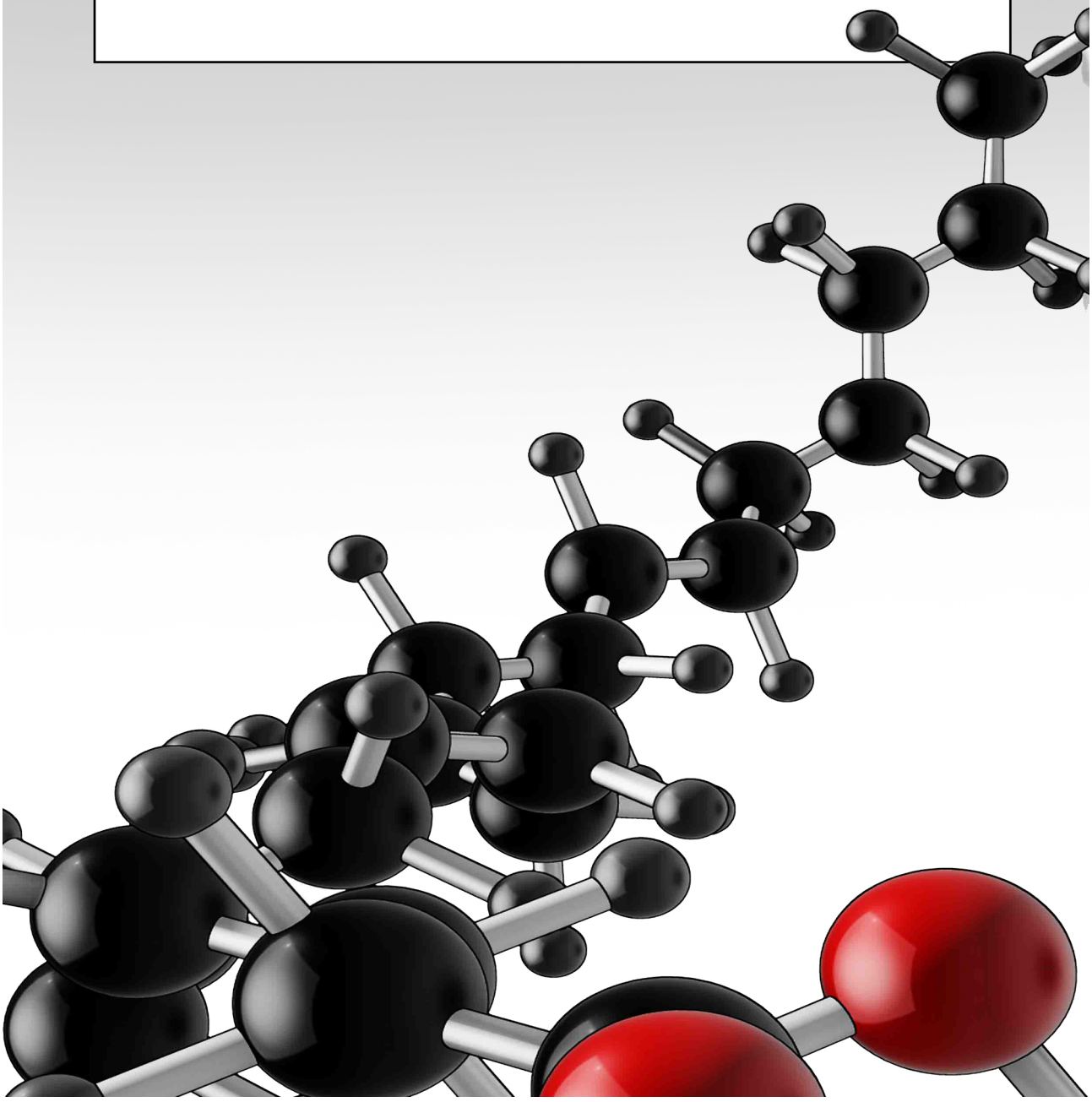
The Arachidonic Acid Pathway: A potential application in the diagnosis and prognosis of prostate cancer

-Giovanny Rodríguez Blanco-



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De arachidonzuur metabole route: Een mogelijke toepassing voor diagnose en prognose van prostaatkanker

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A mi familia

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Chapter 1

General Introduction

Introduction

The prostate is a gland that lies underneath the bladder and surrounds the urethra, the tube through which urine passes from the bladder to the penis (Figure 1). It is only found in men and about the size and shape of a walnut. The prostate gland's main function is to produce prostate fluid, a constituent of semen that protects the sperm cells (1).

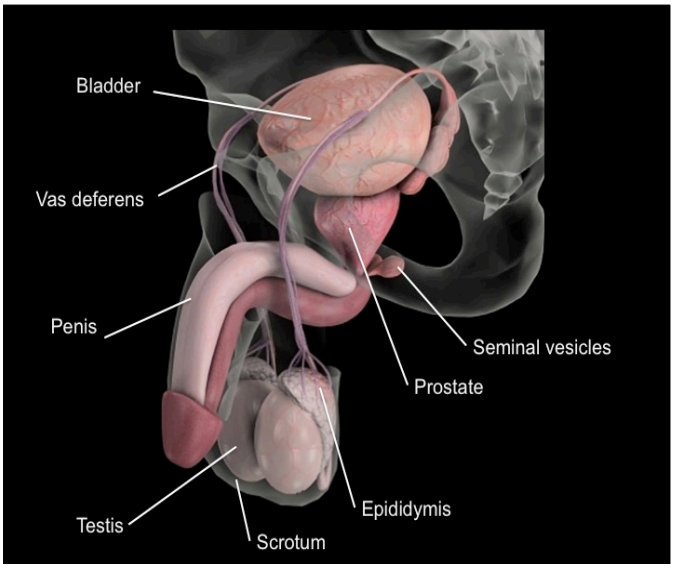

Figure 1. Graphical representation of the prostate gland. This figure is adapted from Turbosquid, (2014), male genitals [ONLINE].
Available at: http://www.turbosquid.com/3d-models/male-reproductive-penis-prostate-3d-model/233868 [Accessed 12 October 14].

Three main conditions can affect the prostate and can be generally associated with aging in men. The most common is called benign prostate hyperplasia (BPH), characterised by non-cancerous prostate tissue overgrowth that can cause pain and trouble with urination. The second condition is called prostatitis; it is caused by either an infection or an inflammation and it can often be treated with antibiotics or other drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) (2). The third condition is prostate cancer (PCa), the second most common type of cancer seen in men nowadays, and the sixth leading cause of cancer death among men worldwide (3, 4).
Some prostate cancers grow very slow and do not cause symptoms unless the subject lives a long life. Other prostate cancers grow quickly and they can result in death if not detected and treated on time. The probabilities of survival are

higher when PCa is diagnosed early. In general, 85% of men survive PCa after five years, but this numbers are affected by the stage, i.e. men with metastatic disease (Stage IV) at diagnosis, could have a 5-year survival rate of around 30% (5, 6). When the cancer is localized (or confined) to the prostate, it can be monitored by active surveillance and/or watchful waiting to avoid unnecessary treatment and possible side effects. PCa can be effectively treated by radical prostatectomy (RP), which is a surgery that removes the entire prostate gland, and also by radiation treatment such as Brachytherapy or External Beam Radiation Therapy (EBRT) in which X-ray beams are directed towards the prostate, thus damaging cancer cells and stopping their growth. Radiotherapy is used when the cancer has spread outside the prostate or when the prostate specific antigen (PSA) rises after removing the prostate (also known as biochemical recurrence BCR). However, the choice of radiotherapy depends also on both patient's health and preference, as this treatment could be drastic when life expectancy is low.
In case of metastatic disease, or when primary treatment is not used, hormone treatments based on the suppression of testicular androgens by either chemical or surgical castration, are the mainstay treatment. They are aimed to reduce the levels of male hormones (androgens: DHT and testosterone) in the body, thus stopping the 'fuel' needed for the cancer cells to grow. Antiandrogen drugs such as flutamide, bicalutamide and nilutamide, block testosterone and DHT binding to the AR and thereby the ability of prostat tissue to use andrigens. However, these treatments do not cure cancer and in most cases become ineffective after a certain period of time (7, 8). Metastatic prostate cancer usually responds well to hormone therapy, but, in some cases, cancer cells adapt themselves to grow in an hormone-reduced environment, thus becoming hormone-resistant PCa.
Chemotherapy involves the use of anti-cancer drugs such as docetaxel or carbazitaxel to kill cancer cells. Although this treatment does not cure PCa, in many patients it slows its growth.
Some risk factors make men more likely to suffer from PCa. The biggest risk factor associated with PCa is generally age, but family history and ethnicity have been also considered as risk factors to develop PCa (9). Lifestyle also might play a role in the development of PCa, associations between PCa and physically inactive lifestyles, consumption of saturated and unsaturated fats, excessive smoking and alcohol drinking, might be present (10-12).

Prostate Cancer diagnosis

The most commonly used diagnosis of PCa are the digital rectal examination (DRE) and the prostate-specific antigen (PSA) test. DRE is used to feel whether any bump or hard/irregular area is present on the prostate. PSA tests measure the amount of the kallikrein-related peptidase (KLK3) in blood. This protein is produced by cells in the prostate and secreted to the seminal fluid in large quantities. An alteration of cellular barriers that normally keep PSA within the

ductal system of the prostate usually occurs during the development of PCa, thus altering blood levels of PSA.
Although the introduction of PSA screening decreased mortality by 4% between 1994 and 2006 (13), the use of PSA as a diagnostic serum marker still presents several drawbacks. The concentration of this protein in the blood increases during the development of cancer but also due to the other above-mentioned prostate alterations: BPH or prostatitis. Therefore, this method suffers from low specificity and, consequently, results in incorrect risk assessment and unnecessary biopsies. It is known that the proportion of men with a positive test result who actually have PCa, using a PSA cut-off value of ≥ 3 ng/mL, is 24% according to European Randomised study for Prostate Cancer (ERSPC) (14). When using a cut-off value of 1.0 ng/mL, the proportion decreases to 10% (15). Moreover, approximately 15% of patients with PCa have PSA values lower than the cut-off value of 4.0 ng/mL, thus leaving many cases undetected. The actual PCa diagnosis is performed by means of an ultrasound guided biopsy of the prostate, and its subsequent histological analysis.

A prostate biopsy involves the removal of tissue core samples mainly from the peripheral zone (the outer part) of the prostate gland. Usually, 6 to 14 cores are taken and analysed by a pathologist and, subsequently, the stage and grade of the disease are determined. Grading of PCa, an estimation of the ductal structure differentiation of the disease, is performed according to the Gleason system. This system is based on the assignment of a grade using numbers (from 1 to 5) to the cells in cancerous tissue depending on how similar they are to normal prostate tissue. Since PCa usually exhibits areas with different grades, the grading is performed on two areas that make up most of the cancer. These two grades are reported together as the Gleason Score, with values ranging from 2-10. Scores are considered non aggressive when the values are within 2-4; mildly aggressive for values between 5-6; moderately aggressive when the score is 7; and very aggressive for scores between 8-10 (16). More recently, the International Society of Urological Pathology (IUSP) proposed a modified classification using a scale from 1-5 where Gleason 6 is considered group 1. Gleason 3+4=7 and 4+3=7 are now considered as prognostic groups 2 and 3, respectively, Gleason 8 is the prognostic group 4 and Gleason 9-10 fall into group 5 (17, 18).

Stage of PCa is an estimation of how extensive or advanced is the PCa and whether it has metastasized, or spread, beyond the prostate gland. The TNM system (Tumour Node Metastasis) is the most common method to stage PCa, involving different clinical tests such as CT scans, MRI or bone scans. TNM provides information about the tumour itself (T), the regional nodes (N) in case the cancer has spread to the lymph nodes or bone, and also about the distant metastasis (M) occurring beyond the pelvis. Clinical outcomes from patients with stages I/II of prostate cancer are good, with up to 99% progression-free survival

after 1 year and over 90% after five years. However, for patients diagnosed at stage III and stage IV, survival decreases to 85% in the first years and 30-40% after 5 years (6, 17, 19).

Taken together, PSA values, Gleason Scores and TNM stages allow the determination of the PCa risk factor for a patient, and constitute a help for clinicians to determine what kind of treatment is more appropriate for a specific condition.

Biochemical recurrence

Among men diagnosed as having PCa, assessment of disease recurrence after primary treatment typically includes periodic measurements of PSA. A detectable PSA level after radical prostatectomy, or an increasing PSA level following radiation therapy, is considered as biochemical recurrence (BCR) or "PSA failure" (20). BCR is associated with increased PCa mortality and it can indicate disease progression years before clinical signs or symptoms appear. After radical prostatectomy, PSA usually becomes undetectable within 6 weeks after surgery because the most important source of PSA (the prostate gland) was removed. However, detectable PSA levels after surgery are observed in approximately 35% of men (21) and it most likely implies residual or recurrent PCa. In general, a cut-off point of 0.2 ng/mL or higher after undetectable PSA is accepted to define BCR after radical prostatectomy (21, 22).

Markers for prostate cancer

A marker can be defined as a characteristic objectively measured molecule or feature that can be evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention (23). In a broad perspective, "an ideal marker for general use in PCa management should be able to differentiate tumour tissue from benign tissue, and an aggressive tumour from an insignificant one, with high specificity and sensitivity" (24). In addition, it should be available at preferably low-cost, and as a non-invasive test to encourage widespread use (25). Markers for PCa can be classified into five categories: risk, diagnostic, prognostic, predictive and monitoring (Table 1), thus allowing an improvement in the management of PCa (26). Technological progress, particularly in the so-called "omics" era of next-generation sequencing and mass spectrometry, continuously provides new options for higher throughput and higher content DNA, RNA, protein and metabolite detection (25, 27-30)

In the following lines, some examples of known markers in serum/plasma, urine and tissue are described.

PSA is one of the fifteen family members of kallikrein-related peptidases, and the analysis of its level in serum revolutionized the management of PCa. PSA shares biochemical similarities with the other gene family members and their

use in the management of PCa has involved the analysis of various molecular forms of PSA (free, intact, complexed PSA, and pro-PSA) (31, 32).
The prostate health index (PHI), aims to diagnose healthy and malignant prostate conditions in men older than 50, and with normal DRE and PSA values lower than 4 ng/mL. This test included the analysis three PSA isoforms followed by the formula [-2] proPSA / fPSA x PSA1/2. Another test, the 4K score, combines the analysis of the three isoforms included in the PHI, together with the kallikrein-related peptide (hK2) assessment, and clinical information such as age and history before biopsy. Although these tests have gained attention in the last years for the detection of PCa, prospectives studies are still needed to evaluate their use as screening tool for the early diagnosis of PCa (33).
In urine, the PCA3 assay involves the analysis of prostate cancer antigen 3 (PCA3), a non-coding gene which is highly expressed in PCa cells and produced only in prostate tissue. The PCA3 assay measures the mRNA in urine after DRE and it is more specific than PSA, which is secreted by all prostate cells and can be influenced by prostate volume (25, 34). Initial findings suggest that the PCA3 assay does identify cancer, but does not discriminate between low risk and aggressive disease (35, 36).
A urinary marker that detects TMPRSS2 fused with ERG has been reported to yield more than 90% specificity and 94% positive predictive value for PCa diagnosis (37), however, the sensitivity is low since only 40-50% tumours carry this fusion. Prognostic performance also based on tissue expression remains controversial (37). In addition, it has been shown that urine TMPRSS2:ERG, in combination with urine PCA3, enhances the utility of serum PSA for predicting prostate cancer risk and clinically relevant cancer on biopsy (37, 38).
In tissue, different multi-gene expression assays have been developed from prostate needle biopsies. The Oncotype DX test measures the expression of 12 cancer-related genes discriminating four biological functions such as androgen signalling, proliferation, cytoskeletal organization and stromal response (39). The Prolaris test, uses expression analysis of 46 genes together with Gleason score and PSA to predict cancer aggressiveness (40).

At protein level, the immunohistological detection of the AMACR protein, which is overexpressed in most prostate cancers (41), has been proposed as a test for detecting PCa. AMACR has been proposed as a novel drug target for PCa and it has been demonstrated that diminution of AMACR protein levels using siRNA methods reduced proliferation of the androgen-dependent LAPC-4 PCa cell line. (41-44). A prognostic assay, ProMark, has been also proposed to predict PCa aggressiveness, by means of the quantitative analysis of 8 proteins (DERL1, CUL2, SMAD4, PDSS2, HSPA9, FUS, phosphorylated S6, and YBOX1) using immunofluoresence on biopsy tissue sections.

Table 1. Current and emerging markers for PCa management. Adapted from the article: Are there usable molecular markers for prostate cancer? Jenster, G., European Urology Today: EUT Congress News. March 2015.

Type of marker	Most common current markers	Less commonly used and emerging molecular markers
Risk	Family history, urinary symptoms, age, race	Single nucleotide polymorphisms (SNPs), rare variants of HOXB13 and germline BRCA mutations, PSA at age 40-50.
Diagnostic	PSA and PSA-based markers (Age-adjusted PSA, PSA doubling time, PSA velocity, free PSA, Percent-free PSA, ProPSA, PSA density), DRE, biopsy histopathology AMACR/p63 or ERG IHC, imaging (MRI, CT, TRUS)	Prostate Health Index (PHI), PCA3, TMPRSS2-ERG, marker profiles of differentially expressed and mutated genes in urine and blood
Prognostic	Gleason score (biopsy), extend of disease in biopsies, imaging (CT, MRI bone scan), after prostatectomy pT stage, Gleason grade and surgical margins	PTEN, c-MYC and expression of other oncogenes and tumour suppressor genes in tissue (biopsies), miRNA profiles in serum, urine and tissue, imaging.
Predictive		Androgen receptor variants (indicating resistance to hormonal therapy), mutations in DNA repair genes.
Monitoring	PSA, imaging (MRI, CT, bone scan)	EpCAM capture and molecular characteristics of CTCs, imaging.

Altered metabolism in PCa

Cancer is a complex genetic disease that results into deregulation of various metabolic mechanisms including bioenergetics. With technological improvements, the focus of cancer research has gradually shifted from changes in an individual biochemical pathway or metabolite, towards changes in the context of the global network of metabolic pathways, in a cell tissue or organism (45).

In multicellular organisms, most normal cells are exposed to a constant supply

of nutrients. In order to prevent an aberrant and uncontrolled cell proliferation, organisms use control growth factors that prevent and regulate the uptake of nutrients when their availability exceeds the levels needed to support cell division. In cancer cells, this growth factor dependence is overcome by acquiring genetic mutations that functionally alter receptor-initiated signalling pathways. These oncogenic mutations can result in the uptake of nutrients, particularly glucose, that meet or exceed the bioenergetics demands of cell growth and proliferation (also known as 'Metabolic reprograming', nowadays recognised as one of the hallmarks of cancer (46-48). It has been observed already by Warburg (1924, Nobel Prize) that cancer cells have different metabolic features, fermenting glucose into lactate even in the presence of enough oxygen to support mitochondrial oxidative phosphorylation (47, 49, 50).

One of the major functions of prostate cells is the production of citrate, PSA, and polyamines, such as spermine and spermidine, which are the major components of prostatic fluid. Prostate cells have a distinct metabolic profile as they produce specific compounds. Metabolic reprogramming in PCa is different compared to the reprogramming observed in other cancers. In normal prostate cells, there is an accumulation of zinc that produces the enzymatic activity inhibition of mitochondrial aconitase (the enzyme that catalyses the conversion of citrate to D-isocitrate) and therefore a truncated TCA cycle. In prostate cancer cells, zinc transporters are down-regulated, resulting in the enzymatic inhibition relieve of aconitase, thus enabling the utilisation of citrate through the mitochondrial TCA cycle, the decreased accumulation of polyamines, and the increased ATP production that provide energy for accelerated proliferation (51, 52).

Oxaloacetate and acetyl-coenzyme A (acetyl-coA) are essential for citrate synthesis, but whereas oxaloacetate is regenerated in the Krebs cycle, acetyl-coA is consumed. It is necessary to maintain elevated rates of citrate oxidation, with the subsequent availability of acetyl-coA, to ensure that cancer cells have the needed energy for rapid proliferation. It has been proposed that to maintain this accelerated citrate oxidation, alterations in the fatty acid metabolism (both saturated and unsaturated) are needed to provide both ATP and acetyl-coA (51, 52). A schematic representation of the major metabolic pathways altered in PCa is shown in Figure 2.

Beyond Krebs cycle and glycolysis, glucose can be degraded through the pentose phosphate pathway. This metabolic pathway involves the production of NADPH and ribose-5-phosphate, which are associated to nucleotide and nucleoside biosynthesis (51). It has been reported that Androgen Receptor signalling increases the levels of glucose-6-phosphate dehydrogenase (G6PD), NADPH, as well as ribose synthesis in hormone-sensitive PCa cells, thus confirming the role of the pentose phosphate pathway in PCa growth (51, 53).

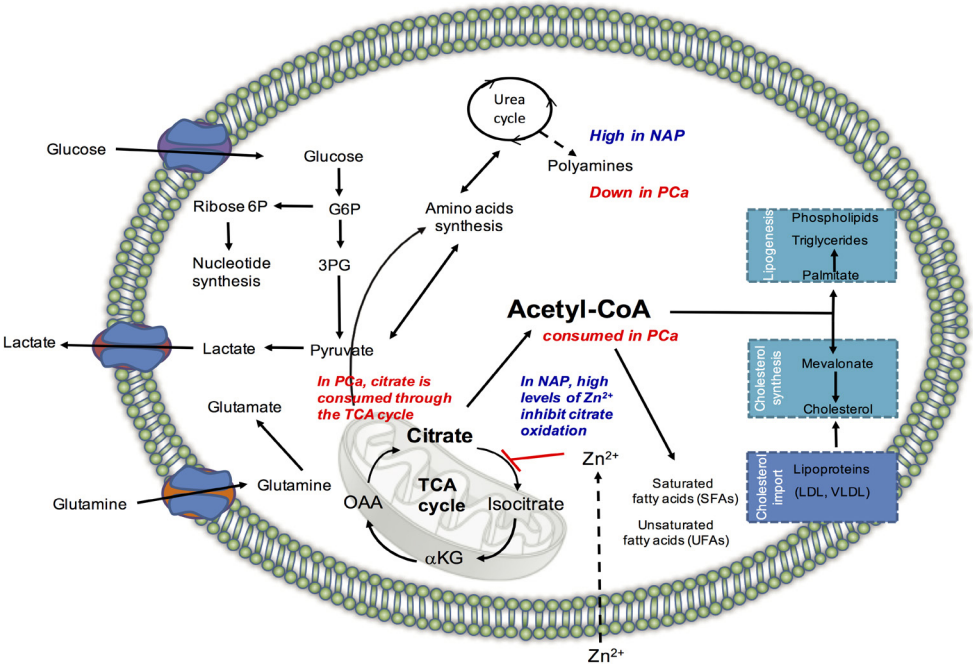

Figure 2. Altered metabolic pathways in PCa (Figure adapted from references 42-44). A major and persistent characteristic that distinguishes normal prostate tissue from malignant PCa tissue is the high level of citrate content, caused by the accumulation of Zn.

Cell proliferation and intercellular signalling are dependent on increased lipid biosynthesis. Acetyl-coA also plays an important role in this metabolic alteration because it is a precursor for lipogenesis and cholesterolgenesis and can be produced by transformation of citrate in the cytosol (51). Sterol regulatory element binding protein 1 (SREBP1) an essential transcription factor for lipogenesis, is also implicated in AR transcriptional regulation. It is known that beyond lipogenesis, SREBP1 also increases reactive oxygen species (ROS) production and the expression of NADPH oxidase, which leads to proliferation, migration, and invasion of PCa cells (54).

In PCa cells, the levels of choline and creatine are increased because of a higher rate of membrane synthesis during cell proliferation (52). Glutamine also has an important role in the maintenance of lipogenesis, as well as to provide intermediates for the Krebs cycle through glutaminolysis. Glutamine transporter and glutaminase have been shown to be overexpressed in PCa, the role of these enzymes in PCa development and progression is still unknown (52).

Metabolomics approaches in PCa research

Metabolites are intermediate and end products of the cellular regulated processes and their levels can be regarded as the biochemical signature of the overall phenotype of a system at a certain stage or phase of development, taking into account both genetic as well environmental influences (45).
Metabolomics aims to comprehensively identify small molecules (metabolites) to gain insight into cellular signalling pathways underlying disease and to discover novel biomarkers for screening, early diagnosis, prognosis and response to specific treatment. Metabolomics is interdisciplinary, driven by basic sciences (analytic biochemistry, biology) and bioinformatics, together with epidemiology and clinical research (55). Metabolomics is an important tool in the study of human diseases because metabolites constitutes the readout of gene-environment interactions and may therefore be complementary biomarkers than single nucleotide polymorphisms (SNPs) or epigenetic markers (56). Mass spectrometry (MS)-based techniques have the specific advantages of being more sensitive and therefore superior in terms of metabolic coverage, compared to nuclear magnetic resonance (NMR). Metabolomic experiments can be divided into targeted and untargeted analysis: the former aims to accurately determine concentrations of determined subset of metabolites, obtained from a defined pathway, whereas the untargeted approach uses a more global approach to cover the maximum number of metabolites that can be detected by an analytical method (45, 55).

Different studies describing metabolic alterations associated to PCa have been reported in literature in the last decade. These studies included metabolomics experiments in body fluids such as urine, plasma or serum from PCa patients, as well as metabolomic profiling in PCa tissue and cell lines. Major findings in the application of metabolomics approaches in PCa are
summarised in Table 2.

Table 2. Applicability of metabolomics in the diagnosis and prognosis of PCa.

Sample	Analytical methodology	Findings: Metabolites or class of metabolites		Cancer Type or condition	Ref.
		Up-regulated	***Down-Regulated***		
Urine, Tissue	GC-LC/MS	Sarcosine, proline, kynurenine, uracil and glycero-3-phosphate		PCa vs Normal	(57)
		Sarcosine		PCa progression to metastasis	(57)
Urine	GC-LC/MS	Sarcosine is not elevated in PCa		PCa vs Normal	(58-60) Reviewed by Trock *et al*, kers in Ref (61)
	HPLC-MS/MS	Kynurenine, Proline, glycerol-3-phosphate and uracil are not reliable markers for detecting or differentiating the aggressiveness of PCa		PCa vs Normal	(62)
	GC-LC/MS		Urea cycle, TCA cycle, amino acids, purines	PCa vs Normal	(63)
	GC/MS	Fatty acids	Pyrimidines, creatinine, purines, glucosides	PCa vs Normal	(64)
	1H NMR	Sarcosine, alanine, pyruvate	Glycine	High-grade and Low-grade PCa vs Normal	(65)
	LC-HRMAS	Tryptophan metabolites	Thymine metabolites, nitrogen metabolites, tri-peptides	PCa vs Normal	(66)
Serum	UHLPC-MS	Hexadecaenoic acid, tryptophan, steroid hormone metabolites	Phenylalanine, lysophospholipids	PCa vs Normal	(26)

Serum	HPLC-FLD	Non-altered tryptophan and kynurenine		PCa Gleason 6&7 vs Normal	(67)
	UHPLC-MS/MS	HETEs	Arachidonic acid	PCa vs Normal and different stages of PCa	(68)
	LC/MS	Deoxycholic acid, glycochenodeoxycholate, tryptophan, docosapentaenoic acid, arachidonic acid, deoxycytidine triphosphate and pyridinoline		PCa patients before and during endocrine therapy	(69)
	LC/MS	38:5 egg phosphatidylcholine (ePC), 40:3 (PC) and 42:4 (PC)		PCa vs Normal	(70)
Tissue	HRMAS 1H NMR		Spermine, citrate	PCa vs Normal, Low -grade PCA vs High-grade PCa	(71)
	GC-LC/MS	Aminoadipic acid, cerebronic acid, glycerophosphoethanolamine, 2-hydroxybehenic acid, isopentenyl pyrophosphate, 7-methylguanine and tricosanoic acid	Gluconic Acid, Maltotriose	PCa vs Normal	(72)
	1H HR-MAS	Lysine degradation, fatty acids, sugars	Mevalonate metabolism, purines	PCa vs Normal	(73)
	UHPLC-MS/MS	Amino acids, carnitines, purines, pyrimidines, choline,	Laureate, malate, mannose, ADP	Extracapsular extension PCA vs organ-confined PCa	(74)
		NAD+, choline derivatives,	Polyamine, fatty acids, sugars	Extracapsular extension PCa vs seminal vesicle PCa	(74)
Prostate cancer cells	LC/MS	Sarcosine, threonine, phenylalanine, alanine, nitrogen metabolites	Tryptophan	PCa vs Normal	(75)
	LC/MS	Homocysteine, polyamines	S-Adenosylmethionine	AR dependent vs Androgen Independent	(76)
	LC/MS	Amino sugars, methylation metabolites, Amino acids	Sugars, energy signaling	AR-dependent PCa vs CRPC	(75)
	LC/MS	17-HpDHA and 17-HDHA are potent inhibitors of proliferation on androgen positive and negative PCa cell lines		Proliferation inhibition	(77)

Arachidonic acid pathway

Polyunsaturated fatty acids (PUFA) play a key role in the structure and physiological properties of cell membranes. Two classes of PUFA, designated as ω-6 and ω-3 (indicating the position of the C=C double bond), normally are present in tissue and body fluids. These fatty acids cannot be synthesized by mammalian cells, and need to be obtained from the diet. It is known that the Western diet usually contains 10- to 20-times more ω-6 than ω-3 PUFA. Plasma and most other tissues are richer in ω-6 fatty acids, but the brain and retina are considered exceptions because they are rich in ω-3 PUFA. Absence of ω-6 PUFA could cause a very serious systemic illness called essential fatty acid deficiency (78).

The need for arachidonic acid (20:4n-6), almost certainly is the primary reason why ω-6 PUFA are essential. Arachidonic acid is the main substrate for synthesis of the eicosanoid mediators it is highly enriched in the inositol phospholipids that are involved in signal transduction (79).

Arachidonic acid (AA, or sometimes ARA) is a polyunsaturated omega-6 fatty acid 20:4(ω-6) (Figure 3). It is the counterpart to the saturated arachidonic acid found in peanut oil. AA is present in phospholipids -especially phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositides- of membranes of the cells, and is abundant in brain, muscles and liver. The four cis double bonds in the arachidonic acid molecule confer it high mobility and flexibility, thus helping it to provide mammalian cell membranes their correct fluidity at physiological temperatures. In addition, the double bonds are responsible for the tendency of arachidonic acid to react with molecular oxygen (78, 79).

Dietary fat intake might be one of the most studied dietary risk factors for PCa, although its role in influencing cancer risk remains controversial (80). It has been observed in PCa cell lines that n-6 fatty acids, such as linoleic acid and arachidonic acid (AA), promote cell proliferation, whereas long-chain polyunsaturated n-3 fatty acids, might inhibit cell proliferation (81). Thus, these promotional and inhibitory effects of n-6 and n-3 fatty acids, respectively, have motivated the study of dietary fatty acids in the development and progression of PCa.

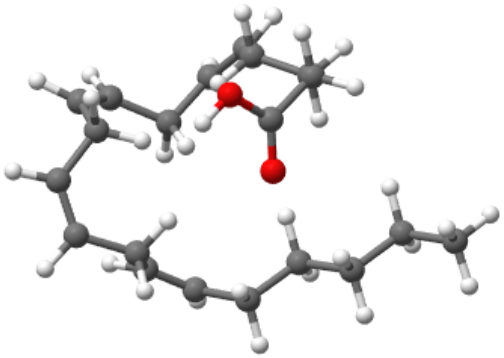

Figure. 3. Chemical structure of arachidonic acid.

AA is released from a phospholipid molecule in cell membranes by the enzyme phospholipase A2 (PLA2), which cleaves off the fatty acid. AA can also be generated from diacylglycerol (DAG) by a DAG-lipase enzyme. AA generated for signalling purposes appears to be derived by the actions of a phosphatidylcholine- specific cytosolic phospholipase A2 (cPLA2), whereas inflammatory AA is generated by the action of a low molecular weight secretory PLA2 (sPLA2) (82).

AA is a precursor in the production of eicosanoids: signalling molecules produced by oxidation of twenty-carbon fatty acids. Three families of enzymes catalyse the oxidation of fatty acids to produce eicosanoids: Cycloxygenases, lipoxygenases and epoxygenases. Cycloxygenases (COX-1 and COX-2) lead to prostaglandin H2 (PGH_2), which in turn is used to produce the prostaglandins (PGs), prostacyclins (PGIs) and thromboxanes (TXs). Lipoxygenases (5-LOX, 12-LOX, 15-LOX and 15-LOX-2) produce hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs). Epoxygenases (including the family of Cytochrome P450 enzymes) produce also HETEs and epoxyeicosatrienoic acids (EETs) (81, 83). The production of these derivatives and their action in the body are collectively known as the AA pathway.

The PGs are synthesized by the action of COX enzymes: the constitutively expressed COX-1 and the inducible COX-2. COX-2 can be stimulated by inflammatory mediators, cytokines, growth factors, and tumour promoters and can be inhibited by steroids and certain nonsteroidal anti-inflammatory drugs. Both AA and PGE_2 stimulate cell proliferation and tumour growth in vitro in PC-3 human PCa cells (84). In PC-3, LNCaP, and DU145 prostate cancer cell lines, upregulation of COX-2 and PGE2 was reported to be inversely correlated with apoptosis (85).

LOX products, such as LTs and hydroxyeicosatetraenoic acids (HETEs), as well as TXs, metabolites of COX enzymes, have been studied during proliferation of

various human cancer cell lines. Expression of 12-LOX was reported to stimulate angiogenesis in human PCa cells (81). The 5-LOX product 5-HETE has been suggested to play role as a potent pro-growth survival factor for human prostate cancer cells (86).

Aim and outline of the thesis

Although the introduction of prostate-specific antigen (PSA) has improved considerably the management of PCa, its lack of specificity in the diagnosis and prognosis has motivated the search for novel and non-invasive molecular markers as well as novel therapeutic targets for treating prostate carcinoma.
In this thesis, we aimed to identify and validate novel metabolites or proteins in tissue or bio-fluids, which might improve the diagnosis and/or the prognosis of PCa. To accomplish this aim, we used both metabolomics and proteomics methodologies, and particularly we used the state of art of mass spectrometry (MS) for the identification and quantitation of both metabolites and proteins in serum and PCa tissue.
Kynurenine, a metabolite produced along the tryptophan pathway, was reported a few years ago as a metabolite-deregulated in PCa. In Chapter 2, we aimed to validate the role of kynurenine as a diagnostic marker for PCa by analysing concentrations of kynurenine (Kyn) and tryptophan (Trp), in serum samples from PCa patients exhibiting different Gleason Scores as well as controls. We demonstrate that neither the concentrations of these metabolites, nor the ratio Kyn/Trp, an indicator of the Indoleamine 2-3 dioxygenase (IDO) activity, cannot be considered as new markers for detecting PCa.
Arachidonic acid (AA) has been proposed to play a role in PCa development and progression. Particularly, lipoxygenases, a type of enzymes responsible of incorporating a hydroxyl group to the arachidonic chain, have been shown to be altered in PCa biopsies and cancer cell lines. In addition, concentrations of AA have been reported to be diminished in PCa tissue. In Chapter 3, we aimed to study concentrations of arachidonic acid, five of its metabolites (HETEs) in serum from PCa patients, and aimed to analyse their potential in the diagnosis and prognosis of the disease. We found that concentrations of HETE metabolites were higher in a selected group of patients at the most advanced stages of the disease. Although our markers do not exhibit a higher sensitivity and therefore, cannot be considered as markers for detecting PCa, it is of interest that concentrations of AA at the time of radical prostatectomy (RP), can predict biochemical recurrence (BCR) after surgery, thus suggesting a potential prognostic role for this metabolite in serum.
In order to get an insight into the proteins of the arachidonic acid pathway and their relationship with PCa, as well as their role in prognosis and prognosis, we performed a global proteomics analysis of PCa tissue. To accomplish this

aim, we first analysed differentially expressed proteins in PCa tissue compared to normal adjacent tissue (NAP). Next, we used both normalised intensities and expression intensities from a PCa tissue microarray (TMA), to identify prognostic properties for two de-regulated proteins: Anterior Gradient 2 (AGR2) and 15-LOX-B. Finally, we mapped the proteins of the AA pathway into the identified and quantified proteins in PCa tissue to obtain a simplified picture of the enzymes of the AA and their de-regulation in PCa.
The results obtained in Chapters 3 and 4, highlighted the role of arachidonic acid, fatty acid metabolism and eicosanoid signalling in PCa. We decided to analyse the mass spectrometry ionisation of fatty acids by the addition of inorganic ions, in order to improve the analysis of these kinds of molecules by mass spectrometry. In Chapter 5, we present a mass spectrometry-based study of the incorporation of carboxylate-substituted super-halogens in the structural characterization of fatty acids by MS. We analysed the addition of Calcium ions in the fragmentation of arachidonic acid and the analysis of each unsaturation on the carbon chain. We proposed this novel technology as a useful tool in the analysis of fatty acids, because it allows a better identification of functional groups in fatty acids, steroids, complex lipids, and other metabolites also possible associated to PCa.
Finally, the findings are summarised and a general discussion is presented.

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Chapter 2

Serum kynurenine/tryptophan ratio is not a potential marker for detecting prostate cancer

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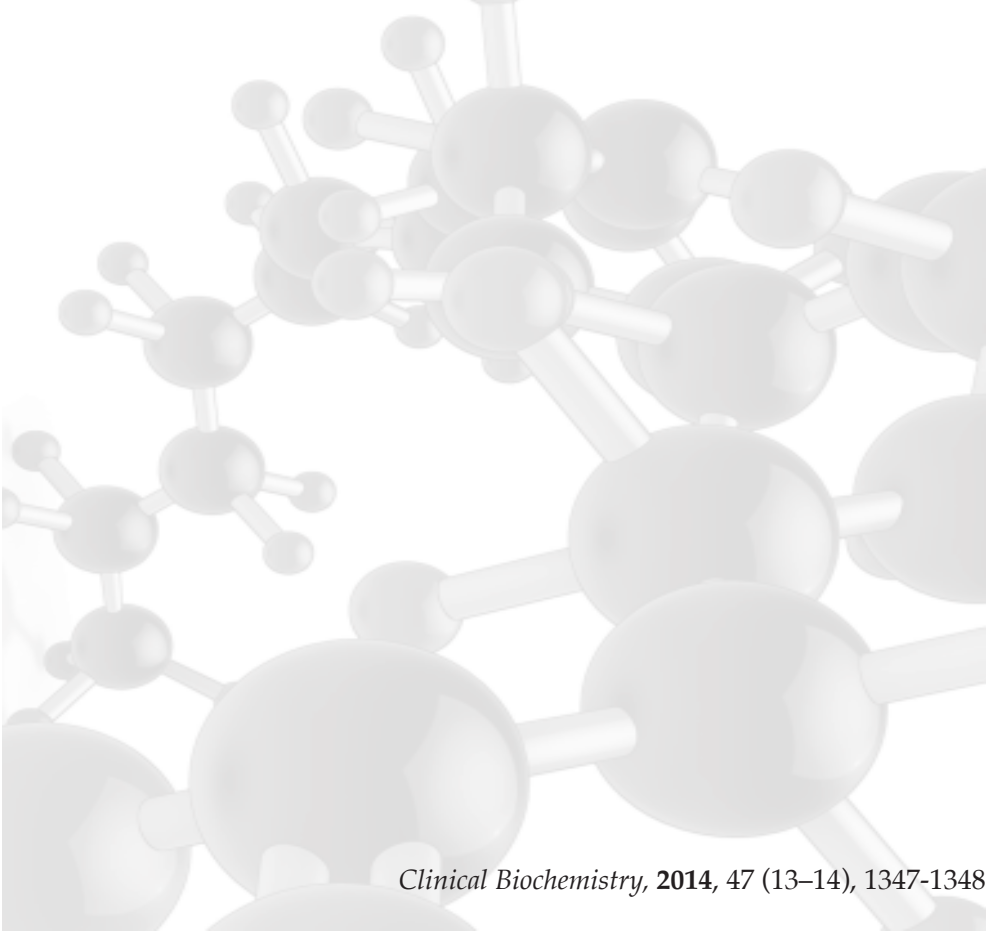

Clinical Biochemistry, **2014**, 47 (13–14), 1347-1348

Tryptophan (Trp) is an essential amino acid in humans that plays an important role in protein synthesis and it is a precursor of many biologically active metabolites, including kynurenine (Kyn)(1). The enzyme that converts tryptophan into kynurenine is indoleamine 2,3- dioxygenase (IDO), which has been associated to immune escape of tumour cells (2). Kynurenine was part of a subset of six metabolites found to be associated to prostate cancer (PCa) progression as described by Srekumar et al. (3) in plasma, urine and tissue. More recently, Kyn was proposed by McDunn et al. (4), as a metabolite that strongly correlates with aggressiveness of this malignancy as measured by Gleason scores. We determined concentrations of Kyn and Trp by fluorescence detection in serum samples from patients diagnosed with PCa, and classified in two groups: Gleason score 6 (n=15) and Gleason score 7 or higher (n=28). We compared these concentrations with the ones in a group of male subjects with low PSA levels and without PCa, denominated as PCa Controls (n=20). Power analysis indicated that these number of samples resulted in 70% of power with 95% confidence and with effect size of 0.8 according to Cohen's suggestions (5). Serum samples from patients diagnosed with colorectal liver metastases (n=10), liver adenoma (n=8), and hepatocellular carcinoma (n=6), as well as serum samples from healthy kidney donors (n=12), were also included as extra groups to generate information about tumour specificity. The use of serum samples for research purposes was approved by the Erasmus MC Medical Ethics Committee according to the medical research involving human subjects Act (MEC-ERSPC). Serum samples were obtained according to the protocols reported by the Rotterdam Centre for the European Randomized Study of Screening for Prostate Centre (ERSPC)(6), and were stored at -80°C until further processing. Sample preparation included thawing of the samples on ice for 1 h, protein precipitation/dilution with trichloroacetic acid at room temperature, and centrifugation at 14.000 g at 4°C for 15 min. Kyn and Trp in the supernatant were separated by liquid chromatography (LC) and detected by fluorescence (FLD) using the standard addition method. This method is used to avoid matrix interferences by means of the addition of known quantities of the analyte of interest, with a concomitant increment in signal response of the instrument. We used zinc-mediated fluorescence for the accurate determination of Kyn (7, 8), and endogenous concentrations of Trp and Kyn were calculated by extrapolation using at least four points in a calibration curve (9). In all cases, the percentage of error was less than 15%, indicating that the small variances in the measurements allow differentiation between cases and controls. Stability was evaluated by consecutive measurements of pooled serum obtained from ten healthy individuals and also of the same pooled serum spiked with known amounts of Kyn and Trp to produce high, medium and low concentrations. Measurements were performed within a period of 20 h, which is the time the samples are stored in the autosampler before analysis. In all cases, relative standard deviations were lower than 5%.

Clinical-pathological characteristics of the patients involved in this study are presented in Table 1.

Table 1. Clinical and pathological characteristics of cases and controls. PCa Controls, PCa Patients.

Characteristic		**Controls/ ERSPC**	**PCa Patients**
		(n=20)	**(n=43)**
Age (years)	Median	68.2	65.3
	Range	58.2-69.4	56.2-69.1
PSA (ng/mL)	Median	0.2	5.4
	Range	0.1-4.4	2.0-15.1
Pathological State		n (%)	n (%)
	pT1	-	5(11.6)
	pT2	-	27(62.8)
	pT3	-	6(13.9)
	pT4	-	4(9.3)
	Unknown	-	1(2.3)
Gleason Score			
	3+3	-	15(34.9)
	3+4	-	21(48.8)
	3+5	-	1(2.3)
	4+3	-	4(9.3)
	4+4	-	2(4.6)

Mean serum concentrations of Kyn for PCa patients were found at 2.1 ± 0.7 μM (mean ± SD) in Gleason 6, and 1.91 ± 1.2 μM in Gleason 7; these concentrations were not statistically different compared to the Control group (2.1 ± 0.7 μM). These values are consistent with reported data using different analytical approaches in healthy individuals (10). Trp concentrations for the PCa patients were also not significantly different (73.0 ± 13.8 μM for Gleason 6 and 70.7 ± 16.8 μM for Gleason 7) compared to those for the Control group (77.7 ± 21.7 μM).

Mean concentrations of Trp and Kyn, as well as the calculated Kyn/Trp ratio are shown in Figure 1a-c. Differences among groups were not significant ($p>0.05$). Possible associations between metabolites concentrations in the serum of the PCa patients and clinical information available such as pathological stage and Gleason Score indicated no statistical significance ($p>0.05$). Our data show that there are no major alterations in extracellular concentrations of Trp and Kyn. Therefore, these parameters or the calculated Kyn/Trp ratio, have no potential as biomarker for the diagnosis of prostate cancer in serum.

Metabolic signatures between PCa tissue and benign prostate tissue have suggested that an increment exists in the concentration of Kyn in PCa tissue (3, 4). Although the number of serum samples used in this study was relatively small, the results presented here indicate that the reported metabolic signatures do not translate to changes in serum. Therefore, although metabolic alterations in kynurenine pathway might be occurring at the level of the prostate, this pathway cannot be considered as a target for serum biomarkers and of clinical applicability.

Acknowledgments

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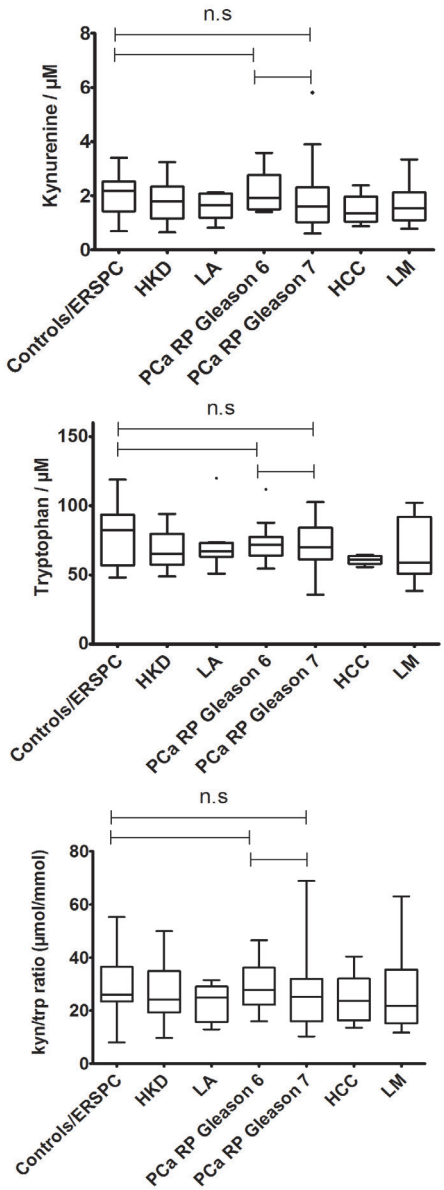

Figure 1. Serum concentrations of a) Tryptophan, b) Kynurenine and c) Kyn/Trp ratio in PCa Controls, PCa Patients Gleason 6 (n=15) and Gleason 7&8 (n=28), HKD: Healthy Kidney Donors (n=12, median age: 57,2 years) , LA: Liver Adenoma (n=8, median age: 33.8), HCC: Hepatocarcinoma (n=6, median age: 56.3), and LM: Liver Metastasis (n=10, median age: 64.2).

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Chapter 3

Serum levels of arachidonic acid metabolites change during prostate cancer progression

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Abstract

Background: Arachidonic acid (AA) pathway has been shown to play a role in the development and progression of prostate cancer (PCa). In this study, we aimed to assess the changes in concentrations of hydroxyeicosatetraenoic acids (HETEs) in serum samples from patients diagnosed with PCa compared to controls.
Methods: HETEs were determined using ultrahigh pressure liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS).
Results: Elevated concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE were observed in 6 out of 20 patients diagnosed with PCa; no statistical differences with controls were observed for 12-HETE and AA in the discovery set. An independent validation set composed of 222 samples divided in five groups ranging from subjects with low PSA and no PCa, to patients with advanced PCa was included. In 30% of the patients in the advanced PCa group, up to ten times higher concentrations of the same set of HETEs were observed with a significant concomitant decrease of the concentration of AA. Logistic regression and Kaplan Meier curves illustrate that a decreased concentration of AA is a predictor of PCa biochemical recurrence after radical prostatectomy (RP).
Conclusions: From the present study, we conclude that a significant association between AA and AA metabolites in serum and PCa progression exists, although serum concentrations of HETEs exhibited low sensitivity towards the diagnosis of PCa. Analysis of RNA microarray data of PCa tissue indicate elevated levels of phospholipase A2, which might explain the elevated levels of HETEs and the significant decrease of AA in advanced PCa.

Introduction

Prostate cancer (PCa) is the second most common cancer in the Western world, and it has been predicted that around 70,000 men will die in 2013 from PCa in Europe (1). Identification of biochemical networks involved in PCa development and progression is helpful for the discovery of novel biomarker candidates, as well as for the establishment of new therapeutic strategies specially for early stages of the disease (2).

The reported association of arachidonic acid (AA) metabolism with PCa indicates that there are various key routes within the AA pathway that can provide possible novel and therapeutic opportunities for treating PCa. Vainio et al. explored the expression patterns of 36 genes involved in AA pathway in approximately 10,000 human tissue samples and they found that six genes from this pathway, ALOX15B, CYP4F8, EPHX2, FAAH, PLA2G2A and PLA2G7, were highly expressed in PCa samples compared to normal tissue (3).Interestingly, one of these genes, arachidonate 15 lipoxygenase type II, or ALOX15B, is a gene that encodes a member of the family of the lipoxygenases which converts exclusively AA to the eicosanoid 15-hydroxyeicosatetraenoic acid (15-HETE) (4).

In the human being, eicosanoids are bioactive metabolites produced mainly from essential C20 fatty acids such as dihomo-ω-linolenic (20:3(n-6)), arachidonic (20:4(n-6)) and eicosapentaenoic acid (20:5(n-3)) (5). These metabolites are obtained by the action of three oxygenated-type enzymes: lipoxygenases (LOX), cyclooxygenases (COX-1 and COX-2) and cytochrome P450. Prostaglandins, leukotrienes, thromboxanes and other oxygenated derivatives such as hydroxyeicosatetraenoic acids (HETEs) are known as biologically active eicosanoids (6, 7).

Their important role in the pathogenesis of different human diseases, including inflammation and cancer has been well documented (6-10). Figure 1 schematically illustrates the eicosanoids produced in the AA metabolism, as well as some of the genes involved in this pathway.

To date, the relationship between AA and the development and progression of PCa has been addressed from different perspectives (6, 7, 12). Several studies have reported expression analysis of COX and LOX in PCa tissue, cell lines and animal models using RT-PCR and western blotting (6, 7). Recently, AA metabolites, and especially those produced by LOX-type oxygenases, have been proposed as a target for treating PCa after studying mRNA and protein expression of LOX and COX in androgen receptor dependent and independent cell lines, xenografts, and biopsy samples of primary tumours (13). In body fluids, determination of urinary concentrations of 12-HETE and 20-HETE have been reported for patients diagnosed with PCa and benign prostate hyperplasia (BPH) (14). However, knowledge on concentrations of HETEs in serum and its association with PCa is still missing.

In this study, we used ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) to compare serum concentrations of AA and HETEs between patients diagnosed with PCa and controls. We report for the first-time serum levels of AA and HETEs in PCa patients at different stages of disease. We conclude that AA serum levels predict biochemical recurrence of PCa after radical prostatectomy.

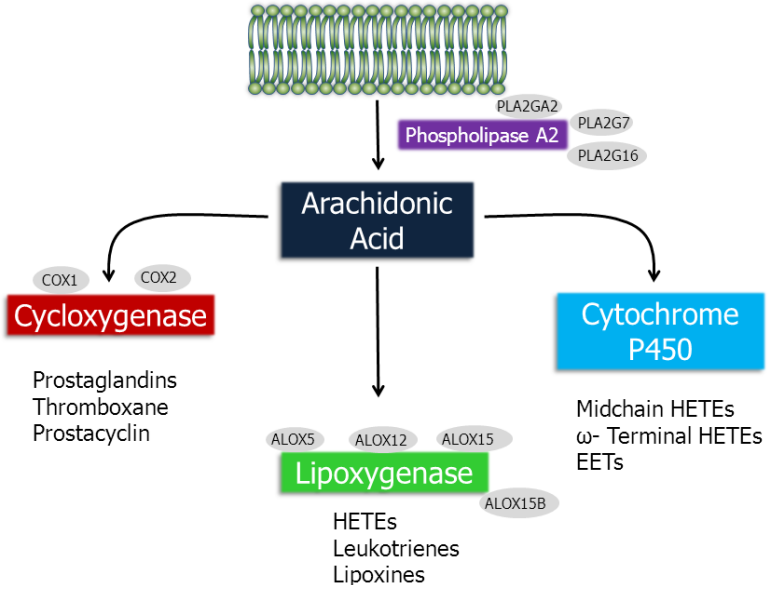

Figure 1. Arachidonic acid pathway. Arachidonic acid is released from phospholipids in the cell membrane by phospholipase A2 enzymes. Next, it is metabolised by one of three major routes: the cycloxygenase (COX), the lipoxygenase (LOX) and the cytochrome P450 epoxygenase route in the generation of eicosanoids. Grey circles indicate names of some of the RNA transcripts analysed in this study. Figure freely adapted from references (7, 11).

Materials and Methods

Clinical samples

Serum samples: Serum samples were collected at time of diagnosis. The discovery set included two sets of serum samples corresponding to patients diagnosed with PCa (n=20) and subjects with PSA values lower than 3.2 ng/mL denominated as < PSA Controls (n=20). Samples were obtained from men included in the Rotterdam Center for the European Randomized Study of Screening for Prostate Center (ERSPC). Samples were supplied by the Urology department, Erasmus MC, Rotterdam. Four sets of sera from patients diagnosed with colorectal liver metastases (n=10), liver adenoma (n=8), hepatocellular carcinoma (n=6) and healthy kidney controls (n=12), were also included in the discovery set. These samples were supplied by the department of Surgery of Erasmus MC, Rotterdam. The validation set included five groups of samples of prostate cancer patients at different stages of the disease. Serum samples were collected between 1994 and 1998 and completed clinical follow up information was available for those patients within a period of 10 years. Subjects with low PSA values (≤1.0 ng/mL) and no evidence of PCa after 9 years of follow up (n=44) were classified in group 1. Subjects with PSA values between 3-10 ng/mL, and repeated negative biopsies confirming no PCa (n=45) were included in group 2. Patients who were diagnosed with insignificant PCa, T1c & T2 and Gleason Score 6 (n=73), and underwent radical prostatectomy and were diagnosed with insignificant PCa, T1c & T2 and Gleason Score 6 (n=73) were classified in group 3. Patients with significant PCa (T2&T3a/b, Gleason 6&7 and tumour volume > 0.5 mL) and no PSA recurrence after radical prostatectomy, (n=41) were part of group 4. Patients with significant PCa (T2&T3a/b and Gleason 6&7) and PSA recurrence after radical prostatectomy, (n=19) were included in group 5. These samples were supplied by the Urology department, Erasmus MC, and were collected at the time of diagnosis for Groups 3-5. Status of the disease was histologically evaluated by an uropathologist of the Erasmus MC department of Pathology. All samples were collected and immediately stored at -80ºC until further analysis. Clinical pathological information of these patients is listed in Table 1.

Chemicals: Arachidonic Acid, (S)-HETE HPLC mixture containing 5(S)-HETE, 8(S)-HETE, 11(S)-HETE, 12(S)-HETE, and 15(S)-HETE, Arachidonic Acid-d8 and 15(S)-HETE- d8 were purchased from Bio-Connect (Huissen, the Netherlands). All solvents used were obtained from commercial sources and were of LC-MS grade (Biosolve, Valkenswaard, The Netherlands). Most stock solutions were prepared by dissolving the substances in ethanol.

LC-MS/MS

The UHPLC used in this study was an automated Dionex Ultimate 3000 (Thermo Fisher Scientific, Amsterdam, the Netherlands) system coupled to an API4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo Ion Spray electrospray ionization (ESI) source.

Experiments were performed using Selective Reaction Monitoring (SRM) in ion negative mode. Source-dependent mass spectrometer (MS) parameters were set at 20 psi for curtain gas, -4000 V for Ion Spray Voltage and 500°C for ion source temperature. Nebulizer 1 and 2 were kept at 20 psi. Compound-dependent MS parameters are given in Supp. Table 1. Chromatographic separation was carried out on a Kynetex analytical column (100 x 2.1 mm, 1.7 μm particle size) from Phenomenex (Aschaffenburg, Germany) at 40°C. Gradient elution conditions were optimized using 0.1% formic acid (FA) in water as solvent A, and 0.1% FA in ACN as solvent B. The analysis time for each sample was 8 min. Data acquisition and peak integration was performed using Analyst software version 1.4.2 (Applied Biosystems, Foster City, CA, USA). Representative chromatogram of the simultaneous determination of AA and HETEs in serum is shown in Supp. Fig 1.

Table 1. Patient clinical and pathological characteristics. Discovery set included <PSA Controls, PCa Patients, HKD: Healthy Kidney Donors, LA: Liver Adenoma, HCC: Hepatocarcinoma, and LM: Liver Metastasis. Validation set included five groups of Prostate Cancer at different stages of the disease.

Discovery	**< PSA Controls**	**PCa Patients**	**HKD**	**LA**	**HCC**	**LM**
	(n=20)	(n=20)	(n=12)	(n=8)	(n=6)	(n=10)
Age (years)						
Median	61.8	67.1	57.2	33.8	56.3	64.2
Range	58.8-70.8	59.7-74.4	34.2-75.3	23.1-76.8	20.4-70.8	43.1-81.4
PSA (ng/mL)						
Median	1.1	3.4				
95% C.I.	1.0-1.8	2.2-8.4				
Pathological State	**n(%)**	**n(%)**				
pT1	-	9(45)				
pT2	-	4(20)				
pT3	-	2(10)				
PT4	-	-				
Unknown	-	5(25)				

Validation	Group 1	Group 2	Group 3	Group 4	Group 5
	(n=44)	(n=45)	(n=73)	(n=41)	(n=19)
Age (years)					
Median	59.6	62.1	63.4	64.9	65.6
Range	54.6-66.8	55.1-66.8	55.0-72.8	55.7-70.4	48.7-71.1
PSA (ng/mL)					
Median	0.5	4.6	4.2	4.7	17.6
95% C.I.	0.4-0.6	4.6-5.8	4.3-5.6	4.7-8.9	10.0-25.1
Pathological State	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**
pT1	-	-	51(68.9)	19(46.3)	2(10.5)
pT2	-	-	23(31.1)	20(48.8)	7(36.8)
pT3	-	-	-	2(4.9)	6(31.6)
pT4	-	-	-	-	1(5.3)
Unknown	-	-	-	-	3(15.8)
Gleason Score					
6	-	-	73(100)	25(61.0)	
7	-	-	-	16(39.0)	12(63.1)
8	-	-	-	-	1(5.3)
9	-	-	-	-	1(5.3)
Unknown	-	-	-	-	5(26.3)

Sample preparation

Serum samples were thawed on ice for 2 hours and 20 μL were immediately transferred to a new Eppendorf cup and were spiked with internal standards. For protein precipitation, cold methanol (-20°C) was used. After centrifugation at 14,000 x g for 10 min, 20 μL of the supernatant were diluted with the mobile phase solvents and injected for UHPLC-MS/MS analysis.

Statistical Analysis

Statistical calculations were performed with GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA) and SPSS version 20.0.0.1 (IBM Corp., Armonk, NY, USA).

The variables evaluated were age, serum concentrations of AA, 5-HETE, 8-HETE, 11-HETE, 12-HETE and 15-HETE, PSA at the time of diagnosis, prostate volume, pathological status, and Gleason Score. Pearson r^2 or Spearman's correlation was performed to evaluate associations between concentration of HETEs, AA, PSA level, Gleason Score or pathological stage. Comparison of biomarkers median values between PCa patients and PCa controls in the discovery set was performed by Mann-Whitney U test. The same test was used to analyse biomarkers within the groups in the validation set. Kruskal-Wallis test was performed to evaluate differences among the additional group of cancer patients, as well as among the PCa patients in different groups in the validation set. Combined predictive effect of HETEs or AA was addressed by logistic regression. Kaplan-Meier (KM) curves were constructed to assess PSA recurrence as a function of time after RP, and log-rank test was used to evaluate differences between curves. In this two-tailed test, a p value of < 0.05 was considered significant.

Results

Concentrations of HETEs are elevated in a sub-group of PCa patients

Clinical and pathological characteristics of patients in this study are summarized in Table 1. Median concentrations of HETEs in the discovery set composed of prostate controls and PCa patients are described in Fig. 2. In 6 out of 20 PCa patients (33%), concentrations up to ten times higher than controls were found for 5-HETE, 8-HETE, 11-HETE and 15-HETE with a significant difference between controls and PCa patients for 5-HETE ($p=0.0090$). The selected sub-group of patients with elevated concentrations of HETEs exhibited the same trend in all the HETEs studied except for 12-HETE and AA. No significant associations between HETEs or AA concentration and pathological stage were found, but at least 4 of the patients in this sub-group exhibited pathological stage T2a or T3a. In Supp. Table 2, concentrations of HETEs and AA in the discovery set are presented. The sub-group of patients with relative extreme elevated concentration of HETEs is included in a different column in the same table for separate analysis.

We decided to include another group of patients diagnosed with other malignancies in order to test the possible specificity of HETEs towards PCa. Median concentrations of HETEs in patients diagnosed with hepatocarcinoma (HCC), liver metastasis (LM), liver adenoma (LA), and health kidney donors (HKD), are presented in Figure 2. For none of the HETEs studied and for AA, a statistical difference among these groups was observed. High concentrations of HETEs compared to the median values in all the groups in the discovery set were found in one patient diagnosed with HCC, however these concentrations are up to five times lower compared to the highest concentrations found in PCa patients.

HETEs and AA are associated with advanced stages in PCa

Concentrations of HETEs were measured in an extended group of subjects without PCa and PCa patients at different stages of the disease to evaluate prognostic properties. Serum HETEs concentrations in the five groups evaluated are presented in Figure 3. Groups were classified according to PSA values and pathological status. In group 1, subjects with PSA values lower than 1.0 ng/mL and no evidence of PCa after more than 9 years of follow up were included. In this group, the median PSA value was 0.5 ng/mL and no correlations were found between concentrations of HETEs or AA and PSA values or age at the time of sampling. Group 2 included subjects with abnormal PSA values but repetitive negative biopsies for PCa. One subject in group 2 exhibited high levels of HETEs, but PCa was not detected in the 9 years following the sampling of the serum. Taking into account that clinical follow up indicated that subjects in groups 1&2 did not show evidence of PCa, these two groups were considered as controls and grouped for further analysis. In group 3 were included patients with insignificant PCa, and the median PSA value was 4.2 ng/mL. There were no statistically significant differences with respect to concentrations of HETEs and AA between groups 1&2 and group 3, thus confirming the results obtained in the discovery set.

Group 4, composed of patients with pathological stage T2 & T3a/b and a tumour volume higher than 0.5 mL, represents an advanced stage of the disease. However, clinical follow up within at least five years after RP indicated no recurrence. In this group, median PSA value was found as 4.7 ng/mL and no statistical differences were found in the comparison between groups 3 and 4, and between groups 1&2 and 4. Similarly to the PCa patients in the discovery set, high concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE were found in 4 out of 41 patients (10%) in group 4.

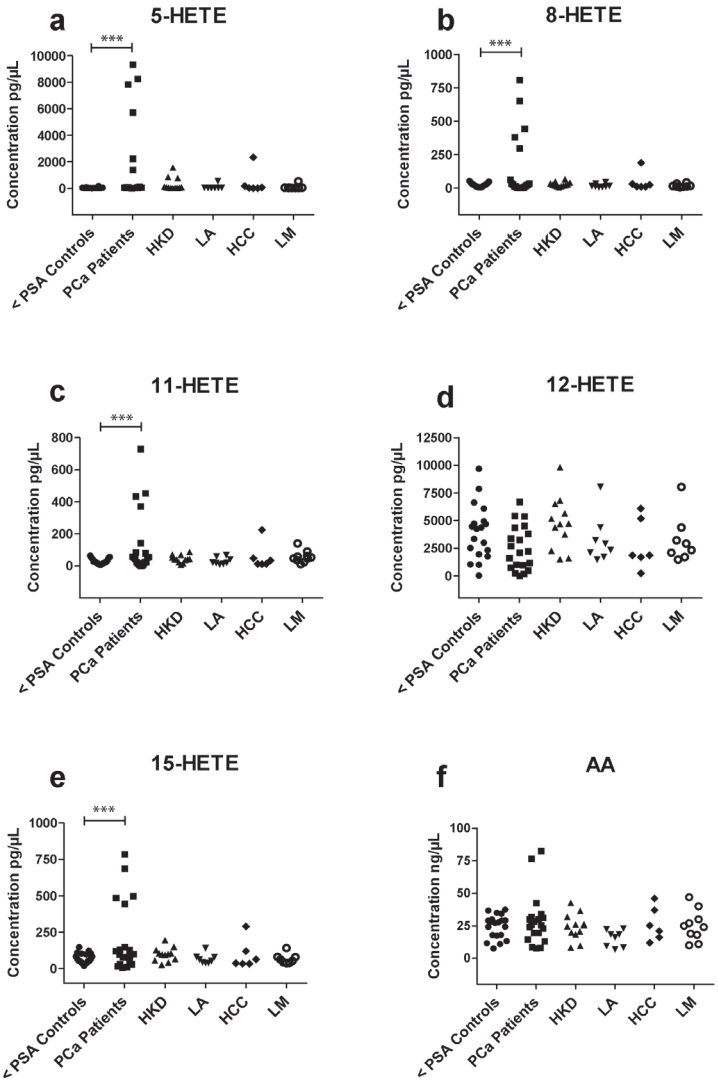

Figure 2. Serum concentrations of eicosanoids in the Discovery Set. Elevated concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE are observed in 6 out of 20 PCa patients. Patients diagnosed with other malignancies are also included to analyse specificity of the markers towards PCa. HKD: Healthy Kidney Donors, LA: Liver Adenoma, HCC: Hepatocarcinoma, and LM: Liver Metastasis patients were included in the discovery set.

Patients with the most advanced PCa status, defined as Gleason score equal or higher than 7 and detectable PSA levels after three months after RP, represented group 5. High concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE were found in 6 out of 19 (32%) of the patients in group 5, and as a consequence, a significant difference was observed between group 3 and 5 for the HETEs. In Figure 3, concentrations of HETEs and AA in the validation set are presented and a clear trend showing that the concentration of HETEs increases with the status of PCa is observed (although this behaviour is seen in less than 35% of the cases under study). Concentrations of AA in group 5 were found up to 5 times lower compared to all the groups under study including the controls and this effect showed a significant difference between these groups ($p<0.0001$). In addition, a Kruskal-Wallis analysis for AA in the five groups, too, indicated a significant difference among them ($H=38.02$, $p<0.0001$).

Logistic Regression confirms serum concentration of AA at the time of diagnosis as a predictor of PSA recurrence after radical prostatectomy

Logistic regression confirmed the negative relationship between the concentration of AA and the PSA recurrence after 10 years' follow-up in the PCa patients who were treated by radical prostatectomy. Analysis was performed for patients in groups 3, 4 and 5, using backward selection. Age, PSA, Gleason Score, and concentration of HETEs and AA were considered as independent variables. Results indicated that the significant variables were PSA, Gleason Score, and AA. However, ROC analysis indicated a small reduction in the AUC when AA is added to the model (0.751 vs. 0.792). The Logistic Regression Model's results are presented in Table 2.

Table 2. Logistic Regression Model.

Sample	Variables in the final model	Odds ratio	95 % C.I.	AUC
Groups 3, 4 and 5	Intercept	0		0.792
	PSA	1.087	1.05 - 1.17	
	Gleason Score	3.008	1.15 - 7.86	
Groups 3, 4 and 5	Intercept	0.001		0.751
	PSA	1.071	0.99 - 1.15	
	Gleason Score	2.231	0.80 - 6.17	
	AA	0.946	0.91 - 1.01	

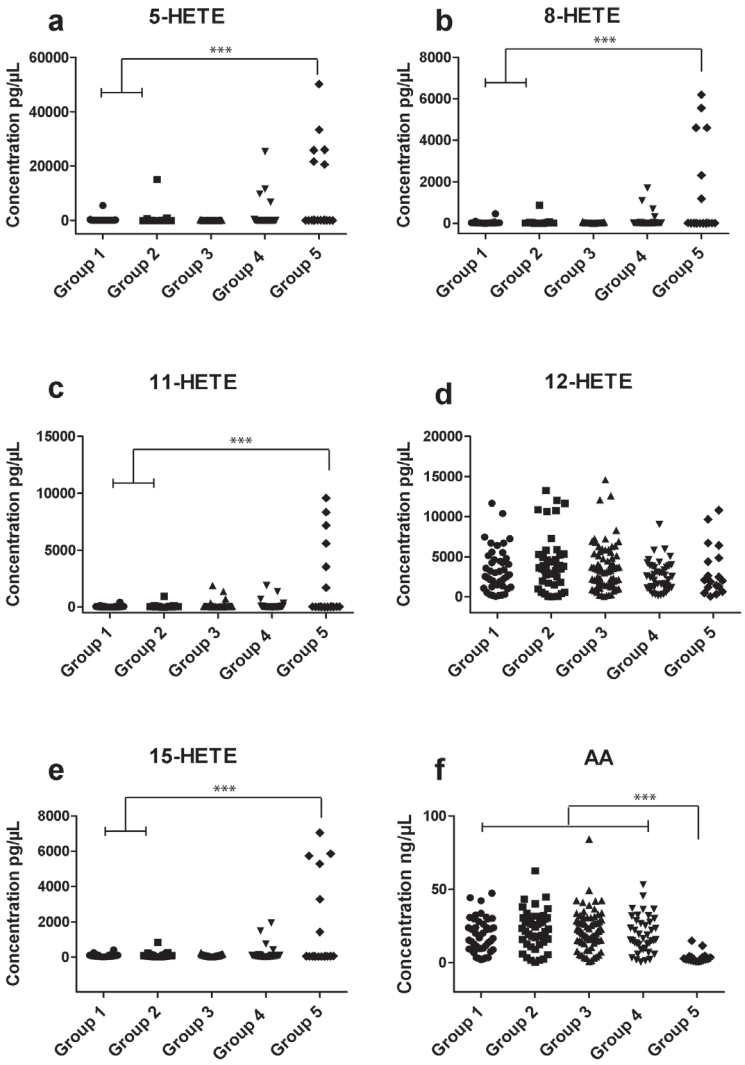

Figure 3. Serum concentrations of eicosanoids in the Validation Set. High concentration of HETEs are observed in 30% of the patients with the most advanced stage of PCa.

PSA recurrence after radical prostatectomy and its relationship with serum HETEs and AA concentration

Kaplan-Meier (KM) curves were constructed to analyse PCa progression after radical prostatectomy as a consequence of biochemical recurrence of the disease (PSA recurrence). In these curves, the independent variable was set as the time when a rise in the PSA level was observed. Observations were censored at the last month of clinical follow up after RP. Figure 4a, shows KM curve of PSA recurrence for those patients in the validation set who exhibited high concentrations of HETEs (9 patients from groups 4&5). Although the number of patients with high concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE is low compared to the whole group of patients, difference between the two curves indicates that this panel of HETEs may be considered as positive predictor of PSA recurrence ($p=0.048$). The same analysis was performed when the predictor factor is PSA at the time of diagnosis and the cut-off value is set as 4 ng/mL. Figure 4b shows KM curves for PSA recurrence after RP in groups 3, 4 and 5 and a statistical significant difference was observed between the two curves ($p=0.022$).

In order to evaluate the effect of AA concentration as a predictor of PSA recurrence, a cut-off value was chosen according to a statistical descriptive analysis for AA concentrations in groups 3-5. Supp. Figure 2 shows KM curves for AA concentrations in the validation set at 25% percentile (9.2 ng/μL, $p=0.001$), AA concentrations between 25-75% percentile (9.2-18.4 ng/μL, $p=0.006$, and AA concentrations in 75% percentile (18.4 ng/μL, $p=0.934$). Thus, cut-off value of 10 ng/μL was chosen and the same above-mentioned conditions for the independent variable were kept for the analysis. In Figure 4c, a KM curve of PSA recurrence as a function of time after surgery and AA concentration as a factor is presented. A statistical significant difference was observed when the curve of PSA progression in those patients with AA concentration lower than 10 ng/μL, is compared to the curve for patients with AA concentrations higher than this cut-off value ($p=0.0003$). Therefore, AA concentrations lower than 10 ng/μL at the time of diagnosis in PCa patients may be considered as potential positive predictor of PSA recurrence and a selective readout of PSA recurrence related to advanced disease.

Figure 4d shows the PSA recurrence after surgery when combined effects of PSA level at diagnosis and AA concentrations were evaluated (blue line: AA concentration > 10 ng/μL, PSA level < 4 ng/mL, red line: AA concentration < 10 ng/μL, PSA level > 4 ng/mL). A statistical significant difference was observed ($p<0.0001$) and the difference between the two lines was found to be even higher compared to Figure 4a-c. These results suggest that this non-invasive parameter aids in the prediction of PCa recurrence after RP.

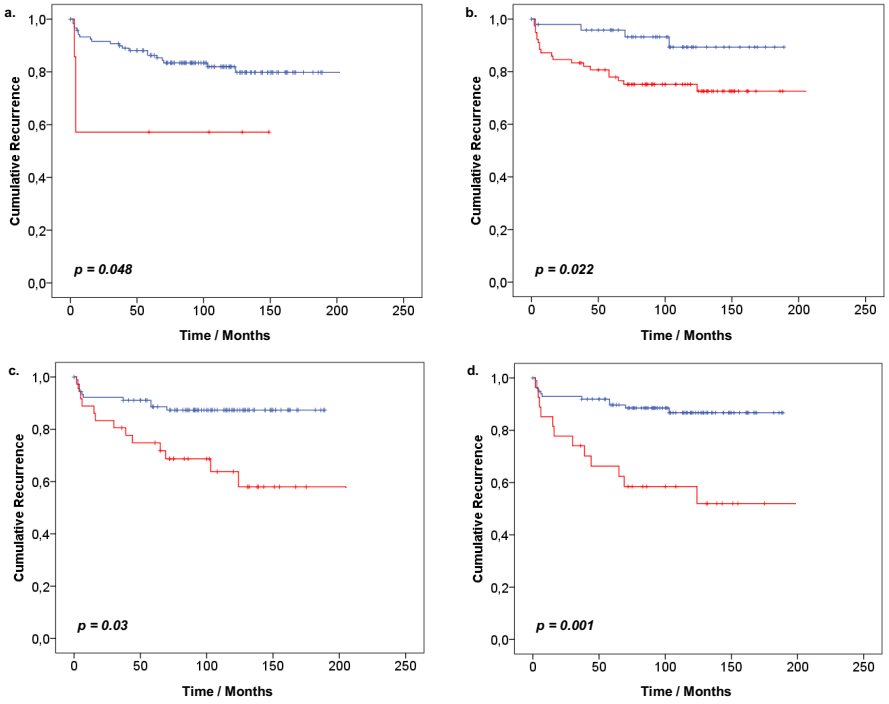

Figure 4. Kaplan-Meier curves assessing the probability of PSA recurrence in groups of patients with PCa discriminated by a: HETEs concentration b: PSA at the time of the diagnosis c: AA concentration and d: Combined AA concentration and PSA level, as a function of time after surgery in months. Blue lines represent: a: low concentration (< 5ng/μL) of HETEs, b: PSA < 4 ng/mL, c: AA concentrations > 10 ng/μL, d: AA concentrations > 10 ng/μL and PSA level < 4 ng/mL. Red lines represent: a: high concentration (> 5ng/μL) of HETEs, b: PSA levels > 4 ng/mL, c: AA concentrations < 10 ng/μL, and d: AA concentrations < 10 ng/ μL and PSA levels > 4 ng/mL.

Discussion

The aim of this work was to determine serum concentrations of HETEs and AA in PCa patients in order to evaluate the correlation with progress of disease. For that purpose, we decided to use a liquid chromatography-based technique because it allows specific, sensitive, reproducible and robust measurements (15), which are essential requirements for clinical applications.

We have used an analytical method that allows the simultaneous measurement of HETEs in serum in 7 minutes and which can be organized in an automated way. Sample preparation was optimized to avoid expensive and relative time consuming procedures such as solid phase extraction (SPE). Thus, extraction with 4 volumes of cold methanol was chosen as the optimal condition in sample preparation for obtaining good linearity (R=0.95 or higher) and good repeatability within runs (<20 % CV). We decided to evaluate only five HETEs due to the availability of the stable isotope labelled standards. This methodology can be extended for further studies for the analysis for instance of ω-terminal HETEs due to the good peak resolution exhibited in the chromatographic profiles (Supp. Fig. 1). In the present study, we have analysed 76 samples in the discovery set and 222 samples in the validation set, in addition to the internal quality controls, and we have obtained coefficients of variation < 20% for the stable isotope compounds (15-HETE d8 and AA d8), as well as good reproducibility in retention time (<5%) and peak shape, indicating a high reliability of the measurements.

We designed the experiments with the aim to profile HETEs in serum samples from the Rotterdam section of the European Randomized Study of Screening for Prostate Cancer (ERSPC), and also included patients diagnosed with other malignancies in order to test specificity towards PCa. Results indicated that a selected subgroup of 6 patients in the discovery set exhibited high concentration of HETEs and two different patients also exhibited elevated concentrations of AA. Serum HETEs concentrations assessed for controls in both the discovery and validation set were found to be in the same order of magnitude as values reported in literature using different analytical approaches in metabolomics (16). We have not found significant differences among concentrations of 12-HETE in all the patients; however, this can be explained by the lack of selectivity of our method to differentiate the metabolite 12(S)-HETE to the optical isomer 12(R)-HETE (17, 18). Further experiments are required to identify possible associations of each enantiomer with PCa and also the role of these two metabolites with the disease progression.

Comparison of concentrations of HETEs and AA between the discovery set and validation set indicated similarity in median values in subjects categorised as Controls. A clear trend indicates an elevated concentration of HETEs as well as a reduced concentration of AA in patients with a more advanced stage of PCa (Group 5). We performed a logistic regression analysis and made Kaplan-Meier curves for groups 3-5 to identify the role of these metabolites as predictors

of PCa recurrence. We have identified that the AA concentration at the time of diagnosis is a negative predictor of PCa recurrence (expressed as an increase in PSA level after radical prostatectomy), and this non-invasive parameter can be a potential marker for biochemical recurrence; however, further and independent studies in extended cohorts, using different analytical methodologies, and with detailed evaluation towards specificity in the model are required to make it clinically applicable. We have observed that AA concentrations notably decrease in those patients with PSA recurrence (Group 5), but patients diagnosed also as an advanced status (Group 4) had similar concentrations even compared with the control groups (Groups 1&2). This makes difficult to propose any hypothesis about how AA is metabolised in PCa patients at the most aggressive stage, and how the AA metabolism in PCa cells may modify eicosanoids concentrations in serum. We have found that concentrations of 5-HETE, 8-HETE, 11-HETE and 15-HETE were high in a selected group of patients having an advanced stage of PCa. It is known that 15-LOX-2 converts AA exclusively to 15-HPETE, which is further reduced by cellular peroxidases to 15-HETE (4). Therefore, a link between tissue mRNA expression and serum concentration of 15-HETE might exist, although this reasoning could be strongly affected by different clearance rates from blood for the various compounds studied and variations of clearance of these compounds among patients. Similarly, 5-LOX catalyses the first step in oxygenation of AA to produce 5-HPETE with subsequent production of 5-HETE and leukotriens. We have found a statistical significant increase of 5-HETE concentrations in PCa patients in the discovery set and in the patients with advanced in the validation set. Although the number of patients involved in this study is relatively small, these findings suggest that 5-HETE may be considered as a potential marker for PCa prognosis for further validation studies, in an extended cohort. Different researchers have reported overexpression of 5-LOX in PCa tissue, and they have also suggested that 5-LOX inhibitors participate in cell growth inhibition through the induction of apoptosis (6, 19-22), suggesting an important role of 5-LOX in PCa progression.

Serum concentrations of 12-HETE were not found to be high in the subgroup of patients with high concentration of the other HETEs determined. This metabolite was proposed recently as a potential marker for PCa progression in tissue (13), and high levels of platelet type (P-) 12-LOX mRNA in 40% of PCa tissue have also been reported (22). It suggests a crucial role of LOX-12 pathway in PCa. In our experiments, we cannot exclude the possibility that endogenous levels of 12-HETE might be also affected by the concentration of the optical isomer 12(R)-HETE because only a reverse-phase analytical column is used for the measurements. Therefore, further experiments validating the correlation of 12(S)-HETE and possible over-expression of LOX-12 in tissue have to be considered.

We also noticed high concentration of 8-HETE and 11-HETE in the selected subgroup of patients. It is known that AA can be metabolized also by

microsomal P450 monoxygenases (Cytochrome P450) by means of a Bis-allylic oxidation (lipoxygenase –like reaction) to generate 6 regioisomer HETEs, where the hydroxyl group is located at the positions 5, 8, 9, 11, 12 or 15 of the chain (23). We hypothesize that high concentration of HETEs may be related with the deregulation of phospholipases-type enzymes that release AA from the phospholipids in the cell membrane (Fig 1) (24). This hypothesis is supported by Schumacher et al. who demonstrated that AA levels in PCa tissue are decreased in comparison to NAP, thus suggesting that AA is metabolised preferably in PCa cells into pro-tumoural eicosanoids (25).

To date, this relative large family of enzymes has been classified into six classes on the basis of their nucleotide and amino acid sequence homology as: sPLA2, cPLA2, iPLA2, PAF-AH, LPLA2 and AdPLA2, and more than 20 members have been identified in mammals (26).

Association of the enzymes of the PLA2 family with PCa has been described in literature recently, and some of them have been proposed as alternative targets in the treatment of PCa (3, 27-30). Recent studies have confirmed that cytosolic phospholipase A2-α plays a role via AA and one of its metabolites, 5-HETE in functioning of protein kinase B (AKT), extracellular signal-regulated kinase (ERK) and androgen receptor (AR) signalling in PTEN-null/mutated prostate cancer cells. These results support that inhibition of cPLA2-α could be an attractive and effective target for treating advanced PCa and also support the results obtained in this study indicating association of serum AA and HETEs and the progression of PCa. However further experiments in vivo or ex vivo have to be performed to analyse whether the production of eicosanoids, and especially the HETEs, is altered by the deregulation of the phospholipase-type enzymes.

Conclusions

We have analysed changes in the concentration of AA and HETEs in serum samples from PCa patients at different stages of the disease. High concentrations of HETEs and a reduction in the concentration of AA was observed in those patients having advanced and aggressive state of PCa. Decreased AA levels in serum predict biochemical recurrence for PCa, but large samples as well as complementary techniques are required for further validation and clinical use. The results shown here also highlight the relevance of studying the arachidonic acid pathway in PCa development and progression, and how it can be considered as an important target for therapeutic strategies.

Acknowledgments

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Chapter 4

Tissue proteomics outlines AGR2, FASN, and members of the arachidonic acid pathway as markers for prostate cancer

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In preparation

Abstract

Prostate cancer (PCa) remains as one of the most common cancer types in men worldwide. We aimed to identify and validate proteins involved in PCa development and progression using nano LC-MS/MS and tissue immunohistochemistry. The proteomics dataset included protein fractions of PCa samples (n=34) and normal adjacent tissue (NAP, n=33), and we used label free quantification (LFQ) to identify differentially expressed proteins between PCa and NAP. Fatty acid synthase (FASN, p=1.05E-07) and Anterior Gradient protein 2 (AGR2, p=7.34E-07) were up-regulated in PCa tissue. In addition, TEBP protein, encoded by PTGES3 gene, was also found highly overexpressed in PCa tissue compared to NAP (p=9.10E-10). Moreover, analysis of 79 arachidonic acid (AA) pathway proteins showed that 15 of them were differentially expressed in PCa compared to NAP (six proteins were down-regulated and nine were up-regulated). Proteins AGR2, FASN and the lipoxygenase LX15B were further validated by immunohistochemistry. LOX5, identified previously as a marker of prostate cancer also part of the AA pathway was also included. Tissue microarray immunohistochemical staining of an independent cohort of patients was performed to study biochemical recurrence (BCR) after radical prostatectomy (RP). It was found that both low percentage of positive tumour cells for AGR2 (HR (95% CI) = 0.61 (0.43-0.93), and low percentage of positive tumour cells for LOX5 expression (HR (95% CI) = 2.53 (1.23-5.22) are predictors of BCR after RP. In conclusion, AGR2, and two proteins of the AA pathway, TEPB and LOX5, are associated to PCa, and can be used as diagnostic and prognostic markers for PCa.

Introduction

Prostate cancer (PCa) remains to date the most commonly diagnosed cancer in men in the Western world (1). Although many patients are cured from this disease after radical prostatectomy (RP) (2), one third of patients will show an increment in serum PSA levels -also known as biochemical recurrence (BCR)-(3). For those patients, more frequent follow-up and adjuvant therapies are often required to limit progression of disease (3, 4). There is a high need for robust molecular markers that can distinguish indolent cases of PCa from those that will recur after initial treatment (3, 4).

Multiple approaches with different techniques, as well as mass spectrometry-based proteomics strategies, have been applied to identify differentially expressed proteins in PCa that can predict the chance of recurrence. Tan et al., isolated PCa and benign tissue by laser micro dissection from FFPE sections of five PCa patients and used multidimensional fractionation with mass spectrometry to identify proteins stratified by ERG (ETS-related gene) expression status (5). More recently, Iglesias-Gato et al. reported quantitative proteomic profiling of 28 prostate tumours using a SILAC approach and a SAX fractionation. They have found that levels Pro-NPY, alone or in combination with ERG status, might serve as marker for disease specific mortality in patients exhibiting low Gleason score (GS) (6).

In our previous study, using a LC-MS/MS-based targeted metabolomics method, we found lower concentrations of arachidonic acid (AA) in serum from PCa patients at the most aggressive stage of the disease (7). In addition, serum levels of hydroxyeicosatetraenoic acid (HETE) metabolites, which are produced by lipoxygenase-type enzymes from AA, were found elevated in part of the patients within the same group of advanced PCa (7). At tissue level, it has been reported that levels of AA in PCa were significantly lower compared to benign prostate tissues (8). In addition, Yang et al. analysed PCa core biopsies and they found that the 15-LOX-2 metabolite 15-HETE, was higher in PCa than in the normal cores (9). These findings suggest that the AA pathway might play an important role in PCa development and progression. However, analysis of proteins of the AA pathway in PCa, as well as their role in diagnosis and prognosis is still unknown. Proteins of the (AA) pathway have been studied recently in RAW264.7 macrophages using a targeted and quantitative assay (10). There are 79 proteins along the eicosanoid signalling pathway, including prostaglandin, cyclooxygenases, lipoxygenases and cytochrome P450 enzymes, which play a role in cell signalling, as well as inflammatory features in different diseases (11).

In this study, we used nano-LC and Orbitrap mass spectrometry to identify proteins in PCa that can be correlated to diagnosis and prognosis. We focused primarily on the proteins of the AA pathway in order to identify expression of its proteins at various stages of the disease.

Materials and Methods

Clinical Specimens
Discovery set
The protein fractions from tissue RNA isolation of 67 samples (33 NAP tissues and 34 PCa tissues) were analysed (MEC-2004-261); PCa samples were previously published (GSE41408) (12), as well as additional cancerous and control samples, accessible via GEO accession number GSE59745 (13). Clinico-pathologic characteristics of the samples used for proteomics profiling are presented in Supplementary Table 1.

Tissue Microarray (TMA)- Evaluation set
A Tissue Microarray (TMA) was constructed including 481 patients diagnosed with PCa from the European Randomized Study of Screening for PCa (ERSPC) (14-16). All patients had undergone RP in Erasmus MC between 1987 and 2010, without previous radiation or hormonal therapy. Clinical follow-up was recorded after each control visit at our outpatient clinic, and data were transmitted to a central study database. Post-operative biochemical recurrence (BCR) was defined as an increment of 0.2 ng/mL in serum PSA after two consecutive measurements, with at least three months between measurements. Clinico-pathologic characteristics and follow-up for patients treated by RP are summarized in Supplementary Table 1.

Sample preparation
Proteomics
Protein fractions kept at -80 °C of the protein interface from tissue RNA isolation with RNA-Bee of 67 PCa tissue samples (33 NAP adjacent tissues and 34 PCa) were selected. For protein digestion, samples were thawed and 50 μL were transferred to a new microcentrifuge tube and precipitated with cold acetone. After spinning down for 10 minutes, the supernatant was removed and the pellet was washed twice with cold acetone. Supernatant was removed and 50 μL of 0.1% RapiGest (Waters Corporation, Milford, MA) in 50 mM NH4HCO3 were added to the protein pellet. The protein pellet was dissolved by external sonication for 5 min at 70% amplitude at a maximum temperature of 25 °C (Digital Sonifier model 450, Branson, Danbury, CT). The proteins were reduced with 10 mM dithiothreitol (DTT) at 60 °C for 30 min. After the mixture was cooled down to room temperature, it was alkylated in the dark with 50 mM iodoacetamide at ambient temperature for 30 min, and digested overnight with 8 μL trypsin 0.1 ug/mL (Promega, Madison, WI). To inactivate trypsin and to degrade the RapiGest, 6 μL of 5% TFA was added and samples were incubated for 30 minutes at 37 °C. Samples were centrifuged at maximum speed for 60 minutes at 4 °C and the supernatant was transferred to a new Eppendorf tube. A fraction of 5 μL was then diluted 40 times and subsequently transferred to LC

vials for LC-MS analysis.

Chromatography Separation and Mass Spectrometric Analysis: Samples were measured using a nano-LC system (Ultimate 3000, Thermo Fisher Scientific, Amsterdam, the Netherlands) coupled online to Q Exactive plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic and mass spectrometry conditions used are described previously (17, 18). Briefly, 2 μL were injected into the nano-LC after preconcentrating and washing of the sample on a C18 trap column (1 mm×300 μm internal diameter) Thermo Fisher Scientific). Peptides were eluted after loading the sample on to a C18 column (PepMap C18, 75 μm ID × 500 mm, 2 μm particle and 100 Å pore size, Thermo Fisher Scientific) using a linear 90 min gradient (4-25% acetonitrile/H2O; 0.1% formic acid) at a flow rate of 250 nL/min. The separation of the peptides was monitored by a UV detector (absorption at 214 nm). Full scan MS spectra (m/z 400-1600) in profile mode were acquired in the Orbitrap with a resolution of 70,000 after accumulation of an AGC target of 1 x 106 using a maximum fill time of 100 ms. The top 12 peptide signals (charge-state 2+ and higher) were isolated (1.6 Da window) and fragmented by HCD (Higher-energy collision, normalized collision energy 28.0) and measured in the Orbitrap with a AGC target of 50,000 a maximum fill time of 60 ms and a resolution of 17,500. Dynamic exclusion was activated; after the first time a precursor was selected for fragmentation it was excluded for a period of 30 seconds using a relative mass window of 10 ppm. Lock mass correction was activated to improve mass accuracy of the survey scan.

Orbitrap-MS/MS Data Processing and Analysis: Data files resulting from the discovery set were extracted and converted into mgf files by using MSConvert of ProteoWizard (version 3.0.06245). All mgf files were analysed using Mascot (version 2.3.02; the Matrix Science, London, UK). Mascot was used to perform database searches against the human subset the uniprot_sprot_2015-10 database; Homo sapiens species restriction; 20,194 sequences) of the extracted MS/MS data. For the database search the following settings were used: a maximum of two miss cleavages, oxidation as a variable modification of methionine, carbamidomethylation as a fixed modification of cysteine and trypsin was set as enzyme. A peptide mass tolerance of 10 ppm and a fragment mass tolerance of 0.02 Da were allowed. An ion score of 40 was used as a cut-off value.
To validate and filter the database results, Scaffold (version Scaffold_4.6.2, Proteome Software Inc., Portland, OR) was used. Peptide identifications were accepted if they could be established at greater than 91.0% probability to achieve an FDR less than 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 13.0% probability to achieve an FDR less than 1.0% and contained at least 2 identified peptides. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Proteins sharing significant peptide evidence were grouped into clusters. Scaffold software package was also used to create a spectrum report that was subsequently used to import the identifications results into Progenesis.
Label free Quantitation was performed using Progenesis LC–MS Software (version 3.0; Nonlinear Dynamics Ltd., Newcastle-upon-Tyne, UK) following our previously reported methodology (18, 19). Proteins where considered differentially expressed when fold change was higher than 1.5 or lower than 0.66 and the protein p-value lower than 0.002 ($p<0.002$, q-value 0.001). Fold change direction (PCa or NAP) for selected proteins was confirmed using the peptide output file. Reported fold change include proteins having at least 90% of peptides having the same direction in up-regulation or down-regulation and less than 5 ppm in mass accuracy. Duplicates in identified sequences as a consequence of peak tailing were removed to avoid false positives.
Technical replicates of each sample were randomly analysed within the measurement period and no significant changes in the number of identified proteins were observed in time for both the replicates and quality control measurements. The workflow for proteomic analysis and further validation steps is outlined in Figure 1.

Pathway Analysis: The list of differentially expressed proteins identified by proteomics, including their statistical significance and fold-change derived from the data analysis, were uploaded into the Ingenuity IPA system version 26127183 [www.ingenuity.com], to identify networks or pathways associated to PCa.

Immunohistochemistry
Tissue slides (5 μm) were mounted on aminoacetyl-silane coated glass slides (Statfrost, Berlin, Germany), deparaffinised in xylene and dehydrated in ethanol. Endogenous peroxidase was blocked by 1% hydrogen peroxide in methanol for 20 min. Samples were pretreated by microwave (700 W) in TRIS-EDTA pH 9.0 or in citrate buffer pH 6.0 for 15 min. The slides were incubated overnight at 4 °C with the following primary antibodies targeting anterior gradient protein 2 (AGR2; 1:100; HPA007912, Sigma); fatty acid synthase (FASN; 1:400; ab22759, Abcam, Cambridge, MA, USA); arachidonate 15-lipoxygenase type B (LX15B 1:2000, ab23691, Abcam, Cambridge, MA, USA), and arachidonate 5-lipoxygenase (LOX5; 1:200 ab169755, Abcam, Cambridge, MA, USA).(16) Chromogenic visualization was performed with the EnVision DAKO Kit (Dako, Glostrup, Denmark). After counterstaining with haematoxylin, slides were thoroughly washed, dehydrated, cleared in xylene and mounted in malinol (Chroma-Geselschaft, Körgen, Germany). Immunohistochemical staining for AGR2, 5-LOX and FASN were visually examined as described previously (20). Staining intensity was scored as negative (0; no staining), weak (1+; only visible at high magnification), moderate (2+; visible at low magnification), and strong (3+; striking at low magnification). If there was heterogeneous

expression, the strongest intensity was used for further analyses. For AGR2, the percentage of positive tumour cells was counted and used for further analyses. For optimization and validation of all immunohistochemical procedures we used appropriate internal and external controls, and omitted first antibodies to exclude non-specific binding (21).

Statistics

Statistical calculations were performed with GraphPad Prism 5 for Windows (GraphPad Software Inc., La Jolla, CA, USA), SPSS version 22 (IBM Corp., Armonk, NY, USA) and R version 3.2.3 (2015-12-10 Vienna, Austria). Multiple testing correction was performed via False Discovery Rate (FDR) using the R package fdrtool (22).

Associations between clinico-pathologic parameters and protein expression in TMA experiments were performed by student t-test or chi-squared test. Survival curves were calculated according Kaplan-Meier (KM), and to detect significant survival differences the Log-Rank test was used. Univariate and multivariate Cox regression were used to determine predictive properties of AGR2, LOX5 and FASN for BCR. A two-sided p-value of ≤ 0.05 was considered significant.

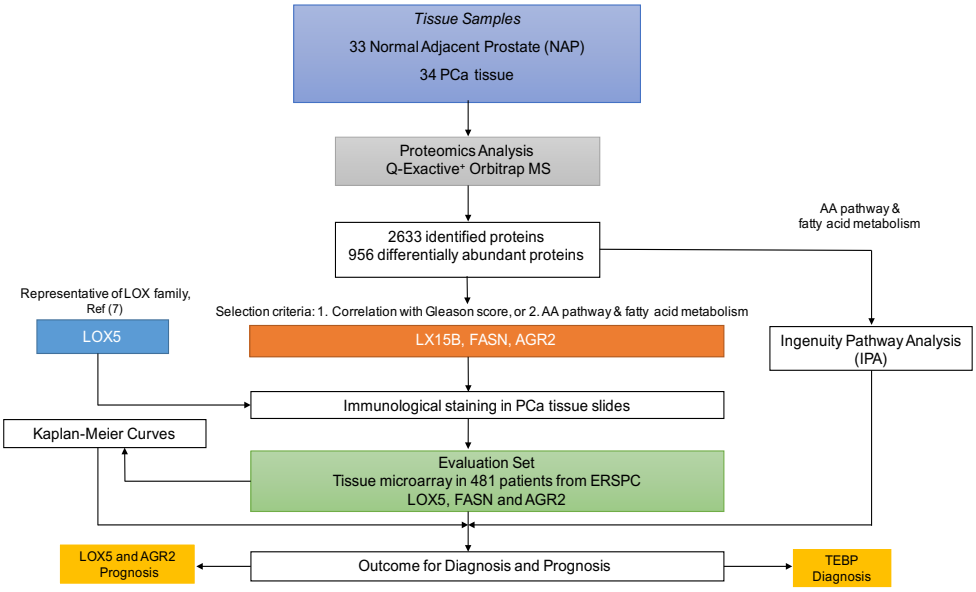

Figure 1. Workflow for the label free quantitation analysis of PCa tissue in fresh-frozen and formalin-fixed paraffin-embedded prostatectomy samples.

Results

Proteomics

In this study, we performed a label free quantification analysis to identify proteins differentially expressed in PCa tissue. After analysing 34 PCa and 33 NAP tissues, 2633 proteins were identified, 1066 proteins had a statistical p-value lower than 0.002, and 956 proteins were considered differentially expressed. A volcano plot of the differentially expressed proteins in the discovery set is presented in Supplementary Figure 1. The list of significant differentially expressed proteins is described in Supplementary Table 2.

In order to identify proteins associated with PCa aggressiveness, we analysed the differences in normalised abundances (using Progenesis) between GS <7 and GS ≥7. We found that 35 proteins were differentially expressed ($p<0.05$, listed in Supplementary Table 3).

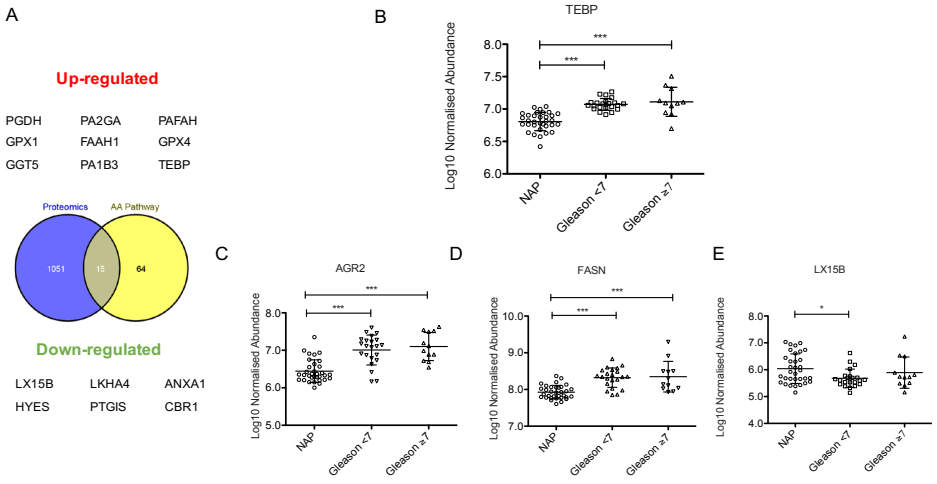

Figure 2A. Representation of the number of differentially expressed proteins in PCa identified in the proteomics dataset, belonging to the AA pathway, as well as the number of proteins in common. B. Normalised abundances of prostaglandin E synthase 3 (TEBP), C. Anterior Gradient 2 (FASN), D. Fatty acid synthase (FASN), and E. 15-Lipoxygenase-2 (LX15Bin PCa patients having different Gleason Scores (GS <7 and GS ≥ 7).

Two proteins: anterior gradient 2, AGR2 (Gene Name (GN): AGR2, p=7.34E-07) and fatty acid synthase, FASN (GN: FASN, p=1.05E-07) were up-regulated (Supplementary Table 2), and were selected for further analysis. To investigate whether the proteins in the AA pathway as described by Sabidó et al., are de-regulated in PCa tissue, we compared the list of differentially expressed proteins with the 79 proteins associated to the AA metabolism (Supplementary Table 4, described in reference (10)), and we found fifteen proteins deregulated in PCa (Figure 2A). PGDH (GN: HPGD, p = 2.9E-02), PA2GA (GN: PLA2G2A, p = 3.2E-2), PAFAH (GN: PLA2G7, p=1.1E-03), GPX1 (GN: GPX1, p=2.9E-03), FAAH1 (GN: FAAH, p=3.8E-03), GPX4 (GN: GPX4, p=7.4E-03), GGT5 (GN: GGT5, p=4.8E-04), PA1B3 (GN: PAFAH1B3, p=8.1E-05), and TEBP (GN: PTGES3, p=9.1E-10) were up-regulated. LX15B (GN: ALOX15B, p=1.5E-2), LKHA4 (GN: LTA4H, p=3.2E-06), ANXA1 (GN: ANXA1, p=5.84E-06), HYES (GN: EPHX2, p=9.40E-06), and PTGIS (GN: PTGIS, p=1.8E-07), and CBR1 (GN: CBR1: p=9.9E-07) were down-regulated. These proteins might delineate a diagnostic role in PCa diagnosis. A schematic representation of protein abundances of arachidonic acid-related proteins: PA2GA, PAFAH, TEBP, LX15B, LKHA4, and HYES is shown in Figure 2 B-G.

Pathway Analysis

Differentially expressed proteins from the proteomics analysis were uploaded into IPA for pathway analysis. Evaluation of canonical pathways indicated that the strongest association corresponds to the EIF2 signalling pathway (p=1.1E-45) (Supplementary Table 5), which is associated to cellular growth, proliferation and development. EIF2 has been associated to AKT, a protein activated after PTEN inactivation, a common genetic defect in metastatic PCa (23).
IPA defines the eicosanoid signalling as the canonical pathway involving the majority of proteins of the AA metabolism (24). In order to identify the number of proteins de-regulated in eicosanoid signalling in our PCa dataset, we initially overlapped the list of proteins differentially expressed between PCa and normal prostate ($p<0.01$) with the eicosanoid signalling pathway. Figure 3 shows that two genes belonging to the PLA2 family (PLA2G2A and PLA2G7), were up-regulated (coloured in red). No other proteins involved in the production of eicosanoids such as HETEs and Leukotrienes were up-regulated in PCa tissue. Three genes (ALOX15B, LTA4H and PGIS) were down-regulated. We then uploaded into IPA the total list of proteins identified and having a p-value lower than 0.05. Interestingly, five genes were de-regulated: GGT1, TXS, PTGES2, PGFS, and PGDS. This highlights the importance of proteins in the AA pathway for the development of PCa (Figure 3).

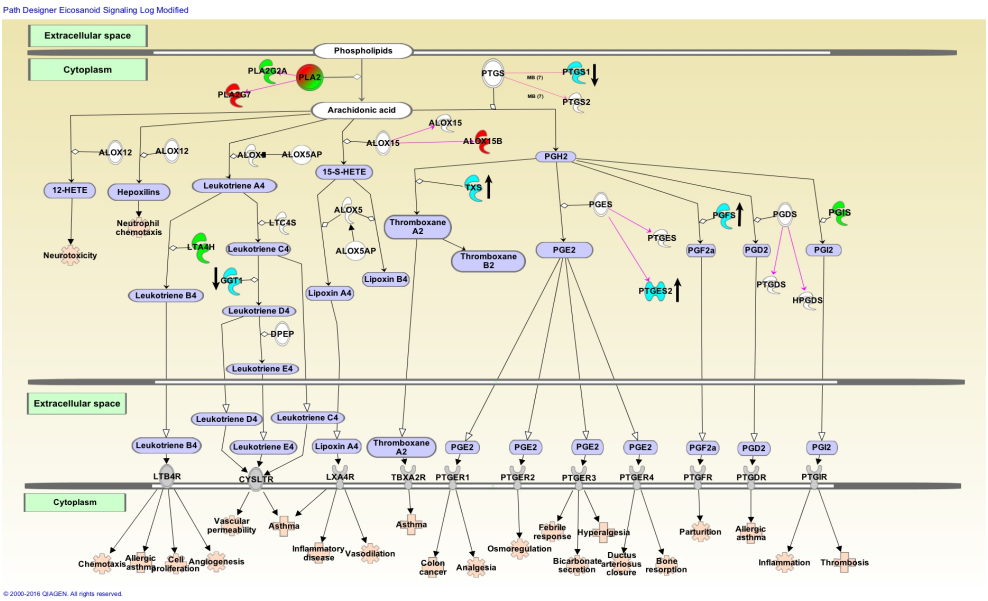

Figure 3. Eicosanoid signalling pathway overlapping with proteomics data when comparing protein expression in normal adjacent prostate (NAP) and PCa. Enzymes coloured in red and green represent proteins up-regulated and down-regulated in PCa, respectively. Enzymes coloured in blue represent identified proteins but not fulfilling the criteria described for differential expression. Protein receptors in the eicosanoid signalling are coloured in grey. Purple text indicates metabolites produced along the AA pathway. Enzymes are represented by helixes and protein complex involved in metabolic reaction are represented by a circle. Proteins or complexes not identified in this work are coloured in white. Possible diseases associated to particular metabolite or enzyme are coloured in pink.

Immunohistochemistry

For immunohistochemical (IHC) validation, we selected from our list of proteins highly expressed in PCa, those targets against which antibodies were available that have been shown to reliably work using formalin-fixed paraffin-embedded (FFPE) tissue. Considering the fact that AGR2, LX15B, and FASN resulted de-regulated in our proteomics datasets, we decided to evaluate these proteins by immunohistochemistry on formalin paraffin embedded tissue sections. Since we previously reported that high concentrations of HETEs are present in serum from men with aggressive PCa (7), we also selected LOX5 for IHC analysis as an additional representative of the LOX family. We were able to robustly detect AGR2, LX15B, LOX5 and FASN using IHC on a small set of prostate and PCa

FFPE tissue sections (Figure 4).
AGR2 showed heterogeneous expression in normal luminal epithelium and PCa. AGR2 staining was strikingly positive in cancer and negative in normal (Figure 4A). Expression of LX15B was generally moderate to strong (2+/3+) and occurred in both cytoplasm and nucleus of both benign luminal cells and PCa. Normal basal epithelium and atrophic prostate epithelium generally showed lower expression (0/1+). Stromal expression was negative (0) to weak (1+) (Figure 4B).
Expression of cytoplasmic FASN was negative to weak and rarely moderate in normal prostate luminal epithelium. Expression in PCa was stronger (1+/2+) than in adjacent normal tissue (0/1+) with locally strong expression (3+) in Gleason grade 4 and 5 areas (Figure 4C).
LOX5 staining was found to be predominantly expressed in the nuclei and cytoplasm of benign basal epithelial cells and atrophic luminal epithelial cells (1+ to 3+). Normal luminal epithelial cells were generally negative (0) or weakly positive (1+). PCa showed enhanced expressions as compared to benign luminal cells varying from weak to strong, but no clear association with Gleason score was observed (Figure 4D).

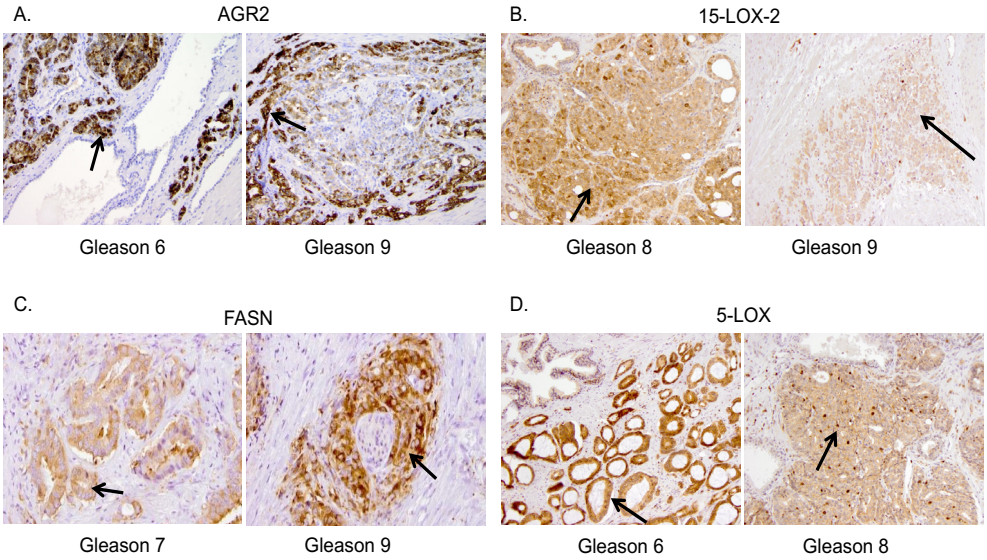

Figure 4. Immunohistochemical staining in PCa tissue for A. Anterior Gradient 2 (AGR2) in Gleason score 6 and Gleason score 9, B. 15-lipoxygenase-2 (LX15B) in Gleason score 8 and Gleason 9, C. Fatty Acid Synthase (FASN) in Gleason 7 and Gleason 9 and D: 5-lipoxygenase (LOX5) Gleason 6 and Gleason 8.

Tissue Microarray

To determine whether expression of AGR2, FASN, and LOX5 might correlate with clinical parameters, we analysed these proteins in 481 samples from RP patients. Cytoplasmic expression of AGR2 occurred in 84% (404/481) of the patients, with 52% of cores showing strong intensity (3+). 74% (299/404) of the cores exhibited staining in 100% of tumour cells. Strong FASN staining occurred in 86% (399/461) of patients. We did not find any expression of LOX5 in 54% of the cores, and both the cytoplasmic and the nuclear intensities were weak (1+) in most cases. The percentage of positive tumour cells stained for LOX5 was lower than 10% in the 224 positive cores.

An association between Gleason score (GS) and the percentage of positive tumour cells and intensity of AGR2 was found (p=0.017, and p=0.032, respectively, as described in Table 1A-B). AGR2 expression occurred more often in patients with lower GS (42% in patients with GS <7 when analysing percentage of tumour cells, and 52.3% when cytoplasm was analysed). FASN expression was higher in GS <7 and GS=7 (49.0% and 34.6% respectively) than in GS>7 (5.4%), but no correlation existed between FASN and PSA, GS or pt. stage (Supplementary Table 6).

Table 1A. Clinico-pathologic correlations in the PCa-TMA and AGR2 (percentage of positive tumour cells). Positive =100% positive cells, Negative = less than 100% positive tumour cells.

	Negative	**Positive**	**Total**	**p-value**
PSA at diagnosis				
≤10 ng/ml	88 (21.8%)	264 (65.3%)	352 (87.1%)	0.141
>10 ng/ml	18 (4.4%)	34 (8.5%)	52 (12.9%)	
Total	106 (26.2%)	298 (73.8%)	404	
Gleason score				
<7	44 (10.9%)	170 (42.0%)	214 (52.9%)	0.017
7	51 (12.6%)	112 (27.6%)	163 (40.2%)	
>7	11 (2.7%)	17 (4.2%)	28 (6.9%)	
Total	106 (26.2%)	299 (73.8%)	405	
pT-stage				
pT2	68 (16.8%)	212 (52.3%)	280 (69.1%)	0.121
pT3/4	38 (9.4%)	87 (21.4%)	125 (30.9%)	
Total	106 (26.2%)	299 (73.8%)	405	

Table 1B. Intensity of positive tumour cells, Negative = weak or no staining, Positive = strong staining intensity.

	Negative	Positive	Total	p-value
PSA at diagnosis				
≤10 ng/ml	9 (2.2%)	343 (84.9%)	352 (87.1%)	0.203
>10 ng/ml	3 (0.8%)	49 (12.1%)	52 (12.9%)	
Total	12 (3.0%)	392 (97.0%)	404	
Gleason score				
<7	2 (0.5%)	212 (52.3%)	214 (52.8%)	0.032
7	8 (2.0%)	155 (38.3%)	163 (40.3%)	
>7	2 (0.5%)	26 (6.4%)	28 (6.9%)	
Total	12 (3.0%)	393 (97.0%)	405	
pT-stage				
pT2	9 (2.3%)	271 (66.9%)	280 (69.2%)	0.465
pT3/4	3 (0.7%)	122 (30.1%)	125 (30.8%)	
Total	12 (3.0%)	393 (97.0%)	405	

A correlation between pT stage and cytoplasm intensity of LOX5 was found (p= 0.044, in Supplementary Table 7A). No other correlation was found when analysing cytoplasmic intensity, nuclear intensity, or percentage of positive tumour cells for LOX5 (Supplementary Table 7 B-C).
We constructed Kaplan Meier (KM) curves to identify the role of AGR and LOX5 in predicting BCR after surgery. A percentage lower than 100% of positive tumour cells (<100%) in AGR2 was predictive for BCR (HR (95% CI) = 0.61 (0.43-0.93); p=0.02), as described in Table 2 and Supplementary Figure 2. Expression for 5-LOX was characterised by a small percentage of positive tumour cells (<10%). KM curves indicated that low percentage of LOX5 positive tumour cells was a predictor of BCR in comparison with negative staining (0%), in a univariate analysis (HR (95% CI) = 2.53 (1.23-5.22); p=0.02), as presented in Table 2 and Supplementary Figure 2.

Table 2. Predictive value of protein marker expression for biochemical recurrence (BCR) after radical prostatectomy.

	Univariate Analysis			Multivariate Analysis		
	HR	**(95% CI)**	**p-value**	**HR**	**(95% CI)**	**p-value**
Age	1.06	(1.01-1.10)	0.02	0.97	(0.91-1.04)	0.46
PSA concentration	3.38	(2.22-5.16)	<0.01	1.38	(0.68-2.82)	0.38
Gleason Score	2.66	(2.00-3.54)	<0.01	2.39	(1.47-3.85)	<0.01
pT-Stage	1.74	(1.47-2.05)	<0.01	1.3	(0.99-1.72)	0.06
Surgical Margins	3.09	(2.12-4.50)	<0.01	1.7	(0.90-3.17)	0.1
AGR2 Percentage of positive tumour cells	0.61	(0.43-0.93)	0.02	1.1	(0.60-2.01)	0.77
LOX5 Percentage of positive tumour cells	2.53	(1.23-5.22)	0.01	2.3	(1.08-4.98)	0.03
FASN intensity	0.84	(0.47-1.47)	0.55	-	-	-

Discussion

Gleason score is an effective indicator of aggressiveness of PCa and therefore an important parameter to determine prognosis. However, a better knowledge of which patients will relapse after radical prostatectomy and/or which patients will respond better to a specific treatment is still a medical need. In this study, we performed a label free proteomics LC-MS quantification methodology using a one-step strategy of enzymatic cleavage of tissue prior to mass spectrometry analysis. We analysed sixty-seven tissues to generate an extensive dataset for the study of proteins associated to PCa. to improve the classification of patients having biochemical recurrence using the proteomics dataset. This is one of the largest studies on PCa to date, identifying a novel panel of protein markers to determine both diagnosis and prognosis of PCa by mass spectrometry. However, limitations of the study include still the number of samples used for proteomic profiling (n=67) and the lack of protein analysis of PCa tissue at the most aggressive stages of the disease.

The AA pathway is a key inflammatory pathway involved in cellular signalling as well as prostate carcinogenesis (25). Arachidonic acid is stored in cell membranes as a phospholipid, it is released by the action of phospholipase A2-type enzymes, and then metabolised by the action of cyclooxygenases (COX),

lipoxygenases (LOX) and P450 cytochromes to produce biologically active eicosanoids, as shown in Figure 3 (9).
Interestingly, we found that the protein TEBP (PTGES3), was up-regulated in PCa tissue, and to the best of our knowledge, TEBP protein is involved in eicosanoid signalling as it produces the Prostaglandin E2, involved in inflammation processes. In addition, it is reported to be an enhancer of androgen receptor activity. It is involved in AR binding to chromatin, which is a critical step in AR signalling and PCa development (26, 27). Further validation is still required, using both quantitative mass spectrometry and immunohistochemistry, to confirm a potential role of this protein in PCa diagnosis and prognosis. In addition, further analysis in-vitro, could address the role of the metabolite prostaglandin E, produced by TEBP along the AA pathway, in PCa development and progression.
Fatty acid synthase (FASN) is known to be a key enzyme in the production of long chain fatty acids from Acetyl-CoA and Malonyl-CoA (28). Overexpression of this protein in PCa tissue has been reported in cell lines (29), tissue microarrays (30), tissue biopsy cores (31) and exosomes (32). FASN-normalised intensity was high in PCa in our proteomics dataset and its expression was independently evaluated by immunohistochemistry and a TMA. Although its expression does not predict biochemical recurrence, inhibition of FASN has been proposed as a therapeutic target because of its increased expression and its relation to both cell cycle arrest and apoptosis (33). Our results reinforce the theory that FASN could be an important target to manipulate the fatty acid and lipid metabolism in cancer and therefore control cancer cell behaviour (34-36).
It has been described that one possible mechanism for the PCa cell proliferation is caused by ω-6 fatty acids, resulting in the production of certain eicosanoids, molecules mediators in the inflammatory response (37). Leukotriene A-4 hydrolase (LKHA4), and bifunctional epoxide hydrolase (HYES) were down-regulated in PCa. Although the normalised intensities for these proteins did not show any predictive value for biochemical recurrence after radical prostatectomy (data not shown), these proteins constitute a new target in understanding the role of AA in PCa progression. LKH4A catalyses the hydrolysis of the epoxide LTA4 to the diol, leukotriene B4 (LTB4), which mainly functions as a chemoattractant and activator of inflammatory cells and therefore has been proposed as a target for cancer prevention and therapy (38). In PCa, it has been shown that LTA4H SNP rs1978331 was inversely associated with the overall disease risk (39). However, little is known about this protein and its specific role in PCa.
We found that the abundance of lipoxygenase 15 type 2 (LX15B), an enzyme encoded by the gene ALOX15B, was lower in PCa than in NAP. Evaluation by immunohistochemistry showed a moderate increased abundance in both cytoplasm and nucleus of both normal luminal cells and PCa when compared to normal basal epithelium. Thus, these results do not support our hypothesis that the previously reported high serum concentration of HETE metabolites,

could be explained by an up-regulation of the lipoxygenase-type enzyme (7). We believe that an overexpression on upstream enzymes, such as the phospholipases (PLA2), could be associated to high concentration of eicosanoids in serum, but further functional studies need to be performed in order to analyse the role of these enzymes, as well as the HETE metabolites, in the development of PCa.
We found that two proteins belonging to the PLA2 family were overexpressed in PCa (PA2GA and PAFAH). Association of different enzymes of this family with PCa has been described in literature recently. Patel et al. (40), studied the expression of cytosolic phospholipase A2 in PCa cells and they reported that increased levels of this enzyme were observed in androgen-insensitive PCa cell lines and they suggested that this enzyme plays a role in cancer cell proliferation and apoptosis. PAFAH (PLA2G7) enzyme was identified by Vainio et al. firstly in a set of 9783 human tissue samples and it was proposed as a potential drug target specially in ERG positive PCa (41). Validation studies performed by the same group indicated a correlation between staining intensity for PAFAH and Gleason Score in 50 % of the cases, thus suggesting both this enzyme as a biomarker for PCa, and the PAFAH inhibition by statins as a therapeutic tool for the management of the disease (25).
AGR2 is a predictor of biochemical recurrence after performing TMA immunostaining in the Evaluation set (Supplementary Figure 2), thus confirming previous reports for this protein as biomarker for PCa (42-44). Bu et al., demonstrated that AGR2 is overexpressed in PCa, particularly in low-grade tumours and also in tumour precursor lesions PIN. In addition, high levels of AGR2 transcript were found in urine sediments from PCa patients (45). Two distinct splice variants of AGR2 in urine exosomes have been also identified as effective markers distinguishing NAP and PCa (46). AGR2 has been reported to be induced by androgens in PCa (47), and its tumorigenic function is associated with cell growth, survival and metastasis, as recently reviewed (48).
Pathway analysis of the differentially abundant proteins indicated a de-regulation in fatty acid metabolism and in the AA pathway (Figure 3). Cancer cells demand energy for proliferation, and as a consequence there might be a metabolic reprograming in the cancer progression process (Warburg effect). It is of interest that the expression of lactate dehydrogenase-A protein (LDHA) was higher in PCa than in NAP. This result might be associated to previous reports for this protein indicating a key role in PCa oncogenesis (49). LDHA executes the final step of aerobic glycolysis and has been reported to be involved in tumour progression (50). It was recently demonstrated that LDHA overexpression is highly linked to local relapse (51). The EIF2 network is identified as the most significantly altered pathway using IPA (p=1,1E-45). Interestingly, this pathway is up-regulated due to several ribosomal proteins de-regulated in our proteomics dataset (Supplementary Table 4). This pathway is involved in synthesis of proteins that are needed for proliferation. Therefore, this pathway might represent an important therapeutic target for PCa.

In conclusion, the experiments in this study allowed the identification of proteins and pathways associated to PCa. We identified a relationship between proteins in the AA pathway and PCa, and we have shown that expression of LOX5 and AGR2 in tissue predict biochemical recurrence after radical prostatectomy. Further validation studies on independent cohorts using different antibodies are needed to analyse the role of TEBP in PCa progression, as well as their clinical applicability. In addition, functional analyses are still required to fully understand their role in cancer cell proliferation, apoptosis and senescence. Altogether, our results confirm the role of AA pathway as an important possible therapeutic target for PCa.

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Supplementary Information

Supplementary Table 1. Clinico-pathological Characteristics and Follow-up of Patients Treated by Radical Prostatectomy for Prostate Cancer

Parameter	Proteomics Discovery set				TMA Evaluation set	
	NAP tissue (n=33)		PCa tissue (n=34)		PCa Tissue (n=481)	
	Mean (range) or n (%)		Mean (range) or n (%)		Mean (range) or n (%)	
Age at RP (years)	60.9	(50.0-71.0)	62.6	(49.0-72.0)	64.8	(55.4-75.14)
PSA level at diagnosis					7.2	(0.3-125.2)
Total	8.4	(2.0-28.9)	14.6	(0.5-64.3)		
≤ 10 ng/mL	22	66.60%	16	41.00%	418	86.90%
> 10 ng/mL	0	30.30%	21	53.80%	62	12.90%
Missing	1	3.10%	2	5.10%	1	0.20%
Tumour Percentage	-	-	84.6%	(60%-100%)		
Gleason Score (RP)						
<7	-	-	22	56.40%	264	54.90%
7	-	-	12	30.80%	188	39.10%
3+4=7	-	-	7	17.90%	153	31.80%
4+3=7	-	-	5	12.80%	35	7.30%
>7	-	-	5	12.80%	29	6.00%
Pathological State						
T2	-	-	12	30.80%	343	71.40%
T3a/b	-	-	12	30.80%	110	22.80%
T4	-	-	12	30.80%	28	5.80%
Missing	-	-	3	7.70%	-	-
Lymph Nodes (RP)						
Positive	-	-	-	-	1	0.20%

Negative	-	-	-	-	480	99.80%
Surgical Margins						
Positive	-	-	-	-	119	24.70%
Negative	-	-	-	-	362	75.30%
BCR						
Positive	23	69.70%	20	51.30%	119	24.70%
Negative	10	30.30%	19	48.70%	362	75.30%
Local Recurrence						
Positive	-	-	-	-	21	4.40%
Negative	-	-	-	-	460	95.60%
Death						
Positive	7	21.20%	16	41.00%	112	23.30%
Negative	26	78.80%	22	56.40%	368	76.50%
Missing	-	-	1	2.60%	1	0.20%
Death from PCa						
Positive	3	9.10%	6	15.40%	12	10.70%
Negative	3	9.10%	13	33.30%	74	66.10%
Unknown	27	82%	20	51.30%	26	23.20%
Follow-up time (Months)	130.5	(45.0-235.0)	138.7	(80.0-204.4)	113.3	(0.0-203.8)

Supplementary Table 2. Differentially expressed proteins in PCa tissue compared to NAP in the discovery set. Proteins having p-value <0.002 and fold change higher than 1.5 and lower than 0.66 were considered as differentially expressed, and coloured in green.

The list of differentially expressed proteins can be provided upon request.

Supplementary Table 3. Differentially abundant proteins between Gleason 6 and Gleason ≥7 in our proteomics dataset.

Accession	Description	Ratio	t-test
		GS 7 /GS6	(p)
P51636	Caveolin-2 OS=Homo sapiens GN=CAV2 PE=1 SV=2	0.6	0.003
P04083	Annexin A1 OS=Homo sapiens GN=ANXA1 PE=1 SV=2	0.77	0.007
Q969G5	Protein kinase C delta-binding protein OS=Homo sapiens GN=PRKCDBP PE=1 SV=3	0.67	0.008
P45880	Voltage-dependent anion-selective channel protein 2 OS=Homo sapiens GN=VDAC2 PE=1 SV=2	1.47	0.014
Q13232	Nucleoside diphosphate kinase 3 OS=Homo sapiens GN=NME3 PE=1 SV=2	3.18	0.018
P49747	Cartilage oligomeric matrix protein OS=Homo sapiens GN=COMP PE=1 SV=2	0.17	0.019
P21397	Amine oxidase [flavin-containing] A OS=Homo sapiens GN=MAOA PE=1 SV=1	3.88	0.020
O96008	Mitochondrial import receptor subunit TOM40 homolog OS=Homo sapiens GN=TOMM40 PE=1 SV=1	1.8	0.020
Q99623	Prohibitin-2 OS=Homo sapiens GN=PHB2 PE=1 SV=2	1.92	0.021
P35232	Prohibitin OS=Homo sapiens GN=PHB PE=1 SV=1	1.95	0.023
O43665	Regulator of G-protein signaling 10 OS=Homo sapiens GN=RGS10 PE=1 SV=2	0.63	0.025
Q6YN16	Hydroxysteroid dehydrogenase-like protein 2 OS=Homo sapiens GN=HSDL2 PE=1 SV=1	1.84	0.025
O75340	Programmed cell death protein 6 OS=Homo sapiens GN=PDCD6 PE=1 SV=1	0.76	0.029
P21796	Voltage-dependent anion-selective channel protein 1 OS=Homo sapiens GN=VDAC1 PE=1 SV=2	1.57	0.029

Q16891	MICOS complex subunit MIC60 OS=Homo sapiens GN=IMMT PE=1 SV=1	1.46	0.029
P30042	ES1 protein homolog, mitochondrial OS=Homo sapiens GN=C21orf33 PE=1 SV=3	1.74	0.029
P47755	F-actin-capping protein subunit alpha-2 OS=Homo sapiens GN=CAPZA2 PE=1 SV=3	0.86	0.032
Q99714	3-hydroxyacyl-CoA dehydrogenase type-2 OS=Homo sapiens GN=HSD17B10 PE=1 SV=3	1.75	0.033
O00159	Unconventional myosin-Ic OS=Homo sapiens GN=MYO1C PE=1 SV=4	0.82	0.033
P07954	Fumarate hydratase, mitochondrial OS=Homo sapiens GN=FH PE=1 SV=3	1.7	0.034
P31040	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial OS=Homo sapiens GN=SDHA PE=1 SV=2	1.5	0.034
Q16853	Membrane primary amine oxidase OS=Homo sapiens GN=AOC3 PE=1 SV=3	0.68	0.034
Q96I99	Succinyl-CoA ligase [GDP-forming] subunit beta, mitochondrial OS=Homo sapiens GN=SUCLG2 PE=1 SV=2	2.08	0.036
P61604	10 kDa heat shock protein, mitochondrial OS=Homo sapiens GN=HSPE1 PE=1 SV=2	2.56	0.04
P21912	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial OS=Homo sapiens GN=SDHB PE=1 SV=3	1.56	0.040
P13987	CD59 glycoprotein OS=Homo sapiens GN=CD59 PE=1 SV=1	0.79	0.042
Q9H2U2	Inorganic pyrophosphatase 2, mitochondrial OS=Homo sapiens GN=PPA2 PE=1 SV=2	2.03	0.043
P10515	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial OS=Homo sapiens GN=DLAT PE=1 SV=3	1.6	0.045
Q6NVY1	3-hydroxyisobutyryl-CoA hydrolase, mitochondrial OS=Homo sapiens GN=HIBCH PE=1 SV=2	2.54	0.046
Q96S97	Myeloid-associated differentiation marker OS=Homo sapiens GN=MYADM PE=1 SV=2	0.7	0.046
P02749	Beta-2-glycoprotein 1 OS=Homo sapiens GN=APOH PE=1 SV=3	0.77	0.047
P45954	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial OS=Homo sapiens GN=ACADSB PE=1 SV=1	2.39	0.048
O43294	Transforming growth factor beta-1-induced transcript 1 protein OS=Homo sapiens GN=TGFB1I1 PE=1 SV=2	0.71	0.049
O94832	Unconventional myosin-Id OS=Homo sapiens GN=MYO1D PE=1 SV=2	0.65	0.049

Supplementary Table 4. List of Proteins in the AA pathway

Entry	Entry name	Protein names
P09917	LOX5_ HUMAN	Arachidonate 5-lipoxygenase (5-LO) (5-lipoxygenase) (EC 1.13.11.34)
P16050	LOX15_ HUMAN	Arachidonate 15-lipoxygenase (15-LOX) (15-LOX-1) (EC 1.13.11.33) (12/15-lipoxygenase) (Arachidonate 12-lipoxygenase, leukocyte-type)
P18054	LOX12_ HUMAN	Arachidonate 12-lipoxygenase, 12S-type (12S-LOX) (12S-lipoxygenase) (EC 1.13.11.31) (Lipoxin synthase 12-LO) (EC 3.3.2.-) (Platelet-type lipoxygenase 12)
Q16873	LTC4S_ HUMAN	Leukotriene C4 synthase (LTC4 synthase) (EC 4.4.1.20) (Leukotriene-C(4) synthase)
P78329	CP4F2_ HUMAN	Phylloquinone omega-hydroxylase CYP4F2 (EC 1.14.13.194) (20-hydroxyeicosatetraenoic acid synthase) (20-HETE synthase) (EC 1.14.13.-) (Arachidonic acid omega-hydroxylase)
P10632	CP2C8_ HUMAN	Cytochrome P450 2C8 (EC 1.14.14.1) (CYPIIC8) (Cytochrome P450 IIC2) (Cytochrome P450 MP-12) (Cytochrome P450 MP-20) (Cytochrome P450 form 1) (S-mephenytoin 4-hydroxylase)
P36969	GPX4_ HUMAN	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial (PHGPx) (EC 1.11.1.12) (Glutathione peroxidase 4) (GPx-4) (GSHPx-4)
P07203	GPX1_ HUMAN	Glutathione peroxidase 1 (GPx-1) (GSHPx-1) (EC 1.11.1.9) (Cellular glutathione peroxidase)
Q16678	CP1B1_ HUMAN	Cytochrome P450 1B1 (EC 1.14.14.1) (CYPIB1)
Q02928	CP4AB_ HUMAN	Cytochrome P450 4A11 (20-hydroxyeicosatetraenoic acid synthase) (20-HETE synthase) (CYP4AII) (CYPIVA11) (Cytochrome P-450HK-omega) (Cytochrome P450HL-omega)
P35354	PGH2_ HUMAN	Prostaglandin G/H synthase 2 (EC 1.14.99.1) (Cyclooxygenase-2) (COX-2) (PHS II) (Prostaglandin H2 synthase 2) (PGH synthase 2) (PGHS-2) (Prostaglandin-endoperoxide synthase 2)
P11712	CP2C9_ HUMAN	Cytochrome P450 2C9 (EC 1.14.13.-) ((R)-limonene 6-monooxygenase) (EC 1.14.13.80) ((S)-limonene 6-monooxygenase) (EC 1.14.13.48) ((S)-limonene 7-monooxygenase)
P20292	AL5AP_ HUMAN	Arachidonate 5-lipoxygenase-activating protein (FLAP) (MK-886-binding protein)
P04798	CP1A1_ HUMAN	Cytochrome P450 1A1 (EC 1.14.14.1) (CYPIA1) (Cytochrome P450 form 6) (Cytochrome P450-C) (Cytochrome P450-P1)
Q14914	PTGR1_ HUMAN	Prostaglandin reductase 1 (PRG-1) (EC 1.3.1.-) (15-oxoprostaglandin 13-reductase) (EC 1.3.1.48) (NADP-dependent leukotriene B4 12-hydroxydehydrogenase)
P51589	CP2J2_ HUMAN	Cytochrome P450 2J2 (EC 1.14.14.1) (Arachidonic acid epoxygenase) (CYPIIJ2)
Q16647	PTGIS_ HUMAN	Prostacyclin synthase (EC 5.3.99.4) (Prostaglandin I2 synthase)

Q08477	CP4F3_ HUMAN	Docosahexaenoic acid omega-hydroxylase CYP4F3 (EC 1.14.13.199) (20-hydroxyeicosatetraenoic acid synthase) (20-HETE synthase) (EC 1.14.13.-) (CYPIVF3) (Cytochrome P450 4F3)
P23219	PGH1_ HUMAN	Prostaglandin G/H synthase 1 (EC 1.14.99.1) (Cyclooxygenase-1) (COX-1) (Prostaglandin H2 synthase 1) (PGH synthase 1) (PGHS-1) (PHS 1) (Prostaglandin-endoperoxide synthase 1)
P15428	PGDH_ HUMAN	15-hydroxyprostaglandin dehydrogenase [NAD(+)] (15-PGDH) (EC 1.1.1.141) (Prostaglandin dehydrogenase 1) (Short chain dehydrogenase/reductase family 36C member 1)
P34913	HYES_ HUMAN	Bifunctional epoxide hydrolase 2 [Includes: Cytosolic epoxide hydrolase 2 (CEH) (EC 3.3.2.10) (Epoxide hydratase) (Soluble epoxide hydrolase) (SEH); Lipid-phosphate phosphatase
P16444	DPEP1_ HUMAN	Dipeptidase 1 (EC 3.4.13.19) (Dehydropeptidase-I) (Microsomal dipeptidase) (Renal dipeptidase) (hRDP)
O15296	LX15B_ HUMAN	Arachidonate 15-lipoxygenase B (15-LOX-B) (EC 1.13.11.33) (15-lipoxygenase 2) (15-LOX-2) (Arachidonate 15-lipoxygenase type II) (Linoleate 13-lipoxygenase 15-LOb)
O75342	LX12B_ HUMAN	Arachidonate 12-lipoxygenase, 12R-type (12R-LOX) (12R-lipoxygenase) (EC 1.13.11.-) (Epidermis-type lipoxygenase 12)
P24557	THAS_ HUMAN	Thromboxane-A synthase (TXA synthase) (TXS) (EC 5.3.99.5) (Cytochrome P450 5A1)
P33261	CP2CJ_ HUMAN	Cytochrome P450 2C19 (EC 1.14.13.-) ((R)-limonene 6-monooxygenase) (EC 1.14.13.80) ((S)-limonene 6-monooxygenase) (EC 1.14.13.48) ((S)-limonene 7-monooxygenase)
P09960	LKHA4_ HUMAN	Leukotriene A-4 hydrolase (LTA-4 hydrolase) (EC 3.3.2.6) (Leukotriene A(4) hydrolase)
P05177	CP1A2_ HUMAN	Cytochrome P450 1A2 (EC 1.14.14.1) (CYPIA2) (Cholesterol 25-hydroxylase) (Cytochrome P(3)450) (Cytochrome P450 4) (Cytochrome P450-P3)
Q9H4A9	DPEP2_ HUMAN	Dipeptidase 2 (EC 3.4.13.19)
Q9HBI6	CP4FB_ HUMAN	Phylloquinone omega-hydroxylase CYP4F11 (EC 1.14.13.194) (3-hydroxy fatty acids omega-hydroxylase CYP4F11) (EC 1.14.13.-) (Cytochrome P450 4F11) (CYPIVF11)
Q7Z449	CP2U1_ HUMAN	Cytochrome P450 2U1 (EC 1.14.14.1)
P13584	CP4B1_ HUMAN	Cytochrome P450 4B1 (EC 1.14.14.1) (CYPIVB1) (Cytochrome P450-HP)
P33527	MRP1_ HUMAN	Multidrug resistance-associated protein 1 (ATP-binding cassette sub-family C member 1) (Leukotriene C(4) transporter) (LTC4 transporter)
Q5TCH4	CP4AM_ HUMAN	Cytochrome P450 4A22 (CYPIVA22) (Fatty acid omega-hydroxylase) (Lauric acid omega-hydroxylase) (Long-chain fatty acid omega-monooxygenase) (EC 1.14.13.205)
Q6NT55	CP4FN_ HUMAN	Cytochrome P450 4F22 (EC 1.14.14.-)
Q9UNU6	CP8B1_ HUMAN	7-alpha-hydroxycholest-4-en-3-one 12-alpha-hydroxylase (EC 1.14.18.8) (7-alpha-hydroxy-4-cholesten-3-one 12-alpha-hydroxylase) (CYPVIIIB1) (Cytochrome P450 8B1) (Sterol 12-alpha-hydroxylase)
P98187	CP4F8_ HUMAN	Cytochrome P450 4F8 (EC 1.14.14.1) (CYPIVF8)

Q6GMR7	FAAH2_ HUMAN	Fatty-acid amide hydrolase 2 (EC 3.5.1.99) (Amidase domain-containing protein) (Anandamide amidohydrolase 2) (Oleamide hydrolase 2)
P49137	MAPK2_ HUMAN	MAP kinase-activated protein kinase 2 (MAPK-activated protein kinase 2) (MAPKAP kinase 2) (MAPKAP-K2) (MAPKAPK-2) (MK-2) (MK2) (EC 2.7.11.1)
O60760	HPGDS_ HUMAN	Hematopoietic prostaglandin D synthase (H-PGDS) (EC 5.3.99.2) (GST class-sigma) (Glutathione S-transferase) (EC 2.5.1.18) (Glutathione-dependent PGD synthase)
Q9H4B8	DPEP3_ HUMAN	Dipeptidase 3 (EC 3.4.13.19)
Q14390	GGTL2_ HUMAN	Gamma-glutamyltransferase light chain 2 (Gamma-glutamyltransferase-like protein 4)
P19440	GGT1_ HUMAN	Gamma-glutamyltranspeptidase 1 (GGT 1) (EC 2.3.2.2) (Gamma-glutamyltransferase 1) (Glutathione hydrolase 1) (EC 3.4.19.13) (Leukotriene-C4 hydrolase)
P36269	GGT5_ HUMAN	Gamma-glutamyltransferase 5 (GGT 5) (EC 2.3.2.2) (Gamma-glutamyl transpeptidase-related enzyme) (GGT-rel) (Gamma-glutamyltransferase-like activity 1)
Q9BX51	GGTL1_ HUMAN	Gamma-glutamyltransferase light chain 1 (Gamma-glutamyltransferase-like activity 4) (Gamma-glutamyltransferase-like protein 6)
P36268	GGT2_ HUMAN	Inactive gamma-glutamyltranspeptidase 2 (GGT 2) (Gamma-glutamyltransferase 2) (Glutathione hydrolase 2)
P41222	PTGDS_ HUMAN	Prostaglandin-H2 D-isomerase (EC 5.3.99.2) (Beta-trace protein) (Cerebrin-28) (Glutathione-independent PGD synthase) (Lipocalin-type prostaglandin-D synthase)
Q15185	TEBP_ HUMAN	Prostaglandin E synthase 3 (EC 5.3.99.3) (Cytosolic prostaglandin E2 synthase) (cPGES) (Hsp90 co-chaperone) (Progesterone receptor complex p23) (Telomerase-binding protein p23)
O14684	PTGES_ HUMAN	Prostaglandin E synthase (EC 5.3.99.3) (Microsomal glutathione S-transferase 1-like 1) (MGST1-L1) (Microsomal prostaglandin E synthase 1) (MPGES-1) (p53-induced gene 12 protein)
P42330	AK1C3_ HUMAN	Aldo-keto reductase family 1 member C3 (EC 1.-.-.-) (17-beta-hydroxysteroid dehydrogenase type 5) (17-beta-HSD 5) (3-alpha-HSD type II, brain) (3-alpha-hydroxysteroid dehydrogenase type 2)
Q8TBF2	PGFS_ HUMAN	Prostamide/prostaglandin F synthase (Prostamide/PG F synthase) (Prostamide/PGF synthase) (EC 1.11.1.20) (Protein FAM213B)
O00519	FAAH1_ HUMAN	Fatty-acid amide hydrolase 1 (EC 3.5.1.99) (Anandamide amidohydrolase 1) (Oleamide hydrolase 1)
Q7L5A8	FA2H_ HUMAN	Fatty acid 2-hydroxylase (EC 1.-.-.-) (Fatty acid alpha-hydroxylase)
P16152	CBR1_ HUMAN	Carbonyl reductase [NADPH] 1 (EC 1.1.1.184) (15-hydroxyprostaglandin dehydrogenase [NADP(+)]) (EC 1.1.1.197) (NADPH-dependent carbonyl reductase 1) (Prostaglandin 9-ketoreductase)
O14880	MGST3_ HUMAN	Microsomal glutathione S-transferase 3 (Microsomal GST-3) (EC 2.5.1.18) (Microsomal GST-III)
Q99735	MGST2_ HUMAN	Microsomal glutathione S-transferase 2 (Microsomal GST-2) (EC 2.5.1.18) (Microsomal GST-II)
Q58HT5	AWAT1_ HUMAN	Acyl-CoA wax alcohol acyltransferase 1 (EC 2.3.1.75) (Diacylglycerol O-acyltransferase 2-like protein 3) (Diacylglycerol acyltransferase 2) (Long-chain-alcohol O-fatty-acyltransferase 1)

P47712	PA24A_ HUMAN	Cytosolic phospholipase A2 (cPLA2) (Phospholipase A2 group IVA) [Includes: Phospholipase A2 (EC 3.1.1.4) (Phosphatidylcholine 2-acylhydrolase); Lysophospholipase (EC 3.1.1.5)]
P05181	CP2E1_ HUMAN	Cytochrome P450 2E1 (EC 1.14.13.-) (4-nitrophenol 2-hydroxylase) (EC 1.14.13.n7) (CYPIIE1) (Cytochrome P450-J) [Cleaved into: Cytochrome P450 2E1, N-terminally processed]
O60733	PLPL9_ HUMAN	85/88 kDa calcium-independent phospholipase A2 (CaI-PLA2) (EC 3.1.1.4) (Group VI phospholipase A2) (GVI PLA2) (Intracellular membrane-associated calcium-independent phospholipase A2 beta)
Q5TZZ9	Q5TZZ9_ HUMAN	Annexin
P04083	ANXA1_ HUMAN	Annexin A1 (Annexin I) (Annexin-1) (Calpactin II) (Calpactin-2) (Chromobindin-9) (Lipocortin I) (Phospholipase A2 inhibitory protein) (p35)
P07355	ANXA2_ HUMAN	Annexin A2 (Annexin II) (Annexin-2) (Calpactin I heavy chain) (Calpactin-1 heavy chain) (Chromobindin-8) (Lipocortin II) (Placental anticoagulant protein IV) (PAP-IV) (Protein I) (p36)
Q9NP80	PLPL8_ HUMAN	Calcium-independent phospholipase A2-gamma (EC 3.1.1.5) (Intracellular membrane-associated calcium-independent phospholipase A2 gamma) (iPLA2-gamma) (PNPLA-gamma)
P0C869	PA24B_ HUMAN	Cytosolic phospholipase A2 beta (cPLA2-beta) (EC 3.1.1.4) (Phospholipase A2 group IVB)
Q9UP65	PA24C_ HUMAN	Cytosolic phospholipase A2 gamma (cPLA2-gamma) (EC 3.1.1.4) (Phospholipase A2 group IVC)
Q68DD2	PA24F_ HUMAN	Cytosolic phospholipase A2 zeta (cPLA2-zeta) (EC 3.1.1.4) (Phospholipase A2 group IVF)
O15496	PA2GX_ HUMAN	Group 10 secretory phospholipase A2 (EC 3.1.1.4) (Group X secretory phospholipase A2) (GX sPLA2) (sPLA2-X) (Phosphatidylcholine 2-acylhydrolase 10)
Q9NZ20	PA2G3_ HUMAN	Group 3 secretory phospholipase A2 (EC 3.1.1.4) (Group III secretory phospholipase A2) (GIII sPLA2) (sPLA2-III) (Phosphatidylcholine 2-acylhydrolase 3)
Q9UNK4	PA2GD_ HUMAN	Group IID secretory phospholipase A2 (GIID sPLA2) (sPLA2-IID) (EC 3.1.1.4) (PLA2IID) (Phosphatidylcholine 2-acylhydrolase 2D) (Secretory-type PLA, stroma-associated homolog)
Q9NST1	PLPL3_ HUMAN	Patatin-like phospholipase domain-containing protein 3 (EC 3.1.1.3) (Acylglycerol O-acyltransferase) (EC 2.3.1.-) (Adiponutrin) (Calcium-independent phospholipase A2-epsilon) (iPLA2-epsilon)
P14555	PA2GA_ HUMAN	Phospholipase A2, membrane associated (EC 3.1.1.4) (GIIC sPLA2) (Group IIA phospholipase A2) (Non-pancreatic secretory phospholipase A2) (NPS-PLA2) (Phosphatidylcholine 2-acylhydrolase 2A)
Q13093	PAFA_ HUMAN	Platelet-activating factor acetylhydrolase (PAF acetylhydrolase) (EC 3.1.1.47) (1-alkyl-2-acetylglycerophosphocholine esterase) (2-acetyl-1-alkylglycerophosphocholine esterase)
Q99487	PAFA2_ HUMAN	Platelet-activating factor acetylhydrolase 2, cytoplasmic (EC 3.1.1.47) (Serine-dependent phospholipase A2) (SD-PLA2) (hSD-PLA2)
P43034	LIS1_ HUMAN	Platelet-activating factor acetylhydrolase IB subunit alpha (Lissencephaly-1 protein) (LIS-1) (PAF acetylhydrolase 45 kDa subunit) (PAF-AH 45 kDa subunit) (PAF-AH alpha) (PAFAH alpha)
P68402	PA1B2_ HUMAN	Platelet-activating factor acetylhydrolase IB subunit beta (EC 3.1.1.47) (PAF acetylhydrolase 30 kDa subunit) (PAF-AH 30 kDa subunit) (PAF-AH subunit beta) (PAFAH subunit beta)

Q15102	PA1B3_ HUMAN	Platelet-activating factor acetylhydrolase IB subunit gamma (EC 3.1.1.47) (PAF acetylhydrolase 29 kDa subunit) (PAF-AH 29 kDa subunit) (PAF-AH subunit gamma) (PAFAH subunit gamma)
Q9H7Z7	PGES2_ HUMAN	Prostaglandin E synthase 2 (Membrane-associated prostaglandin E synthase-2) (mPGE synthase-2) (Microsomal prostaglandin E synthase 2) (mPGES-2) (Prostaglandin-H(2) E-isomerase)
P41247	PLPL4_ HUMAN	patatin-like phospholipase domain-containing protein 4 (EC 3.1.1.3) (Protein GS2)

Supplementary Table 5. Cannonical pathways de-regulated in PCa tissue after Ingenuity Pathway Analysis

	Ingenuity Canonical Pathways	p-value	z-Score
1	EIF2 Signaling	3.44E-17	5.15
2	Regulation of eIF4 and p70S6K Signaling	3.93E-13	
3	Mitochondrial Dysfunction	4.22E-11	NA
4	mTOR Signaling	8.20E-11	1
5	Protein Ubiquitination Pathway	4.63E-10	NA
6	TCA Cycle II (Eukaryotic)	1.28E-9	NA
7	Fatty Acid β-oxidation I	1.42E-9	NA
8	Isoleucine Degradation I	3.12E-9	NA
9	Acetyl-CoA Biosynthesis I (Pyruvate Dehydrogenase Complex)	1.52E-8	NA
10	Caveolar-mediated Endocytosis Signaling	1.86E-8	NA
11	Oxidative Phosphorylation	2.71E-8	NA
12	tRNA Charging	7.33E-8	NA
13	Valine Degradation I	1.39E-7	NA
14	Glutaryl-CoA Degradation	1.86E-7	NA
15	RAN Signaling	2.21E-7	NA
16	ILK Signaling	4.85E-7	-1.34
17	Remodeling of Epithelial Adherens Junctions	5.58E-7	0.44
18	Tryptophan Degradation III (Eukaryotic)	6.00E-7	NA
19	Clathrin-mediated Endocytosis Signaling	6.76E-7	NA
20	Noradrenaline and Adrenaline Degradation	8.22E-7	NA
21	Actin Cytoskeleton Signaling	2.26E-6	-2.13
22	Ethanol Degradation II	2.74E-6	NA
23	Granzyme A Signaling	3.42E-6	NA
24	Glycogen Degradation III	3.42E-6	NA
25	Virus Entry via Endocytic Pathways	4.99E-6	NA
26	Ketolysis	5.45E-6	NA
27	Integrin Signaling	5.79E-6	-3.57
28	Tryptophan Degradation X (Mammalian, via Tryptamine)	7.15E-6	NA
29	NRF2-mediated Oxidative Stress Response	9.76E-6	1.66
30	Pyrimidine Ribonucleotides Interconversion	1.11E-5	NA
31	Leucine Degradation I	1.30E-5	NA
32	Phenylethylamine Degradation I	1.44E-5	NA

33	GDP-glucose Biosynthesis	1.44E-5	NA
34	2-ketoglutarate Dehydrogenase Complex	1.44E-5	NA
35	Regulation of Actin-based Motility by Rho	1.48E-5	0
36	Pyrimidine Ribonucleotides De Novo Biosynthesis	2.55E-5	NA
37	RhoGDI Signaling	3.14E-5	1.5
38	Cellular Effects of Sildenafil (Viagra)	4.35E-5	NA
39	Glucose and Glucose-1-phosphate Degradation	5.43E-5	NA
40	Pyrimidine Deoxyribonucleotides De Novo Biosynthesis I	5.64E-5	NA
41	Ketogenesis	5.86E-5	NA
42	Glycogen Degradation II	5.86E-5	NA
43	Epithelial Adherens Junction Signaling	6.82E-5	NA
44	Putrescine Degradation III	9.31E-5	NA
45	Serotonin Degradation	1.05E-4	NA
46	Mevalonate Pathway I	1.18E-4	NA
47	Thiosulfate Disproportionation III (Rhodanese)	1.45E-4	NA
48	GDP-L-fucose Biosynthesis I (from GDP-D-mannose)	1.45E-4	NA
49	Formaldehyde Oxidation II (Glutathione-dependent)	1.45E-4	NA
50	Adenine and Adenosine Salvage I	1.45E-4	NA
51	4-aminobutyrate Degradation I	1.45E-4	NA
52	Dopamine Degradation	1.51E-4	NA
53	VEGF Signaling	1.58E-4	-1.89
54	RhoA Signaling	1.87E-4	-0.27
55	LXR/RXR Activation	2.03E-4	-2.33
56	Phenylalanine Degradation IV (Mammalian, via Side Chain)	2.21E-4	NA
57	Glycolysis I	2.41E-4	NA
58	Calcium Signaling	2.52E-4	-1.34
59	Germ Cell-Sertoli Cell Junction Signaling	2.75E-4	NA
60	Telomere Extension by Telomerase	4.13E-4	NA
61	DNA Double-Strand Break Repair by Non-Homologous End Joining	4.13E-4	NA
62	Regulation of Cellular Mechanics by Calpain Protease	5.45E-4	-1.34
63	Glutathione-mediated Detoxification	6.00E-4	NA
64	Endoplasmic Reticulum Stress Pathway	6.00E-4	NA
65	Superpathway of Geranylgeranyldiphosphate Biosynthesis I (via Mevalonate)	7.63E-4	NA
66	Oxidative Ethanol Degradation III	7.63E-4	NA
67	Granzyme B Signaling	7.63E-4	2
68	Colanic Acid Building Blocks Biosynthesis	7.63E-4	NA

69	Calcium Transport I	1.08E-3	NA
70	Pyruvate Fermentation to Lactate	1.20E-3	NA
71	Fatty Acid β-oxidation III (Unsaturated, Odd Number)	1.20E-3	NA
72	D-glucuronate Degradation I	1.20E-3	NA
73	γ-linolenate Biosynthesis II (Animals)	1.32E-3	NA
74	Aldosterone Signaling in Epithelial Cells	1.55E-3	NA
75	Pentose Phosphate Pathway	2.37E-3	NA
76	Ethanol Degradation IV	3.93E-3	NA
77	FAK Signaling	4.96E-3	NA
78	Melatonin Degradation II	5.58E-3	NA
79	Glutamate Degradation III (via 4-aminobutyrate)	5.58E-3	NA
80	Branched-chain α-keto acid Dehydrogenase Complex	5.58E-3	NA
81	Arginine Degradation I (Arginase Pathway)	5.58E-3	NA
82	Lipid Antigen Presentation by CD1	1.10E-2	NA
83	Gluconeogenesis I	1.10E-2	NA
84	Purine Nucleotides De Novo Biosynthesis II	1.10E-2	NA
85	AMPK Signaling	1.17E-2	-2.12
86	CDK5 Signaling	1.42E-2	NA
87	Xenobiotic Metabolism Signaling	1.52E-2	NA
88	Agrin Interactions at Neuromuscular Junction	1.52E-2	-2.45
89	Tight Junction Signaling	1.62E-2	NA
90	UDP-N-acetyl-D-glucosamine Biosynthesis II	2.12E-2	NA

Supplementary Table 6. Clinico-pathologic correlations in the PCa-TMA. FASN intensity of positive tumour cells.

	Negative	Positive	Total	p-value
PSA at diagnosis				
≤10 ng/ml	43 (9.3)%	359 (77.7)%	402 (87.0)%	
>10 ng/ml	8 (1.7)%	52 (11.3)%	60 (13.0)%	0.543
Total	51 (11.0)%	411 (89.0)%	462	
Gleason score				
<7	28 (6.0)%	227 (49.0)%	255 (55.1)%	
7	20 (4.3)%	160 (34.6)%	180 (38.9)%	0.998
>7	3 (0.6)%	25 (5.4)%	28 (6.0)%	
Total	51 (11.0)%	412 (89.0)%	463	
pT-stage				
pT2	33 (7.1)%	297 (64.1)%	330 (71.3)%	
pT3a/b	12 (2.6)%	93 (20.1)%	105 (22.7)%	0.177
pT4	6 (1.3)%	22 (4.8)%	28 (6.0)%	
Total	51 (11.0)%	412 (89.0)%	463	

Supplementary Table 7. Clinico-pathologic correlations in the PCa-TMA, LOX5 **A**. Intensity in the cytoplasm. **B**. Intensity in the nucleus. **C**. Percentage of positive tumour cells.

A.

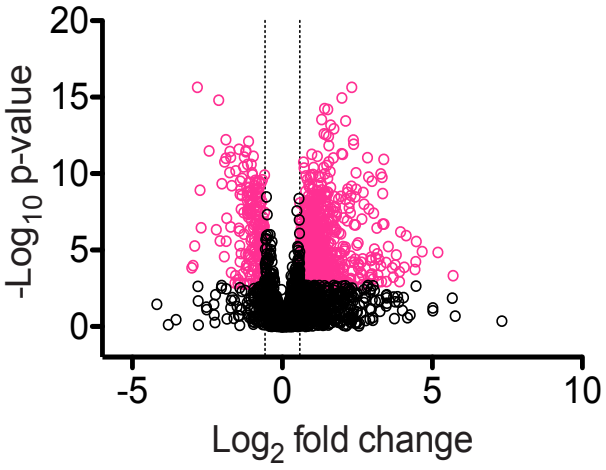
	Negative	Positive	Total	p-value
PSA at diagnosis				
≤10 ng/ml	160 (71.4)%	31 (13.8)%	191 (85.3)%	
>10 ng/ml	28 (12.5)%	5 (2.2)%	33 (14.7)%	0.77
Total	188 (83.9)%	36 (16.1)%	225	
Gleason score				
<7	79 (35.1)%	25 (11.1)%	104 (46.2)%	
7	68 (30.2)%	31 (13.8)%	99 (44.0)%	0.321
>7	18 (8.0)%	4 (1.8)%	22 (9.8)%	
Total	165 (73.3)%	60 (26.7)%	225	
pT-stage				
pT2	108 (48.0)%	40 (17.8)%	148 (65.8)%	
pT3a/b	51 (22.7)%	13 (5.8)%	64 (28.4)%	0.044
pT4	6 (2.7)%	7 (3.1)%	13 (5.8)%	
Total	165 (73.3)%	60 (26.7)%	225	

B.

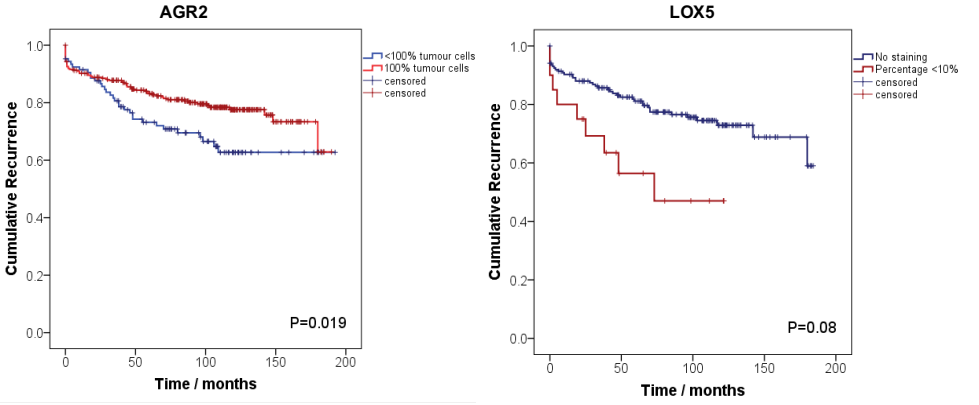
	Negative	Positive	Total	p-value
PSA at diagnosis				
≤10 ng/ml	221 (78.4)%	23 (8.2)%	244 (86.5)%	
>10 ng/ml	33 (11.7)%	5 (1.8)%	38 (13.5)%	0.474
Total	254 (90.1)%	28 (9.9)%	282	
Gleason score				
<7	121 (42.9)%	16 (5.7)%	137 (48.6)%	
7	111 (39.4)%	11 (3.9)%	122 (43.3)%	0.501
>7	22 (7.8)%	1 (0.4)%	23 (8.2)%	
Total	254 (90.1)%	28 (9.9)%	282	
pT-stage				
pT2	168 (59.6)%	21 (7.4)%	189 (67.0)%	
pT3a/b	72 (25.5)%	6 (2.1)%	78 (27.7)%	0.634
pT4	14 (5.0)%	1 (0.4)%	15 (5.3)%	
Total	254 (90.1)%	28 (9.9)%	282	

C.

	Negative	Positive	Total	p-value
PSA at diagnosis				
≤10 ng/ml	154 (75.1)%	17 (8.3)%	171 (83.4)%	
>10 ng/ml	31 (15.1)%	3 (1.5)%	34 (16.6)%	0.841
Total	185 (90.2)%	20 (9.8)%	206	
Gleason score				
<7	89 (43.2)%	8 (3.9)%	97 (47.1)%	
7	83 (40.3)%	8 (3.9)%	91 (44.2)%	0.17
>7	14 (6.8)%	4 (1.9)%	18 (8.7)%	
Total	186 (90.3)%	20 (9.7)%	206	
pT-stage				
pT2	119 (57.8)%	11 (5.3)%	130 (63.1)%	
pT3a/b	56 (27.2)%	6 (2.9)%	62 (30.1)%	0.298
pT4	11 (5.3)%	3 (1.5)%	14 (6.8)%	
Total	186 (90.3)%	20 (9.7)%	206	



Supplementary Figure 1. Volcano plot illustrating the differentially expressed proteins in the Discovery set. Comparison is performed between NAP samples (n=33), and PCa tissue samples exhibiting more than 50% of tumour area (n=34). Y axis corresponds to statistical significance expressed as –log10 and X axis corresponds to Fold Change expressed as log2. Pink dots represent proteins up or down-regulated with a p-value < 0.002, and fold change higher than 1.5 or lower than 0.66.



Supplementary Figure 2. Kaplan-Meier curves assessing the probability of PCa biochemical recurrence after radical prostatectomy by A: AGR2 and B: LOX5. Blue lines represent: AGR2: Percentage of tumour cells <100% B: LOX5: No staining. Red lines represent: AGR2: 100% of positive tumour cells, LOX5: <10% staining of positive tumour cells.

Chapter 5

Generation and dissociation of $RCOOCaCl_2^-$ and other carboxylate substituted superhalogens: CO_2 capture and implications for structure analysis

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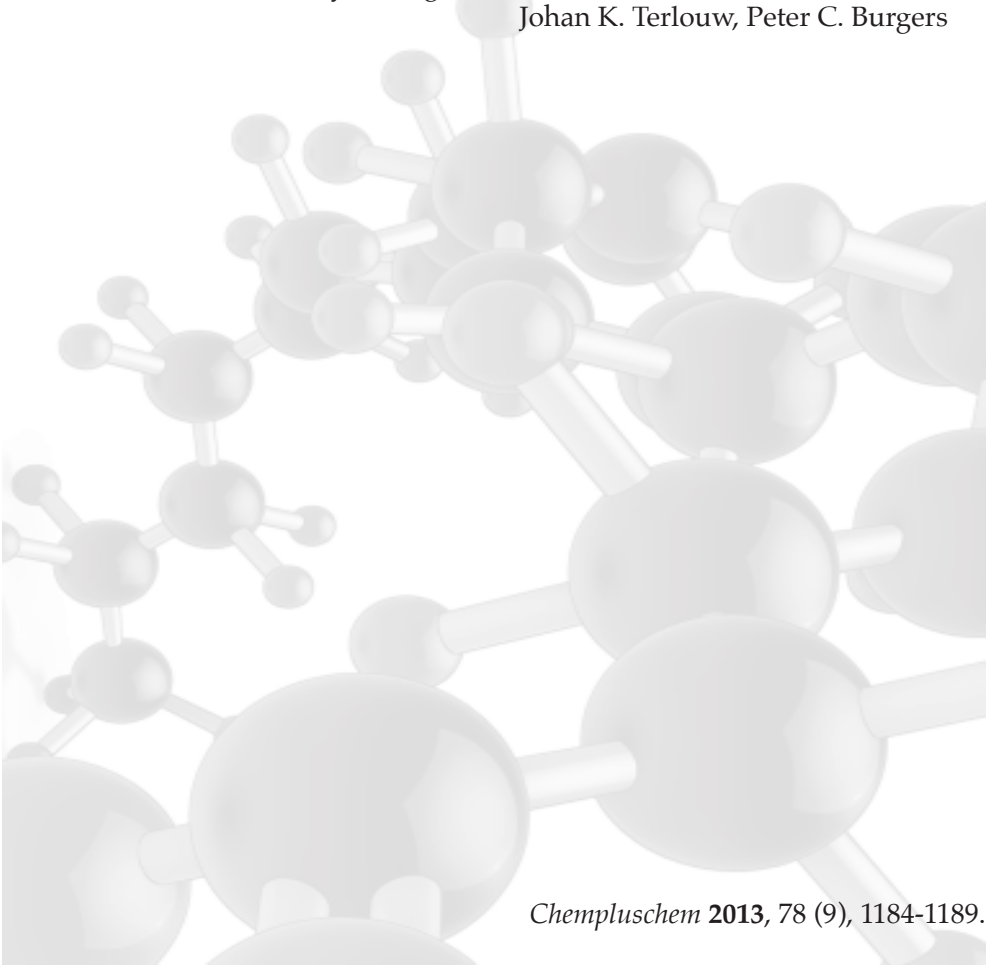

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Abstract

Carboxylate substituted superhalogens of the type $RCOOMX_2^-$ (M = Mg, Ca, Sr, Ba, Mn, Co, Ni, Zn and X = Cl, Br) are easily accessible in the gas-phase by electrospray ionization. Their collision induced dissociation (CID) characteristics have been probed using ion trap and triple quadrupole mass analyzers with particular emphasis on the behaviour of $RCOOCaCl_2^-$ type ions. In the ion trap these appear to readily react with residual water yielding $HOCaCl_2^-$ as the hydrolysis product. In the absence of water, a collision induced McLafferty type rearrangement takes over to produce $HCaCl_2^-$ with the expulsion of an olefin and CO_2. A brief computational analysis using the CBS-QB3 model chemistry provides a satisfactory rationale for these observations.

When complexed with MX_2 (M = Mg, Ca, Sr, Ba) long chain unsaturated aliphatic carboxylate anions undergo various backbone cleavages upon collision. These leads to structure diagnostic olefin losses because the position of the double bonds remains intact. Such cleavages are absent in the bare ion $RCOO^-$.

The long chain ions $RCOOMX_2^-$ also produce the intriguing species $[CO2]MX_2^-\bullet$. These were characterized by CID experiments while theory indicates that they may be viewed as a CO2 molecule captured by the salt anion $MX_2^-\bullet$.

Finally, it is shown that the CID spectra of $RCOOCaCl_2^-$ ions derived from all-trans retinoic acid, a compound of current interest in biochemistry and medicine, shows a unique structure diagnostic dissociation that may greatly aid its qualitative and quantitative analysis.

Introduction

In 1981, Gutsev and Boldyrev introduced the term "superhalogen" to describe complex radicals like $BF4^{\bullet}$ and $AlF4^{\bullet}$, which have a comparatively high Electron Affinity (EA), higher than that of the parent halogen atom (1). Superhalogens have the formula MX_{k+1}, where M is a metal atom with maximum valence *k* while X is a halogen atom. The vertical detachment energy (VDE) of an $[MX_{k+1}]^-$ anion greatly exceeds that of the corresponding halogen anion (2). For example, the VDE of $CaCl_3^-$ (6.62 eV) (3) is much larger than the EA of $Cl^{\bullet}$ (3.617 eV) (4). Superhalogens are promising candidates for oxidizing species with a high ionization energy (such as O_2 and Xe) and for the preparation of organic superconductors (5). Moreover, they allow the synthesis of new classes of ionic compounds like $Xe^+[PtF_6]^-$ (6).
The presence of halogen atoms in superhalogens is not mandatory (7). Many other functional groups may serve as ligands in superhalogen anions (8, 9). This concept has been used to render otherwise electronically unstable anions stable. For example, whereas the EA of $C_2H_5^-$ is negative (0.263 ± 0.089 eV), the VDE of $[C_2H_5MgF_2]^-$ is positive (4.088 eV)(10) and very much so.
Theoretical calculations have shown that electrophilic substituents, such as COOH, too, may lead to species having a large VDE, for example the VDE of $Mg(COOH)_3^-$ has been calculated at 5.2 eV (8).

It occurred to us that carboxylate anions ($RCOO^-$) could also serve as suitable ligands to produce superhalogens of the type $RCOOMX_2^-$ (M = Mg, Ca, Sr, Ba, Mn, Co, Ni, Zn, X = Cl, Br) and that such species may be accessible by Electrospray Ionization (ESI). Since $RCOO^{\bullet}$ radicals have relatively large EAs (for example the EA of $CH_3COO^{\bullet}$ is 3.496 ± 0.0010 eV(4), on a par with the EA of $Cl^{\bullet}$), it may reasonably be expected that $RCOOMX_2^-$ anions, too, will have large VDEs. Thus, in $RCOOMX_2^-$, the electron may well be sufficiently bound to allow unimolecular or collision induced dissociations to take place below the threshold for electron detachment.
This indeed is the case: in the course of this study we observed structure characteristic dissociations of long chain unsaturated fatty acids substituted by superhalogen groups. We also discovered novel species, like the radical anion $[CO2]CaCl_2^{-\bullet}$, whose structure we probed by ab initio calculations.
The $RCOOMX_2^-$ ion can also be viewed as a $RCOO^-$ anion to which a salt molecule MX_2 is attached, that is as a carboxylate anion/salt complex. While preparing this contribution, the groups of R.A.J. O'Hair and J-C. Tabet reported(11) a comprehensive study that deals with the dissociation chemistry of organomagnesates $[RMgX_2]^-$ generated by decarboxylation of $[RCO_2MgX_2]^-$. Their results will be discussed where they overlap with ours.

Chapter 5

Experimental Section and Computational Methods

Experiments

Collision induced dissociation (CID) experiments were performed using a Bruker Esquire ESI-ion trap mass spectrometer. A crucial parameter is the capillary current: it has to be kept below 30 nA to prevent destruction of the metal complexes. CID experiments were also performed on an AB Sciex API 4000 triple quadrupole mass spectrometer with the following conditions: declustering potential 20V, entrance potential 10V, collision exit potential 12V, capillary voltage 5000V. Comparing ion trap and triple quadrupole results allows the identification of ion-molecule reactions that take place with residual water molecules present in the ion trap.
FT-ICR measurements were performed on a Varian 9.4T Fourier Transform Ion Cyclotron Resonance mass spectrometer operated in the positive electrospray mode, at a resolution of 30 000 (FWHM at m/z 150). The $RCOOMX_2^-$ ions were generated as described below, isolated in the ICR cell and then dissociated by sustained off–resonance irradiation collision-induced dissociation (SORI-CID).
The $RCOOMX_2^-$ complexes were generated as follows. A 10 μL 0.01 M RCOOH solution in water or ethanol was mixed with 10 μL of a 0.05 M solution of the salt MX_2 in water. This mixture was diluted with 200 μL of isopropanol/water (1:1) and then infused into the MS at a rate of 240 μL/hr. The observed anions $RCOOMX_2^-$ were then mass selected and subjected to CID experiments.

Theoretical calculations

The calculations were performed with the CBS-QB3 model chemistry (12) using the Gaussian 09, Rev C.01 suite of programs (13) on the SHARCNET computer network at McMaster University. All relative energies presented in the text and in the energy diagram are derived from CBS-QB3 total enthalpies (298 K) in kJmol-1. The complete set of computational results is available from the authors upon request.

Results and Discussion

Formic acid and acetic acid: unimolecular decay versus hydrolysis

Table 1 shows the neutral losses observed for $HCOOMCl_2^-$ and $CH_3COOMCl_2^-$ (M = Ca, Mn, Zn). It is seen that for the formate anion, the $CaCl_2$ adduct eliminates CO, via a putative 1,2-H shift, whereas the $MnCl_2$ and $ZnCl_2$ adducts decarboxylate, via a 1,3-H shift.
Table 2 shows the Hydride Ion Affinities (HIA) and OH– affinities (in $kJmol^{-1}$) of Ca^{2+} and Zn^{2+} calculated by the CBS-QB3 method. It follows that Ca^{2+} prefers to attach OH^- over the binding of H^-, but the reverse is true for Zn^{2+}. This would rationalize the observed difference in behaviour, namely that $HCOOCaCl_2^-$ decarbonylates to produce $HOCaCl_2^-$, whereas $HCOOZnCl_2^-$ decarboxylates to form $HZnCl_2^-$.

Table 1. Reactions observed for $HCOOMCl_2^-$ and $CH_3COOMCl_2^-$

M	$HCOOMCl_2^-$	$CH_3COOMCl_2^-$
Ca	- CO	- CH2CO
Mn	- CO_2	- CO_2
Zn	- CO_2	- CO_2

Table 2. Hydride Ion Affinity (HIA) and OH^- affinity of Ca^{2+} and Zn^{2+} (CBS-QB3, kJmol-1).

Metal ion	HIA	OH^- affinity
Ca^{2+}	1275	1390
Zn^{2+}	1860	1780

However, deuterium labelling experiments indicate that formation of $HOCaCl_2^-$ may not be a unimolecular process. Ions $DCO_2CaCl_2^-$ and $CD_3CO_2CaCl_2^-$ both form $HOCaCl_2^-$ to the exclusion of $DOCaCl_2^-$. A possible explanation is that initially $DOCaCl_2^-$ is formed by decarbonylation and that it undergoes a rapid H/D exchange with residual water in the ion trap. However, ions $HOCaCl_2^-$ dominate the ion trap CID spectrum of various $RCOOCaCl_2^-$ anions, including that of $C_6F_5COOCaCl_2^-$, which has no H atom available for a unimolecular decarbonylation. This leads us to propose that the product ion $HOCaCl_2^-$ actually results from the following hydrolysis reaction with residual water present in the ion trap:

$$RCOOCaCl_2^- + H_2O \longrightarrow RCOOH + HOCaCl_2^- \ldots (1)$$

This hydrolysis reaction is discussed in more detail in the next section. Hydrolysis was also observed in the study of reference 10; for example, $PhCH_2CH_2MgCl_2^-$ abundantly forms m/z 111, $HOMgCl_2^-$.

Propanoic acid

The ion trap CID mass spectrum of $CH_3CH_2COOCaCl_2^-$ presented in Figure 1a displays an intense peak for formation of $HOCaCl_2^-$ at m/z 127. As stated above, this product ion could be due to a unimolecular process, most likely a 1,4-H shift followed by the losses of C_2H_4 and CO (eq. 2) or it could result from a hydrolysis reaction, (eq. 3):

$$CH_3CH_2COOCaCl_2- \longrightarrow CH_2{=}CH_2 + CO + HOCaCl_2^- \ldots\ldots (2)$$

$$CH_3CH_2COOCaCl_2^- + H2O \longrightarrow CH_3CH_2COOH + HOCaCl_2^- \ldots\ldots (3)$$

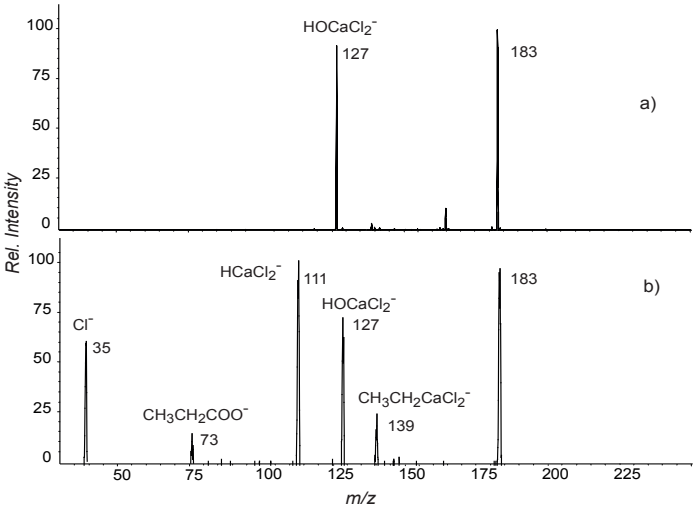

Figure 1. CID mass spectra of $CH_3CH_2COOCaCl_2^-$ obtained on an ion trap (a) and on a triple quadrupole CID mass spectrometer (b).

The CID mass spectrum as recorded on the triple quadrupole is shown in Figure 1b. It is seen that additional reactions appear, viz. formation of Cl^- (loss of $CH_3CH_2COOCaCl$) and formation of $HCaCl_2^-$. That Cl^- is not seen in the ion trap spectrum is because this fragment lies below the mass cutoff (m/z 50). However, another important fragment that is observed in Figure 1b but not in Figure 1a is $HCaCl_2^-$, m/z 111; its formation probably proceeds via a unimolecular McLafferty type rearrange-ment, via a 1,5-H shift, eq. (4):

$$CH_3CH_2COOCaCl_2^- \longrightarrow CH_2{=}CH_2 + CO_2 + HCaCl_2^- \ldots\ldots (4)$$

This raises the question why $HCaCl_2-$ is not observed in the ion trap.
The results of our model chemistry calculations for reactions 2, 3 and 4 are presented in Figure 2. In the presence of water, reaction (3) is the least energy demanding reaction and this hydrolysis reaction, we propose, occurs in the ion trap. In the absence of water (or if the reaction time is significantly smaller as in the triple quadrupole apparatus) reactions (2) and (4) should take over, as indeed is observed, see Figure 1(b). Thus, the fragment ion $HOCaCl_2^-$ observed in the ion trap is due to hydrolysis, but in the triple quadrupole it is the result of a unimolecular reaction. Other reactions that are seen at these elevated internal energies are loss of CO_2 to produce the organometallate $CH_3CH_2CaCl_2^-$ and loss of $CaCl_2$.

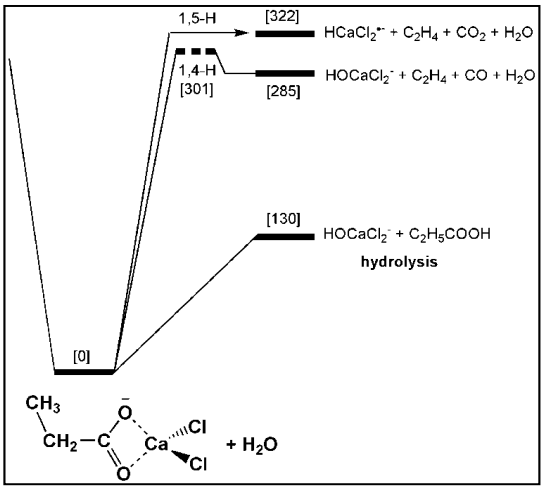

Figure 2. Energy diagram for hydrolysis and dissociation reactions of $CH_3CH_2COOCaCl_2^-$.

The ion $CH_3CH_2COOZnCl_2^-$ was also investigated and it showed, both in the ion trap and in the triple quadrupole apparatus, loss of CO_2 paralleling the behaviour of the formate and acetate complex ions, see above. Thus, the zinc analogue does not undergo hydrolysis in the ion trap but it forms an organometallate via decarboxylation(14).

Long chain aliphatic acids

Long chain saturated aliphatic acids show only one or two dissociation reactions when their deprotonated forms are complexed with $CaCl_2$ and $ZnCl_2$ and are then subjected to CID in the ion trap. Thus, the stearic acid ion $C_{18}H_{35}COOCaCl_2^-$ generates only $HOCaCl_2^-$ probably via hydrolysis whereas the zinc analogue loses CO_2 and $ZnCl_2$. However, when an unsaturation is present, such as in linoleic acid, backbone dissociations are observed for the $CaCl_2$ complex but not for the $ZnCl_2$ complex. An example is provided by the species derived from arachidonic acid, C20:4. The bare carboxylate anion itself, $C_{20}H_{31}COO^-$, predominantly undergoes decarboxylation. This is not a structure characteristic dissociation as many long chain carboxylate anions lose CO2. However, when $CaCl_2$ is attached to the arachidonic anion, a very different situation obtains. Figure 3a shows many odd mass, even electron fragments that correspond to backbone dissociations via olefin losses. Thus, the intense fragment ion at m/z 181 could be CH_2=CH-$COOCaCl_2^-$ produced by a hydrogen shift and C-C cleavage β to the double bond.

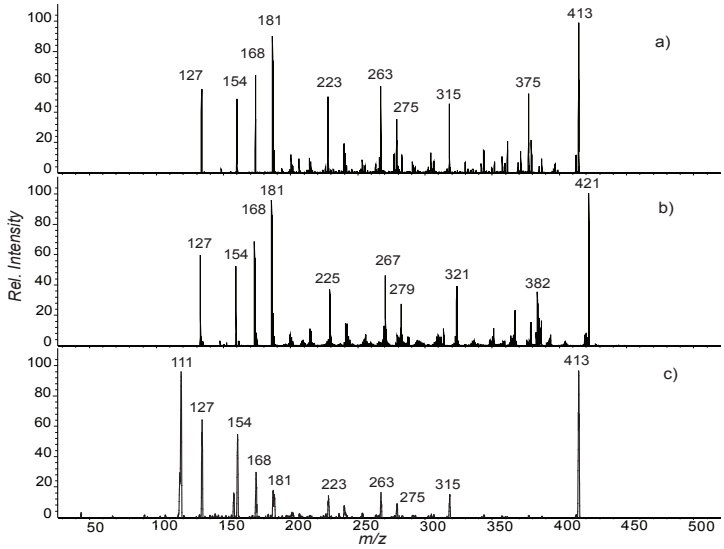

Figure 3. (a) CID mass spectrum of the arachidonic carboxylate anion complexed with $CaCl_2$ on an ion trap; (b) arachidonic d8 carboxylate anion on an ion trap; (c) arachidonic carboxylate anion on a triple quadrupole.

The ion trap CID mass spectrum of $C_{20}H_{23}D_8COOCaCl_2^-$ derived from arachidonic acid-*d*8 where all deuterium atoms are at the double bonds, is shown in Figure 3b. It is seen that the masses of the backbone fragments shift by the number of deuterium atoms present in the fragment ion.
A similar situation pertains to stearidonic acid C18:4 where backbone fragments are seen for β- cleavages. The observed backbone fragments for the stearidonic and arachidic complexes are listed in Table 3 to show that the observed fragment masses follow the double bond positions. These are prime examples of charge-remote fragmentations, which have been extensively studied by Gross (15-17) and others (18).
Arachidonic acid is an essential fatty acid that cannot be synthesised de novo. It is a component of the phospholipids of cells and it favours membrane fluidity due to the four double-cis bonds of its structure (19, 20). In body fluids, levels of arachidonic acid are measured by LC-MS/MS in the negative ion mode, but as mentioned above, the carboxylate anion dissociates by a non-structure characteristic loss of CO_2. However, when MX_2 (M = Mg, Ca, Sr, Ba, X = Cl, Br) is attached to the anion, highly structure characteristic backbone cleavages are seen. For the transition metal ions Mn – Zn, much simpler spectra are obtained: formation of $MCl_2^{-\bullet}$, $HMCl_2^-$ and loss of CO^2 is observed but backbone fragmentations do not occur.

Table 3. Double bond positions and observed fragment masses for $C_{18}H_{27}COO$-$CaCl_2-$ and $C_{20}H_{31}COOCaCl_2-$ derived from stearidonic and arachidonic acid.

Stearidonic acid		Arachidonic acid	
Double bond	**Observed fragment masses (m/z)**	**Double bond**	**Observed fragment masses (m/z)**
6	237	5	223
9	277	8	263
12	289	11	275
15	329	14	315

We note that the CID mass spectrum of Figure 3c recorded on the triple quadrupole instrument shows an intense peak for m/z 111, $HCaCl_2^-$, which is absent in the ion trap mass spectrum of Figure 3a. This is because the mass cutoff in the spectrum of Figure 3a lies at m/z 112 and so ions below this mass are not detected by the ion trap.
We were surprised to find two odd electron species in the CID mass spectra of Figure 3, namely at m/z 154 and at m/z 168 and will briefly discuss the ion at m/z 154. This ion is also observed for other long chain unsaturated carboxylates, e.g. oleic and linoleic acid. A direct bond cleavage would produce $[CO_2]CaCl_2^{-\bullet}$.

In the CID spectrum of $C_{20}H_{31}COOCa_{35}Cl_{37}Cl^-$ the above m/z 154 peak is cleanly shifted to m/z 156 and so the product ion contains two Cl atoms. The exact mass measured for m/z 154 by FTMS (153.8907) is within experimental uncertainty equal to the calculated value for $[CO_2]CaCl_2^{-\bullet}$ (153.8906) and so the elemental composition is [C, O_2, Cl_2, Ca]. In an MS^3 experiment this species was found to dissociate by loss of CO_2 and so we conclude it indeed to be $[CO_2]CaCl_2^{-\bullet}$. Our calculations indicate that this species is stable by 105 $kJmol^{-1}$ with respect to CO_2 loss and that electron detachment to produce CO_2 + $CaCl_2$ requires a further 95 $kJmol^{-1}$. Inspection of the calculated optimized geometries of $[CO_2]CaCl_2^{-\bullet}$ and its Mg counterpart, see Figure 4, indicates that these intriguing species can be viewed as CO_2 captured by the anionic salts $CaCl_2^{-\bullet}$ and $MgCl_2^{-\bullet}$. However, a population analysis suggests that the unpaired electron (and charge) may well reside on the CO_2 moiety.

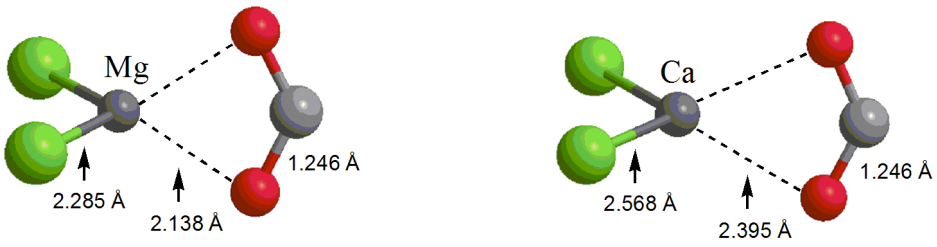

Figure 4. CBS-QB3 optimized structures for $[CO_2]MgCl_2^{-\bullet}$ and $[CO_2]CaCl_2^{-\bullet}$

They are currently the subject of a further study. The related species [CO2]MgX– (X = OH, Cl, Br) have recently been studied by Dossmann et al.(21)

Retinoic Acid (atRA)

Metabolism activates vitamin A (retinol) into all-trans retinoic acid (atRA) (22). Abnormal levels for atRA may cause and/or permit epithelial degeneration, such as xerophthalmia, and neurological disorders, such as schizophrenia, Parkinson's disease, Huntington's disease and Alzheimer's disease (23). Endogeneous atRA levels are assessed by LC-MS/MS operated in the positive ion mode; the negative ion mode is reported to be less sensitive than the positive mode (18). In the negative mode, the retinoic acid carboxylate anion dissociates only by loss of the non-structure characteristic fragment CO_2, see Figure 5a.

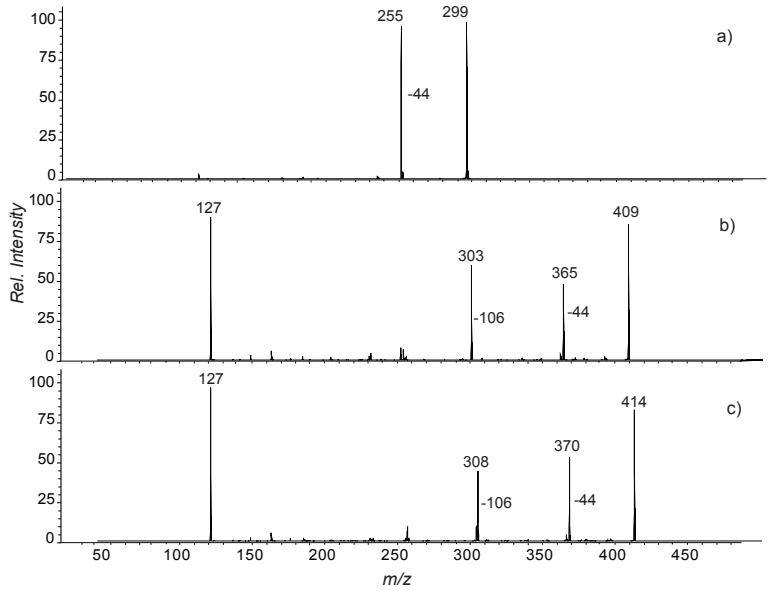

Figure 5. ESI-CID ion trap mass spectra of (a) the retinoic acid carboxylate anion; (b) the anioncomplexed with $CaCl_2$ and (c) the labeled retinoic acid complex ion.

When deprotonated atRA is complexed with $CaCl_2$, loss of CO_2 still occurs - and also the hydrolysis reaction is seen to produce $HOCaCl_2^-$, m/z 127, see Figure 5 - but now an additional intense fragment ion is observed at m/z 303, corresponding to the loss of 106 Da. For the D-labelled analogue retinoic acid-*d*5 this peak shifts to m/z 308 (loss of 106 Da). This leads us to propose that loss of 106 Da corresponds to the loss of C_8H_{10} and a possible extrusion mechanism is given in Figure 6.

Although we do not have definitive evidence, it is tempting to view this dissociation as occurring via a [CO2]CaCl2−• shift, a species observed as an isolated entity in the previous section. Apart from these intriguing mechanistic aspects, attachment of CaCl2 to the retinoic carboxylate anion leads to a highly structure characteristic dissociation product that may greatly aid its qualitative and quantitative analysis.
The observation that the precursor ion can also eliminate CO2 shows that calcium can form an organometallate via decarboxylation(14).

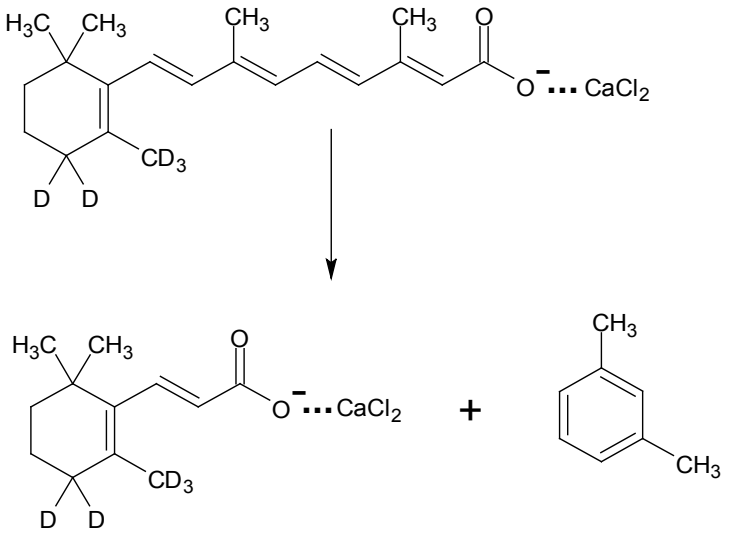

Figure 6. Proposed mechanism for elimination of 106 Da from the retinoic carboxylate anion complexed with $CaCl_2$.

Conclusions

Carboxylate substituted superhalogens $RCOOMX_2^-$, notably $RCOOCaCl_2^-$, can be generated by Electrospay ionization of mixtures of the acids RCOOH acid and salts MX_2 and their collision induced dissociations (CID) can readily be obtained. For the earth alkali metals an important product ion observed in the ion trap is $HOMX_2^-$; this ion is also present when the system contains no hydrogen atoms, as in $C_6F_5COOCaCl_2^-$. In addition, the labeled ions $DCO_2CaCl_2^-$ and $CD_3CO_2CaCl_2^-$ both form $HOCaCl_2^-$ to the exclusion of $DOCaCl_2^-$and so we conclude that formation of $HOCaCl_2^-$ occurs via hydrolysis by residual water present in the ion trap via:

$$RCOOCaCl_2^- + H_2O \longrightarrow RCOOH + HOCaCl_2^-$$

If no water is present (as for the triple quadrupole collision cell) formation of $HOCaCl_2^-$ can still occur, but only if the size of R permits the more energy demanding unimolecular process, a 1,4-H shift that leads to expulsion of an olefin and a CO molecule. A McLafferty type rearrangement followed by loss of an olefin and a CO_2 molecule accounts for the co-generation of $HCaCl_2^-$ ions. When complexed with MX_2 (M = Mg, Ca, Sr, Ba, X = Cl, Br), the CID mass spectra of long chain unsaturated aliphatic carboxylate anions display backbone

cleavages involving the loss of specific olefin molecules which readily reveal the position of the double bond(s). Such long chain ions also produce the species $[CO_2]MX_2^{-\bullet}$ which were characterized by CID experiments and which can be viewed as a CO2 molecule captured by the anionic salt $MX2^{-\bullet}$ species.
Deprotonated retinoic acid complexed with $CaCl_2$ shows an abundant loss of CO_2 showing that $CaCl_2$ can produce organometallates by decarboxylation. In addition, another intriguing dissociation is observed, viz. loss of 106 Da which is proposed to correspond to loss of meta-xylene, a highly structure characteristic dissociation.

Acknowledgements

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Chapter 6

General Discussion

General Discussion

Prostate cancer (PCa) is a common malignancy in men and its incidence continues to rise in many countries. Although the introduction of the PSA test has improved the management of the disease considerably, its low specificity has led to overtreatment of many prostate cancers, including aggressive treatments in older men considered to be at low risk for progression of the disease. Despite known limitations of the PSA test, it is worldwide used as a cancer marker for diagnosis, as well as for monitoring of success of radical prostatectomy and biochemical recurrence.

Searching for novel and/or complementary markers to PSA for the diagnosis and prognosis of PCa is still a relevant topic in PCa research. During the last decade, it has been addressed by using the most advanced technologies at different levels i.e. genes (genomics), transcripts (transcriptomics), proteins (proteomics) and metabolites (metabolomics).

In this thesis, we aimed to identify and validate novel metabolites or proteins in tissue or bio-fluids, which might improve the diagnosis and/or the prognosis of PCa. We used analytical approaches, and particularly mass spectrometry to study the role of some metabolites and proteins, particularly along the arachidonic acid (AA) pathway, in the diagnosis and prognosis of PCa.

Figure 1, describes the main pathways outlined in this thesis, after combining the results from a targeted metabolomics in serum and also a shotgun proteomics approach in PCa tissue. Most of the proteins studied in this thesis belong to the eicosanoid signalling pathway, from phospholipases cleaving AA from cell membranes, to specific hydroxylases such as LX15B, which converts AA to the bioactive molecule 15-HETE.

Primarily, we were interested in developing analytical methods by using liquid chromatography and mass spectrometry to identify statistically significant metabolites in serum samples from PCa patients and controls. In this process, we evaluated the concentrations of tryptophan and kynurenine (indicators of the IDO enzyme activity), as well as six metabolites within the arachidonic acid (AA) pathway (5-HETE, 8-HETE, 11-HETE, 15 HETE and AA), for the diagnosis and the prognosis of PCa.

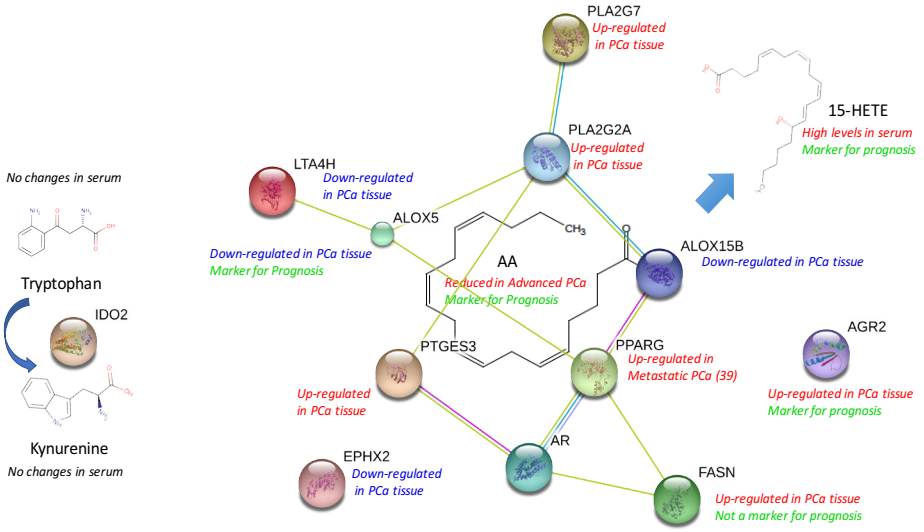

Figure 1. Overview figure illustrating the main metabolites, proteins and pathways discussed in this thesis. A STRING tool (www.string-db.org) was used to show the interaction of proteins along the AA pathway. Two proteins: AGR2 and IDO2, do not belong to this pathway, but they were also discussed in this thesis.

IDO enzyme activity is not a marker for detecting PCa

Considering the list of potential metabolic markers associated to PCa progression reported by Sreekumar et al. in 2009 (1), we decided to explore analytical tools to identify novel markers in serum samples from patients diagnosed with PCa. The first approach was the study of an enzyme activity, expressed as the kynurenine/tryptophan (Kyn/Trp) ratio, for indoleamine 2, 3-dioxygenase (IDO2, Figure 1). This enzyme is produced within tumours and initiates the conversion of tryptophan (Trp) within the kynurenine (Kyn) pathway, resulting in the production of immune-suppressive catabolites known to inhibit T- cell stimulation and to cause T-cell apoptosis (2). In addition, it was demonstrated by Feder-Mengus et al. (3) that IDO2 gene expression is significantly higher in PCa tissue compared to benign prostate hyperplasia (BPH) tissue. Thus, the study of metabolites produced along the kynurenine pathway was of interest in our search for non-invasive markers.

We used a HPLC method with fluorescence detection for the quantitation of tryptophan and kynurenine in serum. We did not find any statistically significant difference between the Controls/ERSPC and the group of PCa patients (**Chapter 2**, Figure 1). Therefore, we concluded that neither serum concentrations of these

metabolites, nor the ratio Kyn/Trp, an indicator on the IDO enzyme activity, can be considered as novel markers for the diagnosis of PCa.
We used a standard addition method, using at least five external calibrators to accurately calculate the concentrations of these metabolites, and in all cases the R2 values were higher than 0.99. We used 1-methyl tryptophan as an internal standard, and we have used both UV and fluorescence detectors because these methods and instrumentation can be used routinely, and can also be independently validated for clinical purposes.
One of the major challenges in metabolomics is the clinical applicability of potential metabolites in the diagnosis or prognosis of the disease. Several studies have demonstrated the potential applicability of metabolomics for PCa diagnosis (**Chapter 1**, Table 2), but unfortunately none of them is used in the clinic so far. The analytical methodology employed to detect/quantify metabolites still remains an important drawback in metabolomics and most of the methods and studies lack validation between different laboratories.
Different studies using metabolomics approaches have reported an increment in the concentration of Kyn in PCa tissue (1, 4). However, we found that this alteration in tissue does not lead to changes in serum. Although metabolic alterations in the tryptophan pathway might be occurring in the prostate, tryptophan catabolism occurs in many cells of the body, thus affecting the concentration of its metabolites in the bloodstream. Metabolites in biofluids are highly affected by diet, physical activity, environment or drug intake. Recent advances in the chemistry of chromatography columns, as well in the commercial use of heavy labelled standard isotopes, might contribute to the improvement in the quantitation of these molecules using mass spectrometry. HILIC chromatography columns have been employed recently for both the untargeted and targeted analysis of polar compounds in biofluids (5, 6). Extended cohorts of individuals, analysing even thousands of samples, might contribute to compensate the variation in biological samples, and they are required to truly evaluate the role of particular metabolites in the diagnosis of PCa.

Arachidonic acid pathway and its role in PCa progression

The Arachidonic Acid (AA) pathway is a well-studied biological pathway that triggers the cell membrane by the action of phospholipase A2-type (PLA2) enzymes (7). The metabolism of AA includes cyclooxygenase (COX), lipoxygenase (LOX) and P450 epoxygenases that generate eicosanoids, including prostaglandins (PGs), leukotriens (LTs), thromboxans (TXs), hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatetraenoic acids (EETs), and hydroxyperoxy-eicosatetraenoic acids (HPETEs) (8, 9).
Our main interest when searching for novel and non-invasive markers for PCa focussed on the use of analytical approaches using liquid chromatography and mass spectrometry. Metabolites produced by lipoxygenase enzymes along the

AA pathway had been proposed as a target for preventing and treating PCa by Yang et al. (10). In addition, Schumacher et al.(11), observed a decrease in the concentration of AA in PCa tumours. Therefore, we decided to develop an analytical method for the simultaneous quantitation of HETEs and AA in serum by using ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

In **Chapter 3**, we described a targeted metabolomics study of AA and some of its metabolites in serum samples from patients with PCa as well as other malignancies. In a discovery phase, we have found that the concentration of hydroxyeicosatetraenoic acids (HETEs) was higher in six out of twenty PCa patients, when compared with patients having low PSA levels. We validated these findings in an extended cohort with more than two hundred patients exhibiting different stages of PCa. We found that six out of nineteen samples in the most advance stage group (Group 5) exhibited high concentrations of the same metabolites, as well as a reduction in the serum concentration of AA.

We performed a partial validation of our analytical methodology, and we found R2 values of 0.99 after constructing calibration curves using commercial standards (AA, 5-HETE, 15-HETE, 12-HETE and AA d8 amd 15-HETE d8) and also when the eicosanoids where spiked into artificial serum. In addition, we analysed the reproducibility of both peak area and retention time over a period of 48 h at 4 ºC, which was the maximum time the samples stayed in the autosampler before MS injection. We also used heavy isotopic labelled standards (AA-d8 and 15-HETE-d8) to ensure an accurate quantitation of metabolites. A serum sample from a healthy individual was used as a Quality Control (QC) sample. Each sixth sample was analysed as an QC to confirm the analytical reproducibility of the measurements.

Several methods (12-16) have been reported for the quantitation of eicosanoids in plasma or serum using mass spectrometry. Some of these methods proposed the use of solid phase extraction cartridges to both clean up and concentrate eicosanoids before MS acquisition. We decided to use a simultaneous protein crash-dilution method using cold methanol, because it allows an efficient recovery of metabolites, and the high throughput required when hundreds of samples are analysed.

We focused on the molecules produced mainly by lipoxygenases-like enzymes and found that concentration of HETE metabolites correlate with the stage of PCa. However, several and different eicosanoids might also be altered as a consequence of PCa progression and they could be potentially analysed using a similar approach. Further experiments are needed to analyse the role of leukotriens (LTs) and thromboxans (TXs) in the diagnosis and the prognosis of PCa. In addition, similar analytical methodologies could be used to study the role of these molecules after in vitro targeting of PCa cells with particular anticancer drugs.

It is known that eicosanoids like HETEs and leukotriens are bioactive fatty acids

involved in the regulation of a diverse set of homeostatic and inflammatory processes (17-19). Therefore, the question whether HETE metabolites are PCa specific, or whether they are side products of tumour progression involving inflammation processes, remains to be answered.

AA pathway in PCa tissue

The search for novel and non-invasive markers for PCa diagnosis and prognosis resulted in a new set of small molecules associated to PCa progression: HETEs. However, several metabolic reactions in the human body, including the processes involved in inflammation, can be responsible for the elevated concentration of these molecules in serum. To further evaluate the role of the AA pathway (the pathway where the HETEs are produced), we set out to identify possible proteome signatures of the enzymes producing these metabolites in PCa tissue. In **Chapter 4**, we aimed to understand whether the deregulated concentration of AA and its metabolites in serum, could be associated with a de-regulation of particular proteins in prostate tumours. In addition, using a proteomics approach, and particularly, a label free quantitation (LFQ) strategy, we aimed to identify novel molecular pathways associated to the PCa progression.
We used Orbitrap technology to identify statistically significant proteins in PCa tissue compared to normal adjacent tissue (NAP) and independently validated the expression of three proteins using tissue microarray with 481 patients. We found that normalised abundances for the proteins: AGR2, and FASN and TEBP, were higher in PCa tissue than in NAP in our proteomics dataset. In addition, we found that normalised abundances of LX15B, LKHA4 and HYES were lower in PCa, and could have a potential clinical applicability for PCa diagnosis. Further validation experiments using tissue immunohistochemistry are still required to confirm the role of these proteins in PCa prognosis.
When analysing the proteins of the AA pathway by tissue immunohistochemistry, we found that expression of LOX5 was higher in PCa, but LX15B expression was almost comparable between PCa and NAP. Although we could not detect the LOX5 protein directly in our proteomics analysis, we evaluated the tissue microarray (TMA) expression data for LOX5. We found that expression of the protein could be an indicator of biochemical recurrence (BCR) after radical prostatectomy, thus indicating a prognostic role for PCa.

Arachidonic Acid and its MS fragmentation pattern

Fatty acids like arachidonic acid (AA) ionise well at low pH to produce mass spectra in the negative mode. Several methods have been reported to quantify AA in different matrices using HPLC tandem mass spectrometry (17), and in most cases a unique fragmentation of m/z: 259.2410 is reported, corresponding to the carboxylate loss in the carbon chain. In **Chapter 5**,

we report a novel approach to study the fragmentation pattern of fatty acids to identify double bonds in the carbon skeleton. We selected AA because of its biological importance in the development of cancer, and particularly PCa. The process called remote charged fragmentation, has been successfully used using Ca^{2+} and Mg^{2+} cations for the structural characterization of peptides using mass spectrometry, and we have shown that the same cations can be used to analyse fatty acids and lipids with mass spectrometers.
Particularly, lipids are complex molecules characterised by having different numbers of carbon chains, and also different numbers of double bonds distributed along the carbon chains. These characteristics make the structural characterisation of lipids a real challenge, especially when there are no differences in mass between two or more isomers. Recently, the use of additives like Li+ and Cs+ salts have improved both the detection and the identification of lipid species in different biological matrices (20). Therefore, the proposed methodology using double charged cations in fatty acids can be extrapolated to the study of lipids with biological relevance to PCa. Further experiments are needed to understand the role of remote charge fragmentation in lipid species in several matrices such as plasma, and tissue cell lines.

AA pathway and PCa progression: A transversal readout

In this thesis, a new set of molecules possibly involved in PCa progression has been described. Despite the lack of direct clinical applicability of the HETEs as markers for PCa diagnosis, HETEs show a potential to predict PCa relapse after radical prostatectomy and these molecules might claim the role of AA pathway as a therapeutic target for the management of prostate carcinoma. We have shown that both metabolomics and proteomics results highlighted differentially expressed molecules associated to PCa (i.e. eicosanoids and proteins involved in arachidonic acid pathway and fatty acid metabolism).
In order to understand the cause of high concentration of HETEs in PCa patients, the biological relevance of the AA pathway, and the correlation of our proteomics data with available mRNA expression data, we analysed publicly available data sets for gene expression from PCa tissue and benign prostate tissue using Oncomine (21). Yang et al (10). previously reported that the mRNA level of COXs and LOXs, and the intracellular levels of the metabolite 12-HETE, are altered among androgen dependent and independent cell lines and their corresponding xenograft models. Thus, in first instance, we checked whether the high concentration of the metabolites HETEs could be caused by any over-expression of the genes belonging to the AA pathway. Six data sets were analysed and checked for RNA expression of lipoxygenases, cyclooxygenases, epoxygenases as well as other enzymes involved in the AA pathway and lipid metabolism.

Analysis of mRNA expression is presented in Supplementary Table 1. In this table, a down regulation of lipoxygenases ALOX5, ALOX12, ALOX15B is observed in PCa compared to benign prostate. For ALOX5, these results were contradictory to previous reports in literature where lipoxygenase-5 was reported as over-expressed in PCa (9, 22). In our proteomics dataset, we found an down-regulation in LX15B, which is in agreement with findings reported by Shapell et al., who proposed it as the most characteristic alteration (down-regulation of LX15B) of AA metabolism in PCa (23). In Oncomine, ALOX15 was found to be over-expressed in only one study (Arredouani (24)), but the combined analysis showed no statistical significance ($p > 0.05$). These results suggested that elevated serum concentrations of 5-HETE and 15-HETE in our PCa patients could not be explained by an up-regulation of mRNA expression of the enzymes converting AA into the above-mentioned metabolites.

By the metabolomics approach (**Chapter 3**), we also found an elevated concentration of the metabolite 11-HETE, a product of the AA metabolism produced by COX-1 and COX-2 (cyclooxygenases, GN: PTGS1 and PTGS2). It has been suggested that cyclooxygenases are associated to PCa progression. Shapell et al. found in human samples from RP specimens that expression of COX-2 was elevated only in high-grade tumours (23). In addition, Khor et al., studied the association of COX-2 expression with outcome in patients with PCa who had radiotherapy, indicating that COX-2 expression was highly associated with biochemical recurrence (25). Amirian et al. conducted a pathway-based analysis to investigate the role of putative functional AA metabolism gene polymorphisms in prostate cancer susceptibility (26). From the single-locus analyses, they have found that one SNP from each of the two major branches of AA metabolism, the prostaglandin synthesis sub pathway (PTGES2 rs10987883) and the leukotriene synthesis sub pathway (LTA4H rs1978331), may be relevant to prostate cancer risk (26).

Our analysis using Oncomine presented in Supplementary Table 1 shows that both COX-1 and COX-2 mRNA expression are down-regulated in PCa (combined analysis $p < 0.05$). Considering the controversial COX-2 expression in human PCa cells reviewed by Patel et al.(9), as well as the down-regulation of COX-2 described in Table 2, we conclude that high concentrations of the metabolite 11-HETE cannot be directly explained by an association with the level of mRNA expression of COXs enzymes.

In our targeted metabolomics study (**Chapter 3**), we found that eicosanoids concentration exhibited an outlier-like profile, so we decided to look into more detail whether the enzyme mRNA profile contains also possible outliers, for all the lipoxygenases the average values were similar between cancer and normal tissue, with the subsequent absence of statistical significance.

Interestingly, Oncomine expression data also describes an up-regulation in PTGES3 (TEBP protein), in agreement with our protemics results presented in **Chapter 3**. To the best of our knowledge, this protein has not been reported

before as marker for PCa diagnosis or prognosis, and it represents a new target for studying PCa progression.

Phospholipases: key enzymes for PCa

In this thesis, we have shown a reduced concentration of AA in serum from PCa patients in the most advanced stage of the disease. In addition, our proteomics study in PCa tissue indicated that cPLA2 is differentially abundant compared to benign prostate tissue The Oncomine results presented in Table 1 show that PA2GA (GN: PLA2G2A, p = 1.24E-5, in combined analysis), PA24A (GN: PLA2GIV, p = 0.018), PAFAH (GN: PLA2G7, p = 3.91E-7, in combined analysis) and PA2GX (GN: PLA2G10, p = 0.014) are overexpressed in PCa, thus confirming the potential role of this family of enzymes in PCa progression.

As mentioned before, it is well known that AA is mobilized from cellular membrane glycerolipid pools by the action of phospholipase A2 enzymes, PLA2, leading to the production of free fatty acids and lysophospholipids. The PLA2 enzymes consist of a family with 20 members identified in mammals. This family can be classified into 4 classes based on their nucleotide and amino acid sequence, namely sPLA2, cPLA2, calcium independent cytosolic and PAF acetyl-hydrolase enzymes (7, 27). Association of different enzymes of the PLA2 family with PCa has been described in literature (28-35). Mirtti et al. determined mRNA and protein expression of PLA2G2A during PCa progression in localized and metastatic prostate tumour. They found that both mRNA and protein expression were significantly higher in Gleason Grade 3+3 carcinoma compared to benign prostate tissues, but metastatic carcinoma expression decreased compared to primary carcinomas (33). Patel et al. studied the expression of cytosolic phospholipase A2 (PA24A) in PCa cells and they reported that the level of this enzyme was increased in androgen-insensitive PCa cell lines and suggested also that this enzyme played a role in cancer cell proliferation and apoptosis (32). PLA2G7 (PAFAH) is highly expressed in PCa (**Chapter 4**). This enzyme was for the first time identified by Vainio et al. in a set of 9783 human tissue samples and it was proposed as a potential drug target specially in ERG positive PCa (36). Further validation studies performed by the same group indicated a correlation between staining intensity for PAFAH and Gleason Score in 50% of the cases, thus suggesting this enzyme as a biomarker for PCa. The same author reported the PLA2G7 inhibition by statins, a widely-used drug to lower cholesterol levels, as a therapeutic tool for the management of the disease (37).

Considering the fact that high serum concentrations of HETEs, as well as a diminished serum concentration of AA in our PCa patients could not be explained by any alteration of mRNA or protein abundance of lipoxygenases and/or cyclooxygenases-type enzymes (PTGES1 and PTGES2); we suggest that the deregulation in concentration of AA metabolites might be associated to the deregulation of phospholipases. Thus, our proteomics results described

in **Chapter 4**, as well as the mRNA expression results presented in Table 1, fit the hypothesis that the phospholipases family can be seen as a relevant target to analyse PCa progression.
It is known that prostate tumours include a heterogeneous group of tumour growth patterns such as fused, ill-defined, cribriform and glomerular glandular structures, particularly in Gleason grade 4, and it has been shown that cribriform growth is a strong prognostic marker for distant metastasis and disease-specific death in patients with Gleason score 7 PCa (38). To date, possible correlations between arachidonic acid pathway and lipid metabolism and the above-mentioned tumour growth patterns are missing, so further experiments in vivo (either using PCa-derived xenografts models or genetically modified mice such as PTENNULL as describe in reference (39)) should be performed to analyse the role of these enzymes in PCa progression. An effective way to identify the molecular mechanisms of enzymes in the AA pathway, or enzymes involved in lipid and fatty acid metabolism, is the selective inactivation/silencing of those genes by RNAi, by the use of advanced gene editing techniques such as CRISPR-Cas9 (40), or by the use of chemical inhibitors (drugs). We have shown that our mass spectrometry methodologies are suitable for the analysis of eicosanoids, so they can be translated into in vivo experiments to further understand the role of these molecules in the development and progression of PCa.

Lipid metabolism in PCa

In **Chapter 4**, we have shown that proteins associated to lipid metabolism were deregulated in PCa tissue. From our mRNA analysis with Oncomine, we have shown that FASN is overexpressed in PCa (p=2.87E-6 in combined analysis), thus confirming the link between fatty acid/lipid metabolism and PCa progression.
FASN is probably the most extensively studied member of the lipogenic enzymes in the context of carcinogenesis. Overexpression of this enzyme has been reported in a variety of cancer including prostate, liver, ovarian, colon, endometrial and breast (41). Results from a number of studies suggest that FASN might serve as biomarker of PCa progression, with increased expression associated with a more aggressive phenotype (42). However, our results reported in Chapter 4, indicate that expression of FASN cannot be used as a predictor of biochemical recurrence, and therefore it is not a suitable prognostic marker for PCa.
It has been shown that transgenic expression of FASN in cultured PCa cells increased the rate of proliferation and inhibited apoptosis(43). Down-regulation of FASN expression by siRNA, on the other hand, attenuated growth and induced apoptosis (44). In a separated study, FASN inhibition not only suppressed cell proliferation but prevented pseudopodia formation and suppressed cell adhesion, migration and invasion. FASN inhibition also suppressed genes involved in production of intracellular second messenger AA and androgen hormones, both promoting tumour progression (45, 46).

Ahmad et al., recently reported that the transcription factor PPARG as a promoter of metastatic PCa through the activation of lipid signalling pathways, including the overexpression of enzymes such as fatty acid synthase FASN (**Chapter 4**), and acetyl co-A carboxylase (ACC). It was also reported that high levels of PPARG correlate with FASN in PCa tissue and that high levels of PPARG/FASN and PI3K/pAKT pathway activation is an indicator poor prognosis(39).

Fatty acid receptors termed peroxisome prolifelator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily of ligand inducible transcription factors. All three isotopes (PPARα, PPARβ and PPARγ) are known modulators of lipid metabolism. The important role of PPARs in carcinogenesis was highlighted by the ability of their ligand to affect cellular proliferation and differentiation or to interfere in apoptosis and angiogenesis. While different subtypes of PPARs may play a role in tumour progression in different cancer types, high level of expression of PPARG has been detected in PCa (47).

It is known that PPARγ is activated by endogenous ligands produced along the both the linoleic and arachidonic metabolism. Some of these ligands are the lipoxygenase products 13(S)-HODE and 15(S)-HETE (48). This could explain the fact that we only found high concentration of HETE metabolites in the group of patients at the most aggresive state of the disease.

Another enzyme associated to lipid metabolism identified in our proteomics in **Chapter 4** is AMACR. It is believed that increased fatty acid oxidation is a predominant phenomenon in PCa because of its role as source of energy. Thus, the overexpression of the perixomal enzyme α-methylacyl-coA racemase (AMACR) required for oxidation of branch chain fatty acids, supports this hypothesis.

PCa cells exhibit increased expression of many lipogenic enzymes, often as a result of stimulation by oncogenic signalling pathways such as PI3K/Akt and HER2 (46). Conversely, increased expression of fatty acid synthesis enzymes has been linked to activation of and nuclear localization of Akt/PKB in clinical tumour samples. Androgens, via the androgen receptor, activated the gene expression of lipogenic enzymes. Analysis of mRNA expression of lipogenic enzymes by Oncomine (Reviewed in (46)), revealed that mRNAs encoding lipogenic enzymes are increased in PCa, including those involved in de novo fatty acid synthesis (ATP citrate lyase (ACLY), acetyl-coA carboxylase-α (ACACA), fatty acid synthase (FASN), and long chain fatty acid acyl-coA synthetases (ACSL1, ACSL2, ACSL3, ACSL5). The transcription factor, sterol regulatory binding protein 1 (SREBP1) that regulates the expression of fatty acids and cholesterol signalling enzymes, was also increased in PCa.

Arachidonic acid and genetic alterations in PCa

In this thesis, we have shown that the AA pathway plays a role in the development of PCa. It is known that AA interacts with different genes and pathways already described to be involved in PCa development. Genomic loss of the PTEN locus, leading to a constitutively active PI3K/Akt pathway, occurs in 30-70% of prostate tumours. In addition, 8q amplification including the Myc gene, is present in 30% of tumours, thus representing the most common genetic alterations known in PCa (49).

Mutations in PTEN have been found in approximately 15% of primary PCa, rising to 50% of expression loss in advanced primary PCa (50), and more than 60% of patients with PCa metastasis have also been associated with alterations in PTEN (50, 51). Oxidation of PTEN by AA metabolism decreased PTEN activity, resulting in elevated PIP3 levels and increased signalling through Akt and its downstream targets (52). The AA pathway contributes to PCa progression by modulating PCa cell proliferation, apoptosis, angiogenesis and metastasis. Growing evidence suggests that the AA pathway can stimulate PI3K/Akt in PCa cells. Incubation of PCa cancer cell lines with AA causes increased prostaglandin E2 synthesis, followed by induction of PI3K-mediated Akt activation, which then leads to increased cell proliferation (53).

Inhibition of the cyclooxygenase-2 (COX-2, described above as an enzyme involved in eicosanoids production) has been shown to induce apoptosis in both androgen-responsive LNCaP and androgen-unresponsive PC-3 cells, by blocking Akt phosphorylation and by down-regulation of cyclin D1(54). As PTEN is non-functional in the LNCaP (mutation/deletion) and PC-3 (deletion) PCa cell lines, these results suggest that AA/eicosanoids could stimulate PI3K/Akt in the absence of PTEN. Several studies examining regulation of PI3K/Akt by AA or eicosanoids were conducted with pharmacological inhibition of AA or eicosanoid production, showing that AA and eicosanoids have a stimulatory effect on PI3K/Akt signalling (49).

Prostate Cancer, inflammation and diet

Arachidonic acid is one major ingredient of animal fats and the biologically active lipids from this substrate have crucial roles in chronic inflammation and cancer (19). Epidemiological, clinical and animal studies provide evidence that activation of the COX and LOX pathways during inflammation and carcinogenesis results in aberrant metabolism of arachidonic acid, which may be one mechanism involved in the contribution of dietary fats to carcinogenesis (19). The COX pathway has been suggested to be involved in PCa development and progression (9). Particularly, COX-2 has been correlated with areas of increased chronic inflammation (high number of T-lymphocytes and macrophages) and increased micro-vessel density. In addition, it has been proposed that cytokines from the local inflammatory infiltrate induces COX-2 expression in stromal

tissues and angiogenesis, and may form a link between chronic inflammation and cancer development (9).

Chronic inflammation is known to be involved in the development of cancer. In PCa, inflammation could be caused by several environmental factors, such as infections, dietary factors, hormonal changes and other environmental exposures that yet remain unknown (55). Diet has been brought forward as one of the environmental factors involved in inflammation and PCa development, although studies also showed contradictory results. High polyunsaturated fat consumption was found to increase the risk of high-grade (Gleason score 8-10) PCa, as shown in reports from the Prostate Cancer Prevention Trial(56). Linoleic acid (LA) is the major component of polyunsaturated fat diets in the Western countries, which turns into AA, the substrate for production of pro-inflammatory prostaglandins E2 and leukotriene B4. Therefore, increased intakes of polyunsaturated fats could be involved in PCa through their role in inflammation (57).

It has been proposed that pro-inflammatory mediators within the prostate can lead to a state of chronic inflammation, resulting in lesions of proliferative inflammatory atrophy that may lead to prostatic intraepithelial neoplasia (PIN) and eventually prostatic carcinoma (57, 58). Dietary fat as a possible source of inflammation is the most frequent risk factor for PCa. Although epidemiologic data on total fat consumption as well as ingested fat quality remains inconclusive, experimental data strongly suggest that essential fatty acids such as omega-6 and omega-3 polyunsaturated fatty acids that must be ingested in the diet play an important role in prostate carcinogenesis (57, 58).

The publications that link total fat consumption to PCa risk or progression remains controversial (59). Although animal studies repeatedly show that reducing dietary fat intake slows tumour growth, multiple case-control studies and cohort studies have not found any association between total fat consumption and PCa risk (59). High consumption of saturated fats such as those found in butter, lard, and animal meats do not seem to be associated with overall PCa risk, but they may be associated with a slight increase in biochemical recurrence after treatment. It is hypothesized that saturated fats may lead to increased circulating IGF-1, which in turn leads to PCa progression, although this remains speculative (59, 60).

Emerging data suggest that omega-3 fatty acids, which are found primarily in cold water oily fish like tuna, salmon, herring, and swordfish, as well as flaxseed, may slow the growth of many tumours, including prostate (61). In vitro and animal studies suggest that these fats induce anti-inflammatory, pro-apoptotic, anti-proliferative, and anti-angiogenic pathways making them a perfect anti-tumour molecule. A phase II prospective randomized trial of a low-fat diet with fish oil supplementation in men undergoing radical prostatectomy showed omega-3 supplementation 4–6 week prior to RP decreased PCa proliferation (56), but further experiments in vitro and in vivo have to be performed to understand

molecular mechanisms of omega-3 fatty acids and tumour progression.
Chronic inflammation that is associated with different types of cancers, is typically associated with increased NF-kB activity and it is casually linked to tumour progression. It is known that PPARg agonist the production of pro-inflammatory signalling proteins such as TNF, IL-6 and MCP-1 could be inhibited by PPARG agonist. PPARG is expressed in tumour cells and infiltrating immune cells, and there is evidence that anti-inflammatory activities are mediated by PPARG in many cell types (62).
Brasky et al. reported that, within the Selenium and Vitamin E Cancer Prevention Trial, plasma phospholipid levels of long-chain omega-3 fatty acids (including eicosatetraenoic acid (EPA), docosapentaenoic acid (DPA), and docosapentaenoic acid (DHA)) measured in blood samples collected at baseline are positively associated with subsequent risk of low-grade, high-grade and total PCa (63). These observations have been considered incompatible with previous hypotheses suggesting that omega-6 fatty acid promotes tumour development; the latter because AA metabolites have pro-inflammatory and pro-angiogenic effects. On the other hand, it is believed that omega-3 fatty acids are thought to protect from cancer because they are substrates for the same metabolic enzymes used by omega-6 derived eicosanoids (64). Further work using animal models should be performed to analyse the role of a diet rich in omega-3 fatty acids on PCa tumour development. Particularly, the use of PCa xenografts models, where the tumour can be genetically and biochemically characterised, could be used to understand molecular mechanisms behind the fatty acid metabolism and PCa progression.

Concluding remarks

In this thesis, we first wondered whether small molecules in serum could distinguish between healthy individuals and PCa patients. Although we only analysed two pathways of the metabolome, we could conclude that the concentrations of tryptophan and kynurenine, both belonging to the tryptophan pathway, cannot be used as diagnostic markers of PCa. On the other hand, and taking advantage of the state of the art analytical techniques such as liquid chromatography and mass spectrometry, we have shown that serum concentrations of hydroxyeicosatetraenoic acids (HETEs), a class of eicosanoids produced along the AA pathway, are associated with the stage of the disease.
We also questioned whether the proteins producing the HETEs eicosanoids, could play a role in the diagnosis and prognosis of PCa, and we performed a proteomics screening in PCa tissue. We found that some enzymes involved in fatty acid and lipid metabolism (FASN, LOX5, LX15B, TEBP, LKHA4 and HYES), are deregulated in PCa tissue, and also that immunostaing of LOX5, and enzyme of the AA pathway, can predict biochemical recurrence of PCa after radical prostatectomy.

We believe that the experimental approaches used in this thesis can be incorporated directly in the study of PCa, by complementing the information obtained by gene sequencing, RNA expression, epigenetic profiling, and all the other techniques used nowadays to understand the origin and the development of prostate cancer. Our contribution to the field is the discovery of novel molecules within the AA pathway, that could be both markers for diagnosis and prognosis, as well as therapeutic targets for the disease. Further research is required to understand the molecular mechanisms associated with the arachidonic acid, the eicosanoids, and the enzymes involved in the transformation of these metabolites.

Although the results described in this thesis do not have any potential application in the clinic so far, we believe that both the eicosanoids and the proteins in the AA pathway will have an impact on future research in the field. Recent technical improvements in mass spectrometry and liquid chromatography, will be of interest for the analysis of eicosanoids from serum or plasma from PCa patients at particular moments of the treatments (diagnosis, before surgery, after surgery, or response to treatment). In addition, targeted proteomics profiling of AA proteins in PCa biopsies should open ways for choosing an optimal treatment for PCa and/or to evaluate the response towards a cancer therapy.

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Supplementary Information

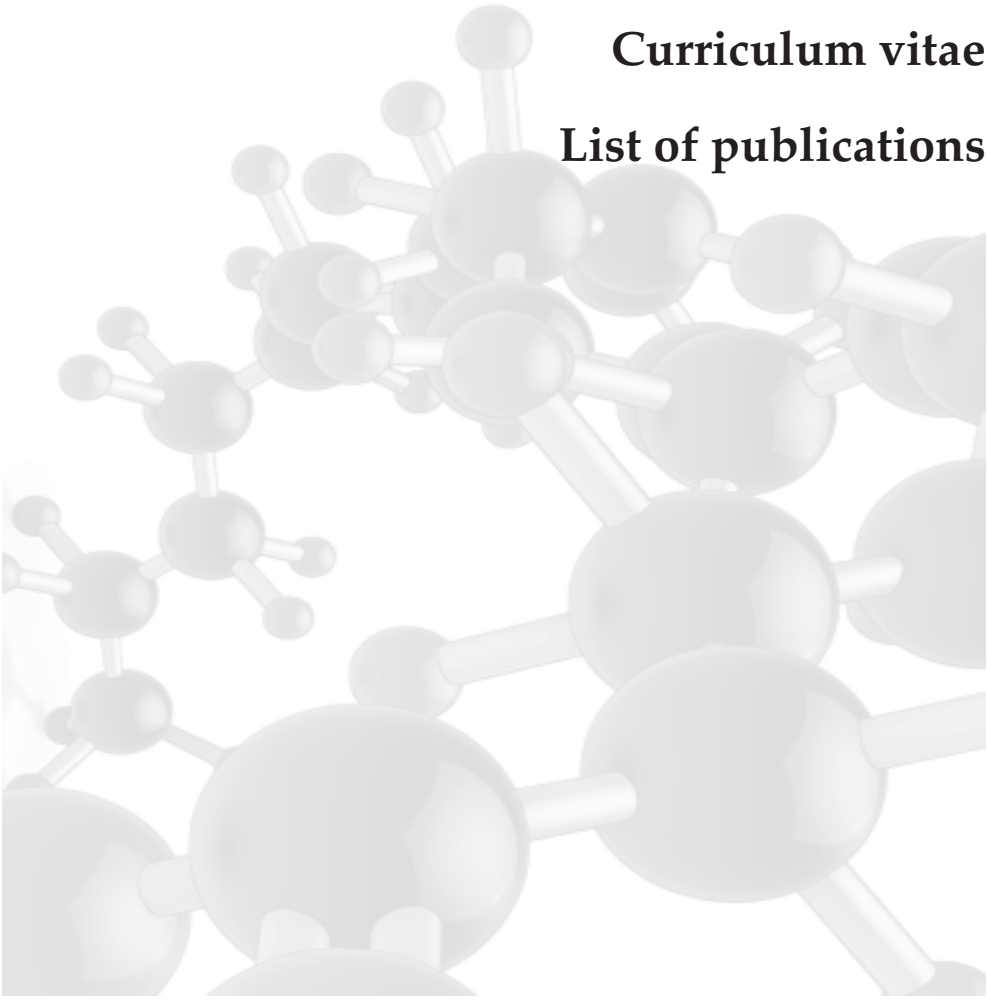
Supplementary Table 1. mRNA expression of Arachidonic acid pathway enzymes in normal and prostate cancer.

Gene	Study	Fold change	p-value	Meta-analysis	PCa	NAP
		PCa/ NAP		p-value	(N)	(N)
ALOX5	LaTulippe (65)	-1.535	0.013		23	3
	Tomlins (66)	-2.241	4.23E-5	0.007	29	22
ALOX12	Luo (67)	-8.838	0.004	NA	15	15
ALOX15	Singh (68)	-1.769	0.016		52	50
	Arredouani (24)	1.582	2.93E-4	0.508	13	8
ALOX15B	Arredouani (24)	-2.905	4.40E-2		13	8
	Lapointe (69)	-1.544	1.22E-4	0.022	60	40
COX-1	Varambally (70)	-10.513	4.27E-4		7	6
	Grasso (71)	-2.803	3.44E-4		59	28
	Singh (68)	-1.672	7.00E-3	0.007	52	50
COX-2	Vanaja (72)	-2.826	1.50E-2		27	8
	Arredouani (24)	-2.497	5.00E-3		13	8
	Wallace (73)	-1.694	6.00E-3		69	20
	Tomlins (66)	-1.724	4.70E-2	0.031	30	22
PTGES3	Lapointe (31)	1.093	0.012		62	40
	TCGA	1.011	0.016		61	45
	Singh (30)	2.419	7.56E-4		50	50
	Tomlins (28)	1.902	0.005		30	23
	Vanaja (34)	1.415	0.012		27	8
	Luo (29)	1.202	0.05	0.027	15	15
LTAH4	LaTulippe (65)	-1.298	4.71E-5		23	3
	Holzbeierlein	-1.257	0.008		35	3
	Wallace (73)	-1.151	0.028	0.018	69	20
EPHX2	TGCA	-1.16	5.12E-6		61	45
	Tomlins (66)	-1.98	0.005		30	23
	Arredouani (24)	-1.707	0.016		13	8
	Grasso (71)	-1.225	0.036	0.005	59	28
PLA2G2A	Singh (68)	4.608	4.23E-6		52	50

	Lapointe (69)	2.922	2.05E-5		62	40
	Tomlins (66)	4.333	6.00E-3		30	23
	Holzbeierlein (74)	2.332	3.00E-3	1.24E-5	35	3
PLA2G4	Welsh (75)	-1.675	4.41E-4		25	9
	Varambally (70)	-1.641	5.00E-2		7	6
	Arredouani (24)	-1.56	1.80E-2	0.018	13	8
PLA2G7	Singh (68)	5.155	3.70E-14		52	50
	Welsh(75)	4.463	7.17E-7		25	9
	Vanaja (72)	2.889	1.16E-7		27	8
	Grasso (71)	2.628	2.21E-6		59	28
	Lapointe (69)	2.238	6.46E-8		41	60
	Taylor (76)	1.917	5.34E-6		131	29
	Liu (77)	1.663	5.31E-5		44	13
	Yu (78)	1.592	9.21E-7		65	23
	Arredouani (24)	2.346	5.00E-3		13	8
	Luo (67)	2.193	1.70E-2		15	15
	Varambally (70)	2.003	5.00E-3		7	6
	Holzbeierlein (74)	1.696	2.63E-4	3.91E-7	31	2
PLA2G10	Varambally (70)	-1.906	1.40E-2	NA	7	6
PTEN	Arredouani (24)	1.515	5.20E-5	NA	13	8
	Lapointe (69)	-1.722	1.57E-10		62	40
	Singh(68)	-1.638	3.18E-4		52	50
	Magee(79)	-1.52	8.00E-3	3.18E-4	8	4
FASN	Singh (68)	5.644	1.96E-6		52	50
	Welsh (75)	3.211	4.90E-8		25	9
	Vanaja (72)	3.198	7.65E-5		27	8
	Yu (78)	2.141	4.19E-6		65	23
	Taylor (76)	1.564	2.87E-6		131	29
	Luo (67)	4.662	4.80E-2		15	15
	Wallace (73)	3.681	1.55E-4		69	20
	Magee (79)	3.243	1.62E-4		8	4
	Varambally (70)	1.852	7.00E-3		7	6
	Liu (77)	1.852	3.00E-3		44	13
	Grasso (71)	1.638	2.00E-3	2.87E-6	28	59
PPARG	Varambaly (70)	-15.837	1.20E-4		7	6
	Grasso (71)	-1.807	4.46E-4		28	59

	Taylor (76)	-1.246	2.00E-3		131	29
	Lapointe (69)	-1.174	6.50E-2	1.00E-3	62	40

Appendices



The Arachidonic Acid Pathway: A potential application in the diagnosis and prognosis of prostate cancer

Summary

Prostate cancer (PCa) is the most common cancer in men, and in 2012 more than 400,000 cases were diagnosed in Europe. Although the introduction of the PSA test improved the management of the disease, its low specificity has led to overdiagnosis and unnecessary treatments for men considered to be at low risk for progression of the disease. Thus, novel and reliable molecular markers are needed to improve the diagnosis of PCa and also to better differentiate indolent cases of PCa from those that will recur after initial treatment.

In this thesis, we aimed to identify and validate novel metabolites and proteins markers in tissue or bio-fluids, which could improve the diagnosis and/or the prognosis of PCa. We used analytical approaches to study the role of selected metabolites and proteins, particularly along the arachidonic acid (AA) pathway.

We used targeted metabolomics approaches in serum to evaluate whether concentrations of molecules in two pathways could be used as markers for PCa. For tryptophan (Trp) and one of its metabolites, kynurenine (Kyn), we used an HPLC method with fluorescence detection on serum samples from older men having low PSA values and no PCa, as well as subjects diagnosed with PCa. We could not identify statistical differences between the groups and thus we concluded that neither the concentrations of these metabolites, nor the ratio Kyn/Trp, could be used as markers for PCa.

Next, we used a UHPLC-MS/MS method to evaluate the concentrations of AA and some of its metabolites in serum from PCa patients at different stages of the disease. We developed a method for the simultaneous quantification of six metabolites belonging to the AA pathway, and we used the results to evaluate PCa diagnosis. Interestingly, we found that a selected group of patients had higher concentrations of hydroxyeicosatetraenoic acid (HETE) metabolites, compared to the control group. We then used the same methodology on an extended and well defined group of patients having different stages of PCa, and we found that concentrations of HETE metabolites are associated with the most aggressive status of the disease. Concomitantly, we also found a reduction in the AA concentration in the same group of patients and these results (which may be related to each other) could be used as an indicator of PCa relapse after radical prostatectomy.

In tissue, we used label free quantitative proteomics to identify proteins in PCa that are associated to diagnosis and prognosis and also to evaluate whether the altered concentrations of AA and its metabolites in serum could be associated with a deregulation of particular proteins in the arachidonic acid (AA) pathway. We analysed the protein fraction from RNA isolation of 67 PCa tissue samples, consisting of 33 normal adjacent tissue (NAP) samples and 34 PCa samples,

using nano-LC high resolution mass spectrometry. In addition, we validated the expression of four proteins by immunohistochemistry and we used tissue microarrays (TMA) to evaluate the prognostic performance of these markers. Remarkably, we found that FASN, AGR2, and one protein of the AA pathway: TEBP, were highly upregulated in PCa. In addition, TMA indicated that both low percentage of positive AGR2 tumour cells and low percentage of positive LOX5 tumour cells , are predictors of biochemical recurrence after radical prostatectomy. These results highlight the role of proteins in the AA pathway in the diagnosis and prognosis of PCa.

We have shown that proteins and metabolites in the AA pathway play a key role in PCa. To complement the structural characterisation of AA in mass spectrometry, we studied the fragmentation of AA in the presence of metal ions such as Ca^{2+} and Mg^{2+}. We showed that the addition of these metal ions improved the fragmentation of AA in the gas-phase, and in particular such fragmentations provide the exact location of the double bonds in the carbon chain. Thus, this strategy could be further used to analyse extended sets of fatty acids and lipids using mass spectrometry, and also their role in diseases like cancer.

We conclude that we have shown that the experimental methodologies used in this thesis help to understand the role of the fatty acid metabolism and arachidonic acid pathway in PCa. Key metabolites such as HETEs and AA, as well as enzymes along this pathway, could improve the diagnosis and prognosis of PCa, and they could also become important therapeutic targets of the disease.

De arachidonzuur metabole route: Een mogelijke toepassing voor diagnose en prognose van prostaatkanke

Samenvatting

Prostaatkanker (PCa) is bij de man de meest voorkomende vorm van kanker, in Europa werden in 2012 meer dan 400.000 gevallen gediagnosticeerd. Hoewel de introductie van de PSA test de behandeling verbeterde, leidt de lage specificiteit van de test tot overdiagnose en dus tot onnodige behandelingen van mannen die een laag risico hebben op progressie van de ziekte. Daarom zijn nieuwe en betrouwbare moleculaire markers nodig om de diagnose van PCa te verbeteren en om indolente patiënten te onderscheiden van hen die metastase krijgen.
In dit proefschrift proberen wij nieuwe marker metabolieten en marker eiwitten te identificeren in weefsel of lichaamsvloeistoffen, die de diagnose en/of prognose van PCa kunnen verbeteren. Daartoe hebben wij analytische methoden ontwikkeld om de rol van sommige metabolieten en eiwitten te onderzoeken, in het bijzonder de moleculen verbonden aan de arachidonzuur metabole route.
Wij hebben gerichte metabolomics benaderingen in serum toegepast om na te gaan of de concentraties van moleculen in twee verschillende metabole routes gebruikt kunnen worden voor de diagnose en prognose van PCa. Voor tryptofaan en voor een van zijn metabolieten (kynurenine) hebben we een HPLC methode toegepast met fluorescentie detectie. Serum monsters zijn gebruikt van enerzijds oudere mannen met lage PSA waarden en geen PCa en anderzijds van mannen met PCa. Er werden geen statistische verschillen gevonden tussen deze twee groepen en wij concluderen dan ook dat de concentraties van deze metabolieten (of hun verhouding) niet gebruikt kunnen worden als markers voor PCa.
Vervolgens hebben wij een UHPLC-MS/MS methode toegepast om de concentraties van arachidonzuur en enige van zijn metabolieten te meten in serum van PCa patiënten in verschillende stadia van de ziekte. Wij hebben een methode ontwikkeld om zes metabolieten van de arachidonzuur metabole route kwantitatief en gelijktijdig te meten en de resultaten hebben we gebruikt om de PCa diagnose te evalueren. In een kleine studie vonden wij dat een groep patiënten hogere concentraties van hydroxyeicosatetraenoic zuur (HETE) metabolieten hadden, vergeleken met de controle groep. Daarna hebben we dezelfde methode toegepast op een grotere en meer gedefinieerde groep patiënten, nu met verschillende stadia van PCa. We vonden dat een aantal patiënten met de meest agressieve vorm van PCa hoge concentraties hadden van deze HETE metabolieten en tegelijkertijd dat deze patiënten een lagere concentratie hadden van het arachidonzuur zelf. Deze, waarschijnlijk aan elkaar gerelateerde resultaten, kunnen gebruikt worden om recidief PCa na behandeling met radicaal prostatectomie te voorspellen.
In PCa en prostaat weefsel hebben wij kwantitatieve proteomics gebruikt om eiwitten te identificeren die geassocieerd zijn met diagnose en prognose. Tevens

hebben wij geëvalueerd of een verandering in concentratie van arachidonzuur in verband gebracht kan worden met een ontregeling van bepaalde eiwitten verbonden aan de arachidonzuur metabole route. Daartoe hebben wij van 67 PCa weefselmonsters RNA isolatie toegepast en in de eiwitfractie daarvan hebben wij, met proteomics technieken, eiwitten geïdentificeerd. Deze weefselmonsters bestonden uit 33 monsters van normaal aangrenzend weefsel en 34 PCa monsters. Van vier gevonden eiwitten hebben wij de expressie gevalideerd met immuunhistochemie en tevens hebben we microarrays van weefsel gebruikt om de prognostische waarde van de gevonden markers te evalueren. Een opmerkelijk bevinding was dat de productie van de eiwitten FASN, AGR2 en één eiwit van de arachidonzuur metabole route, namelijk TEBP, in patiënten met PCa significant gestimuleerd is. De experimenten met de microarrays van weefsels toonden ook aan dat een laag percentage van positieve AGR2 tumorcellen, als ook een laag percentage van LOX5 tumorcellen, voorspellers zijn voor recidief PCa na behandeling met radicaal prostatectomie.
In dit proefschrift hebben wij aangetoond dat bepaalde eiwitten en metabolieten in de arachidonzuur metabole route een belangrijke rol spelen in PCa. Om de karakterisatie van de structuren van de arachidonzuur metabolieten te verbeteren hebben wij de fragmentaties van arachidonzuur en verwante moleculen bestudeerd in aanwezigheid van de metaalionen Ca^{2+} en Mg^{2+}. Toevoeging van deze metaalionen leidde tot structuurkarakteristieke fragmentaties waarbij met name de posities van de dubbele bindingen in de moleculen bepaald kunnen worden. Deze strategie kan verder geoptimaliseerd worden om vetzuren en lipiden beter met massaspectrometrie te identificeren om hun rol in bijvoorbeeld kanker beter in beeld te brengen.
Wij menen te hebben aangetoond dat de experimentele methodieken ontwikkeld in dit proefschrift kunnen bijdragen om de rol van het vetzuurmetabolisme en de rol van de arachidonzuur metabole route in PCa beter te kunnen begrijpen. Het kwantificeren van sleutelmetabolieten, zoals de hydroxyeicosatetraenoic zuren en arachidonzuur, als ook de enzymen geassocieerd met de metabole routes, kunnen de diagnose en prognose van PCa verbeteren en kunnen zij belangrijke therapeutische doelen worden in de bestrijding van PCa.

Acknowledgements

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From Guido's lab, I would like to write some words to acknowledge Mirella, Elena, Ines, Rene, Diederick and Diana. Thanks for your help, and for letting me know the cool things of your research.

Living in foreign country is a difficult thing, but it becomes a little bit easier when you find people taking your own language and face similar challenges. I would like to thank to Francisco, Lady Catherine, Dayan, Adriana B, Carolina, Sandra, Paula, Monica P, Adriana I, Cesar, Ivan, Monica N, Karol and Liz. Thank you all for the support and the fun moments.

En algun momento decidi iniciar un camino lejos de mi casa, sacrificando muchas cosas y luchando dia a dia para lograr mis objetivos. Esto no hubiera sido possible sin el apoyo incondicional de mis padres Leonor y Francisco. Infinitas gracias por estar ahi siempre, y por querer siempre lo mejor para mi. La responsabilidad, el compromiso y la disciplina se aprenden en casa, y ustedes son mi mejor ejemplo para salir adelante a pesar de cualquier adversidad. Los quiero con el alma y esta tesis es para ustedes.

Al comienzo viajaba una vez al mes a Francia a ver a mi hermana Claudia y a mi cuñado Antoine para recargar baterías y volver al lab a trabajar en la tesis. Luego llegó Anais con su sonrisa y mis motivos para ser feliz se multiplicaron por mil. Ahora Santiago es la chispa de picardía que complementa a la perfección esa familia tan bonita que siempre me apoya y me dio su cariño. Mercy beaucoup pequeños!

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Curriculum Vitae

"The Arachidonic Acid Pathway: A potential application in the diagnosis and prognosis of prostate cancer", summarizes several years of dedicated research, which made Giovanny a committed scientist with valuable knowledge in mass spectrometry and its application to key fields like cancer research. However, his passion for science started much earlier.

Giovanny Rodriguez Blanco was born on September 8, 1984 in Bogota, Colombia. He studied Chemistry and obtained a Master degree in Physical Chemistry in 2008. After few years working as a Chemistry assistant teacher in different universities in Colombia, such as Universidad Nacional de Colombia and Universidad de los Andes, he moved to Rotterdam, the Netherlands to start his PhD under supervision of Dr. Theo Luider and Prof. Guido Jenster at the Erasmus MC. Here, he was trained in mass spectrometry for biomedical applications and particularly, he applied this technique to identify and validate novel biomarkers for prostate cancer diagnosis and prognosis. The results of his research at the Erasmus MC form the basis of this thesis.

In 2014, he moved to Edinburgh, UK, and continued his training at the Institute of Genetics and Molecular Medicine, IGMM. In a short period as postdoctoral fellow, he studied the metabolic regulation of epigenetic reactions using mass spectrometry.

In 2015, he joined the Phenome Centre Birmingham, UK, as a postdoctoral fellow and developed the analytical methods for the analysis of metabolites in bio-fluids and tissues using untargeted and targeted metabolomics approaches. Those methodologies are currently used in different institutions worldwide for metabolic phenotyping.

Recently, Giovanny has returned to Scotland, and he has joined the Mass Spectrometry and Proteomics facility at the Beatson CRUK institute in Glasgow. He is involved in several projects deciphering the role of proteins and small molecules in the molecular mechanisms of cancer development and progression.

List of Publications

1. Chetwynd, A. J., Dunn, W. B., and **Rodríguez-Blanco, G**. (2017) Collection and Preparation of Clinical Samples for Metabolomics. *Advances in experimental medicine and biology* 965, 19-44
2. Partolina, M., Thoms, H. C., MacLeod, K. G., **Rodríguez-Blanco, G.**, Clarke, M. N., Venkatasubramani, A. V., Beesoo, R., Larionov, V., Neergheen-Bhujun, V. S., Serrels, B., Kimura, H., Carragher, N. O., and Kagansky, A. (2017) Global histone modification fingerprinting in human cells using epigenetic reverse phase protein array. *Cell Death Discovery* 3, 16077
3. Lawson, T. N., Weber, R. J., Jones, M. R., Chetwynd, A. J., **Rodríguez-Blanco, G.**, Di Guida, R., Viant, M. R., and Dunn, W. B. (2017) msPurity: Automated Evaluation of Precursor Ion Purity for Mass Spectrometry-Based Fragmentation in Metabolomics. *Anal Chem* 89, 2432-2439
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