Identifying Genetic Biomarkers for Diagnosis of Prostate Cancer in South African men

by

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Declaration

I, Samkele Azola Salukazana, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgment indicates otherwise) and that neither the whole work nor any part of it has been, is being, or is submitted for another degree in this or any other university.

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Abstract

Background and Aim: Prostate cancer (PCa) is the leading cancer diagnosis amongst South African men. The incidence of PCa is 68.0 per 100 000 Age Standardized Rate (ASR) and the mortality rates are 27.9 per 100 000 ASR, Globocan 2018. Diagnosis of PCa is based on a combination of digital rectal examination, prostate-specific antigen (PSA) and histology. Several biomarkers have been used to increase the sensitivity and specificity of PSA in distinguishing patients with PCa from those with benign prostatic hyperplasia (BPH). These include fractionated PSA, free/total PSA ratio, −2proPSA, prostate cancer antigen 3 and prostate health index, amongst others. Biomarkers are needed to differentiate BPH from PCa due to a lack of specificity of these markers with PSA levels above 4.0 ng/ml. The aim of this study is to investigate gene expression patterns of South African men in 9 PCa and 10 BPH patients in order to distinguish between the two groups.

Methods: Ethical approval was obtained (HREC 454/2012). Patients scheduled for transurethral resection of the prostate were recruited from the Western Cape. RNA was extracted from prostate tissue using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). Complementary DNA was synthesized from RNA using the SuperScript IV VILO Master Mix (Thermo Fischer Scientific). Gene expression was analyzed with the Human Prostate Cancer RT^2 Profiler PCR Array and SYBR Green Master Mix. Data were analyzed with the GeneGlobe RT^2 and miScript PCR Array Data Analysis Centre from Qiagen.

Results: The cohort included patients from different ethnic groups namely, Caucasians, Mixed- and African ancestry. The PCa group has an age range from 56 to 75 years (mean 65) while the BPH group was slight older ranging from 60 to 76 years (mean 68). PSA levels range from 24 to 5000 ng/ml (mean 1252 ng/ml, median 185) for the PCa group and 11 to 58 ng/mL (mean 25 ng/ml, median 22) for the BPH group. The following genes were downregulated 2-fold in the PCa group with p values <0.05, *IGF1*, *PTEN*, *GSTP1*, *SOCS3*, *EGR3*, *GPX3*, *TIMP3*, *ZNF185*, *DKK3*, *PTGS2*, *FOXO1*, *ARNTL*, *TNFRSF10D*, *CCND1*, and *DLC1*, upregulated genes included *CDH1*, *MKI67*, *TMPRSS2*, *ERG*, *CDKN2A*, *FASN*, and *AR* but were not statistically significant. At a fold-change threshold of 1.5, the following additional genes were downregulated in the PCa group with p values <0.05, *DAXX*, *EGFR*, *RASSF1*, *SOX4*, and *TIMP2*, upregulated genes were *ACACA*, *AR*, *CDKN2A*, *ERG* and *FASN* but were also not statistically significant. The study shows similarly differentially expressed genes as seen in international studies. Of note *PTEN*, *MKI67* and *FASN* which are associated with poor

prognosis. *EGR3* was downregulated in our study and this has been associated aggressive disease and predict relapse after PCa treatment. This could explain the high mortality demonstrated in South African epidemiological studies.

Conclusion: We identified a group of differentially expressed genes that have potential in distinguishing PCa and BPH patients with PSA values above 10 ng/ml. A larger population study is needed to further evaluate the clinical significance of our findings.

Acknowledgements and contributions

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Lastly, to my late mother, Zoliswa Salukazana, the man I am today is a reflection of your sacrifices and love.

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Abbreviations

PCa: Prostate cancer

ASR: Age Standardized Rate

BPH: Benign prostatic hyperplasia

PSA: Prostate-specific antigen

DRE: Digital rectal examination

LUTS: Lower urinary tract symptoms

PHI: Prostate Health Index

PCA3: Prostate cancer antigen 3

TURP: Transurethral resection of the prostate

USC: Urinary sediment cells

RNA: Ribonucleic acid

DNA: Deoxyribonucleic acid

PCR: Polymerase chain reaction

C_T: Cycle threshold

Chapter 1. Introduction and Literature review

1.1. Introduction: Prostate Cancer in South Africa

Prostate cancer (PCa) is the second most common non-cutaneous malignancy diagnosed in men, following lung malignancy. It is the fifth leading cause of cancer death in men worldwide [1]. Similar (high) PCa incidences are reported in America, Europe and some parts of Sub-Saharan Africa, however, the mortality rates reported in America and Europe are low compared to Sub-Saharan regions (South Africa, Zambia and Zimbabwe) and the Caribbean (Barbados, Jamaica, and Haiti), **Figure 1**. PCa incidences vary by race or ethnicity. African Americans have 59% higher incidence rates than Caucasian Americans [2]. Mortality rates are generally higher in African populations [Caribbean, 12.3-48.0 per 100,000 Age Standardized Rate (ASR) and Sub-Saharan Africa, 14.0-36.3 per 100,000 ASR], intermediate in America (7.7-14.0 per 100,000) and lower in Asia (2.9 per 100,000 in South-Central Asia) [1].

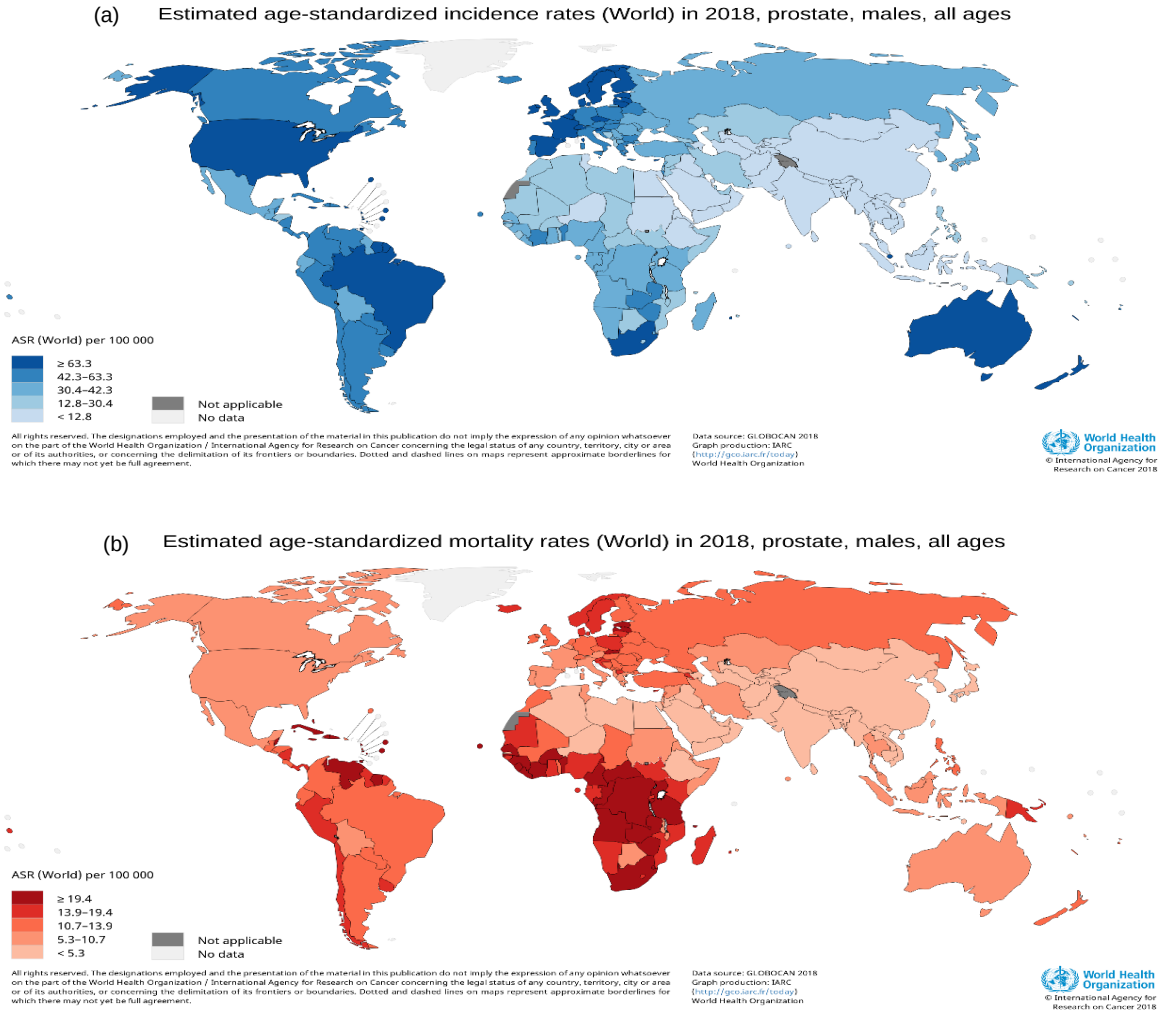

Figure 1. Global maps presenting the estimated (a) the incidence of PCa and (b) age standardized mortality rates, 2018 [1].

PCa is the leading cancer diagnosis amongst South African men. According to the International Agency for Research on Cancer (IARC), Globocan 2018, 12 452 new cases were diagnosed in 2018 [3]. The incidence of PCa is 68.0 per 100 000 ASR, and the mortality rates are 27.9 per 100 000 ASR in South Africa [1]. Black South African men have been shown to have higher PSA values, higher clinical and pathological stages (Gleason score>7 and poorly differentiated tumors) at diagnosis [4, 5]. Reasons for these findings are thought to be multifactorial; these include delayed presentation, different health-seeking behaviors which may be cultural related, lack of screening practices and biological factors which are likely genetic [4, 5].

Higher mortality rates and incidence of PCa reported in populations of African descent (Sub-Saharan Africa) makes the study of PCa genetics in South Africa imperative.

1.2. PCa Diagnosis

The diagnosis of PCa is based on a PSA level and DRE. Even a suspicious DRE has shown a positive predictive value of only 5 to 30% in patients with PSA values below 4ng/ml [6]. Histological verification is, therefore, necessary to confirm the diagnosis of adenocarcinoma on prostate biopsy cores. A prostate biopsy is an invasive procedure that may be associated with complications such as bleeding, urinary retention, prostatitis, fever and may require hospitalization.

Prostatic acid phosphatase was the first biomarker used for the diagnosis of PCa. With the discovery of PSA, it was rapidly replaced because of its poor sensitivity for diagnosis and follow up of PCa [7]. PSA is a glycoprotein primarily produced by prostatic luminal epithelial cells and is expressed by both normal and neoplastic prostate tissue. Higher levels of PSA are seen in PCa patients. Serum PSA levels are used for screening and diagnosis of PCa, with a high sensitivity in predicting PCa but with a low specificity as a marker for PCa [8]. PSA is organ-specific but not cancer-specific, therefore, it may be elevated in benign prostatic hyperplasia (BPH), prostatitis and other non-malignant conditions. Upper limits of normal for PSA are regarded as 4 ng/ml, while levels above 4ng/ml are suspicious for malignancy [9]. For PSA levels between 4.0 and 10.0ng/ml, the positive predictive value is about 25% and nearly 75% of cancers detected in this zone are all confined and potentially curable [10]. The serum PSA is more useful for determining the extent of PCa and assessing the response to PCa treatment. Although its use in screening still remains a controversial topic, it has been associated with early diagnosis and a 21% reduction in mortality on long term follow up studies [11]. BPH is a pathological process characterized by an increase in the number of epithelial

and stromal cells in the periurethral area of the prostate. Similar to PCa, BPH is usually associated with lower urinary tract symptoms (LUTS) caused by bladder outlet obstruction. There is a strong association between serum PSA and prostate volume in men with BPH and the relationship depends on age [12]. Twenty-eight percent of men with a histological proven BPH have serum PSA's above 4ng/ml [13]. Therefore, there is a significant overlap in serum PSA values between men with BPH and clinically localized PCa. Localized PCa may also coexist with BPH.

1.3. Biomarkers for PCa diagnosis

Several biomarkers have been shown to improve the sensitivity and specificity of PSA in the diagnosis of PCa. These include serum-, urine- and tissue-based biomarkers (**Table 1**). Some of these biomarkers can also be used to select for repeat biopsies in men with an elevated risk of PCa and a prior negative biopsy.

1.3.1. Serum-based biomarkers

Free/total PSA ratio, PSA kinetics, and other markers have been used to increase the sensitivity of PSA in diagnosing PCa. The free/total PSA ratio has shown a low sensitivity and specificity alone in diagnosing PCa, with a sensitivity of 70% in men with PSA between 4 and 10ng/ml [14]. PSA velocity (absolute annual increase in serum PSA in ng/ml/year) and PSA doubling time (the time in months for PSA level to double) have limited value in PCa diagnosis. Additional serum tests have also been used to improve the diagnostic accuracy of PSA for PCa. These tests include [-2]proPSA, prostate health index (PHI, total PSA, free PSA and [-2]proPSA) and the 4 kallikrein score (total PSA, free PSA, intact PSA and human kallikrein-related peptide 2). These markers have been shown to be the strongest predictors of PCa on initial or repeat biopsies compared to total PSA and %free PSA alone [15-17].

Table 1: Summary of currently available biomarkers for use in prostate cancer detection or stratification

Name of test	Marker Description	Biomaterial	Indication	FDA	Accuracy
Serum-based biomarkers					
proPSA & Prostate Health Index	Total PSA, fPSA, p2PSA	Blood Serum	Diagnosis/Prognosis	Yes	AUC 0.703 Spec 16% Sens 95%
4K score test	Total PSA, fPSA, intact PSA, hK2	Blood plasma	Diagnosis/Prognosis	No	AUC 0.82
Urine-based biomarkers					
Prostate cancer antigen 3 (Progensa)	PCA3, PSA, mRNA	Post-DRE urine	Diagnosis: re-biopsy setting	Yes	AUC 0.68-0.87
TMPRSS2-ERG	TMPRSS2-ERG	Post-DRE urine	Diagnosis: re-biopsy setting	No	Sens 24.3-37%, Spec 93% PPV 94%
Mi(chigan) Prostate Score	PCA3 and TMPRSS2-ERG mRNA, serum pPSA	Post DRE first void urine	Diagnosis/Prognosis	No	AUC 0.88
Select MDx	HOXC6, DLX1, KLK3 mRNA	Post-DRE first void urine	Diagnosis/Prognosis	No	Sens 91%, Spec 36%, NPV 94%, PPV 27%, AUC 0.76
Tissue-based biomarkers					
ConfirmMDx	DNA methylation of GSTP1, APC, & RASSF1 genes	Benign prostate biopsy	Diagnosis	No	NPV 96%
Oncotype DX	12 cancer related genes, 5 reference genes	Prostate cancer tissue	Prognosis	No	Not reported
Prolaris score	31 cell cycle progression genes	Prostate cancer tissue	Prognosis	No	Not reported
Decipher	22 RNA biomarkers	Prostate cancer tissue	Prognosis	No	AUC 0.79

Abbreviations: 4K, four-kallikrein panel; TMPRSS2-ERG, transmembrane protease serine 2-ERG; hK2, human kallikrein peptidase 2; AUC, area under the curve; FDA, US Food and Drug Administration.

Table compiled from [18] and [19]

1.3.2. Urine-based biomarkers

Several urine biomarkers also have the potential to improve the diagnostic accuracy of PCa, mainly to select men for a repeat biopsy after an initial negative biopsy. These markers include prostate cancer antigen 3 (PCA3), *TMPRSS2-ERG* fusion, SelectMDX, Mi (chigan) Prostate score (MiPS) and ExoDx (**Table 1**). PCA3 is a prostate-specific long non-coding RNA (lncRNA) biomarker that is detectable in urine sediments obtained after three strokes of a prostatic massage during DRE. *TMPRSS2-ERG* fusion, a fusion of the transmembrane protease

serine 2 (*TMPRSS2*) and the *ERG* gene are frequently detected in PCa. *TMPRSS2-ERG* fusion has been shown to be highly specific (93-99%) for predicting PCa and clinically significant PCa on biopsy [20, 21]. When detection of *TMPRSS2-ERG* fusion in urine is added to PCA3 expression and serum PSA (MiPS), cancer prediction improves significantly [21]. The use of this marker in our African population is questionable because the frequency of *TMPRSS2-ERG* fusion in PCa has shown notable racial disparities, the highest prevalence in men of European ancestry (49%), followed by Asian (27%) and African ancestries (25%) [22]. A recent South African PCa study showed a frequency of only 13% for *TMPRSS2-ERG* fusion in tumors from Black South Africa men. Additionally, an inverse relation was found for the acquisition of *TMPRSS2-ERG* fusion with aggressive PCa [23].

The SelectMDX test is similarly based on mRNA biomarker isolation from urine. *HOXC6* and *DLX1* mRNA levels were shown to be good predictors for the detection of high-grade PCa [24]. Currently, both the MiPS-score and ExoDx assay are considered experimental. Combined analysis of androgen-receptor gene (*AR-CAG*) repeat length, the percentage of promoter methylation (PPM) of genes glutathione-S-transferase P1 (*GSTP1*) and Ras associated domain family 1 isoform A (*RASSF1A*) have also been reported to increase the specificity of PSA in PCa diagnosis [25].

1.3.3. Tissue-based biomarkers

Dysregulated gene expression has also been associated with PCa, disease progression and potential to metastasis. α-Methylacyl coenzyme A racemase (*AMACR*) has also been shown to be overexpressed in PCa tissue samples [26]. Hypermethylation of numerous genes including glutathione-S-transferase π (*GSTP1*), adenomatous polyposis coli (*APC*), retinoic acid receptors beta 2 (*RARβ2*), and RAS is A (*RASSF1A*) have also been implicated in the development of PCa [27]. ConfirmMDx is indicated for men who have a documented history of a previous negative prostate biopsy result for cancer or cellular atypia suspicious for malignancy. ConfirmMDx uses the methylation status of three biomarkers (GSTP1, RASSF1, and APC) from prostatic tissue to help determine a patient's chance for having PCa on a subsequent biopsy. ConfirmMDx epigenetic assay was found to be a significant and independent predictor of PCa in repeat biopsies in men with prior negative biopsies with a negative predictive value of 88% [28].

Genetic abnormalities have also been associated with an increased risk of PCa and advanced disease. Mutations in the *p53* gene are reported in about 10 to 20% and 60 to 90% for primary

and advanced prostate cancers, respectively [29, 30]. Germline mutation of the *BRCA2* tumor suppressor gene substantially increases the lifetime risk of developing PCa. *BRCA2*-mutant tumors also exhibit an increased frequency of intraductal carcinoma (IDC), a pathology that predicts adverse outcomes in both familial and sporadic PCa. *BRCA2*-mutants in PCa are uniquely aggressive, they are associated with younger age of onset, higher rates of lymph node and distant metastasis, and increased mortality relative to sporadic compared to non-*BRCA2*-mutant disease [31]. In a small South African study, a germline variation has been observed in African versus European patients, no pathogenic mutations were found in known high penetrance genes such as *BRCA1, BRCA2, ATM, HOXB13 and CHK2* [32]. A higher tumor mutational burden and a frequent gain of the *MYC* gene was seen in treatment naïve, high-risk PCa in an African versus a European cohort [33].

1.3.4. Tissue-based prognostic markers

Gene expression profiling has also been used for risk stratification in men with PCa. These assays include Prolaris (Myriad Genetics), Oncotype Dx (Genomic Health) and Decipher (Genomic Dx Biosciences). The Prolaris test measures the expression of 31 cell-cycle associated genes in biopsy derived PCa tissue. These genes include *FOXM1, CDC20, CDKN3, CDC2, KIF11, KIAA0101, NUSAP1, CENPF, ASPM, BUB1B, RRM2, DLGAP5* and *TK1* amongst others. Cell cycle progression (CCP) score provides prognostic value in terms of progression and death from PCa [34]. Oncotype Dx Genomic Prostate Score (GPS) assay is an RNA-based test based on 12 carcinoma-associated genes and 5 reference genes that can be applied to carcinoma tissue in prostate biopsies to determine the aggressiveness of the carcinoma. The assay profiles multiple gene pathways involved in PCa to predict disease aggressiveness. These pathways include androgen signaling (*AZGP1, FAM13C, KLK2,* and *SRD5A2*), cellular organization (*FLNC, GSN, GSTM2,* and *TPM2),* stromal response (*BGN, COL1A1,* and *SFRP4*), cellular proliferation (*TPX2*) and 5 reference genes [35]. Decipher uses oligonucleotide microarrays to measure 22 RNA expression biomarkers, extracted from formalin fixed paraffin embedded (FFPE) prostate biopsy specimens, to derive a Decipher score and corresponding probability of clinical metastasis in patients with PCa. This genomic classifier includes the following genes *LASP1, IQGAP3, NFIB, S1PR4, THBS2, ANO7, PCDH7, MYBPC1, EPPK1, TNFRSF1,9* and *PBX1* amongst others [36].

1.4. Identification of Biomarkers in the South African context

Only PSA, PCA3 and PHI are FDA approved for PCa diagnosis and management. Except for PSA, none of the other biomarkers are commercially available in our resource-constrained environment. As most biomarker studies are based on European and American populations, it is unclear whether these markers are applicable to South African men as they have not been validated in our population.

The quality and quantity of cell-free RNA and DNA isolated from urine or plasma are low in comparison with that isolated from tissue. Prostate tissue models show greater benefit on analysis with findings very similar to those observed in non-invasive tissue samples, like urinary sediment cells (USC) and cell-free DNA from plasma [25]. For example, positive predictive values of diagnosing PCa in the combined analysis of *GSTP1 + RASSF1A + AR-CAG* ≥ 25 repeats, with PSA ≥ 4 and PPM ≥20 reaching 87.0% and 90.8% in plasma and USC respectively. Negative predictive value 64.5% and 67.5% for plasma and USC respectively. Increased values were seen with PSA ≥10 [25]. Considering this, in our study RNA was isolated from prostate tissue samples.

1.5. Conclusion

PCa is a heterogeneous disease, ranging from indolent to highly lethal disease. Higher mortality rates and incidence of PCa reported in Sub-Saharan African makes the study of PCa genetics in South Africa imperative. The aims of this study are to describe gene expression patterns of 84 key genes [**Appendix 1**] commonly involved in PCa and to distinguish patients with PCa from those with BPH with PSA values above 10ng/ml. BPH patients selected presented with high PSA values similar to patients with PCa. The 84 genes are part of a commercially available Human Prostate Cancer RT² Profiler PCR Array. The array represents genes involved in androgen receptor, PI3 kinase/AKT, and PTEN signaling, as well as the cell cycle and apoptotic pathways. Identification of patterns in RNA expression will aid in biomarker development in a South African context. The study is also likely to identify genetic biomarkers unique to our population and ethnic groups.

Chapter 2. Material and methods

2.1. Study Design

Descriptive study

2.2. Ethics and Cohort description

The study is part of an ongoing project titled *"Novel Markers of Prostate Cancer using Proteomics, Genomics and Lipidomics Techniques: An important Tool for Early Cancer Diagnosis and Treatment monitoring Protocol".* Human research ethics committee approval was obtained from the University of Cape Town, Faculty of Health Sciences (HREC REF: 454/2012).

South African men treated at Groote Schuur, New Somerset and Eerste Rivier Hospital in the Western Cape, who were scheduled for transurethral resection of the prostate (TURP), were included. It should be noted that surgery was performed for medical reasons and not for research purposes. Only a small portion of the prostate chips from the TURP procedure was collected, the remainder were analyzed by the National Health Laboratory Services (NHLS) to ensure accurate diagnosis and staging of the patients. Inclusion criteria were patients with a histological diagnosis of PCa or BPH, patients scheduled for transurethral resection of the prostate or radical prostatectomy as part of their treatment plan and patients able and willing to participate voluntarily and give written informed consent. Exclusion criteria were patients who received treatment for PCa, in the form of chemotherapy, hormonal manipulation or radiation treatment, insufficient material for analysis and patients unable or unwilling to consent.

Informed written consent for enrolment in the study was obtained by members of the International Centre of Genetic Engineering and Biotechnology (ICGEB) and qualified staff of the Urology team at Groote Schuur Hospital. Informed consent was taken in the patient's own language or by using an interpreter to ensure a full understanding of enrolment in the study and to deal with concerns and questions that were raised by patients. Brief genetic counseling was also provided to ensure patient understanding of the nature of the study and their participation.

2.3. RNA Isolation and cDNA synthesis

Prostate tissue collected for the experiments was immediately stabilized with the RNA stabilizing reagent, RNAlater and stored at -80 degrees Celsius (°C). After a histological confirmation by the NHLS of BPH or PCa (at least 80% cancer on a tissue chip), RNA was

extracted from 20mg of frozen prostate tissue using the AllPrep DNA/RNA/miRNA Universal Kit, this was done by following the manufacture's protocol. Tissue lysis was done in 600 µl lysis buffer (RLT Plus) with a TissueLyser LT (QIAGEN), using 2 stainless steel 4 mm beads operated for 25 minutes at 40 Hz. The homogenized lysate was then transferred to an AllPrep DNA Mini spin column. To elute RNA, 20 µl RNase-free water was added directly to the spin column membrane and centrifuged at ≥ 8000 x *g* for 1 minute, step repeated once, for a total of 40 µl RNase-free water per specimen. RNA was quantified using a NanoDrop (Thermo Scientific). RNA quality was measured using ultraviolet–visible spectroscopy (agarose gel electrophoresis) which measures absorbance of a diluted RNA sample. The Beer-Lambert law equation expresses the relation between absorption and concentration of nucleic acids:

$$A = \epsilon Cl$$

A = *absorbance*, ϵ = *molar extinction coefficient* [0.025(µg/ml) cm^{-1}], C = *concentration* (in the units corresponding to ϵ) and l = *light pathlength*.

To avoid RNase contamination, the specimens were handled with aseptic techniques and all surfaces were treated with RNaseZAP (SIGMA). Complementary DNA (cDNA) was synthesized from 1 µg of RNA using the reverse transcription synthesis kit, SuperScript IV VILO Master Mix (Thermo Fischer Scientific). To remove possible genomic DNA contamination, the RNA samples were treated with DNase enzyme that is part of the SuperScript IV VILO Master Mix kit. cDNA was stored at -80 °C.

2.4. PCR Array and Data Analysis

RT^2 Profiler PCR Array (QIAGEN) is a complete system for pathway-focused gene expression analysis. RT^2 Profiler PCR Arrays were done on a 96-well plate using Format F which is suitable for the real-time PCR Roche Light Cycler 480 machine. RT^2 Profiler PCR Array analyzed 84 key genes involved in PCa, 5 housekeeping genes, a genomic DNA control, 3 reverse-transcription controls, and 3 positive PCR controls. The plate outline is shown in **Figure 2** and the genes and their descriptions are listed in **Appendix 1**. These genes are commonly involved in PCa development. The procedure was done following the manufacture's protocol, in short: The PCR component mix was prepared in a 15 ml tube to a final concentration of 1x RT^2 SYBR Green Master Mix, 1x cDNA synthesis reaction (prepared from 1 µg RNA) and RNase-free water to a total of 2.7mls. The RT^2 Profiler PCR Array was tightly sealed with an optical adhesive film and centrifuged at 1000 x *g* for 1 minute at room temperature to remove bubbles. PCR cycling programs were as follows 1) heat activation for 10 minutes at 95 °C, 1 cycle (ramp rate 4.8 °C/s), 2) quantification for 15 seconds at 95 °C

(ramp rate 1.5 °C/s) and 1 minute at 60 °C (ramp rate 1.5 °C/s), 45 cycles. The melting curves were obtained by decreasing the temperature to 60 °C before continuously increasing the temperature to 95 °C with a ramp rate of 0.03 °C/s. Data were captured and analyzed using a Second Derivative Maximum analysis method, this is an algorithm based on the kinetics of a PCR reaction. Cycle threshold (C_T) values obtained were used to calculate gene expression patterns.

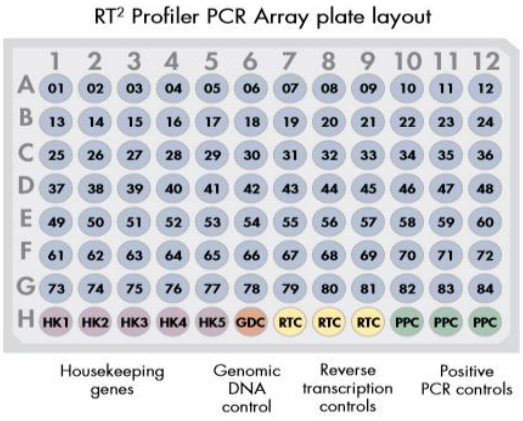

Figure 2. 96-well format, RT2 Profiler PCR Array plate layout

Data were analyzed with the GeneGlobe RT^2 and miScript PCR Array Data Analysis Center from Qiagen. The results of the C_T values were uploaded on the specified excel sheet format. The integrated web-based platform automatically calculates the fold-change results for gene expression using the equations explained in **section 2.4.1**. Samples were assigned to controls (BPH) and test group (PCa). C_T values were normalized based on the 5 housekeeping/reference genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1* and *RPLP0*). The C_T cut-off was set to 35. Results are illustrated in scatter plot, volcano plot, clustergram, tabular and heat map formats. The p values of fold-change were calculated based on a Student's t-test of the replicate 2^ (- Delta C_T) values for each gene in the BPH and PCa group. The p-value calculation used is based on parametric, unpaired, two-sample equal variance, two-tailed distribution.

2.4.1. Detailed mathematical explanation of $\Delta\Delta C_T$ data analysis method

The data analysis web portal calculates fold-change/regulation using delta-delta C_T method, in which delta C_T is calculated between the gene of interest (GOI) and an average of reference genes (HKG), followed by delta-delta C_T calculations [delta C_T (Test Group)-delta C_T (Control Group)]. Fold-change is then calculated using 2^ (-delta delta C_T) formula.

Due to the inverse proportional relation between C_T and the original gene expression level, expression levels for each gene of interest is expressed as:

$$L = 2^{-CT}$$

To normalize expression levels:

$$\frac{2^{-C_T(GOI)}}{2^{-C_T(HKG)}} = 2^{-[C_T(GOI) - C_T(HKG)]} = 2^{-\Delta C_T}$$

To determine fold-change in gene expression:

$$\frac{2^{-\Delta C_T(expt)}}{2^{-\Delta C_T(ctrl)}} = 2^{-\Delta\Delta C_T}$$

Where $\Delta\Delta C_T$ is equal to ΔC_T (expt) - ΔC_T (ctrl)

The complete equation:

$$\frac{\frac{2^{-\Delta C_T(GOI)}expt}{2^{-\Delta C_T(HKG)}expt}}{\frac{2^{-\Delta C_T(GOI)}ctrl}{2^{-\Delta C_T(HKG)}ctrl}} = \frac{2^{-[C_T(GOI) - \Delta C_T(HKG)]}expt}{2^{-[C_T(GOI) - \Delta C_T(HKG)]}ctrl} = \frac{2^{-\Delta C_T expt}}{2^{-\Delta C_T ctrl}} = 2^{-\Delta\Delta C_T}$$

Abbreviations: GOI, gene of interest; HKG, housekeeping gene; expt, experimental sample; ctrl, control sample.

Chapter 3. Results

3.1. Cohort description

For this pilot cohort study, patients were selected from the cohort based on their self-reported ethnicity (Caucasians, Mixed- and African ancestry), histological confirmed diagnosis of PCa or BPH, PSA levels > 10 ng/ml, age and good quality of RNA extracted from prostate tissue samples (**Table 2**). The BPH patients with PSA above 10 ng/ml were selected because this group is the most difficult to distinguish from patients with PCa. Most patients in the BPH group had previous prostate biopsies to exclude PCa except for 3 patients (BPH184, BPH223 and BPH234). Informed consent was obtained, and brief genetic counseling was provided to patients before enrollment. The cohort included 9 patients with PCa (2 Caucasians, 4 Mixed- and 3 African ancestry) and 10 patients with BPH (2 Caucasians, 5 Mixed- and 3 African ancestry). The PCa group has an age range of 56 to 75 years (mean 65, median 66), while the BPH group had a range of 60 to 76 years (mean 68, median 67). PSA levels range from 24.1 to 5000.0 ng/ml (mean 1252.0 ng/ml, median 185.0) for the PCa group and 11.0 to 58.1 ng/mL (mean 25.0 ng/ml, median 22.0) for the BPH group.

Table 2. Cohort description

Patient Number	Agee	Race	PSA	Diagnosis	Gleason Score
PCa037	67	MA	185.0	PCa	4+5
PCa070	66	MA	34.0	PCa	5+4
PCa077	71	C	24.1	PCa	4+5
PCa108	56	A	82.6	PCa	3+4
PCa159	75	A	5000.0	PCa	4+5
PCa180	58	A	5000.0	PCa	5+5
PCa183	63	MA	34.0	PCa	5+4
PCa191	61	C	576.0	PCa	5+4
PCa226	70	MA	332.3	PCa	5+4
BPH148	60	MA	21.7	BPH	
BPH184	76	MA	38.8	BPH	
BPH194	67	A	12.3	BPH	
BPH215	67	MA	58.1	BPH	
BPH217	69	MA	22.7	BPH	
BPH223	65	A	20.3	BPH	
BPH234	71	C	14.1	BPH	
BPH240	63	MA	27.5	BPH	
BPH144	66	C	11.0	BPH	
BPH190	74	A	11.3	BPH	

Abbreviations: C, Caucasian; MA, Mixed ancestry; A, African

3.2. RNA Isolation and cDNA synthesis

RNA quantification was done using the NanoDrop (Thermo Scientific). RNA concentration in the PCa group ranging from 221.1 to 2653.8 ng/µl (mean 647.7), and 84.5 to 655.5 ng/µl (mean 318.8) for the BPH group (**Table 3**).

The A_{260}/A_{280} ratio was used to determine protein contamination of nucleic acid samples. The ratio for a good quality RNA should be above 2. A_{260}/A_{280} ratios for the PCa and BPH group were similar, ranging from 2 to 2.1 and A_{260}/A_{230} ratios (measure of organic contamination) were ranging 1.7 to 2.2 (mean 1.9) for the PCa group and 0.9 to 1.9 (mean 1.6) in the BPH group. RNA quality was also assessed using agarose gel electrophoresis. RNA degradation was seen and this was because of time intervals from collecting prostate tissue specimens in theatre to adding RNA stabilization agent (RNAlater) in the lab, **Figure 3.**

Table 3. RNA isolation results

Patient Number	**Diagnosis**	**RNA isolation**		
		ng/µl	**$A_{260}/_{280}$**	**$A_{260}/_{230}$**
PCa037	PCa	606.3	2.0	1.9
PCa070	PCa	316.4	2.1	2.0
PCa077	PCa	339.1	2.1	2.0
PCa108	PCa	221.1	2.0	1.7
PCa159	PCa	232.0	2.0	1.9
PCa180	PCa	326.8	2.0	1.8
PCa183	PCa	356.9	2.1	2.0
PCa191	PCa	776.5	2.1	2.0
PCa226	PCa	2653.8	2.1	2.2
BPH148	BPH	397.9	2.1	1.8
BPH184	BPH	357.2	2.1	1.6
BPH194	BPH	110.3	2.1	1.1
BPH215	BPH	336.6	2.0	1,5
BPH217	BPH	84.5	2.0	1.9
BPH223	BPH	345.7	2.0	1.5
BPH234	BPH	153.0	2.0	0.9
BPH240	BPH	655.5	2.0	1.8
BPH144	BPH	128.0	2.0	1.6
BPH190	BPH	410.8	2.1	1.8

$A_{260}/_{280}$ protein contamination; **$A_{260}/_{230}$** organic contamination

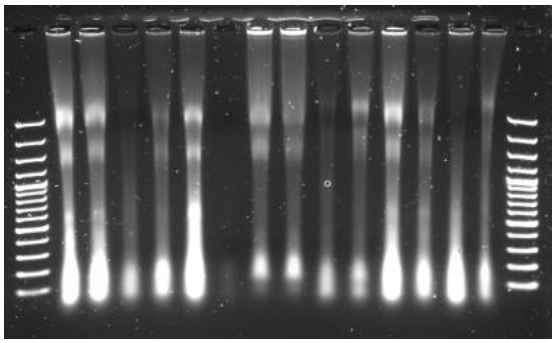

Figure 3. Agarose gel electrophoresis

3.3. PCR Array and Data Analysis

The RT^2 Profiler PCR Array include quality control to assess PCR array reproducibility, reverse transcription efficiency and genomic DNA contamination. For PCR array reproducibility, the positive PCR control of all the plates must have a C_T value of 20±2 and the C_T of two arrays should not differ with more than 2 units. All the positive PCR controls had C_T values between 19.47 and 19.84, therefore reproducibility of the arrays was acceptable.

The RT^2 Profiler PCR Array test for genomic DNA contamination, with a C_T cut-off set at 35 all the arrays except for 4 (PCa226, C_T 33.46, BPH194, C_T 34.83, BPH215, C_T 34.31 and BPH240, C_T 34.96) had values above 35 indicating no genomic DNA contamination. During cDNA synthesis, a DNase digestion step was included to remove contaminate DNA. In further experiments the genomic DNA contamination can be addressed by increasing incubation time of the DNase digestion step.

Housekeeping / reference genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, and *RPLP0*) were used to normalize the data, **Appendix 2**. **Figure 4** and **Appendix 3** shows the gene expression profiles of the PCa and BPH groups. When considering a 2-fold change and a p value of <0.05, the following genes were downregulated in the PCa group compared to the BPH group *ARNTL*, *CCND1*, *DKK3*, *DLC1*, *EGR3*, *FOXO1*, *GPX3*, *GSTP1*, *IGF1*, *PTEN*, *PTGS2*, *SOCS3*, *TIMP3*, *TNFRSF10D* and *ZNF185*, upregulated genes included CDH1 and MKI67 but were not statistically significant, **Figure 5, 6**, **7** and **8.**

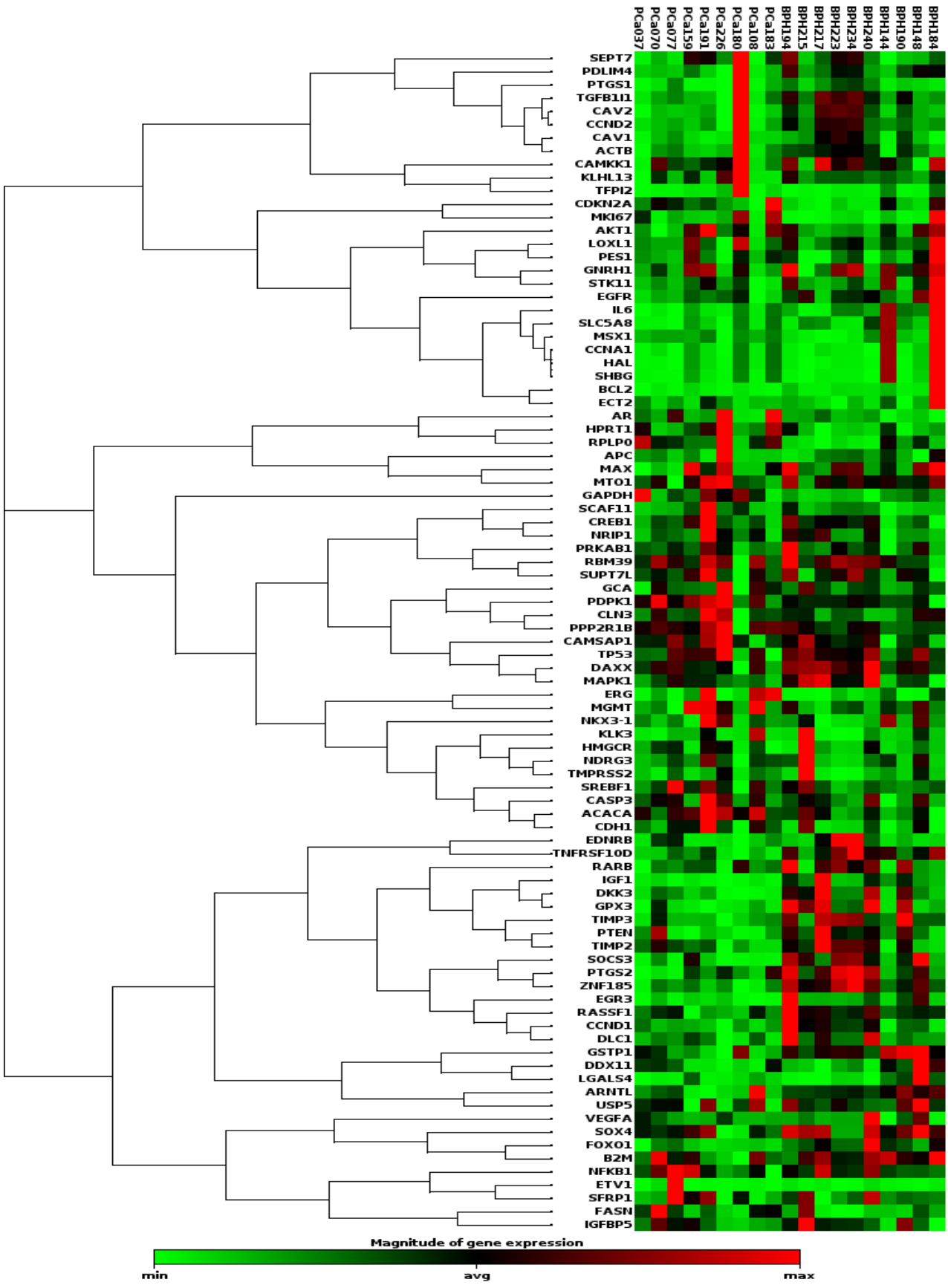

Figure 4. 1-D Clustergram: Gene expression patterns of the PCa and BPH group (2-fold change)

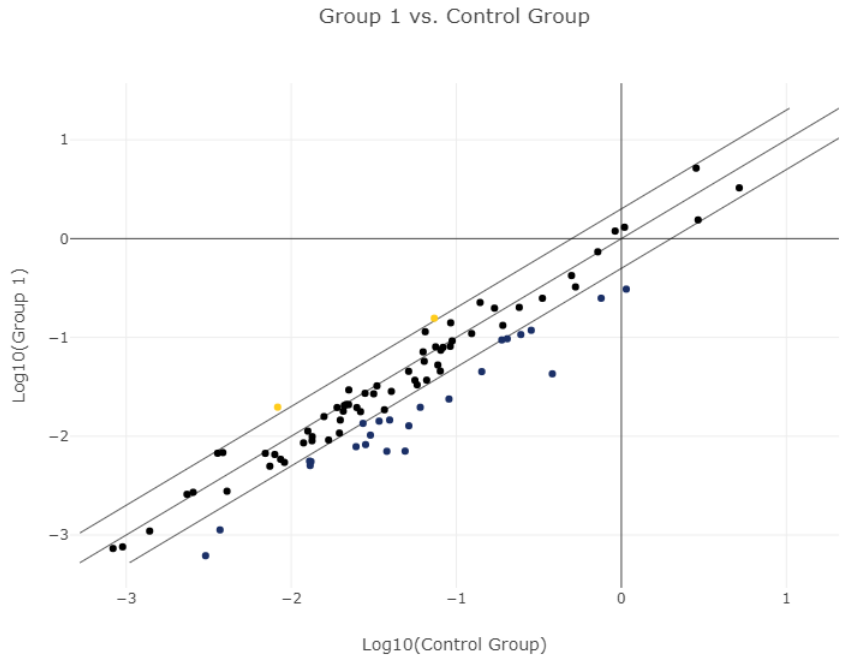

Figure 5. Scatter Plot: Gene expression patterns in the PCa versus the BPH group (2-fold change, p value <0.05)

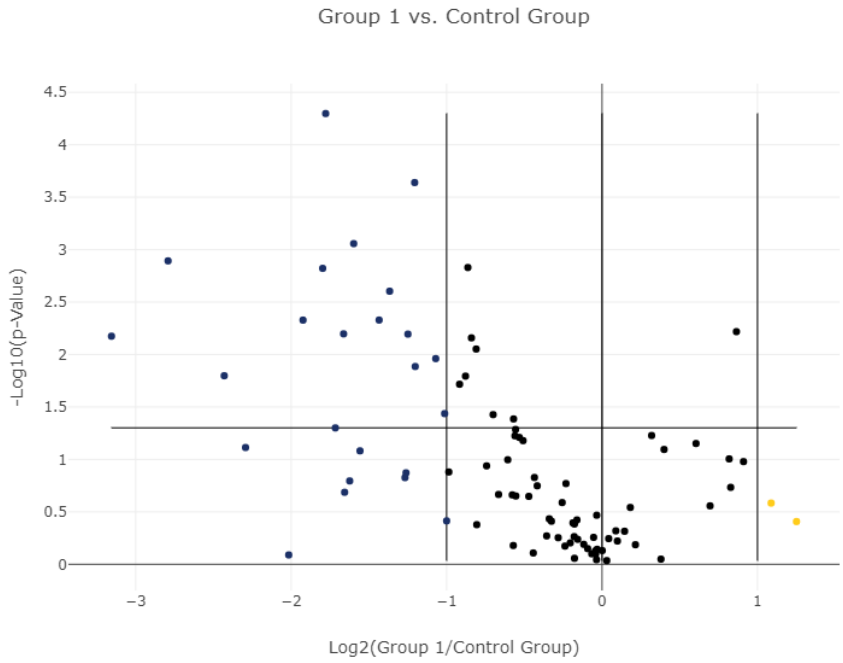

Figure 6. Volcano Plot: Gene expression patterns in the PCa versus the BPH group (2-fold change, p value <0.05)

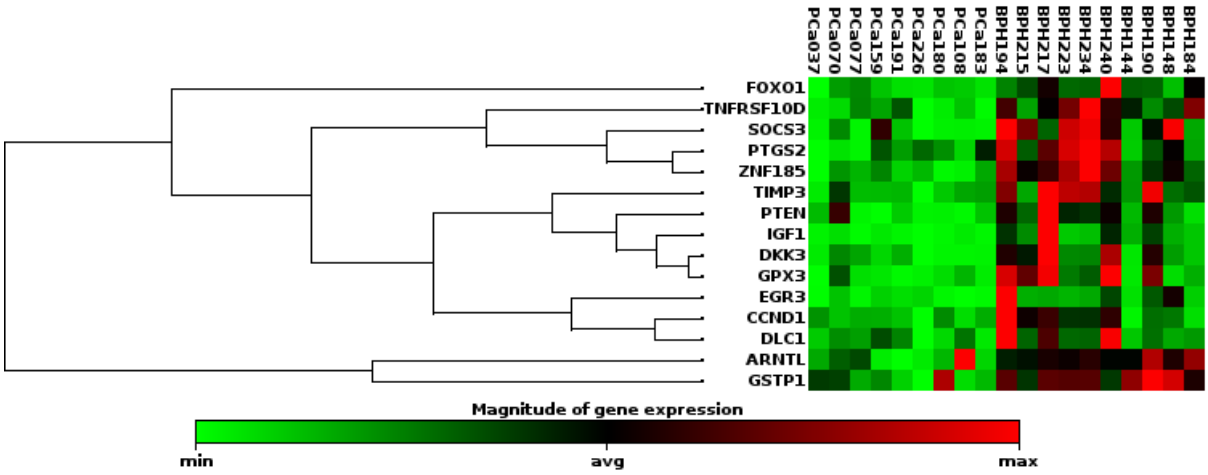

Figure 7. 1-D Clustergram: illustrating the expression of genes in the PCa versus the BPH group (2-fold change, p value <0.05)

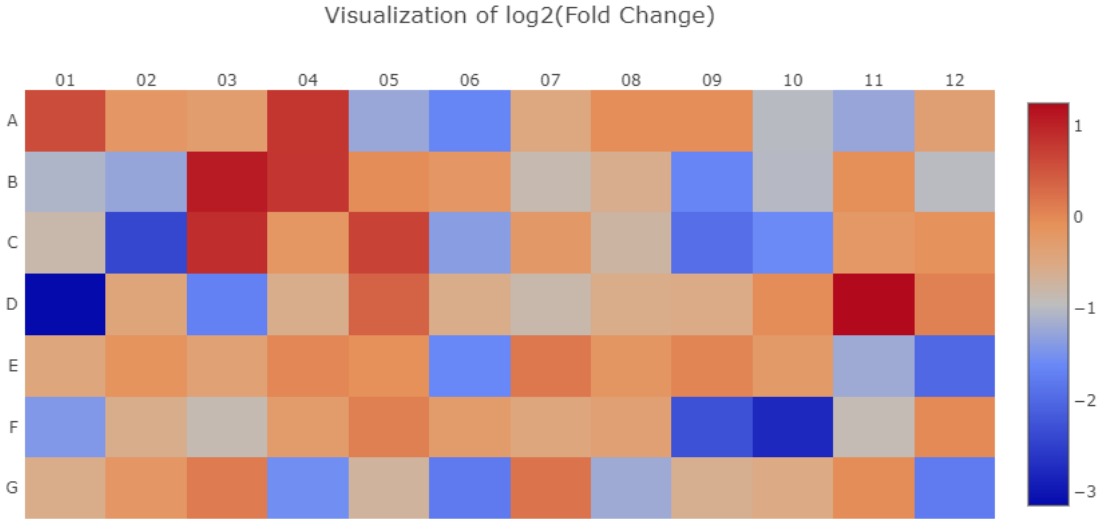

Figure 8. Heat Map: Differential expression of genes in the PCa and the BPH group (2-fold change)

At a fold-change threshold of 1.5, the following additional genes were downregulated in the PCa group with p values <0.05 *DAXX*, *EGFR*, *RASSF1*, *SOX4*, and *TIMP2*, additional upregulated genes were *ACACA*, *AR*, *CDKN2A*, *ERG* and *FASN* but were also not statistically significant, **Figure 9**, **10** and **11**. Although there is no clear consensus on reporting fold-change thresholds, most studies use either 1.5 or 2 with p values of less than 0.05.

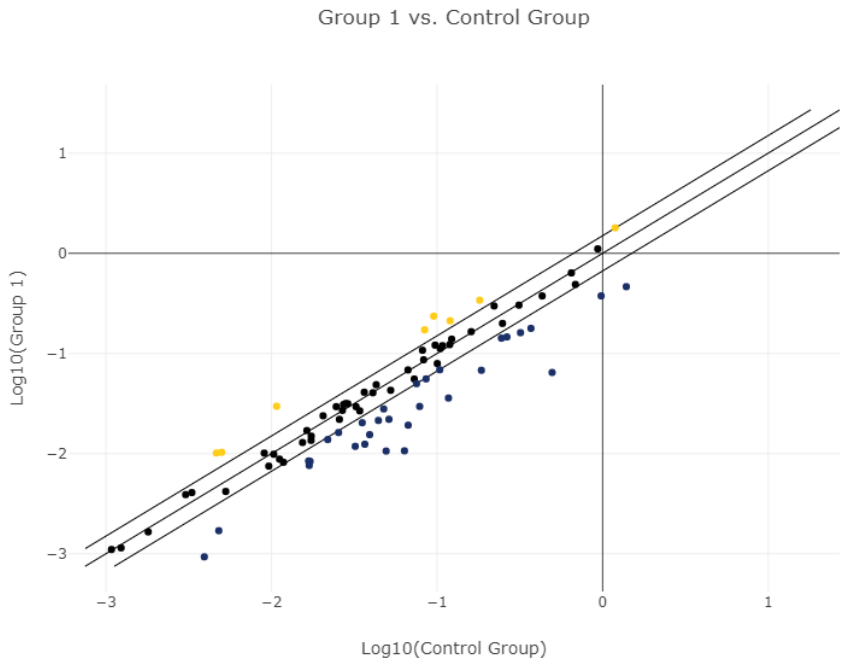

Figure 9. Scatter Plot: Gene expression patterns in the PCa versus the BPH group (1.5-fold change)

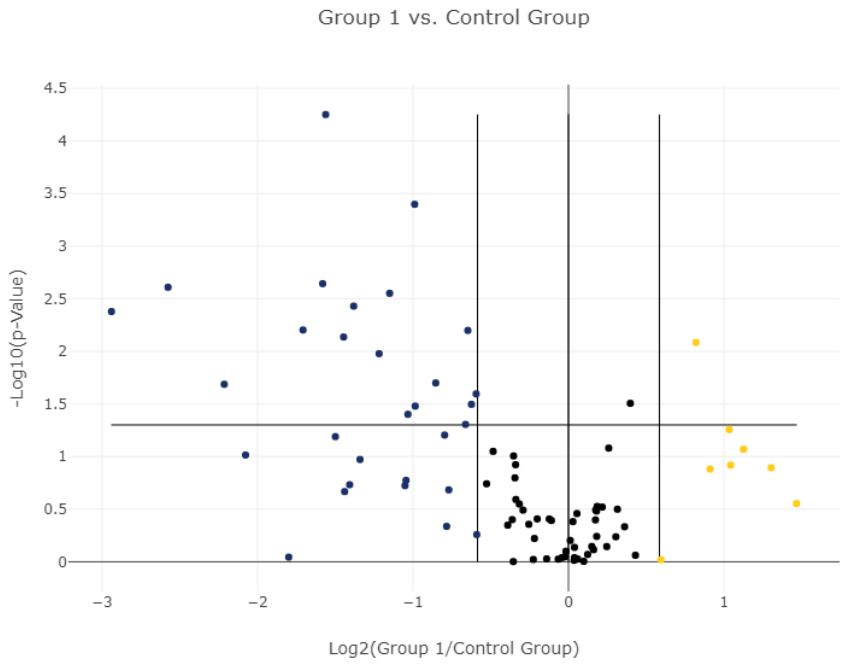

Figure 10. Volcano Plot: Gene expression patterns in the PCa versus the BPH group (1.5-fold change)

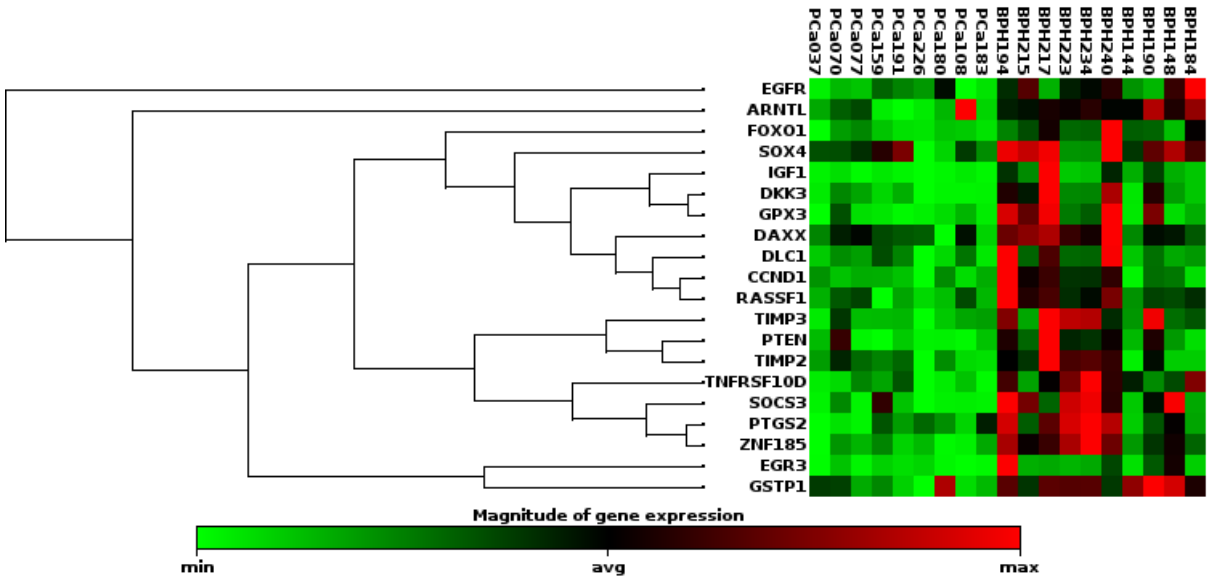

Figure 11. 1-D Clustergram: illustrating the expression of genes in the PCa versus the BPH group (1.5-fold change, p value <0.05)

Chapter 4. Discussion

4.1. Summary of findings

To our knowledge, there are currently no studies reporting on gene expression profiling in a South African cohort. Our study is the first to report differential gene expressions patterns between PCa and BPH with PSA levels > 10ng/ml in South Africa. Other studies in the South African population have looked at germline mutations, tumor mutational burden and genomic-wide associations [32, 37]. PCa is the leading cancer diagnosis amongst SA men and has been associated with higher mortalities compared to Europe and America. Early and accurate diagnosis is, therefore, imperative to institute appropriate and early treatment for patients with PCa.

PSA remains the most commonly used serum-based biomarker for diagnosis and screening of PCa in South Africa. Its low sensitivity and specificity for PCa diagnosis has led to several other biomarkers being explored to try and improve its diagnostic accuracy. These biomarkers include serum (PHI, 4K score test), urine (PCA3, *TMPRSS2-ERG* fusion) and tissue-based biomarkers (ConfirmMDx). Only PCA3 and PHI are FDA approved, but none of these biomarkers are available or used for PCa diagnosis in South African men. These biomarkers have also not been validated for use in our unique population. This was a pilot study of 19 patients (9 PCa and 10 BPH) from different ethnic groups in a Western Cape cohort. We describe a comprehensive analysis of gene expression patterns of 84 key genes commonly involved in PCa to differentiate between PCa and BPH in patients with PSA levels > 10ng/ml.

The RT^2 Profiler PCR Arrays were the preferred system for array-based gene expression profiling because of their high sensitivity, specificity and reproducibility amongst different users. RNA degradation was seen on agarose gel electrophoresis, this was because of the time intervals from collecting prostate tissue specimens to RNA stabilization with RNAlater, and an effort to decrease the time has been made. Opitz *et al.,* 2010 found that degraded RNA can still be used to assess gene expression patterns since a higher biological variance between patients is observed compared to the effect of RNA degradation [38]. In our samples, RNA degradation was similar. To address this in future studies, an RNA stabilization agent should be added in theatre at the time of collection.

Several genes were downregulated in PCa compared to BPH group, these genes include tumor suppressor genes *PTEN, DKK3, DLC1, FOXO1* and *RASSF1*. These genes were similarly

downregulated in keeping to findings in most studies [39-41]. Phosphatase and tensin homologue (*PTEN*) is a tumor suppressor gene and its function is to inhibit cell cycle progression and induce a G1 arrest. Loss of expression occurs in about 40% of patients and is associated with adverse pathological findings, Gleason scores of 7 or higher and advanced pathological stages [42, 43]. This gene has also been associated with a higher risk of advanced disease and poor prognosis [42, 43]. Other downregulated genes in our study include, Glutathione S-transferase pi (*GSTP1*) and Prostaglandin-endoperoxide synthase 2 (*PTGS2*). *GSTP1* is a cytosolic isoenzyme within the glutathione S-transferase family enzymes which is important in inactivation of electrophilic carcinogens by conjugation with glutathione, loss of this enzyme is seen in greater than 90% of patients with PCa. Silencing of this gene results in *GSTP1* promoter methylation which is seen in early tumorigenesis and PCa progression [44, 45]. Chronic inflammation has also been implicated in the pathogenesis of PCa. *PTGS2*, also known as cyclooxygenase-2 (COX-2) has been evaluated as a prognostic predictor marker in patients treated for PCa and studies have shown a better prognosis in patients with lower expression levels [46].

Differences in expression were seen with Cyclin D1 (*CCND1*) and Early growth response 3 (*EGR3*). These genes were also found to be downregulated in our study, but were reported to be overexpressed in other international studies. *CCND1* is a critical regulator of androgen-dependent transcription and cell cycle progression in PCa cells. This nuclear protein is involved in cell cycle transition from G1 (growth) to S-phase (synthesis) in both normal and neoplastic tissue. Overexpression of this gene has been associated with adverse pathological findings, high Gleason scores >7 and perineural invasion [47]. *EGR3* is a member of the EGR family of transcription factors that play diverse functions in response to cellular stimuli, including growth, stress and inflammation. *EGR3* is commonly upregulated in PCa and this is usually seen in men with less aggressive disease, while a low expression is associated with aggressive disease and predict relapse after PCa treatment. This can be used as a marker for diagnosis and prognosis of PCa [48]. In our study, most patients had high Gleason scores which could have contributed to the low *EGR3* expression observed.

Upregulation of the Antigen identified by monoclonal antibody Ki-67 (*MKI67*), Fatty acid synthase (*FASN*) and V-ets erythroblastosis virus E26 oncogene homolog (ERG) were similarly seen in our cohort. *MKI67* and *FASN* overexpression are usually associated with progression, metastasis and a lower biochemical failure-free survival [49-53]. Androgen

receptor (*AR*) expression is higher in patients with PCa and is associated with primary PCa, disease progression and a lower recurrence-free survival [54].

E-Cadherin (*CHD1*) is a Ca^{2+} dependent homotypic cell adhesion molecule and this tumor suppressor gene has been reported to be underexpressed in literature and its loss is associated with loss of homotypic cellular adhesiveness which results in tumor cell invasion and metastasis [55].

Low expression of *PTEN* and *EGR3* and overexpression of *MKI67* and *FASN* are seen in patients with adverse pathological outcomes and these have been associated with poor prognosis in patients diagnosed with PCa, this could explain the high mortality demonstrated in South African epidemiological studies.

4.2. Limitations

We recognize limitations in our study and these include a smaller sample size with only 9 PCa patients and 10 BPH patients. The sample size may not be representing a true reflection or be generalized to Sub-Saharan Africa as the study only included patients treated in the Western Cape. We could not demonstrate differences in expression profiles amongst the different ethnic group because of a smaller representation of the different groups. The Western Cape also consists of a highly heterogeneous population which could have an impact in interpreting results.

Most patients in our study had high risk PCa based on Gleason scores, our cohort did not include patients with low risk PCa. The gene expression patterns may differ in low risk PCa, a follow-up study is needed to address this.

4.3. Conclusion

The aims of the study were successfully achieved. We identified a group of differentially expressed genes that have potential in distinguishing PCa and BPH patients with PSA values above 10 ng/ml. The study shows similarly differentially expressed genes as seen in international studies. Of note *PTEN*, *MKI67* and *FASN* which are associated with poor prognosis. *EGR3* is commonly upregulated in PCa, but in our study it was downregulated and this is has been associated with aggressive disease and predict relapse after PCa treatment [48]. Differences seen in expression profiles of *CCND1* and *CHD1* in our study compared to those

reported in other studies need to be further evaluated and their clinical significance is unknown in our population. A larger and more representative population study is needed to further evaluate the clinical significance of our findings in a South African cohort.

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Appendices

Appendix 1: Description of the Genes in the RT^2 Profiler PCR Array for Prostate cancer

Position	Unigene	Refseq	Symbol	Description
A01	Hs.160556	NM_198834	ACACA	Acetyl-CoA carboxylase alpha
A02	Hs.525622	NM_005163	AKT1	V-akt murine thymoma viral oncogene homolog 1
A03	Hs.158932	NM_000038	APC	Adenomatous polyposis coli
A04	Hs.76704	NM_000044	AR	Androgen receptor
A05	Hs.65734	NM_001178	ARNTL	Aryl hydrocarbon receptor nuclear translocator-like
A06	Hs.150749	NM_000633	BCL2	B-cell CLL/lymphoma 2
A07	Hs.8417	NM_032294	CAMKK1	Calcium/calmodulin-dependent protein kinase kinase 1, alpha
A08	Hs.522493	NM_015447	CAMSAP1	Calmodulin regulated spectrin-associated protein 1
A09	Hs.141125	NM_004346	CASP3	Caspase 3, apoptosis-related cysteine peptidase
A10	Hs.74034	NM_001753	CAV1	Caveolin 1, caveolae protein, 22kDa
A11	Hs.212332	NM_001233	CAV2	Caveolin 2
A12	Hs.417050	NM_003914	CCNA1	Cyclin A1
B01	Hs.523852	NM_053056	CCND1	Cyclin D1
B02	Hs.376071	NM_001759	CCND2	Cyclin D2
B03	Hs.461086	NM_004360	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)
B04	Hs.512599	NM_000077	CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)
B05	Hs.534667	NM_000086	CLN3	Ceroid-lipofuscinosis, neuronal 3
B06	Hs.516646	NM_004379	CREB1	CAMP responsive element binding protein 1
B07	Hs.336916	NM_001350	DAXX	Death-domain associated protein
B08	Hs.443960	NM_004399	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11
B09	Hs.292156	NM_015881	DKK3	Dickkopf homolog 3 (Xenopus laevis)
B10	Hs.134296	NM_006094	DLC1	Deleted in liver cancer 1
B11	Hs.518299	NM_018098	ECT2	Epithelial cell transforming sequence 2 oncogene
B12	Hs.732046	NM_000115	EDNRB	Endothelin receptor type B
C01	Hs.488293	NM_005228	EGFR	Epidermal growth factor receptor
C02	Hs.534313	NM_004430	EGR3	Early growth response 3
C03	Hs.473819	NM_182918	ERG	V-ets erythroblastosis virus E26 oncogene homolog (avian)
C04	Hs.574240	NM_004956	ETV1	Ets variant 1
C05	Hs.83190	NM_004104	FASN	Fatty acid synthase
C06	Hs.370666	NM_002015	FOXO1	Forkhead box O1
C07	Hs.377894	NM_012198	GCA	Grancalcin, EF-hand calcium binding protein
C08	Hs.82963	NM_000825	GNRH1	Gonadotropin-releasing hormone 1 (luteinizing-releasing hormone)
C09	Hs.386793	NM_002084	GPX3	Glutathione peroxidase 3 (plasma)
C10	Hs.523836	NM_000852	GSTP1	Glutathione S-transferase pi 1
C11	Hs.190783	NM_002108	HAL	Histidine ammonia-lyase
C12	Hs.643495	NM_000859	HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase
D01	Hs.160562	NM_000618	IGF1	Insulin-like growth factor 1 (somatomedin C)

D02	Hs.607212	NM_000599	IGFBP5	Insulin-like growth factor binding protein 5
D03	Hs.654458	NM_000600	IL6	Interleukin 6 (interferon, beta 2)
D04	Hs.348262	NM_033495	KLHL13	Kelch-like 13 (Drosophila)
D05	Hs.171995	NM_001648	KLK3	Kallikrein-related peptidase 3
D06	Hs.5302	NM_006149	LGALS4	Lectin, galactoside-binding, soluble, 4
D07	Hs.65436	NM_005576	LOXL1	Lysyl oxidase-like 1
D08	Hs.431850	NM_002745	MAPK1	Mitogen-activated protein kinase 1
D09	Hs.285354	NM_002382	MAX	MYC associated factor X
D10	Hs.501522	NM_002412	MGMT	O-6-methylguanine-DNA methyltransferase
D11	Hs.689823	NM_002417	MKI67	Antigen identified by monoclonal antibody Ki-67
D12	Hs.424414	NM_002448	MSX1	Msh homeobox 1
E01	Hs.347614	NM_012123	MTO1	Mitochondrial translation optimization 1 homolog (S. cerevisiae)
E02	Hs.437338	NM_022477	NDRG3	NDRG family member 3
E03	Hs.618430	NM_003998	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
E04	Hs.55999	NM_006167	NKX3-1	NK3 homeobox 1
E05	Hs.155017	NM_003489	NRIP1	Nuclear receptor interacting protein 1
E06	Hs.424312	NM_003687	PDLIM4	PDZ and LIM domain 4
E07	Hs.459691	NM_002613	PDPK1	3-phosphoinositide dependent protein kinase-1
E08	Hs.517543	NM_014303	PES1	Pescadillo homolog 1, containing BRCT domain (zebrafish)
E09	Hs.269128	NM_002716	PPP2R1B	Protein phosphatase 2, regulatory subunit A, beta
E10	Hs.741184	NM_006253	PRKAB1	Protein kinase, AMP-activated, beta 1 non-catalytic subunit
E11	Hs.729457	NM_000314	PTEN	Phosphatase and tensin homolog
E12	Hs.201978	NM_000962	PTGS1	Prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase)
F01	Hs.196384	NM_000963	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
F02	Hs.654490	NM_000965	RARB	Retinoic acid receptor, beta
F03	Hs.476270	NM_007182	RASSF1	Ras association (RalGDS/AF-6) domain family member 1
F04	Hs.282901	NM_004902	RBM39	RNA binding motif protein 39
F05	Hs.210367	NM_004719	SCAF11	SR-related CTD-associated factor 11
F06	Hs.191346	NM_001788	SEPT7	Septin 7
F07	Hs.213424	NM_003012	SFRP1	Secreted frizzled-related protein 1
F08	Hs.632235	NM_001040	SHBG	Sex hormone-binding globulin
F09	Hs.444536	NM_145913	SLC5A8	Solute carrier family 5 (iodide transporter), member 8
F10	Hs.527973	NM_003955	SOCS3	Suppressor of cytokine signaling 3
F11	Hs.643910	NM_003107	SOX4	SRY (sex determining region Y)-box 4
F12	Hs.592123	NM_004176	SREBF1	Sterol regulatory element binding transcription factor 1
G01	Hs.515005	NM_000455	STK11	Serine/threonine kinase 11
G02	Hs.6232	NM_014860	SUPT7L	Suppressor of Ty 7 (S. cerevisiae)-like
G03	Hs.438231	NM_006528	TFPI2	Tissue factor pathway inhibitor 2
G04	Hs.513530	NM_015927	TGFB1I1	Transforming growth factor beta 1 induced transcript 1
G05	Hs.633514	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2

G06	Hs.644633	NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3
G07	Hs.439309	NM_005656	TMPRSS2	Transmembrane protease, serine 2
G08	Hs.213467	NM_003840	TNFRSF10D	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain
G09	Hs.437460	NM_000546	TP53	Tumor protein p53
G10	Hs.631661	NM_003481	USP5	Ubiquitin specific peptidase 5 (isopeptidase T)
G11	Hs.73793	NM_003376	VEGFA	Vascular endothelial growth factor A
G12	Hs.16622	NM_007150	ZNF185	Zinc finger protein 185 (LIM domain)
H01	Hs.520640	NM_001101	ACTB	Actin, beta
H02	Hs.534255	NM_004048	B2M	Beta-2-microglobulin
H03	Hs.592355	NM_002046	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H04	Hs.412707	NM_000194	HPRT1	Hypoxanthine phosphoribosyltransferase 1
H05	Hs.546285	NM_001002	RPLP0	Ribosomal protein, large, P0
H06	N/A	SA_00105	HGDC	Human Genomic DNA Contamination
H07	N/A	SA_00104	RTC	Reverse Transcription Control
H08	N/A	SA_00104	RTC	Reverse Transcription Control
H09	N/A	SA_00104	RTC	Reverse Transcription Control
H10	N/A	SA_00103	PPC	Positive PCR Control
H11	N/A	SA_00103	PPC	Positive PCR Control
H12	N/A	SA_00103	PPC	Positive PCR Control

Appendix 2: House keeping genes

Gene Symbol									
	PCa108	PCa180	PCa226	PCa191	PCa159	PCa077	PCa070	PCa183	PCa037
ACTB	23.3	23.49	18.57	23.78	25.16	19.1	19.77	25.22	22.53
B2M	22.79	27.99	20.48	24.89	25.26	20.14	19.68	26.59	22.81
GAPDH	23.96	26.07	19.62	24.2	26.52	20.85	21.86	27.61	22.15
HPRT1	29.77	32.59	24.32	29.75	31.74	26.85	27.48	31.49	28.07
RPLP0	22.01	25.82	16.83	23.18	24.67	18.83	19.2	24.28	20.14
Average	**24.37**	**27.19**	**19.96**	**25.16**	**26.67**	**21.15**	**21.60**	**27.04**	**23.14**

Gene Symbol										
	BPH194	BPH190	BPH144	BPH240	BPH234	BPH223	BPH217	BPH215	BPH148	BPH184
ACTB	21.89	19.44	27.76	17.12	21.64	21.71	17.63	17.85	23.75	27.65
B2M	23.85	20.63	26.83	17.89	23.33	23.35	18.5	18.82	24.54	26.98
GAPDH	24.23	21.88	28.67	20.18	24.52	24.53	20.49	20.57	25.45	29.01
HPRT1	29.07	28.11	33.66	25.53	30.11	30.24	26.42	25.93	31.47	34.48
RPLP0	23.07	20.29	26.06	18.92	23.49	23.32	19.65	19.3	23.34	27.68
Average	**24.42**	**22.07**	**28.60**	**19.93**	**24.62**	**24.63**	**20.54**	**20.49**	**25.71**	**29.16**

Appendix 3: Fold-change, p values and fold regulation of the Genes in the RT^2 Profiler PCR Array for Prostate cancer.

Position	Symbol	Fold-Change (comparing to BPH)		p-value	Up-Down Regulation (comparing to BPH)	
		PCa			PCa	
		Fold-Change	Comments		Fold Regulation	Comments
A01	ACACA	1.52		0.070552	1.52	
A02	AKT1	0.88		0.876927	-1.13	
A03	APC	0.82	B	0.558069	-1.22	B
A04	AR	1.76		0.098906	1.76	
A05	ARNTL	**0.42**		**0.006391**	**-2.38**	
A06	BCL2	**0.32**	A	0.205867	**-3.15**	A
A07	CAMKK1	0.72	B	0.225160	-1.39	B
A08	CAMSAP1	0.97		0.745045	-1.03	
A09	CASP3	0.97		0.795802	-1.03	
A10	CAV1	**0.50**		0.385606	**-2.00**	
A11	CAV2	**0.42**	A	0.134373	**-2.40**	A
A12	CCNA1	0.80	B	0.388522	-1.25	B
B01	CCND1	**0.48**		**0.010943**	**-2.10**	
B02	CCND2	**0.42**		0.149267	**-2.41**	
B03	CDH1	**2.13**		0.261015	**2.13**	
B04	CDKN2A	1.77	B	0.184702	1.77	B
B05	CLN3	0.98		0.904331	-1.02	
B06	CREB1	0.88		0.545092	-1.13	
B07	DAXX	0.56		**0.006940**	-1.79	
B08	DDX11	0.67	B	0.217945	-1.49	B
B09	DKK3	**0.32**		**0.006357**	**-3.17**	
B10	DLC1	**0.50**	A	**0.036597**	**-2.02**	A
B11	ECT2	0.96	B	0.553942	-1.04	B
B12	EDNRB	0.50		0.131846	-1.98	
C01	EGFR	0.57		**0.008872**	-1.75	
C02	EGR3	**0.19**	A	**0.015944**	**-5.39**	A
C03	ERG	1.88	B	0.104968	1.88	B
C04	ETV1	0.89	B	0.377959	-1.12	B
C05	FASN	1.62		0.277313	1.62	
C06	FOXO1	**0.39**		**0.002492**	**-2.58**	
C07	GCA	0.87		0.625418	-1.15	
C08	GNRH1	0.60	B	0.115291	-1.67	B
C09	GPX3	**0.26**		**0.004700**	**-3.79**	
C10	GSTP1	**0.33**		**0.000877**	**-3.03**	
C11	HAL	0.88	B	0.402755	-1.14	B
C12	HMGCR	0.94		0.708993	-1.07	

D01	IGF1	**0.11**		**0.006692**	**-8.91**	
D02	IGFBP5	0.75		0.178500	-1.34	
D03	IL6	**0.30**	B	0.050118	**-3.28**	B
D04	KLHL13	0.67	B	0.662372	-1.49	B
D05	KLK3	1.30		0.893238	1.30	
D06	LGALS4	0.68	B	0.223418	-1.47	B
D07	LOXL1	0.57		0.419141	-1.75	
D08	MAPK1	0.68		0.051867	-1.47	
D09	MAX	0.69		0.061756	-1.45	
D10	MGMT	0.98		0.719628	-1.02	
D11	MKI67	**2.38**	A	0.391302	**2.38**	A
D12	MSX1	1.06	B	0.479996	1.06	B
E01	MTO1	0.74	B	0.148761	-1.35	B
E02	NDRG3	0.92		0.645384	-1.08	
E03	NFKB1	0.78		0.536526	-1.28	
E04	NKX3-1	1.02		0.922412	1.02	
E05	NRIP1	0.96		0.794337	-1.05	
E06	PDLIM4	**0.32**		0.160525	**-3.08**	
E07	PDPK1	1.13		0.287236	1.13	
E08	PES1	0.90		0.578037	-1.11	
E09	PPP2R1B	1.03		0.569266	1.03	
E10	PRKAB1	0.85		0.170024	-1.17	
E11	PTEN	**0.43**		**0.013015**	**-2.30**	
E12	PTGS1	**0.25**	A	0.814788	**-4.04**	A
F01	PTGS2	**0.37**	A	**0.004694**	**-2.70**	A
F02	RARB	0.67		**0.041142**	-1.48	
F03	RASSF1	0.55	A	**0.001482**	-1.82	A
F04	RBM39	0.84		0.257963	-1.19	
F05	SCAF11	1.07		0.600118	1.07	
F06	SEPT7	0.85		0.671082	-1.18	
F07	SFRP1	0.74	A	0.779941	-1.36	A
F08	SHBG	0.79	B	0.368882	-1.27	B
F09	SLC5A8	**0.20**	B	0.077062	**-4.90**	B
F10	SOCS3	**0.14**	A	**0.001280**	**-6.93**	A
F11	SOX4	0.54	A	**0.016079**	-1.84	A
F12	SREBF1	1.00	B	0.740337	1.00	B
G01	STK11	0.68	B	0.059605	-1.47	B
G02	SUPT7L	0.88		0.413387	-1.13	
G03	TFPI2	1.11	B	0.484275	1.11	B
G04	TGFB1I1	**0.34**	A	0.082887	**-2.94**	A
G05	TIMP2	0.62		**0.037454**	-1.63	
G06	TIMP3	**0.29**		**0.001509**	**-3.48**	
G07	TMPRSS2	1.16		0.650910	1.16	
G08	TNFRSF10D	**0.43**		**0.000229**	**-2.31**	

G09	TP53	0.66		0.100786	-1.52	
G10	USP5	0.70		0.066257	-1.42	
G11	VEGFA	0.98		0.340633	-1.02	
G12	ZNF185	**0.29**	A	**0.000050**	**-3.43**	A

Comments:

A: This gene's average threshold cycle is relatively high (> 30) in either the control or the test sample and is reasonably low in the other sample (< 30).

These data mean that the gene's expression is relatively low in one sample and reasonably detected in the other sample suggesting that the actual fold-change value is at least as large as the calculated and reported fold-change result.

This fold-change result may also have greater variations if p value > 0.05, therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

B: This gene's average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the p-value for the fold-change is either unavailable or relatively high (p > 0.05).

This fold-change result may also have greater variations, therefore, it is important to have a sufficient number of biological replicates to validate the result for this gene.

Appendix 4: Human research ethics committee approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone (021) 406 6492
Email: sumayah.arieftien@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

31 October 2019

HREC REF: 454/2012

A/Prof L Zerbini
Division of Urology
E-26 NGSH

Dear A/Prof Zerbini

PROJECT TITLE: NOVEL MARKERS OF PROSTATE CANCER USING PROTEOMICS, GENOMICS AND LIPIDOMICS TECHNIQUES: AN IMPORTANT TOOL FOR EARLY CANCER DIAGNOSIS AND TREATMENT MONITORING

Sub-study for MMED candidate Dr Samkele Azola Salukazana: Identifying genetic Biomarkers for Diagnosis of prostate Cancer in South African Men
Supervisor: Dr Lisa Kaestner

Thank you for your letter to the Faculty of Health Sciences Human Research Ethics Committee (HREC) dated 18 October 2019.

The HREC has approved the above-mentioned sub study. Approval is granted until 30 November 2019 as per the main study 454/2012.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

The HREC acknowledge that the student: Dr Samkele Salukazana will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

Yours sincerely

Signature removed to avoid exposure online

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE

HREC 454/2012

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938
NHREC-registration number: REC-210208-007

This serves to confirm that the University of Cape Town Human Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC-SA), Food and Drug Administration (FDA-USA), International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use: Good Clinical Practice (ICH GCP), South African Good Clinical Practice Guidelines (DoH 2006), based on the Association of the British Pharmaceutical Industry Guidelines (ABPI), and Declaration of Helsinki (2013) guidelines. The Human Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

HREC 454/2012