Analysis of the Bacterial Microbiome from Prostate Tissue of South African Men

Pieter Hendrik Bouwman

A dissertation submitted in partial fulfilment of the requirements of the degree of M.Sc. Genetics in the Faculty of Natural and Agricultural Sciences, Department of Genetics, University of Pretoria, Pretoria

July 2019

Supervisor: Prof DA Cowan

Co-Supervisors: Dr A Valverde

Prof MS Bornman

Prof VM Hayes

Abstract

Cancer is a major cause of death globally and the incidence of the disease is expected to increase in coming years. It is predicted that 20 million new cases of cancer will be observed annually as early as 2025, rising from 14.1 million new cases in 2012. Prostate Cancer (PCa) was found to have the second highest incidence among male cancers globally, and it is expected that the absolute number of men with PCa will increase.

Contrary to global trends, PCa has a higher incidence and mortality rate than lung cancer in Sub-Saharan African men. According to the National Cancer Registry of South Africa, the incidence of PCa was three times greater than the incidence of lung cancer amongst men in 2010. Furthermore, disparities have been reported in the presentation and outcomes of PCa between racial groups. PCa has been found to be more common amongst men with African ancestry, and Black South African men have been shown to present a more aggressive disease phenotype. The South African Prostate Cancer Study (SAPCS) was established in 2008 to investigate clinical presentation, epidemiological risk factors, and associated microbial pathogenic contributions to PCa within Black South Africans from rural and urban localities.

Chronic inflammation has been associated with the development of cancer. Inflammatory responses include increased cellular proliferation, the production of growth factors, as well as factors that are known to damage DNA. Together, these responses create an environment which can promote the development of neoplasia. With regard to PCa in particular, chronic inflammation of benign prostate tissue has been associated with high-grade PCa. Bacterial infection represents one potential source of such long term inflammation. Studies on mouse models have found bacteria to be capable of inducing chronic inflammation with prostatitis which can still be detected in 40% of the subjects up to 1 year post-infection.

The present study investigated the bacterial communities identified from prostate biopsy tissue taken from South African men suffering from prostate cancer or benign prostatic hyperplasia (BPH). A population of 50 men were enrolled in the study representing 26 BPH patients and 24 PCa patients.

Bacterial communities were shown to be highly diverse for each patient, but the total bacterial communities did not differ significantly between the PCa group and the BPH group. However, significant associations were identified between specific taxa and the two patient groups. These taxa included known pathogens, and bacteria previously associated with diseases of the prostate and various cancers. Taken together, these results suggest a contribution of specific bacterial taxa, to the aggressive PCa disease observed amongst South African men.

Declaration

I, Pieter Hendrik Bouwman, declare that the dissertation 'Analysis of the Bacterial Microbiome from Prostate Tissue of South African Men' which I hereby submit for the degree MSc at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Full names:	Pieter Hendrik Bouwman
Student number:	11319608
Date submitted:	2019/06/06
Degree:	M.Sc. Genetics
Topic of work:	Analysis of the Bacterial Microbiome from Prostate Tissue of South African Men
Supervisor:	Prof Donald A. Cowan, Department of Genetics
Co-supervisors:	Prof Angel Valverde, University of the Free State
	Prof Riana S. Bornman, Department of Urology
	Prof Vanessa M. Hayes, Garvan Institute of Medical Research

Signature: _____

Acknowledgements

I would like to acknowledge and thank the National Research Foundation (NRF) and the Genomics Research Institute (GRI) for the funding they provided this project.

I wish to acknowledge Prof Don Cowan for his support and guidance throughout my degree. A big thank you for everything from the initial introduction that sparked this collaboration, to all the energy you put into CMEG. Similarly, I would like to thank Dr Angel Valverde for the many hours he spent guiding me through this project. Your assistance with the statistics, motivation during the long waits, and positive attitude are truly appreciated. To Prof Vanessa Hayes, my thanks for the incredible opportunity to visit Australia. Visiting your facilities and taking part in PCWC 2015 are memories I will carry with me for many years. Thank you for your commitment to the SAPCS and also for the trolley incident.

To Prof Riana Bornman, my heartfelt thanks for managing the Pretoria branch of the SAPCS with the passion that you do. You showed me what it means to truly care for the patients, and what true inspiration and hard work can achieve. Dankie Prof, u invloed word inniglik waardeer, deur almal wat die voorreg kry om met Prof te werk.

To the Heroin Puppies, thanks for Soccer Physics tournaments and chats over coffee. You are all legends. A special thank you to Big Mike for the many years of friendship. Your support has been unwavering, and you are a constant inspiration to me Boet. Finally, to the other two members of the PCR, thank you. I am forever grateful for your friendship, and I know we will have many more adventures to share.

My greatest thanks go out to my family and loved ones. The examples you all set for me, the steadfast support you provide, and the love you give me are the foundation of my life. To mom and dad, thank you for always fighting for me and showing me what is really important in life. LYA. To Nikkie, you really are the Bestest Sisterest Everest. I can't thank you enough for everything you did for me through my degree, and all of my life. I'm very lucky to have you. And finally, my thanks to Rochelle for the support you show me every single day, for waiting with me through all of these years, and for believing in me even when I can't anymore. I am incredibly blessed to have you in my life.

Dedication

This dissertation is dedicated to Rochelle, my parents, and my sister. Love you all.

Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	Definition
ADT	Androgen deprivation therapy
ANOVA	Analysis of Variance
BP	Bacterial prostatitis
bp	Base pairs
BPH	Benign prostatic hyperplasia
°C	Degrees Celcius
DNA	Deoxyribonucleic acid
DRE	Digital rectal exam
EAU	European Association of Urology
EBRT	External-beam radiation therapy
et al	*et alia*
EtOH	Ethanol
GS	Gleason score
HIV	Human immunodeficiency virus
HMP	Human Microbiome Project
IARC	International Agency for Research on Cancer
PGM™	Ion Personal Genome Machine™
ISUP	International Society of Urological Pathology
LUTS	Lower urinary tract symptoms
µl	Micolitre
ml	Millilitre

ng	Nanogram
NBP	Nonbacterial prostatitis
nMDS	Non-metric multidimensional scaling
NIH	National Institutes of Health
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
PCa	Prostate Cancer
PCA	Principal Component Analysis
PERMANOVA	Permutational multivariate analysis of variance
PSA	Prostate specific antigen
QIIME	Quantitative Insights Into Microbial Ecology
rDNA	Ribosomal deoxyribonucleic acid
RP	Radical prostatectomy
rRNA	Ribosomal ribonucleic acid
SAPCS	South African Prostate Cancer Study
TUIP	Transurethral incision of the prostate
TURP	Transurethral resection of the prostate

Chapter 1: Literature Review

1.1 Global cancer burden

Cancer is a leading cause of death worldwide and this burden is expected to increase (TORRE *et al.* 2015). According to the GLOBOCAN project, 14.1 million new cases of cancer were estimated to have occurred globally in 2012 along with 8.2 million deaths, with the incidence predicted to approach 20 million new cases annually as early as 2025 (FERLAY *et al.* 2013).

While cancer is a global problem, medium- and low-income countries are facing an increasing proportion of the burden (VINEIS AND WILD 2014). Developing countries represent 57% of cases and 65% of the deaths attributed to cancer in 2012, yet these countries frequently lack the population-based cancer registries that record cancer prevalence (TORRE *et al.* 2015). The true impact of cancer in these regions may be further obscured by other confounding factors including a younger age structure and competing causes of death such as infection. It is predicted that medium- and low-income countries will experience 78% and 93% increases in cancer incidence respectively, from 2008 to 2030 (BRAY *et al.* 2012).

The global incidence of Prostate Cancer (PCa) in men is second only to lung cancer (14.8% vs 16.8% of cancer in men in 2012). It is expected that by 2030 there will be 1.85 million new cases of PCa and over 500 000 deaths (FERLAY *et al.* 2013). While the mortality rate of PCa seems to be decreasing, it is expected that the absolute number of men with PCa will increase (SFANOS AND DE MARZO 2012).

1.2 Anatomy of the prostate

The prostate is an accessory gland of the male reproductive system and plays a role in the urinary system (CANADIAN CANCER SOCIETY 2017). It has an oval shape with a pointed tip similar to an inverted pyramid (AMIS 1994). It is located directly below the bladder surrounding the urethra (**Figure 1-A**) and it is surrounded by a layer of connective tissue known as the prostatic capsule. The prostate is heterogeneous in nature comprising equal parts of fibromuscular and glandular tissue by weight. The prostate is subdivided into three compartments known as the peripheral, transition and central zones (**Figure 1-B**).

The prostate contains 20-40 tubuloalveolar glands which have excretory ducts leading into the urethra as it passes through the prostate and it is closely associated with the seminal vesicles (AMIS 1994). These are paired organs located between the lower bladder and the rectal wall above the prostate which further contribute to the male ejaculate.

The prostatic zones are of particular interest with regards to prostatic disease as they typically display different frequencies of the three most prevalent prostate conditions (Amis 1994). These diseases defined broadly are prostatitis and adenocarcinoma which typically originate in the peripheral zone and benign prostatic hyperplasia (BPH, formerly described as benign prostatic hypertrophy), which has been reported to originate in the transition zone.

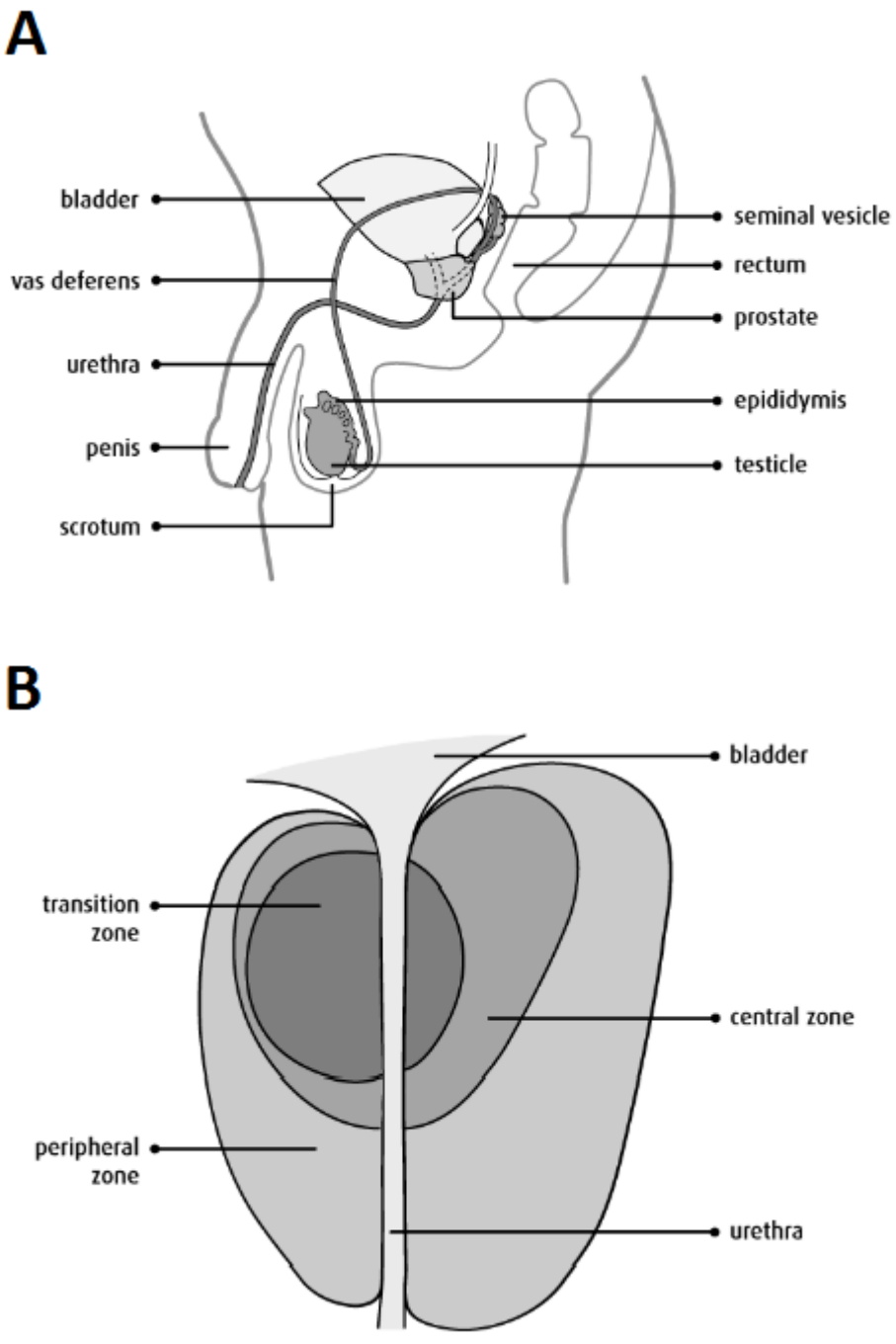


Figure 1-A. The male reproductive system. 1-B. Zones of the prostate. Taken from The Canadian Cancer Society on 2016/04/03**.**

1.3 Diseases of the prostate

1.3.1 Prostatitis

Prostatitis is a common disorder of the prostate. It is estimated that 10% of all men are affected by the disease at any time (NATIONAL CENTER FOR HEALTH STATISTICS 1995). The precise prevalence of prostatitis is unknown, but a 2003 analysis of global epidemiological data estimated a range as high as 15-16% (KRIEGER *et al.* 2003). Symptoms of the disease include pain in the perineum, scrotum, and even the back. The disease may also produce various urinary symptoms such as hesitancy, a weak stream and painful urination; with up to 25% of affected patients reporting impotence or ejaculatory dysfunction (TANNER *et al.* 1999). Prostatitis has been sub-classified into the four categories shown in **Table 1** by the National Institutes of Health (NIH) based on clinical presentation (KRIEGER *et al.* 1999).

Table 1. NIH definition and classification of prostatitis. Table from GILL AND SHOSKES (2016).

Category I	Acute bacterial prostatitis
Category II	Chronic bacterial prostatitis
Category III	Chronic prostatitis/chronic pelvic pain syndrome
Category IV	Asymptomatic inflammatory prostatitis

The first two categories may be referred to as bacterial prostatitis (BP), while cases from the remaining categories are called nonbacterial prostatitis (NBP). The majority of cases are assigned to category III or IV; however, some studies suggest that these cases may still have an infectious origin (TANNER *et al.* 1999; NICKEL 2017).

The diagnosis into BP groups is reliant on culturing of organisms from urine or expressed prostatic secretions, or a urine gram stain (SHARP *et al.* 2010). The use of culturing as a tool for microbial identification, and more accurate methods of microbial analysis are discussed in section 1.7 of this review. TANNER *et al.* (1999) used a molecular method (sequencing) to identify bacterial DNA from the expressed prostatic secretions of patients diagnosed with BP, as well as patients diagnosed with NBP. They were able to identify three patients who had been diagnosed with NBP but produced positive bacterial DNA signals, and successfully treated these patients with antibiotics. Antibiotic treatment of Type III prostatitis was shown to be effective in improving symptoms (NICKEL *et al.* 2003; ALEXANDER *et al.* 2004), and improved NIH-Chronic Prostatitis Symptom Index scores (ZHOU *et al.* 2008; REES *et al.* 2015).

1.3.2 Benign prostatic hyperplasia (BPH)

The prevalence of BPH has been shown to correlate strongly with age (**Table 2 below**). Some authors report global occurrence rates as high as 90% among men in their 80's based on various studies (PAOLONE 2010).

Table 2. Histologic Prevalence of BPH with Age.
Adapted from PAOLONE (2010).

Age (Decade)	Histologic Prevalence
30s	0%
50s	50%
70s	80%

Voiding symptoms

- Hesitancy is a delay in the onset of micturition which shows the time required by the detrusor muscle to overcome outlet resistance.
- Symptoms of poor urinary flow and straining to void develop insidiously, so that the patient may not notice the decreasing flow. A flow rate less than 10 mL/s is suggestive of obstruction.
- The sensation of incomplete bladder emptying indicates that the bladder is unable to empty itself completely, causing residual urine to develop.
- The symptoms of terminal or post-micturition dribbling are associated with age-related weakness in the bulbo-spongiosus muscle, which aids urethral emptying.
- Prolonged urination is seen when the outlet is obstructed, because the reduced flow rate results in an increased time taken to void

Storage symptoms

- Healthy daytime urination takes place less than seven times per day. Urinary frequency is defined as voiding too frequently during the day.
- Nocturia is defined as having to wake at night to void and can greatly impair the quality of life of the patient and their partner. In addition to these causes, it also arises in cardiovascular disease, hypertension, and diuretic treatment when a reversal of the normal diurnal rhythm of urinary concentration may be seen.
- Urgency is a sudden compelling desire to void, which is difficult to defer. It tends to arise in men who also have frequency and nocturia, and can be caused by BPH or by idiopathic detrusor over-activity.

Figure 2. LUTS categories. Adapted from THORPE AND NEAL (2003).

BPH is a non-malignant growth of the prostate gland causing it to become enlarged and many men may never develop any symptoms from the condition or require any treatment for it (ROEHRBORN 2004; SIMON AND ZIEVE 2012). The most common set of symptoms that a patient will experience are collectively referred to as lower urinary tract symptoms (LUTS) and are categorised as either voiding or storage symptoms, summarised in **Figure 2**.

The review from THORPE AND NEAL (2003) further mentions that BPH can lead to acute or chronic urinary retention which may necessitate surgical intervention. The American Urological Association (AUA) published a detailed guideline for diagnosis and treatment of BPH (AUA PRACTICE GUIDELINES COMMITTEE 2003). **Figure 3** is adapted from the framework that the authors created to address diagnosis and treatment of the disease.

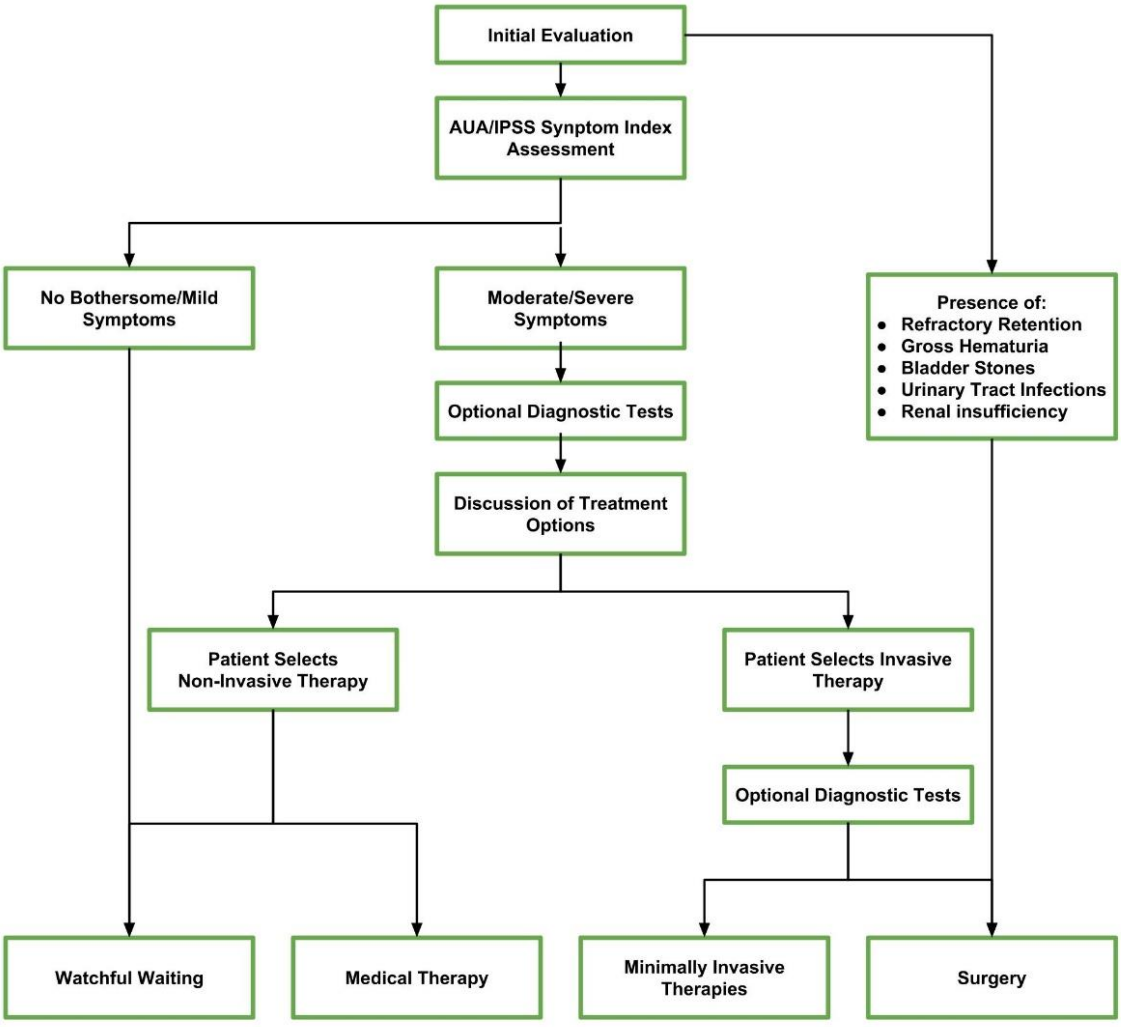


Figure 3. Diagnosis and treatment framework for BPH. Adapted from AUA PRACTICE GUIDELINES COMMITTEE (2003).

The four treatment options differ in risk to the patient as well as intended outcome. Watchful waiting is the treatment option which presents the lowest risk but it has no curative intent. It is a management strategy where the patient is advised to make lifestyle changes to reduce their symptoms such as reducing alcohol and caffeine intake (AUA PRACTICE GUIDELINES COMMITTEE 2003). Additionally the patient will be monitored by repeat visits for re-examination and the treatment decision will be adjusted if the symptoms worsen. This is especially important as recent literature suggests that BPH is progressive in nature (WIYGUL AND BABAYAN 2009). Watchful waiting is suitable for patients with no bothersome symptoms.

Table 3. Summary of minimal-invasive surgeries. (CHUNG AND WOO 2014).

Technology	Commercially available	Local anaesthesia possible?	Outpatient treatment possible?	Routine catheterization essential?	Clinical trial data	Adverse events
Intraprostatic botulinum toxin injection	Yes	Yes	Yes	No	Phase 2 trial completed and published.	Not significant
Intraprostatic ethanol injection	Yes	Yes	Yes	No	Phase 2 trial completed and published.	Infrequent but catastrophic bladder necrosis reported
Intraprostatic NX-1207 injection	No	Yes	Yes	No	Phase 2 trial completed and published. Phase 3 trial in progress	Not significant
Intraprostatic PRX302 injection	No	Yes	Yes	No	Phase 2 trial completed and published.	Not significant
Urolift	Yes	Yes	Yes	No	Phase 3 trial completed and published.	Not significant
Urolume stent	Yes	Yes	Yes	No	Phase 3 trial completed and published.	Migration/explantation
Memokath stent	Yes	Yes	Yes	No	Phase 3 trial completed and published.	Migration/explantation
Allium stent	Yes	Yes	Yes	No	None published.	Unknown
Rezum	No	Yes	Yes	No	None published.	Post-procedure acute urinary retention in 50%
Histotripsy	No	Unknown	Unknown	Unknown	Pilot study in progress.	Unknown
Aquablation	No	Unknown	Unknown	Unknown	Pilot study in progress.	Unknown

However, medical treatment is advised in the event of moderate to severe symptoms. Medical therapies aim to reduce the symptoms that the patient experiences, but they have a lower efficacy than surgical intervention as they are not curative treatments (MADERSBACHER *et al.* 2007). The range

of medical therapies available include: phytotherapeutic agents, α1-adrenergic receptor-blockers, 5α-reductase inhibitors as well as antimuscarinic agents (PAOLONE 2010). A combination therapy which uses different classes of treatments may also be considered.

Contrasting with non-invasive therapies, invasive therapy does have a curative intent. Many minimally invasive therapies have been investigated and used with varying success. A few recent techniques are summarised in **Table 3.** The goal of minimally invasive therapy is to decrease bladder outlet obstruction and relieve the LUTS (PAOLONE 2010). In addition to more severe cases, minimally invasive treatments are also recommended when patients have adverse effects to medical therapy, or when taking the drugs becomes too bothersome or expensive for the patient (ROEHRBORN 2005). This treatment avenue is an intermediate which offers lower symptom improvement than ablative surgical options, but negates the requirement of general anaesthesia and with minimal significant morbidity (CHUNG AND WOO 2014).

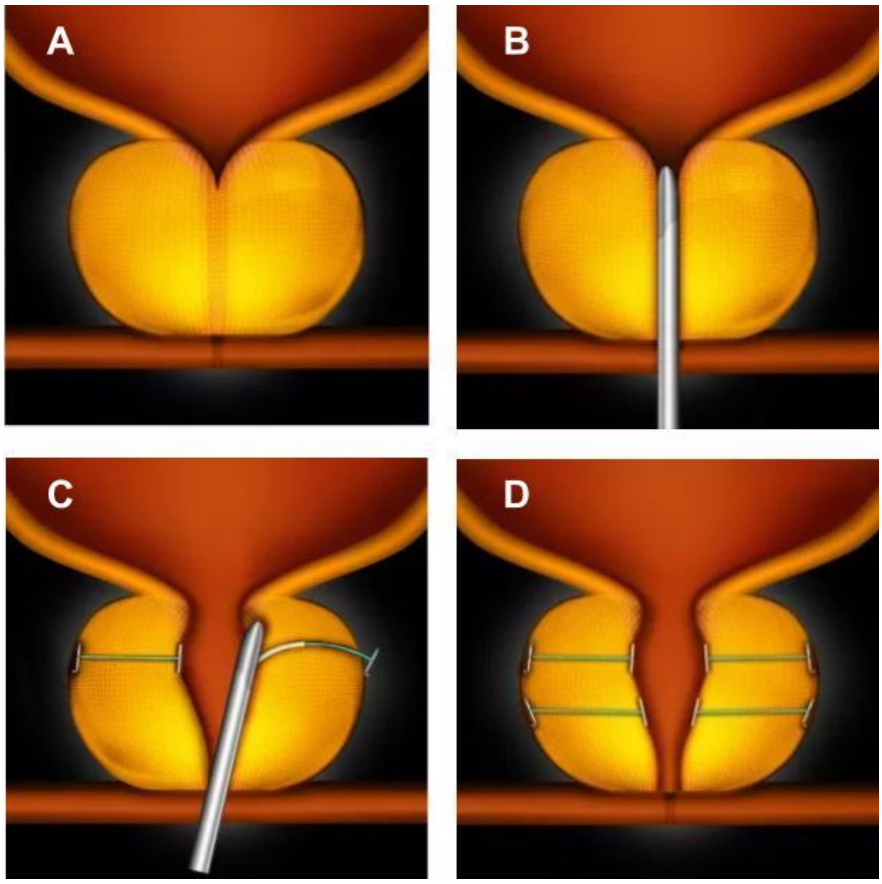


Figure 4. Urolift system. The Urolift system as a treatment for an enlarged prostate (4-A). The delivery device is inserted into the enlarged prostate (4-B) and Urolift implants are placed in the prostate to hold back the tissue (4-C) producing an open urethra (4-D). From NEOTRACT INC (2017).

Minimally invasive therapies use amongst others- heat therapy, transurethral needle ablation, intraprostatic injection, or the insertion a device such as a stent or the newer Urolift (AUA PRACTICE GUIDELINES COMMITTEE 2003; ROEHRBORN 2005; CHUNG AND WOO 2014). The Urolift device's functioning is displayed in **Figure 4**. The procedure can be performed by a urologist in his offices under local anaesthetic (NEOTRACT INC 2017).

In the most severe cases of BPH, surgery would be recommended. The ideal option for surgical relief of BPH is a transurethral resection of the prostate (TURP), where an electrified loop is used to resect obstructive prostate tissue and cauterize sites of bleeding (PAOLONE 2010). The procedure is performed via endoscopy through the urethra and requires no external skin excision. TURP is comparable to watchful waiting with regards to rates of urinary incontinence and decline in sexual function (WASSON *et al.* 1995). Transurethral incision of the prostate (TUIP) is a similar procedure, where incisions are made in the prostate and prostate capsule rather than excision of prostatic tissue. This procedure has a lower risk of side effects, but TURP offers a greater chance of success and superior symptom improvement (PAOLONE 2010).

Open prostatectomy is reserved for patients with a massively enlarged prostate where it is unlikely that transurethral approaches will suffice (PAOLONE 2010). The prostatectomy may be performed with a retro- or suprapubic approach, during which the transition zone of the prostate is removed. This procedure is effective for BPH, but as the peripheral zone of the prostate is left behind, the procedure is not an adequate treatment option for PCa, the next condition to be discussed.

1.3.3 Prostate cancer

PCa is similar to BPH as it rarely affects younger men, with less than 1% of cases reported in men under 50 (FERLAY *et al.* 2013). The incidence of PCa may not be as high as that of BPH, but the cumulative risk of developing PCa by the age of 74 years is 8.8% globally and this rate is expected to increase (SFANOS AND DE MARZO 2012; TORRE *et al.* 2015). This increase is attributed to growing populations and the aging of these populations.

Cancer occurs when cells begin to divide uncontrollably despite signals regulating the cell cycle. These cells may infiltrate other tissues, and the disease will often kill the host organism if left untreated, as they do not respond to density-dependant inhibition and lack anchorage dependence (CAMPBELL AND REECE 2008). Additionally, some cancer cells are able to continue dividing indefinitely when provided with sufficient nutrients.

The disease requires a normal cell to become transformed to a cancerous cell (CAMPBELL AND REECE 2008). This process typically requires multiple somatic mutations described as the multistep model

of carcinogenesis (WEINBERG 1989). These cells then begin proliferating to form a mass, which is defined as a benign tumour if it remains at the original site without invading nearby tissue or metastasizing. Malignant tumours occur when the mass begins to impair the function of the organism's organs and in advanced cancer; some cells may split off from the tumour and travel to other sites in the body. The cancer is described as metastatic if these cells begin to develop into secondary tumours. PCa most commonly arises as an adenocarcinoma, meaning that it is a malignancy originating from glandular cells (AMIS 1994).

Grading of PCa is achieved with the Gleason score (GS), however metrics such as the serum prostate-specific antigen (PSA) may also be used to describe the disease severity. The GS was developed between 1966 and 1974 to describe prostatic carcinoma based on the architectural pattern of the cells (BAILAR 3RD *et al.* 1966; GLEASON 1992; EPSTEIN *et al.* 2005). This system has formed the backbone of PCa grading but has been revised over time to include the opinions of experts in the field of urology with regard to the inclusion of different cell types.

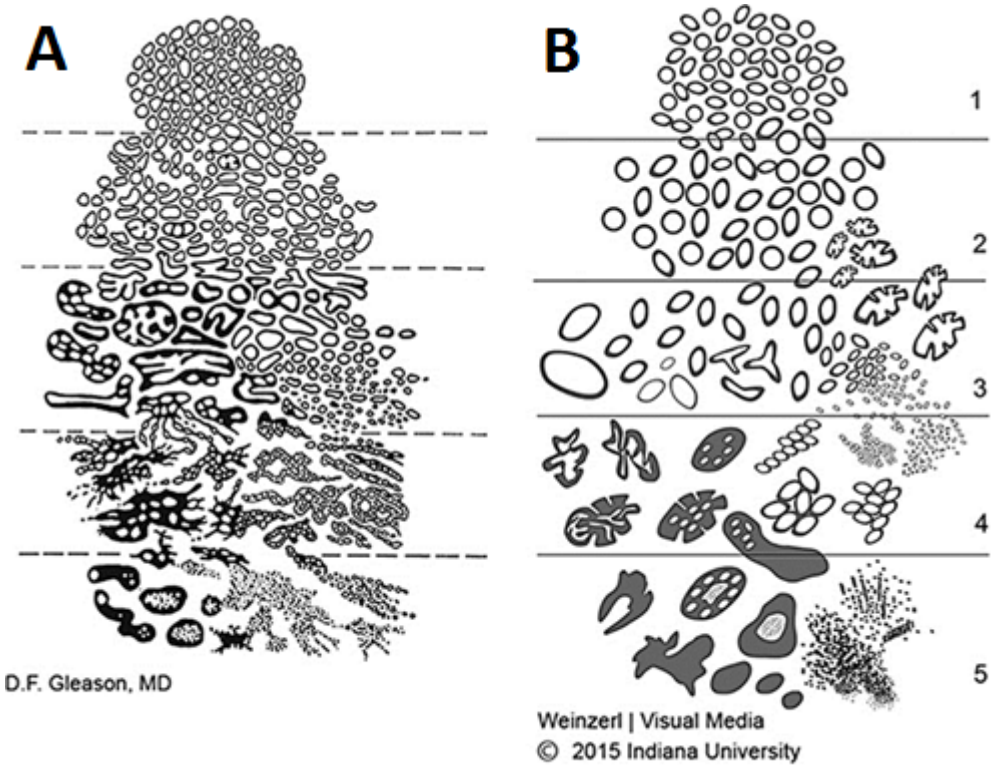


Figure 5. Histologic patterns of prostatic adenocarcinoma. (A). Original Gleason classifications. (B). Revised 2015 ISUP Gleason classifications. Taken from EPSTEIN *et al.* (2016).

These modifications are revised by the International Society of Urological Pathology (ISUP) Consensus Conferences, where the nature of the grading system is edited to reflect patient's outcome and aid their understanding of the assigned grade (EPSTEIN *et al.* 2016). **Figure 5-A** shows the original

classification and **Figure 5-B** reflects the 2015 modified ISUP Gleason classifications. The most important changes were that cribriform glands would be assigned to a Gleason score 4 regardless of morphology. Previously round and regular cribriform glands would have been assigned to a GS 3. Similarly the participants of the consensus meeting agreed that glomeruloid glands should always be assigned to a GS 4. It was decided that mucinous carcinoma should not be graded as a GS4, but that the underlying growth pattern should determine the grading.

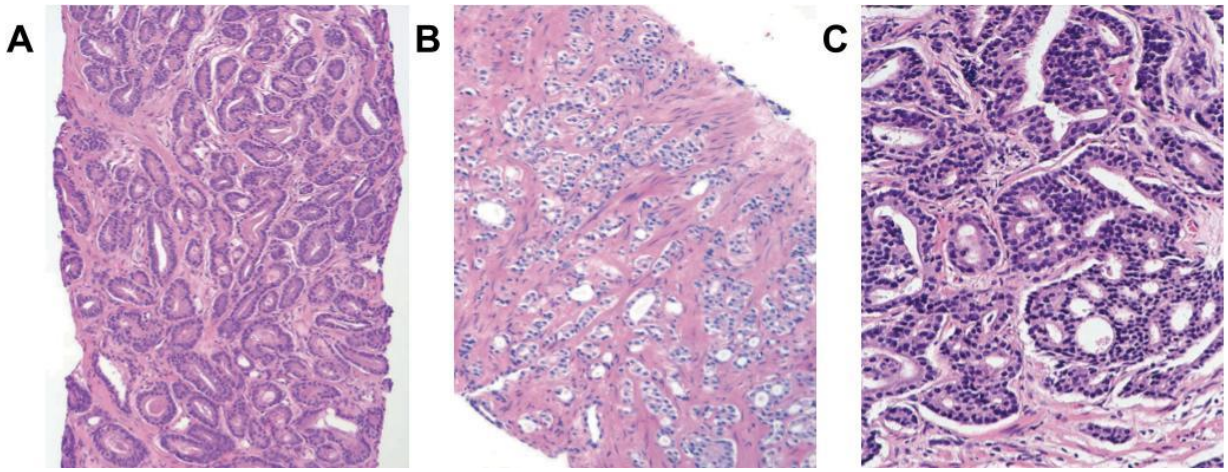


Figure 6. Examples of prostatic adenocarcinoma for pathology. Taken from EPSTEIN *et al.* (2005)

Traditional Gleason scores are selected by a pathologist who scores the prostate based on the first and second most common patterns observed in the tumour tissue, producing a score between two and ten. For example- a biopsy core with mostly well differentiated cells (Gleason 3) but a few poorly differentiated cells (Gleason 4) is given a Gleason score of 3 + 4 = 7. Examples of prostatic adenocarcinoma are shown in **Figure 6**, where the samples A-C were graded as a 3 + 3 = 6 grade tumour, a 4 + 3 = 7 tumour, and a 4 + 4 = 8 tumour respectively. **Figure 6-A** displays small and discrete glands that are variably sized. In contrast, the majority of the biopsy in **Figure 6-B** displays poorly formed glands. The final panel demonstrates a biopsy containing cribriform glands which are large and irregular (**Figure 6-C**)

The scale used in the Gleason grading system did however have some weaknesses. Firstly, it was observed that Gleason scores between two and five were rarely assigned to patients and that the use of these assignments were in decline (EPSTEIN *et al.* 2016). As such, the lowest clinical grade being assigned was a six out of ten. This score may mislead the patient into thinking that the severity of their cancer is in the middle of the grading scale rather than the lowest possible grade. This impacts negatively on their emotional state. Another major drawback was the grade combinations that were being used for prognosis and therapy.

Table 4. Definition of New Grading System. Adapted from EPSTEIN *et al.* (2016)

New Grade Groups	Gleason score
I	≤ 6
II	3 + 4 = 7
III	4 + 3 = 7
IV	4 + 4 = 8 Or 5 + 3 = 8 Or 3 + 5 = 8
V	9-10

The goal of this grouping is to stratify patients into low-, intermediate- and high-risk groups, but these assignments were not being made consistently, which prohibited comparisons between studies. Additionally, Gleason grade seven tumours were shown to have significantly worse prognoses if the patient was a 4 + 3 rather than a 3 + 4 (CHAN *et al.* 2000; STARK *et al.* 2009). In other words, tumours with a majority of poorly differentiated cells vs mostly well differentiated cells. As a consequence, a new grading system was sought to divide patients into the lowest number of grades such that each would have a unique prognosis (**Table 4**). It was presented by the 2014 ISUP consensus conference, and more accurately reflects the disease progression and state than the traditional Gleason scoring.

In contrast to the invasive nature of the biopsy required for Gleason scoring of a patient's prostate, PSA can be measured from a blood test. PSA is a glycoprotein that is produced by the prostate and can be detected in the blood serum. PSA levels may be elevated as a result of PCa, but also increase in response to other prostatic diseases such as prostatitis and BPH, procedures such as a biopsy or

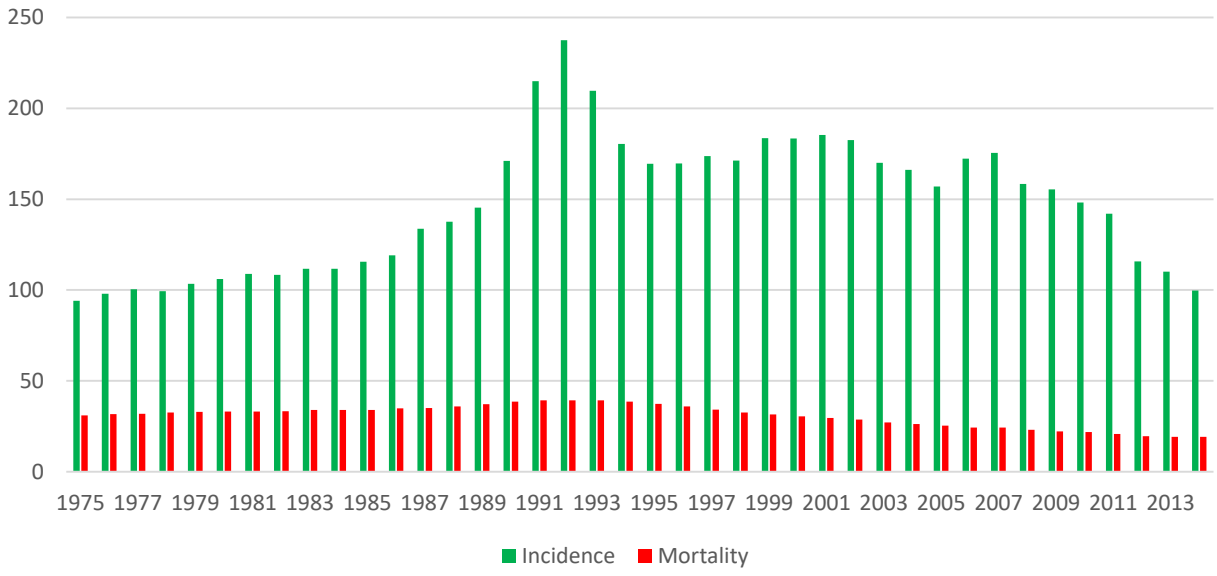


Figure 7. US PCa Incidence and Mortality rates per 100 000 1975-2014. Data from the SEER Program (HOWLADER *et al.* 2017).

transurethral prostatectomy, and even ejaculation (HERSCHMAN *et al.* 1997; BARRY 2001). The PSA test has become highly controversial with regards to screening for PCa, but the test is also used for diagnosis of PCa and throughout treatment to describe the severity of the disease and success of therapy.

The US incidence and mortality rates from 1975 to 2014 are shown in **Figure 7**, data taken from the Surveillance, Epidemiology and End Results (SEER) Program (HOWLADER *et al.* 2017). The introduction of the PSA test in the late 1980's caused the reported incidence of PCa to rise dramatically (BARRY 2001). The increase is attributed to an over-diagnosis and over-treatment of patients with lower grade PCa. This period was followed by a sharp decrease, which was attributed to the identification of men with a pre-existing condition. Despite highly variable incidence rates, the mortality rate remained near constant over this period. The incidence rate had little fluctuation for the following this decrease from 1995, but decreased again after 2011 when the US Preventative Services Task Force announced its recommendation against PSA screening for PCa (ETZIONI AND GULATI 2016).

This decision is based on the opinion that "-there is moderate or high certainty that the service has no net benefit or that the harms outweigh the benefits-" (MOYER 2012). The Task Force based the recommendation on their observations that: the test has a high false-positive rate, a high rate of complications are associated with the diagnostic biopsy, a there is a large risk of over-diagnosis and the accompanying overtreatment. Additionally, they concluded that the mortality benefits of the screening after 11 years were still small to non-existent.

The European Association of Urology (EAU) have published a set of guidelines on PCa with the most recent update available in 2014. They recommend that the decision surrounding PSA testing should be made by the patient and his physician after discussing the advantages and disadvantages of the test

Table 5. Risk of PCa in relation to low PSA values. Adapted from (THOMPSON *et al.* 2004)

PSA level, ng/ml	% Risk of PCa
0-0.5	6.6
0.6-1	10.1
1.1-2	17.0
2.1-3	23.9
3.1-4	26.9

(HEIDENREICH *et al.* 2014). This concept of shared decision making does however have its own challenges. Men from the Health Information National Trends Survey were interviewed for a study by LEYVA *et al.* (2016). They identified 811 men who were between the ages of 50 and 74, with no prior history of PCa. Of these men, 55% reported that they had undergone a PSA-test. The researchers determined that 81% of these men had discussed whether or not to do the test with their physicians, but that only 33% were told that there is uncertainty surrounding the test's validity. Additionally, it was found that black men or less educated men were even less likely to be provided with this information. The EAU guidelines provide a framework for PSA-based screening, depending on patient age and baseline PSA if available (HEIDENREICH *et al.* 2014). **Table 5** shows the risk of PCa with low levels of PSA (≤4ng/ml) of men who were in the placebo arm of a PCa Prevention Trial spanning 7 years (THOMPSON *et al.* 2004). The results indicate that men with a low PSA level are still at risk of PCa.

The final examination to suspect PCa is a digital rectal exam (DRE). In this test a physician will investigate the size of the prostate and will also search for any bumps or irregularities of the prostate with a lubricated finger inserted in the rectum (CANCER.NET EDITORIAL BOARD 2012). DRE has been found to be suggestive of PCa, regardless of the patient's PSA level (HEIDENREICH *et al.* 2014). It has been found that as many as 30% of patients who have a PSA level below 4 ng/ml but a suspicious DRE, may be diagnosed with PCa (CARVALHAL *et al.* 1999). Therefore, a hard nodular prostate on DRE is suggestive of PCa, but a normal DRE does not exclude PCa.

The EAU guidelines (HEIDENREICH *et al.* 2014) recommend that the decision to perform a biopsy be made on the basis of the PSA of a patient, a suspicious DRE and various patient information, such as age, comorbidities and the consequences of treatment. The standard practice is to perform a transrectal ultrasound-guided (TRUS) or transperineal laterally directed 18G core biopsy. During the procedure, 10-12 cores of prostatic tissue are removed from the prostate and sent for histopathologic examination (Gleason scoring).

The Prostate Cancer Foundation of South Africa has published a set of Prostate Cancer Diagnostic and Treatment Guidelines (COETZEE *et al.* 2013). Similar to the EAU guidelines, detection and diagnosis of PCa is recommended via DRE and PSA. Additionally, a prostate cancer antigen 3 (PCA3) test is recommended to stratify the risk categories for suspected PCa cases. A prostate biopsy is indicated following an abnormal DRE, or when the PSA passes an age-specific threshold or displays an increased PSA velocity.

The severity of the tumour is a key consideration when selecting the treatment or treatment combination for PCa (HEIDENREICH *et al.* 2014). The least invasive treatment option is active

surveillance which is suitable in cases of very low risk PCa, and addresses concerns of overtreatment. These patients will be carefully monitored, but further treatment will be avoided until the disease shows signs of progression. KLOTZ *et al.* (2014) observed that well differentiated PCa patients have an excellent long-term PCa-specific survival rate. This ongoing study reports that men with low-risk prostate cancer experienced 10 and 15 year survival rates of 98.1% and 94.3% respectively, and 55% of the patients avoided any other treatment. The authors conclude that these rates can be further increased through more accurate early diagnosis of the PCa. Patients for whom active surveillance is recommended typically have a Gleason score ≤ 6 (New Grade I), and a PSA < 10 ng/ml (HEIDENREICH *et al.* 2014).

Some practitioners may prescribe watchful waiting for low-risk PCa but it has been shown that radical prostatectomy (RP) provides better PCa-specific survival rates (HOLMBERG *et al.* 2012). The authors report an absolute risk reduction of 6.6% in overall mortality between patients assigned to RP, compared with watchful waiting. The EAU guideline calls for RP of low- to intermediate-risk localised PCa, preferably nerve-sparing RP which aims to preserve sexual potency and urinary continence (CATALONA AND BIGG 1990). Recent literature cites robot-assisted RP (RARP) as the gold standard approach to perform the procedure (PORPIGLIA *et al.* 2013; HEIDENREICH *et al.* 2014; HU *et al.* 2014). Patients treated with RARP were found to require less use of additional cancer therapy across 24 months after the procedure when compared with open RP. Another study found that RARP patients had superior functional outcomes of continence and recovery of erectile function than patients undergoing laparoscopic RP.

RP is typically included in treatment for intermediate- and high-risk PCa (HEIDENREICH *et al.* 2014). Such patients who underwent RP typically show good 5- and 10- year survival rates including cases where lymph node metastases were detected. GONTERO *et al.* (2011) reported on cancer-specific survival rates at 5- and 10-years. They observed a poorer outcome for patients with increased PSA with survival rates dropping from 96% and 91% respectively for a pre-operative PSA >20,1-50 ng/ml, to 88% and 80% for patients with >100ng/ml serum PSA.

Radiation therapy may also be recommended for patients with low-risk PCa who wish to avoid the side effects of surgery. The most common treatment is 3D conformal radiation therapy which is a technique that allows precise delivery of radiation to localised tumours, while avoiding healthy tissues around it (ZELEFSKY *et al.* 1998). This system has been optimised in development of the intensity-modulated radiation therapy (IMRT) which can escalate dosage without increasing the associated toxicity (ZELEFSKY *et al.* 2002). Finally, brachytherapy has been shown to be a safe and effective option with STOCK *et al.* (2006) reporting a 96% disease-specific survival rate after 10 years. This

procedure involves the implantation of radioactive particles into the prostate to deliver direct radiation to the tumour, and may be used as a monotherapy, or in conjunction with other treatments (DAVIS *et al.* 2012).

External-beam radiation therapy (EBRT) should be considered for intermediate- to high-risk PCa patients (HEIDENREICH *et al.* 2014). Immediate postoperative radiation therapy has been shown to increase 5-year survival rates by approximately 20%. The therapy may also be considered as a monotherapy, or in conjunction with brachytherapy or androgen deprivation therapy (ADT).

ADT is a treatment initially intended for the treatment of metastatic disease. This therapy aims to reduce the levels of androgens in the body, as they have been shown to be involved with prostate cancer growth (HARRIS *et al.* 2009). ADT may be performed surgically through orchiectomy, or medically through the administration of gonadotropin-releasing hormone agonists. The latter is currently preferred in clinical practice. ADT has been shown to improve quality of life in metastatic disease, despite being associated with various adverse effects including hot flashes, metabolic changes and decreased sexual function (SHARIFI *et al.* 2005).

Table 6. Cohort studies of the association between BPH and PCa

Study	Sample Size	Outcome
ARMENIAN *et al.* (1974)	300 men with PCa, 300 men with BPH	4-5 fold increased risk of PCa
GREENWALD *et al.* (1974)	800 men with BPH	Null association
SIMONS *et al.* (1993)	4 800 men with BPH	Null association
CHOKKALINGAM *et al.* (2003)	87 000 men with BPH	Interim results showed a 1.2-1.7 fold increased risk of PCa incidence and mortality
SCHENK *et al.* (2011)	1000 men with BPH, 1 200 men with PCa	Null association
ØRSTED *et al.* (2011)	187 000 men with BPH, 53 000 men with PCa	Increased risk of PCa incidence (2-3 fold) and PCa-related mortality (2-8 fold)

There is controversy with regards to the association between BPH and PCa with published evidence both supporting and rejecting a relationship between the diseases. **Table 6** summarises these studies as per the review by ØRSTED AND BOJESEN (2013).

The two largest studies reported an increased risk of PCa incidence and mortality with BPH (CHOKKALINGAM *et al.* 2003; ØRSTED *et al.* 2011). This association is attributed to shared characteristics of the diseases such as inflammation (ØRSTED AND BOJESEN 2013). A relationship was found between chronic inflammation and the severity of LUTS and it has further been shown that C-reactive protein, a marker of inflammation, was associated with rapid worsening of this symptom set (NICKEL *et al.* 2008; SAUVER *et al.* 2009). These observations suggest that chronic inflammation is associated with the development of BPH. The role of inflammation in PCa is described later in this review.

In addition to inflammation, BPH and PCa have been found to share hormonal influences. Dutasteride is a form of 5α-reductase inhibitor which is intended to block the conversion of testosterone into dihydrotestosterone. Treatment with the drug reduced the risk of PCa incidence amongst men in the trial and was further found to increase outcomes associated with BPH, such as a 73% reduction observed in BPH requiring surgery (ANDRIOLE *et al.* 2010).

These observations amongst others suggest that BPH is associated with an increase in PCa incidence and mortality.

1.4 Prostate cancer in South Africa

PCa has been shown to be more severe in Sub-Saharan Africa, where it replaces lung cancer as the most common cancer in both incidence and mortality in men (BRAY *et al.* 2012). This is attributed to

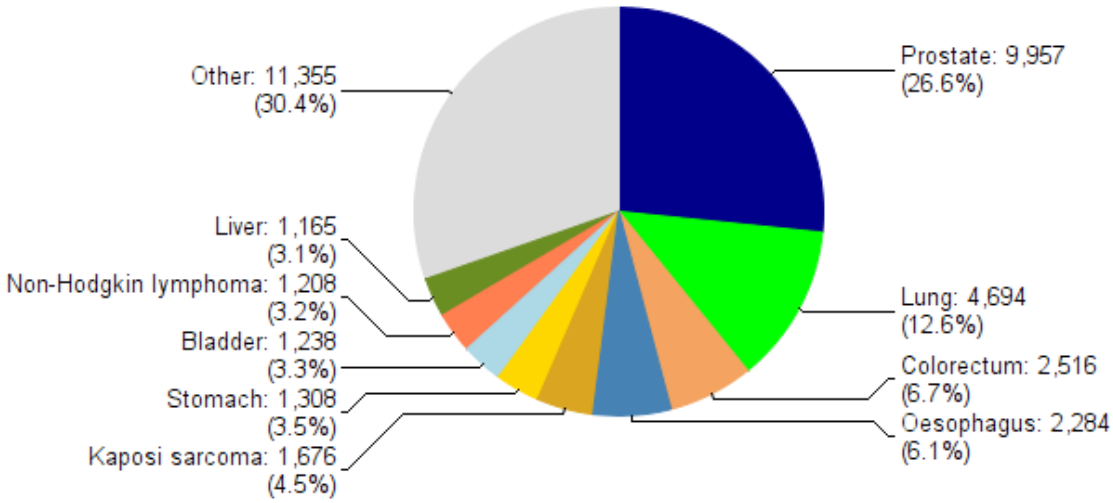


Figure 8. Pie chart of the estimated number of cancer cases of all ages of South African men. Taken from GLOBOCAN 2012 (IARC).

an inherited susceptibility to PCa development, as well as environmental and lifestyle exposures unique to these areas. In South Africa PCa is not only the most common cancer amongst men (NATIONAL CANCER REGISTRY: CANCER IN SOUTH AFRICA 2010), it has an incidence nearly three times more than that of lung cancer (**Figure 8**).

The South African Prostate Cancer Study (SAPCS) was established to assess the impact of PCa on Black South Africans in light of the disparity in PCa presentation between Southern Africa and the rest of the world. In addition to being more common amongst Black South African men, PCa also presents a more aggressive phenotype (TINDALL *et al.* 2014). Aggressive disease was defined by a Gleason score >7, and a PSA ≥20 ng/ml. The study reported that 83.0% (404/487) of men presenting with PCa had a PSA ≥20 ng/ml of which 60.4% (244/404) had a PSA ≥98 ng/ml.

Another study analysed the PCa incidence and mortality of men who are of African descent from different populations (REBBECK *et al.* 2013). The study reported a significantly greater proportion of tumours with high Gleason scores or high tumour stage for tumours in Africa when compared to those from populations of African men from USA or the UK. Further, the mortality:incidence rate ratio of cancers in Africa was highest at 0.71, compared to 0.41 in the Caribbean and only 0.14 in the USA. The authors attribute this to a substantial underreporting of PCa in Sub-Saharan Africa and the Caribbean. Other data from the study showed that PCa incidence correlated positively with the percentage of GDP spent on health care, the number of physicians per 10 000 of the population, as well as PCa mortality.

1.5 Inflammation in cancer

Chronic inflammation was first hypothesised to be involved in cancer initiation by Virchow in 1863 (COUSSENS AND WERB 2002). Characteristics of inflammation include increased cellular proliferation, the production of reactive oxygen and nitrogen species, as well as the production of growth/survival factors that support the growth of cells which have sustained DNA damage. Taken together, these elements provide an environment favouring the development of neoplasia. The excellent reviews by COUSSENS AND WERB (2002) as well as GRIVENNIKOV *et al.* (2010) thoroughly discuss the association between inflammation and cancer, and highlights from these reviews will be presented in this section.

Inflammation is a normal host response to tissue damage intended to heal the site of an injury. The response involves a complex network of immune and inflammatory cells as well as chemical signalling that makes use of chemotactic cytokines, or chemokines, to regulate the inflammatory response. Such inflammation is usually acute and self-limiting. In contrast to this, chronic

inflammation occurs after dysregulation of the inflammatory response either by continuous production of initiation factors or a failure of factors such as anti-inflammatory cytokines that are meant to terminate the inflammatory response (BALKWILL AND MANTOVANI 2001). The contrast is visualised in **Figure 9**. **Figure 9-A** represents typical wound healing characterised by a highly organised and segregated architecture. The inflammation is tightly regulated by signalling chemotactic factors and will subside after healing, moderated by reciprocal signalling. **Figure 9-B** is a representation of invasive carcinoma. The tissue is less organised, and blood vessels and lymphatics are arranged chaotically.

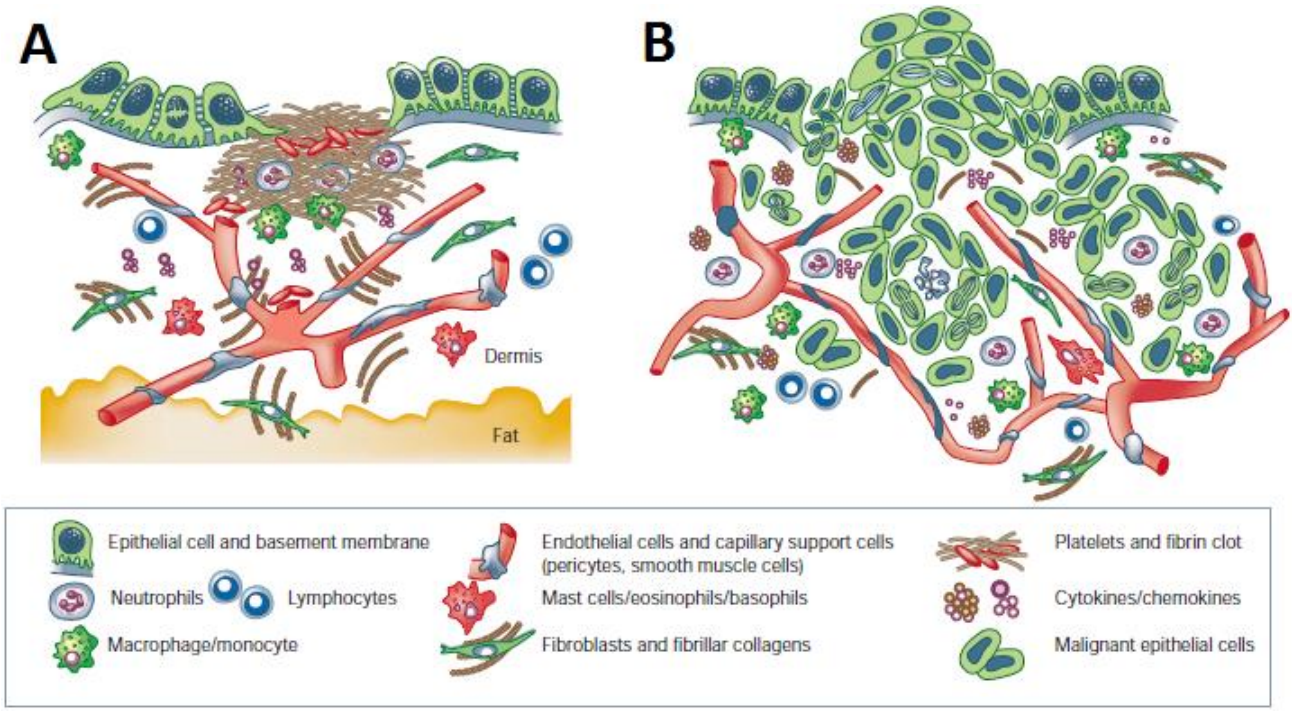


Figure 9. Wound healing compared with invasive tumour growth. (A). Typical wound healing. (B). Invasive carcinoma. Taken from Coussens and Werb (2002).

Chronic inflammation can have various sources. Certain bacterial and viral pathogens have been shown to subvert host immunity, and create long-term, but low-level chronic inflammatory regions which can progress to neoplasia. The role of infections in cancer are discussed in depth later in this literature survey. Other sources of inflammation linked to an increased cancer risk include obesity which increases the risk of all cancers analysed, and inhaled pollutants such as asbestos (BALKWILL AND MANTOVANI 2001; WOLK *et al.* 2001; LADOU 2004). Tobacco smoke contains carcinogens which are believed to initiate cancer through chronic inflammation (LEE *et al.* 2009).

Once tumour development is initiated, the nature of the inflammation changes to one that has pro-tumorigenic properties. Under these conditions oncogenes such as *RAS* and *MYC* may become

activated, initiating a transcriptional program that drives cancer progression through tissue remodelling, angiogenesis and the recruitment of mast cells (SPARMANN AND BAR-SAGI 2004; SOUCEK *et al.* 2007). As the tumour grows it outpaces it's blood supply and lacks sufficient nutrients and oxygen (GRIVENNIKOV *et al.* 2010). The resultant metabolic or hypoxic stress will result in the continuous death of small numbers of cells. This necrosis induces production of chemokines such as Interleukin-1 and High Mobility Group Box 1 protein, which are chemokines able to initiate neoangiogenesis and produce additional growth factors for the remaining cancer cells (VAKKILA AND LOTZE 2004). While these effects are not observed in apoptotic cell death, necrotic cell death has been shown to initiate stromagenesis and promote epithelial proliferation which are necessities for tumour growth. The chemokines produced by neoplastic cells are mitogenic, driving tumour growth and angiogenesis, and enabling the metastatic spread through the venous or lymphatic system.

1.6 Infections in Cancer

It has been well established that certain pathogens can lead to cancer development in humans. KUPER *et al.* (2000) declared that, "Following tobacco use, infections as a group may be the most important preventable cause of cancer in humans-". In their review, they describe the three main mechanisms through which an infection can cause cancer in a host.

The first mechanism involves long term chronic inflammation by a pathogen. Carcinogenesis is driven by production of reactive oxygen species as well as reactive nitrogen species by phagocytes which damage the cell membranes, proteins, but also the DNA of nearby cells. In addition, these cells are stimulated to have a high proliferation rate to compensate for the cell damage. The combination of these two factors (DNA damage and a high proliferation rate) drives the development of malignant cells.

The second mechanism involves pathogens that can actively transform a cell by introducing an oncogene into the host genome, stimulating mitosis, or disrupting tumour suppressor genes.

The final mechanism describes infectious agents that induce carcinogenesis through immunosuppression. Perhaps the most well documented example of this is the human immunodeficiency virus (HIV) which has been associated with the development of Kaposi's sarcoma, as well as high grade non-Hodgkin's Lymphoma, amongst others. The authors mention that this route of carcinogenesis typically results in a very aggressive cancer course.

Pinpointing an infectious agent associated with cancer can be very difficult. The pathogen is typically one that is highly prevalent but only induces carcinogenesis in a small proportion of infected individuals, and often after a long period of infection. An example is *Helicobacter pylori* which is

estimated to affect half of all people on earth (HUNT *et al.* 2011). The bacterium was responsible for as much as 89.0% of all non-cardia gastric carcinoma in 2008, which represents approximately 6.2% of all cancer cases globally for the year (PLUMMER *et al.* 2015).

Helicobacter pylori was first declared a Group I (Definite) human carcinogen in 1994 by the International Agency for Research on Cancer (IARC). Many studies have confirmed the relationship between the infection and cancer, with odds ratios from 1.92-2.56 in six meta-analyses the investigated (LOCHHEAD AND EL-OMAR 2007).

Infection with the bacterium results in apoptosis of affected gastric epithelial cells. The chronic loss of these cells induces an increase in turnover rate, which is thought to increase the risk of accumulation of genetic changes, particularly those involved in survival and proliferation (HATAKEYAMA 2004). The authors present detailed explanations of the mechanisms underlying the infection, and highlight that the CagA-SHP2 complex involved in cell cycle disruption can only be detected in gastric mucosa from patients suffering from chronic atrophic gastritis, but not from patients who have progressed to carcinoma (YAMAZAKI *et al.* 2003). This indicates that *H. pylori* infection may play a role in the early stages of gastric carcinogenesis.

A recent review on global trends in gastric cancer mortality found declines in almost all countries (FERRO *et al.* 2014). The authors note that countries with a low prevalence of *H. pylori* infection in the 1990's exhibited the largest decreases, as observed in USA and other high-income countries. On the other hand, countries such as Japan which had higher infection rates in the 1990's still have higher mortality rates attributed to gastric cancer. This trend is a result of the infection typically being acquired during childhood. The implication is that it may take three decades for Japan, and two decades for Europe, to reach the low levels of gastric cancer mortality currently observed in the USA.

1.7 Inflammation and infection in prostate cancer

It has been shown that the presence of chronic inflammation in benign prostate tissue is associated with prostate cancer, and particularly high-grade prostate cancer (GUREL *et al.* 2014). Factors that contribute to prostatic inflammation include diet, infection and physical trauma (SFANOS AND DE MARZO 2012). Dietary mutagens known as heterocyclic amines are produced when meat is cooked at a high temperature, and diets rich in well-cooked meat have been associated with prostate cancer risk (RODRIGUEZ *et al.* 2006; SINHA *et al.* 2009). It has also been shown that one such molecule, 2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) is particularly associated with the rat prostate, but not all studies confirm these findings (SHIRAI *et al.* 1997; SANDER *et al.* 2011).

The physical trauma is thought to occur as a result of small bodies within the prostate. These are called corpora amylacea and calculi, and are common to the adult prostate (SFANOS AND DE MARZO 2012). It is thought that these bodies are remnants of previous acute inflammation as they are mostly comprised of proteins involved with this response.

The role of infection in prostate cancer is controversial. In 2013 a group of researchers published a literature survey of studies published from 1980 - December 2011, and concluded that there was no support for an association between PCa and the pathogens their survey focussed on (HRBACEK *et al.* 2013). The analysis however included only 74 articles out of an initial pool of 1 509 entries identified by a Medline/PubMed search of "PCa AND infection". In the same year, SFANOS *et al.* (2013) published a review countering this opinion. These authors conclude that there is evidence that bacterial infections are capable of initiating long-term chronic inflammation of prostatic tissue. They found inflammatory responses lasting up to a year post-infection in rodent prostates and further suggest that the organism/s responsible may not be detected as this inflammation is not dependant on the persistence of the pathogen. This explains the findings of other researchers who failed to identify infectious organisms in most tissue samples using molecular techniques (YOW *et al.* 2014).

Researchers have shown that infection with uropathogenic *E. coli* produced a significant inflammatory response in a mouse prostate (BOEHM *et al.* 2012). In two cases, the bacteria persisted in a low-titer colonization throughout the study period. Another study used an *E. coli* strain from a human patient with bacterial prostatitis, again in a mouse model. Chronic inflammation with prostatitis was identified in 89%, 85% and 40% of the mice after 8 weeks, 6 months and 1 year post-infection respectively. While the infection was not sufficient to initiate cancer in the wild-type C57BL/6J mice used initially, it was found to significantly accelerate prostate cancer progression in Hi-Myc mice (SIMONS *et al.* 2015; ELLIS *et al.* 2016).

More recently it has been realised that there is little information regarding the bacterial communities that co-inhabit the prostate, as most investigations tend to focus on individual species (PUHR *et al.* 2016).

1.8 The microbiome and metagenomics

It has previously been suggested that only 10% of the cells found in the human body are human cells, while the remaining 90% are prokaryotic and eukaryotic microbial cells (SAVAGE 1977). More recent estimates put the ratio of bacterial:human cells closer to 1:1, yet it remains clear that humans are host to complex microbial communities (SENDER *et al.* 2016).

The human microbiome was described by LEDERBERG AND MCCRAY (2001) as "-the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space-". It is thought that these microbial communities have been co-adapting with their hosts for millions of years, and are now involved in a range of body functions such as reproduction and metabolism (CHO AND BLASER 2012; FRANASIAK AND SCOTT 2015).

Different sites of the body vary with regards to the microbes they host. **Figure 10** shows the relative proportions of sequences assigned to different bacterial phyla at eight anatomical sites. Additionally, the two profiles indicated for the stomach show the effect that a single microbial species can have on the total community. In this study the authors found that individuals with stomachs positive for the presence of *H. pylori* displayed drastically reduced bacterial diversity, with 93-97% of reads obtained representing this genus, although not all disruptions are as dramatic (ANDERSSON *et al.* 2008). It is thought that some disease states may be caused by variations in the microbial populations, rather than just single organisms (CHO AND BLASER 2012).

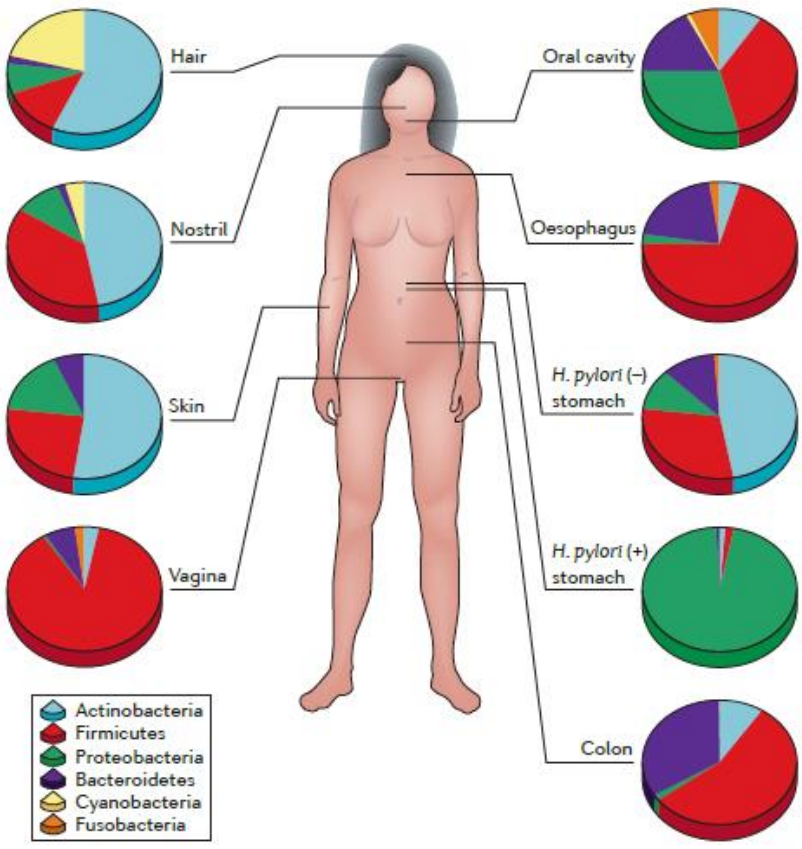


Figure 10. Compositional differences in the microbiome by anatomical site. Taken from CHO AND BLASER (2012).

Traditional approaches to investigate microorganisms were dependant on culturing, and it has long been understood that many microorganisms cannot be cultured in the laboratory (WALKER *et al.*

2014). Metagenomics is a tool initially developed for analysis of microbes from marine or soil environments where it was estimated that less than 1% of microbes are culturable. When applied to faecal samples it was found that as little as 20% of the Operational Taxonomic Units (OTUs) from 16S rRNA gene-based studies could be matched to cultured organisms (ECKBURG *et al.* 2005). Recent literature has contested these long-held views regarding the fraction of microbes which are culturable (MARTINY 2019). The author contests that a much larger fraction of the major bacterial lineages across diverse biomes are found in culture than the "1% culturability paradigm" allows for, particularly with recent advances in culturing techniques. Martiny does concede that extensive knowledge of the nutritional requirements of certain members would be required to achieve a successful culture. These requirements bias culture-based approaches. Finally, Martiny concedes that culture-based approaches have the potential to miss rare members of the communities.

Metagenomics is a culture-independent molecular approach used for analysis of microbial populations (HUGENHOLTZ AND TYSON 2008). It is particularly powerful when rare or low abundance members are to be included in analyses. The approach seeks to investigate the genomic sequences of a community of organisms taken from an environment. It is a relatively unbiased approach that enables analysis of the community structure as well as the functional capacity of a community.

Typically the process begins with DNA extraction from an environmental sample, and this is followed by shotgun (non-targeted) or amplicon (targeted) sequencing approaches to produce sequenced data called reads (WALKER *et al.* 2014). Amplicon sequencing includes an amplification step where a specific region is selectively amplified prior to sequencing. The reads are subjected to quality control and then clustered together based on the similarity of their composition (PACHTER 2007). These clusters represent highly similar sequences and are called OTUs. Species assignment is typically performed on these clusters by comparing the sequences to a database of bacterial genes. Species assignment is typically performed based on a 97% sequence identity for the 16S rRNA gene sequence (WEINSTOCK 2012).

This study used amplicon sequencing of the 16S rRNA genes as low fractions of bacterial DNA were expected. This technique has been vital to microbial community investigations over the last 25 years, and remains relevant thanks to ongoing methodological advances (TRINGE AND HUGENHOLTZ 2008). This gene is particularly suited for these analyses as it is ubiquitous amongst bacteria, it has high sequence conservation, as well as a high information content (LANE *et al.* 1985).

The data generated by metagenomics can be used for various applications such as the mining of novel enzymes and studies of microbial ecology. The biggest drawbacks to the metagenomics approach are that it requires immense computational power, and that sequence analysis requires accurate

characterisation of the genes it identifies (HUGENHOLTZ AND TYSON 2008). Despite these restrictions, the use of metagenomics is increasing as sequencing costs continue to drop, and the bioinformatics used to analyse the data becomes more efficient (LI *et al.* 2012).

Metagenomics has been used to characterise and compare the microbiome of the human gut, and various organs, in healthy and diseased individuals (GILL *et al.* 2006; TURNBAUGH *et al.* 2007; ARUMUGAM *et al.* 2011). The Human Microbiome Project (HMP) is an initiative to summarise the ongoing projects investigating human microbes (PETERSON *et al.* 2009). The HMP has 3 main goals, firstly the characterisation of the microbiomes across the body of healthy adults to generate a reference dataset. Secondly, the project is constructing a database of microbial genome sequences of reference strains, and finally an investigation of the properties of those microbes that are associated with specific diseases (PROCTOR 2011).

The project was initiated with the greater objective of demonstrating that: "… there are opportunities to improve human health through monitoring or manipulation of the human microbiome." (PETERSON *et al.* 2009).

Chapter 2: Material and methods

2.1 Participant Selection

Sampling was conducted throughout 2015 at Polokwane Hospital in Limpopo, South Africa (**Figure 11)**. A total of 80 participants were recruited for the present study. These men were patients attending the hospital for a urological complaint, and had consented to be enrolled in the South African Prostate Cancer Study. Each participant completed a questionnaire and relevant medical information was collected. Once histology reports became available for the patients, the participants were divided into two groups; namely BPH and PCa. BPH assignment required a histological report concluding the presence of prostate tissue, with hyperplasia present. Patients were assigned to the PCa group upon a histological report of prostate tissue with an adenocarcinoma, irrespective of the GS reported. For this pilot study, each participant who enrolled in the SAPCS was included in the sample population.

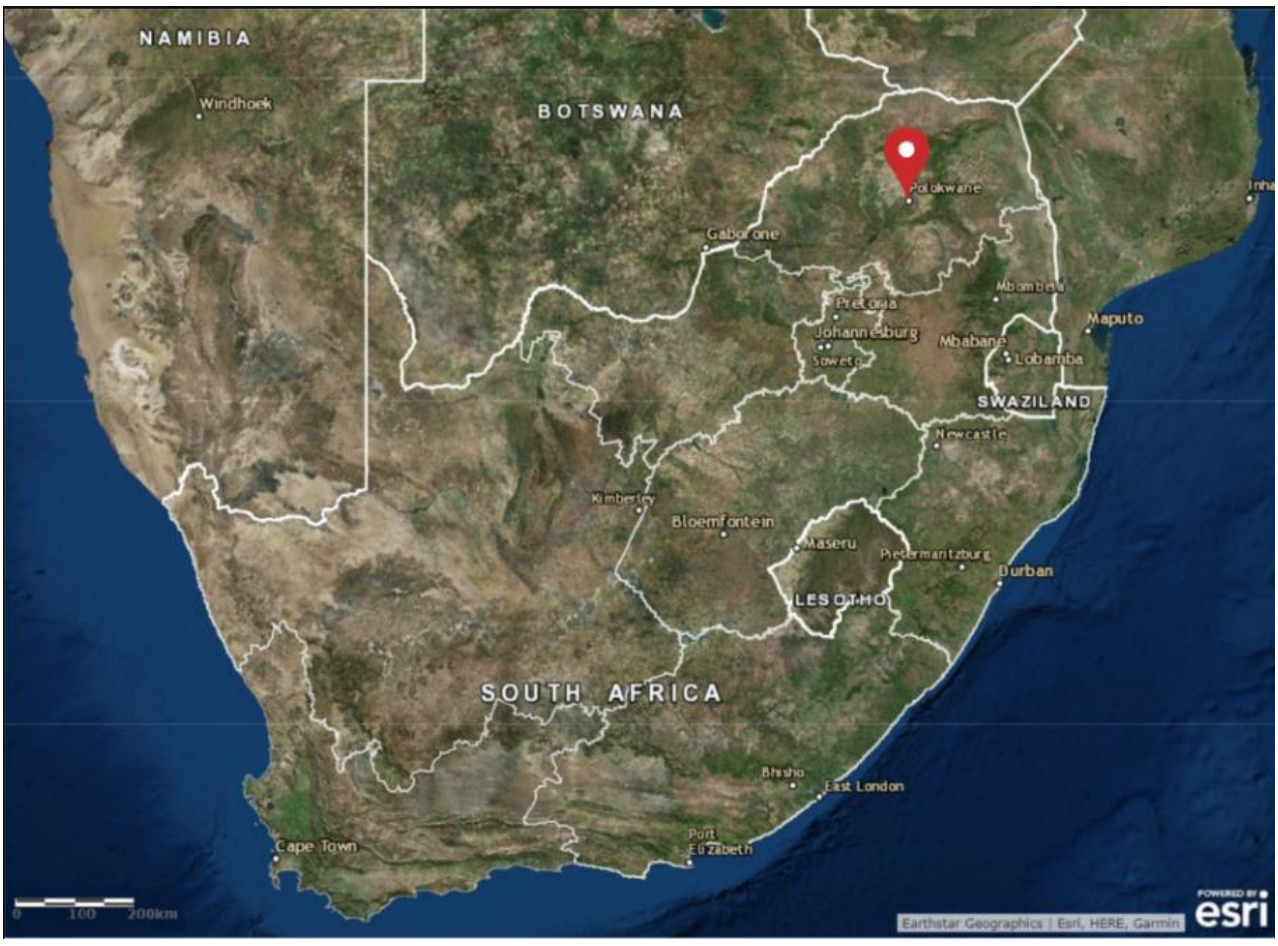


Figure 11. Map of Southern Africa created in ArcGIS (ESRI 2011). The location of Polokwane Hospital is indicated with a red marker.

Each participant underwent transrectal ultrasound-guided biopsy to collect prostate tissue for pathological scoring. The first biopsy core from each participant was immediately transferred to a sterile Eppendorf tube and snap-frozen in liquid nitrogen. The samples were kept in a portable -80°C freezer and transported to the Faculty of Health Sciences in Pretoria, and stored at -80°C until DNA extraction was performed.

Ethical approval for the project was granted by Prof CW van Staden, Chair of the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria. Amendments for the full scope of work performed for this dissertation were included in Protocol no. 43/2010 V3, protocol: Genetic risk factors of prostate cancer risk in indigenous African population groups.

2.2 Total DNA Extraction

Total DNA was extracted from the prostate tissue using the QIAamp DNA mini kit (Qiagen, Hilden, Germany, Catalog #51304) at the University of Pretoria. The extraction protocol was altered to optimise the yield of bacterial DNA through the addition of a lysozyme (Sigma Aldrich, Catalog #L6876) digestion step. The prostate biopsies were removed from the freezer and allowed to thaw before 162 µl of ATL buffer was added to the reaction tube together with 18 µl of a 200 mg/ml lysozyme solution. The solution was vortexed for 30 seconds and incubated at 37°C with agitation for 30 minutes. Following the kit instructions; 20 µl of proteinase K was added before the reaction was again vortexed and incubated at 56°C with shaking for 30 minutes, or until complete digestion of the tissue was observed.

The solution was centrifuged and 200 µl of Buffer AL was added to the reaction. The solution was incubated in a heating block at 70°C for 10 minutes and centrifuged again. The solution was vortexed after the addition of 200 µl of 100% ethanol (EtOH), and centrifuged to remove any condensation from the tube lid. The solution was transferred to a spin column inserted into the provided collection tube. Centrifugation was performed at 6 000 x *g* for one minute before the column was transferred to a new collection tube. The DNA bound to the column was washed by the addition of 500 µl of Buffer AW1, followed by another centrifugation at 6 000 x *g* for one minute. The column was transferred to a final collection tube before centrifugation at 15 000 x *g* for one minute to remove any remaining Buffer AW1 from the column. The column was transferred to a sterile 2-ml Eppendorf tube before 200 µl of Buffer AE was added directly to the top of the column. The reaction was left to stand for one minute to allow the eluent to disperse across the column top, after which the reaction was centrifuged at 6 000 x *g* for one minute. This elution was repeated with a new 2-ml Eppendorf tube. The quantity and purity of the total DNA were assessed using the NanoDrop™ 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA) against Buffer AE. The DNA solutions

were stored at -20°C prior to shipment to the Garvan Institute of Medical Research in Sydney, Australia for 16S rRNA gene sequencing.

2.3 16S rRNA Gene Sequencing

The DNA sequencing was performed on the Ion Personal Genome Machine™ (PGM™) System from Thermo Scientific at the Garvan Institute. Amplification of the bacterial DNA was achieved using the Ion 16S Metagenomics Kit (Life Technologies, Grand Island, New York, USA, Catalog #A26216). This kit is optimized for detection of complex microbial populations with an Ion Torrent approach. The 16S primer set V3-6, 7-9 (Life Technologies, Grand Island, New York, USA, Product #100026496) was used for the amplification, together with Ion Xpress™ Barcode Adapters kits 1-16, 17-32, 33-48, 49-64 and 65-80 (Life Technologies, Grand Island, New York, USA, Catalog #4471250, #4474009, #4474518, #4474519, #4474520) to permit multiplexing of the samples. The corresponding P1 adapter was included to prevent bias introduced from the barcodes. The reactions were pooled and sequenced using a 318v2 chip (Life Technologies, Grand Island, New York, USA, Catalog #4484355).

Initial quality filtering of the reads was performed by the Ion Torrent Suite software on the Ion PGM™ System, with the Ion Torrent Suite software provided with this system. Reads that were detected as being polyclonal, primer dimers, or of a low quality (Q<20) were removed.

2.4 16S rRNA OTU assignment

Further analysis was performed with the Quantitative Insights Into Microbial Ecology (QIIME version 1.8.0) analysis pipeline (CAPORASO *et al.* 2010). QIIME is an open-source pipeline that is targeted at analysis of sequence data from viral, bacterial, fungal and archaeal communities (KUCZYNSKI *et al.* 2012).

The pipeline began with sequence data in the FASTQ format. The barcodes were identified from the sequence data so that the data set could be demultiplexed into samples. Additionally, this step removed reads that had low quality defined as those with; more than three consecutive low quality base calls before truncation should occur, less than 75% consecutive high quality base calls as a fraction of the read length, or any unknown (N) bases within the read.

The QIIME pipeline next identified chimeric sequences from the data sets using usearch61 approach (EDGAR 2010). Chimeric sequences are PCR products that originate from two transcripts (HAAS *et al.* 2011). These artefacts occur when incomplete elongation during one cycle of the PCR reaction yields a fragment of DNA that anneals to an improper template in a subsequent cycle, and acts as a

primer. The product is a chimeric sequence containing information from different templates. The usearch61 algorithm has the ability to detect chimeric sequences through comparisons to a provided database (i.e., Greengenes), as well as *de novo* chimera detection using the unclustered data. Clustering with the algorithm was performed at a 97% identity threshold. These chimeric sequences were removed from the data set.

Chimera removal was followed by OTU assignment. This process began by selecting OTUs from the input files by comparing the reads to a database. The Greengenes database was selected for the current study as it is chimera-checked and contains full-length, annotated 16S rRNA gene sequences (DeSantis *et al.* 2006). Reads that failed to match the database were filtered out and randomly subsampled. This set of reads was clustered *de novo* using the UCLUST algorithm (Edgar 2010), and these clusters were used to generate new OTUs. The total failure set was then assigned an OTU against this new set of OTUs, and failures were subjected to *de novo* OTU picking. A representative sequence from each OTU was selected and used to produce a final OTU map. Each of the representative sequences in the map was assigned a taxonomy from the database. The final quality filtering involved removing low abundance OTUs from the dataset, by filtering out any OTUs accounting for less than 0.005% of the total sequence count.

2.5 Diversity analysis

Statistical analyses were performed using the vegan package (Oksanen *et al.* 2015) in R (Ihaka and Gentleman 1996). Reads for every sample were randomly subsampled to the lowest number of reads observed for a single data set.

For each participant, the total number of OTUs were assessed and reported for α-diversity analysis. This metric is a measure of the within-sample diversity. The values obtained for each sample were used to perform a two sample t-test in R, to investigate whether a significant relationship existed between the observed values and the participant groups (BPH and PCa). The number of OTUs identified for all samples within each participant group was investigated for γ-diversity. These values were divided by the average of the α-diversity for both participant groups to determine the β-diversity. The β-diversities were used to perform an Analysis of Variance (ANOVA) in R. A Venn diagram was produced from the rarefied OTU data to gain insight into the distribution of the taxa identified in the two participant groups.

Comparisons continued with the taxonomic information generated from the QIIME pipeline. A barchart was produced in R to display the relative abundances of phyla detected within each sample. These taxonomic data were used to generate a heat map of the phylum-level proportions with the

heatmaps.2 function in the gplots (v3.0.1) R package (WARNES *et al.* 2016). The 12 most abundant taxa were included for the lower taxonomic levels; namely class, order, family and genus.

2.6 nMDS and PCA

The bacterial OTU data were transformed using the Hellinger transformation in the decostand function of the Vegan package in R. This transformation returns the square root of the quotient of each data point with its row sum from that data frame. Previous studies have found that this transformation is suitable for species abundance data as it assigns rare species in the data set a low weight (LEGENDRE AND GALLAGHER 2001).

A Bray-Curtis dissimilarity matrix (BRAY AND CURTIS 1957) of these data were generated to allow for the comparison of the bacterial communities in the two groups. This matrix was used to generate a 2-dimensional non-metric multidimensional scaling (2D-nMDS) ordination. The ordination was generated in R using the Vegan and ggplot2 packages (GINESTET 2011). The bacterial community comparisons were next interrogated through means of Principal Component Analysis (PCA), with the ordination once again constructed in R using the Vegan and ggplot2 packages.

2.7 Indicator species Analysis

Indicator species analysis was performed on the taxonomic bacterial data at each taxonomic level studied (i.e. phylum, class, order, family and genus). The analysis was performed in R using the indval function from the labdsv package (v1.8.0).

This analysis assigns an indicator value to each taxon for each site (DUFRENE AND LEGENDRE 1997). A high indicator value suggests that a taxon and the related site have a high specificity and a high fidelity for one another. Specificity refers to the proportion of sites of one type that have the taxon present, while fidelity is the proportion of the abundance of the taxon that is present in that type of site. As such, a high indicator value indicates a taxon that shows a high occurrence in a particular site together with a site type that contains a high proportion of that taxon. For example, a high indicator value for species i and site type j, suggests that species i is present in a high proportion of sites belonging to type j (specificity) and that sites of type j contain a high proportion of the reported abundance of species i (fidelity).

Chapter 3: Results and Discussion

3.1 Participants selected for 16S rRNA gene sequence analysis

A total of 50 participants were selected from the patient group, for whom all relevant data were known. A participant was excluded if any of the compared variables was unknown. The variables included in the current study were the disease of the patient, the grade of the tumor of the PCa, the age of the participant and finally the PSA of the participant (**Table 7)**. The population included 26 men diagnosed with BPH and 24 men with PCa.

The mean age of the participants was 71.8 years (range from 50 to 85). We report PSA levels ranging from 0.3-1530 ng/ml (mean 244 ng/ml). The PSA results were stratified into five categories as summarised in **Table 8** to allow the comparison in the paragraph below. It is apparent that men with PCa tend to present a higher PSA, while a greater proportion of men with BPH were found to have an intermediate PSA level.

Our results we similar to those reported by TINDALL *et al.* (2014) in their study. The authors investigated a population of men from Limpopo as well as a Gauteng population, representing rural and urban groups respectively. Amongst the Limpopo population the authors report an average age of 71.8 years for PCa participants and 70.8 for BPH participants. In line with our own data, a greater proportion of men with high PSA were observed amongst cancer participants than amongst BPH participants. These trends are expected as numerous studies have shown that higher PSA levels are associated with a more aggressive disease course (STAMEY *et al.* 1987; GROSSKLAUS *et al.* 2002; LILJA *et al.* 2008).

With regard to GS, our sample population was found to present a higher proportion of aggressive disease than the participants of the study mentioned above. The majority of men were found to have a GS > 7, accounting for 19/24 (79%) of the PCa individuals compared to 39.5% of cases in the previous study. The remainder were assigned to a GS = 7, while no men with PCa were assigned a score below 7. This difference is attributed to the smaller sample size utilized for the present study. Additionally, only participants for whom all relevant patient history data were known were included in the study and it is possible that participants with less severe GS's may have been removed as a result.

Table 7. Summary of 50 men selected for 16S rRNA sequencing analysis.

Patient number	Disease	GS	Grade Group	Age	PSA
N0001	Hyperplasia	-	-	73	12
N0002	Cancer	4 + 5	V	66	77
N0003	Hyperplasia	-	-	76	13.7
N0004	Hyperplasia	-	-	66	21
N0005	Cancer	4 + 5	V	65	1138
N0006	Cancer	4 + 5	V	72	153
N0007	Hyperplasia	-	-	73	17.4
N0008	Hyperplasia	-	-	89	114
N0009	Hyperplasia	-	-	71	18
N0010	Cancer	4 + 4	IV	80	84.3
N0011	Cancer	4 + 4	IV	71	1500
N0012	Cancer	4 + 3	III	68	39.9
N0013	Cancer	4 + 4	IV	72	49.3
N0014	Cancer	4 + 5	V	69	1500
N0015	Hyperplasia	-	-	64	22.6
N0016	Cancer	4 + 5	V	64	1232
N0017	Hyperplasia	-	-	72	1359
N0018	Hyperplasia	-	-	74	25
N0019	Cancer	4 + 4	IV	67	519
N0020	Hyperplasia	-	-	68	18.7
N0021	Cancer	4 + 4	IV	65	12.1
N0022	Cancer	4 + 4	IV	80	195.5
N0023	Hyperplasia	-	-	76	18
N0024	Cancer	4 + 5	V	68	294.2
N0025	Cancer	4 + 3	III	59	42.8
N0026	Hyperplasia	-	-	74	8.4
N0027	Hyperplasia	-	-	85	6.2
N0028	Hyperplasia	-	-	83	107
N0029	Hyperplasia	-	-	80	17
N0030	Hyperplasia	-	-	65	39.5
N0031	Hyperplasia	-	-	67	107
N0032	Cancer	4 + 4	IV	74	188
N0033	Hyperplasia	-	-	60	4.6
N0034	Hyperplasia	-	-	68	21.6
N0035	Cancer	4 + 4	IV	84	9.2
N0036	Hyperplasia	-	-	76	2
N0037	Cancer	4 + 3	III	71	40.37
N0038	Hyperplasia	-	-	74	17.2
N0039	Cancer	4 + 5	V	95	108
N0040	Cancer	4 + 4	IV	71	261
N0041	Cancer	4 + 5	V	70	781
N0042	Hyperplasia	-	-	71	23.4
N0043	Hyperplasia	-	-	79	13.8
N0044	Hyperplasia	-	-	60	17.4
N0045	Cancer	4 + 4	IV	76	1530
N0046	Cancer	4 + 4	IV	76	286.7
N0047	Cancer	3 + 4	II	81	119.8
N0048	Cancer	3 + 4	II	75	16.2
N0049	Hyperplasia	-	-	50	0.3
N0050	Hyperplasia	-	-	56	6.5

Table 8. Stratification of observed PSA levels amongst (A) PCa participants and (B) BPH participants.

A

PSA range	Number of patients (%)
<4 ng/ml	0 (0)
≥4<10 ng/ml	1 (4.2)
≥10<20 ng/ml	2 (8.3)
≥20<98 ng/ml	6 (25)
≥98 ng/ml	15 (62.5)

B

PSA range	Number of patients (%)
<4 ng/ml	2 (7.7)
≥4<10 ng/ml	4 (15.4)
≥10<20 ng/ml	10 (38.5)
≥20<98 ng/ml	6 (23.1)
≥98 ng/ml	4 (15.4)

3.2 Sequencing results

A total of 2.98 million reads were produced after initial quality filtering by the Ion Torrent Suite software on the PGM™ machine. This initial filtering removed reads that were detected as being polyclonal, primer dimers, or of a low quality (Q<20). There were an average of 59 570 reads per sample with 32 633 and 86 878 as the minimum and maximum read counts, respectively. The average of the mean read lengths reported per sample was 187 bp.

After QIIME analysis, a total of 2 003 unique OTUs (97% identity) were found across the 50 participants. Following the quality control performed here, the remaining reads were found to range from 23 412- 67 392 reads per sample.

Table 9. OTU diversity metrics

Condition	Sample size	α-diversity	γ-diversity	β-diversity (γ/α)
BPH	26	593 (±127)	1949	3.29
PCa	24	602 (±93)	1940	3.22

Table 9 presents diversity metrics of the unique OTUs identified between the two participant groups using the vegan package in R. Both groups display similar and high α-diversity scores, which is the mean of the number of unique OTUs observed within that particular group. BPH participant were found to harbour 593 total unique OTUs on average, while 602 OTUs were identified on average amongst the PCa participant. A two sample t-test performed on the individual α-diversity scores showed that the results did not differ significantly between the groups ($P > 0.05$). Subsequently the γ-diversity was determined, which is a metric representing the total number of unique OTUs within

each group. We report similar and high scores for the metric, with the BPH and PCa groups containing 1949 and 1940 total unique OTUs respectively. The final metric included is β-diversity which measures the total diversity observed within a group divided by the average for that group. This score was found to be 3.29 and 3.22 for BPH and PCa participants respectively. The results for the two groups were found not to differ significantly by ANOVA in the R vegan package ($P > 0.05$).

The high α-diversity scores suggest that the samples represent highly diverse bacterial communities. Previous studies have often reported low occurrences of bacterial sequences within prostate tissue or even none at all (MÄNDAR 2013). The high γ-diversity and β-diversity values indicate that the communities observed here show high variation from another. This may be an artefact of host-immunity controlling the bacteria that colonise each participant, or the bacteria may be specific to the region of the prostate that was sampled, as explained later in this section.

A range of technologies have been employed to analyse the bacterial diversity present in the human prostate. Early approaches involved culturing of bacteria from prostate tissue, and these methods frequently had low detection rates. BERGER *et al.* (1997) were able to culture bacteria from 32% of prostatic biopsies, while LEE *et al.* (2003) reported positive cultures from 38% of participants suffering from chronic pelvic pain syndrome and 36% from healthy controls. Near equal distributions between the two groups suggested to the authors that bacterial colonization of the prostate may be an intermittent or even continuous event even in healthy men.

Molecular-based approaches such as the analysis of PCR products were also utilized. These analyses have delivered contradictory results (MÄNDAR 2013). For instance, a study of 16S rDNA sequences by RILEY *et al.* (1998) found that culturing produced negative results in 61% of samples where bacterial rDNA was detected. The authors concluded that diverse and related 16S rDNA sequences were present in the prostate, but that culture-based methods failed to detect these microbes. KRIEGER *et al.* (2000) reported on a comparative study between the bacterial sequences detected in the prostates of men with PCa versus those with chronic prostatitis. The authors were able to amplify bacterial DNA sequences from 19.6% of samples from men with PCa, and 46.4% of men with chronic prostatitis.

The contradictions arose when HOCHREITER *et al.* (2000) failed to detect bacterial sequences in any samples removed during the autopsies of individuals free from prostate disease, and only 27% of participants they analysed diagnosed with BPH or PCa (grouped). It is interesting to note that while they identified bacterial DNA sequences in all of the samples with evidence of inflammation from participants, this was not the case for the autopsy samples that exhibited inflammation. The authors deduced that the high levels of bacterial presence observed in previous studies may be a result of

increased inflammation in these participants, and that their study suggests that the healthy prostate does not harbour bacterial flora.

These findings were supported when LESKINEN *et al.* (2003) reported on only one sample out of 20 testing positive for the presence of 16S rDNA. The study did however use primers that were designed to identify pathogens, and specifically avoid contamination by detecting only highly abundant genera. The authors note that their study does not rule out low abundances of bacteria or their DNA.

A different group of researchers later found at least one prostate biopsy core to test positive for bacterial DNA in 87% of PCa participants investigated (SFANOS *et al.* 2008). The authors used a more conserved universal 16S rRNA primer set than that used in the previous study. Additionally, the authors noted that culture-based approaches yielded different and reduced bacterial diversity when compared to molecular-based techniques. A final observation to highlight from the study is that while the majority of men had at least one biopsy core test positive for bacterial DNA, only 37% of the total cores examined were positive. The authors hypothesised that bacterial presence in the prostate is limited to "lesional" zones.

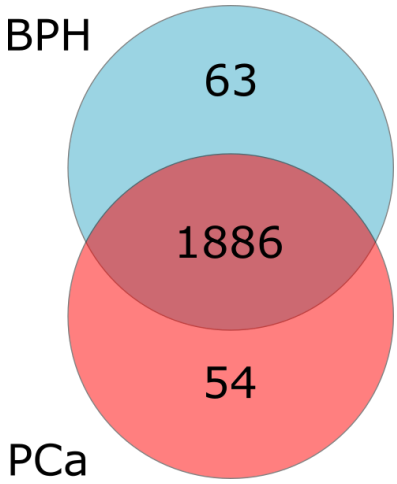


Figure 12. Venn diagram of unique OTU counts of participants with BPH and participants with PCa.

A recent review on inflammation and the microbiome on PCa development by the same authors echoed these conclusions (SFANOS *et al.* 2017). They hypothesise that a range of bacteria may contribute to prostatic inflammation and the associated micro-environment which is thought to drive development into PCa. Additionally, they propose that these organisms are not ubiquitous, but rather localised to focal regions of infections.

The present study identified bacterial DNA from every biopsy core analysed. It is important to note that no healthy controls were available and as such both groups represent diseased prostate tissue.

Additionally, the primers used were designed to detect a broad range of bacteria from complex and mixed populations. Previous studies often used primers that target only one, or a few organisms of interest.

Figure 12 displays the large overlap in the number of OTUs between the two participant groups, with 94% of the observed OTUs shared. These data support the hypothesis of a complex bacterial microbiome present in the prostate. Together with the diversity metrics from **Table 9,** we conclude that diverse bacteria were detected in the prostate, and that these communities do not associate significantly with either disease type. The high overlap between the OTUs detected in the sample groups suggests that a core microbiome is present in the prostate. Further analysis investigating tissue from healthy volunteers would be needed to support this conclusion.

3.3 Bacterial community composition at the phylum level

Taxonomic identities were assigned to the 2 003 OTUs identified. This was achieved by comparing those OTUs to the Greengenes (DeSantis *et al.* 2006) database using the UCLUST method in QIIME.

The proportions of the bacterial phyla detected in each sample are displayed in **Figure 13**. It is apparent that the communities were typically dominated by Firmicutes (55% of the total reads) and

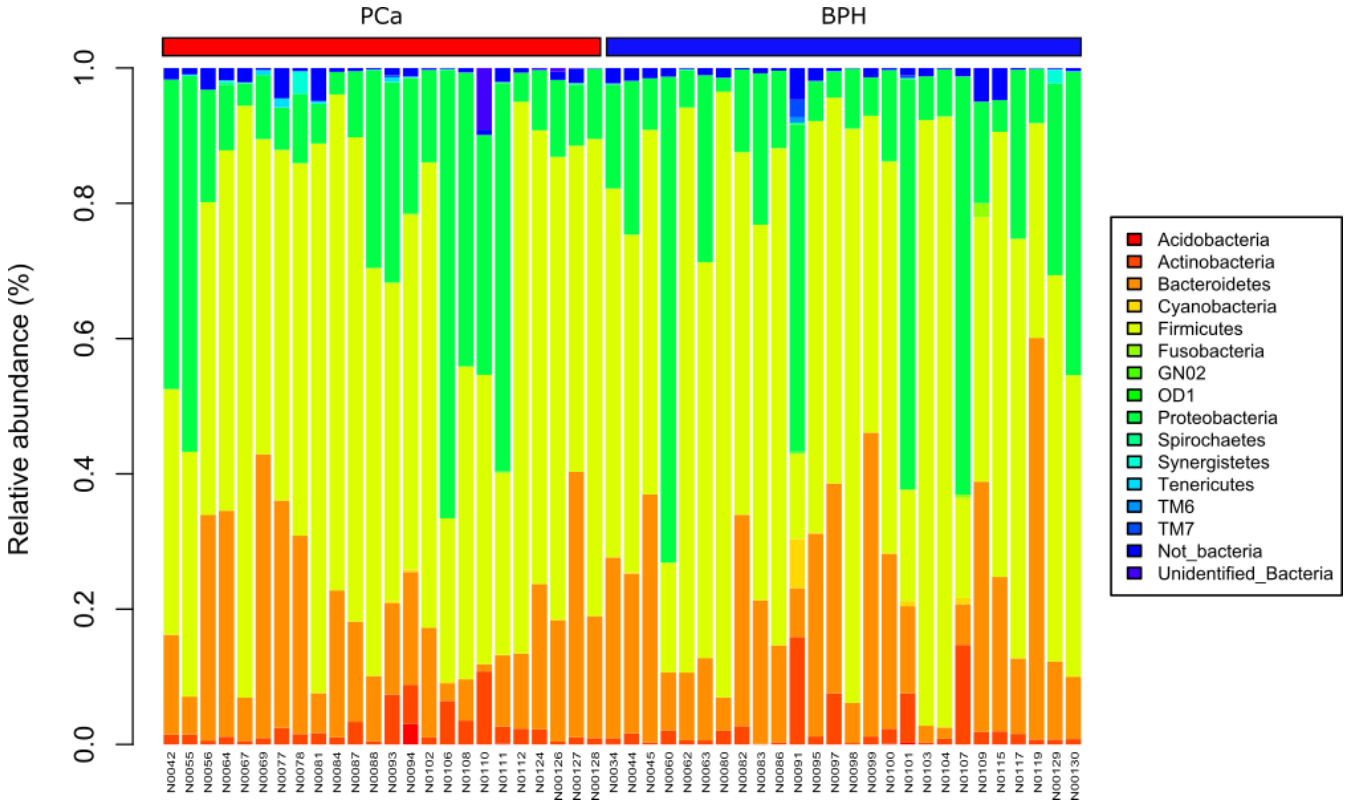


Figure 13. Barchart of bacterial phyla abundances for each sampled participant

Proteobacteria (21%). Several samples also showed particularly high representations of Bacteroidetes (19%). It can be observed that there is no apparent relationship between the participant groups and the proportions of sequences assigned to a particular phylum.

One major concern with this type of study is contamination during the biopsy collection. As a transrectal ultrasound-guided biopsy was performed, faecal contamination must be avoided. To this end, all samples used for this study represent the initial biopsy core harvested from each participant. Additionally, the core was immediately removed from the housing in the needle and transferred to a sterile Eppendorf tube which was snap-frozen in liquid nitrogen.

The phylum-level profiles shown here suggest that contamination was kept to a minimum. Faecal microbiomes have been shown to be dominated by Firmicutes which were highly prevalent in our samples, but the next most abundant phyla in these environments are Bacteroidetes and Actinobacteria (TURNBAUGH *et al.* 2009). **Figure 13** shows proportions of Proteobacteria which are highly atypical for faecal microbiomes. Finally it is expected that these communities should share some similarity, as it is thought that bacteria infiltrate the prostate via the rectal wall. Our findings support the hypothesis of a prostatic bacterial microbiome comprised of a diverse range of bacteria, which may be undetected when only searching for particular pathogens using targeted approaches.

3.4 Heatmaps of Bacterial OTUs

The rarefied OTU data were used to generate heatmaps of the bacterial diversity at five taxonomic levels. **Figure 14.A** shows the complete diversity at the Phylum level which reflects the results displayed in **Figure 13**.

The remaining heatmaps were cleared of non-informative data to simplify the illustrations. This was achieved by trimming the data at each taxonomic level to only the 12 most abundant taxa within that taxon on average. **Figure 14.B** displays the distribution of classes of bacteria in the sequence data. These distributions reflect the trend of Firmicute dominance that was observed at the phylum level, as Clostridia was found to be the dominant class. Clostridia were found to have an average abundance of 52.0% across the data set, followed by Bacteroidia (18%) and Gammaproteobacteria (15%) which are representatives of Bacteroidetes and Proteobacteria respectively. Similarly the heatmap of bacterial orders was dominated by Clostridiales at an average abundance of 52%. Bacteroidales (18%) was found to be the next most dominant order contributing to the observed Bacteroidetes abundance, while Enterobacteriales (10%) and Pseudomonadales (3%) together contribute to the high abundance of Gammaproteobacteria observed at the class level.

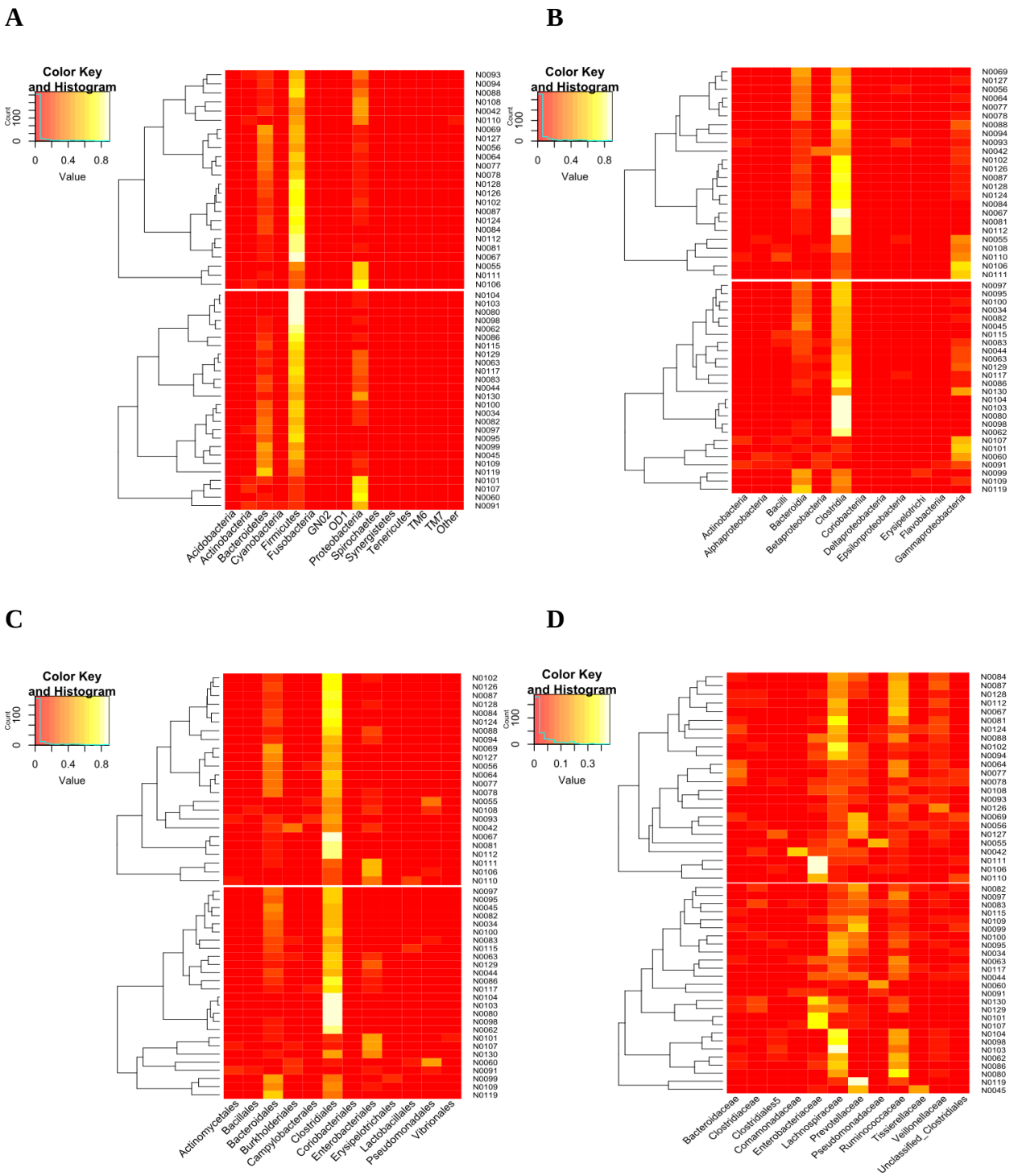
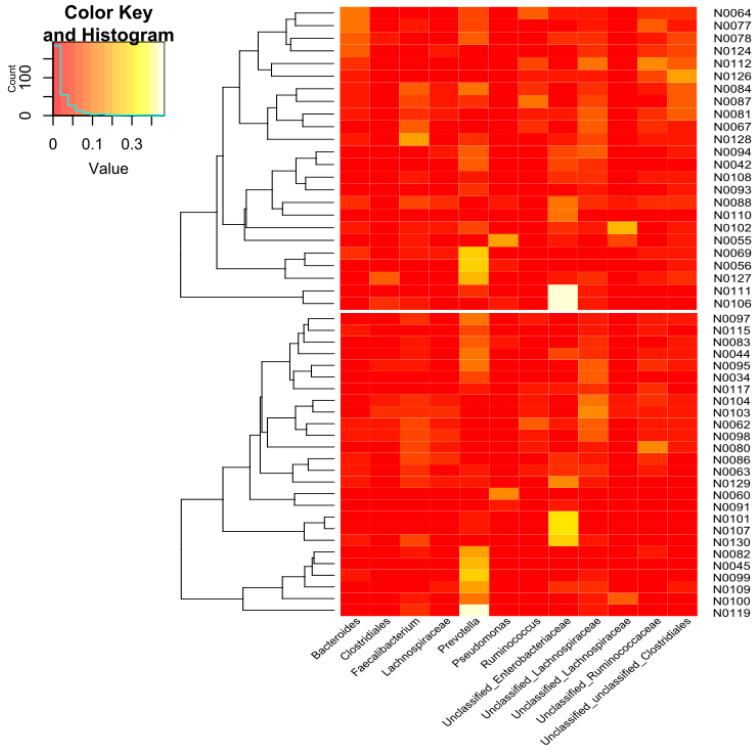


Figure 14. Heatmaps displaying bacterial proportion at different taxonomic levels. For each heatmap the top cluster represents bacteria from PCa participants, and the lower cluster represents BPH participants. A. Proportions of bacteria across all Phyla investigated. The remaining heatmaps display the distributions of the 12 most abundant (B) classes, (C) orders, (D) families and (E) genera of bacteria observed.

Figure 14.E. (cont.)



Average abundances were more diverse at the family level than the previous taxonomic levels (**Figure 14.D**) as four families were found to have average abundances >10%. The two most abundant families were found to be Lachnospiraceae (19.1%) and Ruminococcaceae (15.0%) which are both representatives of the order Clostridiales. These were followed by Prevotellaceae (11.2%) and Enterobacteriaceae (10.1%) which are members of Bacteroidales and Enterobacteriales respectively.

The major contributors to observed patterns at higher taxonomic levels are identified in (**Figure 14.E**) the heatmap of genus diversity. This distribution indicates that the dominance of only one or two taxa across the majority of samples does not extend to the genus level. The highest average abundance for a genus was observed for *Prevotella* (11.2%) closely followed by an unclassified genus of Enterobacteriaceae (8.3%). This genus however shows less consistent dispersion across the data set with only a few individuals harbouring a greatly increased proportion of this genus. The dominance of Lachnospiraceae at the family level is mirrored in the genus dispersion by an unknown genus of this family showing the third highest average occurrence (7.7%). None of the remaining genera identified achieved more than 5% average occurrence, which indicates a high level of diversity at this taxonomic level.

3.5 nMDS and PCA of OTUs

Another method that is commonly employed for bacterial community comparisons is the non-metric multidimensional scaling ordination. These representations are used to visualise the compositional differences between bacterial communities.

The OTU data of the bacterial communities were square root transformed and matrices of the Bray-Curtis dissimilarity were generated. Square-root transformation was selected as this technique has been shown to maintain the relative abundance information of data but it is also capable of reducing the effect of highly abundant taxa (THORNE *et al.* 1999; CARSON *et al.* 2009). Bray-Curtis dissimilarity was employed to generate a matrix of dissimilarities (BRAY AND CURTIS 1957; CLARKE 1993). These dissimilarities are a measure of the differences between two bacterial communities and take both species abundance and identity into account. These scores were used to produce a 2-dimensional non-metric multidimensional scaling model (nMDS) shown in **Figure 15**. This analysis is non-metric as it uses the dissimilarity scores rather than the Euclidean distances between the bacterial communities.

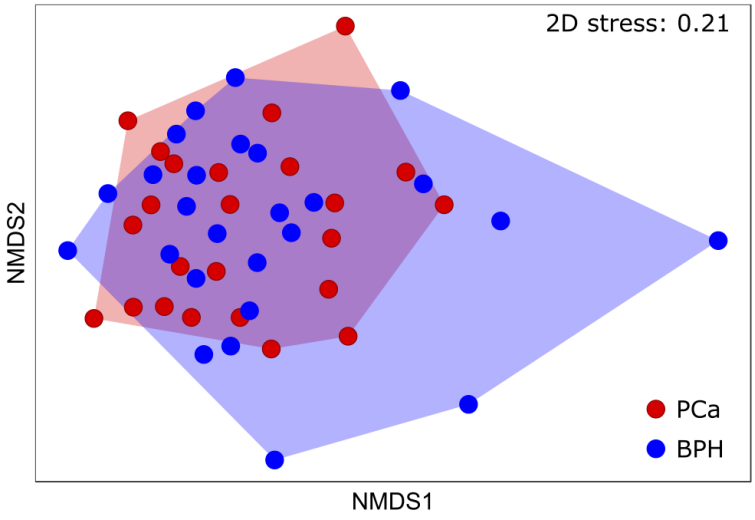


Figure 15. 2-Dimensional non-metric multidimensional scaling representation of bacterial community OTUs

The stress value of an nMDS plot indicates the difference between the visualised points and their calculated multivariate dissimilarities. A stress value <0.05 indicates that an ordination is an accurate depiction of the dissimilarities. The stress value of 0.21 achieved here suggests that the visualisation may be dangerous to interpret. Typically a stress value near 0.35-0.4 indicates that an ordination has very little relation to the original dissimilarities (CLARKE 1993). The visualisation indicates that there are individual communities that exhibit high dissimilarity from other communities displayed by points that are distant from each other. It is further important to note that the two groups were found

to have a large overlap, with no clear separation. We conclude that there is no significant relationship between the OTU data and the participant groups, and this is confirmed by PERMANOVA ($P > 0.05$).

These results indicate that the bacterial communities show greater variation within these groups than they do between groups. This supports the view that the prostate harbours diverse bacteria, but that these total communities are not separated by the disease condition.

Following the nMDS ordination, the bacterial community's OTU data were used to perform a Principal Component Analysis (PCA). The PCA aims to reduce the number of dimensions in a dataset and achieves this through means of Principal Components, which are linear combinations of the variables investigated. Principal Component 1 (PC1) is the linear combination of the available data that achieves a maximum variance of the data. As such, it presents the maximum variation possible across the data set. That is, it best separates the individual samples from each other by making use of the total data per sample. PC2 is the linear combination that maximises the remaining variation, given that it has no correlation to PC1.

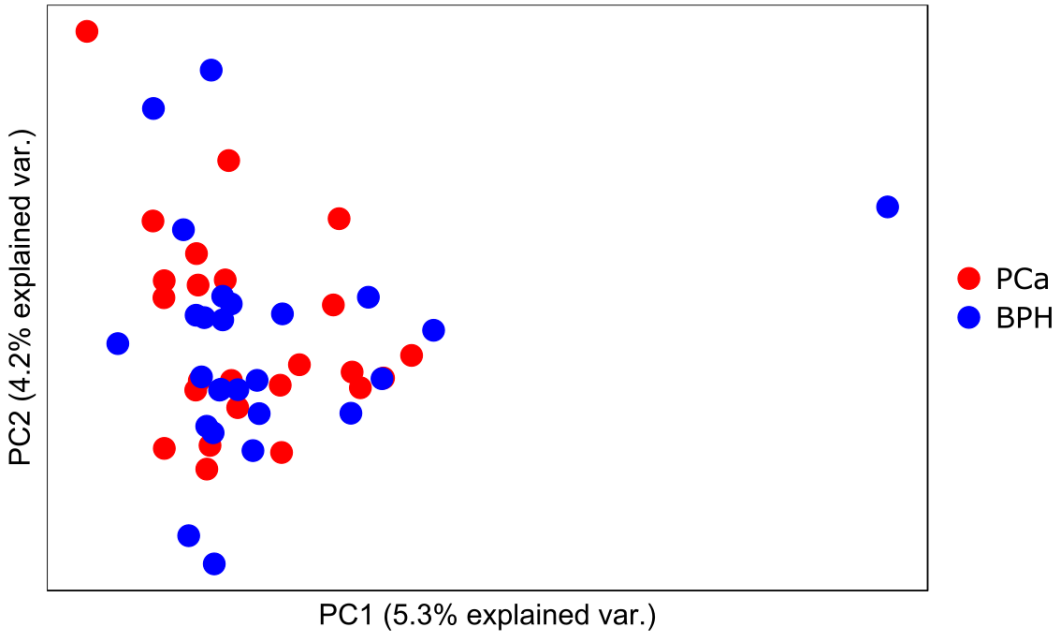


Figure 16. Principle Component Analysis of the bacterial communities.

Figure 16 shows Principal Components 1 and 2 for the bacterial OTUs. The colours denoting participant groups are assigned based on identity, not position on the plot. The ordination once again displays a large overlap of the two groups, which suggests that the two cannot be distinguished on the basis of the bacterial OTUs present. These components together explain only 9.5% of the variance within the data which indicates that the communities are highly complex.

3.6 Indicator Species Analysis

Statistical analysis of the taxonomic data by PERMANOVA found no significant associations with the participant groups ($P > 0.05$ at all taxonomic levels). This analysis however includes the total community data. To gain insight into meaningful relationships between the sample groups and the individual taxa observed in those groups, indicator species analysis was performed. The analysis was proposed by DUFRENE AND LEGENDRE (1997) as a novel method of identifying species or species assemblages that are characteristic of particular sites. This approach was an improvement on previous methods as the scores that are generated are independent of the other species' relative abundances and it eliminates the use of pseudospecies. Additionally the indicator values are independent of the classification method.

The OTU data were subjected to this analysis which identified significant associations at the order, family and genus taxonomic levels. These results are presented in **Table 10**. No significant associations were observed for any phyla or classes in the data set. A total of ten bacterial genera were found to associate significantly with a particular group, of which seven were found to associate significantly with the cancer populations. Two unclassified genera groupings were found to have the strongest association with the cancer type, namely a cluster of unclassified Clostridiales and one of Barnesiellaceae. These associations were found to remain significant at the family level where once again an Unclassified Clostridiales and the family Barnesiellaceae were found.

Clostridiales is an order of bacteria belonging to the phylum Firmicutes, of which GW-34 and *Sarcina* are also members. These organisms represent a highly polyphyletic order of obligate anaerobes that are widespread in environments such as soil, lake sediments as well as the intestinal tracts of warm-blooded animals (MADIGAN *et al.* 2012). Previous research has shown an association of members of this order with urological diseases, bacterial vaginosis in South African women (AFRICA *et al.* 2014). Most interestingly however, a study investigating the effect of dietary change on the gut microbes of overweight or obese men with PCa undergoing radical prostatectomy, reported a positive association between faecal *Clostridium* and the GG of PCa participants (FRUGÉ *et al.* 2016).

The family Barnesiellaceae is classified within the phylum Bacteroidetes and has also been associated with the gut microbiome. The family was shown to occur more frequently amongst women who have a more sedentary lifestyle, and could be predicted using the percentage of body fat in the subjects (BRESSA *et al.* 2017). To our knowledge, no association with PCa or any urological condition have been described for this family. A noteworthy association was recorded by MONTASSIER *et al.* (2016). The authors found that the presence of Barnesiellaceae was associated with a reduced risk of bloodstream infection associated with chemotherapy used during hematopoietic stem cell therapy.

They propose that these bacteria are able to prevent oxygen-tolerant bacteria such as *Enterococcus* from becoming established in the bloodstream of these participants.

The next significant association was identified between CF231 and the PCa participant group. CF231 are members of the Prevotellaceae family that have mainly been identified in studies on ruminant gut microbiota (ZHAO *et al.* 2015; WANG *et al.* 2017). A closely related member of the family is *Prevotella*, which we identified as the genus with the highest average abundance in our study. This genus has been associated with conditions such as autism (KANG *et al.* 2013), Type 1 Diabetes (BROWN *et al.* 2011) and more recently it was shown to have significantly reduced levels in the gut of men with chronic prostatitis, when compared with healthy controls (SHOSKES *et al.* 2016). The authors suggest that these microbes may reduce inflammation in the gut, and so protect against chronic prostatitis.

Members of the genus *Dermabacter* have been cultured from the urinary tracts of men, but no association has been described with PCa (SHANNON *et al.* 2006). Similarly, *Rothia* has not previously been associated with a prostatic malignancy as we have shown here, but it is known to cause endocarditis and other infections (SCHAFER *et al.* 1979; SCHIFF AND KAPLAN 1987; MORLEY AND TUFT 2006).

The strongest association between a genus and the BPH group was observed for *Sutterella*. This genus is also responsible for the significant association of the Alcaligenaceae family to which it is assigned, with BPH. *Sutterella* have been isolated from faeces, and these populations have been shown to respond to dietary changes (JAVUREK *et al.* 2017). Mice fed high-fat diets were found to have a reduced abundance of these microbes in their faeces compared to control mice. These studies primarily investigated the seminal fluid microbiome, and included the faecal microbiome as a comparison (JAVUREK *et al.* 2016). *Sutterella* was not identified in the seminal fluid in these studies.

The *Haemophilus* genus showed an association with BPH bacterial communities, as did the family and order it belongs to, Pasteurellaceae and Pasteurellales respectively. This was the only association that remained significant to the level of order. A review is available on this genus and the closely associated *Aggregatibacter*, which described *Haemophilus* as a genus with a range of pathogenicity (NØRSKOV-LAURITSEN 2014). While it may lead a non-pathogenic lifestyle, the genus is known to be a human pathogen and has been implicated in a range of diseases such as endocarditis, pneumonia, and it has been found to be responsible for prostatitis in rare cases (GOETZ AND CRAIG 1982; AL-MOHIZEA AND ALOTAIBI 2014; FUJII *et al.* 2017). An increased proportion of Pasteurellaceae in faeces

is commonly associated with individuals suffering from inflammatory bowel diseases (DUBINSKY AND BRAUN 2015).

Table 10. Indicator Values at (A) Genus, (B) Family and (C) Order taxonomic levels

A

Genus	Group	Indicator value	Probability
Unclassified Clostridiales	Cancer	0.6759	0.002
Unclassified Barnesiellaceae	Cancer	0.6419	0.034
CF231	Cancer	0.5853	0.007
Dermabacter	Cancer	0.4977	0.008
GW-34	Cancer	0.4411	0.049
Rothia	Cancer	0.2327	0.045
Sarcina	Cancer	0.1667	0.044
Sutterella	Hyperplasia	0.603	0.046
Haemophilus	Hyperplasia	0.4195	0.041
Propionivibrio	Hyperplasia	0.3815	0.018

B

Family	Group	Indicator value	Probability
Unclassified Clostridiales	Cancer	0.6759	0.002
Barnesiellaceae	Cancer	0.6419	0.034
Alcaligenaceae	Hyperplasia	0.6093	0.030
Pasteurellaceae	Hyperplasia	0.4571	0.037

C

Order	Group	Indicator value	Probability
Pasteurellales	Hyperplasia	0.4571	0.033

The final genus found to associate with BPH significantly was *Propionivibrio*. A member of this genus has been shown to be involved with bioremediation, particularly as a glycogen accumulating organism present in a biological phosphorus removal plant (ALBERTSEN *et al.* 2016). It is also worth

noting that members of the Rhodocyclaceae family to which *Propionivibrio* belong were identified in the semen microbiome discussed earlier (JAVUREK *et al.* 2016).

We identified a range of bacterial taxa that were significantly associated with PCa or BPH communities. Many of these taxa have previously been identified in investigations of gut microbiota, and may represent contamination from the biopsy process. Further study is needed to investigate prostate tissue collected from radical prostatectomy to confirm the presence of these organisms in the prostate. As previously stated however, the phylum-level community structure observed in this study does indicate that contamination was limited. Additionally, as it is hypothesised that bacteria infect the prostate through the rectal wall, these correlations are not unexpected. The taxa identified here included pathogenic, and non-pathogenic organisms and should be the topic of further studies to confirm their presence in the prostate, as well as investigate the inflammatory responses they elicit in the host.

3.7 Conclusion

We report here on the results of bacterial community analysis performed on DNA extractions from prostate biopsy tissue from South African men suffering from prostate cancer or benign prostatic hyperplasia. A total of 50 men were enrolled in the study representing 26 BPH participants and 24 PCa participants. The study population was similar to a population reported on previously with regards to age and PSA distribution, although analysis of GG did reveal a PCa population with more aggressive disease.

Bacterial communities were identified for each prostate biopsy, and were shown to be highly diverse with high numbers of unique OTUs attributed to each participant. The diversities were further found to be high for both BPH and PCa participants, with a high degree of overlap between the groups. The nature of the bacterial taxa that were identified at higher taxonomic levels suggest that contamination was kept to a minimum in this study.

Analyses were performed at different taxonomic levels to profile the bacterial communities. Firmicutes and Proteobacteria were found to be the most abundant phyla on average in both participant groups. Class-level analyses showed that both groups were dominated by Clostridia which was found to represent an average of 52% of the OTU abundance across the participant data sets. This taxonomic dominance became less pronounced at lower levels. Only eight classes and eight orders of bacteria were found to have an average abundance ≥1%, while 17 families and 20 genera had average abundances above this level.

Statistical analyses supported the hypothesis that the bacterial communities are not significantly different between the PCa group and the BPH group. Both nMDS and PCA ordinations failed to separate the groups of communities, yet indicated that individual populations within the groups were highly dissimilar.

Significant relationships were found between specific taxa, and one or the other participant groups. Ten genera, four families and one order were found to significantly associate with one participant group more than the other. Among these taxa were various known pathogens, and bacteria known to associate with obese or sedentary individuals. As obesity has been shown to be a risk factor of various cancer's investigated (WOLK *et al.* 2001) we feel that these bacteria in particular warrant further study.

Future research should investigate the precise identities of the bacteria identified only at higher taxonomic levels to associate with a particular participant condition. We hypothesise that the microbes described earlier in this chapter may play a role in driving the aggressive PCa observed in the participant group through inflammation, or that those associated with BPH participants may attenuate this inflammation to prevent the development of an aggressive PCa phenotype. Further studies should include disease-free controls to identify the true core prostate bacteria microbiota. Additionally, future studies could include culture-based techniques for characterisation of the associated bacteria identified here, and their pathologies.

Reference List

Africa, C. W., J. Nel and M. Stemmet, 2014 Anaerobes and bacterial vaginosis in pregnancy: virulence factors contributing to vaginal colonisation. International Journal of Environmental Research and Public Health 11**:** 6979-7000.

Al-Mohizea, M. M., and F. E. Alotaibi, 2014 An unusual case of chronic prostatitis caused by Haemophilus influenzae in an elderly Saudi patient: A case report and literature review. Journal of Family & Community Medicine 21**:** 193.

Albertsen, M., S. J. McIlroy, M. Stokholm-Bjerregaard, S. M. Karst and P. H. Nielsen, 2016 "Candidatus Propionivibrio aalborgensis": A Novel Glycogen Accumulating Organism Abundant in Full-Scale Enhanced Biological Phosphorus Removal Plants. Frontiers in Microbiology 7.

Alexander, R. B., K. J. Propert, A. J. Schaeffer, J. R. Landis, J. C. Nickel *et al.*, 2004 Ciprofloxacin or tamsulosin in men with chronic prostatitis/chronic pelvic pain syndrome: a randomized, double-blind trial. Annals of Internal Medicine 141**:** 581-589.

Amis, E. S., 1994 Anatomy and Physiology of the Prostate, pp. 167-169 in *Radiology of the Lower Urinary Tract*, edited by E. K. Lang. Springer Berlin Heidelberg, Berlin, Heidelberg.

Andersson, A. F., M. Lindberg, H. Jakobsson, F. Bäckhed, P. Nyrén *et al.*, 2008 Comparative analysis of human gut microbiota by barcoded pyrosequencing. PLOS One 3**:** e2836.

Andriole, G. L., D. G. Bostwick, O. W. Brawley, L. G. Gomella, M. Marberger *et al.*, 2010 Effect of dutasteride on the risk of prostate cancer. New England Journal of Medicine 362**:** 1192-1202.

Armenian, H., A. Lilienfeld, E. Diamond and I. J. Bross, 1974 Relation between benign prostatic hyperplasia and cancer of the prostate: a prospective and retrospective study. The Lancet 304**:** 115-117.

Arumugam, M., J. Raes, E. Pelletier, D. Le Paslier, T. Yamada *et al.*, 2011 Enterotypes of the human gut microbiome. Nature 473**:** 174-180.

AUA Practice guidelines committee, 2003 AUA guideline on management of benign prostatic hyperplasia (2003). Chapter 1: Diagnosis and treatment recommendations. The Journal of Urology 170**:** 530-547.

Bailar 3rd, J., G. Mellinger and D. Gleason, 1966 Survival rates of patients with prostatic cancer, tumor stage, and differentiation--preliminary report. Cancer Chemotherapy Reports 50**:** 129.

Balkwill, F., and A. Mantovani, 2001 Inflammation and cancer: back to Virchow? The Lancet 357**:** 539-545.

Barry, M. J., 2001 Prostate-specific–antigen testing for early diagnosis of prostate cancer. New England Journal of Medicine 344**:** 1373-1377.

Berger, R. E., J. N. Krieger, I. Rothman, C. H. Muller and S. L. Hillier, 1997 Bacteria in the prostate tissue of men with idiopathic prostatic inflammation. The Journal of Urology 157**:** 863-865.

Boehm, B. J., S. A. Colopy, T. J. Jerde, C. J. Loftus and W. Bushman, 2012 Acute bacterial inflammation of the mouse prostate. The Prostate 72**:** 307-317.

Bray, F., A. Jemal, N. Grey, J. Ferlay and D. Forman, 2012 Global cancer transitions according to the Human Development Index (2008–2030): a population-based study. The Lancet Oncology 13**:** 790-801.

Bray, J. R., and J. T. Curtis, 1957 An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs 27**:** 325-349.

Bressa, C., M. Bailén-Andrino, J. Pérez-Santiago, R. González-Soltero, M. Pérez *et al.*, 2017 Differences in gut microbiota profile between women with active lifestyle and sedentary women. PLOS One 12**:** e0171352.

Brown, C. T., A. G. Davis-Richardson, A. Giongo, K. A. Gano, D. B. Crabb *et al.*, 2011 Gut Microbiome Metagenomics Analysis Suggests a Functional Model for the Development of Autoimmunity for Type 1 Diabetes. PLOS one 6**:** e25792.

Campbell, N. A., and J. B. Reece, 2008 *Biology: International Edition*. Pearson Benjamin Cummings.

Canadian Cancer Society, 2017 Anatomy and Physiology of the Prostate, pp. in *Prostate Cancer*. Canadian Cancer Society, http://www.cancer.ca/en/cancer-information/cancer-type/prostate/prostate-cancer/the-prostate/?region=on.

Cancer.Net Editorial Board, 2012 Digital Rectal Exam (DRE), pp. in *Cancer.net Articles*. American Society of Clinical Oncology (ASCO) - Cancer.Net.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman *et al.*, 2010 QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7**:** 335-336.

Carson, J. K., L. Campbell, D. Rooney, N. Clipson and D. B. Gleeson, 2009 Minerals in soil select distinct bacterial communities in their microhabitats. FEMS Microbiology Ecology 67**:** 381-388.

Carvalhal, G. F., D. S. Smith, D. E. Mager, C. Ramos and W. J. Catalona, 1999 Digital rectal examination for detecting prostate cancer at prostate specific antigen levels of 4 ng./ml. or less. The Journal of Urology 161**:** 835-839.

Catalona, W., and S. Bigg, 1990 Nerve-sparing radical prostatectomy: evaluation of results after 250 patients. The Journal of Urology 143**:** 538-543; discussion 544.

Chan, T. Y., A. W. Partin, P. C. Walsh and J. I. Epstein, 2000 Prognostic significance of Gleason score 3+ 4 versus Gleason score 4+ 3 tumor at radical prostatectomy. Urology 56**:** 823-827.

Cho, I., and M. J. Blaser, 2012 The human microbiome: at the interface of health and disease. Nature Reviews Genetics 13**:** 260-270.

Chokkalingam, A. P., O. Nyrén, J. E. Johansson, G. Gridley, J. K. McLaughlin *et al.*, 2003 Prostate carcinoma risk subsequent to diagnosis of benign prostatic hyperplasia. Cancer 98**:** 1727-1734.

Chung, A., and H. H. Woo, 2014 What's truly minimally invasive in benign prostatic hyperplasia surgery? Current Opinion in Urology 24**:** 36-41.

Clarke, K. R., 1993 Non-parametric multivariate analyses of changes in community structure. Austral Ecology 18**:** 117-143.

Coetzee, L. J. E., P. T. Paradza, M. Haffejee, M. Mackenzie and G. F. G. O. de Muelenare, 2013 Prostate Cancer Diagnostic and Treatment Guidelines The Prostate Cancer Foundation of South Africa Publication Date: June 2013. The Prostate Cancer Foundation of South Africa.

Coussens, L. M., and Z. Werb, 2002 Inflammation and cancer. Nature 420**:** 860-867.

Davis, B. J., E. M. Horwitz, W. R. Lee, J. M. Crook, R. G. Stock *et al.*, 2012 American Brachytherapy Society consensus guidelines for transrectal ultrasound-guided permanent prostate brachytherapy. Brachytherapy 11**:** 6-19.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie *et al.*, 2006 Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology 72**:** 5069-5072.

Dubinsky, M., and J. Braun, 2015 Diagnostic and prognostic microbial biomarkers in inflammatory bowel diseases. Gastroenterology 149**:** 1265-1274. e1263.

Dufrene, M., and P. Legendre, 1997 Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecological Monographs 67**:** 345-366.

Eckburg, P. B., E. M. Bik, C. N. Bernstein, E. Purdom, L. Dethlefsen *et al.*, 2005 Diversity of the human intestinal microbial flora. Science 308**:** 1635-1638.

Edgar, R. C., 2010 Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26**:** 2460-2461.

Ellis, L., S. Ku, Q. Li, G. Azabdaftari, J. Seliski *et al.*, 2016 Generation of a C57BL/6 MYC-Driven Mouse Model and Cell Line of Prostate Cancer. The Prostate 76**:** 1192-1202.

Epstein, J. I., W. C. Allsbrook Jr, M. B. Amin, L. L. Egevad and I. G. Committee, 2005 The 2005 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma. The American Journal of Surgical Pathology 29**:** 1228-1242.

Epstein, J. I., L. Egevad, M. B. Amin, B. Delahunt, J. R. Srigley *et al.*, 2016 The 2014 International Society of Urological Pathology (ISUP) consensus conference on Gleason grading of prostatic carcinoma: definition of grading patterns and proposal for a new grading system. The American Journal of Surgical Pathology 40**:** 244-252.

ESRI, R., 2011 ArcGIS desktop: release 10. Environmental Systems Research Institute, CA.

Etzioni, R., and R. Gulati, 2016 Recent trends in PSA testing and prostate cancer incidence: a look at context. JAMA Oncology 2**:** 955-956.

Ferlay, J., I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser *et al.*, 2013 GLOBOCAN 2012 v1.0, Cancer incidence and mortality worldwide: IARC CancerBase No. 11. Available from: http://globocan.iarc.fr.

Ferro, A., B. Peleteiro, M. Malvezzi, C. Bosetti, P. Bertuccio *et al.*, 2014 Worldwide trends in gastric cancer mortality (1980–2011), with predictions to 2015, and incidence by subtype. European Journal of Cancer 50**:** 1330-1344.

Franasiak, J. M., and R. T. Scott, 2015 Reproductive tract microbiome in assisted reproductive technologies. Fertility and Sterility 104**:** 1364-1371.

Frugé, A. D., T. Ptacek, Y. Tsuruta, C. D. Morrow, M. Azrad *et al.*, 2016 Dietary Changes Impact the Gut Microbe Composition in Overweight and Obese Men with Prostate Cancer Undergoing Radical Prostatectomy. Journal of the Academy of Nutrition and Dietetics.

Fujii, M., H. Gomi, H. Ishioka and N. Takamura, 2017 Bacteremic renal stone-associated urinary tract infection caused by nontypable Haemophilus influenzae: A rare invasive disease in an immunocompetent patient. IDCases 7**:** 11-13.

Gill, B. C., and D. A. Shoskes, 2016 Bacterial prostatitis. Current Opinion in Infectious Diseases 29**:** 86-91.

Gill, S. R., M. Pop, R. T. DeBoy, P. B. Eckburg, P. J. Turnbaugh *et al.*, 2006 Metagenomic analysis of the human distal gut microbiome. Science 312**:** 1355-1359.

Ginestet, C., 2011 ggplot2: Elegant Graphics for Data Analysis. Journal of the Royal Statistical Society: Series A (Statistics in Society) 174**:** 245-246.

Gleason, D. F., 1992 Histologic grading of prostate cancer: a perspective. Human Pathology 23**:** 273-279.

Goetz, M. B., and W. A. Craig, 1982 Haemophilus influenzae prostatitis. JAMA 247**:** 3118-3118.

Gontero, P., M. Spahn, B. Tombal, P. Bader, C. Y. Hsu *et al.*, 2011 Is there a prostate-specific antigen upper limit for radical prostatectomy? BJU International 108**:** 1093-1100.

Greenwald, P., V. Kirmss, A. K. Polan and V. S. Dick, 1974 Cancer of the prostate among men with benign prostatic hyperplasia. Journal of the National Cancer Institute 53**:** 335-340.

Grivennikov, S. I., F. R. Greten and M. Karin, 2010 Immunity, inflammation, and cancer. Cell 140**:** 883-899.

Grossklaus, D. J., J. A. Smith, S. B. Shappell, C. S. Coffey, S. S. Chang *et al.*, 2002 The free/total prostate-specific antigen ratio (% fPSA) is the best predictor of tumor involvement in the radical prostatectomy specimen among men with an elevated PSA, pp. 195-198 in *Urologic Oncology: Seminars and Original Investigations*. Elsevier.

Gurel, B., M. S. Lucia, I. M. Thompson, P. J. Goodman, C. M. Tangen *et al.*, 2014 Chronic inflammation in benign prostate tissue is associated with high-grade prostate cancer in the placebo arm of the prostate cancer prevention trial. Cancer Epidemiology and Prevention Biomarkers 23**:** 847-856.

Haas, B. J., D. Gevers, A. M. Earl, M. Feldgarden, D. V. Ward *et al.*, 2011 Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research 21**:** 494-504.

Harris, W. P., E. A. Mostaghel, P. S. Nelson and B. Montgomery, 2009 Androgen deprivation therapy: progress in understanding mechanisms of resistance and optimizing androgen depletion. Nature Clinical Practice Urology 6**:** 76-85.

Hatakeyama, M., 2004 Oncogenic mechanisms of the Helicobacter pylori CagA protein. Nature Reviews Cancer 4**:** 688-694.

Heidenreich, A., P. J. Bastian, J. Bellmunt, M. Bolla, S. Joniau *et al.*, 2014 EAU guidelines on prostate cancer. Part 1: screening, diagnosis, and local treatment with curative intent—update 2013. European Urology 65**:** 124-137.

Herschman, J. D., D. S. Smith and W. J. Catalona, 1997 Effect of ejaculation on serum total and free prostate-specific antigen concentrations. Urology 50**:** 239-243.

Hochreiter, W. W., J. L. Duncan and A. J. Schaeffer, 2000 Evaluation of the bacterial flora of the prostate using a 16S rRNA gene based polymerase chain reaction. The Journal of Urology 163**:** 127-130.

Holmberg, L., A. Bill-Axelson, G. Steineck, H. Garmo, J. Palmgren *et al.*, 2012 Results from the Scandinavian Prostate Cancer Group Trial Number 4: a randomized controlled trial of radical prostatectomy versus watchful waiting. Monographs-Journal of the National Cancer Institute 2012**:** 230.

Howlader, N., A. Noone, M. Krapcho, D. Miller, K. Bishop *et al.*, 2017 SEER Cancer Statistics Review, 1975-2014.

Hrbacek, J., M. Urban, E. Hamsikova, R. Tachezy and J. Heracek, 2013 Thirty years of research on infection and prostate cancer: No conclusive evidence for a link. A systematic review, pp. 951-965 in *Urologic Oncology: Seminars and Original Investigations*. Elsevier.

Hu, J. C., G. Gandaglia, P. I. Karakiewicz, P. L. Nguyen, Q.-D. Trinh *et al.*, 2014 Comparative effectiveness of robot-assisted versus open radical prostatectomy cancer control. European Urology 66**:** 666-672.

Hugenholtz, P., and G. W. Tyson, 2008 Microbiology: metagenomics. Nature 455**:** 481-483.

Hunt, R., S. Xiao, F. Megraud, R. Leon-Barua, F. Bazzoli *et al.*, 2011 Helicobacter pylori in developing countries. World Gastroenterology Organisation global guideline. Journal of Gastrointestinal and Liver Diseases 20**:** 299-304.

Ihaka, R., and R. Gentleman, 1996 R: a language for data analysis and graphics. Journal of Computational and Graphical Statistics 5**:** 299-314.

Javurek, A. B., W. G. Spollen, A. M. M. Ali, S. A. Johnson, D. B. Lubahn *et al.*, 2016 Discovery of a novel seminal fluid microbiome and influence of estrogen receptor alpha genetic status. Scientific Reports 6**:** 23027.

Javurek, A. B., W. G. Spollen, S. A. Johnson, N. J. Bivens, K. H. Bromert *et al.*, 2017 Consumption of a high-fat diet alters the seminal fluid and gut microbiomes in male mice. Reproduction, Fertility and Development 29**:** 1602-1612.

Kang, D.-W., J. G. Park, Z. E. Ilhan, G. Wallstrom, J. LaBaer *et al.*, 2013 Reduced Incidence of Prevotella and Other Fermenters in Intestinal Microflora of Autistic Children. PLOS One 8**:** e68322.

Klotz, L., D. Vesprini, P. Sethukavalan, V. Jethava, L. Zhang *et al.*, 2014 Long-term follow-up of a large active surveillance cohort of patients with prostate cancer. Journal of Clinical Oncology 33**:** 272-277.

Krieger, J. N., L. Nyberg Jr and J. C. Nickel, 1999 NIH consensus definition and classification of prostatitis. Jama 282**:** 236-237.

Krieger, J. N., D. E. Riley, P. Y. Cheah, M. L. Liong and K. H. Yuen, 2003 Epidemiology of prostatitis: new evidence for a world-wide problem. World Journal of Urology 21**:** 70-74.

Krieger, J. N., D. E. Riley, R. L. Vesella, D. C. Miner, S. O. Ross *et al.*, 2000 Bacterial DNA sequences in prostate tissue from patients with prostate cancer and chronic prostatitis. The Journal of Urology 164**:** 1221-1228.

Kuczynski, J., J. Stombaugh, W. A. Walters, A. González, J. G. Caporaso *et al.*, 2012 Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Current Protocols in Microbiology**:** 1E. 5.1-1E. 5.20.

Kuper, H., H. O. Adami and D. Trichopoulos, 2000 Infections as a major preventable cause of human cancer. Journal of Internal Medicine 248**:** 171-183.

LaDou, J., 2004 The asbestos cancer epidemic. Environmental Health Perspectives 112**:** 285.

Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin *et al.*, 1985 Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences 82**:** 6955-6959.

Lederberg, J., and A. T. McCray, 2001 Ome SweetOmics--A Genealogical Treasury of Words. The Scientist 15**:** 8-8.

Lee, G., T. C. Walser and S. M. Dubinett, 2009 Chronic inflammation, chronic obstructive pulmonary disease, and lung cancer. Current Opinion in Pulmonary Medicine 15**:** 303-307.

Lee, J. C., C. H. Muller, I. Rothman, K. J. Agnew, D. Eschenbach *et al.*, 2003 Prostate biopsy culture findings of men with chronic pelvic pain syndrome do not differ from those of healthy controls. The Journal of Urology 169**:** 584-588.

Legendre, P., and E. D. Gallagher, 2001 Ecologically meaningful transformations for ordination of species data. Oecologia 129**:** 271-280.

Leskinen, M. J., K. Rantakokko-Jalava, R. Manninen, M. Leppilahti, T. Marttila *et al.*, 2003 Negative bacterial polymerase chain reaction (PCR) findings in prostate tissue from patients with symptoms of chronic pelvic pain syndrome (CPPS) and localized prostate cancer. The Prostate 55**:** 105-110.

Leyva, B., A. Persoskie, A. Ottenbacher, J. G. Hamilton, J. D. Allen *et al.*, 2016 Do men receive information required for shared decision making about PSA testing? Results from a national survey. Journal of Cancer Education 31**:** 693-701.

Li, W., L. Fu, B. Niu, S. Wu and J. Wooley, 2012 Ultrafast clustering algorithms for metagenomic sequence analysis. Briefings in Bioinformatics**:** bbs035.

Lilja, H., D. Ulmert and A. J. Vickers, 2008 Prostate-specific antigen and prostate cancer: prediction, detection and monitoring. Nature Reviews Cancer 8**:** 268-278.

Lochhead, P., and E. M. El-Omar, 2007 Helicobacter pylori infection and gastric cancer. Best Practice & Research Clinical Gastroenterology 21**:** 281-297.
Madersbacher, S., M. Marszalek, J. Lackner, P. Berger and G. Schatzl, 2007 The long-term outcome of medical therapy for BPH. European Urology 51**:** 1522-1533.
Madigan, M. T., J. M. Martinko, D. Stahl and D. Clark, 2012 *Brock Biology of Microorganisms*. Pearson Education Inc.
Mändar, R., 2013 Microbiota of male genital tract: impact on the health of man and his partner. Pharmacological Research 69**:** 32-41.
Martiny, A. C., 2019 High proportions of bacteria are culturable across major biomes. The ISME Journal**:** 1.
Montassier, E., G. A. Al-Ghalith, T. Ward, S. Corvec, T. Gastinne *et al.*, 2016 Pretreatment gut microbiome predicts chemotherapy-related bloodstream infection. Genome Medicine 8**:** 49.
Morley, A. M., and S. J. Tuft, 2006 Rothia dentocariosa isolated from a corneal ulcer. Cornea 25**:** 1128-1129.
Moyer, V. A., 2012 Screening for prostate cancer: US Preventive Services Task Force recommendation statement. Annals of Internal Medicine 157**:** 120-134.
National Cancer Registry: Cancer in South Africa 2010, 2015 South African National Cancer Registry.
National Center for Health Statistics, 1995 *Advance data from vital and health statistics*. US Dept. of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Center for Health Statistics.
NeoTract Inc, 2017 Urolift System, pp., http://urolift.com/urolift-system/.
Nickel, J. C., 2017 Is chronic prostatitis/chronic pelvic pain syndrome an infectious disease of the prostate? Investigative and Clinical Urology 58**:** 149-151.
Nickel, J. C., J. Downey, J. Clark, R. W. Casey, P. J. Pommerville *et al.*, 2003 Levofloxacin for chronic prostatitis/chronic pelvic pain syndrome in men: a randomized placebo-controlled multicenter trial. Urology 62**:** 614-617.
Nickel, J. C., C. G. Roehrborn, M. P. O'Leary, D. G. Bostwick, M. C. Somerville *et al.*, 2008 The relationship between prostate inflammation and lower urinary tract symptoms: examination of baseline data from the REDUCE trial. European Urology 54**:** 1379-1384.
Nørskov-Lauritsen, N., 2014 Classification, identification, and clinical significance of Haemophilus and Aggregatibacter species with host specificity for humans. Clinical Microbiology Reviews 27**:** 214-240.
Oksanen, J., F. Blanchet, M. Friendly, R. Kindt, P. Legendre *et al.*, 2015 vegan: Community Ecology Package. Ordination methods, diversity analysis and other functions for community and vegetation ecologists. Version 2.3-3 (2016), pp.
Ørsted, D. D., and S. E. Bojesen, 2013 The link between benign prostatic hyperplasia and prostate cancer. Nature Reviews Urology 10**:** 49-54.
Ørsted, D. D., S. E. Bojesen, S. F. Nielsen and B. G. Nordestgaard, 2011 Association of clinical benign prostate hyperplasia with prostate cancer incidence and mortality revisited: a nationwide cohort study of 3 009 258 men. European Urology 60**:** 691-698.
Pachter, L., 2007 Interpreting the unculturable majority. Nature Methods 4**:** 479-481.
Paolone, D. R., 2010 Benign prostatic hyperplasia. Clinics in Geriatric Medicine 26**:** 223-239.
Peterson, J., S. Garges, M. Giovanni, P. McInnes, L. Wang *et al.*, 2009 The NIH human microbiome project. Genome Research 19**:** 2317-2323.
Plummer, M., S. Franceschi, J. Vignat, D. Forman and C. de Martel, 2015 Global burden of gastric cancer attributable to Helicobacter pylori. International Journal of Cancer 136**:** 487-490.
Porpiglia, F., I. Morra, M. L. Chiarissi, M. Manfredi, F. Mele *et al.*, 2013 Randomised controlled trial comparing laparoscopic and robot-assisted radical prostatectomy. European Urology 63**:** 606-614.
Proctor, L. M., 2011 The human microbiome project in 2011 and beyond. Cell Host & Microbe 10**:** 287-291.
Puhr, M., A. De Marzo, W. Isaacs, M. S. Lucia, K. Sfanos *et al.*, 2016 Inflammation, microbiota, and prostate cancer. European Urology Focus 2**:** 374-382.
Rebbeck, T. R., S. S. Devesa, B.-L. Chang, C. H. Bunker, I. Cheng *et al.*, 2013 Global patterns of prostate cancer incidence, aggressiveness, and mortality in men of African descent. Prostate Cancer 2013.
Rees, J., M. Abrahams, A. Doble, A. Cooper and P. E. R. Group, 2015 Diagnosis and treatment of chronic bacterial prostatitis and chronic prostatitis/chronic pelvic pain syndrome: a consensus guideline. BJU International 116**:** 509-525.

Riley, D. E., R. E. Berger, D. C. Miner and J. N. Krieger, 1998 Diverse and related 16S rRNA-encoding DNA sequences in prostate tissues of men with chronic prostatitis. Journal of Clinical Microbiology 36**:** 1646-1652.

Rodriguez, C., M. L. McCullough, A. M. Mondul, E. J. Jacobs, A. Chao *et al.*, 2006 Meat consumption among Black and White men and risk of prostate cancer in the Cancer Prevention Study II Nutrition Cohort. Cancer Epidemiology and Prevention Biomarkers 15**:** 211-216.

Roehrborn, C., 2004 Benign prostatic hyperplasia. Textbook of Benign Prostatic Hyperplasia**:** 295.

Roehrborn, C. G., 2005 Benign prostatic hyperplasia: an overview. Reviews in Urology 7**:** S3.

Sander, A., J. Linseisen and S. Rohrmann, 2011 Intake of heterocyclic aromatic amines and the risk of prostate cancer in the EPIC-Heidelberg cohort. Cancer Causes & Control 22**:** 109-114.

Sauver, J. L. S., A. V. Sarma, D. J. Jacobson, M. E. McGree, M. M. Lieber *et al.*, 2009 Associations between C-reactive protein and benign prostatic hyperplasia/lower urinary tract symptom outcomes in a population-based cohort. American Journal of Epidemiology**:** kwp085.

Savage, D. C., 1977 Microbial ecology of the gastrointestinal tract. Annual Reviews in Microbiology 31**:** 107-133.

Schafer, F. J., E. J. Wing and C. W. Norden, 1979 Infectious endocarditis caused by Rothia dentocariosa. Annals of Internal Medicine 91**:** 747-748.

Schenk, J. M., A. R. Kristal, K. B. Arnold, C. M. Tangen, M. L. Neuhouser *et al.*, 2011 Association of symptomatic benign prostatic hyperplasia and prostate cancer: results from the prostate cancer prevention trial. American Journal of Epidemiology**:** kwq493.

Schiff, M. J., and M. H. Kaplan, 1987 Rothia dentocariosa pneumonia in an immunocompromised patient. Lung 165**:** 279-282.

Sender, R., S. Fuchs and R. Milo, 2016 Revised estimates for the number of human and bacteria cells in the body. PLOS Biology 14**:** e1002533.

Sfanos, K. S., and A. M. De Marzo, 2012 Prostate cancer and inflammation: the evidence. Histopathology 60**:** 199-215.

Sfanos, K. S., W. B. Isaacs and A. M. De Marzo, 2013 Infections and inflammation in prostate cancer. American Journal of Clinical and Experimental Urology 1**:** 3-11.

Sfanos, K. S., J. Sauvageot, H. L. Fedor, J. D. Dick, A. M. De Marzo *et al.*, 2008 A molecular analysis of prokaryotic and viral DNA sequences in prostate tissue from patients with prostate cancer indicates the presence of multiple and diverse microorganisms. The Prostate 68**:** 306-320.

Sfanos, K. S., S. Yegnasubramanian, W. G. Nelson and A. M. De Marzo, 2017 The inflammatory microenvironment and microbiome in prostate cancer development. Nature Reviews Urology.

Shannon, B. A., R. J. Cohen and K. L. Garrett, 2006 Polymerase chain reaction-based identification of Propionibacterium acnes types isolated from the male urinary tract: evaluation of adolescents, normal adults and men with prostatic pathology. BJU International 98**:** 388-392.

Sharifi, N., J. L. Gulley and W. L. Dahut, 2005 Androgen deprivation therapy for prostate cancer. JAMA 294**:** 238-244.

Sharp, V. J., E. B. Takacs and C. R. Powell, 2010 Prostatitis: diagnosis and treatment. American Family Physician 82**:** 397-406.

Shirai, T., M. Sano, S. Tamano, S. Takahashi, M. Hirose *et al.*, 1997 The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) derived from cooked foods. Cancer Research 57**:** 195-198.

Shoskes, D. A., H. Wang, A. S. Polackwich, B. Tucky, J. Altemus *et al.*, 2016 Analysis of gut microbiome reveals significant differences between men with chronic prostatitis/chronic pelvic pain syndrome and controls. The Journal of Urology 196**:** 435-441.

Simon, H., and D. Zieve, 2012 Benign prostatic hyperplasia, pp. in *In-depth patient education notes*, edited by H. Simon, university of Maryland Medical Centre.

Simons, B. D., A. S. Morrison, R. H. Young and W. Verhoek-Oftedahl, 1993 The relation of surgery for prostatic hypertrophy to carcinoma of the prostate. American Journal of Epidemiology 138**:** 294-300.

Simons, B. W., N. M. Durham, T. C. Bruno, J. F. Grosso, A. J. Schaeffer *et al.*, 2015 A human prostatic bacterial isolate alters the prostatic microenvironment and accelerates prostate cancer progression. The Journal of Pathology 235**:** 478-489.

Sinha, R., Y. Park, B. I. Graubard, M. F. Leitzmann, A. Hollenbeck *et al.*, 2009 Meat and meat-related compounds and risk of prostate cancer in a large prospective cohort study in the United States. American Journal of Epidemiology**:** kwp280.

Soucek, L., E. R. Lawlor, D. Soto, K. Shchors, L. B. Swigart *et al.*, 2007 Mast cells are required for angiogenesis and macroscopic expansion of Myc-induced pancreatic islet tumors. Nature Medicine 13**:** 1211-1218.

Sparmann, A., and D. Bar-Sagi, 2004 Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell 6**:** 447-458.

Stamey, T. A., N. Yang, A. R. Hay, J. E. McNeal, F. S. Freiha *et al.*, 1987 Prostate-specific antigen as a serum marker for adenocarcinoma of the prostate. New England Journal of Medicine 317**:** 909-916.

Stark, J. R., S. Perner, M. J. Stampfer, J. A. Sinnott, S. Finn *et al.*, 2009 Gleason score and lethal prostate cancer: does 3+ 4= 4+ 3? Journal of Clinical Oncology 27**:** 3459-3464.

Stock, R. G., J. A. Cesaretti and N. N. Stone, 2006 Disease-specific survival following the brachytherapy management of prostate cancer. International Journal of Radiation Oncology 64**:** 810-816.

Tanner, M. A., D. Shoskes, A. Shahed and N. R. Pace, 1999 Prevalence of corynebacterial 16S rRNA sequences in patients with bacterial and “nonbacterial” prostatitis. Journal of Clinical Microbiology 37**:** 1863-1870.

Thompson, I. M., D. K. Pauler, P. J. Goodman, C. M. Tangen, M. S. Lucia *et al.*, 2004 Prevalence of prostate cancer among men with a prostate-specific antigen level≤ 4.0 ng per milliliter. New England Journal of Medicine 350**:** 2239-2246.

Thorne, R. S. J., W. P. Williams and Y. Cao, 1999 The influence of data transformations on biological monitoring studies using macroinvertebrates. Water Research 33**:** 343-350.

Thorpe, A., and D. Neal, 2003 Benign prostatic hyperplasia. The Lancet 361**:** 1359-1367.

Tindall, E. A., L. R. Monare, D. C. Petersen, S. van Zyl, R. A. Hardie *et al.*, 2014 Clinical presentation of prostate cancer in Black South Africans. The Prostate 74**:** 880-891.

Torre, L. A., F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent *et al.*, 2015 Global cancer statistics, 2012. CA: A Cancer Journal for Clinicians 65**:** 87-108.

Tringe, S. G., and P. Hugenholtz, 2008 A renaissance for the pioneering 16S rRNA gene. Current Opinion in Microbiology 11**:** 442-446.

Turnbaugh, P. J., M. Hamady, T. Yatsunenko, B. L. Cantarel, A. Duncan *et al.*, 2009 A core gut microbiome in obese and lean twins. Nature 457**:** 480-484.

Turnbaugh, P. J., R. E. Ley, M. Hamady, C. Fraser-Liggett, R. Knight *et al.*, 2007 The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature 449**:** 804.

Vakkila, J., and M. T. Lotze, 2004 Inflammation and necrosis promote tumour growth. Nature Reviews Immunology 4**:** 641-648.

Vineis, P., and C. P. Wild, 2014 Global cancer patterns: causes and prevention. The Lancet 383**:** 549-557.

Walker, A. W., S. H. Duncan, P. Louis and H. J. Flint, 2014 Phylogeny, culturing, and metagenomics of the human gut microbiota. Trends in Microbiology 22**:** 267-274.

Wang, J., H. Fan, Y. Han, J. Zhao and Z. Zhou, 2017 Characterization of the microbial communities along the gastrointestinal tract of sheep by 454 pyrosequencing analysis. Asian-Australasian Journal of Animal Sciences 30**:** 100.

Warnes, G. R., B. Bolker, L. Bonebakker, R. Gentleman, W. H. A. Liaw *et al.*, 2016 gplots: various R programming tools for plotting data. R package version 3.0. 1. The Comprehensive R Archive Network.

Wasson, J. H., D. J. Reda, R. C. Bruskewitz, J. Elinson, A. M. Keller *et al.*, 1995 A comparison of transurethral surgery with watchful waiting for moderate symptoms of benign prostatic hyperplasia. New England Journal of Medicine 332**:** 75-79.

Weinberg, R. A., 1989 Oncogenes, antioncogenes, and the molecular bases of multistep carcinogenesis. Cancer Research 49**:** 3713-3721.

Weinstock, G. M., 2012 Genomic approaches to studying the human microbiota. Nature 489**:** 250-256.

Wiygul, J., and R. K. Babayan, 2009 Watchful waiting in benign prostatic hyperplasia. Current Opinion in Urology 19**:** 3-6.

Wolk, A., G. Gridley, M. Svensson, O. Nyrén, J. K. McLaughlin *et al.*, 2001 A prospective study of obesity and cancer risk (Sweden). Cancer Causes and Control 12**:** 13-21.

Yamazaki, S., A. Yamakawa, Y. Ito, M. Ohtani, H. Higashi *et al.*, 2003 The CagA protein of Helicobacter pylori is translocated into epithelial cells and binds to SHP-2 in human gastric mucosa. Journal of Infectious Diseases 187**:** 334-337.

Yow, M. A., S. N. Tabrizi, G. Severi, D. M. Bolton, J. Pedersen *et al.*, 2014 Detection of infectious organisms in archival prostate cancer tissues. BMC Cancer 14**:** 579.

Zelefsky, M., S. Leibel, P. Gaudin, G. Kutcher, N. Fleshner *et al.*, 1998 Dose escalation with three-dimensional conformal radiation therapy affects the outcome in prostate cancer. International Journal of Radiation Oncology 41**:** 491-500.

Zelefsky, M. J., Z. Fuks and S. A. Leibel, 2002 Intensity-modulated radiation therapy for prostate cancer, pp. 229-237 in *Seminars in Radiation Oncology*. Elsevier.

Zhao, L., Q. Meng, L. Ren, W. Liu, X. Zhang *et al.*, 2015 Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. Asian-Australasian Journal of Animal Sciences 28**:** 1433.

Zhou, Z., L. Hong, X. Shen, X. Rao, X. Jin *et al.*, 2008 Detection of nanobacteria infection in type III prostatitis. Urology 71**:** 1091-1095.

Appendix A- SAPCS Questionnaire

Appendix A- SAPCS Questionnaire

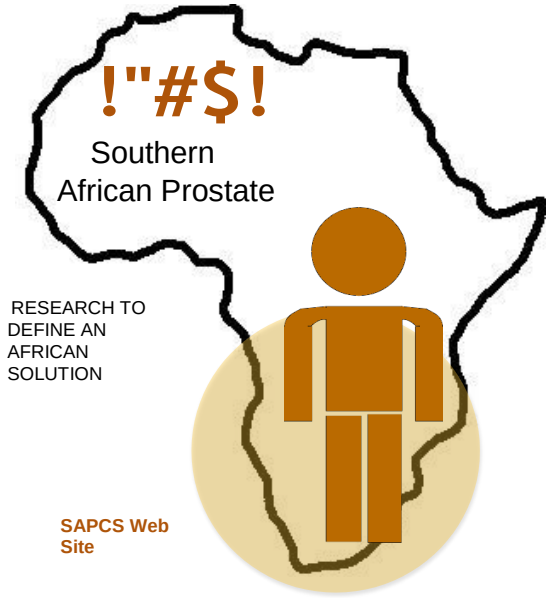
OFFICE USE ONLY: STUDY ID

SA-SAPCS QUESTIONNAIRE updated 1 May 2013

SA-SAPCS: South African SOUTHERN AFRICAN PROSTATE CANCER STUDY

Thank you for your participation in this global effort to understand the environmental and genetic drivers of prostate cancer disparities around the world. Your participation within the South African arm of the SAPCS is a first-of-its-kind study, which is allowing for researchers to address prostate cancer needs within Southern Africa. The information you provide will contribute to a large study to begin to understand what is causing and driving prostate cancer within the region. Your participation may be as a 'case' (diagnosed with prostate cancer) or as a 'control' (no diagnosis of prostate cancer). Both arms of the study are important. Please complete this questionnaire as thoroughly as possible. If you do not know an answer then rather state 'unknown'. You will be provided a unique study ID, which will be used for all future research purposes. No researcher will have access to your identity, which will be placed in a password secured database and only accessible to the Study Manager and Team Leaders named on this document. Your confidentiality is important to us.

PLEASE REMOVE AND KEEP THIS COVER SHEET FOR YOUR RECORDS



www.SAPCS.Webs.com

PCDN STUDY MANAGER:
Melissa Vincent: m.vincent@garvan.org.au
PCDN TEAM LEADER:
Vanessa Hayes: v.hayes@garvan.org.au
SA-SAPCS TEAM LEADER:
Riana Bornman: Riana.Bornman@up.ac.za

The SAPCS is a study within the **PROSTATE CANCER DISPARITIES NETWORK (PCDN)** a global network of clinical and basic researchers working on finding genomic and environmental solutions to observed global prostate cancer disparities (i.e. why men from certain parts of the world are more susceptible than others to prostate cancer and/or poor disease outcomes). www.PCDN.Webs.com

Appendix A- SAPCS Questionnaire

OFFICE USE ONLY: STUDY ID

SA-SAPCS QUESTIONNAIRE

FAMILY DEMOGRAPHICS

Birth demographics

Date of birth: ___/___/_____ Country of birth: _____ Town/village of birth: _____

Where were you born? (select the most applicable answer): At home ☐ At a hospital ☐ Unknown ☐

Family structure [Relative to all live births]

Number of brothers: _____ Number of sisters: _____

Position in the family (example first born, or number 4 of six children etc): _____

Are/were your parents related? Yes ☐ No ☐ Unknown ☐

If YES, first cousins? Yes ☐ No ☐

OR second cousins? Yes ☐ No ☐

OR Other Explain: _____

Residential history

Current residential location:

Town/village: Country: Number of years:

List places you have lived for any extended period of time (> 5 years):

Town/village: Country: Number of years:

Town/village: Country: Number of years:

Town/village: Country: Number of years:

Town/village: Country: Number of years:

Offspring demographics [Relative to all live births]

Do you have or have you fathered any children of your own? Yes ☐ No ☐

If yes, for how many children [live births] are/were you the biological father? _____

Appendix A- SAPCS Questionnaire

OFFICE USE ONLY: STUDY ID

POPULATION DEMOGRAPHICS

Population identifier (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

Population identifier of your <u>MOTHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ OTHER ☐ specify below

_____

Population identifier of your <u>FATHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

Population identifier of your <u>MATERNAL GRANDMOTHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

Population identifier of your <u>MATERNAL GRANDFATHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

Population identifier of your <u>PATERNAL GRANDMOTHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

Population identifier of your <u>PATERNAL GRANDFATHER</u> (select all applicable):
amaNdebele ☐ amaZulu ☐ amaXhosa ☐ BaPedi ☐ Basotho ☐ Baster ☐ Batswana ☐ Cape Malay ☐ Chinese ☐ Coloured ☐ European Afrikaaner ☐ English White South African ☐ German ☐ Indian ☐ VaTsonga ☐ VhaVenda ☐ Shangana ☐ Shona ☐ siSwati ☐ **OTHER ☐ specify below**

_____

OFFICE USE ONLY: STUDY ID

OCCUPATION

Your occupation history

Current occupation: _____ Number of years: _____

List all previous occupations and number of years in that profession/trade:

Previous occupation: _____ Number of years: _____

Previous occupation: _____ Number of years: _____

Previous occupation: _____ Number of years: _____

Have you ever worked on a farm? Yes ☐ No ☐ *If NO go to next section*

What type of farm? _____

How many years? _____

Describe the type of work did _____

Have you ever worked directly with pesticides? Yes ☐ No ☐ Unknown ☐

If YES, can you name which pesticides?: _____

Parent's occupations

What was the occupation of your **FATHER** (list if more than one): _____

What was the occupation of your **MOTHER** (list if more than one): _____

EDUCATION

Parent's education

Did your **FATHER** attend school? Yes ☐ No ☐

If YES mark the closest represented year attained:

Year 5 (standard 3) ☐ Year 7 (standard 5) ☐ Year 10 (standard 8) ☐ Year 12 (Matric) ☐

Did your **MOTHER** attend school? Yes ☐ No ☐

If YES mark the closest represented year attained:

Year 5 (standard 3) ☐ Year 7 (standard 5) ☐ Year 10 (standard 8) ☐ Year 12 (Matric) ☐

OFFICE USE ONLY: STUDY ID

Your education

Did you attend school? Yes ☐ No ☐ *If NO go to next section*

If YES mark the closest represented year attained:

Year 5 (standard 3) ☐ Year 7 (standard 5) ☐ Year 10 (standard 8) ☐ Year 12 (Matric) ☐

Did you live at the school during those years? Yes ☐ No ☐

Did you receive school meals in Years 1-7 (Junior School): Yes ☐ No ☐

Did you receive school meals in Years 8-12 (High School): Yes ☐ No ☐

Best describe how you got to school:

Walk ☐ Bicycle ☐ Donkey/horse ☐ Car/bus/train ☐

Did you participate in school sport? Yes ☐ No ☐

If YES, which sports _____

Do you have any post school education? Yes ☐ No ☐ *If NO go to next section*

If YES, mark what best reflects your post school education:

Trade certificate ☐ Technicon ☐ University ☐

CHILDHOOD YEARS - NUTRITION

NOTE THE ANSWERS TO THESE QUESTIONS SHOULD BEST REFLECT YOUR **CHILDHOOD YEARS** - PROVIDE THE MOST SUITABLE ANSWER [select only one answer, unless more than one applicable]

How accessible where you during your **childhood years** to a supermarket?

Too far away to access ☐ Accessible once a month via motor transport ☐

Accessible once a week via motor transport ☐ Readily accessible ☐

Your diet would be best described during your **childhood years** as: Traditional ☐ Western ☐

Did you ever go hungry during your **childhood years**? Yes ☐ No ☐

On average how many meals did you eat a day as a **child**? One ☐ Two ☐ Three ☐

As a **child** when was your largest meal? Breakfast ☐ Lunch ☐ Dinner ☐

During your **childhood years** you ate **RED meat** (***excluding chicken, ostrich and fish***):

Every day ☐ Every second day ☐ Once a week ☐

Twice a month ☐ Once a month ☐ Never ☐

OFFICE USE ONLY: STUDY ID

What was the most common source of **RED meat** during those years: _____

How was the **RED meat** most commonly prepared during your **childhood years**?

Raw ☐ Braaied (open fire) ☐ Stew (pooitjie) ☐ Fried in fat/oil ☐ Dried (biltong)

As a **child** did your family have: Cattle? Yes ☐ No ☐

Goats? Yes ☐ No ☐

Sheep? Yes ☐ No ☐

During your **childhood years** did you gather plant food from the bush? Yes ☐ No ☐

If YES, what was the most common **bush food** during those years? _____

_____

During your **childhood years** did you grow your own food? Yes ☐ No ☐

If YES, what types of food did you grow? _____

_____

During your **childhood years** was your food prepared with salt?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

During your **childhood years** was your food prepared with Aromat?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

During your **childhood years** was your food prepared with sugar?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

During your **childhood years** who prepared most of your food for you?

Mother ☐ Father ☐ Grand-mother ☐ Grand-father ☐ Aunty ☐ Uncle ☐

School ☐ Yourself ☐ Other ☐ specify _____

During your **childhood years** how often did you go to a restaurant?

Twice a week ☐ Once a week ☐ Twice a month ☐ Once a month ☐ On special occasions ☐ Never ☐

OFFICE USE ONLY: STUDY ID

How often did you eat these foods during your CHILDHOOD YEARS?:

1. Mopani worms	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
2. Ostrich	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
3. Pig trotters	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
4. Chicken (meat)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
5. Walkie-Talkies (chicken heads / feet)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
6. Fish (fresh)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
7. Tinned fish (e.g. tuna, sardines, pickled fish, etc)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
8. Boxed frozen fish	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
9. Samosa (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
10. Biryani	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
11. Bobotie	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
12. Curry (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
13. Mala Mogodu	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
14. Skilpadjies	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
15. Boerewors	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
16. Poitjiekos (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
17. Eggs	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
18. Hard cheese	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
19. Polony	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
20. Vienna sausage	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
21. Milk (full cream)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
22. Amasi (Maas)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
23. Mageu (amaHewu)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
24. Umvubo	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
25. Pap	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
26. Phutu	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
27. Isidudu	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
28. Umngqusho	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
29. Umphokoqo	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
30. Porridge	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
31. Boxed cereal	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
32. Potatoes	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
33. Sweat potatoes	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
34. Mielies (whole)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
35. Rice	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
36. Pasta	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
37. Brown Bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
38. White Bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
39. Mielie-bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
40. Potbrood	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
41. Rusks (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
42. Pastries(bought)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
43. Margarine	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
44. Butter	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
45. Chakalaka	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐

46. Tomato bredie Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
47. Baked beans Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
48. Spinach Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
49. Pumpkin Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
50. Gem squash Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
51. Carrots Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
52. Green peas Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
53. Tinned vegetables (any)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
54. Citrus fruit fresh (orange, naartjie, grapefruit, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
55. Other fruit fresh (banana, apple, guava, pear, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
56. Tinned fruit (any) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
57. Jams Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
58. Fruit juices (any) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
59. Yoghurts (any) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
60. Ice cream Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
61. Smagwinya Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
62. Malva pudding Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
63. Melktert Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
64. Koeksisters Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
65. Vetkoek Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
66. Custard Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
67. Desert (other) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
68. Peanut butter Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
69. Syrup Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
70. Honey Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
71. Biscuits (e.g. marie, tennis, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
72. Peanuts Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
73. Nuts other (e.g. walnuts, peacan, cashew, almond, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
74. Muesli Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
75. Chocolate Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
76. Sweets Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
77. Packed chips Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
78. Sugar (raw) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
79. Rooibos tea Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
80. Tea other Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
81. Instant coffee Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
82. Sugar Soda (eg. Coke, Pesi, Fanta, Cream-soda, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
83. Diet Soda (e.g. Diet Coke, TAB, Diet Pepsi, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐

OFFICE USE ONLY: STUDY ID

ADULT YEARS - NUTRITION

THE ANSWERS TO THESE QUESTIONS SHOULD BEST REFLECT YOUR **ADULT / CURRENT DIETARY PREFERENCES** - PROVIDE THE MOST SUITABLE ANSWER [select only one answer, unless more than one applicable]

Your diet would be best described as: Traditional ☐ Western ☐

Compared to your childhood years your diet is: The same ☐ Different ☐

How accessible are you currently to a supermarket?

Too far away to access ☐ Accessible once a month via motor transport ☐

Accessible once a week via motor transport ☐ Readily accessible ☐

On average how many meals did you eat a day? One ☐ Two ☐ Three ☐

What time of the day is your largest meal? Breakfast ☐ Lunch ☐ Dinner ☐

Do you eat **RED meat** (***excluding chicken, ostrich and fish***):

Every day ☐ Every second day ☐ Once a week ☐

Twice a month ☐ Once a month ☐ Never ☐

What is your most common source of **RED meat** currently: _____

How do you prepare your **RED meat**?

Raw ☐ Braaied (open fire) ☐ Stew (pooitjie) ☐ Fried in fat/oil ☐ Dried (biltong)

Do you currently have for your own use: Cattle? Yes ☐ No ☐

Goats? Yes ☐ No ☐

Sheep? Yes ☐ No ☐

Do you gather plant food from the bush? Yes ☐ No ☐

If YES, what is the most common **bush food** you gather and eat? _____

_____

Do you grow your own food? Yes ☐ No ☐

If YES, what types of food do you grow? _____

_____

OFFICE USE ONLY: STUDY ID

Who prepares your food most often?

Wife ☐ Yourself ☐ Mother/Mother-in-law ☐ Father/Father-in-law ☐ Other ☐ specify _____

How is your food prepared most often (mark more than one if frequently used)?

Over the open fire/coals ☐ In a pooitjie pot over the coals ☐ In a pot on stove ☐ In the oven ☐

Cold raw salads ☐ Other ☐ specify _____

Do you own a microwave? Yes ☐ No ☐

If YES, what do you use it for and how often? _____

_____

Is your food prepared with salt?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

Is your food prepared with Aromat?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

Is your food prepared with sugar?

All of the time ☐ Most of the time ☐ Sometimes ☐ Rarely ☐ Never ☐ unknown ☐

How often do you go to a restaurant?

Twice a week ☐ Once a week ☐ Twice a month ☐ Once a month ☐ On special occasions ☐ Never ☐

How often do you eat these foods?:

1. Mopani worms Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
2. Ostrich Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
3. Pig trotters Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
4. Chicken (meat) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
5. Walkie-Talkies (chicken heads / feet)
   Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
6. Fish (fresh) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
7. Tinned fish (e.g. tuna, sardines, pickled fish, etc)
   Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
8. Boxed frozen fish Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
9. Samosa (any) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
10. Biryani Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
11. Bobotie Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
12. Curry (any) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
13. Mala Mogodu Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
14. Skilpadjies Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐

OFFICE USE ONLY: STUDY ID

15. Boerewors	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
16. Poitjiekos (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
17. Eggs	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
18. Hard cheese	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
19. Polony	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
20. Vienna sausage	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
21. Milk (full cream)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
22. Amasi (Maas)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
23. Mageu (amaHewu)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
24. Umvubo	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
25. Pap	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
26. Phutu	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
27. Isidudu	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
28. Umngqusho	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
29. Umphokoqo	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
30. Porridge	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
31. Boxed cereal	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
32. Potatoes	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
33. Sweat potatoes	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
34. Mielies (whole)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
35. Rice	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
36. Pasta	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
37. Brown Bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
38. White Bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
39. Mielie-bread	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
40. Potbrood	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
41. Rusks (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
42. Pastries (bought)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
43. Margarine	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
44. Butter	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
45. Chakalaka	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
46. Tomato bredie	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
47. Baked beans	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
48. Spinach	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
49. Pumpkin	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
50. Gem squash	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
51. Carrots	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
52. Green peas	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
53. Tinned vegetables (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
54. Citrus fruit fresh (orange, naartjie, grapefruit, etc)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
55. Other fruit fresh (banana, apple, guava, pear, etc)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
56. Tinned fruit (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
57. Jams	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
58. Fruit juices (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐
59. Yoghurts (any)	Daily ☐	Couple of times a week ☐	Couple of times a month ☐	Never ☐

OFFICE USE ONLY: STUDY ID

60. Ice cream Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
61. Smagwinya Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
62. Malva pudding Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
63. Melktert Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
64. Koeksisters Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
65. Vetkoek Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
66. Custard Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
67. Desert (other) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
68. Peanut butter Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
69. Syrup Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
70. Honey Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
71. Biscuits (e.g. marie, tennis, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
72. Peanuts Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
73. Nuts other (e.g. walnuts, peacan, cashew, almond, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
74. Muesli Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
75. Chocolate Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
76. Sweets Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
77. Packed chips Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
78. Sugar (raw) Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
79. Rooibos tea Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
80. Tea other Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
81. Instant coffee Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
82. Sugar Soda (eg. Coke, Pesi, Fanta, Cream-soda, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
83. Diet Soda (e.g. Diet Coke, TAB, Diet Pepsi, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
84. Energy drinks (e.g. red bull, energizer, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐

Alcohol use

How often do you drink from the following list?

Commercial Beer (e.g. Castle Lager, Black Label, Hansa, Amstel, Windhoek, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Umqombothi Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐ Cider
(e.g. Hunter's Gold, Savanna Dry, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐ Red
wine Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐ White
wine Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Sparkling wine Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Fortified wines (e.g. brandy, sherry, port, madeira, marsala, vermouth)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Flavoured alcoholic beverages / cocktail drinks (e.g. Smirnoff ice, vodka, gin and tonic, martini, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Creamy spirits (e.g. Amarula, Irish cream, Don Pedro, etc)
Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐
Witblits/mampoer Daily ☐ Couple of times a week ☐ Couple of times a month ☐ Never ☐

OFFICE USE ONLY: STUDY ID

MEDICAL GENERAL

Primary health care practices

Do you seek medical advice from a Sangoma? Yes ☐ PREFERENCE_____ No ☐

Do you seek medical advice from a Inyanga? Yes ☐ PREFERENCE_____ No ☐

Do you seek medical advice from a western doctor? Yes ☐ PREFERENCE_____ No ☐

If you answered YES for more than one above then please provide a PREFERENCE ORDER from 1 first to be consulted to 3 last to be consulted.

Major surgery

Have you ever undergone major surgery? Yes ☐ No ☐

If YES, what and at what age? _____

_____

Circumcision

Have you been circumcised? Yes ☐ No ☐

If YES, was this for cultural / religious reasons? Yes ☐ No ☐

If YES, was the circumcision performed in a hospital? Yes ☐ No ☐

Smoking history

Do you smoke? Yes ☐ No ☐

If YES, what age did you start smoking? _____

What do you smoke? Cigarettes ☐ Cigars ☐ Pipe ☐

How often / how many cigarettes do you smoke per day? _____

Childhood-related medical related questions

Was your childhood house sprayed for malaria control purposes? Yes ☐ No ☐ Unknown ☐

Describe methods used to avoid mosquito bites as a child? _____

Did you have your childhood vaccinations? Yes ☐ No ☐ Unknown ☐

As a child did you seek medical advice from a Sangoma? Yes ☐ No ☐

As a child did you seek medical advice from a Inyanga? Yes ☐ No ☐

As a child did you seek medical advice from a western doctor? Yes ☐ No ☐

As a child did you have acne? Yes ☐ No ☐

OFFICE USE ONLY: STUDY ID

Cancer history

Have you ever been given a diagnosis of cancer? Yes ☐ No ☐

If YES, what cancer type? _____

What was your reason for visiting the urologist today? _____

Family medical history

Is your **FATHER** alive? Yes ☐ No ☐ If NO, at what age did he pass? _____

Cause of death? _____

Is your **MOTHER** alive? Yes ☐ No ☐ Of NO, at what age did she pass? _____

Cause of death? _____

Was your **FATHER** ever diagnosed with **prostate** cancer? Yes ☐ No ☐ Unknown ☐

If YES, at what age? _____

Was your **FATHER** ever diagnosed with **any** cancer? Yes ☐ No ☐ Unknown ☐

If YES, what cancer type? _____

Do you have a **BROTHER** diagnosed with **prostate** cancer? Yes ☐ No ☐

If YES, at what age? _____

Do you have a **UNCLE** diagnosed with **prostate** cancer? Yes ☐ No ☐

If YES, is/was he your FATHER or MOTHER's brother? _____

Was your **MOTHER** ever diagnosed with **breast** cancer? Yes ☐ No ☐ Unknown ☐

If YES, at what age? _____

Was your **MOTHER** ever diagnosed with **ovarian** cancer? Yes ☐ No ☐ Unknown ☐

If YES, at what age? _____

Was your **MOTHER** ever diagnosed with **cervical** cancer? Yes ☐ No ☐ Unknown ☐

If YES, at what age? _____

Do you have a **SISTER** diagnosed with **any** cancer? Yes ☐ No ☐ Unknown ☐

If YES, what type? _____

OFFICE USE ONLY: STUDY ID

Any other family history of cancer, please state (cancer type and relation to you): _____

_____

_____

_____

_____

_____

Medical history (non-cancer)

Have you ever been diagnosed with or treated for any of the following **NON-CANCER diseases** (mark all that are relevant)?

Allergies ☐ Arthritis ☐ Asthma ☐ Eczema ☐ Celiac Disease ☐ Cardiac Disease ☐

Diabetes ☐ ***IF YES FOR DIABETES ONLY*** Type 2 ☐ Type 1 ☐ Unknown ☐

Depression ☐ Encephalitis ☐ Gonorrhea ☐ Hay Fever ☐ Hepatitis A ☐ Hepatitis E ☐

High Blood Pressure ☐ High Cholesterol ☐ HIV/AIDS ☐ Lupus ☐ Malaria ☐

Meningitis ☐ Multiple Sclerosis ☐ Porphyria ☐ Polio ☐ Sexually transmitted infection ☐

Syphilis ☐ TB ☐ Typhoid Fever ☐ Yellow Fever ☐ Other ☐ specify _____ **Use of medicines**

Do you ever use any of these pain relief tablets (mark the most appropriate for each)?

Yes weekly ☐ Yes monthly ☐ Never ☐

Yes weekly ☐ Yes monthly ☐ Never ☐

Are you currently taking any medications? Yes ☐ No ☐

If YES, name the medication: _____

Vitamin usage

Do you take vitamin supplements? Yes ☐ No ☐

If YES, name which vitamins: _____

OFFICE USE ONLY: STUDY ID

HORMONE-RELATED QUESTIONS

Do you have chest hair? Yes ☐ No ☐

Do you have 'male breasts' (associated with increased age)? Yes ☐ No ☐

Do you have polythelia (extra nipples)? Yes ☐ No ☐

Are you balding? Yes ☐ No ☐

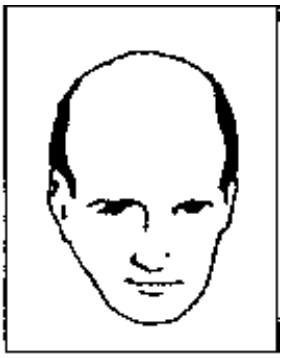
Circle which pattern most describes your hair loss:

NO BALDING

FRONTAL / RECEDING

VERTEX

FRONTAL + VERTEX



At what age did you start to loose your hair?

20-29 years ☐ 30-39 years ☐ 40-49 years ☐ 50-59 years ☐

60-69 years ☐ 70-79 years ☐ >80 years ☐

SEXUAL ACTIVITY

At what age did you become sexually active (first sexual encounter)?

10-14 years ☐ 15-19 years ☐ 20-24 years ☐ 25-29 years ☐

30-34 years ☐ 35-39 years ☐ Never ☐

Before you turned 20 years, how sexually active would you have rated yourself?

Every day ☐ 3-4 times a week ☐ 1-2 times a week ☐ Twice a month ☐

Once a month ☐ Couple of times a year ☐ Once a year ☐ Never ☐

Between 20-30 years, how sexually active would you have rated yourself?

Every day ☐ 3-4 times a week ☐ 1-2 times a week ☐ Twice a month ☐

Once a month ☐ Couple of times a year ☐ Once a year ☐ Never ☐

OFFICE USE ONLY: STUDY ID

Between 30-40 years, how sexually active would you have rated yourself?

Every day ☐ 3-4 times a week ☐ 1-2 times a week ☐ Twice a month ☐

Once a month ☐ Couple of times a year ☐ Once a year ☐ Never ☐

After you turned 40 years, how sexually active would you rate yourself?

Every day ☐ 3-4 times a week ☐ 1-2 times a week ☐ Twice a month ☐

Once a month ☐ Couple of times a year ☐ Once a year ☐ Never ☐

Are you currently sexually active? Yes ☐ No ☐

If NO, for how long have you NOT been sexually active?

Month ☐ 6 months ☐ 1 year ☐ 2 years ☐

2-5 years ☐ 5-10 years ☐ 10-15 years ☐ >15 years ☐

Do you have erectile problems? Yes ☐ No ☐

If YES, at what age did the erectile problems begin?

20-29 years ☐ 30-39 years ☐ 40-49 years ☐ 50-59 years ☐

60-69 years ☐ 70-79 years ☐ >80 years ☐

EXERCISE

Do /did you own a car? Yes ☐ No ☐ Do /did you own a motorbike? Yes ☐ No ☐ Do

/did you own a bicycle? Yes ☐ No ☐ Do /did you own a donkey/horse? Yes ☐ No ☐

Which mode of transport do you use **MOST days** (mark all appropriate):

Car/taxi ☐ train ☐ bus ☐ motorbike ☐ bicycle ☐ donkey/horse ☐ walk ☐

Do you participate in a team sport currently? Yes ☐ No ☐

If YES, which sport/s? _____

Do you perform any cardio exercise to stay fit (e.g. running, cycling, swimming, etc)? Yes ☐ No ☐

If YES, how many times a week? _____

Do you belong to a gym? Yes ☐ No ☐

If YES, do you do weight training? Yes ☐ No ☐

OFFICE USE ONLY: STUDY ID

If YES, do you attend classes? Yes ☐ No ☐

If YES, do you use the cardio machines? Yes ☐ No ☐

If YES, how many days do you go to gym a week? _____

Do you own a television? Yes ☐ No ☐

If YES, provide the scenario that best fits. I watch TV

Every night ☐ Three days a week ☐ Once a week ☐ Hardly ever ☐

Childhood activity

Did you participate in sport as a child? Yes ☐ No ☐

If YES, list the sports: _____

_____

Did your parent's own a car? Yes ☐ No ☐

OFFICE USE ONLY: STUDY ID

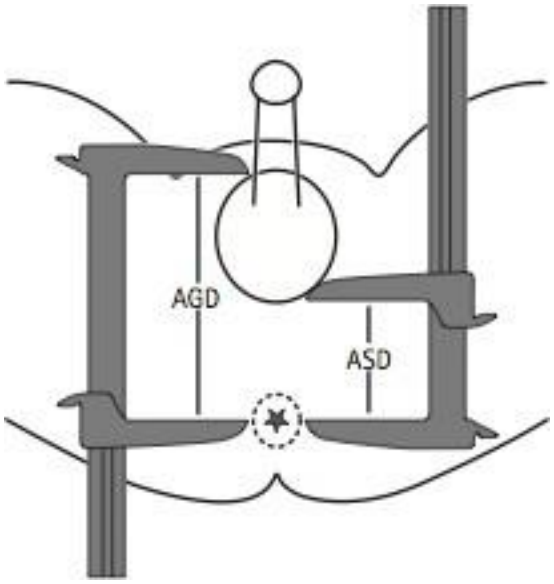
MEDICAL MEASUREMENTS AND SAMPLING – TO BE PERFORMED BY TREATING UROLOGIST/NURSE

Clinic location _____ Treating urologist _____

Reason (medical complaint) for attending clinic _____

Height _____ Weight _____ Waist circumference: _____

Hip circumference: _____ Resting heart rate _____



ASD measurement _____

AGD measurement _____

PLEASE TRACE THE RIGHT HAND [SEE NEXT PAGE]

AT DIAGNOSIS

Date _____ Age at diagnosis _____

PSA level _____ Clinical stage (TNM, T1-T4) _____

Name of diagnosing histopathologist _____

Gleason grades _____+_____ Pathalogical stage _____

BPH present? Yes ☐ No ☐

Prostatitis present? Yes ☐ No ☐

Biopsy core made available for research? Yes ☐ No ☐

First course of treatment _____

OFFICE USE ONLY: STUDY ID

TRACE RIGHT HAND

OFFICE USE ONLY: STUDY ID

FOLLOW-UP – PLEASE KEEP THIS PAGE IN PATIENTS FILE AND COMPLETE ON FOLLOW-UP

Follow-up 1:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 2:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 3:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 4:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 5:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 6:

Date _____ PSA level _____ Diagnosis _____

Treatment _____

Follow-up 7:

Date _____ PSA level _____ Diagnosis _____

Treatment _____