Methylation of *glutathione S-transferase pi 1* as an epigenetic biomarker for prostate cancer detection in black South African men

by

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Declaration

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Summary

African ancestry is a major risk factor for prostate cancer (PCa), contributing to a 1.6-fold increased incidence and a 2.5-fold increased mortality in African Americans compared to European Americans. These are further exacerbated in Black men from South Africa, who experience double the disease burden compared to their African American counterparts, as addressed in **Chapter 2**. Hypermethylation of the *Glutathione S-transferase pi 1* (*GSTP1)* gene, the focus of this thesis, has been shown to be a valuable diagnostic and prognostic biomarker for PCa with higher accuracies than the widely used and 'golden standard' prostate-specific antigen (PSA) screening test. Epigenetic biomarkers, like differential *GSTP1* methylation, have the potential to improve disease outcomes by allowing for early, accurate diagnosis and prompt treatment of aggressive PCa. However, the lack of validation studies in Black South Africans makes it difficult to know whether this biomarker is applicable within this high-risk population. Reviewing in **Chapter 2** different methylation-based methodologies, the aim of this dissertation was to determine whether *GSTP1* could be used as an epigenetic biomarker for PCa detection in Black South African men.

In **Chapter 3**, *GSTP1* methylation-specific primers and probes were assessed to determine their suitability to detect the methylation status in prostate tissue derived from men of African ethnicity, specifically Black South Africans. Identifying low-frequency African-specific single nucleotide polymorphisms (SNPs) within published primer/probes, I designed a novel African-specific assay. Ultimately, I tested and standardised the new African-relevant *GSTP1* methylation primer/probe sets for their feasibility using my selected method, digital PCR (dPCR), discussing the advantages, limitations and cost-effectiveness, to be used within a routine South African relevant diagnostic/prognostic setting.

In **Chapter 4**, I tested the feasibility of the optimised African-specific *GSTP1* dPCR methylation assay developed in **Chapter 3**, to distinguish PCa from commonly occurring non-cancerous benign prostatic hyperplasia (BPH) in 100 South African men. My cohort consisted of 66 men with a clinicopathological diagnosis of PCa and 34 with a diagnosis of no PCa with BPH. While methylation status, along with age (a known PCa risk factor) and PSA, were not significant predictors of PCa risk in this study, I observed significant differential hypermethylation in prostate tissue derived from men with PCa over men with BPH ($P < 0.001$). The designed *GSTP1* dPCR methylation assay was able to distinguish between PCa and BPH in Black South African men, with an area under the receiver operating characteristic curve (AUC) of 0.907, which was further enhanced when combined with PSA (AUC = 0.957). Here I show the significant potential of *GSTP1* hypermethylation as a PCa biomarker in the South African setting.

While other studies have mainly focused on *GSTP1* methylation in Europeans, in this dissertation I provided

a unique, African perspective on *GSTP1* methylation in aggressive PCa. As such, I highlighted important African variants that need to be considered when designing *GSTP1* differential methylation diagnostic/prognostic assays, I discussed the different methodologies available when considering cost-effective testing, while I tested the applicability of one of the most recent technical approaches, namely dPCR. Data presented provided substantial evidence that *GSTP1* is a suitable target for PCa screening in tissue biopsies from Black South Africans. Furthermore, I showed that this methylation-based biomarker has the potential to complement PSA screening for improved diagnosis of clinically significant PCa, specifically differentiating PCa from age-related BPH, with significant potential to not only provide early diagnosis, but importantly reduce associated mortality rates. With further improvements, as discussed in **Chapter 5**, translation into liquid biopsies, and large-scale validation across different ethnic groups in diverse South Africa, *GSTP1* can be a cornerstone for better management of aggressive PCa and could help combat the health disparity.

Keywords: Prostate cancer, *GSTP1*, methylation, South Africa, biomarker, PSA, BPH, dPCR, African variants, aggressive disease.

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List of abbreviations

AA	African American
ACTB	Actin beta
ADCY4	Adenylate cyclase 4
AOX1	Aldehyde oxidase 1
AP-1	Activator protein 1
APC	Adenomatous polyposis coli
AR	Androgen receptor
ARF	Alternative reading frame
ASR	Age standardised rates
ATP	Adenosine triphosphate
AUC	Area under the curve
Bp	Base pair
BPH	Benign prostatic hyperplasia
BRCA	Breast cancer
C1orf114	Chromosome 1 open reading frame 114
CAGE	Cap analysis gene expression
CDH1	Cadherin 1
CDH44	Cadherin 44
CDKN2	Cyclin dependent kinase inhibitor 2
CGI	CpG island
CHR	Chromosome
CI	Confidence interval
COBRA	Combined bisulfite restriction analysis
CRIP3	Cysteine rich protein 3
CTC	Circulating tumour cells
ctDNA	Circulating tumour DNA
CXCL14	C-X-C motif chemokine ligand 14
CYP1B1	Cytochrome P450 family 1 subfamily B member 1
DAPK	Death associated protein kinase 1
DBCAT	Database of CpG islands and analytical tool
DCR	Decoy receptor

ddPCR	Droplet digital PCR
DLEC1	Deleted in lung and esophageal cancer 1
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTP	Deoxynucleoside triphosphate
dPCR	Digital PCR
DRE	Digital rectal exam
dsDNA	Double stranded DNA
EA	European American
EDNRB	Endothelin receptor type B
EPHA3	Ephrin-A3
ER	Estrogen receptor
ESR1	Estrogen receptor 1
EVL	Enah/Vasp-Like
EXCL	Excluding
FDA	Food and drug administration
GAS6	Growth arrest-specific 6
GCK	Glucokinase
GDF15	Growth differentiation factor 15
GFRA2	GDNF family receptor alpha-2
GS	Gleason score
GSTP1	Glutathione S transferase pi 1
HAPLN3	Hyaluronan and proteoglycan link protein 3
HCC	Hepatocellular carcinoma
HDACS	Histone deacetylases
HEMK1	HemK methyltransferase family member 1
HIC1	HIC ZBTB transcriptional repressor 1
HIST1H4K	Histone H4
HOX	Homeobox
HPSE	Heparanase
HREC	Human research ethics committee
HSPA20	Heat shock protein family A member 20
IGF2	Insulin-like growth factor 2
INDEL	Insertion/deletion

ISUP	International society of urological pathology
KIFC2	Kinesin family member C2
KLK10	Kallikrein-related peptidase 10
LINE1	Long interspersed nuclear element-1
LOD	Limit of quantification
LOQ	Limit of quantification
MAF	Minor allele frequency
MALDI-TOF MS	Matrix assisted laser desorption ionisation time of fight mass spectrophotometry
MAP	MBD affinity purification
MBD	Methyl-CpG-binding domain
MBP	Methyl-CpG binding protein
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylation DNA immunoprecipitation assay
MGMT	O-6-methylguanine-DNA methyltransferase
µl	Microliter
µM`	Micromolar
Ml	Millilitre
Min	Minute
MiR-	MicroRNA
MLH1	MutL homolog 1
MOXD1	Monooxygenase DBH like 1
mRNA	Messenger RNA
MS-HRM	Methylation sensitive high-resolution melting
MSP	Methylation specific PCR
MSRE	Methylation restriction enzyme
MST1R	Macrophage stimulating 1 receptor
MSTTR	Mean segmental type-token ratio
MYOD1	Myogenic differentiation 1
NEUROG1	Neurogenin 1
NF-kB	Nuclear factor-kappa B
ng	Nanogram
NKX2-5	NK2 homeobox 5
No.	Number
NTC	No template control

NTRK3	Neurotrophic receptor tyrosine kinase 3
Oligos	Oligonucleotides
OR	Odds ratio
PCa	Prostate cancer
PCDH17	Protocadherin 17
PCR	Polymerase chain reaction
PDX1	Pancreatic and duodenal homeobox 1
Pg	Picogram
PiRNA	Piwi-interacting RNA
PITX2	Paired like homeodomain 2
PIWIL4	Piwi-like RNA mediated gene silencing 4
PLAU	Plasminogen activator, urokinase
PPi	Pyrophosphate
PSA	Prostate specific antigen
PTGS2	Prostaglandin-endoperoxide synthase 2
QPCR	Quantitative PCR
RARB	Retinoic acid receptor beta
RASSF1	Ras association domain family member 1
RB1	Retinoblastoma protein
RFS	Relapse free survival
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RUNX3	Runt-related transcription factor 3
s	Seconds
SAHPRA	South African health products regulatory authority
SAPCS	Southern African prostate cancer study
SFN	Stratifin
SHANK2	SH3 and multiple ankyrin repeat domains 2
SLC18A2	Solute carrier family 18 member A2
SNP	Single nucleotide polymorphism
SNPRN	Small nuclear ribonucleoprotein polypeptide N
SNV	Single nucleotide variant
SOP	Standard operating procedure

SP1	Specificity protein 1
SPARC	Secreted protein acidic and cysteine rich
SPSS	Statistical package for the social sciences
SSA	Sub-Saharan Africa
Ta	Annealing temperature
TCF21	Transcription factor 21
TBX15	T-Box transcription factor 15
TFAP2E	Transcription factor AP-2 epsilon
TFBS	Transcription factor binding site
TGFβ2	Transforming growth factor beta 2
TIMP3	TIMP metallopeptidase inhibitor 3
Tm	Melting temperature
TMEFF2	Transmembrane protein with EGF like and two follistatin like domains 2
TMS1	Target of methylation-induced silencing
TRUS	Trans-rectal ultrasound-guided
TSS	Transcription start site
UCSC	University of California, Santa Cruz
USA	United States of America
USPSTF	US preventative services task force
V	Volts
VAT	Value added tax
VIM	Vimentin
VS	Versus
WGA	Whole genome amplified
WGBS	Whole genome bisulfite sequencing
WGS	Whole genome sequencing
XAP1	X-associated protein 1
XIST	X inactive specific transcript

Chapter 1: General introduction

Prostate cancer (PCa) affects millions of men every year, making this a major public health concern, worldwide.[1,2] African ancestry is a significant risk factor for PCa and has led to a two-fold increase in PCa mortality seen in African Americans (AA) compared to European Americans (EA).[3] This disparity is worse in Black South Africans who suffer a further two-fold aggressive disease over AA (further addressed in **Chapter 2**).[4] Effective screening strategies that allow for early detection and thus prompt treatment of aggressive disease, are crucial for better disease outcomes.[2]

Prostate-specific antigen (PSA), digital rectal exams (DRE), and tissue biopsies are currently used for screening of PCa, however, DRE mostly detects advanced disease, while PSA is associated with overdiagnosis of clinically insignificant PCa.[5,6] Furthermore, PSA levels are elevated by benign prostatic hyperplasia (BPH), a non-cancerous enlargement of the prostate that occurs with increasing age, leading to false positives.[7] While the US Preventative Services Task Force (USPSTF) has recommended against regular PSA screening in men above the age of 70,[7] in South Africa, Black men show elevated PSA levels, irrespective of the presence of PCa (PCa-free median PSA = 9.1 µg/L, PCa median PSA = 98.8 µg/L, range < 4 µg/L - ≥ 98 µg/L *vs* AA with PCa: median PSA = 7.4 µg/L).[4,8]

Trans-rectal ultrasound-guided tissue biopsies (TRUS), and histological grading, are the gold standard used in South Africa to confirm diagnosis in suspected PCa cases. A Gleason score (GS) which describes how abnormal cells look under a microscope, and how quickly they will grow and spread, is assigned to two areas of biopsied tissue.[6] Pattern 1 was originally used to describe cells that looked mostly normal i.e., well-differentiated, small, uniform glands. Pattern 2 described cells that were more loosely arranged and variable in size and shape. In 2005 it was decided that patterns 1 and 2 should not be assigned to tissue biopsies.[9] Pattern 3 is defined as having variable patterns of single cells and cells masses, and invasion of surrounding prostatic tissue by malignant glands.[9] Pattern 4 is defined as being poorly differentiated with considerable invasion while, pattern 5 looks nothing like normal cells, they resemble clumps of non-identical cells.[9] The scores from the two areas are added together to give the GS.[6] Low-grade tumours have a GS = 6 while GS = 7 describes intermediate-grade cancer and a GS of 8-10 is indicative of highly aggressive cancer. Gleason score has now been replaced by International Society of Urological Pathology (ISUP) grading which consists of 5 grades: grade 1 (GS < 3+3), grade 2 (GS 3+4), grade 3 (GS 4+3), grade 4 (GS 4+4, 3+5, and 5+3) and grade 5 (GS 9-10).[9] The problem with TRUS is that it is highly invasive and many initial biopsies (approximately 60-70%) fail to detect PCa.[10] Which is why repeat biopsies are usually necessary, however, this increases healthcare costs and exposes men to risk of infection and treatment complications such as erectile dysfunction and loss of bladder control.[7,11]

The limitations of current screening methods have prompted investigations into less invasive, more accurate screening tests.[12] Hypermethylation of the promoter region of *glutathione S transferase pi 1* (*GSTP1*), the focus of this dissertation, is a common event in PCa, occurring in around 90% of PCa cases.[13,14] Published methylation-based assays targeting the CpG island (CGI) situated around the promoter region can distinguish between PCa and BPH with ~95% specificity, even in biopsied tissue at a distance from the tumour itself, which reduces the need for repeat biopsies.[10,15,16] Furthermore, studies report that when combined with PSA, and other methylated genes, even higher accuracies are achieved.[17,18] Epigenetic biomarkers can further overcome the problem of invasiveness associated with tissue biopsies since aberrant methylation can be detected in blood, urine, and other bodily fluids.[17,19]

The problem is that *GSTP1* methylation assays are mainly developed for use in European populations and, while few studies have validated *GSTP1* in AA men with PCa,[10,20,21] no study has validated this biomarker in Black South Africans. Molecular differences have previously been reported for AA and EA (further discussed in **Chapter 2**) and these can have implications for certain biomarkers.[10,22] The genetic and epigenetic landscape in Black South Africans is less well studied so, while *GSTP1* is an informative biomarker in EA, in Black South Africans, it is unknown whether *GSTP1*; (i) is differentially methylated in PCa and BPH, (ii) methylation is associated with PCa risk, (iii) is able to complement current screening methods, and (iv) contains African germline variants (single nucleotide variants and insertions and deletions), that would affect the binding of published assays and result in inappropriate screening in African PCa.

The **primary aim** of this dissertation was to determine whether hypermethylation of *GSTP1* could be used as an epigenetic biomarker for PCa detection in Black South African men. The **first objective** was to assess the relevance of published assays to detect differential methylation in Africans, while designing and evaluating a new assay that captured African-specific germline variants. The **second objective** was to test the novel assay in a cohort of 100 South Africans to uncover the methylation pattern and determine the suitability of *GSTP1* hypermethylation as a diagnostic biomarker.

The **methodology** involved a small bioinformatics workflow but was mainly focused on molecular biology techniques, including appropriate assay design and standardisation. Since this dissertation focused on developing an assay appropriate for cost-effective, routine PCa screening in South Africans, current methodologies available for methylation analysis were reviewed in **Chapter 2**. The advantages and limitations of different approaches was discussed to decide on the best possible technology that would result in an appropriate screening test applicable to clinical practice. This meant that the chosen method had to be highly accurate and consistent, easy to use, inexpensive, and allow for minimally invasive testing. A cost analysis of other essential lab components such as primer design software, control DNA and, kits was also included.

This dissertation is presented in two experimental chapters. The first section, **Chapter 3**, addresses the first objective. In this chapter, published assays targeting differential methylation in the CpG island (CGI) of *GSTP1* were sourced from the literature[23] and mapped to the *GSTP1* gene sequence. African germline variants from 1000 Genomes[24] and genomAD[25] databases and whole-genome sequencing of Black South Africans[4] were mapped to this region and assays that overlapped with single nucleotide polymorphisms (SNPs) i.e., present in more at least 1% of the population, were identified. The second part of this chapter involved designing primers and probes which were initially tested in real-time PCR and the best performing assay was then further optimised using digital PCR (dPCR), the selected method.

The second objective is addressed in **Chapter 4**. In this chapter, the assay designed in **Chapter 3**, was used in dPCR to measure methylation in a cohort of 100 South Africans either with histopathologically confirmed PCa (n = 66) or no PCa with BPH (n = 34). I determined whether *GSTP1* methylation was significantly different between PCa and BPH groups and whether the odds of having PCa significantly increased in the presence of *GSTP1* methylation. Furthermore, receiver operating characteristic curves (ROC) were used to determine the diagnostic accuracy of the newly designed, African appropriate assay, relative to the golden standard PSA screening test and in combination with PSA. Lastly, the differences in methylation between ethnic groups and the relationship between methylation and other factors such as PSA and age (a known PCa risk factor) were also assessed.

As discussed in **Chapter 5**, by assessing *GSTP1* assays on the genetic and epigenetic level, I ultimately provided a clearer understanding of the utility of this epigenetic biomarker in African PCa. Furthermore, by applying this epigenetic marker to a carefully selected, cost-effective technology, I hoped to ensure its suitability in a clinical setting. Epigenetic tests, like *GSTP1* differential methylation, that have the potential to improve disease screening should be applied to African populations, where there is a substantial need. By improving disease screening, we can hopefully reduce high mortality rates and work towards eliminating health disparities.

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Chapter 2: Literature review

2.1. Epigenetics

Epigenetics is the study of heritable alterations in gene expression that occur without alterations to the DNA sequence.[1] Genomic DNA is wrapped around histones to form a nucleosome which is then condensed into chromatin.[2] Loosely packed chromatin, referred to as euchromatin, is associated with active genes because transcription factors and RNA polymerase are able to access the DNA.[2] If the chromatin is tightly packed, referred to as heterochromatin, the DNA is less accessible and thus genes are inactive.[2] Chromatin structure is influenced by different epigenetic mechanisms including histone modifications, non-coding RNA's, and DNA methylation.[3]

2.1.1. DNA methylation

DNA methylation refers to the transfer of a methyl group (CH_3), by DNA methyltransferase (DNMT) enzymes, to a cytosine that occurs within a CpG context.[2] CpG-rich regions are called CpG islands (CGI) and these are generally located around the transcription start site (TSS) of promoters.[4] Promoter CGI's are usually hypomethylated while intergenic regions and repetitive elements are hypermethylated.[4] However, there are some instances where promoter CGI's are hypermethylated for example during embryonic development, in genomic imprinting, and X-chromosome inactivation which is completely normal.[5] Hypermethylation of CGI's may provoke gene silencing by preventing transcription factors from binding to the DNA, and by attracting methyl-CpG binding domain (MBD) proteins including MeCP2 and MBD 1,2,3,4, which recruit other repressive factors that condense chromatin and lead to loss of expression.[2,6]

2.1.2. DNA methylation in cancer

Aberrant DNA methylation in the form of genome-wide hypomethylation and focal hypermethylation is linked to carcinogenesis (Fig 2.1).[7–9] Loss of methylation at intergenic regions and repetitive elements leads to genomic instability.[8] While, silencing of growth suppression genes and apoptotic genes, via promoter hypermethylation, can lead to uncontrolled cell growth and resistance to cell death.[10] Accumulation of these abnormal cells forms a tumour that can invade nearby tissues and metastasize to other parts of the body.[11] Aberrant methylation is not only necessary for cancer development and progression but can also be used as a target for disease screening.[1]

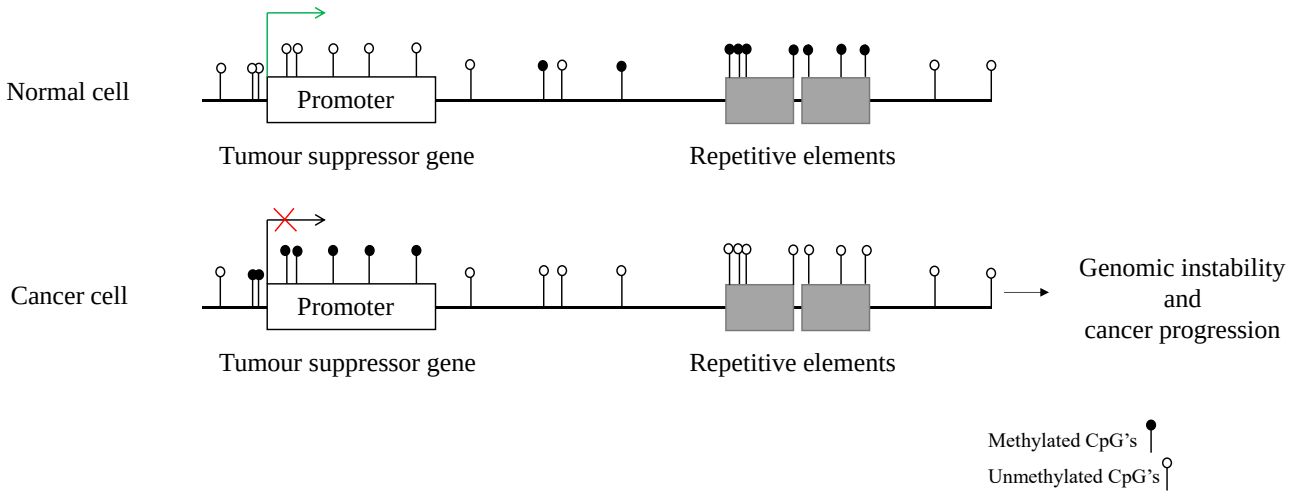
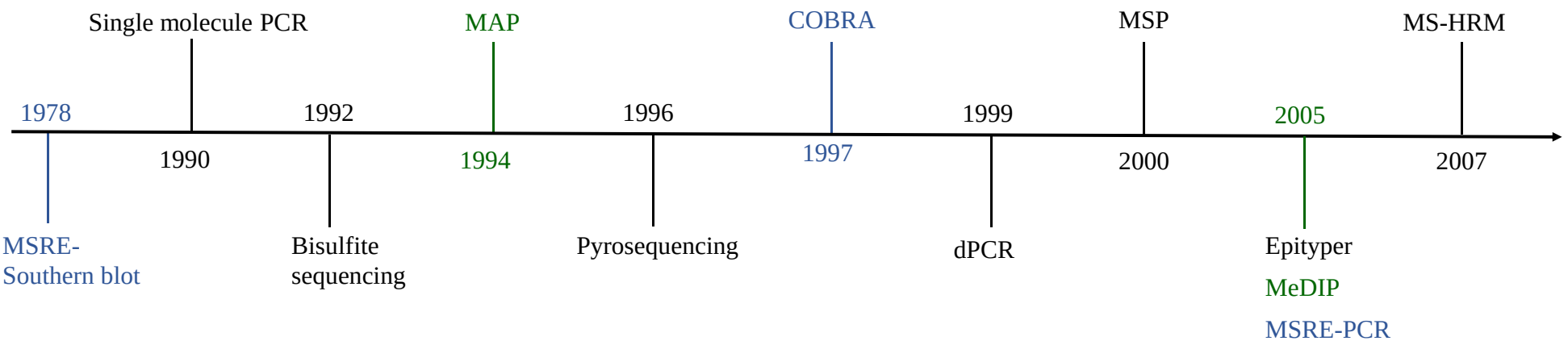


Fig 2.1. Methylation patterns in normal cells *vs* cancerous cells. Tumour suppressor gene promoters, which are unmethylated and active in normal cells, become methylated in cancerous cells which inhibits their expression and leads to tumorigenesis. Repetitive elements become hypomethylated in cancer cells which leads to genomic instability. Adapted from Lopez-Serra *et al.*, 2012.[10]

2.1.3. Methods of studying DNA methylation

Various methods can be used to analyse methylation at the level of a single gene for the purpose of cancer screening. These can be divided into three main groups namely; (i) sodium bisulfite-based approaches, (ii) restriction enzyme-based approaches, and (iii) affinity-based approaches (Fig 2.2), with bisulfite approaches being the most popular.[12] PubMed search on 1/12/2021 for the terms; "DNA Methylation" AND "bisulfite" resulted in 7,934 hits; "DNA Methylation" AND "restriction enzyme" 3,123 hits; and "DNA Methylation" AND "affinity enrichment" 96 hits.



Combined bisulfite restriction analysis (COBRA)
Digital PCR (dPCR)
MBD affinity purification (MAP)
Methylation DNA immunoprecipitation assay (MeDIP)
Methylation sensitive restriction enzymes (MSRE)
Methylation sensitive-high resolution melting (MS-HRM)
Methylation specific PCR (MSP)

Fig 2.2. Timeline of methylation detection strategies. Affinity enrichment-based approaches are green, restriction enzyme-based approaches are blue and bisulfite-based approaches are black. Adapted from Pajares *et al.*, 2020.[12]

Restriction enzyme approaches are based on digestion by methylation sensitive restriction endonucleases (MSRE) which specifically recognize and cut unmethylated restriction sites but do not cleave methylated sites.[4] Downstream analysis, such as PCR or Southern blotting, can then be used to differentiate between methylated and unmethylated DNA.[12] The problem with this method is that it is costly, it is prone to false positives, it requires large amounts of high quality DNA, there is low flexibility in terms of selecting a target region, since it is only applicable to loci with defined CGIs, and analysis is limited to few CpG's.[4,12] Affinity enrichment approaches use anti-methylcytidine antibodies or MBD proteins to separate methylated DNA from the rest of the sample.[12] The main limitations of affinity-based approaches are low resolution and bias toward observations of methylated DNA.[13] This approach requires a high density of methylated cytosines/CpG's, therefore low-density regions can be misinterpreted as being unmethylated.[13] Bisulfite based approaches are likely the most popular because they overcome limitations associated with these other methods. Bisulfite approaches offer high resolution, they require low amounts of input DNA, they are highly specific and provide quantitative results, these methods are also relatively low cost and there is flexibility in selection of target regions.[4,12,14] For these reasons, I decided to focus on bisulfite-based approaches.

2.1.3.1. Bisulfite based methods

Frommer *et al.*, 1992 was the first to use bisulfite conversion for the analysis of 5-methylcytosines.[15] This approach involves the treatment of DNA with sodium bisulfite prior to methylation analysis. Bisulfite conversion changes unmethylated cytosines to uracil while methylated cytosines remain unaffected (Fig 2.3).[15] Ultimately, this will cause a methylated locus to look different from an unmethylated locus and this sequence difference can then be detected using various downstream techniques such as bisulfite sequencing (7,866 studies listed in PubMed as of 1/12/2021) and methylation-specific PCR (MSP) (5,136 studies listed in PubMed as of 1/12/2021), which are the most commonly used.[12]

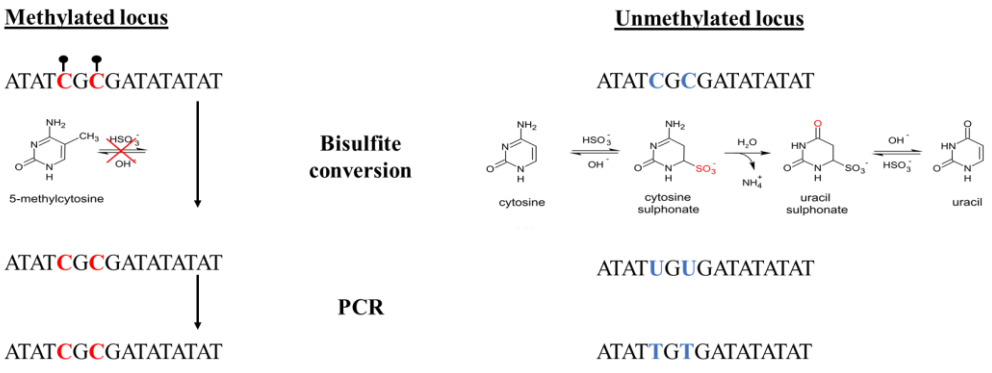


Fig 2.3. Bisulfite conversion Methylated cytosines (📍) are protected from deamination during the conversion process, while unmethylated cytosines are not. This results in a sequence difference between methylated (CpG) and unmethylated (TpG) loci. Adapted from Clark *et al.*, 1994.[26]

2.1.3.1.1. Bisulfite sequencing

Bisulfite-treated DNA samples are amplified by PCR using primers that do not overlap with CpG's, to amplify both methylated and unmethylated DNA.[14] The PCR products are then Sanger sequenced and compared to the sequence of an untreated DNA sample to determine the methylation pattern for a particular region.[12] Alternatively, a cloning step can be added after PCR and the cloned alleles Sanger sequenced.[16]

Direct sequencing of bisulfite PCR products yields poor sequencing quality, which can make it difficult to quantify methylation, therefore it is recommended that a cloning step be included.[12] However, cloning is labour intensive and costly.[16] Sometimes, it can also be difficult to design primers that target a CGI but do not include CG dinucleotides in their sequence.[16] The main advantage of bisulfite sequencing is the high resolution.[12] Methylation can be analysed at the resolution of a single base meaning the methylation status at every CpG covered can be determined.[14] Additionally, bisulfite sequencing is easy to perform.[14]

2.1.3.1.2. Pyrosequencing

In this method, bisulfite converted DNA is PCR amplified using biotin-labelled primers. After PCR, the non-biotin-containing complementary strand is removed and a sequencing primer anneals to the single-stranded DNA template. One at a time, a single deoxynucleoside triphosphate (dNTP) will be dispensed, and complementary dNTPs will be incorporated by DNA polymerase. Incorporation causes pyrophosphate (PPi) to be released which is then converted to adenosine triphosphate (ATP), by ATP sulfurylase. The ATP is used to convert luciferin to oxyluciferin that generates visible light which is displayed as a peak in the pyrogram. Unincorporated dNTPs and any residual ATP are degraded by aparase. The ratio of cytosine peaks to cytosine + thymine peaks is used to calculate the methylation percentage.[17]

Pyrosequencing is a highly accurate, quantitative method of methylation analysis that is easy to perform and is suitable for high throughput analysis since assays can be carried out in 96 well plates.[17] Pyrosequencing allows for analysis and quantification of methylation at individual CpG sites.[12] A limitation of this method is that it requires high-quality primer design which can be difficult.[4,12] The target amplicon also needs to be small, around 150 bp, thus only a limited number of CpG's can be analysed in a single reaction.[17] Pyrosequencing also requires specific instrumentation.[4,18]

2.1.3.1.3. Methylation-specific PCR (MSP)

Methylation-specific PCR (MSP) makes use of two primer sets that cover multiple CpG's.[19] One primer set specifically amplifies methylated DNA (CpG) while the other set specifically amplifies unmethylated DNA (TpG). Methylation is then detected using gel electrophoresis (Fig 2.4 A) or amplification can be

measured in real-time using either SYBR green technology (MethylQuant) or probe-based technology (Methylight) (Fig 2.4 B).[12]

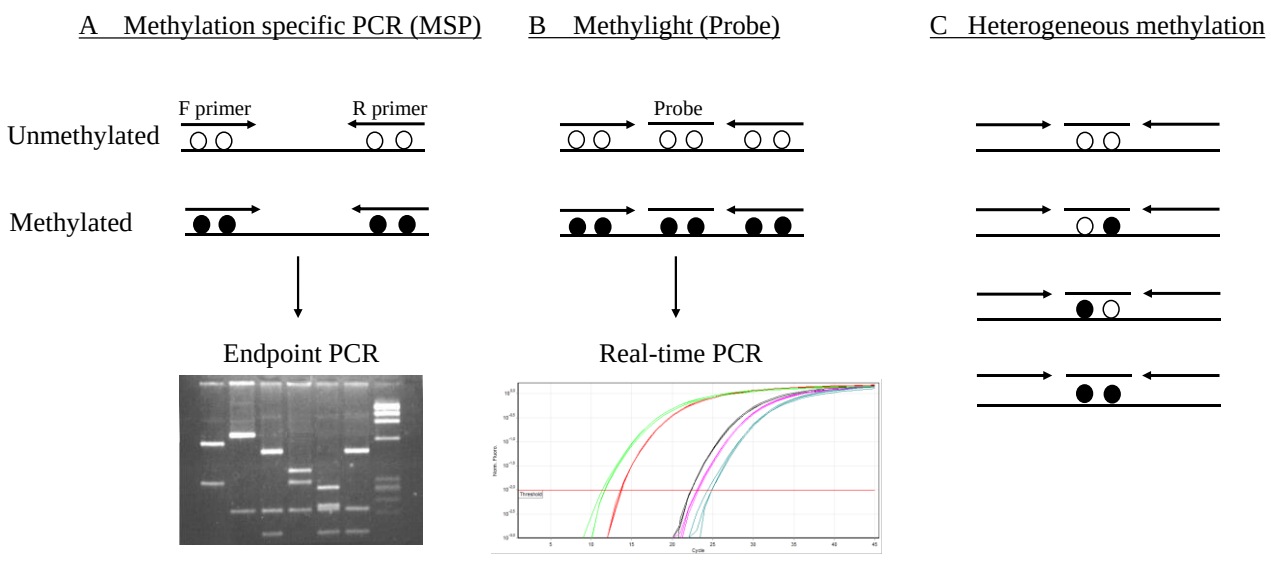


Fig 2.4 Methylation-specific PCR (MSP) primer design A. MSP uses two sets of primers, specific to the methylated and unmethylated sequence respectively. **B**. Methylight makes use of primers and probes that cover CpG's. **C.** Oligonucleotides that cover two CpG's can have four different patterns of methylation. Adapted from Eads *et al.*, 2000.[21]

Methylight assays make use of probes that have a fluorescent reporter at the 5' end and a quencher at the 3' end. When the probe is intact and the quencher and reporter are close to each other, fluorescence is suppressed.[20] During PCR amplification the probe hybridizes to the template DNA, DNA polymerase will then cleave the probe, separating the reporter from the quencher.[20] The fluorescent signal can then be detected by the real-time PCR instrument. Methylation at a particular locus can be quantified by including a methylated control.[12]

Oligonucleotides (oligos) can cover zero to multiple CpG dinucleotides and the more CpG's covered, the more sequence variants possible.[21] For example, an oligo that covers two CpG's can have four sequence variants; either both CpG's can be fully methylated or fully unmethylated or, the first CpG can be methylated and the second unmethylated and vice versa (heterogeneous methylation) (Fig 2.4 C). If each primer and the probe cover two CpG's each then the number of possible permutations is 64 (2^6 = 64).[21] Methylation-specific PCR-based assays are designed to detect one particular pattern of methylation, either fully methylated or fully unmethylated, which means if the other possible permutations are present, the

signal will be low.[20] You can design multiple sets of primers and probes to detect every possible outcome but instead, methylation studies use the ratio between the methylated reaction and unmethylated reaction or the ratio between the methylated reaction and a control (e.g., *ACTB, Alu-C4, MYOD1*, etc) to normalise the input DNA.[22–25] This provides a suitable measurement of the prevalence of methylated molecules at a certain gene locus.[21]

Methylation-specific PCR is highly accurate and easy to perform.[12,19] Furthermore, there is flexibility when it comes to selecting a target region.[12] A limitation of this technique is that only a limited number of CpG's can be analysed at a time since small amplicons are required.[26] Another drawback is that this method cannot detect heterogeneous methylation and lastly, probes can also be expensive.[12]

2.1.3.1.4. Epityper

Epityper makes use of a T7 promoter tagged reverse primer to amplify bisulfite converted DNA. The PCR product is then transcribed into RNA which is recognized and cleaved by RNase after each thymine. The cleaved products from methylated DNA will have different nucleotide composition and mass from unmethylated DNA. Methylated DNA containing C's will be heavier in mass than unmethylated DNA that have T's. Cleavage products are measured by mass spectrometry (matrix assisted laser desorption ionization time of flight mass spectrophotometry, MALDI-TOF MS) and the DNA methylation percentage is calculated and displayed as an epigram. [27]

An advantage of this technique is that it can be used to study heterogeneously methylated alleles.[28] Furthermore, this method is accurate, fast, reproducible, quantitative and allows for analysis of single and multiple CpGs.[12] This method is also suitable for different types of samples including liquid biopsies, fresh frozen tissue, buccal cells, hair etc.[12] Limitations include misinterpretations in the presence of single nucleotide polymorphisms (SNPs) and the presence of contamination peaks can further affect analysis.[12] Knowledge of mass-spectrophotometry is also critical.[12]

2.1.3.1.5. Methylation sensitive high-resolution melting (MS-HRM)

Methylation sensitive high-resolution melting (MS-HRM) uses primers that do not overlap with CpG's and this method is based on the comparison of melting profiles. After bisulfite conversion, the DNA templates are amplified by PCR in the presence of a DNA intercalating fluorescent dye like SYBR green which binds selectively to double stranded DNA and emits a fluorescent signal proportionate to the amount of methylated or unmethylated DNA present. As PCR amplifies the DNA, more fluorescence is produced. The DNA is then subjected to an increase in temperature which causes the PCR products to dissociate into single strands. The dye is then released which results in a drop in the emitted fluorescence. The PCR products from a methylated allele will have a different melting profile from an unmethylated allele because

after bisulfite conversion the methylated alleles are more C rich. Because cytosine is bound to guanine by a triple hydrogen bond, a higher melting temperature is needed to dissociate complementary strands. Thymine and an adenine are only linked by a double hydrogen bond so unmethylated alleles have lower melting temperatures. By comparing melting profiles of unknown samples with profiles from methylated and unmethylated control DNAs the methylation status can be determined.[18,29]

This method is sensitive, cost effective and not very labour intensive.[12] It can also be used to detect heterogeneously methylated samples as they produce a unique melting pattern.[18] The disadvantages of this method are that it cannot provide information on the methylation status of single CpG sites and the accuracy can be influenced by amplicon length, DNA quality etc.[18,29] This method is also susceptible to amplification bias so proper primer design is crucial.[30]

2.1.3.1.6. Digital PCR (dPCR)

Methylation-specific PCR assays can also be applied to digital PCR (dPCR). This method works by sub-partitioning the reaction mixture into thousands of tiny reactions prior to amplification to isolate individual DNA molecules into different compartments.[31] During PCR amplification a fluorescent signal is generated within each compartment according to its DNA content i.e., methylated copies or unmethylated copies (positive) and empty (negative) (Fig 2.5). The known volume of each partition and the proportion of positive partitions to total partitions are used to calculate the target concentration.[32]

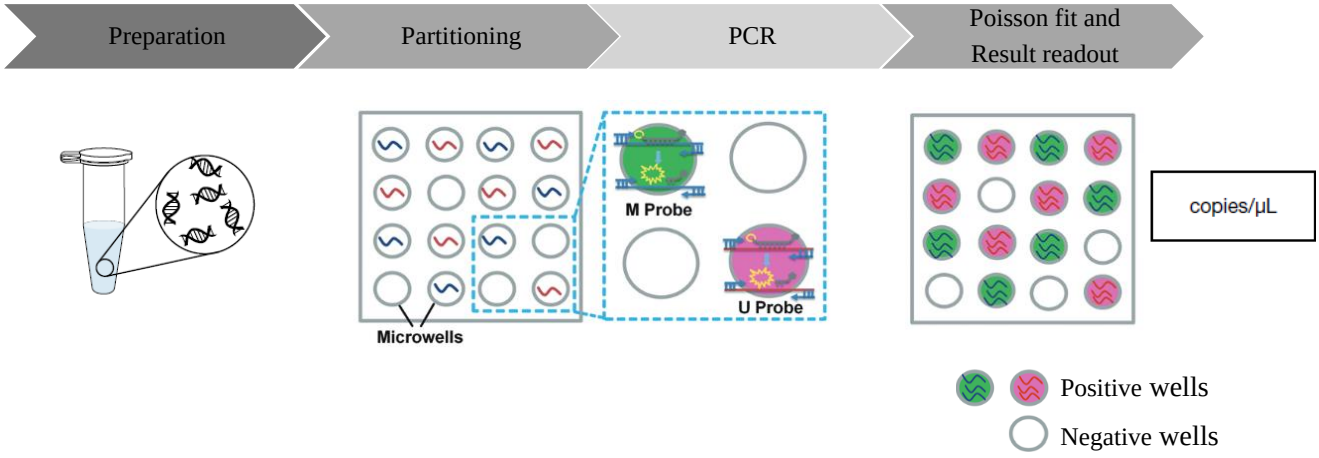


Fig 2.5. Concept of digital PCR. The reaction is partitioned into thousands of thousands of microwells so that each compartment contains a single copy of DNA. Probes specific to methylated (M probe) and unmethylated DNA (U probe) with different fluorescent labels will anneal and generate a fluorescent signal which can be quantified as copies/µl. Adapted from Cui *et al.*, 2018.[36]

The first attempts at this concept were in 1990 and it was then referred to as single molecule PCR.[33] With technical developments later on, sub-partitioning was improved.[31] The term dPCR was coined in 1999[34] and in 2003, one of the pioneering dPCR platforms BEAMing (beads, emulsions, amplifications and magnetics) was applied to cancer research.[35] Since then, various platforms have been developed including Thermo Fisher Scientific's ® chip-based Quantstudio ™ 3D dPCR system[36], Bio-Rad's droplet-based QX-100 droplet digital PCR (ddPCR) system,[37] RainDance Technologies' RainDrop dPCR[38] and Fluidigm's microfluidic chamber based BioMark dPCR system.[39]

Droplet digital PCR, particularly the BioRad ddPCR platform, has gained significant interest in cancer applications and is the most widely used platform today.[31] This is because it is less time consuming and labour intensive than other platforms.[31] The only problem is that the BioRad ddPCR instrument is very expensive. In 2020 Lasec quoted R 5,117,915.45 for the complete Bio-Rad QX-100 ddPCR system while Thermo Fisher Scientific quoted R 520,628.00 (a 10-fold difference) for the complete Quantstudio ™ 3D dPCR system including consumables for 96 reactions. Because the Quantstudio ™ 3D dPCR system was more affordable, this platform was chosen for experiments performed in **Chapters 3** and **4**.

Digital PCR allows for absolute quantification of methylation without the need for calibration curves.[31,32] Digital PCR has also been proven to have superior sensitivity and accuracy over real-time PCR, with Yu *et al.*, 2015 reporting a 25-fold lower limit of quantification (LOQ), i.e. lowest detectable ratio of methylated molecules in unmethylated total molecules, (0.8% *vs* 0.032% for the *NTRK3* gene) and a 20-fold lower limit of detection (LOD), i.e. the lowest detectable concentration of sample, (1250 pg *vs* 62.5 pg for the *EVL* gene).[25] Cui *et al.*, 2018 further compared the performance of real-time PCR *vs* dPCR and observed 10^3-10^4-fold improvements in the LOD and 1-16-fold improvements in LOQ for *GCK* and *PDX1* genes.[40] Digital PCR has also been applied to liquid biopsies, including urine,[41] plasma,[40] serum,[42] cerebrospinal fluid,[43] sputum,[44] and stool samples.[45] There are some technical limitations to this method, for example, reduced precision and quantification uncertainty can occur when there is variation in the partition volume, when a large number of partitions are present or at extremes when most partitions are either positive or negative.[32]

2.1.4. Considerations for epigenetic assay design

2.1.4.1. Bisulfite conversion kits

There are some limitations associated with the bisulfite conversion process, including DNA degradation, incomplete DNA recovery, and incomplete conversion.[46] Bisulfite converted DNA is also less stable thus it requires special storage which can be expensive.[46] The manufacturer's protocol in ZymoResearch kits recommends that bisulfite-converted DNA be stored at -80°C. Ultra-low temperature freezers can cost

between R 127,061.76 (Labex (Pty) Ltd) and R 189,213.24 (Inqaba Biotechnical Industries (Pty) Ltd) for a ~ 400 L freezer (quotes obtained in 2020).

Bisulfite conversion kits from Qiagen and ZymoResearch are arguably the most commonly used in epigenetic studies. Qiagen offers two different kits, the EpiTect Bisulfite Kit and the EpiTect Fast Bisulfite Kit (Qiagen, Hilden, Germany). The most popular ZymoResearch kits are the EZ DNA Methylation-Gold kit and the EZ DNA Methylation-Lightning kit (ZymoResearch, USA).

A study by Kint *et al.,* 2018 compared the performance of 12 different bisulfite conversion kits, including the four kits mentioned above.[47] These kits were compared in terms of DNA fragmentation, recovery and conversion efficiency. In this study, the EZ DNA Methylation Gold kit (ZymoResearch, USA) showed the best recovery, and most appropriate conversion out of all 12 kits while the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) performed the best in terms of fragmentation.[47] This study suggests that the EZ DNA Methylation Gold kit (ZymoResearch, USA) is the best option for methylation analysis of short fragments.[47] While, the EZ DNA Methylation Lightning kit (ZymoResearch, USA) may also be a suitable choice since it allows for slightly faster conversion times and comes with a premade conversion mix, minimizing pipetting errors and allowing for higher throughput.[47] For these reasons, I decided to use this kit in experiments performed in **Chapter 3** and **4**. In terms of cost, the ZymoResearch kits are also cheaper than the Qiagen kits (Table 2.1).

Table 2.1. Prices of bisulfite conversion kits.

Bisulfite conversion kit	Catalog no.	Supplier	No. of reactions	Price excl VAT	Year quoted
EZ DNA Methylation-Gold kit	ZR D5006	Inqaba biotec	200	R 10,437.21	2020
EZ DNA Methylation-Lightning kit	ZR D5031	Inqaba biotec	200	R 11,487.23	2020
EpiTect Bisulfite kit	QIA/59104	Qiagen	48	R 6,489.00	2021
EpiTect Fast Bisulfite kit	QIA/59802	Qiagen	10	R 2,048.00	2021

2.1.4.2. Oligonucleotide design software

The MethPrimer software is a freely available software for designing PCR primers specific to bisulfite converted DNA.[48] This software is easy to use and allows the user to choose from a range of design

strategies including MSP (Fig 2.4A), bisulfite sequencing primers, unbiased primers with a methylation specific probe (Fig 2.4C) etc. MethPrimer also allows for degeneracy in the primers which gives some flexibility when the desired target region is CpG rich, but you opt for an unbiased approach i.e., primers that do not cover CpG's.[48]

Primer Express® Software v3.0.1 (Applied Biosystems™, USA) is useful for designing primers and probes however it is not made specifically for designing methylation-based primers and probes thus, some manual manipulation may be needed to meet your desired design strategy.[24] This software is also expensive, a single-user license (catalog no. 4363991) costs R 8,940.00 excl VAT and a multi-user license (catalog no. 4363993) costs R 14,930.00 excl VAT (2021 prices).

2.1.4.3. Control DNA

Primers and probes designed for methylation analysis are usually tested on fully methylated and fully unmethylated control DNA. There are several commercially available control DNA's (Table 2.2), including EpiTect control DNA (Qiagen, Hilden, Germany), CpGenome Universal Methylated DNA (Sigma-Aldrich, USA) Roche Human Genomic DNA (Merck, Germany) and ZymoResearch also offers a few different options for control DNA.

The problem with purchasing Human Genomic DNA (Merck, Germany) is that you have to apply for permits to import biological material into South Africa from the United States of America. The Department of Health can take up to three months to approve your application and issue import permits therefore lead times for this product can be long.

Table 2.2. Prices of control DNA.

Control DNA	Catalog no.	Supplier	Size	Price excl VAT	Year quoted
EpiTect control DNA set	QIA/59695	Qiagen	100	R 8,423.00	2020
ZymoResearch Human methylated + Non-methylated (WGA) DNA set	ZR D5013	Inqaba biotec	5 µg/20 µl	R 7,106.39	2020
CpGenome methylated DNA	S7821	Merck	10 µg	R 6,456.00	2021
Human Genomic DNA		Merck	100 µg	R 3,394.00	2021

2.1.5. Application of epigenetic biomarkers in disease screening

DNA methylation changes are an early event in tumorigenesis and often occur prior to phenotypic changes, meaning epigenetic assays can be used for early detection of cancer.[49] Furthermore, aberrant methylation can occur at a distance from the tumour itself, referred to as the epigenetic field effect.[50] The epigenetic field effect is useful for identifying cancer in histologically normal tissue biopsies which allows for improved diagnostic accuracy, while reducing the need for repeat biopsies to rule out the possibility of a false negative result.[50–52] Some epigenetic biomarkers can help distinguish between aggressive and indolent tumours.[53] Such biomarkers are useful in that they aid the selection of appropriate treatment interventions. This allows for a more personalized follow-up, which can improve the effectiveness of long-term management of cancer.[52–54]

A limitation of biopsies is that they are highly invasive and in the case of prostate cancer (PCa) for example, can cause an increased risk of infection.[55] Epigenetic biomarkers are able to overcome this limitation, since aberrant methylation can be detected non-invasively using body fluids, this is known as a liquid biopsy. Necrosis, apoptosis, or lysis of circulating tumour cells (CTC) leads to cell-free tumour DNA being released into circulation.[56] Circulating tumour DNA (ctDNA) can then be used to study DNA methylation.[56–58] The non-invasive nature of liquid biopsies makes it possible to do frequent testing to monitor tumour burden, treatment response, and relapse.[56]

Although epigenetic biomarkers have shown promising results, majority are still not used in clinical practice today. This is largely due to the lack of standardised protocols and the absence of large-scale validation studies.[49,56] Extensive, multicentric, prospective studies are needed so that these biomarkers can be implemented into clinical practice and contribute to improved routine disease screening in highly prevalent cancers like PCa.[57,59]

2.2. Prostate cancer

Prostate cancer (PCa) is the second most incident cancer among men globally.[60] According to the Global Cancer Observatory, the estimated number of new cases in 2020 was 1,414,259 worldwide.[60] Prostate cancer is ranked fifth for cancer-related deaths among men worldwide with an estimated 375,304 deaths in 2020.[60] Family history, age and African ancestry are the most significant, non-modifiable, risk factors of PCa development.[61] These risk factors are influenced by germline (inherited) and somatic (acquired) alterations which induce PCa tumorigenesis.[62]

2.2.1. Risk factors for prostate cancer

2.2.1.1. Family History

Men with a first-degree male relative i.e., father or brother, diagnosed with PCa have a 2-3-fold higher risk of developing PCa, while men with two or more first degree relatives with PCa have a 4.4-fold higher risk.[63] Furthermore, those with a relative with breast cancer have a 21% higher risk of being diagnosed with PCa, and have a 34% increased risk of fatality.[64] Germline mutations in the *BRCA2* gene are associated with a 8.6-fold higher risk of PCa.[65] While, abnormal expression due to hypermethylation of the promoter of *BRCA1* has been associated with a higher probability of aggressive disease and poor survival.[66]

2.2.1.2. Age

After the age of 50, the chance of developing PCa rapidly increases, due to somatic changes.[61] Kwabi-Addo *et al.*, 2007, showed a significant increase in promoter methylation of five genes (*RARβ, RASSF1A, GSTP1, NKX2-5*, and *ESR1*) in normal prostate tissue samples which were correlated with age.[67] A positive association has further been observed between increasing age and methylation of *TIG1*, *CDH12*, *EGFR5*, *MCAM*, *SLIT2*, *ESR1*, and *DLC-1*.[68–70] Lastly, loss of genomic imprinting at the well-known imprinted gene *IGF2* in aging prostate tissue is linked to PCa susceptibility.[71]

2.2.1.3. African ancestry

African American (AA) men are more likely to develop PCa (1.6-fold increased risk) and they are roughly three years younger at diagnosis compared to European Americans (EA).[72] Furthermore, AA men suffer from a more aggressive disease than EA, characterised by higher prostate-specific antigen (PSA) (median PSA: 7.4 µg/L *vs* 6.6 µg/L), higher histopathological tumour grades (further discussed in **Chapter 1**) (Gleason score (GS) of 7: 38% *vs* 36%) and faster growing tumours, which makes them more likely to die

from the cancer (2.5-fold increase).[72–74] In AA men, tumours are also commonly located anteriorly which makes it challenging to obtain a biopsy sample representative of the tumour.[75]

Prostate cancer is a major public health concern within the African continent, with the PCa mortality rate expected to increase by 119% to 103,000 between 2020 and 2040 (Northern America expected to increase by 84% to 68,300).[76] Lower incidence and mortality rates (16.6 and 8.2 age standardised rates (ASR) per 100,000) have been observed in northern parts of Africa, while men from Southern and middle parts of Africa suffer from a more aggressive disease, compared to men from Northern America, leading to higher mortality (22 and 24.8 *vs* 8.3 ASR per 100,000).[60,77,78]

Within South Africa, PCa is ranked as the number one cancer with 13,152 new cases in 2020.[60] The estimated number of PCa deaths in South Africa in 2020 was 3,896 which was the second-highest cause of cancer-related deaths in males, lung cancer being the first.[60] Black South African men present with higher histopathological grading (GS > 7: 36% *vs* 17%) and increased PSA levels (PSA ≥ 20 µg/L: 83.2% *vs* 17.2%) at diagnosis compared to AA men, leading to a 2-fold increase in aggressive diasease.[79] It was recommended, by The Prostate Cancer Foundation of South Africa, that men of African ancestry above the age of 40 should receive routine PSA testing, however, many men do not get tested, which may be why Black South Africans are diagnosed around 5 years later than Americans.[74,79] More aggressive, poorly differentiated tumours have also been observed in rural localities compared to urban areas, which could be due to later diagnosis (approximately 3 years later than urban areas).[79]

This health disparity may be due to a lack of PCa awareness and reluctance to seek medical care, clinics being far away, lack of trained urologists, and shortages of resources and infrastructure, which results in a focus on emergency care rather than preventative care.[74,79] Furthermore, many South Africans use traditional healers as their primary source of care, which presents an obstacle for preventing advanced disease and which is further exacerbated by lack of clinical symptoms.[74] The latter calls for a critical need to establish both diagnostic and prognostic tools that are appropriate for all South Africans, while elucidating the genetic, including epigenetic, and non-genetic contributions to aggressive PCa presentation.

2.2.2. Prostate cancer risk factors in Black South Africa men

Not surprisingly, Tindall *et al.*, 2013 found that previously defined genetic risk factors (8q24, 17q24 and 2p21 regions) were not significant predictors of PCa within the Southern African Prostate Cancer Study (SAPCS)[79] cohort, reflecting the bias in both allele representation and identification currently biased towards non-African populations.[80] Conversely, this study found ethnolinguistic identity (Odds ratio (OR) = 1.81, P = 0.0473) and lifestyle factors such as diabetes (OR = 1.83, P = 0.0161) and erectile dysfunction (OR = 1.54, P = 0.0187), to be better predictors of PCa risk in men with African Ancestry, while PSA levels were found to be elevated (≥ 4 µg/L) in men both with (median PSA = 98.8 µg/L) and without PCa (median

PSA = 9.1 µg/L).[80] Recently, Conti *et al.*, 2017 identified African specific risk alleles on chromosomes 13q34 (rs75823044, OR = 1.55, P = $6.10x10^{-12}$) and 22q12 (rs78554043, OR = 1.62, P = 7.10×10^{-10}) however these alleles have not been validated in Black South Africans.[81] Furthermore, methylation-based contributors of PCa risk are even less well studied in the South African population.

2.2.3. Methylation in prostate cancer

In PCa, genes involved in growth suppression (*APC, RARβ*), DNA damage repair (*GSTP1* and *MGMT*), hormonal response (AR, ER), cell adhesion (*CDH1*, *CD44*), cell cycle control (*RASSF1, CDKN2*), and apoptosis (*DCR,1,2, XAF1, TMS1*) are commonly affected by hypermethylation.[1] While, genes such as *LINE1, XIST, PLAU,* and *HPSE* are hypomethylated in PCa.[62] DNA hypomethylation arises later than CGI hypermethylation and contributes to metastatic PCa.[82] (Table 2.3)

Table 2.3. Genes with aberrant methylation in prostate cancer.

Hypermethylated genes	Hypomethylated genes
APC, RARβ, GSTP1, MGMT, AR, ER, CHD1, CD44, RASSF1, CDKN2, XAF1, TMS1, DCR1, DCR2, MLH1, BRCA1, VHL, RB1, DAPK, TIMP, PTGS2, HOXD3, RUNX3, SLC18A2, HIC1, SFN, miR-193b, miR-34b/c, KLK10, TGFβ2, TBX15, SPARC, NKX2-5, HIST1H4K, P16, EDNRB, ARF, DLEC1, PITX2, C1orf114, HOXB5, HOXD9, HOXD10, HOXA7, HOXD8, PCDH17, TCF21, GDF15, ADCY4, TMEFF2, HSPA20, VIM, AOX1rc, KIFC2, CXCL14, EPHA3, GFRA2, HEMK1, MOXD1, NEUROG3, NODAL, RASSF5, RASSF2, TFAP2E, P14, HAPLN3, CRIP3, GAS6	*LINE1, XIST, PLAU, HPSE, CAGE, CYP1B1*

2.2.4. Status of epigenetic screening in prostate cancer

Biomarkers can be classified as either diagnostic, predictive or prognostic. A prognostic biomarker is used to determine the likelihood of a clinical event such as disease recurrence or progression in patients with

confirmed disease. While, predictive biomarkers are used to identify patients who are more likely to respond to a certain treatment. A diagnostic biomarker is used to detect the presence of disease in an individual.[83]

Sensitivity and specificity are important parameters used to assess the accuracy of a diagnostic biomarker. Specificity is the proportion of non-diseased patients correctly identified as such while sensitivity refers to the amount of true positives.[46] Sensitivity and specificity depend on a chosen cut-off point that defines normal *vs* diseased.[84] The receiver operating characteristic (ROC) curve is used to choose an optimal cut-off value that maximises sensitivity and specificity.[84] Furthermore, the area under the ROC curve (AUC) measures how well a marker can discriminate between patients with and without disease, for example, a very good marker will have perfect discrimination i.e. 100% specificity and 100% sensitivity, and would yield an AUC of 1, while a poorer marker will have an AUC closer to 0.5.[84]

To date, ConfirmMDx (MDxHealth, Irvine, USA) is the only commercially available methylation-based diagnostic assay used in PCa.[85] ConfirmMDx (MDxHealth, Irvine, USA) measures DNA methylation of *APC, RASSF1,* and *GSTP1* in histologically negative tissue biopsies, to detect an epigenetic field effect.[86] A combination of DNA methylation of the three genes, relative to the *ACTB* reference gene, and clinical parameters such as age, elevated serum PSA levels, abnormal digital rectal examination (DRE), and histopathology of the initial biopsy are used to estimate the likelihood of finding PCa upon repeat biopsy, in men positive for methylation, and this is used to help guide the decision of whether or not to do a repeat biopsy.[86] Although this assay was *originally* developed and tested on Europeans, Waterhouse *et al.,* 2019 did recently validate this assay in 211 AA men and showed that sensitivity and specificity were not significantly different (P = 0.235, P = 0.697) for the different ethnic groups.[51,87,88]

Such validation studies are critical because acquired epigenetic differences have been observed between ethnic groups which could have implications for certain biomarkers.[62] Woodson *et al.,* 2003 showed that *CD44* methylation differed in Black *vs* White Americans but did not observe a significant difference for *GSTP1*.[89] Similarly, Kwabi-Addo *et al.,* 2010 reported no difference in *GSTP1* methylation between AA and EA but did find significant differences in methylation of multiple genes including: *RARβ2, SPARC, TIMP3* and *NKX2-5*.[90] One study reported that AA with *GSTP1* methylation are more likely to have PCa compared to EA.[91] More recently, Devaney *et al.,* 2015 reported differences in promoter methylation of four genes (*SNPRN, SHANK2, MST1R, ABCG5*) between AA and EA men.[92] Methylation of *APC* has also been reported to be different among AA and EA.[93,94]

I chose to focus on a single gene for the purpose of this dissertation. *GSTP1* was chosen as the exclusive target gene because it is part of the ConfirmMDx (MDxHealth, Irvine, USA) panel and is the most extensively studied biomarker in this panel. Furthermore, *GSTP1* has superior specificity over *RASSF1* and *APC*. Lastly, it has been reported by other studies to be similarly methylated in men of African and European descent.

2.3. Glutathione S-transferase pi 1 (GSTP1)

There are five classes of Glutathione S-transferases (GSTs) namely alpha, mu, pi, sigma and theta with the pi class being the most well studied.[95] The pi class *(GSTP1)* was first described in prostate cancer (PCa) in 1994[96] and since then, there have been 164 publications that list this gene as a PCa biomarker. PubMed search terms on 1/12/2021 (("Prostatic Neoplasms"[Mesh]) AND "Biomarkers"[Mesh]) AND GSTP1. This is more than double the number of studies of other PCa biomarkers (*APC*: 81 studies, *RASSF1*: 35 studies, *RARB*:12 studies and *HOXD3*: 9 studies). What makes *GSTP1* an ideal biomarker is the fact that (i) it is well-studied (ii) it has diagnostic, prognostic and predictive value, ii) it has shown acceptable performance in liquid biopsies (iii) PCR based analysis of *GSTP1* methylation is rapid, cost effective and easy to interpret and lastly, (iv) it has extremely high specificity.

2.3.1. GSTP1 Function

The GST family of enzymes catalyse detoxification reactions by conjugating carcinogens and electrophiles with reduced glutathione to form a less toxic complex that can be metabolised.[59] *GSTP1* acts as a caretaker, protecting cells from damage.[97] Loss of expression results in an increase of reactive oxygen species (ROS) and oxidative stress which can lead to DNA damage and cancer initiation.[98]

2.3.2. GSTP1 gene

The *GSTP1* gene is located on the long arm of chromosome 11. This gene consists of seven exons which code for a 741bp mRNA product (NM_000852). A CpG island (CGI) overlaps with the *GSTP1* promoter region and the first three exons.[95] The promoter consists of an AP-1 binding site, two SP1 binding sites (G/C boxes), and a TATAA box (Fig 2.6).[95] The SP1 sites are important for maintaining an unmethylated state in the CGI and when there is loss or interference of SP1 binding, this can result in loss of transcription.[99] More specifically, Moffat *et al.,* 1996 reported that disruption to the distal SP1 site (- 54 to -46) has more of an effect on promoter activity than the proximal SP1 site.[100] Upstream of the AP-1 binding site is a NF-kB like element, which is a negative regulatory element that has been shown to suppress transcription.[101] Upstream of the CGI is an $(ATAAA)_{19\text{-}24}$ repeat sequence which Millar *et al.,* 2000[99] suggested may act as a barrier to methylation in normal prostate cells however, Platz *et al.,* 2002 did not find this repeat region to play an important role in PCa incidence.[102]

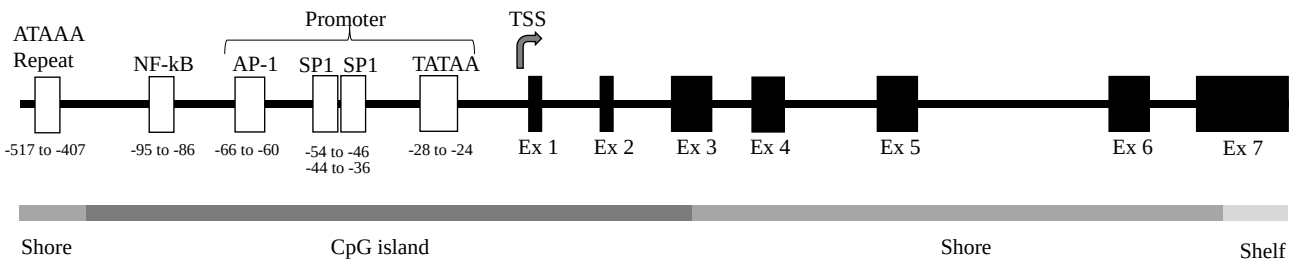


Fig 2.6. *GSTP1* gene The black boxes represent exons 1-7. Upstream of the transcription start site (TSS +1) is the promoter region along with its position, relative to the TSS. The position of the CpG island, shore, and shelf is also shown below the gene. Adapted from Moffat *et al.*,1996.[100]

2.3.3. Methylation of GSTP1 in cancer

Aberrant *GSTP1* methylation has been observed in a variety of cancers including PCa, lung, stomach, hepatocellular carcinoma (HCC), breast etc. Hypermethylation occurs in approximately 25-45% of breast tumours.[59] *GSTP1* hypermethylation is an early even in breast carcinomas meaning it could provide a valuable marker for early disease screening.[103] Increased hypermethylation was observed in breast carcinomas and was associated with gene silencing, while in normal tissue *GSTP1* remained unmethylated and expressed.[103] Furthermore, *GSTP1* hypermethylation may be an indicator of an aggressive phenotype and could serve as a prognostic biomarker for breast cancer.[104,105] Arai *et al.*, 2006 reported *GSTP1* methylation to be significantly associated with large tumours ($P < 0.01$), metastases ($P < 0.05$) and poorer relapse-free survival (RFS) (5 years from treatment to relapse: 60% of those with methylation *vs* 86% of those without methylation).[105]

Approximately 50% of hepatocellular carcinomas (HCC) have *GSTP1* hypermethylation.[59] While some studies report a lack of methylation in normal tissue and an association between methylation and HCC development, others showed methylation in adjacent, non-cancerous tissue.[106,107] The diagnostic performance of *GSTP1* ranges (sensitivities: 50-75% and specificities: 70-91%)[59] and it has been reported that its performance is improved when used in gene panels.[108] The prognostic performance is unclear with some studies reporting prognostic potential while others refute this.[109–111] For example, Qu *et al.*, 2015 found that *GSTP1* was associated ($P < 0.05$) with tumour invasion and metastasis while, Anzola *et al.*, 2003 did not observe differences ($P = 0.2747$) in RFS for patients with *vs* patients without *GSTP1* methylation.[109,110]

GSTP1 hypermethylation is a less frequent event in lung (0-25%), bladder (1-15%), and urothelial cancers (1.4-20.6%).[59] Interestingly, some cancers types including colorectal, stomach, esophageal, lung thyroid and breast cancers have increased *GSTP1* expression.[95,112] Suggesting that *GSTP1* may have a dual role in addition to being a tumour suppressor.[59,112] For example, upregulation of *GSTP1* in thyroid cancer is involved in carcinogenesis and tumour growth.[113] While, in esophageal cancer, *GSTP1* expression reduces chemosensitivity of cancer cells.[114]

The presence of *GSTP1* methylation is significantly associated with increased PCa risk (OR = 18.58, 95%, $P < 0.001$) and approximately 90% of PCa cases are positive for *GSTP1* methylation, which is likely why this is the most extensively studied PCa biomarker.[112,115] Hypermethylation of *GSTP1* is an early event in PCa development.[95] In PCa cells, hypermethylation occurs throughout the CGI and both alleles are abnormally methylated which leads to loss of gene expression.[95] While in normal tissue and benign prostatic hyperplasia (BPH) tissue the CGI remains unmethylated.[96]

Stirzaker *et al.*, 2004 suggested a model to explain how the *GSTP1* CGI is hypermethylated in PCa.[116] They explain that in a PCa cell, *GSTP1* is inactivated which results in methylation spreading from random "seeds" of methylation. These seeds initiate MDB2 binding which then recruits histone deacetylases (HDACS) resulting in histone deacetylation. MBD2 recruits DNMTs which promotes the spread and maintenance of methylation across the CGI.[117] After extensive methylation, MeCP2 binds and recruits histone methyltransferases leading to methylation of lysine 9 on histone 3 (H3K9me) which induces a heterochromatin state. [116,118]

A more recent study found Piwi-interacting RNA 31470 (piR-31470) to be highly expressed in PCa cells.[119] This study suggests that piR31470 could form a complex with piwi-like RNA mediated gene silencing 4 (PIWIL4/piR-31470) which then binds to nascent RNA transcripts and recruits DNMT1, DNMT3a and MBD2 to initiate and maintain *GSTP1* hypermethylation and silencing.[119]

2.3.4. Performance of GSTP1 as a prostate cancer biomarker

According to a meta-analysis study conducted in 2012, which included 35 studies resulting in a pooled cohort of 2,389 PCa patients and 1,082 controls, *GSTP1* had an overall specificity of approximately 95% and a sensitivity of 82% in tissue.[120] Another study reported a sensitivity of 25.2% and a specificity of 86.7%,[121] while more recently, Fiano *et al.*, 2019 reported a sensitivity of 14% and a specificity of 98%.[122] Numerous studies have also commented on the value of *GSTP1* methylation in negative biopsies, explaining that it can predict missed cancer with high specificity.[51,121–123] The performance of *GSTP1* has further been compared between urine and tissue with Woodson *et al.*, 2008 reporting higher sensitivities for tissue (91% *vs* 75%), and higher specificities for urine (98% *vs* 88%).[124]

Shedding of genetic material into urine makes this a precious source for interrogating aberrant methylation and is less invasive than using tissue.[59] In addition to urine, other body fluids including plasma, serum and ejaculate can also be used for PCa screening. However, Payne *et al.*, 2009 reported better sensitivities for *GSTP1* in whole urine than in plasma (63% *vs* 17%).[125] Wu *et al.*, 2011 conducted a meta-analysis on studies that assessed *GSTP1* performance in plasma, serum and urine.[126]They reported a pooled sensitivity of 52%, while the specificity in plasma, serum, and urine was 89%.[126]

The *GSTP1* biomarker has better specificity than another well-established blood-based biomarker, PSA (78.6% specificity *vs* 5.8%),[127] however, as mentioned above, sensitivities can sometimes be low which is why it is commonly used in a panel combination with other genes.[59] For example, when combined with *HOXD3,* the sensitivity increased from 52.5% to 57.1%.[52] While, a gene panel including *APC, GSTP1* and *RARB2* had 100% sensitivity in tissue and 94.3% in urine.[53] When combined with *HAPLN3* and *GAS6*, a sensitivity of 94% was obtained.[9] And, the combination of *GSTP1* with *RASSF1, RARB,* and PSA yielded a sensitivity of 56%.[128] Gene panels have the potential to compliment PSA testing for better detection of clinically significant PCa and allow for increased prognostic potential.[52,127]

Mahon *et al.*, 2014 showed that *GSTP1* methylation has a prognostic role in metastatic castration-resistant PCa.[129] This group explained that *GSTP1* methylation was associated with poorer survival and further showed that this biomarker can be used as a therapeutic efficacy marker for chemotherapy.[129,130] Another study showed that *GSTP1* methylation could be used to identify patients that will relapse (AUC = 0.765).[53] Higher concentrations of *GSTP1* methylation, along with *RASSF1a, APC,* and *RARβ,* in circulation were also associated with disease progression.[131] A gene panel including *GSTP1* and *APC* was able to differentiate between aggressive tumours and indolent ones.[132] The *GSTP1, APC CRIP3* and *HOXD8* gene panel is used to reclassify patients under active surveillance.[133]

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Chapter 3: Designing a *GSTP1* epigenetic assay for African prostate cancer screening

As discussed in **Chapter 2**, *GSTP1* can be a useful epigenetic biomarker for prostate cancer screening. However, developed *GSTP1* assays are designed for European populations and have not been tested in Black South Africans. This chapter will therefore firstly assess the feasibility of published assays in Black South Africans and secondly, design and evaluate a new African relevant digital PCR assay. Developing epigenetic assays applicable to Africans are critical to improve disease screening and outcomes within high-risk populations.

In this chapter, I would like to acknowledge Prof Liza Bornman (University of Pretoria and Ampath) for providing the necessary equipment and lab supplies needed to perform my experiments. I would like to thank the lab of Prof Vanessa Hayes (Garvan Institute of Medical Research) for providing access to relevant unpublished genomic data and in particular, Jue Jiang for assisting with data transfers. Lastly, I would like to thank A/Prof Clare Stirzaker (Garvan Institute of Medical Research) for advice in designing primers and Nicky Boulter and David Murray (Garvan Institute of Medical Research) for advice in probe design.

3.1. Introduction

Hypermethylation of CpG islands (CGI) in the promoter of tumour suppressor genes is a well-established prostate cancer (PCa) feature.[1] In particular, the CGI in *glutathione S transferase pi 1 (GSTP1)* (further outlined in **Chapter 2**) is almost always hypermethylated in PCa but remains unmethylated in healthy tissues and benign prostatic hyperplasia (BPH).[2] For this reason, *GSTP1* is the most extensively studied epigenetic biomarker for accurate discrimination between patients and controls.[3] With a high specificity of approximately 95% in tissue,[4] the probability of a false negative is very small, making this biomarker extremely attractive.

African-American (AA) men have a 1.6 times higher risk of developing PCa and they are 2.4 times more likely to succumb to the cancer compared to European-Americans (EA).[5] Within Africa, the disease burden is even worse, with Black South Africans suffering from a two times more aggressive PCa compared to AA men.[6,7] Current *GSTP1* assays have been developed based largely on European genomic data and although some of them have been tested in AA men,[8,9] they have not been validated in Black South Africans. This is problematic because if African single-nucleotide polymorphisms (SNPs) were overlooked, this may affect the efficiency of the assay in a population where there is a substantial need for such a test.

Furthermore, methylation assays are now being applied to digital PCR (dPCR) (further outlined in **Chapter 2**), a more sensitive technology capable of accurately detecting low levels of methylation (< 0.05%)[10] and to date, no methylation based dPCR assays have yet been developed for PCa screening in Black South Africans.[10,11]

The aim of this chapter was to firstly assess the feasibility of published *GSTP1* assays for Black South Africans, and secondly design an African relevant dPCR assay appropriate for routine PCa screening. By considering South African variants during assay design, *GSTP1* can be made applicable to this high-risk population. By using the latest innovative technology, we can ensure the best possible assay is designed for improved disease screening in South Africa.

3.2. Methods and Materials

3.2.1. GSTP1 annotation

The CpG island (CGI) overlapping with *GSTP1* was identified using UCSC,[12] Roadmap Epigenome Browser[13] and DBCAT.[14] The Hg38 CGI sequence was downloaded from UCSC[12] and bisulfite converted (*in silico*) using MethPrimer.[15] Regulatory elements such as promoters and transcription factor binding sites (TFBS) were identified using the literature and mapped to the CGI.[16,17] DNase sensitivity sites, which are indicative of open chromatin and thus transcriptional activity, were identified using UCSC[12] and mapped to the CGI sequence.

3.2.2. African-relevant GSTP1 variant data

Germline variant data (including single nucleotide variants (SNV) and insertions and deletions < 50 bases (INDELS)) from whole-genome sequencing (WGS) of 117 Black South Africans with PCa, was derived from the Southern African Prostate Cancer Study (SAPCS),[6] with data generated and obtained from the Garvan Institute of Medical Research, for region hg38_chr11:67583395-67583994 of *GSTP1*. These were compared to African variants, in the same region, derived from the gnomAD database[18] and 1000 Genomes project.[19]

African samples in the 1000 Genomes database[19] are described as Gambian in Western Division, African Ancestry in Southwest US, African Caribbean in Barbados, Yoruba in Ibadan Nigeria, Luhya in Webuye Kenya, Mende in Sierra Leone, Esan in Nigeria and Biaka in Central African Republic. The gnomAD database[18] is made up of contributions from a number of different projects including 1000 Genomes,[19] Human Genome Diversity Project[20] (which has information on populations in Central African Republic, the Democratic Republic of Congo, Senegal, Malawi, Ethiopia and some southern Bantu-speakers from South Africa)[21] and many more,[19] therefore these African samples also have a broad geographic range.

Single nucleotide variants (SNV's) present in at least 1% of the population are regarded as SNPs. Common SNP's have a minor allele frequency (MAF) >5%, low-frequency variants (SNP) have a MAF between 1–5% and variants with a MAF <1% are considered as rare. Here, the same parameters were used to classify INDELS. If the SAPCS-identified variant was not in the dbSNP database,[22] it was regarded as novel.

3.2.3. Primer/probe mapping

A review article published by Gurioli *et al*., 2018[3] listed papers that used *GSTP1* as a liquid biopsy biomarker for cancer detection. The primers and probes used in studies on PCa are listed in Table 3.2. A Primer Blast was used to align these primers and probes against the bisulfite converted sequences to (i) identify target regions, (ii) determine whether published primers and probes targeted a functional region and (iii) whether they overlapped with African variants.

3.2.4. Standard DNA samples

The Human Methylated and Non-Methylated whole-genome amplified (WGA) DNA Set (ZymoResearch, USA) was used as control DNA.

3.2.5. Bisulfite conversion

DNA was bisulfite converted using the EZ DNA Methylation Lightning kit (ZymoResearch, USA), with some modifications to the manufacturer's instructions:

The M-Wash Buffer was prepared by adding 96 ml of 100% ethanol to 24 ml M-Wash Buffer concentrate.

Lightning Conversion Reagent was added to 20 µl of DNA (400 ng) in a PCR tube to obtain a final volume of 150 µl. Each sample completed a run in a Bio-Rad T100 thermal cycler (Bio-Rad, USA) set at 98°C for 8 min, 54°C for 1 hour, 4°C hold. A Zymo-Spin™ IC Column was placed into a collection tube and 600 µl of M-Binding Buffer was added to the column, followed by the sample. The reaction was mixed and centrifuged at 15,990 x g in a Hermle Z167 M centrifuge (Hermle, Germany) for 30 s. The flow-through was discarded and 100 µl of M-Wash Buffer was then added to the column. The reaction was centrifuged again and 200 µl of L-Desulphonation Buffer was added. The reaction was left to stand at room temperature for 15 min. After incubation, the reaction was centrifuged, 200 µl of M-Wash Buffer was added to the column and it was centrifuged again for 30 s. This wash step was repeated but this time the sample was centrifuged for 1 min. The column was placed into a 1.5 ml microcentrifuge tube and 10 µl of M-Elution Buffer was added. The sample was left to incubate for three minutes at room temperature and then finally the DNA was eluted by centrifugation for 30 s at 15,990 x g.

3.2.6. SYBR based real-time PCR

Primers were optimised using standard DNA samples (Human Methylated and Non-Methylated (WGA) control DNA (ZymoResearch, USA)) and real-time PCR. Real-time PCRs were prepared in a final volume of 10 µl consisting of 50 ng of bisulfite converted DNA, 5 µl of 2X PowerUp SYBR green master mix (Applied Biosystems™, USA) and 0.5-0.8 µM of forward and reverse primers (IDT, USA). Real-time PCRs were performed in a QuantStudio™ 3 Real-Time PCR instrument (Applied Biosystems™, USA) under the following conditions: 50°C for 2 min, 95°C for 2 min, 40 cycles of 95°C for 15 s, 58-60°C for 15 s and 72°C for 1 min. If the annealing temperature was higher than 60°C then the elongation step of 72°C for 1 min was excluded. After PCR, the instrument was set to perform a default dissociation step at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s.

3.2.7. Probe based real-time PCR

Probes were evaluated using TaqMan SNP genotyping technology on standard DNA samples. Real-time PCR reactions were performed in a final volume of 25 µl consisting of 100 ng bisulfite converted standard control DNA, 0.5-0.8 µM of each primer, 0.1-0.3 µM of methylated and unmethylated probe, and 12.5 µl of 2X Taqpath ProAmp master mix (Applied Biosystems, USA). Genotyping was performed on a QuantStudio™ 3 Real-Time PCR instrument (Applied biosystems™) under the following conditions: 60°C for 30 s, 95°C for 5 min, 40 cycles of 95°C for 15 s and 60°C for 1 min and a final 60°C for 30 s.

3.2.8. Digital PCR

Digital PCR reactions were performed in duplicate. The dPCR reaction mixture was made up to a final volume of 34.8 µl consisting of 400 ng bisulfite converted DNA, 0.8 µM of each primer, 0.3 µM of methylated and unmethylated probe, and 17.4 µl of 2X QuantStudio™ 3D Digital PCR Master Mix v2 (Applied Biosystems™, USA).

The QuantStudio™ 3D dPCR 20K chip (Applied Biosystems™, USA) was placed into the chip nest of the QuantStudio™ 3D dPCR Chip Loader (Applied Biosystems™, USA) and the sample loading blade was attached. The lid was then locked in place. The reaction mixture (14.5 µl) was transferred into the sample loading port of the loading blade. The loading blade then distributed the reaction mixture across the chips 20, 000 reaction wells. After loading, immersion fluid was added directly onto the chip until the entire surface was covered. The chip was sealed with the chip lid and the chip case was filled with immersion fluid. The chip case was tightly sealed, and the chips were placed into the Proflex ™ 2x Flat PCR system (Applied Biosystems™, USA). PCR was performed with the following program: 96°C for 10 min, 39-50 cycles (as suggested in the QuantStudio™ 3D dPCR optimisation guidelines) of 58-62°C for 2 min and 98°C for 30 s, and finally 60°C for 2 min.

Once the dPCR run was complete, the chip was loaded into the dPCR instrument and the results were read. The dPCR instrument reads the fluorescent signal in all the wells and displays the data as a count of copies per microliter for FAM and VIC. The data was further analysed using the QuantStudio™ 3D AnalysisSuite™ software.

3.3. Results

3.3.1. Interrogating GSTP1 for African-relevant variation.

African relevant variants (SNV and INDEL) present in at least two datasets are summarized in Table 3.1 and depicted in Fig 3.1. Nine germline variants, identified during WGS, overlapped with the region chr11:67583395-67583895. Of these, six were common variants i.e., had a MAF > 5%, and three were rare variants i.e., had a MAF < 1%. Two of these three rare variants (rs8191438 and rs8191439) were classified as low frequency SNPs by the 1000 Genomes[8] and gnomAD databases.[18] In this region, an additional three and 23 rare variants were identified by the 1000 Genomes[19] and gnomAD[18] databases respectively. Additional variants in the gnomAD database[18] are to be expected due to the large study size (n = ~41,000). The small sample size (n = 117) could explain some of the differences between the South African cohort and other Africans. Furthermore, there is huge genetic diversity across Africa with Southern African populations being the most diverse which could also contribute to these differences.

Table 3.1. African relevant variants (SNVs and INDELS) present in region hg38_chr11:67583395-67583895. Variants were identified by gnomAD, 1000 Genomes and the SAPCS along with their allele frequencies and their classification i.e., rare (R) (MAF < 1%), low frequency (L) (MAF 1-5%) and common (C) (MAF > 5%). The SAPCS looked at 117 genomes which represented nine different populations. gnomAD looked at ~41,000 genomes and 1000 Genomes looked at 1,322 African genomes which represented eight populations.

Variant ID	Position	Reference allele	Alternate allele	Allele frequency					
				gnomAD (count)		1000 Genomes (count)		SAPCS (count)	
rs17593068	11:67583461	G	T	0.381 (41,852)	C	0.38 (1,322)	C	0.726 (117)	C
rs45447591	11:67583483	G	T	0.071 (41,844)	C	0.09 (1,322)	C	0.128 (117)	C
rs45457391	11:67583484	C	T	0.071 (41,810)	C	0.091 (1,322)	C	0.128 (117)	C
rs576504247	11:67583499	T	C	0.003 (40,154)	R	0.005 (1,322)	R	0.009 (117)	R
rs11311625	11:67583526	TT	T	0.385 (41,820)	C	0.384 (1,322)	C	0.726 (117)	C
rs36211088	11:67583528	C	A	0.385 (41,828)	C	0.384 (1,322)	C	0.726 (117)	C
rs36211089	11:67583533	C	T	0.385 (41,828)	C	0.384 (1,322)	C	0.726 (117)	C
rs8191438	11:67583625	C	G	0.019 (42,032)	L	0.014 (1,322)	L	0.009 (117)	R
rs560171297	11:67583675	G	C	0.0002616 (42,052)	R	0.001 (1,322)	R		
rs8191439	11:67583826	G	A	0.020 (42,060)	L	0.014 (1,322)	L	0.009 (117)	R
rs45439104	11:67583865	T	G	0.005 (42,004)	R	0.005 (1,322)	R		

3.3.2. Assessment of published assays

Published assays are summarized in Table 3.2, with primers and probes depicted in Fig 3.2. Specifically, Methylight assays (green arrows) are situated around 200 bp to ~ 50 bp upstream of the transcription start site (TSS) and do not overlap with the promoter. MSP assays (purple arrows) target a ~90 bp region slightly upstream of the TSS which covers some TFBS. Bisulfite sequencing primers (black arrows), target a larger region, from approximately -200 to + 150, including the TSS, the promoter, TFBS and DNase hypersensitivity sites. Methylation Sensitive Restriction Enzyme (MSRE) qPCR assays (blue arrows) cover the TSS, the promoter and TFBS. Assays are generally positioned around the TSS to increase the likelihood of targeting a location where DNA methylation will have a functional effect.[23] None of the published assays were found to overlap with any common African SNP, however, the forward primer of the Hoque *et al.*, 2005 assay did overlap with a rare SAPCS variant (low frequency SNP according to 1000 Genomes[19] and gnomAD[18]), which may compromise the efficiency of this assay within other Africans, with less of an effect in the South African population.

Two other rare variants, identified both in the gnomAD[18] and 1000 Genomes[19] databases but not in SAPCS,[6] overlapped with the Woodson *et al.*, 2008 and Goessl *et al.*, 2001 forward primers and Hoque *et al.*, 2005 probe. Since these variants are rare, they should not compromise the assay efficiency in majority of Africans.

Table 3.2. Hg38 Primer and probe sequences previously used to detect differential methylation in *GSTP1*. The colours in the table correlate with the coloured arrows shown in Fig 3.1.

Primer	Sequence 5'-3'	hg38_Start	hg38_Stop	Product size (bp)	Method	Reference
F primer	AGT TGC GCG GCG ATT TC	chr11:67583623	chr11:67583639	140	Methylight	[24,25]
R primer	GCC CCA ATA CTA AAT CAC GAC G	chr11:67583762	chr11:67583741			
Probe	FAM-CGG TCG ACG TTC GGG GTG TAG CG-TAMRA	chr11:67583663	chr11:67583685			
F primer	AGA GGG AAA GGT TTT TTC GGT T	chr11:67583601	chr11:67583622	116	Methylight	[26]
R primer	GCG AAC TCC CGC CGA	chr11:67583716	chr11:67583702			
Probe	FAM-TGC GCG GCG ATT TCG GG-TAMRA	chr11:67583626	chr11:67583642			
F primer	GAT TTG GGA AAG AGG GAA AGG	chr11:67583591	chr11:67583612	391	Bisulfite sequencing	[27]
R primer	CTA AAA ACT CTA AAC CCC ATC C	chr11:67583960	chr11:67583982			
F Primer	GAC CTG GGA AAG AGG GAA AG	chr11:67583591	chr11:67583611	259	MSRE-qPCR	[28]
R Primer	ACT CAC TGG TGG CGA AGA CT	chr11:67583850	chr11:67583831			
F primer	CGG TCC TCT TCC TGC TGT CT	chr11:67583547	chr11:67583566	306	MSRE-qPCR	[29]
R primer	CGT ACT CAC TGG TGG CGA AG	chr11:67583853	chr11:67583834			
F Primer	GGG ACC CTC CAG AAG AGC	chr11:67583717	chr11:67583734	133	MSRE-qPCR	[30]
R primer	ACT CAC TGG TGG CGA AGA CT	chr11:67583850	chr11:67583831			
F primer(M)	TTC GGG GTG TAG CGG TCG TC	chr11:67583672	chr11:67583688	90	MSP	[31–35]
R primer (M)	GCC CCA ATA CTA AAT CAC GAC G	chr11:67583741	chr11:67583741			
F primer (U)	GAT GTT TGG GGT GTA GTG GTT GTT	chr11:67583684	chr11:67583672	78		
R primer (U)	CCA CCC CAA TAC TAA ATC ACA ACA	chr11:67583639	chr11:67583762			

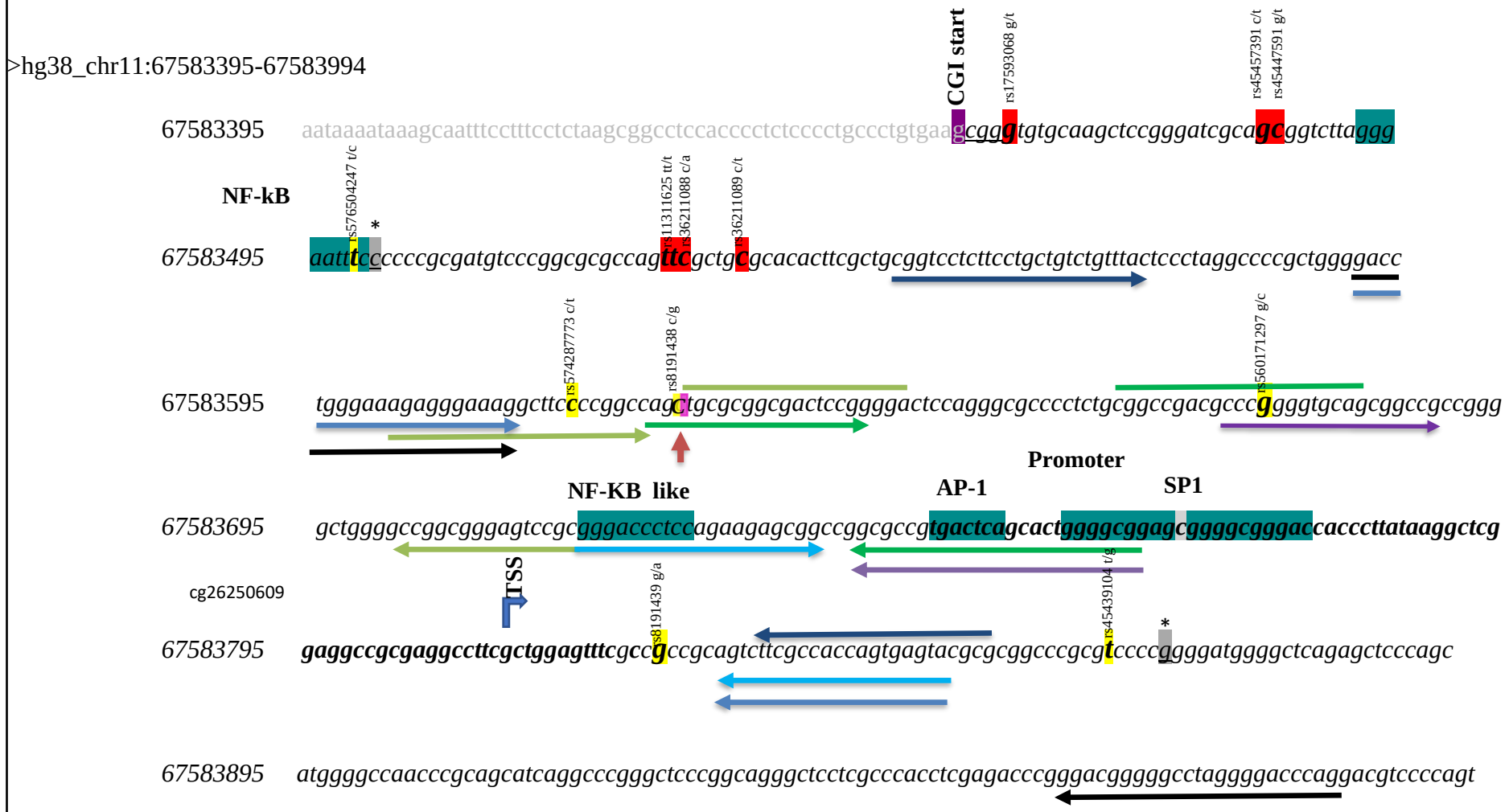


Fig 3.1. Annotated *GSTP1* target region TFBS are highlighted in green and bold text is used to indicate the promoter. The CGI is indicated in italics and the TSS is depicted by a blue arrow. The start and end of DNase hypersensitivity sites are indicated by an asterisk. African-relevant variants, present in at least two datasets, defined as: common variants are highlighted in red, low frequency variants are highlighted in pink and rare variants are highlighted in yellow. Published primers and probes are indicated by coloured arrows. Different colours correlate with different studies listed in table 3.2.

3.3.3. Primer and Probe design

Considering African-relevant variant data and previously published assays, three primer sets, and two probe sets were designed, as outlined in Table 3.3. Multiple primers and probes were designed so that the best design could be chosen for use on clinical samples. The primers were designed to avoid CpG's; methylation detection occurred at the level of the probe only. In cases where CpG's were unavoidable, a degenerate base (N) was used at the CpG site and the CpG was situated near the 5' end. The METH-GSTP1 primer pair and MOD-GSTP1 reverse primer were designed using MethPrimer software;[15] the MOD-GSTP1 forward primer, is a published oligonucleotide sequence[11] modified to avoid CpG's. The MOD-GSTP1 primer set has a larger amplicon (157 bp) which covers both the AP-1 and SP1 TFBS, while METH-GSTP1 has a smaller amplicon (127 bp) and only covers the proximal SP1 site (Fig 3.2). The OWN-GSTP1 primer set was designed 'free hand' without the use of a design software package and this set covers the NF-kB like element, AP-1 and SP1 TFBS.[36,37]

To ensure primers would specifically amplify bisulfite-converted DNA, one or more non-CpG C's were included near the 3' end.[38] The product length was maintained between 50-200 bp to ensure efficient PCR amplification and fluorescence.[15,39,40] The T_m of the forward and reverse primers were matched within 1°C. Primers were evaluated using BiSearch to check for non-specific products.[41] OligoAnalyzer™ (IDT, USA) and OligoEvaluator™ (Sigma-Aldrich, USA) were used to determine the T_m of the primers and were also used to check for secondary structures and primer dimers.

Two sets of probes were designed which could be used interchangeably with the METH-GSTP1 and MOD-GSTP1 primers. The probes specific to methylated DNA (M) was labelled with a FAM fluorescent reporter dye at the 5'end and the probes designed to bind to unmethylated (U) CpG's was labelled with HEX. Although the Applied Biosystems™ dPCR instrument is only compatible with FAM and VIC, HEX can be used as an alternative since it has an emission estimate of 535 which is comparable to that of VIC which is 538.[42] From here onwards, HEX will be referred to as VIC. Both probes had Iowa Black® FQ quenchers attached to the 3' end and internal ZEN quenchers which helps provide greater overall dye quenching, lowers background, and increases signal detection.

The probes were designed to have a Tm at least 5°C above that of the primers although 8-10°C higher was preferable. Probes did not have a G at the 5' as to ensure proper fluorescence.[40] The probes were designed to cover several non-CpG C's to ensure specificity for bisulfite-converted DNA and had a G/C content between 30–80%.[40] Once again, OligoAnalyzer™ (IDT, USA) and OligoEvaluator™ (Sigma-Aldrich, USA) were used to determine the T_m of the probes and were used to check for secondary structures.

Table 3.3. Oligonucleotide sequences

Primer/probe	Sequence (5'-3')	Tm (°C)	Product (bp)
METH-GSTP1_Forward	GGGNGGGATTATTTTTATAAGG	49.9	158
METH-GSTP1_ Reverse	TACTAAAAACTCTAAACCCCATCC	52.3	
MOD-GSTP1_Forward	GNGTNGTGATTTAGTATTGGGG	53.2	127
MOD-GSTP1_Reverse	CATACTAAAAACTCTAAACCCCATCC	53.6	
OWN-GSTP1_forward	GAGTTNGNGGGATTTTTTAGAAG	51.8	90
OWN-GSTP1_reverse	CCTCNGAACCTTATAAAAATAATCC	50.7	
Probe 1M	/56-FAM/ACTACGAYG/ZEN/ACGAAACTCCAACG/3IABkFQ/	68.1	
Probe 1U	/5HEX/AAAACTACA/ZEN/AYAACAAAACTCCAACAAAAACCT/3IABkFQ/	67.2	
Probe 2M	/56FAM/ACGAAAACC/ZEN/TCGCGACCTCCG/3IABkFQ/	73.3	
Probe 2U[1]	/5HEX/AACTCCAAC/ZEN/AAAAACCTCACAACCTCCA /3IABkFQ/	72.2	

[1] M = Methylated probe, U = Unmethylated probe

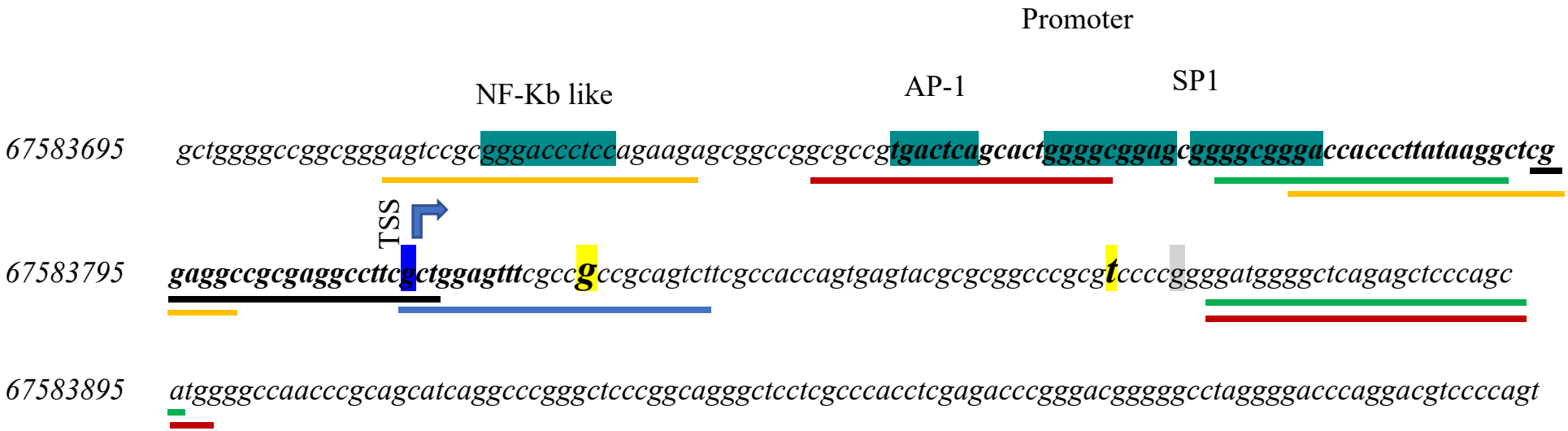


Fig 3.2. A map to show where the primers lie relative to the *GSTP1* CGI. Forward and reverse primers of the OWN-GSTP1 primer set are underlined in yellow. METH-GSTP1 primers are underlined in green and MOD-GSTP1 primers are underlined in red. Probe 2M/U is underlined in black and Probe 1M/U is underlined in blue.

3.3.4. Optimisation of the METH-GSTP1 primer set

A gradient PCR, with annealing temperatures ranging from 52°C to 58°C, (PCR conditions further outlined in Materials and Methods) was used to determine the optimal annealing temperature that produced the best results i.e., acceptable cycle threshold (Ct) value and devoid of primer dimers. At 58°C, amplification crossed the Ct at 31 cycles but the melt curve was still broad and irregular in shape, so the next step was to optimise the primer concentration. A final concentration of 0.3 µM, 0.5 µM and 0.8 µM was used. A well-defined melting curve with a narrow peak was observed when 0.8 µM of each primer was used at an annealing temperature of 58°C (Fig. 3.3A). Optimised METH-GSTP1 primers were tested on fully methylated, fully unmethylated and 50/50 mix of control DNA, demonstrating robust amplification of both methylated and unmethylated DNA (Fig. 3.3B).

A **B**

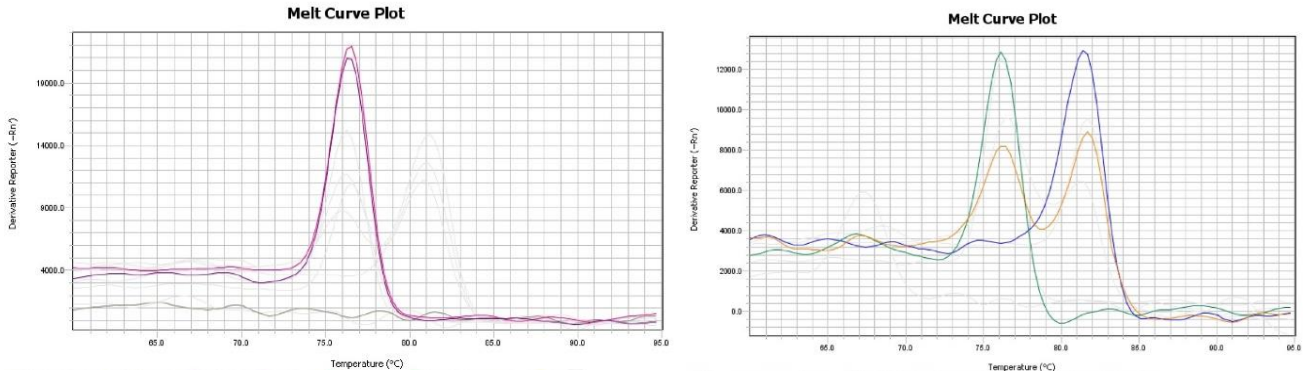


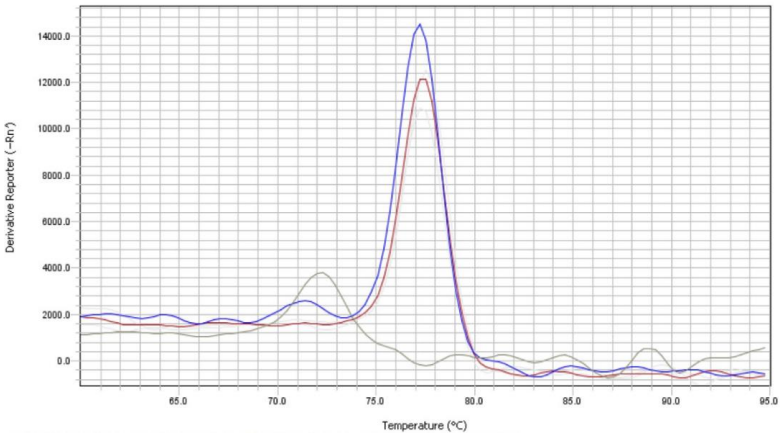
Fig 3.3. Melt curve of the METH-GSTP1 primer pair at 58°C using 0.8 µM of each primer. **A.** Pink and Purple peaks: sample replicates and Grey peak: NTC (non-template control). **B.** Green peak: Unmethylated control DNA, Blue peak: Methylated control DNA and Orange peak:50/50 control DNA.

3.3.5. Optimisation of the MOD-GSTP1 primer set

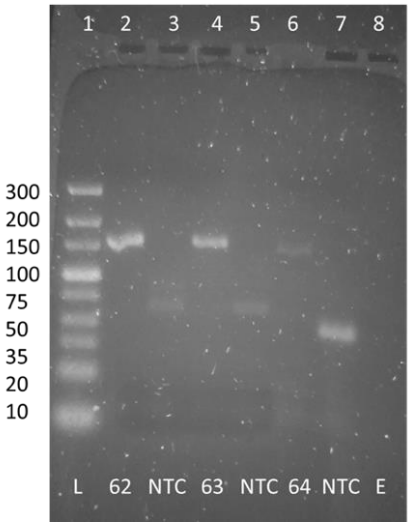
The annealing temperature and primer concentration were optimised using annealing temperatures ranging from 53°C to 66°C and primer concentrations of 0.3 µM, 0.5 µM and 0.8 µM. At 62°C using 0.5 µM of primer, a cycle threshold (Ct) of 30 was obtained (Fig. 3.4). Notably, a small peak was observed at around 72°C, just before the main melt peak (Fig. 3.4A). This same peak was observed in the NTC indicating a

primer dimer. Primer dimers have unique melting peaks that are different to the melting peaks of a desired template. Because primer dimers are smaller, around 30-80 bp they melt at lower temperatures meaning their melt peak will appear before the desired melt peak. They also usually produce a slightly broader peak. To confirm that it was indeed primer dimers, gel electrophoresis was performed (Fig. 3.4B). Five microliters of the amplified products were electrophoresed on a 3% agarose gel at 100 V for 1 hour, visualized with SYBR green (Invitrogen™, USA) gel stain under a UV light and photographed. In the lanes where DNA was present, a single band of 150 bp was observed. A faint band was observed for the reaction that ran at 64°C, which confirms reduced amplification efficiency seen in the melt curves of PCR's that ran at temperatures higher than 62°C. The NTC wells produced bands around 50 bp, which is indicative of primer dimers. MOD-GSTP1 primers were further tested on methylated, unmethylated, and 50/50 control DNA, and while amplification was observed, there was a bias towards amplification of unmethylated DNA (Fig 3.4C). Once again, a single peak was produced in the NTC representing a primer dimer.

A



B



C

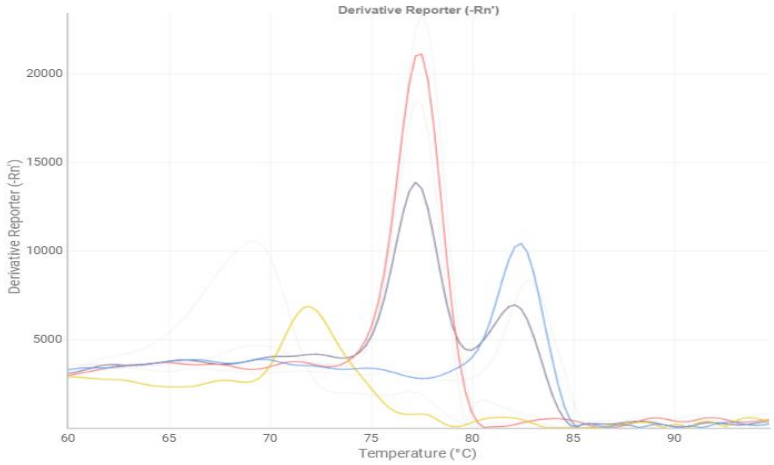


Fig 3.4. MOD-GSTP1 primer pair optimisation using 0.5 µM of each primer. A. Melt curve of the MOD primer pair at 62°C. Blue and Red peaks: sample replicates and Grey peak: NTC (non-template control). **B.** PCR optimisation of MOD primers at varying temperatures. 3% Agarose gel. Lane 1: Ultra low range DNA ladder. Lane 2: 62°C. Lane 3: NTC run at 62°C. Lane 4: 63°C. Lane 5: NTC run at 63°C. Lane 6: 64°C. Lane 7: NTC run at 64°C. Lane 8: Empty. **C.** Melt curve of the MOD primer pair at 62°C. Blue peak: Methylated control DNA, Pink peak: Unmethylated control DNA, Purple peak:50/50 control DNA, Yellow peak: NTC

3.3.6. Optimisation of the OWN-GSTP1 primer set

Annealing temperatures ranging from 52°C to 64°C were used for this primer pair along with varying primer concentrations (0.3 µM – 0.8 µM). At all tested temperatures and primer concentrations, no amplification was observed (Fig. 3.5). No bioinformatics tool was used in designing these primers, instead, the processes outlined in section 3.3.3 were used to create the OWN-GSTP1 primer set. OligoAnalyzer™ (IDT, USA) revealed a heterodimer between the forward and reverse primers with a delta G of -15.36 kcal/mole. Delta G refers to the amount of energy needed to break a secondary structure. A delta G smaller than -9 kcal/mole is considered to be problematic which could explain why this primer pair failed to amplify the desired PCR product. Although these primers may have benefitted from possible redesign and further optimisations, the OWN-GSTP1 primer set was rather excluded from further experiments due to time constraints.

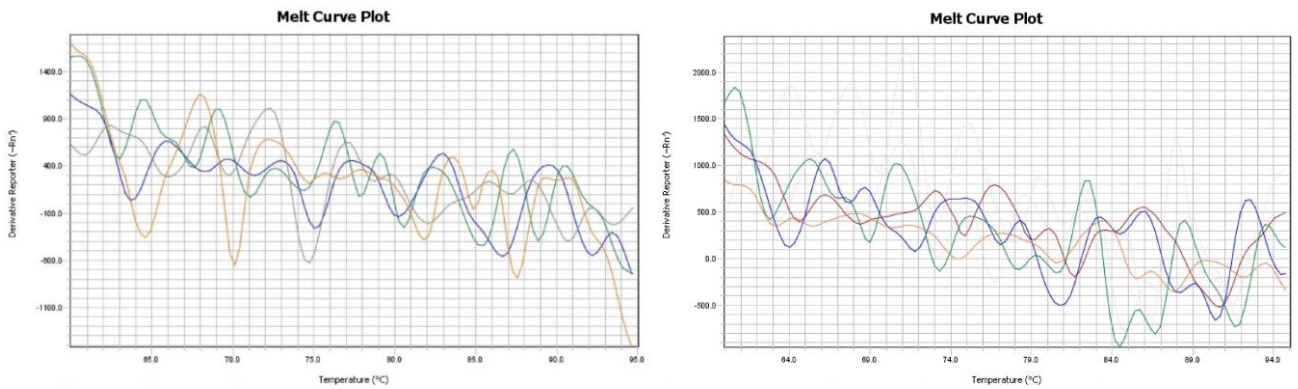


Fig 3.5. Melt curve of the OWN-GSTP1 primer pair using 0.3 µM of each primer at varying temperatures. A. Orange peak: 58°C, Green peak: 55°C, Blue peak: 52°C and Grey peak: NTC (non-template control). **B.** Red peak: 60˚C, Blue peak: 62˚C, Green peak: 64˚C, Orange peak: NTC

3.3.7. Primer selection and cautionary observations

While the METH-GSTP1 primer set showed optimal amplification, the MOD-GSTP1 primer pair did not amplify methylated and unmethylated DNA equally, with a bias towards unmethylated DNA. PCR bias is a concept that was first explained by Warnecke *et al.*, 1997.[43] After bisulfite conversion, methylated DNA sequences will have a higher GC content compared to unmethylated sequences. This may cause the melting temperature to increase and the likelihood of secondary structures to become greater which reduces PCR efficiency.[43] Thus in a mixture of methylated and unmethylated DNA, the unmethylated DNA will have greater amplification after PCR.

When using unbiased primers in real-time quantitative PCR, it is important that methylated and unmethylated DNA are proportionally amplified so that methylation can be accurately measured.[38] Some recommendations have been made to ensure equal amplification; for example, Stoffel fragment, a DNA polymerase that amplifies different DNA sequences simultaneously, helped with PCR bias in some circumstances but Warnecke *et al*., 1997 explains that it is not a universal solution.[43] Tomasz *et al.*,2008 explains that they were able to avoid bias by including a limited number of CpG's in their primers and optimising annealing temperatures.[38,44]

Importantly, in this dissertation using dPCR, while a lack of PCR bias is preferable, it is not critical for a robust dPCR assay. This is due to the fact that in dPCR, individual DNA molecules are partitioned into different wells and each well will amplify individually. Compartmentalisation eliminates competition, therefore if methylated molecules do not amplify as well as unmethylated molecules that will not matter because the probe will still detect the sequence and determine whether it is methylated or unmethylated.[45] The fact that my primers amplified both methylated and unmethylated DNA is the most important thing.

The METH-GSTP1 and MOD-GSTP1 primers were also tested on non-bisulfite treated DNA, to confirm specificity for converted DNA. Both primers failed to amplify non-bisulfite treated DNA, proving that they specifically only amplify bisulfite converted DNA.

3.3.8. Optimisation of Probe 1M/U and Probe 2M/U with the METH-GSTP1 primer pair

Genotyping reactions were performed in triplicate using either probes 1M/U or probes 2M/U. Reactions consisted of 0.8 µM of METH-GSTP1 forward and reverse primers, and 0.1 µM - 0.3 µM of each probe. Better results were observed among samples that had higher probe concentrations. At 0.2 µM, both probe 1M/U and probe 2M/U failed to amplify a single sample but when 0.3 µM of each probe was used, all samples were correctly called as methylated or unmethylated (Fig 3.6). Probe 2M/U exhibited better clustering of replicate samples compared to probe 1M/U and fluorescence was also higher, as indicated by the axes (Fig 3.6B and D). Furthermore, reactions containing probe 2M/U had greater efficiency, amplifying the sample around 2-5 cycles earlier than reactions containing probe 1M/U.

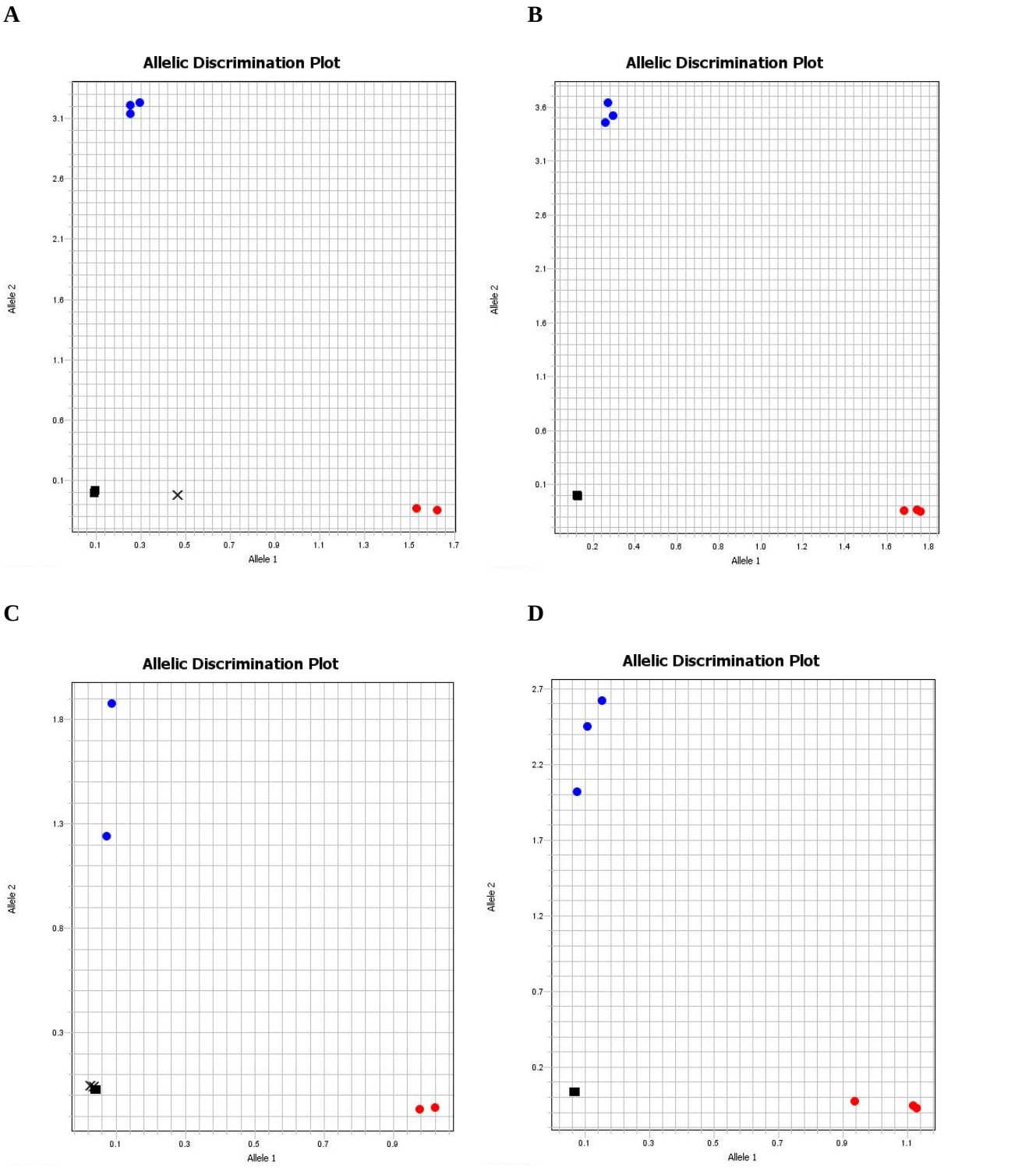


Fig 3.6. Genotyping reactions using different concentrations of probe. A. 0.2 µM of probe 2M/U. **B.** 0.3 µM of probe 2M/U. **C.** 0.2 µM of probe 1M/U. **D.** 0.3 µM of probe 1M/U**.** Allele 2 on the Y axis of the allelic discrimination plot represents FAM and allele 1 on the X axis represents VIC. Blue dots: samples positive for FAM fluorescence, Red dots: samples positive for VIC fluorescence, Black squares: NTC, Crosses: samples undetermined at a quality value of 90%.

3.3.9. Optimisation of Probe 1M/U with the MOD-GSTP1 primer pair

Triplicate reactions consisting of 0.5 µM of each primer (MOD-GSTP1) and 0.1 µM – 0.3 µM of Probe 1M/U were performed for methylated and unmethylated control DNA respectively. No amplification was observed in reactions containing 0.1 µM and 0.3 µM of probe. The samples that were successfully genotyped, using 0.2 µM of probe (Fig 3.7), were not tightly clustered together showing inconsistency among replicate samples; the fluorescence was low, and the PCR efficiency was poor (Ct = ~36).

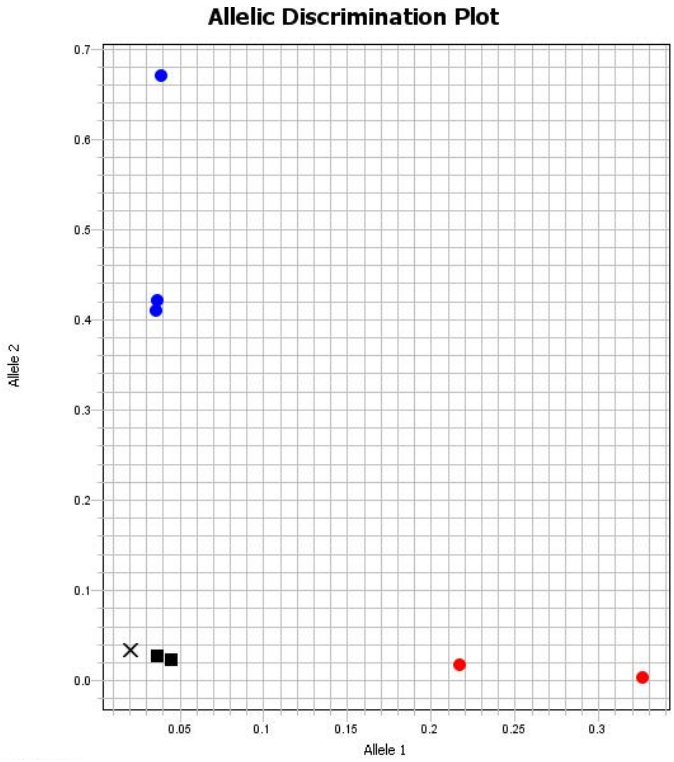


Fig 3.7. **Genotyping reactions using 0.2 µM of probe 1M/U.** Allele 2 on the Y axis of the allelic discrimination plot represents FAM and allele 1 on the X axis represents VIC. Blue dots: samples positive for FAM fluorescence, Red dots: samples positive for VIC fluorescence, Black squares: NTC, Crosses: samples undetermined at a quality value of 90%.

3.3.10. Probe selection and cautionary observations

The METH-GSTP1 primer pair is the preferred primer set because it produces a smaller product compared to MOD-GSTP1 and results in a more robust PCR. The METH-GSTP1 primer pair showed better clustering, fluorescence and amplification efficiency when used in combination with Probe 2M/U, rather than probe 1M/U (Fig 3.6B and Fig 3.6D). Furthermore, probe 1M/U covers an African variant (Table 3.3) which is not ideal. Although I tried to account for this by adding Y (C/T) in the probe, I later realized that if the "a" variant is present, the cytosine will no longer be present in the CpG context therefore it will become a thymine so I should have included "YR" to account for this. Probe binding and/or PCR efficiency may therefore be affected if this variant is present. For these reasons, dPCR experiments were performed with 0.8 µM of the METH-GSTP1 primer set and 0.3 µM of probe 2M/U.

3.3.11. Optimisation of the designed assay in digital PCR

Initial dPCR reactions were performed at 60°C for 39 cycles (cycle conditions advised by the Thermo Fisher Scientific dPCR user guide) using a wide range of DNA concentrations (0.25 ng/µl – 13.9 ng/µl) (standard DNA samples) to determine the optimal amount of input DNA. The dPCR user guide suggests using 2.62 ng/µl - 6.94 ng/µl of genomic DNA which would allow for each individual partition to contain 0.6 - 1.59 copies of the target sequence. Because bisulfite converted DNA is single stranded, and only the forward strand is targeted, double the amount of DNA would be needed, hence a range of 5.24 - 13.9 µl of DNA was used. Since other studies[11] have used as little as 0.195×10^{-3} pg/µl of DNA, a diluted DNA sample of 0.25 ng/µl was also attempted. Very little to no amplification was observed at lower DNA concentrations therefore 13.9 ng/µl of DNA (final concentration) was used for further optimisations.

The dPCR optimisation guidelines recommend adding an additional 5-10 cycles when amplification is poor. Therefore, the number of cycles was increased to 45 cycles (Fig 3.8A) and then again to 50 cycles (Fig 3.8B) which resulted in better amplification. The annealing temperature was increased to 62°C which further improved the amplification (Fig 3.8C); however, this was at the expense of clear separation of positive and negative droplets. Annealing temperatures of 58°C, 61°C, and 63°C were also used which resulted in poor amplification. Since many wells were still failing to amplify, an extension step of 72°C for 30 s was included (Fig 3.8D) but this did not improve amplification.

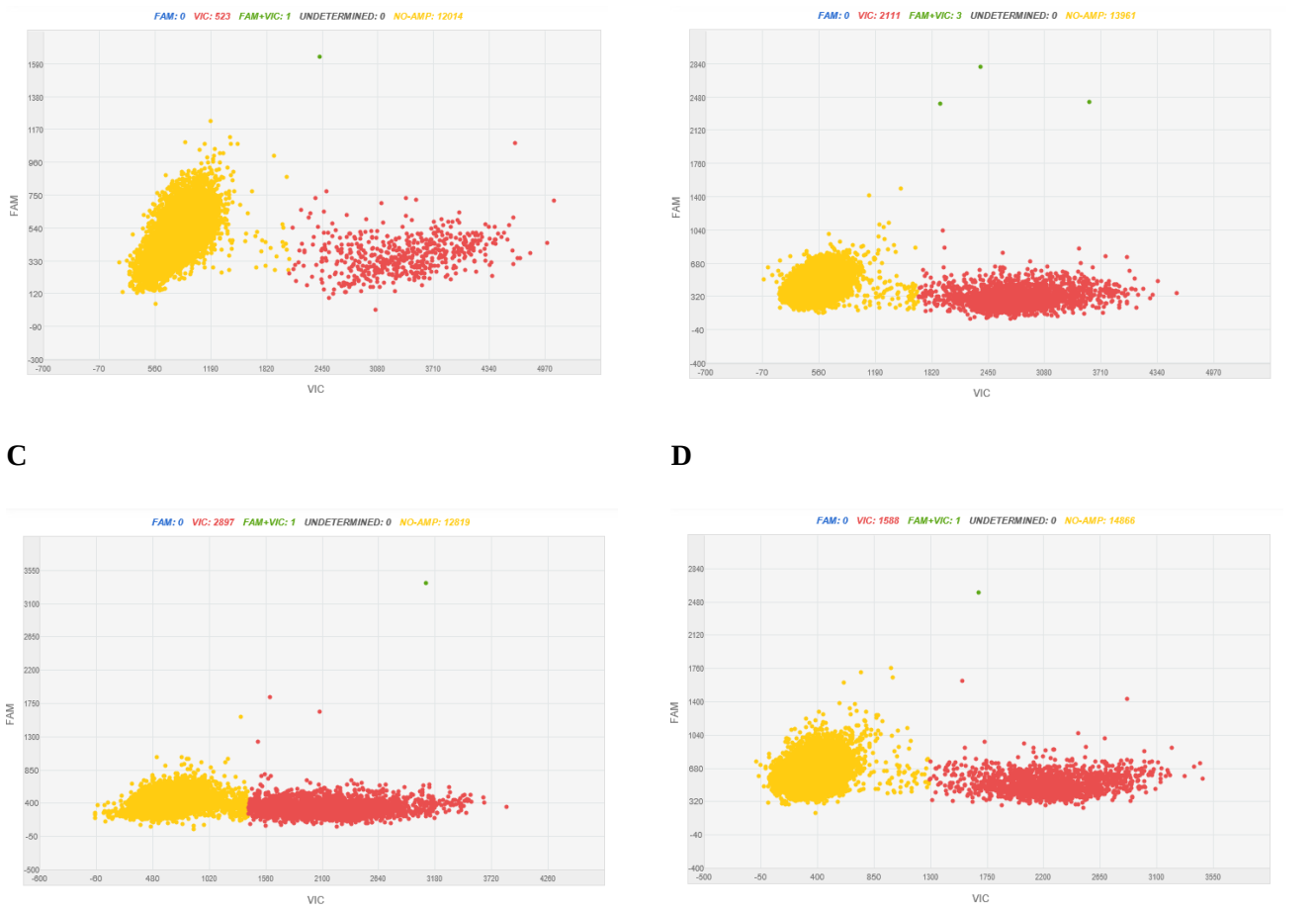
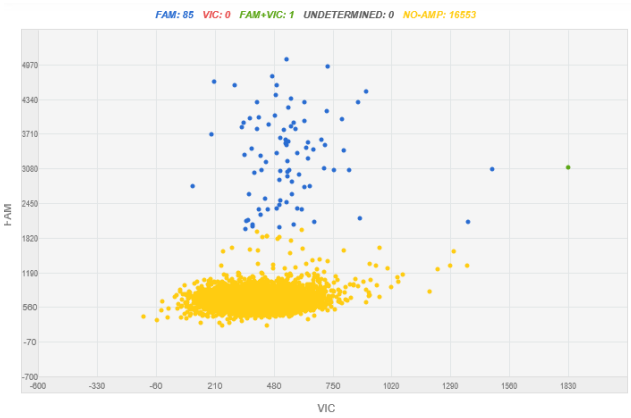


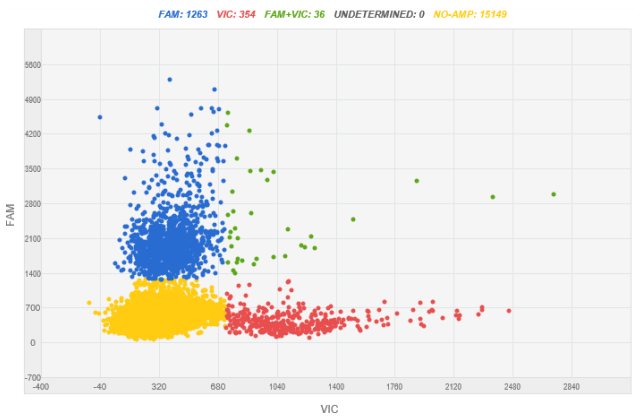
Fig 3.8: Digital PCR optimisations using 13.9 ng/µl of unmethylated control DNA A. 60°C for 45 cycles **B.** 60°C for 50 cycles **C.** 62°C for 50 cycles **D.** 62°C for 50 cycles with an added extension step. Red dots: wells with VIC fluorescence. Green dots: wells with FAM and VIC fluorescence. Yellow dots: wells with no amplification.

Using the conditions that produced the best amplification (62°C for 50 cycles), the assay was tested on control samples with methylation percentages of 100%, 75%, 50%, 25%, and 0% and it was able to predict the methylation percentage within 2.4% (Fig 3.9).

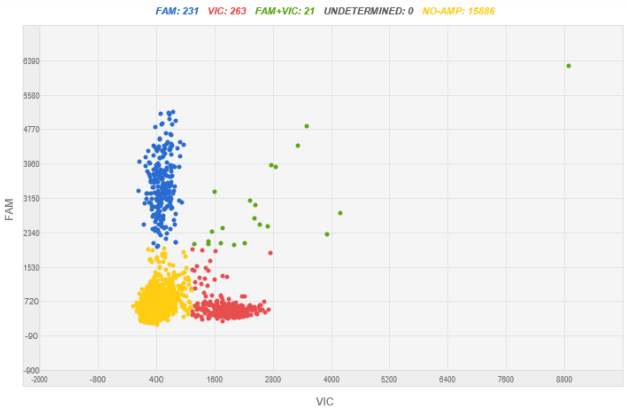
A 98.854%



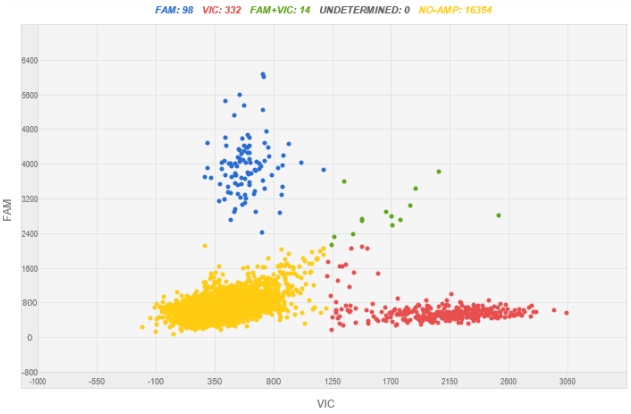
B 77.418%



C 46.99%



D 24.318%



E 0.087%

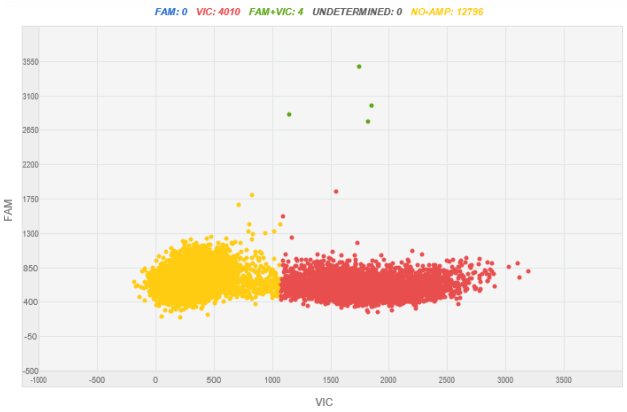


Fig 3.9. **Digital PCR using different percentages of methylated DNA. A.** 100% methylated DNA, **B.** 75% methylated DNA, **C.** 50% methylated DNA, **D.** 25% methylated DNA, **E.** 0% methylated DNA. Blue dots: wells with FAM fluorescence, Red dots: wells with VIC fluorescence, Green dots: wells with FAM and VIC fluorescence and yellow dots: wells with no amplification. The measured methylation percentages are also shown above each figure.

Assay performance was evaluated on two PCa patient samples (UP2099 and UP2004) and two BPH samples (UP2140 and UP2324) (further explained in **Chapter 4**) to determine whether it could be translated from control DNA to clinical samples (Fig 3.10). Amplification was observed in both the PCa cases and BPH samples with similar efficiency as the control DNA (Fig 3.10). Furthermore, BPH samples exhibited mostly VIC fluorescence (unmethylated) while cancer samples had a mixture of FAM and VIC fluorescence in agreement with previous studies that showed *GSTP1* to be methylated in PCa but not in BPH.[2]

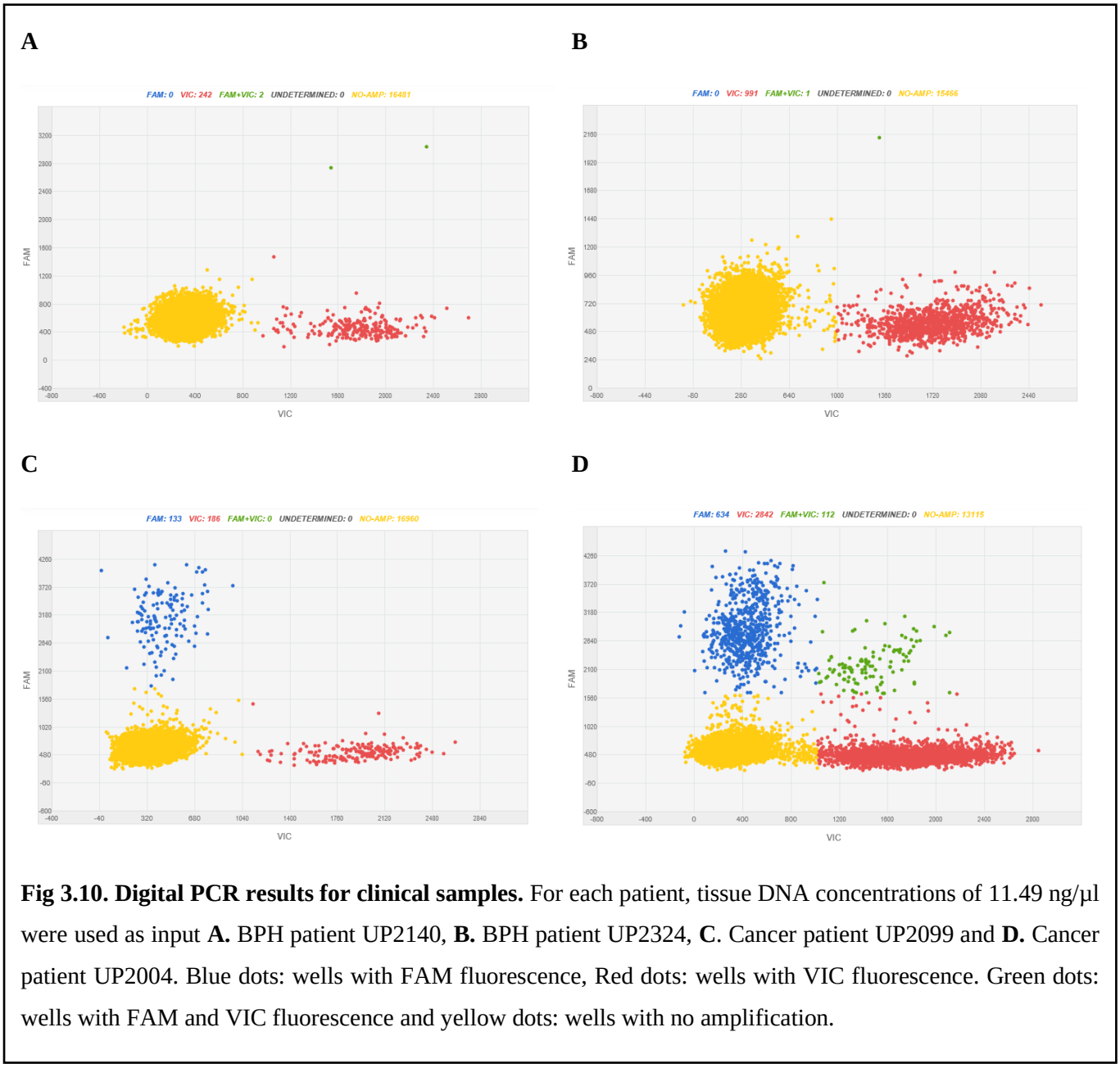


Fig 3.10. Digital PCR results for clinical samples. For each patient, tissue DNA concentrations of 11.49 ng/µl were used as input **A.** BPH patient UP2140, **B.** BPH patient UP2324, **C**. Cancer patient UP2099 and **D.** Cancer patient UP2004. Blue dots: wells with FAM fluorescence, Red dots: wells with VIC fluorescence. Green dots: wells with FAM and VIC fluorescence and yellow dots: wells with no amplification.

3.4. Discussion

Previous research has focused on developing *GSTP1* methylation assays for European populations, with limited biomarkers[9,46,47]validated in Africans. In this chapter, I showed that the primers and probes of some published assays overlapped with African SNPs. While this is not ideal, these are not common variants and a single mismatch closer to the 5'end is less likely to affect annealing.[48] Still, these assays should be tested in Africans because if the binding of primers and probes is affected, this may result in failure or compromise the efficiency of the assay and render it unsuitable for diagnosis of aggressive PCa in Africans. Here, an African relevant assay, that accounts for African-specific SNPs was developed, with the final standard operating procedure (SOP) illustrated in Fig 3.11, and was able to successfully amplify clinical samples.

This chapter has contributed to a clearer understanding of African relevant variants that should be considered when designing *GSTP1* differential methylation diagnostic/prognostic assays. This is also the first dPCR assay to be developed specifically for PCa screening in Black South Africans. However, it should also be applicable to other African populations because it does not overlap with any common African SNP. The assay developed here will potentially inform future studies and is a stepping stone in the pursuit of a diagnostic test for routine screening in high-risk populations.

While majority of cancer studies make use of the BioRad droplet digital PCR (ddPCR) instrument[10,49–51] (further discussed in **Chapter 2**), we chose to use the QuantStudio chip-based dPCR platform from Thermo Fisher Scientific because it was cheaper. However, the ddPCR may allow for simpler, faster analysis of methylation. The main limitation of our assay currently is the amount of DNA required. A previous study[11] was able to detect methylated copies with as little as 0.195 x 10^{-3} pg/µl of DNA using the same dPCR platform (QuantStudio), whereas this assay required an extremely large amount (11.49 ng/µl) of DNA to achieve interpretable results. This is a significant issue because in a clinical setting, DNA is often limited. Since rigorous optimisations were performed, it is suspected that the issue lies within the assay design itself and further refinement of primer and probe design are warranted. Another limitation is that the clinical samples were not quantified prior to dPCR analysis (quantified in Australia before being shipped to South Africa, see **Chapter 4**), therefore the starting concentration could only be assumed.

Although this dPCR assay does not amplify as efficiently as I had hoped, reflected by the amount of DNA needed and the large number of unamplified wells (acceptable range 20-80%),[52] and therefore requires further optimisation, importantly it was still able to amplify the desired PCR product and probes differentiated between methylated and unmethylated copies in clinical samples. In the next chapter, this assay was applied to a larger cohort to determine whether *GSTP1* is indeed a valid biomarker for the purpose of PCa screening in Black South Africans.

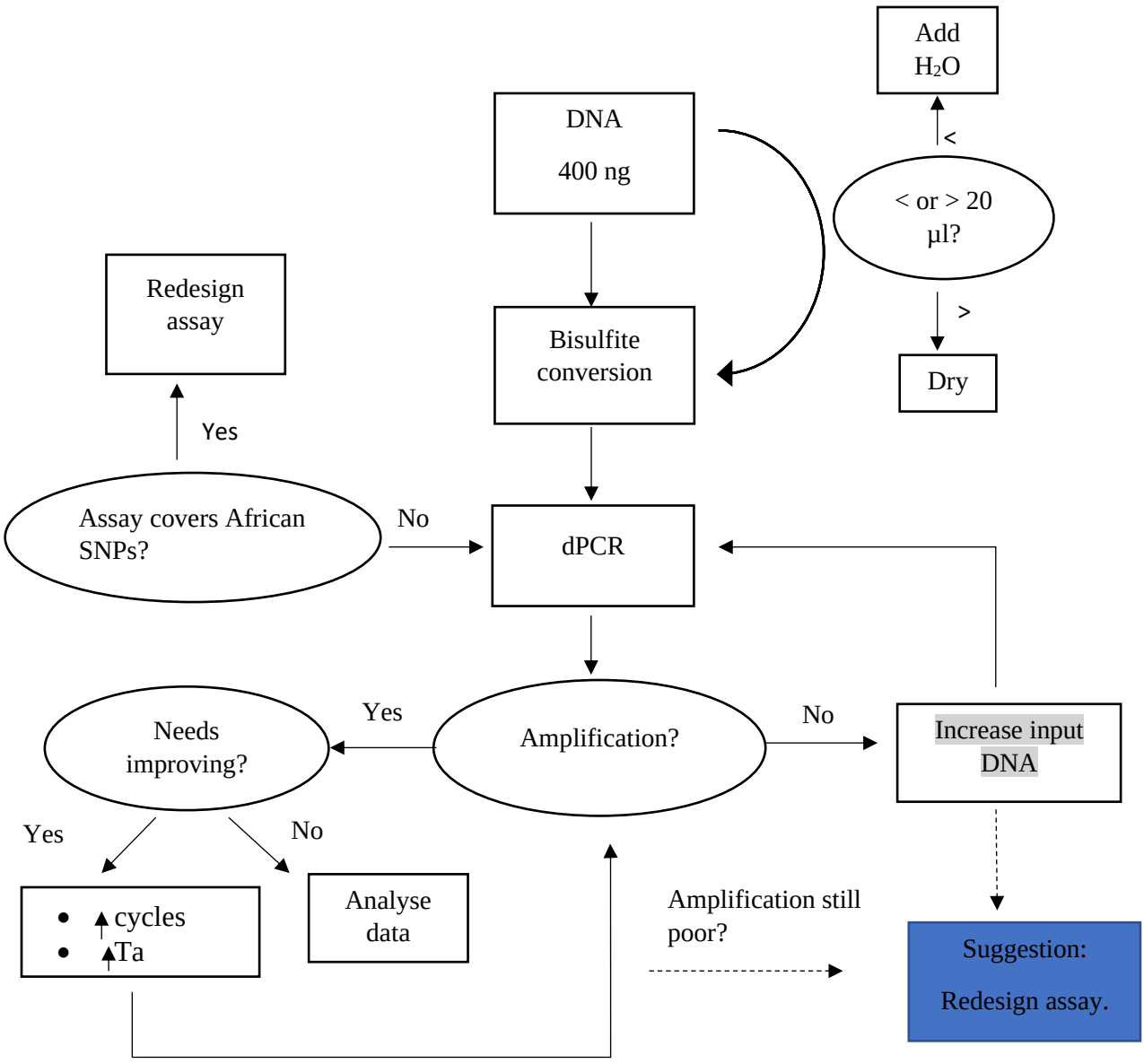


Fig 3.11. SOP for designing an African relevant assay. If suggested optimisation steps fail, then the assay may need to be redesigned. Inefficiencies of my assay are highlighted in grey.

3.5. References

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Chapter 4: Evaluating the suitability of *GSTP1* as a biomarker for African prostate cancer

In **Chapter 3**, an African relevant epigenetic assay was designed to target differential methylation in *GSTP1*. In this chapter, the optimised assay will further be tested on a larger cohort of 100 South African patients, either with PCa (n = 66) or commonly occurring, non-cancerous BPH (n = 34), with the aim of determining whether *GSTP1* truly is an informative biomarker in Black South Africans. Furthermore, this chapter will highlight the methylation differences between South African BPH and PCa patients, while evaluating the diagnostic capabilities of the newly designed, African appropriate assay. The results from this chapter will help inform future studies on the utility of *GSTP1* in African PCa and whether or not to include this biomarker in screening assays.

In this chapter, I would like to thank the patients who donated their tissue samples to the SAPCS, as well as all the urologists and clinical staff, led by Prof. Riana Bornman (University of Pretoria), who contributed to this valuable resource. I would also like to thank Ruth Lyons (Garvan Institute of Medical Research) for extracting the DNA and Dr Sean Patrick (University of Pretoria) for organising import and export permits, ensuring precious DNA samples made it safely to South Africa. Once again, I would like to acknowledge Prof Liza Bornman (University of Pretoria and Ampath) for providing the necessary equipment and lab supplies needed to perform my experiments.

4.1. Introduction

Prostate cancer (PCa) is ranked as the number one cancer affecting men in South Africa with 13,152 incident cases in 2020, according to the Global Cancer Observatory.[1] Prostate specific antigen (PSA) is the golden standard marker for regular PCa screening; however, some controversy surrounds this biomarker.[2,3] PSA levels can be elevated by non-cancerous conditions including, infections, trauma, and age related benign prostatic hyperplasia (BPH), which makes it is prone to false positives and this can end up doing more harm than good.[3–5] This has prompted investigations into alternate markers, including epigenetic biomarkers.

While *GSTP1* has performed exceptionally as an epigenetic biomarker for PCa detection in Europeans,[6] the question remains as to whether this biomarker is applicable to Africans. Africa is extremely diverse and while studies have started to realise this genetic diversity,[7–10] the epigenetic landscape and its clinical implications remain largely unknown. In Europeans, it is clear that *GSTP1* is methylated in PCa and unmethylated in BPH[11] but to date, no study has shown this distinct methylation pattern in Black South Africans. Multiple genes have different methylation profiles in EA *vs* AA men with PCa[12–15] (further discussed in **Chapter 2**). Therefore, it is plausible that *GSTP1* could show different methylation in Black South Africans, rendering it uninformative for discrimination between BPH and PCa in the South African population.

While the previous chapter, highlighted African relevant variants that need to be acknowledged when designing an epigenetic *GSTP1* diagnostic/prognostic assay, here the focus will be shifted towards the epigenetic profile of South Africans. This chapter aims to ascertain whether methylation differences exist between PCa and BPH patients. Furthermore, the African relevant assay (designed in **Chapter 3**) will be evaluated for its diagnostic capabilities. By determining whether *GSTP1* truly is an informative biomarker, we can inform future studies on whether or not to target this gene for PCa screening in Black South Africans.

4.2. Materials and Methods

4.2.1. Clinical sample selection and ethics

The samples used in this dissertation were obtained from the established Southern African Prostate Cancer Study (SAPCS)[16] cohort (#43/2010). The SAPCS[16] was initiated in 2008, with the purpose of studying PCa in Black South Africans.[2,16] To date, approximately 3,000 South African men have been recruited from local clinics and they have been classified as case or control by PSA testing, urological examination and tissue biopsies.[16] Histopathologic Gleason score ≥7 cancer tissue (n = 66), made up the case samples and BPH tissue (n = 34) was used as controls (Fig 4.1). This study was approved by the University of Pretoria Human Research Ethics Committee (HREC #22/2021).

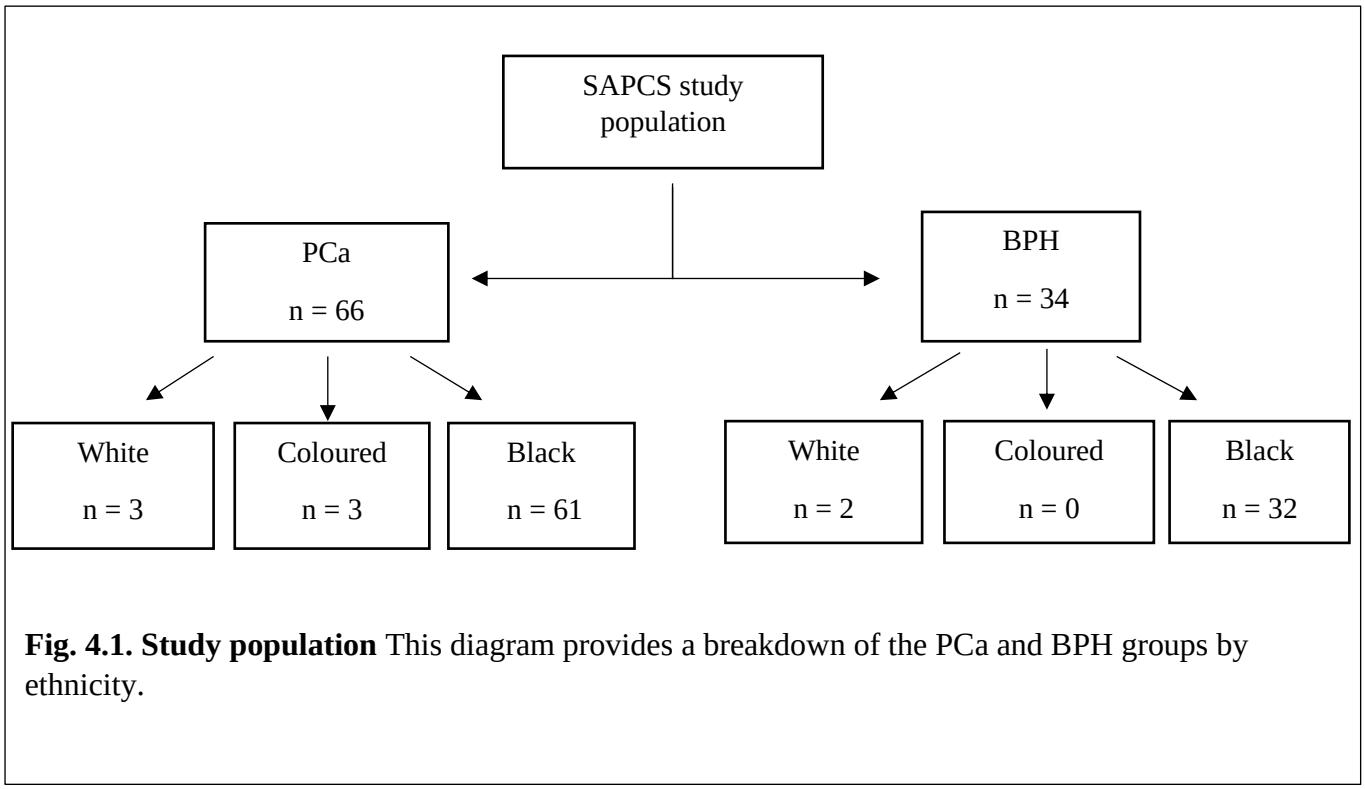

Fig. 4.1. Study population This diagram provides a breakdown of the PCa and BPH groups by ethnicity.

4.2.2. DNA isolation

Somatic DNA was extracted from case and control, fresh-frozen prostate tissue samples by collaborators at the Garvan Institute of Medical Research in Australia using the Qiagen DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA was quantified using a QuBit dsDNA high sensitivity assay (Invitrogen, USA) on a QuBit 2.0 Fluorometer (Invitrogen, USA) according to the manufacturer's instructions. The extracted DNA (400 ng) was shipped to South Africa in accordance with the National Health Act, 2003 (Act No. 61 of 2003), for methylation analysis.

4.2.3. Bisulfite conversion

DNA was bisulfite converted using the EZ DNA Methylation Lightning kit (ZymoResearch, USA), as mentioned in **Chapter 3**. Clinical samples with a volume less than 20 µl were compensated with molecular grade water. Those with a volume greater than 20 µl were vacuum dried at room temperature using the Thermo Scientific™ Savant™ SpeedVac™ DNA130 vacuum concentrator (Thermo Fisher Scientific, USA).

4.2.4. Digital PCR

Digital PCR was performed on 66 PCa samples and 34 BPH samples, according to the protocol mentioned in **Chapter 3**.

4.2.5. Statistical analysis

The Mann-Whitney *U* test was used for comparisons between two groups. Spearman non-parametric test was performed to assess correlations between methylation levels and patients' age or PSA. Multiple logistic regression was performed to identify predictors of PCa risk. A Receiver Operating Characteristic (ROC) curve was generated for *GSTP1* and the area under the curve (AUC) was calculated to estimate the diagnostic performance of this biomarker in Africans. A cut off value was selected using Youden's *J* index (sensitivity + specificity -1) which maximises sensitivity and specificity.[17] Statistical analysis was performed using SPSS v.28.0 (SPSS, Chicago, IL) and P-values < 0.05 were considered statistically significant.

4.3. Results

4.3.1. Digital PCR

Digital PCR provided the concentration (copies/ul) for FAM and VIC as well as the % target/total which is the FAM concentration divided by the total concentration multiplied by 100. The number of positive wells (wells with FAM, VIC, FAM+VIC fluorescence) and negative wells (wells with no amplification) was also observed. Thirty-two samples were excluded from the analysis after dPCR because they had less than 25 positive wells (Fig 4.2A and B). Although all samples were quantified prior to shipment, it is possible that the DNA was degraded resulting in a lack of sufficient template in the dPCR reaction, causing sample dropout. Furthermore, it is possible that the samples may have contained PCR inhibitors which would have prevented amplification. An additional three samples were excluded because they showed more than 80% differences between replicate chips (Fig 4.2C and D). This could be attributed to a problem with the manufactured chip itself or to differences that occurred during loading of replicate chips. A third replicate would have provided a clearer result; however, DNA was limited.

Further analysis was done on the remaining 17 BPH samples and 48 PCa samples, with results presented in Table 4.1. A mixture of methylated and unmethylated copies was observed in the PCa patients, which was expected since biopsied tissue could be contaminated with surrounding normal tissue (Fig 4.2G and H). *GSTP1* was largely unmethylated in BPH patients (Fig 4.2 E-F).

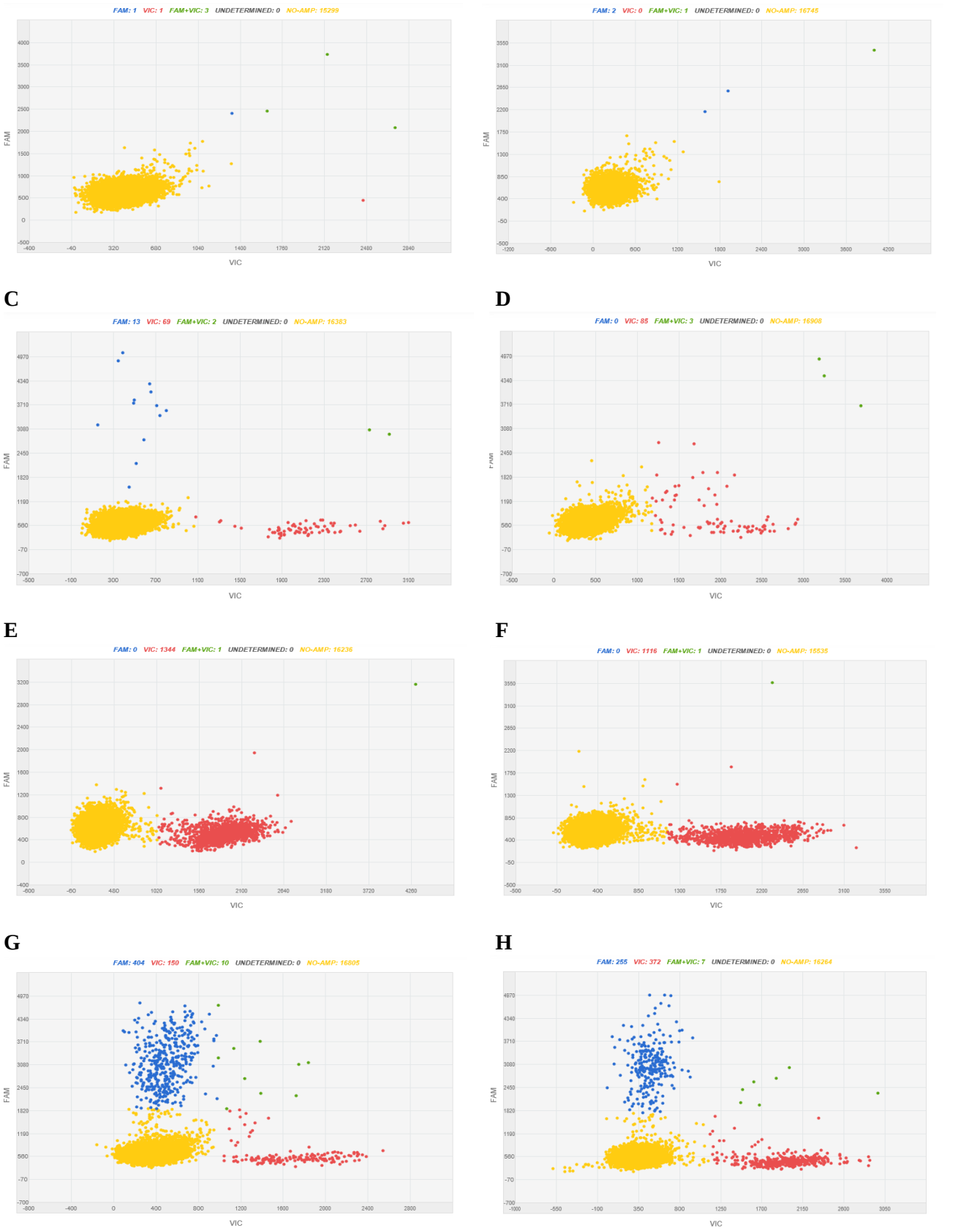

Fig 4.2. Digital PCR results. Chips with no amplification: **A.** BPH patient SMU092 and **B.** PCa patient UP2329. PCa patient UP2035 with >80% differences between replicate chips: **C.** and **D.** Examples of chips with good amplification: **E.** BPH patient UP2371, **F.** BPH patient UP2320, **G**. PCa patient N0015 and **H.** PCa patient UP2116. Blue dots: wells with FAM fluorescence, Red dots: wells with VIC fluorescence. Green dots: wells with FAM and VIC fluorescence and yellow dots: wells with no amplification.

There were some cases where *GSTP1* was unmethylated in a PCa patient and fewer cases where a BPH patient had methylated *GSTP1* copies (Fig 4.3). Methylated copies in the BPH samples (Fig 4.3C) could possibly indicate the presence of a tumour that was undetected by histopathological analysis of biopsied tissue but detected by this assay, due to field effect (further explained in **Chapter 2**). Because the SAPCS[16] does not have follow up data, it is not possible to validate this.

For the PCa samples that show only unmethylated *GSTP1* (Fig 4.3A and B), a possible explanation could be that these samples are heterogeneously methylated (further explained in **Chapter 2**). Since the probe was designed to bind to fully methylated and fully unmethylated alleles, a heterogeneously methylated locus could prevent the probe from annealing and this would lead to loss of FAM fluorescence in the cancer sample. One way to check this would be to perform bisulfite sequencing of the region to get single-nucleotide resolution of the methylation status of the region.

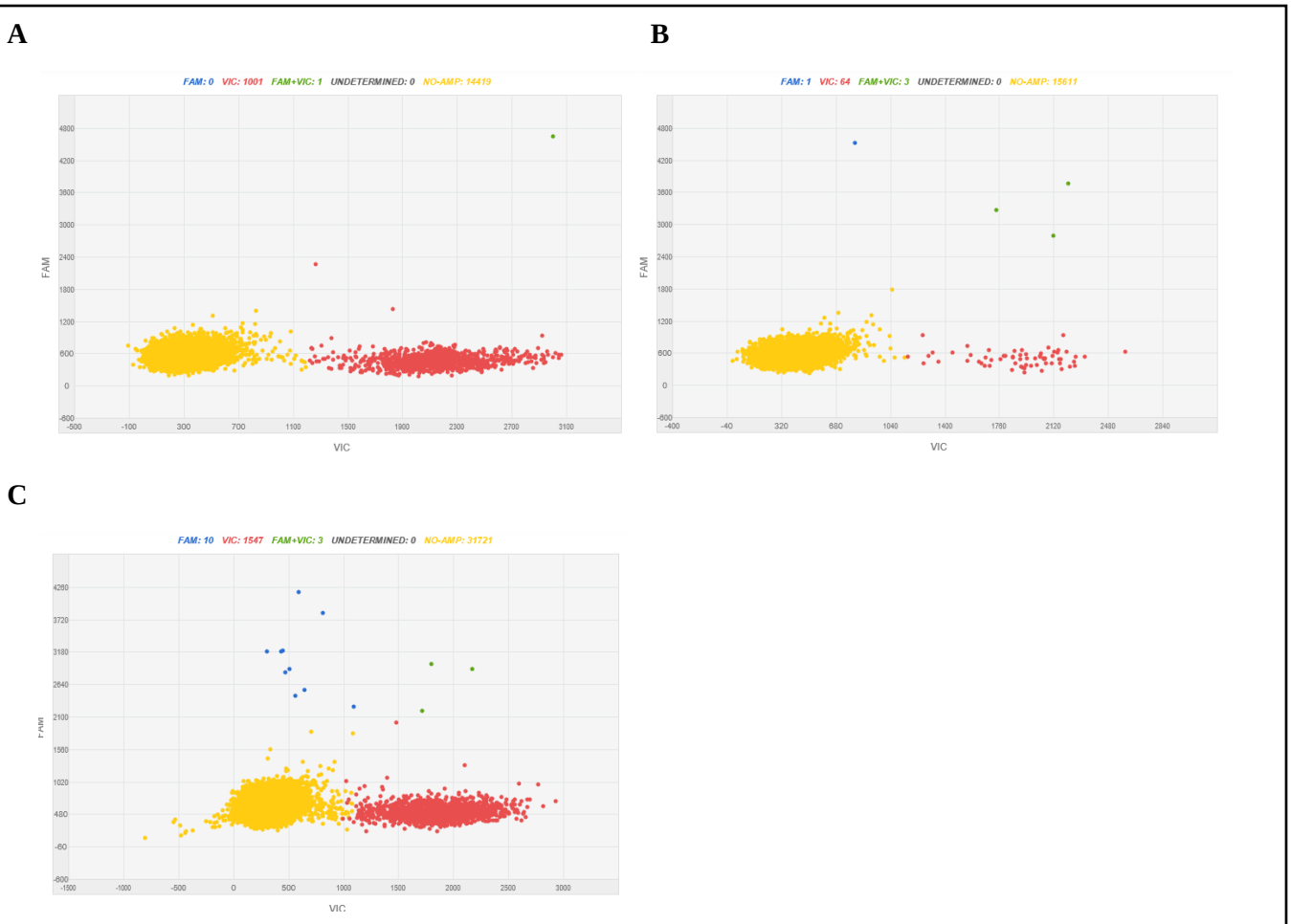

Fig 4.3. Cases with unexpected methylation results. A. PCa patient UP2034, **B.** PCa patient UP2092 and **C.** BPH patient UP2147. Blue dots: wells with FAM fluorescence, Red dots: wells with VIC fluorescence. Green dots: wells with FAM and VIC fluorescence and yellow dots: wells with no amplification.

Table 4.1. Characteristics of patients included in this dissertation and their corresponding digital PCR results.

Sample ID	Ethnicity	Disease	Age	PSA	Fam[2]	VIC[3]	Target/total[4]
SMU081	African	BPH	63	7,58	0.207	6.216	3.244
UP2019	African	BPH	76	6,50	0.457	3.265	6.966
UP2140	African	BPH	65	15,80	0.252	36.942	0.709
UP2147	African	BPH	55	7,50	0.75	111.05	0.651
UP2226	African	BPH	63	29,60	0.372	59.841	0.556
UP2234	African	BPH	57	23,40	0.316	188.48	0.168
UP2293	African	BPH	66	21,00	0.676	3.83	9.196
UP2335	African	BPH	64	32,40	0.0783	42.248	0.188
UP2366	African	BPH	66	22,00	0.231	47.746	0.385
UP2371	African	BPH	67	29,10	0.0758	96.238	0.079
UP2373	African	BPH	70	20,00	0.18	10.214	1.412
UP2359	White	BPH	59	1,80	0.411	47.57	0.832
UP2324	African	BPH	66	6,56	0.38	95.45	0.243
UP2320	African	BPH	67	16,70	0.0777	89.791	0.0865
UP2151	African	BPH	77	10.2	0.417	28.357	1.134
UP2290	African	BPH	59	19.2	0.649	10.004	6.797
UP2337	African	BPH	69	18	0.357	4.519	4.376
N0001	African	PCa	75	22,90	15.437	18.351	45.558
N0002	African	PCa	76	193,46	29.022	15.549	65.125
N0007	African	PCa	78	NA	5.018	23.546	17.569
N0010	African	PCa	NA	NA	4.624	167.54	2.688
N0015	African	PCa	69	NA	31.141	11.323	73.413
N0048	African	PCa	70	83,34	16.034	63.13	20.271
N0053	African	PCa	71	31,50	29.656	284.55	9.533
SMU068	African	PCa	71	64,00	3.712	2.064	41.533
SMU157	African	PCa	64	33,50	3.351	4.836	0.821
SMU161	African	PCa	66	NA	54.21	399.18	48.492
TSH005	African	PCa	56	100,00	3.845	55.967	45.537
UP2003	African	PCa	76	3459,00	15.678	4.582	51.687

[2] FAM copies/ul
[3] VIC copies/ul
[4] FAM concentration/total x 100

UP2004	African	PCa	70	100,00	55.84	14.656	16.938
UP2050	African	PCa	65	581,00	40.779	274.17	41.347
UP2089	African	PCa	55	183,00	16.363	57.846	84.587
UP2092	African	PCa	70	14,00	0.408	3.002	6.99
UP2099	African	PCa	76	100,00	10.686	5.268	40.79
UP2101	African	PCa	57	75,00	5.411	15.553	53.921
UP2109	African	PCa	63	10,00	0.595	4.586	2.905
UP2113	African	PCa	88	123,00	54.08	9.222	75.261
UP2116	African	PCa	99	65,00	20.338	17.458	37.85
UP2119	African	PCa	54	9,00	0.806	33.441	28.479
UP2133	African	PCa	58	100,00	1.376	1.9	19.91
UP2160	African	PCa	94	98,00	13.781	4.952	46.303
UP2212	African	PCa	67	120,00	12.442	16.063	24.957
UP2383	African	PCa	72	NA	25.244	37.686	36.438
UP2207	African	PCa	88	85,80	81.515	43.162	60.009
UP2355	African	PCa	75	18,00	0.126	52.495	0.679
UP2052	African	PCa	64	9,90	34.259	16.654	25.199
UP2221	African	PCa	65	975,00	16.499	102.5	68.239
UP2172	African	PCa	64	23,50	0.482	7.744	2.538
UP2230	Coloured	PCa	68	1043,00	0.332	18.576	0.359
UP2192	African	PCa	63	12,70	3.353	60.171	6.432
UP2034	African	PCa	74	56,00	0.182	48.582	0.149
UP2261	White	PCa	70	212,40	3.054	104.39	19.275
KAL0021	African	PCa	NA	NA	54.805	12.988	49.457
UP2090	African	PCa	61	26,00	1.674	50.97	1.627
KAL0003	African	PCa	NA	NA	0.589	94.893	3.638
UP2159	African	PCa	68	11,90	4.167	11.434	1.507
UP2213	African	PCa	67	25,80	10.395	260.39	13.21
UP2255	African	PCa	59	19,00	30.059	65.54	58.009
UP2298	White	PCa	75	90,00	0.241	21.412	0.136
UP2367	African	PCa	73	243,00	8.273	159.64	7.794
UP2323	African	PCa	67	97,00	40.97	98.822	29.909
UP2301	African	PCa	75	94,84	0.385	96.179	5.115
SMU050	African	PCa	64	831.95	1.156	2.064	34.926
KAL0074	African	PCa	67	18.57	5.482	41.028	10.255
UP2112	African	PCa	56	67	3.715	4.221	47.655

4.3.2. Patient characteristics

The two groups, BPH and PCa, were compared in terms of age and PSA, and the relationship between these characteristics and methylation was assessed. Median age was not significantly different between PCa and BPH groups (P = 0.069) while median PSA was significantly different (P < 0.001) (Table 4.2). No correlation was observed between *GSTP1* methylation (FAM) and PSA levels in PCa (PSA: Spearman's rho = -0.050, P = 0.755) nor in BPH (PSA: Spearman's rho = 0.453, P = 0.068) (Table 4.3). Similarly, methylation was not correlated with age in either of the groups (BPH: Spearman's rho = -0.225, P = 0.385; PCa: Spearman's rho = -0.152, P = 0.320).

Table 4.2. Comparison of characteristic features in PCa and BPH patients.

Characteristic	PCa (n = 48)	BPH (n = 17)	P-value
Age			0.069
Median	68	66	
Range	54-99	55-77	
PSA			<0.001
Median	83.34	18	
Range	9-3459	1.80-32.30	

Table 4.3. Correlations between methylation and PSA or age.

	PCa (Rho, P-value, CI)	BPH (Rho, P-value, CI)
FAM_Age	ρ = -0.152, P = 0.320, -0.433-0.157	ρ = -0.225, P = 0.385, -0.646-0.301
FAM_PSA	ρ = -0.050, P = 0.755, -0.361-0.270	ρ = 0.453, P=0.068, -0.773-0.050
VIC_Age	ρ = 0.199, P = 0.190, -0.109-0.472	ρ = -0.425, P = 0.089, -0.758-0.086
VIC_PSA	ρ = 0.207, P = 0.194, -0.117-0.491	ρ = 0.199, P = 0.445, -0.326-0.629
%Target/total_Age	ρ = -0.218, P = 0.150, -0.488-0.089	ρ = 0.175, P = 0.502, -0.348-0.614
%Target/total_PSA	ρ = -0.266, P = 0.093, -0.537-0.055	ρ = -0.370, P = 0.144, -0.730-0.150

4.3.3. GSTP1 promoter methylation levels

GSTP1 methylation was compared between PCa patients and BPH controls. The two groups were significantly different in terms of *GSTP1* FAM concentration ($P < 0.001$) and % target/total ($P < 0.001$) however, they were not significantly different in terms of VIC concentration ($P = 0.881$).

Methylation was compared between the three ethnic groups (African, White and Coloured) with PCa. No significant differences were observed for FAM ($P = 0.079$), VIC ($P = 0.678$) or % target/total ($P = 0.106$); however, the sample size for Coloured ($n = 1$) and White ($n = 2$) individuals included in the analysis was very small.

4.3.4. Biomarker performance

The assay designed in **Chapter 3** was evaluated for its diagnostic capabilities and compared to that of PSA, the golden standard for PCa screening. The two markers were combined to determine whether this further improved the overall performance. The *GSTP1* biomarker (FAM) had an AUC = 0.907 ($P < 0.001$, CI 0.832-0.981) (Table 4.4, Fig 4.4A). At a cut off of 0.778, the sensitivity was 0.805 and specificity was 1. In comparison, PSA had an AUC = 0.846 ($P < 0.001$, CI 0.749-0.943). At a cut off of 32.9, PSA sensitivity was 0.659 and specificity was 1. When methylation (FAM) and PSA were combined, the predictive capability was improved (AUC = 0.957, $P < 0.001$, CI 0.908-1).

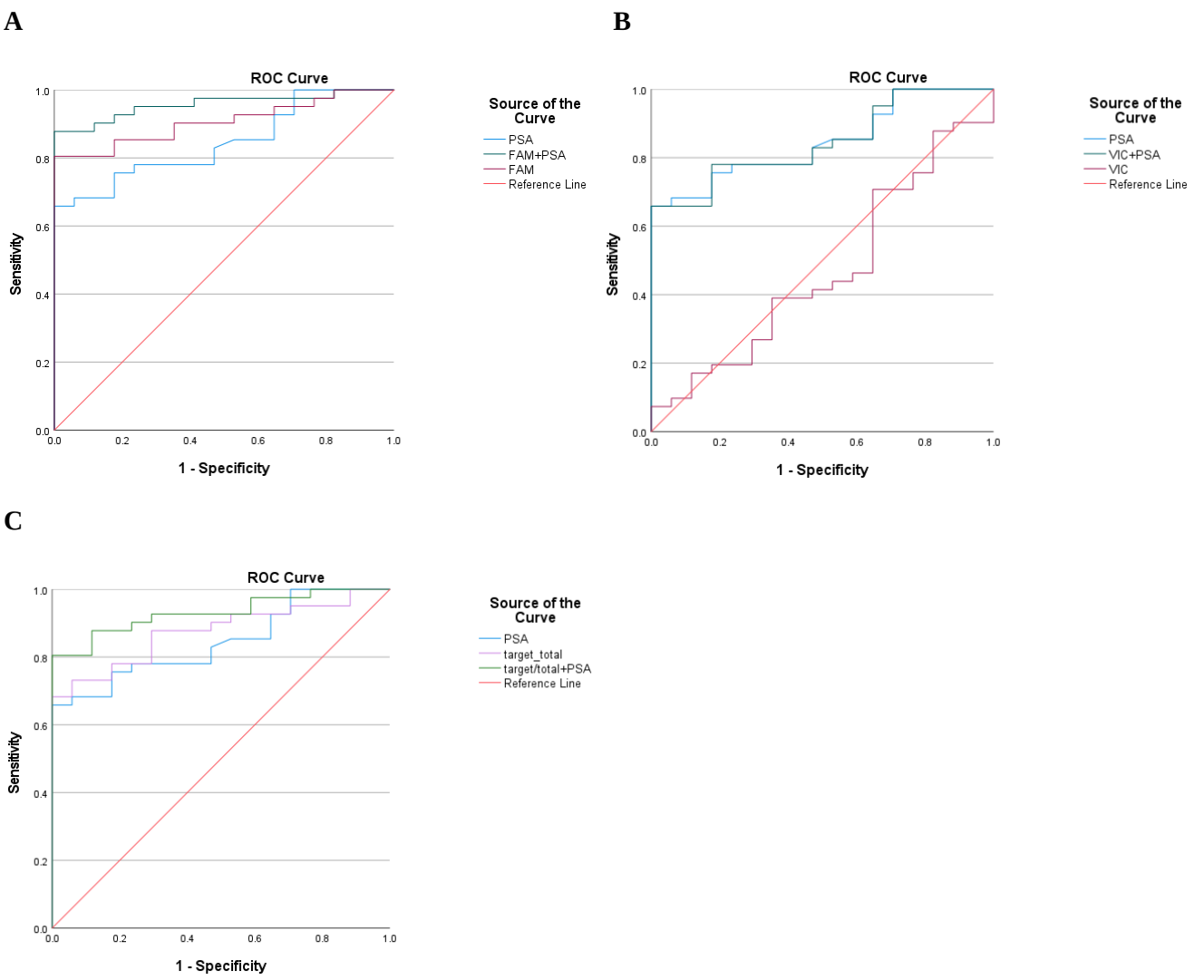

Fig 4.4. Receiver operating characteristic (ROC) curves for the *GSTP1* biomarker compared to PSA alone and combined with *GSTP1* (FAM, VIC, % target/total). A. FAM, **B.** VIC, **C.** Target/total. The purple line represents sensitivities and specificities at different cut offs for *GSTP1*, while the blue line represents PSA and the green line represents a combination of PSA and *GSTP1*.

Table 4.4. Biomarker performance of *GSTP1*, PSA and a combination, for PCa screening in South African patients.

Marker	AUC	CI	P-value	Cut off	Sensitivity	Specificity
GSTP1-FAM	0.907	0.832-0.981	< 0.001	0.7780	0.805	1
PSA	0.846	0.749-0.943	< 0.001	32.9	0.659	1
GSTP1-FAM+PSA	0.957	0.908-1	< 0.001	0.6264285	0.878	1
GSTP1-VIC	0.468	0.305-0.631	0.701	224.435	0.073	1
GSTP1-VIC+PSA	0.845	0.747-0.943	< 0.001	0.7161068	0.659	1
GSTP1-TARGET/TOTAL	0.875	0.788-0.962	< 0.001	9.3645	0.683	1
GSTP1-TARGET/TOTAL+PSA	0.931	0.869-0.944	< 0.001	0.7754894	0.805	1

4.3.5. Predictors of disease

PSA, age and *GSTP1* methylation (FAM, VIC, % target/total) were evaluated as predictors of PCa risk; however, none of these variables were significantly associated with overall PCa risk (Table 4.5).

Table 4.5. Odds ratios (OR) and 95% confidence intervals (CI) for age, PSA and *GSTP1* methylation, estimated using multiple logistic regression.

Predictor	P-value	OR	CI
Age	0.372	1.105	0.887-1.377
PSA	0.115	1.089	0.979-1.212
FAM	0.207	21.923	0.182-2640.652
VIC	0.620	0.989	0.948-1.032
Target/total	0.259	1.134	0.912-1.409

4.4. Discussion

In Europeans, there is a clear distinction between PCa and BPH patients in terms of *GSTP1* methylation,[11] making this a highly accurate biomarker for discrimination between diseased patients and controls.[6,18] However, to date, no study has shown that this distinct methylation difference exists in the South African population, thus the suitability of the *GSTP1* biomarker for Black South Africans is unclear.

The data presented in this chapter indicates that there are significant differences in *GSTP1* methylation between PCa and BPH patients in South Africa with PCa patients having a mixture of methylated and unmethylated copies, while *GSTP1* is largely unmethylated in BPH patients. This data confirms the results seen in European populations[11,19] and offers an African perspective.

The African relevant epigenetic assay, developed in **Chapter 3**, showed greater diagnostic performance (FAM AUC = 0.907) in this cohort than the golden standard, PSA (AUC = 0.846). The performance was further improved when PSA and *GSTP1* methylation were combined (FAM+PSA AUC = 0.957). These results suggest that PCa screening could be enhanced by combining PSA screening with a methylated *GSTP1* test. Previous studies have reported similar sensitivities (81.8% ± 8.8%) and specificities (94.9% ± 2.4%) for *GSTP1*, as the ones reported here, suggesting that the developed assay is on par with published assays.[20]

A limitation of the African-relevant assay is that it was developed and tested on tissue samples. Epigenetic assays are mostly being used in liquid biopsies (further discussed in **Chapter 2**) for a more non-invasive, easy to perform, diagnostic test.[21–23] Future studies should therefore validate this test in blood and/or urine. Although few White and Coloured patients were included as controls, this assay should be tested on a larger cohort to ensure its suitability for all South Africans. Lastly, a diagnostic assay should ideally include a control, such as *ACTB* or *C-less-C1*, that will be amplified regardless of the methylation status and therefore normalize for input DNA.[24–27] The assay developed here can therefore be improved by using the FAM probe to target methylation in *GSTP1* and designing the VIC probe to target a reference gene rather than unmethylated *GSTP1*.[25] This chapter could have further been improved by performing a blinded analysis instead of knowing which samples were PCa and BPH, which would have given the results more value.

The data presented in this chapter has contributed to an understanding of the methylation profile in Black South Africans. Furthermore, this is the first study to provide preliminary data to demonstrate the suitability of differential *GSTP1* methylation as a target for PCa screening in Black South Africans. These results further show that my African relevant, methylation-based *GSTP1* assay has the potential to improve PCa screening in South Africans, by differentiating PCa from age-related BPH, with potential to provide early diagnosis and reduce associated mortality rates, and warrants further investigation in larger clinical studies.

4.5. References

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Chapter 5: General discussion and conclusion

Epigenetic assays have the potential to complement prostate-specific antigen (PSA) screening and lead to improved prostate cancer (PCa) diagnosis and better disease outcomes.[1–3] However, these valuable assays are biased towards European populations[4–8] while African populations, specifically Black South Africans, present with higher risk and mortality rates.[9] In this dissertation, I aimed to determine whether a popular PCa epigenetic biomarker, namely *Glutathione S-transferase pi 1* (*GSTP1)* hypermethylation, could be used for PCa detection in Black South African men. My first task was to review the current methodology in **Chapter 2** that would be appropriate for cost-effective routine PCa screening within the South African context. Secondly, I assessed in **Chapter 3** the relevance of published assays to detect methylation differentiation in Africans, while designing and evaluating a new assay appropriate for the selected method, namely digital PCR (dPCR). Thirdly in **Chapter 4**, I tested the methylation pattern for *GSTP1* in a cohort of 100 South Africans diagnosed either with PCa (n = 66) or benign prostatic hyperplasia (BPH) and without any pathological evidence for PCa (n = 34). The aim of the latter, to determine whether significant differences existed which could be used to accurately differentiate between diseased *vs* healthy patients.

In this dissertation, I found that some published assays[10–12] overlapped with low-frequency and rare African-specific single nucleotide polymorphisms (SNPs). While this is not ideal, a mismatch nearer to the 5' end of oligonucleotides is less likely to prevent amplification,[13] however, further studies should test these assays in Africans to completely understand the effect of these SNPs on assay efficiency. This analysis has highlighted important African variants that should be accounted for during *GSTP1* hypermethylation assay design, which is important because this can help ensure the suitability, of future assays, to different ethnic groups.

A dPCR assay, that considered African-specific SNPs, was designed to ensure suitability for high-risk populations. While dPCR is meant to be highly sensitive[14] and require small amounts of DNA,[15,16] my assay required an unusually large amount of DNA and even then, many wells did not amplify, suggesting that the primers and probes may require further optimisation. A next step could be to further optimise the primer and probe concentrations on the dPCR instrument, try different cycling conditions and possibly revisit the primer and probe design. In my experience, I found the QuantStudio™ 3D dPCR system to be highly labour intensive and time-consuming. In a clinical setting, screening tests should ideally be high throughput, therefore I would argue that the single chip format of the QuantStudio™ dPCR system is not ideal. Furthermore, the many manual steps involved introduces opportunities for contamination and variation among results. For example, excess immersion fluid can cause chips to leak, while bubbles, debris, or condensation on the chip can further affect imaging and lead to varying results among replicate chips. Other

platforms like the BioRad droplet digital PCR (ddPCR) system,[17] which allows for a 96 well plate format, may be a better option because, as mentioned in **Chapter 2**, this platform is simpler and already widely used in cancer studies.[14] That being said, I do think that ddPCR has the ability to improve diagnostics, especially in liquid biopsies where genetic material is limited and high sensitivity is essential (although not tested within the scope of this dissertation).[16]

Importantly, the African relevant assay was able to show that *GSTP1* methylation is significantly different in South African patients with PCa *vs* non-cancerous, age-related BPH. However, none of the variables analysed were significant predictors of PCa risk. I demonstrated that this assay could differentiate between patients and controls with better accuracy than the golden standard PCa screening marker, PSA (Area under the receiver operating characteristic curve (AUC) = 0.907 *vs* 0.846) and that when combined, overall performance was further improved (AUC = 0.957). These results are significant because, with further investigation, differential *GSTP1* methylation could potentially provide accurate, early diagnosis which would lead to reduced mortality rates in the South African population and alleviate the burden on South Africans health systems.

As mentioned in **Chapter 2**, *GSTP1* has been shown to be an effective biomarker for early diagnosis[18] and can detect PCa in negative biopsies.[19] Because the samples used in this dissertation were high-grade PCa tissue samples, we cannot comment on these two points. Furthermore, due to lack of available follow-up data for the test cohort, the prognostic value remains unknown. Future studies should address these points in African populations in order to fully understand the benefits and limitations of this biomarker in aggressive PCa.

Although this dissertation has shown promising results for *GSTP1* in Black South Africans, this biomarker needs to be translated into a liquid biopsy before it can be considered for implementation into routine screening. With Black South Africans experiencing such aggressive disease,[9] they might benefit from frequent testing to monitor disease progression which, will allow for better PCa management.[20] Non-invasive testing is therefore essential, as men are more likely to subject themselves to regular screening if it is easy to perform and causes minimum discomfort. Furthermore, for frequent testing to occur it is important that the assay be applicable to satellite labs near rural areas to ensure it is highly accessible to Black South African men. Lastly, it is important that the *GSTP1* biomarker be incorporated into a larger panel of genes and tested across multiple groups of people, to prove its effectiveness in diverse South Africa,[21] before it can be used in routine PCa screening.

Precision medicine aims to customise healthcare to the individual patient, eliminating a one size fits all approach.[22] Panels of epigenetic biomarkers with prognostic capabilities, used in liquid biopsies, have the potential to provide a personalized snapshot of disease and allow for tailored PCa treatment.[23] Genome-

wide methylation analysis can be used to identify informative African-specific biomarkers and aid in the development of disease screening panels with enhanced sensitivity and specificity.[24,25] Genome-wide approaches such as whole-genome bisulfite sequencing (WGBS) allows for analysis of the entire methylome.[24] Although this method is highly informative, WGBS is costly, it requires technical expertise, and data interpretation can be challenging which is why this method is not suitable for a clinical setting.[26] For now, targeted methylation analysis of specific genes is more accessible and perhaps better for a clinical setting because it allows for higher throughput while genome-wide approaches have a place in the identification of prognostic biomarkers worth targeting.

Implementation of new assays into clinical practice can be a long and costly process with many barriers, which is likely why ConfirmMDx (MDxHealth, Irvine, USA) is the only commercially available epigenetic assay for PCa. For the assay developed here to be implemented into clinical practice, it would need to undergo clinical trials and would have to be reviewed by a regulatory body such as the Food and Drug Administration (FDA) or the South African Health Products Regulatory Authority (SAHPRA).[27] Before it can be used in a diagnostic setting, there would need to be proof that this assay is superior and offers substantial benefits over current diagnostic techniques.[27] Furthermore, medical professionals, who are often reluctant to move away from the gold standard, would need to be convinced to adopt the new test.[27] To move forward, more studies, addressing the extent of the benefits of this biomarker and confirming improvement over PSA testing in Black South Africans are needed to provide compelling evidence for entrance into routine practice. A highly accurate epigenetic assay has the potential to significantly impact Black South African men's attitude towards traditional medicine and encourage frequent screening. This will then have an economic impact since unnecessary, expensive treatments can be avoided.

In conclusion, my results provide preliminary evidence that demonstrates *GSTP1* hypermethylation is a suitable target that can be used to screen for PCa in Black South Africans. While most studies have focused on indolent disease in developed countries,[25] this dissertation has provided important insight into *GSTP1* methylation in aggressive PCa in developing South Africa which, will hopefully have a substantial impact on PCa awareness in South Africa, inform future studies and ultimately result in better screening tests that help combat the PCa health disparity.

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