1)

![Linoleic acid (LA, C18:2, omega-6)](image2)

Figure 14: The two essential, poly-unsaturated fatty acids; α-linolenic acid/omega-3, and linoleic acid/omega-6.
Linoleic acid is the omega-6 compound which is considered to be the “parent” of several $n$-6 compounds varying in length from 14 to 40 carbons and thus would be the essential fatty acid (EFA) [Cunnane, 2003]. Several studies have indicated an excess of omega-6 and its derivatives as instigators of inflammation and also PCa [Kelavkar et al., 2006, 2009; Ukoli et al., 2010]. One of the more known $n$-6 EFAs arachidonic acid (AA); 20:4 ω6, is a precursor in the production of eicosanoids which are compounds involved in the regulation of inflammation and immunity [Funk, 2001]. The $n$-3 eicosapentaenoic acid (EPA) competes with AA for prostaglandin and leukotriene synthesis at the cyclooxygenase (COX) and lipoxygenase (LOX) level [McEntee et al., 2010]. However, the term EFA is ambiguous and the metabolism of essential and non-essential fatty acids is very complex and more interconnected than previously recognized. The requirements and health effects of EFAs are individual and dependent on variables such as: age and tissue type, but also on genetic differences in metabolism [Simopoulos, 2010]. α-linolenic acid and linoleic acid cannot be synthesized endogenously “de novo”, but it seems that this is possible with the 16 carbon precursors; 16:3 ω3, and 16:3 ω6 [Cunnane, 2003]. Both ω6 and ω3 are readily β-oxidized and recycled into cholesterol and palmitic acid, which is the most common saturated fatty acid in mammals [Beare-Rogers et al., 2001], and, less easily replaced in tissue lipids than the non-essential common fatty acids such as palmitic and stearic acid [Cunnane et al., 2003].

HRMAS NMR detects freely rotating compounds in the semi-solid and intact tissue specimens investigated. Furthermore, a filter to suppress signals from macro-molecules was used therefore limiting the detection of compounds tightly bound to membranes. As arachidonic acid is the most biologically important PUFA of cell membranes and, when released by phospholipase, directly affect cell signaling pathways and serve as substrate in the COX and LOX pathways, a hypothesis is therefore that this $n$-6 PUFA was released from membrane phospholipids due to inflammatory activation. Other options include: the conversion of PUFAs for the, apparently, protective properties of membrane lipid saturation [Rysman et al., 2010], or simply from a PUFA buildup needed for the generation of unsaturated fatty acids or cholesterol to be used for the increased synthesis of cellular membranes due to increased proliferation.

**Paper II**

This study was designed to be a comparative investigation where both human and rat prostate tissues were scrutinized. The human specimens originated from the peripheral zone only, and the rat specimens were
obtained from three different lobes, whereas two of them were combined into one entity. In addition, a subcutaneous experimental rat PCa tumor was also included in the design. The HRMAS NMR spectrum provides an image depicting the local metabolism found in the specimen analyzed, hence, comparison of tissues obtained from different species is straightforward. Our result revealed obvious spectral differences between the diverse prostate tissue types, indicating metabolic distinctions.

Rat, mouse, and man share approximately 99% of the same genes [Gibbs et al., 2004], which clarifies the popularity of using rodents as experimental PCa models [Shirai et al., 2000]. However, a similar genome does not, unfortunately, provide the key to perform realistic comparisons [Buttyan et al., 1993; Lamb et al., 2005]. The rat lateral prostate is the closest analogue to the human peripheral zone where most cancers occur. Furthermore, the LP, but not the DP, contains citrate producing cells. As it is difficult to separate the LP from the DP these lobes are usually combined into the DLP entity, which is what we used in our study. One of the finding was the presence of a 2.8 ppm resonances stemming from omega-6 fatty-acids, in all of the Dunning tumors specimens and in some of the DLP specimens. This is in line with our findings in Paper I, i.e., this 2.8 ppm resonance was found in some PCa specimens. One of the questions here is to why the completely healthy DLP show similar characteristics as the Dunning tumors and human PCa tissues. One answer is suggested: the Dunning tumor is believed to originate from the DP and consequently both should therefore share similar metabolic characteristics. Furthermore, the DP is the lobe in the DLP entity that lacks citrate accumulating cells. When comparing the spectra obtained from the DLP and the Dunning tumor it is evident that they share similar metabolic characteristics as compared with the VP which expresses a vastly different metabolism. The Dunning tumor spectra most closely resemble the spectra obtained from human PCa tissue. The one important feature that indicates cancer in both human PCa and the Dunning tumor is the increased resonance of (GPCho+PCho) over the free choline resonance. However, this sign is also evident in completely healthy DLP. Therefore, it would be of great value to study the metabolism of the DP and the LP separately. Another important reason for this is to further study and to clarify the functions of these two lobes in regards to hormonally induced PCa and senescence. Obviously, it is not optimal to compare a prostate tissue sampled from a 65 years old man with one obtained from a 12 wks old rat. This is of special interest in this study as age-dependent overgrowth and pathological changes that leads to cancer usually arises in the DLP but not in the VP. Interestingly, the COX-2 pathway expression is both higher and dependent on the ageing process in the DLP as it increases with age, but seems to be unaffected in the VP. [Badawi et al., 2004]. It is logical that inflammation increases with age.
As already discussed above in Paper I, the COX enzymes convert the omega-6 fatty acid arachidonic acid into the pro-inflammatory, and also pain producer, prostaglandin E2 (PGE2). There exist two forms of the COX enzyme; COX-1, which is expressed in most tissues and organs, and the inducible enzyme COX-2 which is primarily localized in inflammatory cells. COX-2 is therefore considered to be the key enzyme in fatty acid metabolism and inflammation. To inhibit the conversion of AA to PGE non-steroidal anti-inflammatory drugs (NSAIDs), including COX-2 inhibitors, are frequently used, in spite of significant negative side effects [Seibert et al., 1994]. Omega-3 is believed to function as a natural inhibitor of COX-2 and by reducing inflammation indirectly decreases the risk of PCa. Importantly, however, this potential effect appears to be modified by genetic variation in COX-2 [Fradet et al., 2009; Simopoulos, 2010]. Furthermore, a recent study showed that dietary modification that increased tumor EPA and LA (n-3) content enhanced responses to hormone ablation therapy. Conversely, a relapse to androgen independent growth (measured by PSA and tumor volume) positively correlated with tumor AA (n-6) content [McEntee et al., 2008]. As mentioned earlier, the optimal study would be to separately scrutinize the DP and the LP obtained from senescent rats and compare with human PCa tissue samples. In addition, a thorough dietary investigation is recommended to be included in the study to evaluate the therapeutic effects of PUFAs.

**Paper III**

The third study entailed a more comprehensive metabolomic investigation, based upon the knowledge generated from study I and II. The aim was to discover spectral bio-markers that correlate/relate to the various dependents obtained from the clinical examinations of human prostate tissues. In this study, bio-markers known to be part of the prostate metabolism were investigated by exploiting peak ratios and their variation due to metabolic perturbations, using the basic deconvolution method. This approach is termed as “the use of biochemical prior knowledge” and is used to decrease uncertainties with overlapping resonances [Inouye et al., 2010]. This method, using ratios with arbitrary units, circumvent the problems involved in finding the optimal standard reference and leak-proofing the rotors, and the need for T₂ value adjustments, all in order to make accurate quantitative calculations [Swanson et al., 2006; Albers et al., 2009].

Histological evaluation of needle biopsy cores from the prostate is the initial step in diagnosis and treatment decision making. A representative biopsy may provide an indication as to the nature of the cancer in the prostate gland.
based on the Gleason score and the volume of disease. Cores containing sufficient amount of cancer are essential for the evaluation. However, there is no imaging method available that can guide towards the most malignant part of the prostate. Because more men are now being diagnosed with local PCa, due to increased PSA-testing, it is necessary to evaluate and improve diagnostic options with the aim of minimizing under grading of biopsies. As many prostate tumors are latent and will not pose a threat, it is desirable to find a biomarker than is able to differentiate between the indolent and aggressive types in order to avoid overtreatment. Therefore, the aim of this study was to identify potential bio-markers for the discrimination of aggressive vs. the more latent type of cancer and also to estimate the fraction of tumor cells in any given prostate tissue specimen.

As most PCa studies have focused on the tumor, and as it is not possible to accurately detect tumors in-vivo by the imaging methods currently available, we used an opposite approach, i.e., by searching for bio-markers in the non-malignant tissue. The metabolomic bio-marker that was prominent in our study was the naturally occurring nutrient inositol which apparently has the potential of sensing tumors and their corresponding grades elsewhere in the organ. Inositol can be phosphorylated in seven variations to form phosphoinositides that act as cellular mediators of signal transduction, metabolic regulation, and overall growth [Mathews et al., 2000].

By exploiting the ratio of myo-inositol/scyllo-inositol discrimination between GS 6 and GS 7 was achieved. This is potentially a very important finding as GS 6 tumors usually have a better prognosis than GS 7. There are also different treatment approaches for these two different types of prostate cancer. This novel finding needs to be explored further as bio-markers that have the power to detect tumors and also estimate their Gleason score in non-malignant tissue is highly desired. Such feature would minimize the number of biopsy cores sampled as it would therefore not be necessary to aim for the tumor itself. This type of bio-marker is currently lacking and is essential for successful focal therapy and/or active surveillance [Lazzeri et al., 2010]. This finding suggests protective properties of inositol which is in line with numerous other studies [Vucenic et al., 2003, 2006; Fardet, 2010]. The detection of loss/failure of cellular protection at an early stage has the potential of being the basis for preventative medicine.

Curiously, we did not find an equivalent bio-marker in malignant prostate tissue. The ratio of citrate/creatine came close to statistic significance when not adjusting for clustering (uneven sampling among the patients in our study). The focal point here is the complexity of biochemical pathways in all living organisms and the importance of viewing each patient as an individual
rather than a gross average, as there are many factors that have an effect on the metabolome. A tumor marker was identified in a previous study (Paper II); (GPCho+PCho)/Cre. An increased choline metabolism is a well known marker for cancer and is verified in other PCa studies. Our study investigated the correlative strength of this bio-marker ratio; (GPCho+PCho)/Cre, and the fraction of tumor cells in any given prostate tissue sample. Indeed, we found statistically significant correlation. We also found a significant, although very weak, correlation with the proliferation marker Ki67 and (GPCho+PCho)/Cre. This raises questions about the underlying reasons for the increased choline metabolism, which in fact, is poorly understood [Moestue et al., 2010], and as it might not to be correlated with proliferation [Gillies et al., 2005], our observation with Ki67 and (GPCho+PCho)/Cre might be a coincidence. Our study showed large variations in the concentration of choline compounds, especially in prostate specimens containing >50 % cancer cells. A recent study where choline metabolic profiles in breast cancer xenograft models was investigated found associations with differences in gene expression for basal-like vs. luminal-like breast cancer [Moestue et al., 2010]. However, there are no such types of prostate cancer.

Despite uncertainties regarding the exact function of choline metabolites its increase is still a marker for tumors. This feature is valuable for tumor verification and localization and may be used parallel with other bio-markers such as inositol.
Chapter 8: Discussion

A few abnormal cells found in a small piece of prostate tissue are most consequential for a man’s future.

As thoroughly discussed in this dissertation, the prostate is a very difficult organ to diagnose [Chun et al., 2010; Colleselli et al., 2011] thus the concerns about incorrect diagnosis and treatments are most valid. Prostate health awareness, involving PSA-testing and biopsy, has increased the number of early stage and localized PCa [Wolf et al., 2010; Wever et al., 2010]. Treatment options for this diagnosis are radical therapy such as surgery and/or radiotherapy and brachytherapy where there are side effects such as urinary and sexual dysfunctions. The alternative option is active surveillance with the risk of under treatment but also the psychological burden involved. Prostate sparing focal therapy is an alternative treatment option although its effects are not determined yet [Lazzeri et al., 2010]. As PCa more often than not is multifocal, focal therapy has a higher demand for increased biopsy sensitivity and precise tumor localization [Meiers et al., 2007; Scheenen et al., 2011]. Bio-markers with the power of accurately discriminating between aggressive and indolent prostate tumors are most important as this diagnosis will be the basis for therapy choice [Xu et al., 2010]. Therefore, innovative methods to increase the precision of prostate diagnostic are of essence in the field of prostate research. The ex-vivo and non-destructive method of HRMAS NMR spectroscopy, in combination with histopathology, shows promise in this area as it provides metabolomic information, both spectral and morphologic, with the potential of being used as pre-clinical information for the development of clinical diagnostic imaging methods for MRSI and PET [Spratlin et al., 2009]. HRMAS NMR also holds the potential of being used as a complement to clinical histopathological evaluations, although this has to be investigated further [van Asten et al., 2008; Santos et al., 2010].

There are many positive aspects and strengths in this approach using HRMAS NMR in combination with histopathological analysis as is presented here. Firstly, this is a non-destructive method, thus detecting both lipophilic and hydrophilic compound in their present form simultaneously and the tissue is preserved for post-NMR analysis and verification under light microscope. This strength is exploited in Paper I: “Detection of polyunsaturated omega-6 fatty acid in human malignant prostate tissue by 1D and 2D high-resolution magic angle spinning NMR spectroscopy”, where both fatty acids and small organic, water soluble compounds such as citrate are discussed. Furthermore, Paper I presents the versatility of HRMAS NMR where unexpected resonances in the spectra arising from
unknown compounds may be identified by various 2D methods. The spectrum depicts the metabolism as it is and this is correlated with results from histopathology, and thus metabolic profiling is possible. This offers the characterization and visualization of the diverse metabolism in different species, organs, anatomical regions, and different types of cells. This type of characterization is presented in Paper II: “Detection of Local Prostate Metabolites by HRMAS NMR Spectroscopy: A Comparative Study of Human and Rat Prostate Tissues”. Such information is of key value when designing experiments [Costello et al., 2006], especially in translational research [Ruttenberg et al., 2007]. Paper II further discusses spectrally derived bio-markers which are applicable on humans may not be suited for experimental rodent models. HRMAS NMR detects cellular perturbations due to disease vs. health, in other words; the dynamics of cellular homeostasis. The loss of homeostasis, detected at an early stage, is an important key in the understanding of disease [van der Greef, et al 2005]. This type of perturbation/loss is presented in Paper III: “$^1$H HRMAS NMR Derived Bio-markers Related to Tumour Grade, Tumour Cell Fraction, and Cell Proliferation in Prostate Tissue Samples”, where it appears as the homeostasis of non-malignant cells in the prostate, with tumors elsewhere in the organ, is being disturbed. The key metabolite here is the common phyto-nutrient inositol which apparently possesses protective properties [Vucenik et al., 2003, 2006; Roy et al., 2009; Bacić et al., 2010] and is presently under clinical trials investigations [www.ClinicalTrials.gov]. Thus, jointly with the finding in Paper I regarding essential PUFA omega-6, HRMAS NMR provides a window into the area of nutrition. This is of particular interest as lifestyle choices, especially diet, have impact on prostate health and disease [Moyad et al., 2008; Gonzales et al., 2010]. Thus, chemoprevention of prostate cancer/disease using dietary means, and other preventative measures, is a promising area in prostate research [Silberstein et al., 2010].

This approach, using HRMAS NMR in combination with subsequent histopathology, is an excellent way of detecting the metabolome, however, it cannot be stressed enough that this method, like with any other study involving ex-vivo biological tissues, requires strict handling and a robust study protocol as there are many possible short-comings. Biological tissues without the enzymatic control and exposed to oxygen are at high risk of oxidation and degradatation. The time from the freezer to post-NMR handling must be as short and efficient as possible to avoid metabolic break down and steady, low temperature is of essence [Beckonert et al., 2010]. The shimming of the NMR probe to generate a homogeneous magnetic field is equally important as this affects the line-width and thus resolution which is critical for line-fitting. Tuning and matching for the correct nucleus is equally important factor to increase the signal-to-noise ratio [Kreis, 2004]. As both
shimming and tuning are manually adjusted it is important to decrease operator dependent errors. This may be done by using the same operator for all the experiments as we did in our study. Operator dependent errors should also be considered when processing the spectra.

The results presented here are merely a fraction of what can be examined using the metabolomic approach of HRMAS NMR spectroscopy combined with histopathology.
Chapter 9: Suggestions for the Future

“Akufukuzae hakwambii toka”*

*Swahili kanga writing

When reviewing my years involved in studying the prostate, I realize that the most valuable knowledge has been gained simply by trials and errors. I have also come to the conclusion, like many others, that this is a very complex, highly multi-faceted, organ to study. It requires skills in many, widely, separate areas such as, urology, pathology, chemistry, biology, radiology, physics, statistics, demography, sociology, politics etc., and most importantly, a thorough knowledge about the anatomy and function of both human and experimental prostate models. This realization has made me question the current approach of performing prostate cancer research (and research in general), i.e., by separate entities of research groups specializing in proprietary areas with very little, if any, intra-communication. This is unfortunate since it is, simply, necessary in order to gain complete understanding of this complex organ.

Interdisciplinary research is, however, becoming more popular and should be highly encouraged. My one suggestion for the future is, therefore, consequently, to encourage separate research groups to communicate and share and discuss findings and not view conflicting results as intimidating but as valuable information to be used for collective improvements that will ultimately benefit the single most important factor – the patient.

*Inner feelings are mostly communicated through attitude and behavior and much less through words.
Chapter 10: References


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