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Investigating the DNA methylome of prostate cancer in a Ghanaian cohort and novel pathways that

link Schistosome infections to prostate cancer

By

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**THIS THESIS IS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL
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DEGREE IN MOLECULAR CELL BIOLOGY OF INFECTIOUS DISEASES**

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INTEGRI PROCEDAMUS

DECLARATION

I, Djomkam Zune Alexandra Lindsey, certify that the work provided in this thesis is entirely my own original research, except for properly cited information taken from other sources. This thesis does not contain any work that has been submitted for a degree to this University or to any other institution.



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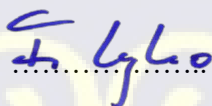
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DEDICATION

To God almighty

To the Djeumbi family

To the Tioguem family,

To my wonderful parents Mr. and Mrs. Djomkam

To my lovely and supportive husband Stephane Tekam



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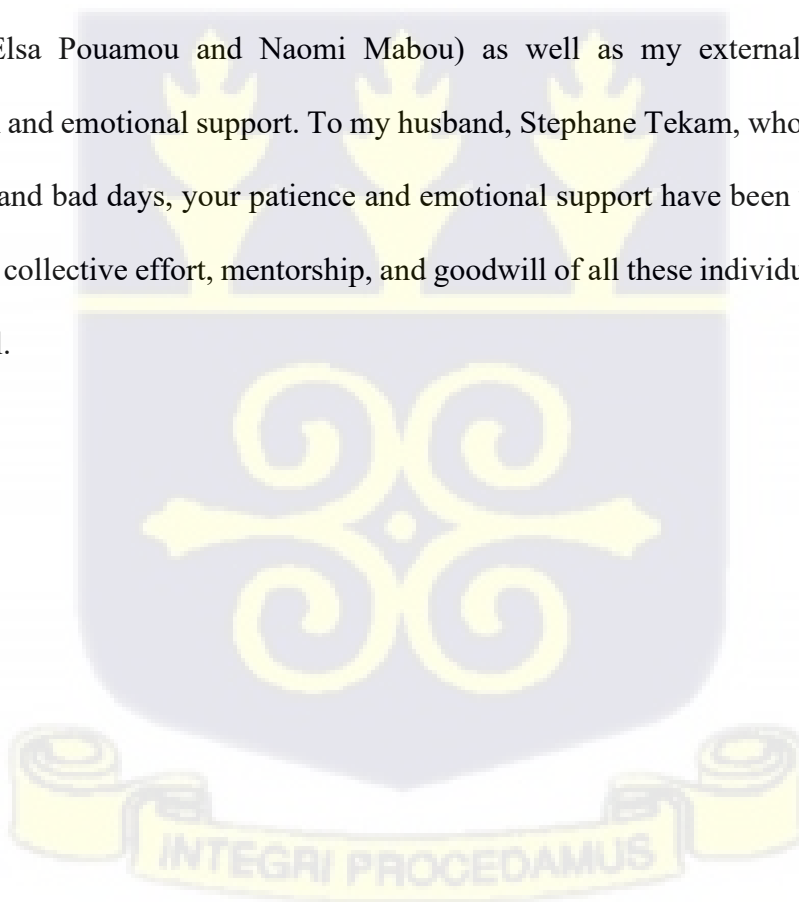


Table of Contents

DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
LIST OF FIGURES	ix
LIST OF TABLES	xii
LIST OF ABBREVIATIONS	xiii
ABSTRACT	xvi
CHAPTER ONE	1
INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement	4
1.3 Rationale	5
1.4 Research questions	6
1.5 Aim	6
1.6 Objectives	6
CHAPTER TWO	8
LITERATURE REVIEW	8
2.1 Prostate Cancer	8
2.1.1 The prostate gland	8
2.1.2 Incidence and mortality	9
2.1.3 Racial disparity	13
2.1.4 Risk factors	17
2.2 Infection, inflammation in PCa pathophysiology	24
2.2.1 Non-infectious causes of inflammation	25
2.2.2 Infectious causes of inflammation	27
2.3 Schistosomiasis and PCa	28
2.4 Epigenetics in PCa Development and Progression	30
2.4.1 Histone modifications in PCa	31
2.4.2 MicroRNA and Epigenetic Alterations in PCa	31
2.4.3 DNA methylation in PCa	32
2.5 Mechanisms by which Pathogens Induce Cancer-Associated Epigenetic Alterations	33
2.6 Challenges in parasite – induced carcinogenesis research and the choice of the experimental approach	35

2.7 Summary	36
CHAPTER THREE	38
DNA METHYLATION PATTERNS ASSOCIATED WITH PROSTATE CANCER AGGRESSIVENESS IN A GHANAIAN PATIENT COHORT	38
3.1 Introduction.....	38
3.2 Methods.....	40
3.2.1 Sample collection.....	40
3.2.2 DNA methylation profiling.....	40
3.2.3 Data analysis	40
3.2.4 Network propagation.....	42
3.3 Results.....	42
3.3.1 Clinicopathological Characteristics of the Study Group.....	42
3.3.2 Consistent offset DNA methylation pattern in samples from patients with high tumour grade.....	44
3.3.3 Differentially methylated probes associated with aggressive PCa.....	47
3.3.4 TP53 targets expression profiles distinguish between indolent and aggressive tumours.....	50
3.3.5 Novel methylation markers of PCa aggressiveness.	53
3.4 Discussion.....	58
3.5 Conclusion	62
3.6 Supplementary data.....	62
CHAPTER FOUR.....	68
COMPUTATIONAL PREDICTION OF SCHISTOSOMA-MEDIATED PROSTATE CARCINOGENESIS PATHWAYS	68
4.1 Introduction.....	68
4.2 Methods.....	69
4.2.1 Network propagation.....	69
4.2.2 Clustering of propagated network.....	70
4.2.3 Integrating genomic data from prostate cancer patients.....	71
4.2.4 Gene set over-representation analyses.	72
4.3 Results.....	73
4.3.1 Putative human targets of <i>Schistosoma haematobium</i> and <i>Schistosoma mansoni</i>	73
4.3.3 Identification of high impact subnetworks associated with PCa based on gene alteration frequencies in cBioportal.	77
4.3.4 Selected sub-networks show relevance in Schistosoma-associated cancers.	78
4.3.5 Top mutated haematobium and mansoni DEGs in PCa genomics data.....	79
4.4 Discussion.....	83

4.5 Conclusion	88
4.6 Supplementary data.....	89
CHAPTER FIVE	99
ROLE OF SCHISTOSOMA HAEMATOBIIUM AND MANSONI SOLUBLE EGG ANTIGENS IN PROSTATE CARCINOGENESIS	99
5.1 Introduction.....	99
5.2 Methods.....	101
5.2.1 Cell culture.....	101
5.2.2 Preparation of <i>Schistosoma haematobium</i> , <i>mansoni</i> and combined Soluble Egg Antigens	101
5.2.3 Determination of the schSEA Effect on Human Normal prostate epithelial Cells	101
5.2.4 Bioinformatics and statistical analysis	106
5.3 Results.....	106
5.3.1 schSEA increases cell proliferation of exposed PNT2 and RWPE1 cells.	106
5.3.2 schSEA exposure of PNT2 cells increases the rate of programmed cell death.....	108
5.3.3 Relative increased expression of the angiogenesis marker <i>VEGFA</i> in schSEA mansoni exposed cells	109
5.3.4 Dynamic gene expression of cytokines in schSEA-treated prostate epithelial cells.....	110
5.3.5 Proteomic Analysis of schSEA-Induced Effects on Normal Prostate Epithelial Cells.....	113
5.4 Discussion	126
5.5 Conclusion	132
5.6 Supplementary	132
CHAPTER SIX.....	134
GENERAL DISCUSSION, CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS.....	134
6.1 General discussion	134
6.2 Conclusions.....	139
6.3 Limitations, recommendations, and prospects	140
References.....	142



LIST OF FIGURES

Figure 2.1 Sagittal section of the pelvis.....	7
Figure 2.2 Global incidence and mortality trends of PCa.....	10
Figure 2.3 Incidence and mortality rates of PCa.....	11
Figure 2.4 Incidence and mortality rates across ethnicities in the USA.....	13
Figure 2.5 Immune profile in PCa varies across race.....	15
Figure 2.6 Prostate cancer friendly microenvironment.....	24
Figure 2.7 Global distribution of schistosomiasis highlighting Ghana.....	28
Figure 2.8 Postulated mechanisms of pathogen induced epigenetic alterations driving carcinogenesis.....	34
Figure 3.1 DNA methylation pattern between malignant and adjacent non-neoplastic tissues....	43
Figure 3.2 Cell deconvolution analyses reveal relative high levels of luminal cells in Ghanaian samples.....	45
Figure 3.3 DNA methylation distinguishes between indolent and aggressive tumours.....	47
Figure 3.4 DMPs are associated with cell division related pathways.....	49
Figure 3.5 DMPs-associated TP53 targets in publicly available genomic data.....	50
Figure 3.6 TP53 targets distinguish between indolent and aggressive tumours in the TCGA Fire-house cohort.....	51
Figure 3.7 DMRs associated with PCa aggressiveness.....	54
Figure 3.8 Network propagation of DMRs-associated genes reveals functional clusters with distinct biological Functions.....	56
Figure 4.1. Network modeling to probe the role of <i>S. haematobium</i> and <i>S. mansoni</i> in PCa development.....	74
Figure 4.2 Network propagation for <i>S. haematobium</i> DEGs.....	76

Figure 4.3 Frequency of Gene Alterations in PCa Patients of Subnetwork Clusters.....	77
Figure 4.4 Alteration frequencies of top five clusters' nodes across the most common cancers in men.....	78
Figure 4.5 Integrating PCa genomics data into the <i>S. haematobium</i> DEG propagated Network..	80
Figure 4.6 Integration of the top mutated <i>S. haematobium</i> DEGs into the <i>S. haematobium</i> DEG propagated subnetwork clusters.	82
Figure 4.7 Gene set enrichment analysis for propagated <i>S. haematobium</i> , <i>S. mansoni</i> or coinfection subnetwork clusters with connections to the top mutated <i>S. haematobium</i> and <i>S. mansoni</i> DEGs.	83
Figure 5.1 schSEA promotes cell proliferation.....	108
Figure 5.2 Evaluation of the effect of schSEA on Apoptosis.....	109
Figure 5.3 Evaluation of the effect of schSEA on Angiogenesis.....	111
Figure 5.4 Relative cytokine gene expression of schSEA exposed RWPE1 cells	113
Figure 5.5 Proteomics analyses of schSEA-treated PNT2 cells.....	114
Figure 5.6 Boxplot of ISG protein intensities by treatment.....	115
Figure 5.7 Principal component analyses of schSEA treated cells.....	116
Figure 5.8 Differential protein abundance associated with various schSEA treatments.....	119
Figure 5.9 Heatmaps illustrating protein expression patterns across schSEAc, schSEAh and schSEAm treatments.....	121

Figure 5.10 Gene alteration frequencies and protein-protein interactions for Clusters 1 and 4.....	123
Figure 5.11 Gene ontology analyses of DEPs in cluster 1 and cluster 4.....	125
Figure 5.12 Analysis of DEPs cell cycle-related genes in schSEA-treated PNT2 cells.....	126
Figure 6.1 Proposed model of mechanisms potentially exploited by <i>Schistosoma spp</i> s in prostate carcinogenesis.....	138



LIST OF TABLES

Table 3.1. Patient clinicopathologic characterization.....	42
Table 3.2. Tumour grade distribution across samples.....	44
Table 3.3. DMR-associated genes.....	54
Table 5.1 RT-qPCR targeted genes and primer sequences.....	103

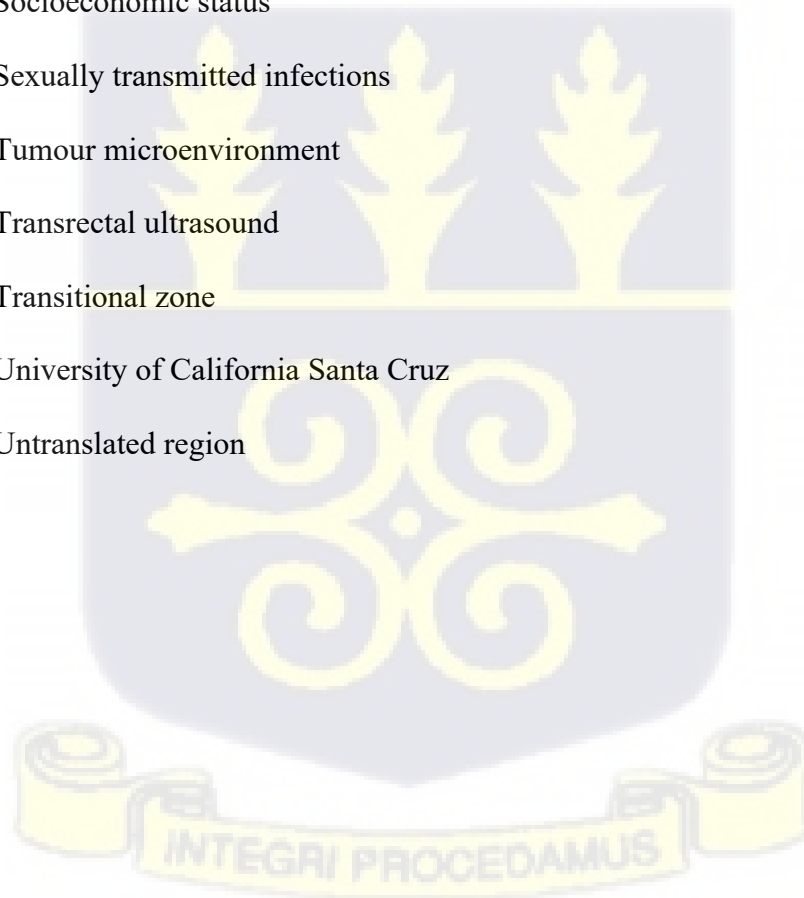


LIST OF ABBREVIATIONS

AA	African American
AAM	Amino acid metabolism
AFMS	Anterior fibromuscular stroma
AR	Androgen receptor
ASR	Age standardized rates
ATCC	American type culture collection
BMI	Body mass index
CBME	Collagen biosynthesis and modifying enzymes
CME	Clathrin-mediated endocytosis
COPI-MAT	COPI-mediated anterograde transport
CORUM	Comprehensive resource of mammalian protein complexes
CSIR	Council for scientific and industrial research
CZ	Central zone
DHT	Dihydrotestosterone
DMPs	Differentially methylated probes
DMRs	Differentially methylated regions
DRE	Digital rectal examination
DROS	Detoxification of ROS
DTT	Dithiothreitol
EA	European American
EBV	Epstein-Barr virus
ECM	Extracellular matrix
ER	Endoplasmic reticulum

ERG	ETS related gene
ESPEN	Expanded special project for elimination of neglected tropical diseases
FBS	Fetal bovine serum
FDR	False discovery rate
FFPE	Formalin fixed paraffin embedded
FWER	Family wise error rate
GEO	Gene expression omnibus
GO	Gene ontology
GSEA	Gene set enrichment analyses
HPV	Human papillomavirus
HR-HPV	High risk HPV
HSV	Herpes simplex virus
IARC	International agency for research on cancer
IGF	Insulin growth factor
KEGG	Kyoto encyclopedia of genes and genomes
LI	Laminin interactions
NMIMR	Noguchi memorial institute for medical research
NSAID	Non-steroidal anti-inflammatory drugs
PAP	prostatic acid phosphatase
PBS	Phosphate buffer saline
Pca	Prostate cancer
PCICP	Processing of capped intron containing pre-mRNA
PIA	Proliferative inflammatory atrophy

PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PZ	Peripheral zone
RGAF	Rho GTPases activate Formins
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RWR	Random walk with restart
schSEA	Schistosome soluble egg antigens
SES	Socioeconomic status
STI	Sexually transmitted infections
TME	Tumour microenvironment
TRUS	Transrectal ultrasound
TZ	Transitional zone
UCSC	University of California Santa Cruz
UTR	Untranslated region



ABSTRACT

Prostate cancer (PCa) is of significant public health concern in Africa. The incidence and mortality rates are notably higher, and the disease often presents at a more advanced stage. This disparity is influenced by a combination of genetic, environmental, and lifestyle factors that are not yet fully understood. DNA methylation studies offer a promising avenue for understanding the epigenetic changes that promote PCa risk and progression. Additionally, the study of schistosomes, parasitic flatworms prevalent in Africa and previously associated with bladder cancer, is crucial as they have been associated with and represent a potential risk factor for PCa development. The present study aimed at profiling the DNA methylation patterns of PCa in sub-Saharan African men and schistosome infections as a potential risk factor for PCa.

The study used the Illumina Infinium EPIC array, a high throughput state-of-the-art platform to profile the DNA methylation profile of the study cohort. To evaluate schistosoma exposure as a risk factor for PCa development, a series of *in vitro* assays, rt-qPCR, and omics approaches (mass spectrometry) were employed.

A high degree of interindividual heterogeneity was observed in DNA methylation patterns between benign and malignant prostate samples. This heterogeneity was attributed to the genomic diversity inherent in African populations. Notably, clustering based on tumour grade was evident, implicating cellular structure, division pathways, and oxidoreductase activities in PCa aggressiveness. Intriguingly, samples from high-grade tumours exhibited elevated levels of luminal cells, suggesting that inflammatory stimuli, potentially from oxidative stress, mechanical injury or infectious agents like *Schistosoma haematobium*, could contribute to PCa aggressiveness. Given Africa's high burden of schistosomiasis, the study further explored the role of *Schistosoma spp* in PCa development. Network propagation techniques were employed, identifying several pathways that could be exploited by the parasites to induce carcinogenesis, including clathrin-

mediated endocytosis, alpha defensins and degradation and organization of the extracellular matrix.

In vitro assays and multi-omics approaches used to validate the predictions on the pathways perturbed by schistosome exposure of normal prostate epithelial cells revealed several insights. Firstly, significant dose-dependent cell proliferation was observed, particularly with schistosome soluble egg antigens from *S. haematobium* or *S. haematobium* plus *S. mansoni* (schSEAh and schSEAc). Secondly, elevated levels of proinflammatory cytokines and angiogenic markers were also noted, supporting the hypothesis that schistosome infections could induce chronic inflammation and promote vascularization, key elements in cancer development. Finally, schSEA treatments triggered differentially expressed proteins (DEPs) associated with proteins associated with cellular metabolism and cell cycle. Metabolic reprogramming and cell cycle alterations are known to be involved in prostate cancer development and progression. Differential expression of MX2, NSUN2 and DLG1 correlated with high alteration frequencies in their corresponding genes in publicly available in TCGA PCa genomic data.

Despite its insights, the study acknowledges limitations such as the small sample size for DNA methylation profiling and the absence of long-term *in vitro* exposure models. Future research would aim to address these gaps to provide a more conclusive understanding of the DNA methylation profile of PCa in Africa and a robust understanding of the complex interplay between schistosome infections and PCa development in African men.

CHAPTER ONE

INTRODUCTION

1.1 Background

Cancer represents a significant global public health challenge. Prostate cancer (PCa) is the second most common malignancy in men worldwide, with approximately 1.4 million new cases and 375,000 deaths in 2020 (Sung *et al.*, 2021a). Although PCa trends exhibited a decline and stability between the years 2000 - 2019 in developed countries, PCa is on the rise and is the most prevalent malignancy in men in Sub-Saharan Africa (Seraphin *et al.*, 2021; L. Wang *et al.*, 2022). In Ghana, 21.4% of all new cancer cases in men were attributed to PCa in 2020 (International Agency for Research on Cancer, 2019).

Most PCa cases are effectively treatable if they are detected at early stages. However, the challenge lies in attaining early diagnosis and efficient treatment due to a myriad of causalities of the disease and the significant infrastructural costs (Rebbeck, 2020). In the context of PCa, diagnosis relies on a triad of tests and examination viz, prostate specific antigen (PSA) serological test, digital rectal examination (DRE) and biopsy, which are nonspecific, subjective and invasive, respectively (Song *et al.*, 2005). Although the PSA test is sensitive, it lacks specificity in diagnosing PCa and (TRUS)-guided prostate biopsy often yield false negatives due to the considerable variability PCa inter- and intra-heterogeneity (Schoenfield *et al.*, 2007). It is therefore essential to discover biomarkers that can lead to precise diagnosis, successful treatment, and novel strategies for preventing PCa.

Establishment of powerful management tools for a disease largely relies on the knowledge of disease biology. The aetiology of PCa is complex and not fully understood (Rawla, 2019), however, several factors contribute to an increased risk of PCa, amongst which the most well-established are; advanced age, ethnicity, family history, genetic and epigenetic factors (Bostwick *et al.*, 2004) and largely unexplored factors such as infections.

In addition to their involvement in infectious diseases, the contribution of pathogens to non-communicable diseases, including cancers, is gaining recognition. Presently, infections are estimated to be responsible for about 25% to 50% of all human malignancies (Knoll *et al.*, 2018). Infectious carcinogens that affect humans encompass every category of microbial pathogens viz, bacteria, fungi, viruses, and parasites. These pathogens include, hepatitis B & C viruses, the Epstein-Barr virus (EBV), high-risk HPV (HR-HPV) genotypes (White *et al.*, 2014), *H. pylori* (Moss, 2016) and *S. haematobium* (Ishida & Hsieh, 2018).

Infection-induced cancers have mostly been associated with specific epigenetic changes (Maekita *et al.*, 2006; Van Tine *et al.*, 2004). In certain cases, the infectious organism itself can directly cause epigenetic modifications, whereas in other situations, the epigenetic changes might occur as a result of inflammation that the infection has provoked (Herceg, 2007). Schistosomiasis ranks second after malaria as the most socioeconomically devastating parasitic disease globally. Post-mortem studies have shown prostate infections with *S. japonicum*, *S. mansoni*, and *S. haematobium* in endemic areas (Y. Lai *et al.*, 2013; Okani *et al.*, 2013; Patil & Elem, 1988) hinting at the possibility that prostate schistosomiasis might be frequently occurring. The pathophysiological consequences of prostate schistosomiasis along with its possible role in PCa onset and progression remain unknown. In contrast, for bladder cancer, it's well-established that *S. haematobium* is a class I carcinogenic factor (International Agency for Research in Cancer, 1994). Given that inflammation and infections cause about 13% of cancers globally (de Martel *et al.*, 2020), it is important to understand the potential role of schistosome infections prostate carcinogenesis. Moreover, it has been reported that schistosome infections of the prostate elicit severe inflammation (Choto, Mduluzza, Sibanda, *et al.*, 2020; Peiffer *et al.*, 2019).

A substantial amount of research indicates that chronic inflammation is a hallmark of cancer (Hanahan, 2022). Inflammation is often noted within the prostate's microenvironment and is

speculated to play a role in the onset and progression of PCa (Sfanos *et al.*, 2018). Although the role of inflammation in prostate carcinogenesis is not fully understood, some studies have shown that DNA methylation alterations is associated with inflammation. For example, Stenvinkel and colleagues have reported an association between global DNA hypermethylation and inflammation in the context of chronic kidney disease (Stenvinkel *et al.*, 2007). Also, several studies provide evidence of inflammation and inflammatory signaling in DNA methylation aberration in cancers (Maeda *et al.*, 2017; Pekow *et al.*, 2019; Stoyanov *et al.*, 2015; Yamashita *et al.*, 2019).

DNA methylation is the most investigated epigenetic mechanism in PCa. Studies indicate that changes in DNA methylation topography are the most recurrent events in PCa (J. Luo *et al.*, 2013), suggesting its relevance in PCa development and progression. Several genes have been described to have hypermethylated promoters, including O⁶-methylguanine DNA methyltransferase (*MGMT*), cyclin-dependent kinase inhibitor 2A (*CDKN2A*), death-associated protein kinase (*DAPK*), and tissue inhibitors of metalloproteinases (*TIMPS*) (Padar *et al.*, 2003; Yamanaka *et al.*, 2003). These genes perform vital biological functions and are involved in important cellular pathways such as hormonal response, tumour cell invasion/metastasis, cell cycle control, apoptosis, and DNA damage repair.

Despite the major role of DNA methylation aberration in PCa development, progression, and aggressiveness, the methylomes of resident Ghanaians and Africans in general have not been profiled. Research on epigenetics in PCa has predominantly focused on Caucasian populations and, to a smaller degree, African Americans (Hobbs & Ramsay, 2015). Understanding the role of DNA methylation in Africa often relies on studies carried out among African Americans, which may not be directly applicable to indigenous African populations because of genetic diversity resulting from population admixture (Campbell & Tishkoff, 2008; Xia *et al.*, 2014). Methylation aberration can be driven by several factors including infections. Africa is characterized by a

different environment which harbours unique pathogens, and therefore, it is imperative to profile the DNA methylation in resident Africans. As a result, Africans residing in Africa face a higher exposure to pathogenic infections in comparison to Africans living abroad. For example, Sub-Saharan Africans have the highest burden of *Schistosoma* infections globally (Hong, 2023). Several studies have reported aberrant methylation patterns in *Schistosoma* associated bladder cancers, particularly promoter hypermethylation of tumour suppressor genes (Akpabio *et al.*, 2023; Conti *et al.*, 2015; Gutiérrez *et al.*, 2004; Zhong *et al.*, 2013). Given that *Schistosoma mansoni* and *haematobium* can infect the prostate, have inflammation potential and that inflammation and inflammatory signaling can lead to aberrant DNA methylation, it is important to profile the DNA methylome of Africans who live in *Schistosoma*-endemic areas. This will contribute to the understanding of the potential role of these infections on prostate carcinogenesis and improve the management of PCa including schistosoma-induced PCa in Africa.

Since the DNA methylome of PCa in African men has never been profiled, this study therefore sought to firstly, profile the DNA methylation of PCa in an African population (Ghana) and to investigate the potential of *S. haematobium* and *S. mansoni* in prostate carcinogenesis. Employing a multidisciplinary approach encompassing multiomics analyses, cell culture techniques, network modeling, among other methods, this study sought to unravel the intricate molecular mechanisms underlying PCa development in the context of the Ghanaian population, shedding light on the role played by these schistosome infections in influencing carcinogenesis.

1.2 Problem Statement

PCa remains a critical public health issue globally, with significant morbidity and mortality, particularly in Sub-Saharan Africa, where it is the most prevalent malignancy among men. Although treatable when detected early, existing diagnostic tools such as PSA testing, DRE, and biopsies are limited by poor specificity and sensitivity, as well as invasive procedures, leading to

suboptimal outcomes. The underlying molecular mechanisms of PCa are complex, with DNA methylation recognized as a key epigenetic driver of its onset and progression. However, research into DNA methylation in PCa has predominantly focused on non-African populations, leaving the epigenetic landscape of indigenous African populations largely unexplored.

Moreover, infections, particularly with *S. haematobium* and *S. mansoni*, which are endemic in Africa, have been associated to cancer development. While *S. haematobium* infections are well-established as carcinogenic factors in bladder cancer, their potential role in PCa remains uncharacterized. Chronic inflammation caused by these parasites may contribute to aberrant DNA methylation patterns, thereby influencing prostate carcinogenesis. The lack of DNA methylome profiling specific to African populations hinders the development of tailored diagnostic, preventive, and therapeutic strategies for PCa in these regions.

1.3 Rationale

Given the unique genetic and environmental contexts of African populations, reliance on findings from studies on African Americans or Caucasians may not provide applicable insights. Therefore, understanding the epigenetic underpinnings of PCa in indigenous African populations, such as Ghana is critical. Furthermore, Schistosoma infections, which are endemic in Africa, may act as key contributors to PCa through inflammation-induced epigenetic modifications, but their precise role remains unexplored. Profiling the methylome of PCa in Ghanaian patients will not only add to knowledge on the disease but contribute targets that could be tailored for population-specific drug discovery once these markers are confirmed. Furthermore, uncovering activated cellular pathways triggered by Schistosoma soluble egg antigen exposure will further deepen our insight into the underlying molecular mechanisms of potential schistosoma-mediated prostate carcinogenesis. Findings from this study have the potential to pave a way for enhanced clinical strategies and innovative therapies.

1.4 Research questions

1. What is the DNA methylome profile of prostate cancer within the Ghanaian population?
2. Could chronic inflammation arising from prostate schistosomiasis bring about modifications to the DNA methylome, ultimately contributing to the onset of prostate cancer?
3. Could Schistosomes co-opt pathways relevant to cellular transformation via protein interactions leading to prostate carcinogenesis?

1.5 Aim

The aim of this project was to characterize the DNA methylome of prostate cancer in Ghana and investigate the role of *Schistosoma haematobium* and *mansoni* in prostate carcinogenesis.

1.6 Objectives

- 1) To characterize the DNA methylation profile of prostate cancer in Ghanaian patients.
- 2) To predict potential biological processes hijacked by *Schistosoma spp* in prostate carcinogenesis.
- 3) To elucidate the role of *Schistosoma haematobium* infections in the development of prostate cancer.
 - i. To determine the effect of *Schistosoma mansoni* and *haematobium* SEA exposure on oncogenic hallmarks in normal prostate epithelial cells.
 - ii. To determine the transcriptomic profile in SEA-exposed prostate epithelial cells.
 - iii. To investigate the DNA methylation modifications associated with SEA-exposed prostate epithelial cells.
 - iv. To determine activated pathways upon SEA exposure in prostate epithelial cells.



CHAPTER TWO

LITERATURE REVIEW

2.1 Prostate Cancer

2.1.1 The prostate gland

Prostate cancer (PCa) is a malignant growth that originates from the prostate gland. The prostate is a male organ with a walnut shape located below the bladder and surrounding the urethra (Figure 2.1). It weighs between 20-30 grams, measures 3-4 cm in length and is 3-5 cm in width (Patel, 2010). It is anatomically divided into distinct zones such as the anterior fibromuscular stroma (AFMS), central zone (CZ), peripheral zone (PZ), and transitional zone (TZ), each with unique characteristics and functions (Mcneal, 1988). The glandular epithelium consists of various cell types, including basal, luminal, and neuroendocrine cells, which have specific markers and functions. For instance, luminal cells are androgen-dependent and secrete prostate-specific antigen (PSA), while basal cells lack secretory vesicles and express low levels of androgen receptors (Toivanen & Shen, 2017).

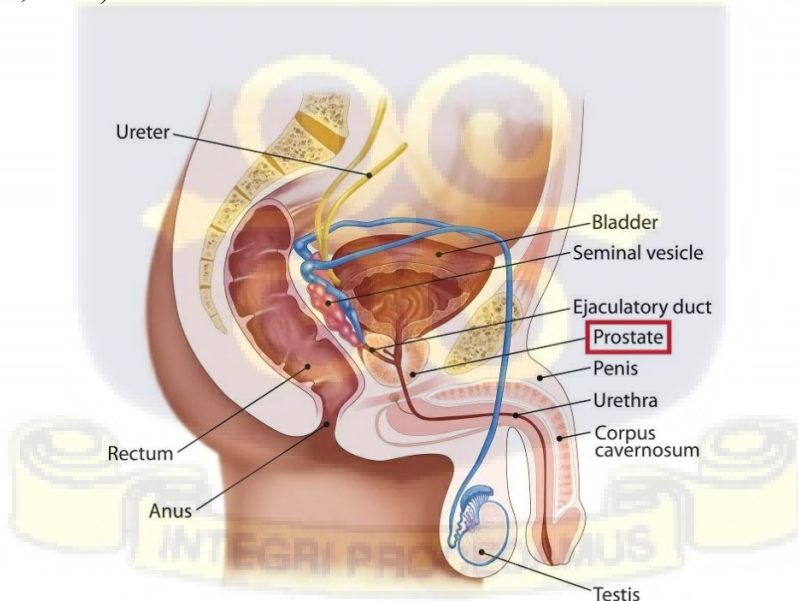


Figure 2.1 Sagittal section of the pelvis provides anatomical view of the position of the prostate gland. Adapted from an image obtained from Adobe Stock (Stock ID: 34775160)

The prostate's primary function is to produce and secrete prostatic fluid. The prostatic fluid contains several essential components including; prostate specific antigen (PSA), prostatic inhibin, prostatic acid phosphatase (PAP), zinc, citric acid and spermine with distinct roles (M. Sharma *et al.*, 2017). For instance, the PSA serves as a biomarker for PCa and plays a role in liquefying seminal coagulum (M. Sharma *et al.*, 2017). PAP may have a role in fertility and concentrations of PAP are higher in PCa patients (Kong & Byun, 2013; R. Sharma *et al.*, 2013).

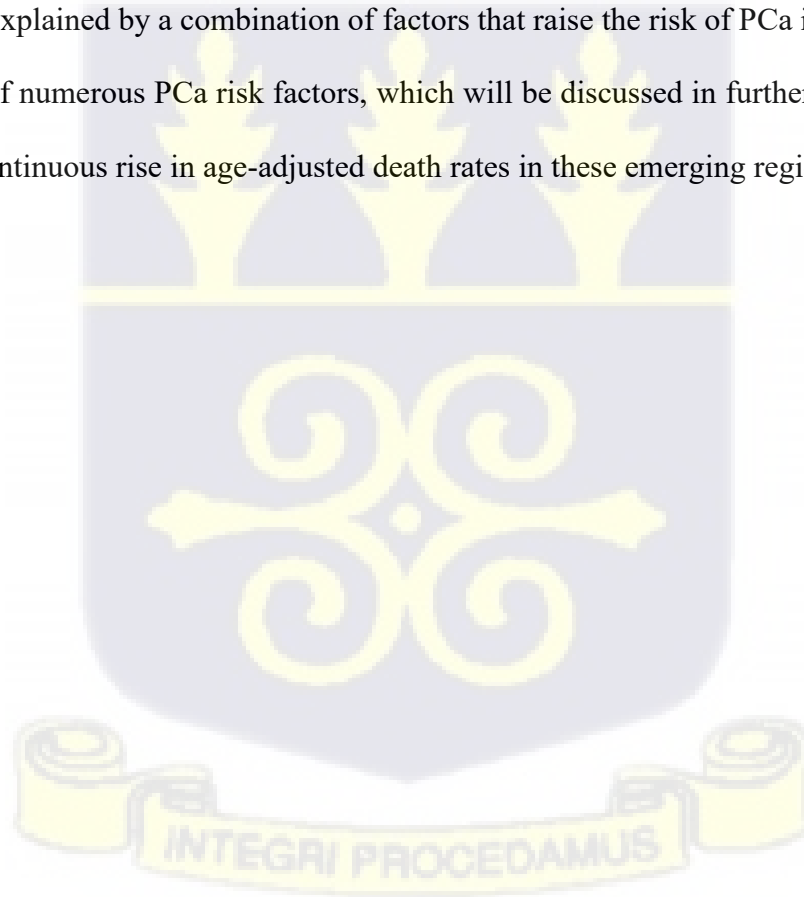
Hormones, growth factors, and other signaling molecules interact to control prostatic activity. Androgens like testosterone and dihydrotestosterone (DHT) regulate gene expression through androgen receptors, which is essential for prostatic growth and function (Davies & Eaton, 1991; P. S. Nelson *et al.*, 2002). Oestrogens have both proliferative and anti-proliferative effects on the tissue of the prostate through the oestrogen receptors alpha (ERa) and beta (ERb) (Risbridger *et al.*, 2007). Prolactin and oxytocin are crucial for ductal morphogenesis and cell proliferation. Thyroid hormones and growth factors including IGF, EGF, and TGF- further regulate prostatic growth and homeostasis (Anguiano *et al.*, 2022; M. Sharma *et al.*, 2017). These elements collectively contribute to the prostate's healthy functioning and are also linked to pathological conditions like prostate cancer.

2.1.2 Incidence and mortality

Millions of men worldwide are affected by PCa, which has a large global impact. It accounts for 7.3% of all new cancer diagnoses in men globally and represent the second most prevalent cancer, following lung cancer in men. Every year, about 1.4 million incident cases are recorded, and an estimated 375,000 deaths are reported (Figure 2.2), the fifth most common cause of cancer mortality in men (Rebello *et al.*, 2021; Sung *et al.*, 2021b). Notably, age is a key risk factor, with people 60 and older accounting for nearly 85% of new cases (Bray *et al.*, 2018).

Due to their ageing populations, the regions with long life expectancies, like the USA and the UK, struggle with higher PCa incidence (Bray *et al.*, 2018). The highest incidence rates worldwide are seen in New Zealand, Australia, North America, and Europe. It is vital to remember that low-income areas now report the lowest incidence rates of PCa, including Central Asia, sub-Saharan Africa, and South Asia (Sung *et al.*, 2021b). However, the annual increase in frequency in these locations is startling (Rebello *et al.*, 2021). Nevertheless, this may be related to growing awareness made possible by easier access to diagnostic screening (Loeb *et al.*, 2014).

Regrettably, the highest age-standardized rates of PCa-related death are found in areas with a significant concentration of underdeveloped countries, such as those in sub-Saharan Africa. This pattern may be explained by a combination of factors that raise the risk of PCa in various regions. The escalation of numerous PCa risk factors, which will be discussed in further detail, may be to blame for the continuous rise in age-adjusted death rates in these emerging regions.



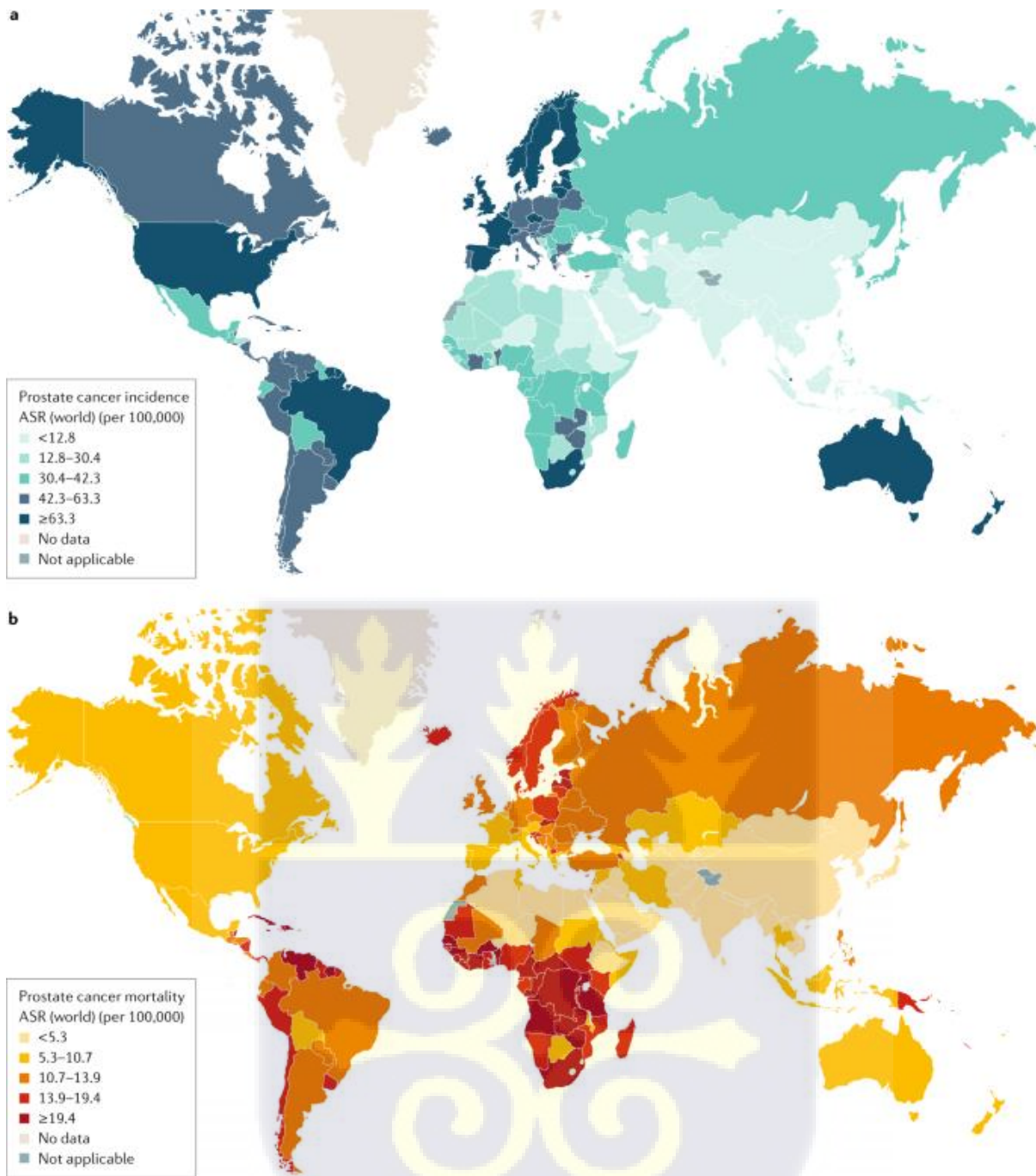
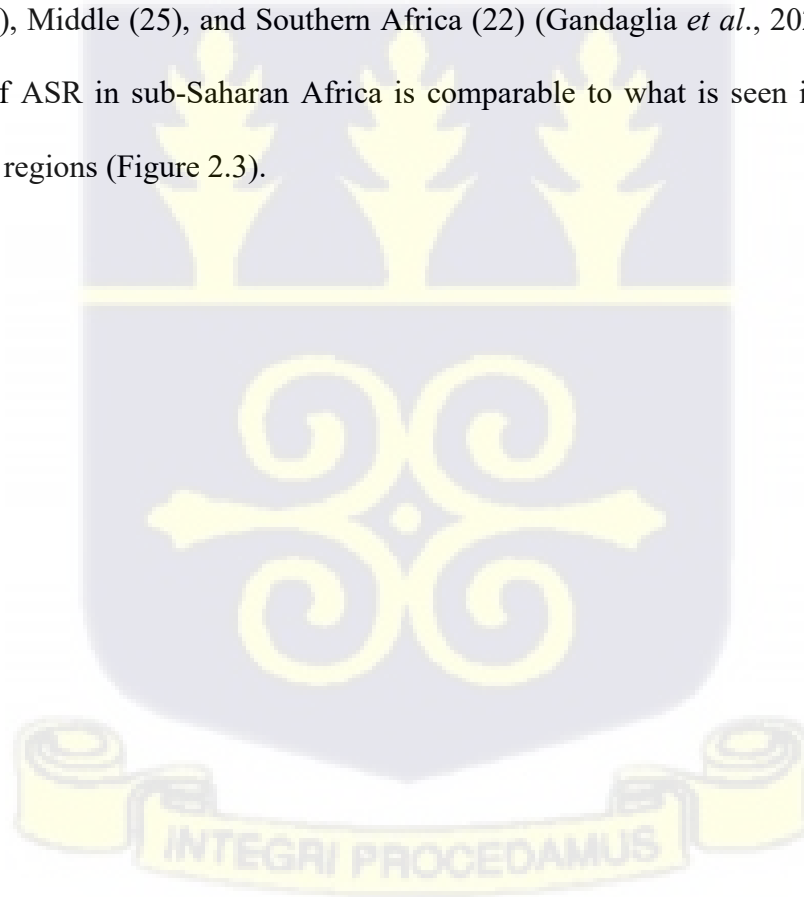


Figure 2.2 Global incidence and mortality trends of PCa. (a) World incidence of PCa in 2018. (b) World mortality from PCa in 2018 are shown as age-standardized rates (ASR), which are adjusted to the World Standard Population to compensate for varying age demographics across regions. The African region is characterized by a disproportionate mortality rate compared to other continents (From Rebello *et al.*, 2021).

It should be emphasized that the overall burden of PCa in Africa does not reflect the regional numbers as there is wide intervariability among its countries. Estimations from 2020 indicate that within the African region, there were 93,171 new cases of PCa and 47,249 fatalities resulting from the disease, with all-age incidence and mortality ASRs being 30 and 16 per 100,000 males, respectively. (Gandaglia *et al.*, 2021; Sharma *et al.*, 2022). Interestingly, in Northern (17), Middle (41), and Southern Africa (66), the all-age incidence ASR significantly differed, ranging from 71 per 100 000 in Zimbabwe to 5.6 per 100 000 in Niger (Gandaglia *et al.*, 2021). The all-age mortality ASR varied widely from 42 per 100 000 in Zimbabwe to 3.8 in Niger and was different in Northern (8.2), Middle (25), and Southern Africa (22) (Gandaglia *et al.*, 2021). This suggests that incidence of ASR in sub-Saharan Africa is comparable to what is seen in Europe and the Americas WHO regions (Figure 2.3).



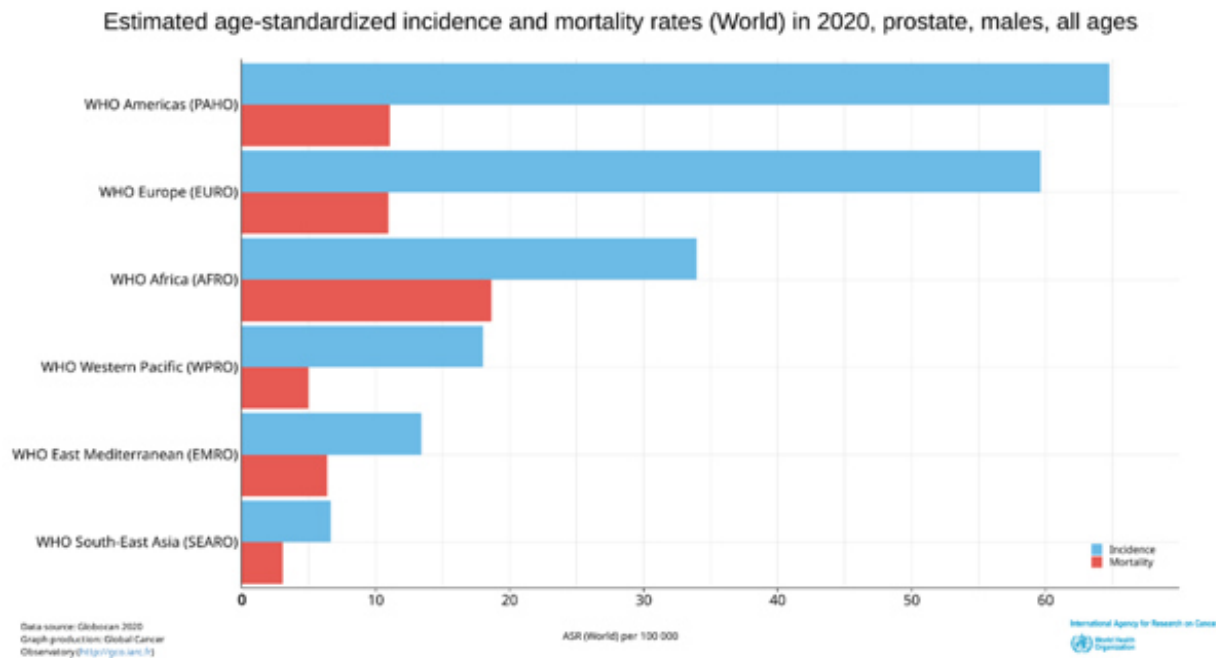


Figure 2.3 Incidence and mortality rates of PCa. The global incidence in the WHO Africa region is far less than that of the Americas and Europe (from cancer today, <http://gco.iarc.fr/today/home>, accessed on 24/08/2023).

2.1.3 Racial disparity

PCa has the largest male cancer health disparity. Black men have a greater than two-fold higher mortality risk compared to other races, while exhibiting an earlier onset and more aggressive disease (DeSantis *et al.*, 2019; Gandaglia *et al.*, 2021; Lowder *et al.*, 2022). Socioeconomic status (SES), environmental exposures, and genetics or biology are a few of the many interconnected elements that contribute to these discrepancies (Lowder *et al.*, 2022; Thorpe *et al.*, 2020). For instance, disparities in PCa outcomes have been attributed mostly to inequities in the healthcare system. However, recent research highlights the crucial role of biology and claims that expanding access to healthcare alone will not completely eliminate racial health disparities. Records show that in 2019, approximately 9.6% of Black men did not have health insurance, in contrast to 16.7%

of Hispanic men and 5.2% of non-Hispanic White men, according to statistics on health coverage in the United States (Keisler-Starkey & Bunch, 2022). Notably, Black men experience the greatest rates of PCa incidence and mortality of any race/ethnic group, even though Hispanic men also face major hurdles to healthcare access (Figure 2.4). In spite of that observation, men of Hispanic descent do not exhibit the same disparity (Simone Giona, 2021).

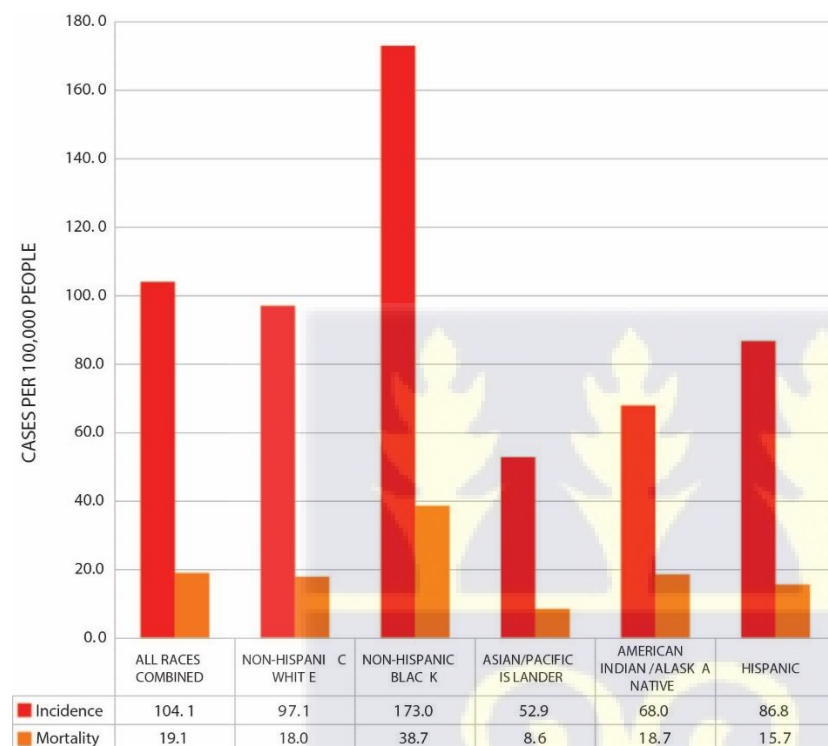


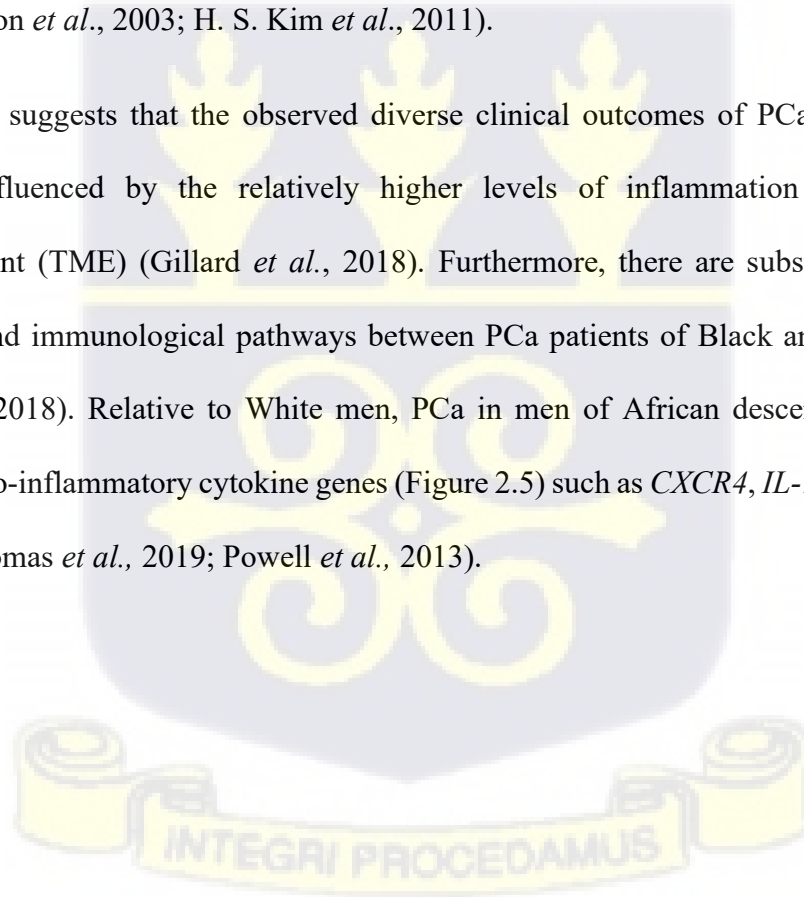
Figure 2.4 Incidence and mortality rates across ethnicities in the USA. Incidence and fatality rates for PCa according to race from 2012 – 2017 in the USA. Non-Hispanic blacks exhibit the highest incidence and mortality rates. (Simone Giona, 2021)

PCa biology varies significantly between ethnicities, and these differences may be a factor in the disparities in health caused by PCa. Black men with PCa display elevated androgen receptor (AR) signaling (Gaston *et al.*, 2003; H. S. Kim *et al.*, 2011; Richard *et al.*, 2014), metabolic

dysregulation, genomic instability, and inflammatory and cytokine signaling in comparison to White men.

For instance, men of African descent have different patterns of signaling for the AR, a transcription factor that is ligand-regulated and essential for the growth and function of the normal prostate as well as the onset and spread of PCa (Loneragan & Tindall, 2011). According to several studies, Black men's free testosterone levels are greater (by as much as 4.9%) than those of White men (Richard *et al.*, 2014). Dihydrotestosterone (DHT) and testosterone are ligands that promote AR signaling. Even after adjusting for Gleason score, studies have observed that Black men with PCa have higher AR protein levels than White men, as well as higher AR somatic and germline hypermutations (Gaston *et al.*, 2003; H. S. Kim *et al.*, 2011).

Recent research suggests that the observed diverse clinical outcomes of PCa in Black men is significantly influenced by the relatively higher levels of inflammation in their tumour microenvironment (TME) (Gillard *et al.*, 2018). Furthermore, there are substantial changes in inflammatory and immunological pathways between PCa patients of Black and White ancestry (Gillard *et al.*, 2018). Relative to White men, PCa in men of African descent exhibits higher expression of pro-inflammatory cytokine genes (Figure 2.5) such as *CXCR4*, *IL-1*, *IL-6*, *IL-8*, *TNF*, and *MMP9* (Thomas *et al.*, 2019; Powell *et al.*, 2013).



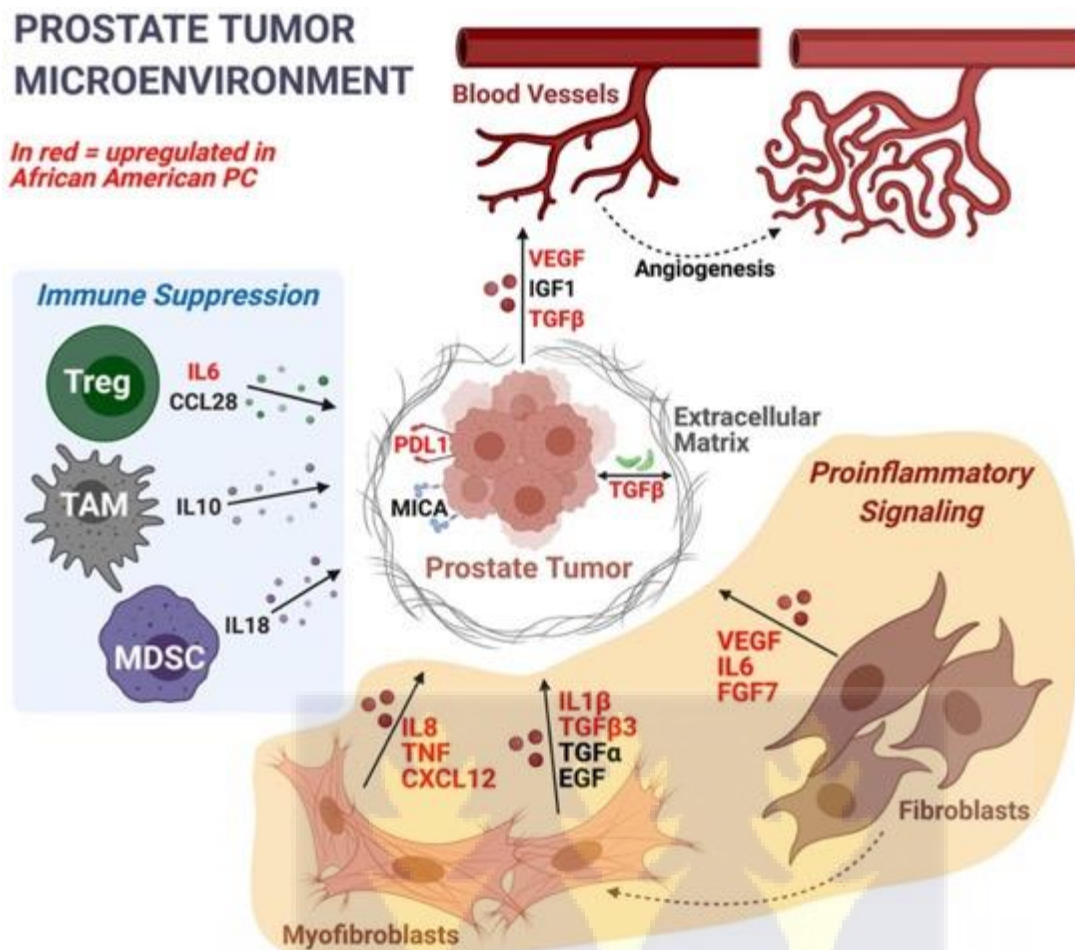


Figure 2.5 Immune profile in PCa varies across race. Most immune/inflammatory genes that are elevated in PCa in Black men have proinflammatory rather than immunosuppressive activities. There is a higher expression of the genes highlighted in red in men of African descent compared to their Caucasian counterparts with PCa. Proinflammatory cytokines such IL-1, IL-6, IL-8, TGFβ, and TNF characterizes the PCa tumour microenvironment. (From Lowder *et al.*, 2022).

Furthermore, several studies have shown that different races and ethnic groups exhibit DNA hypermethylation at different rates in normal and PCa cells (J. W. Kim *et al.*, 2012; Kwabi-Addo *et al.*, 2010; Woodson *et al.*, 2003), these changes may be linked to racial inequalities in PCa. For example, the genes *APC*, *AR*, *ANXA2*, *ANKX2*, *CAV1*, *CD44*, *CDH1*, *EDNRB*, *GSTP1*, *RASSF1*,

RARB, *SPARC*, *TIMP3*, and *AR2*), are extensively more hypermethylated in AA men than in EA men (Kumar et al., 2018). Epigenetic variables are therefore potentially contributing to the racial inequalities in PCa among AA and EA populations and could be targeted for cancer treatment.

Racial disparities in PCa add another layer of complexity to the disease as the causes of these are not fully understood. However, these disparities are closely related to the risk factors associated with PCa as some risk factors are more likely to be present in some populations than others.

2.1.4 Risk factors

Risk factors can be categorized into two: modifiable variables (e.g. lifestyle factors where adjustment may be possible) and non-modifiable factors (such as known family history). Although not well established, some factors could fall in both categories for example, blood-based indicators such as androgen levels and IGF-1, which are affected by hereditary as well as environmental factors (Cuzick *et al.*, 2014).

2.1.4.1 Non-modifiable risk factors

Age

Age is a well known risk factor for PCa, observed across both developed and developing nations. The rapid increase in the incidence of this malignancy is associated with advancing age and is particularly rare in individuals below 40 years (Seraphin *et al.*, 2021). Reports support the link between ageing and prostate cancer as the likelihood of developing this cancer rises from a mere 0.005% in men under 39 to a staggering 13.7% in those aged between 60 and 79 (Stangelberger *et al.*, 2008). Moreover, histological diagnoses reveal that half of the men in the 70-80 age bracket show evidence of malignant prostate cells, although most of these cases are low-grade and follow an indolent course (H. B. Carter et al., 1990).

Additionally, the ageing prostate is a hub for mutagenic activity. Studies show that adult tissues of the prostate, which include histologically normal cells, accumulate mutations over time and also experience tissue dysregulation involving the stromal cells (C. S. Cooper *et al.*, 2015; Freeland *et al.*, 2021). These cells expand with age and influence the growth and function of adjacent epithelial cells through complex crosstalk mechanisms. Changes in the prostatic stroma associated with age have been implicated in the dysregulation of epithelial progenitor cells, thereby contributing to prostate carcinogenesis (Freeland *et al.*, 2021; Untergasser *et al.*, 2005).

Furthermore, aging exerts a profound impact on the prostate's extracellular matrix (ECM), composed mainly of fibrillar collagen (Levesque & Nelson, 2018). While collagen abundance remains relatively stable across age groups, the organization of the ECM deteriorates, giving rise to fragmented fibrils (Bianchi-Frias *et al.*, 2010). The molecular and structural alterations in the ECM and stromal cells not only indicate aging but also contribute to PCa pathogenesis (Freeland *et al.*, 2021).

Age is therefore a complex biological variable that interacts with genetic, molecular, and cellular elements to influence PCa onset and progression.

Race

PCa incidence and mortality rates show significant variation among different countries and geographical regions, and this variation is notably influenced by ethnicity and race (Taitt, 2018). Studies have indicated that men of African ancestry may have a genetic predisposition to PCa, which could play a role in the elevated incidence rates observed in this demographic (Johnson *et al.*, 2021; Samtal *et al.*, 2022). Genome-wide association studies (GWAS) have pinpointed specific susceptibility loci that are independently linked with PCa risk. These findings provide insights into

how germline variations may influence the epidemiological disparities observed across various ethnicities and populations (Al Olama *et al.*, 2014; Du *et al.*, 2020; Takata *et al.*, 2019).

In the United States, the disparities in PCa incidence and mortality rates are particularly striking. African American men exhibit incidence and mortality rates that are approximately double those of Caucasian men and about threefold greater than those observed in Asian Americans. (Siegel *et al.*, 2019). These statistics highlights the necessity of acknowledging race and ethnicity as important risk factors in the diagnosis and management of PCa. The genetic susceptibility loci identified in GWAS offer a potential explanation for these disparities, yet more investigation is required to comprehensively grasp the complex interaction between genetic and environmental factors.

Family history and genetic predisposition

Family history is an important risk factor for PCa, with about 20% of diagnosed men having a paternal or fraternal history of the disease (Hemminki, 2012). The risk of developing PCa is approximately doubled if any first degree relative is affected (Albright *et al.*, 2015). For instance, Grill and colleagues showed that having one first-degree relative diagnosed before the age of 60 increases the risk by 2.5-fold, compared to a 1.6-fold increase for relatives diagnosed at or after 60 years of age (Grill *et al.*, 2015). A study analyzing a Swedish registry indicated that the age-specific likelihood of high-grade disease for men with one affected brother was 3% at age 65 and increased to 8.9% at age 75. Men with two affected brothers faced a 14% chance of developing high-risk cancer at the age of 75, with the odds being greater for those with a stronger family history (Bratt *et al.*, 2016).

Hereditary factors further compound the risk. Having three or more first-degree relatives with PCa, or cases spanning three consecutive generations, or two first-degree relatives diagnosed with early-

onset PCa (<55 years), characterizes a hereditary predisposition to PCa (B. S. Carter *et al.*, 1993). Such hereditary cases can constitute up to 10% of all PCa diagnoses and are typically associated with early-onset disease (Giri & Beebe-Dimmer, 2016).

This genetic predisposition includes uncommon mutations in the *BRCA1*, *HOXB13*, *NBS1*, and *CHEK2* genes that increase the risk of developing PCa (Ni Raghallaigh & Eeles, 2022). Genome-wide association studies have pinpointed over 100 susceptibility loci with low penetrance, each with allele odds ratios (OR) varying from 1 to 13 and possessing high frequencies of these alleles. (Dadaev *et al.*, 2018). For example, men carrying a hereditary mutation in the *HOXB13* gene are at a significantly increased lifetime risk of PCa (Nyberg *et al.*, 2019). When combined in panels, if these loci have a multiplicative effect, they could enhance risk stratification. Apart from their potential as starting points to predict carcinogenic mechanisms, these loci have minimal direct significance on their own. (Dadaev *et al.*, 2018). Family history is a robust predictor of PCa risk, with both familial and hereditary factors contributing to the disease's onset and progression (Beebe-Dimmer *et al.*, 2020).

2.1.4.2 Modifiable risk factors

Several modifiable risk factors have been identified to be associated with PCa. Amongst the plethora of factors are diet, obesity, smoking, alcohol consumption and exposure to infectious agents.

Diet, nutrition, and Obesity

Diet, nutrition, and obesity have been acknowledged as risk factors for PCa (John *et al.*, 2011; Laurent *et al.*, 2016; Perez-Cornago *et al.*, 2017; Vidal *et al.*, 2014; Z. Zhao *et al.*, 2023). For example, a Western-style diet rich in animal products may increase the risk, especially for

aggressive forms of PCa (P.-H. Lin *et al.*, 2015). A fat-rich diet may accelerate PCa progression, while a healthy dietary pattern, such as a Mediterranean-style diet, is associated with a decreased risk (Kenfield *et al.*, 2014). High calcium intake has been positively linked to PCa risk (Aune *et al.*, 2015). On the other hand, adherence to a healthy lifestyle, which includes a balanced diet, may lower the risk of lethal PCa (Plym *et al.*, 2023).

Obesity has been shown to augment the risk of aggressive forms of the disease while decreasing the risk of non-aggressive forms. High body mass index (BMI) at diagnosis and obesity at age 40 are also predictors of biochemical failure in PCa patients (Strom *et al.*, 2005). Diet and lifestyle changes may reduce the risk of PCa progression and death. To sum up, a balanced diet and healthy lifestyle choices can mitigate the risk of PCa, while obesity and certain dietary patterns can exacerbate it.

Smoking and Alcohol consumption

The association between smoking, alcohol intake, and PCa is a subject of ongoing research, and the findings are inconsistent. Some studies have suggested a link between these lifestyle factors and PCa, while others present inconclusive or contradictory evidence.

Various studies have pointed to smoking as a possible risk factor for PCa (Coughlin *et al.*, 1996; Islami *et al.*, 2014; Jochems *et al.*, 2023). For instance, a study by Coughlin *et al.* In 1996 found that cigarette smoking may be a predictor of death from PCa (Coughlin *et al.*, 1996). In alignment with the previous study, Jochems and colleagues reported poor survival rates among patients who smoke, particularly obese smokers (Jochems *et al.*, 2023). However, the precise mechanisms via which smoking influences PCa risk remain unclear. It is hypothesized that the carcinogens in cigarette smoke may promote PCa development or progression (Shui *et al.*, 2016).

The role of alcohol in PCa is even more ambiguous. Some studies, such as the one by Sesso and colleagues found that moderate alcohol consumption is positively associated with the risk of PCa (Sesso *et al.*, 2001). Reports have also indicated that, though alcohol intake is unlikely to affect PCa incidence, it may contribute to disease progression (Brunner *et al.*, 2017). Contrarily, Platz *et al.*, suggest that moderate or greater alcohol consumption is not a strong contributor to PCa risk (Platz *et al.*, 2004).

Given the existing reports, the relationship between smoking, alcohol consumption, and PCa should be addressed cautiously. While smoking appears to have a more definitive link with PCa, the role of alcohol remains debatable. Both factors may interact with other lifestyle and genetic variables, making it challenging to isolate their individual impacts. Therefore, further research is needed to clarify these relationships.

Physical activity

Physical activity appears to have a protective effect against advanced forms of PCa. Extensive physical exercise in early adulthood may lower the incidence of advanced PCa, according to studies (Hrafnkelsdóttir *et al.*, 2015). Similarly, recreational physical exercise has been linked to a lower risk of advanced PCa and PCa-related mortality (Y. Wang *et al.*, 2017). The specific kind and intensity of physical activity is said to be important. Vigorous activities, as opposed to moderate or light activities, have been shown to lower the risk of metastatic PCa (Dai *et al.*, 2019). Nonetheless, it should be mentioned that the overall influence of physical activity on the occurrence of PCa appears to be weak (Y. Liu *et al.*, 2011).

Interventions aimed at increasing physical activity have been proposed to reduce PCa risk reviewed in (Hallward *et al.*, 2020). Moreover, adhering to physical activity guidelines post-diagnosis can significantly reduce mortality risk (Y. Wang *et al.*, 2017). In a nutshell, while

physical activity may not substantially influence the overall incidence of PCa, it appears to offer protective benefits against advanced forms of the disease and improve outcomes for those already diagnosed. Given the inconsistency in the existing literature, further research is needed to establish definitive links between physical activity and PCa risk.

Occupational and chemical exposure

Just like many modifiable risk factors for PCa, the relationship between occupational and chemical exposure and the risk of PCa development has been a subject of extensive research. Some studies suggest that individuals exposed to specific occupational chemicals may be at an elevated risk for PCa. For example, a study observed that exposure to certain occupational chemicals including pesticides could increase PCa risk, although it calls for further research to identify the specific agents involved (Parent & Siemiatycki, 2001). Similarly Krstev and Knutsson reported that pesticides and chromium are amongst occupational risk factors that could be causally linked to PCa (Krstev & Knutsson, 2019). They found that occupational exposures to pesticides and chromium elevated the relative risk of PCa by 1.15 and 1.19 respectively (Krstev & Knutsson, 2019). However, not all research aligns with these findings. A study conducted in Western Australia by Fritschi et al. found no evidence to suggest that selected occupational exposures, including pesticides and metals, are risk factors for PCa (Fritschi *et al.*, 2007). Similarly, Boers and colleagues found that occupational exposure to pesticides was negatively associated with PCa (Boers *et al.*, 2005).

The scientific evidence on occupational and chemical exposure as risk factors for PCa remains inconclusive. While some studies point to a potential increase in risk due to specific occupational exposures, others find no such association or even suggest a protective effect. This inconsistency

calls for more comprehensive, longitudinal studies to better understand the nuanced relationships between occupational and chemical exposures as risk factors for PCa.

Infections and Inflammation

Infections and inflammation in the prostate gland are increasingly being studied for their role in PCa. Examples include chronic prostatitis due to *Escherichia coli*, sexually transmitted infections like *Trichomonas vaginalis* and Human Papillomavirus (HPV), recurrent urinary tract infections (UTIs), and parasitic infections such as schistosomiasis (Choto, Mduluza, Sibanda, *et al.*, 2020; Lawson & Glenn, 2022; Sfanos *et al.*, 2013). These infections and inflammatory conditions can lead to cellular mutations and DNA damage, contributing to PCa onset and progression (Kay *et al.*, 2019; Stark *et al.*, 2015).

2.2 Infection, inflammation in PCa pathophysiology

Inflammation represents the inherent immune reaction to potential harmful stimuli, including infectious agents, tissue injury, and metabolic stress (Antonelli & Kushner, 2017) and is essential for defense. Persistent chronic inflammation can however lead to further tissue damage, releasing reactive oxygen and nitrogen species which will increase cancer risk (Lu *et al.*, 2006). Inflammation involves the activation of signaling pathways, secretion of mediators and cytokines, recruitment of immune cells, and increased vascular permeability (Aguilar-Cazares *et al.*, 2019). The NF- κ B pathway for example is a key inflammatory signaling route, impacting cancer development via cytokine regulation whose activation is linked to various malignancies, including PCa, promoting progression, resistance, and metastasis (Sikka *et al.*, 2021; Staal & Beyaert, 2018; Thomas-Jardin *et al.*, 2020). Inflammation is detected in early PCa onset via proliferative inflammatory atrophy (PIA), which transitions to prostatic intraepithelial neoplasia (PIN) and later

stages including PCa, see Figure 2.6 (W. G. Nelson et al., 2022). The use of anti-inflammatory agents like NSAIDs has shown a reduction in PCa risk, emphasizing the role of inflammation in PCa pathophysiology (Doat *et al.*, 2017).

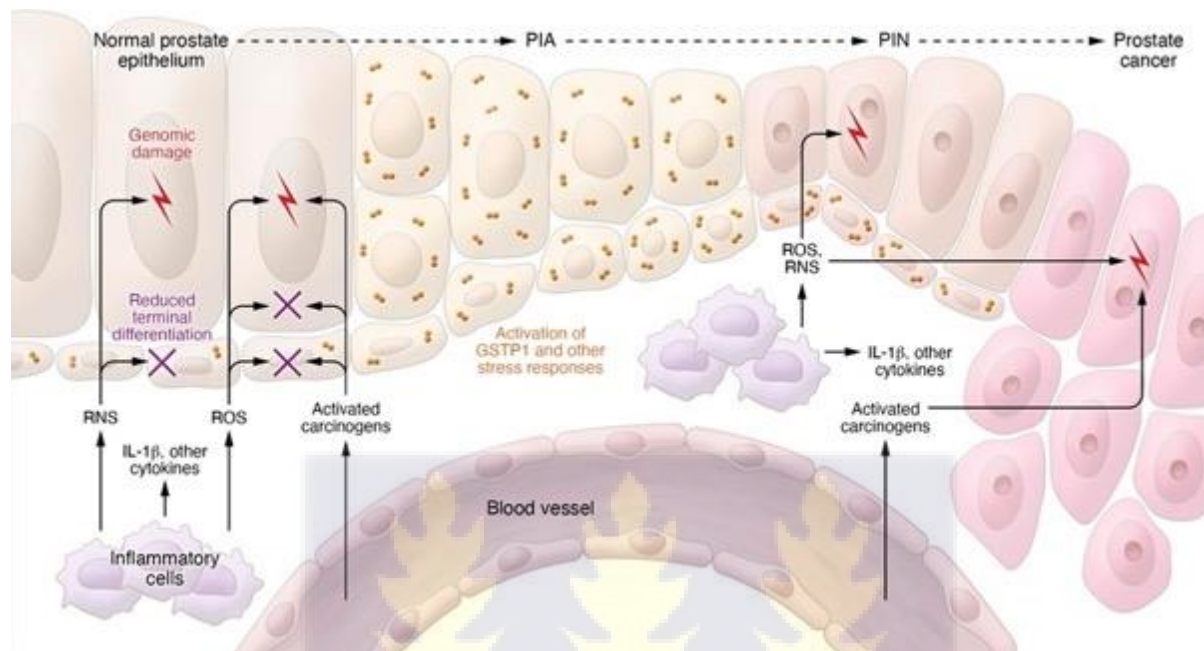


Figure 2.6 PCa friendly microenvironment. Activated dietary carcinogens, inflammatory cytokines, reactive nitrogen species (RNS), and ROS attack the prostate epithelium. The resulting cell and genome damage causes stress response pathways to be activated, reducing terminal differentiation and promoting regenerative growth, which is a hallmark of the PCa precursor proliferative inflammatory atrophy (From Nelson *et al.*, 2022).

The sources that lead to inflammation in the prostate are diverse and can be divided into infectious and non-infectious causes.

2.2.1 Non-infectious causes of inflammation

Prostate inflammation, influenced by a convergence of factors, plays a pivotal role in the onset of prostate diseases, including cancer. Hormonally, sex hormones like androgens and their signaling

orchestrate normal prostate function, while their interplay with inflammatory processes can shape the prostate microenvironment and potentially trigger tumorigenesis (Archer *et al.*, 2020). Androgens also function in regulating or reducing inflammatory mediators. And as individuals age, declining androgen levels and escalating levels of inflammatory cytokines disrupt this balance, setting the stage for chronic inflammation within the prostate (Mohamad *et al.*, 2019; Traish *et al.*, 2018).

Dietary habits also emerged as a relevant contributor to prostate inflammation. Charred meat and fish has been shown to contain carcinogenic compounds like heterocyclic amines (Felton *et al.*, 2007), that have been linked to heightened inflammation-associated PCa (Bylsma & Alexander, 2015), as well as breast and colorectal cancers risk (Chiavarini *et al.*, 2017; Fu *et al.*, 2011). This increased risk was suggested to be caused by chronic inflammation triggered by the major heterocyclic amine compound 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) in a study demonstrating local prostate inflammation in rats (Borowsky *et al.*, 2006; Nakai *et al.*, 2007). Obesity and high-fat diets compound the issue by inducing systemic inflammation that infiltrates prostate tissues, fostering an environment conducive for the onset and progression of cancer (Hsing *et al.*, 2007; Huang *et al.*, 2014). Moreover, traumatic events, such as urine reflux, exacerbate inflammation by inducing the release of pro-inflammatory cytokines within the prostate epithelium, perpetuating a cycle of damage and chronic inflammation (Funahashi *et al.*, 2017).

Genetic predisposition further underscores the multifaceted nature of prostate inflammation. Variants in genes like *RNASEL*, *MSRI*, and *MIC1* have been identified as potential links to heightened PCa risk, with their roles in inflammation and immune function offering insights into disease susceptibility (Archer *et al.*, 2020; W. G. Nelson *et al.*, 2004; Zuo *et al.*, 2017). The *GSTP1* gene, responsible for guarding cells against oxidative stress triggered by inflammation, experiences methylation-related changes that might contribute to PCa's initiation (Martignano *et*

al., 2016; W. G. Nelson *et al.*, 2004). In summary, prostate inflammation can be caused by a number of non-infectious factors that shape the complex relationship between prostate health and disease.

2.2.2 Infectious causes of inflammation

Inflammation of the prostate, often triggered by infectious agents, stands as a critical factor in prostate-related pathologies including cancer. Bacterial infections, such as sexually transmitted infections (STIs), can promote prostatitis, a source of inflammation within the prostate (Sutcliffe *et al.*, 2006). Several bacterial infections have been associated with PCa in one way or the other, including, but not limited to chlamydia and syphilis (Baseri *et al.*, 2021; Hayes *et al.*, 2000; Petyaev *et al.*, 2019). While not all STIs lead to prostatitis, *Neisseria gonorrhoeae*, notably among African American men, has shown an association with an elevated risk of PCa compared to Whites (Lian *et al.*, 2015). Also, the bacteria *Propionibacterium acnes*, has drawn a lot of attention as to its potential role in PCa development (Dadashi *et al.*, 2018; Davidsson *et al.*, 2016; Radej *et al.*, 2022). Davidsson and colleagues for example, showed that *P. acnes* was significantly ($p = 0.001$) more prevalent in men with PCa than in controls, with the bacteria cultured in 60 % of the cases as opposed to 26 % of the controls (Davidsson *et al.*, 2016).

Unlike bacteria, the interplay between viruses like human papillomavirus (HPV) and herpes simplex virus (HSV) and PCa is not well understood, with conflicting conclusions on their potential role in prostate carcinogenesis (Bartoletti *et al.*, 2014; Doble *et al.*, 1991; Ge *et al.*, 2013; Taylor *et al.*, 2005). The emergence of the COVID-19 pandemic has unveiled the potential link between systemic inflammation propelled by viral infections, like SARS-CoV-2, and a heightened risk of tumour development, including within the prostate (Darif *et al.*, 2021; Djomkam *et al.*, 2020; Qudus *et al.*, 2023). Inflammatory cytokines like IL-6, IL-8, and TNF- α , often elevated in response to the virus, have been implicated not only in poor COVID-19 outcomes (Darif *et al.*,

2021) but also in the progression of PCa (Nakashima *et al.*, 2000; J. Sharma *et al.*, 2014). The interaction between the virus and the *TMPRSS2* gene, modulated by androgen signaling, unveils a mechanism for inflammation-triggered genetic alterations that could escalate PCa risk (Chakravarty *et al.*, 2020; Djomkam *et al.*, 2020).

Beyond viruses and bacteria, even though less explored parasitic agents like *Trichomonas vaginalis* and *Schistosoma spp* have also been reported to contribute to prostate inflammation. Considering the disparity in the epidemiology of Schistosomiasis, is it possible that *Schistosoma spp* could contribute to the differences in PCa in Africans? As our understanding evolves, the complex web of infectious causes underlying prostate inflammation continues to unravel, spotlighting opportunities for both heightened awareness and potential therapeutic overlaps in addressing prostate health and infectious diseases.

2.3 Schistosomiasis and PCa

It is estimated that at least 90% of Schistosomiasis cases are in Sub-Saharan Africa, see (World Health Organization, 2022) Figure 2.7. Schistosomiasis ranks as the second most socioeconomically impactful parasitic disease worldwide, trailing only malaria. In the year 2000, the World Health Organization (WHO) estimated that schistosomiasis was responsible for 200,000 deaths annually around the world. (Mbah *et al.*, 2013).



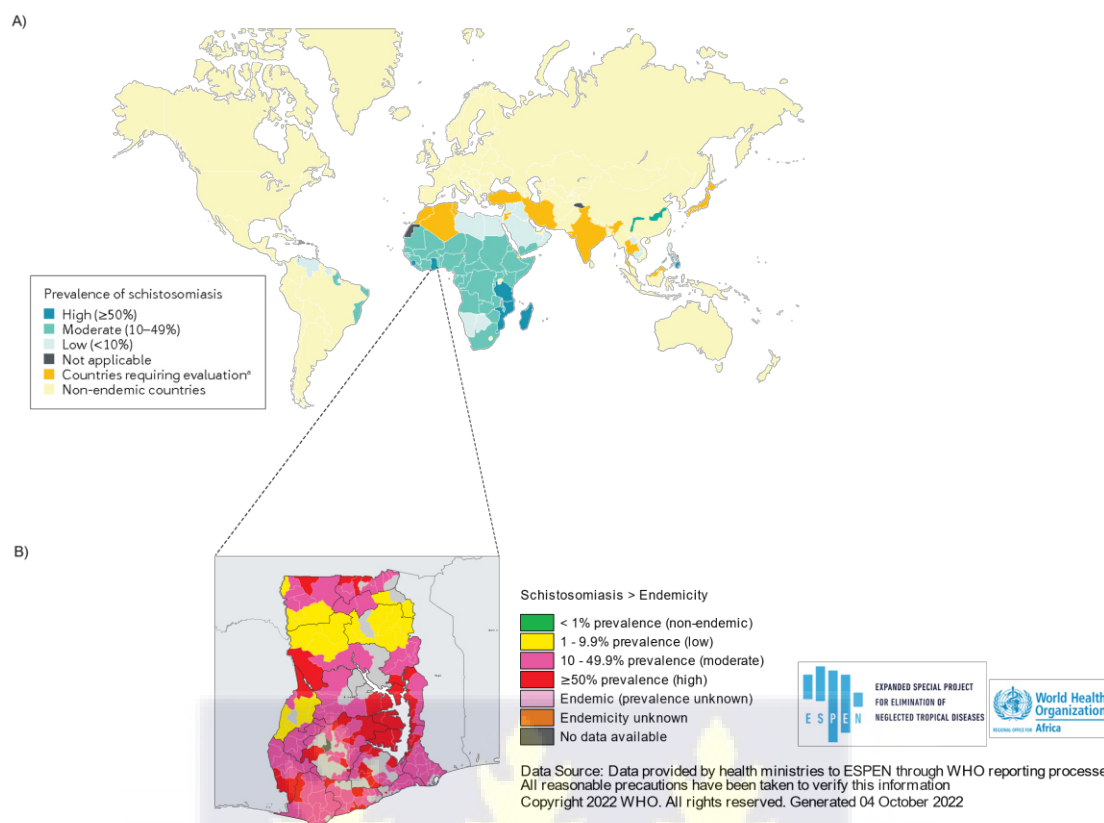


Figure 2.7 Global distribution of schistosomiasis highlighting Ghana. Africa has the highest prevalence of schistosomiasis with Ghana and most regions having a prevalence of 10 – 49%. (Adapted from McManus *et al.*, 2018 and Expanded special project for elimination of neglected tropical diseases (ESPEN), <https://espen.afro.who.int/countries/ghana>).

The urinary causative agent of Schistosomiasis, *Schistosoma haematobium*, has been categorized as a group 1 carcinogen by the International Agency for Research of Cancer (IARC) due to its association with squamous cell carcinoma of the urinary bladder 1 development (IARC Working Group, 1994). Post-mortem studies have shown prostate infections with *S. japonicum*, *S. mansoni*, and *S. haematobium* in endemic areas (Y. Lai *et al.*, 2013; Okani *et al.*, 2013; Patil & Elem, 1988). Findings from these studies mutually propose that prostate schistosomiasis might be a fairly frequent occurrence, ranging from 20% to 50% of cases in two extensive autopsy series of men in Africa (Gelfand *et al.*, 1970; Patil & Elem, 1988). Being a neglected tropical disease, unfortunately

limited research like the previously mentioned studies have been done. There are however a series of case reports reviewed by Choto *et al.*, and Peiffer *et al.*, associating PCa and schistosomiasis (Choto, Mduluz, Sibanda, *et al.*, 2020; Peiffer *et al.*, 2019). In 2018, Tuffour *et al.*, demonstrated in an *in vitro* study that *Schistosoma* soluble egg antigens induces oncogenic hallmarks such as reduced apoptosis in normal prostate epithelial cells (Tuffour *et al.*, 2018). The question that however remains is, do schistosomes have a causal relationship with PCa development or progression? If yes, what are the mechanisms involved?

2.4 Epigenetics in PCa Development and Progression

Although genetic studies have contributed to delineating PCa aetiology, genetics does not fully account for the development and progression of PCa. Also, PCa lack genes that are recurrently mutated in more than one-seventh of cases unlike some other common cancers (Massie *et al.*, 2017). While some mutations like 8p deletions (40% of cases) as well as TMPRSS2-ERG fusions (50% of cases) recur more often, several prostate tumours lack clear genomic driver events (Ross-Adams *et al.*, 2015; Tomlins *et al.*, 2005). Therefore, earlier events preceding somatic mutations and the acquisition of structural variants and copy number alterations could be more valid in the context of PCa. It is now evident that epigenetic alterations present an alternate avenue in understanding PCa aetiology (Dobosy *et al.*, 2007).

The epigenome constitutes a tissue-specific landscape of histone modifications, DNA methylation, nucleosome remodeling and non-coding RNA-associated gene expression changes. PCa is suggested to be a model of ‘epigenetic catastrophe’, especially given the extensive alterations noted in DNA methylation patterns. (Yegnashubramanian *et al.*, 2004). This indicates that epigenetic mechanisms have an important role in PCa pathogenesis. In addition, epigenetics is suspected to be involved in PCa racial disparities (Rai *et al.*, 2018).

2.4.1 Histone modifications in PCa

Histones are crucial components of nucleosomes, the fundamental units of chromatin, and they interact with DNA through electrostatic interactions (Korolev *et al.*, 2007). Post translational changes like acetylation, ADP-ribosylation, methylation, phosphorylation, and ubiquitination occur at specific sites on histone N-terminal tails, influencing chromatin structure and interactions with proteins. These modifications are collectively referred to as "histone code", adding complexity to cellular processes influenced by DNA sequences (R. Liu *et al.*, 2023).

For more than a decade, increasing evidence suggested that histone modification plays a crucial part in PCa onset and advancement. In 2010 for example, Bianco-Miotto *et al.* observed high global levels of H3K4me2 and H3K18Ac which tallied with a 3-fold rise in the risk of PCa reoccurrence in primary and metastatic PCa samples (Bianco-Miotto *et al.*, 2010). Another study indicated significantly reduced global levels of H3K4me1, H3K9me3, H3K9me2, H3Ac and H4Ac in PCa compared to benign prostate hyperplasia and normal prostate tissues (Ellinger *et al.*, 2010). Other studies also showed increasing evidence of modified histone-modifying enzymes expression like the histone methyltransferase, Enhancer of Zeste Homolog 2 (EZH2) alterations in PCa (Gorodetska *et al.*, 2019; Wu *et al.*, 2019) and is now being investigated as a drug target for PCa (Gan *et al.*, 2018). No such studies have however been conducted in a Black African population.

2.4.2 MicroRNA and Epigenetic Alterations in PCa

Epigenetic regulation in cancers including PCa has been shown in the past few decades to be influenced by microRNAs (miRNAs). So far, more than 50 miRNAs have been found dysregulated in PCa (Tang *et al.*, 2011; Wach *et al.*, 2012). In 2010, Cao and colleagues identified that miRNA-101 (miR-101) responds in PCa cell lines, as the androgen agonist R1881 stimulated miR-101 expression. They also showed regulation of EZH2 by miR-101 which is overexpressed in PCa and

acts as an oncogene (Cao *et al.*, 2010). Several miRNAs have been shown to act as oncogenic miRNAs in PCa. Overexpression of miR-221/222 was shown to result in p27 downregulation in LNCaP cells and promoted cellular proliferation (Galardi *et al.*, 2007). There is growing interest in the function of miRNAs and their contribution to carcinogenesis and progression.

2.4.3 DNA methylation in PCa

DNA methylation involves the attachment of methyl groups to cytosine residues, primarily occurring at CpG dinucleotides within gene promoter regions (Shin *et al.*, 2023). This modification can result into gene silencing, interrupting normal cellular processes and promoting cancer development (S. Sharma *et al.*, 2010). DNA methylation has emerged as a significant regulatory mechanism in PCa development and progression (Massie *et al.*, 2017).

Studies have demonstrated aberrant DNA methylation patterns associated with key genes that are integral to cell cycle control, tumour suppression, and DNA repair mechanisms. For example, hypermethylation of DNA repair genes e.g. *GSTP1* and *MGMT* (Kang *et al.*, 2004; Konishi *et al.*, 2002; Lee *et al.*, 1994); cell cycle genes e.g. *CDKN2A* and *RASSF1A* (Jerónimo *et al.*, 2004; L. Liu *et al.*, 2002); growth suppression e.g. *APCA* and *RARb* (Kang *et al.*, 2004; Maruyama *et al.*, 2002); apoptosis-related genes e.g. *TMS1* and *DcR1* (Collard *et al.*, 2006; Das *et al.*, 2006; Shivapurkar *et al.*, 2004); and cell adhesion molecule genes e.g. *CDH1* and *CD44* (L.-C. Li *et al.*, 2001; Lou *et al.*, 1999) have all been identified to be associated with prostate carcinogenesis and/or progression. Additionally, global hypomethylation has been observed, potentially resulting in genomic instability. Some of the genes hypomethylated in PCa include *PLAU*, *HPSE* and *CYP1B1* (Nakashima *et al.*, 2000; Ogishima *et al.*, 2005; Pulukuri *et al.*, 2007). The importance of DNA methylation as a potential biomarker for early detection, prognosis, and therapy is underscored by its capacity to discriminate between benign and prostate cancer tissues. This has been demonstrated particularly for *GSTP1* whose hypermethylation has proven recurrent, found mostly

in malignant tissues and can happen as early as the PIN stage in prostate cell transformation (Brooks *et al.*, 1998; Henrique & Jerónimo, 2004; Yoon *et al.*, 2012). However, it is crucial to acknowledge that these findings predominantly stem from studies conducted on populations of European descent.

Notably absent from the current literature is a comprehensive investigation of DNA methylation patterns in PCa within African populations. Given the genetic diversity across races and geographical regions (Zakharia *et al.*, 2009), it is imperative to extend these studies to African individuals. Africa harbors a unique genetic landscape that might contribute to distinct epigenetic alterations, impacting disease manifestation and treatment outcomes. A comprehensive understanding of DNA methylation patterns in PCa within African populations could offer novel insights into the molecular mechanisms underlying the disease, potentially uncovering biomarkers specific to this population. Such studies would not only address the glaring gap in research representation but also hold the promise of advancing precision medicine approaches tailored to diverse patient populations.

2.5 Mechanisms by which Pathogens Induce Cancer-Associated Epigenetic Alterations

Numerous pathogens have been associated with the emergence of particular malignancies (Morales-Sánchez & Fuentes-Pananá, 2014) and these associations often include distinct epigenetic modifications (Maekita *et al.*, 2006; Van Tine *et al.*, 2004). Sometimes, these epigenetic changes can be directly induced by the pathogen itself, whereas in other cases, they may arise as a consequence of inflammation initiated by the infection. (Djomkam Zune *et al.*, 2021), see Figure 2.8.

Pathogens induce epigenetic changes through diverse mechanisms depending on their classes such as bacteria, fungi, parasites, and viruses (Djomkam Zune *et al.*, 2021). One possible mechanism

involves the induction of aberrant DNA methylation directly by pathogens or indirectly via inflammation triggered by infection. For example, the hepatitis B virus protein X (HBx) directly promotes DNA methylation by upregulating DNA methyltransferases (DNMTs), impacting genes in the Ras pathway and angiogenesis (Calvisi *et al.*, 2007). Also, oncogenic viruses disrupt histone deacetylases (HDACs) resulting to tumour suppressor gene suppression and cell predisposition to tumorigenesis (Chakraborty *et al.*, 2014; Rivière *et al.*, 2015). In contrast, Bacteria like *H. pylori* may directly modify the DNA methylome by inducing intracellular signaling, which causes DNMTs to be activated and injected into gastric epithelial cells via a bacterial type IV secretion system (Low *et al.*, 2014; Sepulveda *et al.*, 2010), or indirectly by inducing inflammation as a result of *H. pylori* infection. *H. pylori* infection indirectly causes chronic inflammation that results in abnormal DNA methylation by impinging on the function of ten-eleven translocation (TET) proteins and other components that contribute to passive and active DNA demethylation via signals from macrophages or nitric oxide (Hattori & Ushijima, 2016). Additionally, certain Gram-positive anaerobic bacteria present in the human colon generate butyrate, which inhibits various HDAC subclasses and might promote carcinogenesis processes (Folkerts *et al.*, 2020).

The field of research focusing on parasite-induced epigenetic changes in human hosts is relatively nascent, with limited but growing evidence. Studies have begun to explore how parasites like *Leishmania*, *Toxoplasma*, and *Theileria* control host cells via epigenetic modulation of host's gene expression (McMaster *et al.*, 2016). For instance, in the medial amygdala of male rats, infection with *Toxoplasma gondii* causes an alteration in the DNA methylation of the arginine vasopressin gene promoter. The hypomethylated arginine vasopressin gene promoter in infected animals increases the expression of this peptide (Hari Dass & Vyas, 2014). Further research is however essential to gain a comprehensive understanding of the epigenetic alterations triggered by parasites and their role in cancer development.

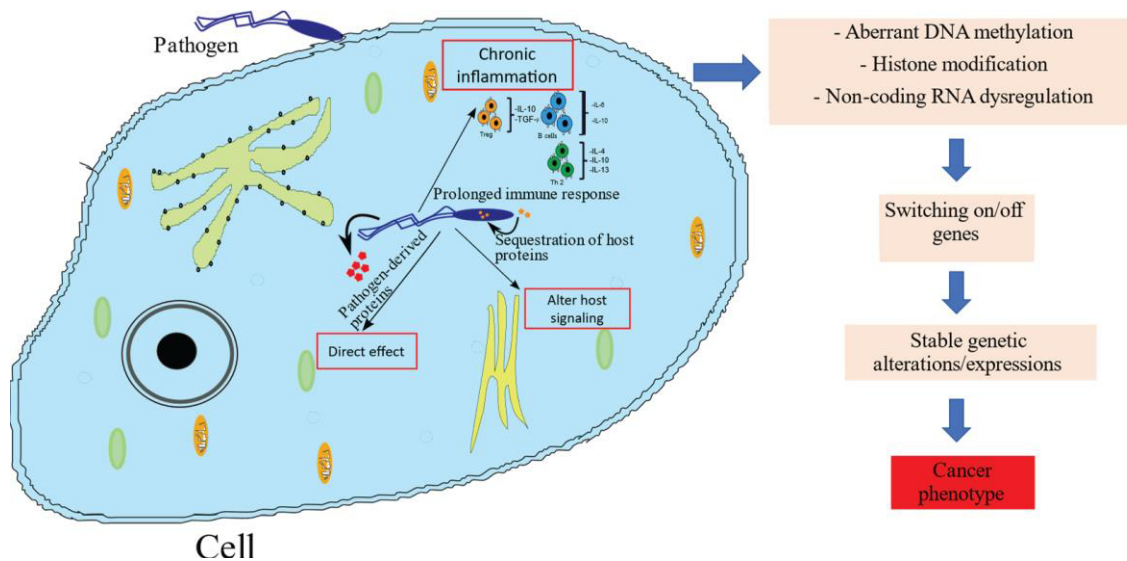


Figure 2.8 Postulated mechanisms of pathogen induced epigenetic alterations driving carcinogenesis. Infectious agents can have a direct or indirect effect on host signaling pathways. In a direct scenario, pathogen proteins will interact with host proteins altering signaling pathways while inflammatory responses to the pathogens can indirectly alter signaling pathways through the alteration of the epigenome (Djomkam Zune *et al.*, 2021).

2.6 Challenges in parasite-induced carcinogenesis research and the choice of the experimental approach.

Establishing a definitive link between inflammation, infection, and PCa poses challenges, as in known associations between infections, chronic inflammation, and other cancers. One key hurdle lies in the relationship between the time of initial infection with a microbial agent, the onset of chronic inflammation, and cancer development. Unlike scenarios where causative infectious agents are highly detectable when cancer emerges (for example HPV in cervical cancer or *H. pylori* in gastric cancer), this might not hold true for PCa (Hansson *et al.*, 1995; Pett & Coleman, 2007).

The hypothesis emerges that in the case of PCa, early-life prostatic infections could induce long-lasting chronic inflammation, independent of the persistent presence of the infectious agent (Sfanos *et al.*, 2013). This concept aligns with evidence from animal models employing *Propionibacterium acnes* to induce prostatic inflammation, revealing the potential for persistent inflammation, possibly even when the infectious agent has subsided (Shinohara *et al.*, 2013). This evolving hypothesis underscores the intricate interplay between inflammation, infection, and PCa, beckoning for further research and understanding.

Therefore, in this study, mindful of this complexity, the experimental approach was to use a more controlled system (*in vitro*) to expose normal prostate epithelial cell lines to *Schistosoma* soluble egg antigens. Then, determine if the pathways activated or modified are similar to what is observed in the DNA methylation profile of PCa patients living in a schistosomiasis endemic country.

2.7 Summary

The reviewed literature highlights the persistent concern of PCa disparities, with men of African descent experiencing more aggressive forms of the disease. Environmental factors, which differ across populations and geographical regions, are crucial to understanding the racial disparity in PCa initiation and aggressiveness. DNA methylation studies have improved the understanding of PCa development and progression, and alterations in the methylome of PCa is thought to contribute to the racial disparity observed. However, Blacks residing in Africa which are at the centre of this disparity (focusing on incidence to mortality ratio) have not had their DNA methylome profiled. Infections also stand out among PCa risk factors as potential contributors to racial disparity observed in PCa due to the biased geographical distribution of some pathogens. Unlike well-established links between infections like *H. pylori* and gastric cancer or HPV and cervical cancer, no infectious agent has been definitively linked to PCa. While there's a well-established connection between *Schistosoma haematobium* and bladder cancer, there are only a few studies that explore

the link between *Schistosoma spp* and PCa. These studies have primarily focused on associations rather than direct causation. The hypothesis is that schistosome infection of the prostate could induce chronic inflammation, leading to modifications in the methylation patterns of the DNA that promote prostate development and/or alterations in signaling pathways that could promote prostate carcinogenesis. Therefore, it is essential to first profile the DNA methylome of PCa in Black men, a gap in the current research and then further investigate the implication of *Schistosma spp* as a PCa risk factor. These endeavors will collectively enhance our understanding of racial disparities in PCa among men of African descent, thereby improving patient management, risk stratification, and prevention strategies.



CHAPTER THREE

DNA METHYLATION PATTERNS ASSOCIATED WITH PROSTATE CANCER AGGRESSIVENESS IN A GHANAIAN PATIENT COHORT

3.1 Introduction

Prostate cancer (PCa) incidence is increasing in sub-Saharan Africa (Necku *et al.*, 2019). PCa is more common than all other malignancies among men in Ghana, with a mortality rate of about 75%, which is partially attributed to late presentation to healthcare facilities (Owoo *et al.*, 2022). Moreover, PCa is the primary cause of cancer-related mortality in Ghanaian men. PCa is known to vary both geographically and in its racial distribution. Men of African ancestry, have been associated with a more aggressive disease and worse outcomes compared to men of European ancestry (Badal *et al.*, 2020).

PCa can progress from an indolent, confined disease to an aggressive metastatic form (Sakellakis *et al.*, 2022). The time of progression in the latter varies from one individual to the other and is important in classifying patients for treatment plans based on predicted prognosis and survival rate (M. Sharma & Miyamoto, 2018). It has been reported that African American (AA) men with indolent PCa progress more rapidly to aggressive disease compared to European Americans (Abern *et al.*, 2013; Iremashvili *et al.*, 2012; Sundi *et al.*, 2015). However, several reports suggest that biological factors like genetic and epigenetic drivers are crucial (reviewed in (Powell & Bollig-Fischer, 2013). These include 8q24 SNPs, *PTEN* deletion, *SPINK1* overexpression, *SPOP* mutation, and *ERG* rearrangement (Khani *et al.*, 2014; Troutman *et al.*, 2012). AA men have higher testosterone levels compared to Caucasian American (CA) men, which may contribute to the increased risk of PCa (Abate & Salini, 2022). Furthermore, AA men exhibit substantial immune-related tumour biology (Wallace *et al.*, 2008) and alterations in epithelial to mesenchymal transition (EMT) signaling in tumour-adjacent stroma which may be related to their aggressive PCa phenotypes (Kinseth *et al.*, 2014; Vijayakumar *et al.*, 2017). These genetic changes might

contribute to the divergence of the cellular niche, which could differentially predispose various populations to PCa aggressiveness.

PCa aetiology and disease progression have been linked to epigenetic regulation, notably abnormal DNA methylation (reviewed in (W. G. Nelson *et al.*, 2009). A common cause of this tumorigenic phenomena is the promoter methylation-mediated silencing of tumour suppressor genes (Rai *et al.*, 2019). The understanding of the global epigenetic processes underlying racial inequality has benefited from certain DNA methylation analyses of both gene specific and genome-wide nature among AA and CA men with PCa (Enokida *et al.*, 2005; Kwabi-Addo *et al.*, 2010; W. Luo *et al.*, 2013). These results collectively stress the existence of race-specific methylation events that are related with either aggressiveness or biochemical recurrence, and may offer prognostic value in determining race-specific treatment regimens and clinical outcomes. Nevertheless, such investigations have not been conducted in an unadmixed African population.

In the present study, a genome-wide DNA methylation analysis was conducted to characterize the DNA methylation patterns in PCa in a ‘pure’ African population, a Ghanaian cohort. The potential mechanisms by which differentially methylated genes may be involved in PCa risk and aggressiveness were investigated by examining pathway enrichment of differentially methylated probes and regions. In addition, associated gene expression analyses based on published datasets as well as network propagations to visualize the network map or interacting partners of methylation associated genes were also examined. Novel differentially methylated genes identified were linked to PCa aggressiveness. Importantly, we further show that these methylation alterations are associated with inflammation-related mechanisms. Together, our results could contribute to understanding racial disparity and PCa management and provide seminal data for further research.

3.2 Methods

3.2.1 Sample collection

Institutional approval was obtained from the Korle-Bu Teaching Hospital after ethical clearance by the Ethics Committee for Basic and Applied Sciences (ECBAS). Archival formalin-fixed paraffin-embedded (FFPE) tissues from treatment naïve patients at initial diagnosis were collected from the Pathology without border (PWB), Accra, Ghana. The slides were reviewed by a pathologist to confirm representative tumour tissue blocks. FFPE blocks with at least 80% tumour cellularity were dissected to represent tumour samples. Non-neoplastic prostate samples were obtained from the adjacent tissues on blocks demonstrating no tumour involvement.

3.2.2 DNA methylation profiling

Genomic DNA was isolated from FFPE samples using the Zymo Research Quick-DNA™ FFPE Kit, according to the manufacturer's instructions. Extracted DNA was quantified by spectrophotometry using a Nanodrop ND-1000 (Nanodrop®). The purity of the DNA was evaluated based on the absorbance ratios at A260/280 and A260/230. DNA samples with an absorbance ratios of approximately 1.70 to 1.90 were considered pure for further experiments. The DNA samples were also visualized on a 1.0% agarose gel, which had been pre-stained with Ethidium Bromide, to estimate the size of the DNA fragments by comparing them with a 1 Kb DNA ladder. (as seen in the supplementary figure denoted as 'S', Figure S3.1).

Following bisulfite conversion, DNA methylation analysis of the samples was performed using the Illumina Infinium methylation EPIC bead chip array, according to the manufacturer's protocol.

3.2.3 Data analysis

The resulting methylated and unmethylated signal intensity data (Idat files) were imported into R 3.4.2 for analysis. Functional normalization was performed using *funnorm* normalization tool with

background correction of the *minfi* package v1.40.0 (Aryee *et al.*, 2014). Probes that had a detection p-value > 0.01 , as well as those identified to have single nucleotide polymorphisms at the CpG interrogation or at the single nucleotide extension sites, and cross-reactive probes, were excluded. The degree of methylation at each probe site was quantified using a beta value, which is derived from the proportion of methylated signal to the sum of methylated and unmethylated signals, with a scale from 0 (no methylation) to 1 (full methylation). A linear regression model analysis tool, the *limma* package v3.50.3 (Ritchie *et al.*, 2015) was used to identify the differentially methylated probes by testing the association of every CpG site with the tumour phenotype (malignant or adjacent non-neoplastic). The design matrix took into consideration the paired nature of the samples. The *p-values* generated were adjusted by employing the eBayes function within the *limma* package, and the Bonferroni method was applied to account for multiple testing. Probes with an adjusted *p-value* < 0.05 and a change in methylation $> 20\%$ was considered to be statistically significant. The *dmpfinder* function from *minfi*, was used to determine differential methylated probes (DMPs) between indolent (low grade, ≤ 7) and aggressive (high grade, ≥ 8) PCa. The Gene set enrichment analysis was carried out using the *Gometh* and *GSAmeth* tool in the *missMethyl* library in R (Phipson *et al.*, 2016) to identify the pathways associated with the DMPs. Furthermore, genes from the most relevant pathways were evaluated for their ability to distinguish between indolent and aggressive tumours in external datasets from the cBioportal database, <https://www.cbioportal.org/> (E. Cerami *et al.*, 2012; Gao *et al.*, 2013).

The *bumphunter* package was used to detect differentially methylated regions (DMRs) via a technique known as bump hunting (Jaffe *et al.*, 2012). This analysis considered regions exhibiting more than a 20% methylation difference between aggressive and indolent tumour samples, with the condition that neighbouring CpGs be no more than 500 base pairs apart. Consistent with the package's recommendations, 1,000 bootstrapping iterations were carried out to determine the

family-wise error rate (FWER). Only regions with at least three consecutive probes and an FWER < 0.1 were selected. Genes associated-DMRs were identified using the UCSC Genome Browser platform, <https://genome.ucsc.edu/> (Kent *et al.*, 2002).

3.2.4 Network propagation

Network propagation analysis for the DMRs-associated genes using the Pathway commons (PC) (Cerami *et al.*, 2011) as the base network was performed. Specifically, a heat-diffusion kernel similar to random walk with restart (RWR, also called insulated diffusion and personalized PageRank) was used, which captures best the local topology of the interaction network compared to a general heat diffusion process. An empirical p-value was calculated for each gene by comparing to 10,000 random permutations in which gene labels were randomly scrambled. Lastly, the 650 most significant genes were selected from the propagation results, induced into a subgraph, clustered into subnetworks using a diffusion state distance metric paired with hierarchical clustering, followed by gene set enrichment analysis on each subnetwork.

3.3 Results

3.3.1 Clinicopathological Characteristics of the Study Group

The study cohort comprised of 16 samples of archival prostate gland tissue whose diagnosis was confirmed by a licensed pathologist. This was composed of 8 diagnoses of prostate adenocarcinoma and 8 matched adjacent non-neoplastic tissues as the control group. The tumour blocks were selected to ensure a minimum of 80% cancer involvement, following the pathologist's review. Subsequently, the tumour samples were categorized into indolent (grade < 4) or aggressive (grade ≥ 4) tumours. There was no overall statistically significant difference between the age of the subjects in the indolent (68.8 ± 8.8) and the aggressive tumour groups (76.5 ± 6.5) (where $p < 0.05$ was considered statistically significant) which implies age was not a confounding factor

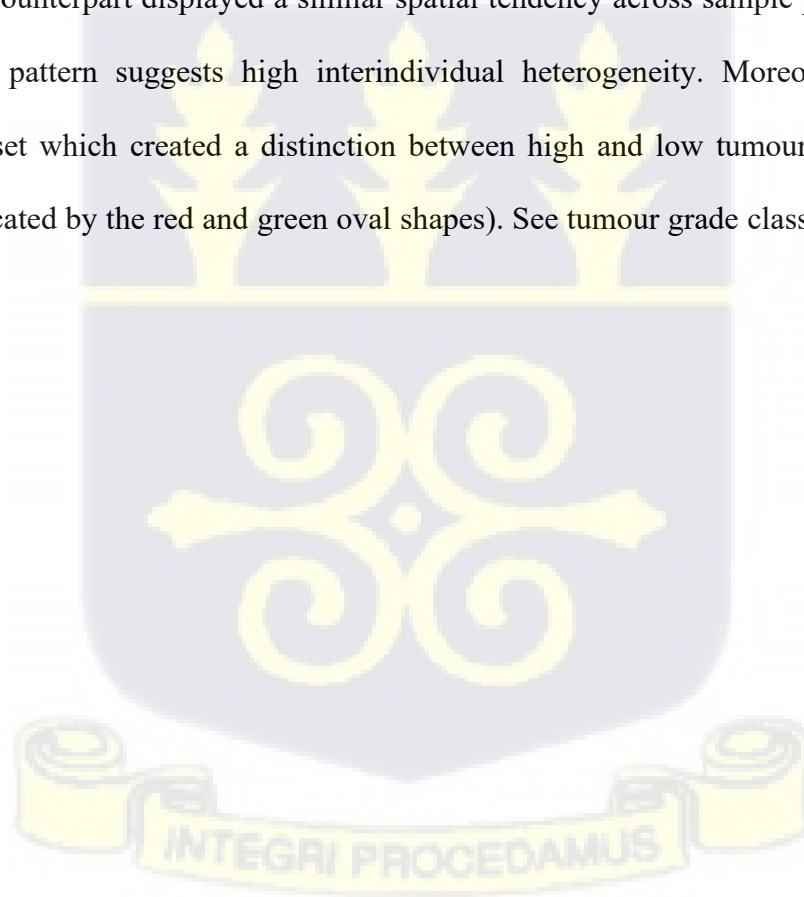
(Table 3.1). Moreover, PSA was significantly different between high and low tumour grades which supports the subsequent findings about the role of inflammation in PCa aggressiveness in this cohort.

Table 3.1. Patient clinocopathologic characterization

Variable	High-risk (Grade ≥ 8)	Low risk (Grade ≤ 7)	p-value
Age at diagnosis			0.4
Mean (years)	76.5	68.8	
Median (years)	76.5	72	
Range age (years)	69-84	56-75	
Preoperative PSA (ng/mL)			0.0188
<4	-	-	
4-10	-	1	
10-20	-	1	
20-100	-	2	
>100	4	-	
Gleason score			0.0058
<7	-	3	
7 (3+4)	-	1	
7 (4+3)	-	-	
>7	4	-	

3.3.2 Consistent offset DNA methylation pattern in samples from patients with high tumour grade.

Altogether, 8 tumour and 8 adjacent non-neoplastic paired FFPE samples were analyzed using the Infinium 850k methylation arrays. The resulting dataset underwent quality evaluation, functional normalization, and was filtered to remove potentially confounding probes (SNP-containing, cross-reacting, and those located in sex chromosomes), and subsequently analyzed using Minfi package (Aryee et al., 2014). A principal component analysis (PCA) performed on all post-filtered 865859 probes could not separate the tumours from adjacent non-neoplastic samples, however, a consistent pattern across each paired sample was observed. The tumour samples with respect to the adjacent non-neoplastic counterpart displayed a similar spatial tendency across sample pairs (Figure 3.1). This consistent pattern suggests high interindividual heterogeneity. Moreover, there was a methylation offset which created a distinction between high and low tumour grade as seen in Figure 3.1 (indicated by the red and green oval shapes). See tumour grade classification in **Table 3.1**.



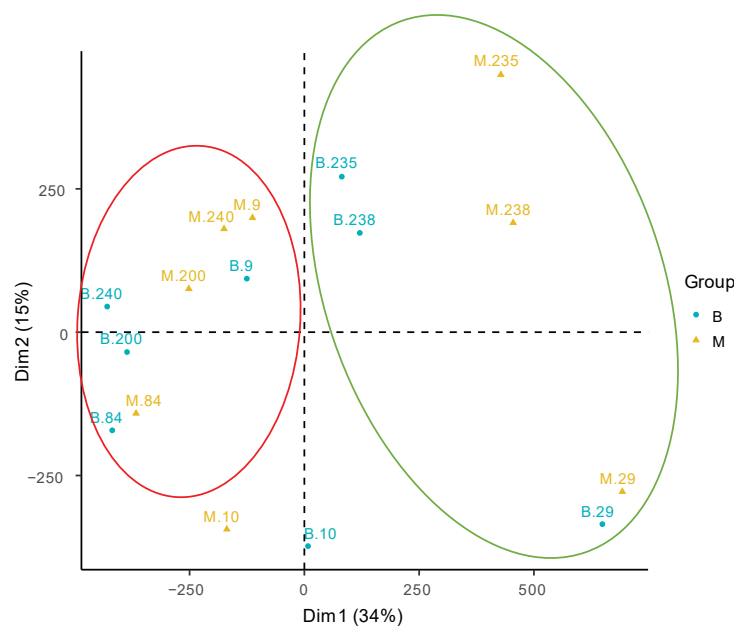


Figure 3.1 DNA methylation pattern between malignant and adjacent non-neoplastic tissues.

PCA based on 865859 probes of 16 samples in our study shows no distinct clusters between tumour (gold triangles, n = 8) and adjacent non-neoplastic (cyan circles, n = 8) tissue groups. The red and green oval indicate clusters of samples from indolent and aggressive cases respectively.

Table 3.1. Tumour grade distribution across samples

Sample	Grade	High	Low
A200	1		✓
A240	1		✓
B9	1		✓
B84	2		✓
A235	4	✓	
A238	5	✓	
B10	4	✓	
B29	4	✓	

To understand the offset of about half of the samples, the *EpiSCORE* tool version 0.9.5 was used to determine the cell-type deconvolution of the samples. This algorithm performs cell-type deconvolution on bulk-tissue mRNA and DNA methylation datasets using a DNA methylation atlas library of tissue-specific mRNA and DNA methylation reference matrices built from high-resolution single-cell RNA-sequencing datasets. The output revealed that samples from patients with tumour grade greater than or equal to 8 had a statistically significant (Wilcoxon Rank sum test, p -value = 0.046) composition of luminal cells compared to the indolent cases in the study cohort (Figure 3.2).

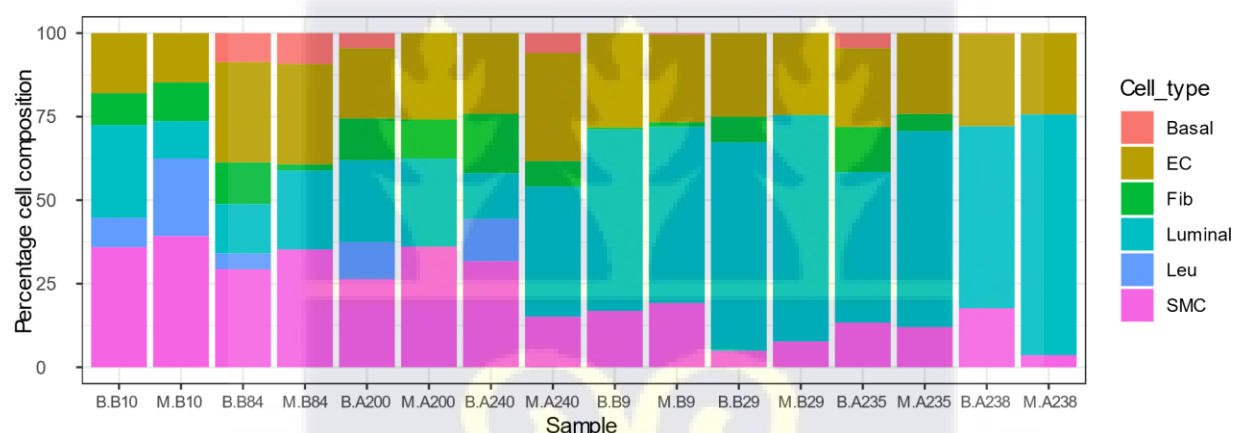


Figure 3.2 Cell deconvolution analyses reveal relative high levels of luminal cells in Ghanaian samples. Episcore tool assessment of cell type composition of study samples. Labels on the x-axes represent individual samples. The cell types assessed are Basal, Endothelial cells (EC), Fibroblasts (Fib), Luminal, Leukocytes (Leu) and smooth muscle cells (SMC).

Luminal cells are secretory cells characterized by their ability to produce PSA and inflammation-related events and have been reported to trigger Luminal cell proliferation. Moreover, these

samples with an offset methylation pattern, relatively high luminal cells are samples from patients with high tumour grade and significantly higher PSA. This suggests that inflammation-related mechanisms could be associated with the offset and phenotype of aggressive tumours. Having observed the clustering of samples based on tumour aggressiveness (Figure 3.1), all subsequent analyses therefore focused on comparing the methylation patterns between indolent and aggressive tumours.

3.3.3 Differentially methylated probes associated with aggressive PCa

To better appreciate the differences between samples associated with aggressiveness observed in Figure 3.1, we calculated a PCA model for stratified tumour samples identified as aggressive (high-risk) and indolent (low risk). All samples with tumour grade ≥ 4 were considered aggressive tumours while those with tumour grade < 4 were grouped as indolent tumours. The PCA plot shows clustering of aggressive and indolent cases apart, with dim1 indicating that 36.3% of the probes can explain the difference observed (Figure 3.3A). Also, it was of interest to determine the potential significance of these DMPs based on the genomic localization enriched. For example, differential methylation in genomic regulatory regions is most likely to have some relevance in the expression of downstream associated genes. This was assessed by calculating the ratio of DMPs to the number of available probes for each region. These analyses demonstrated that the DMPs were enriched in open sea (CpG site not in an island or annotated gene) and 5'UTR genomic regions (Figure 3.3B). Enrichment of DMPs in open sea are associated with intergenic enhancers as opposed to CpG islands which are associated with promoters. Also, open sea DMP-enrichment could influence alternative splicing and subsequently the diversity and function of proteins produced. The DMPs were further characterized into hyper and hypomethylation and thus 89% of DMPs were hypermethylation. Per the results, hypermethylation was the most dominant methylation pattern across all genomic localizations (Figure 3.3C). This may suggest that most

DMP-associated genes related to aggressiveness in the study cohort are potentially silenced or downregulated. In terms of cancer biology, genes that become most likely downregulated or silenced are tumour suppressor genes. These genes are recognized for controlling cell growth and division, as well as pathways that could lead to cancer development and advancement.

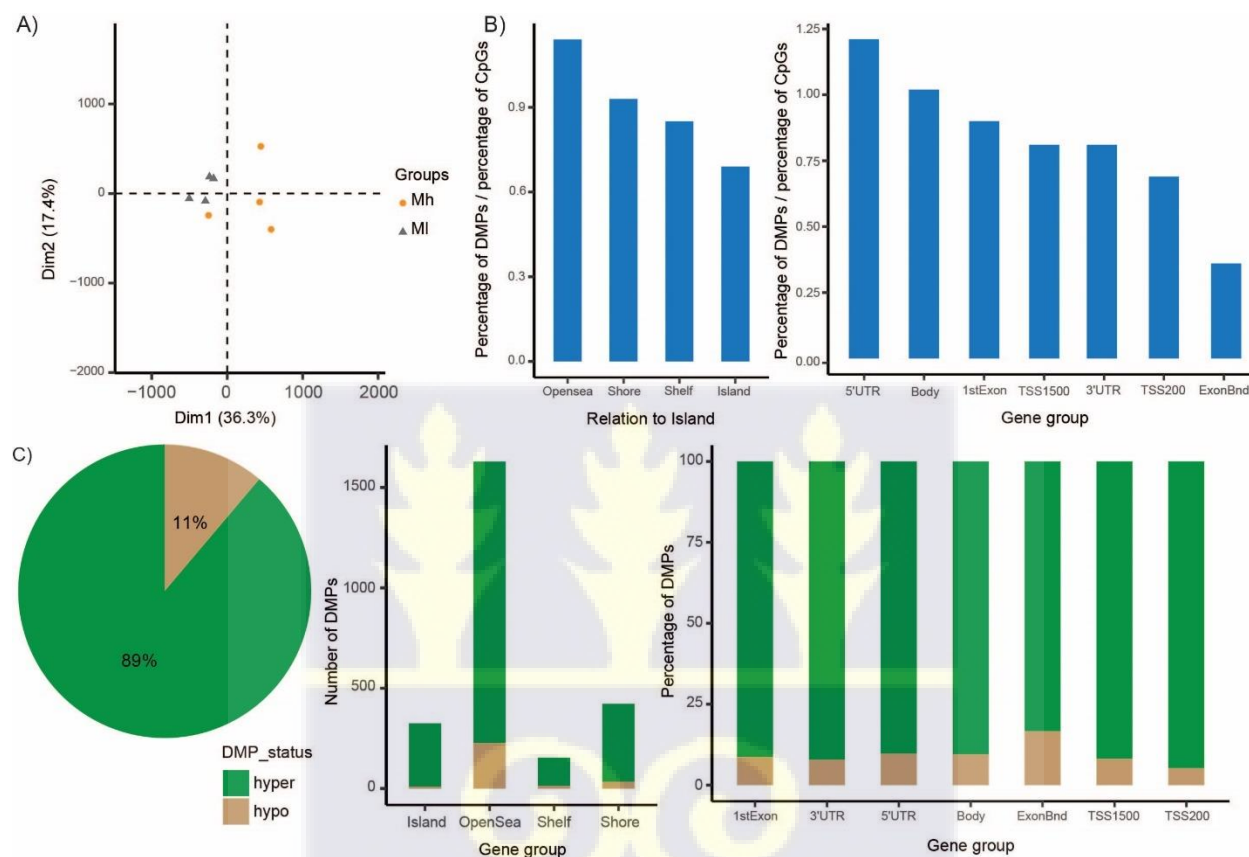


Figure 3.3 DNA methylation distinguishes between indolent and aggressive tumours. (A)

Principal Component Analysis (PCA) shows a separation between indolent (grey) and aggressive tumours (orange). (B) The enrichment of DMPs in different genomic region types and distance from the promoter¹. The enrichment is based on the formular: percentage of DMPs in "group" /

¹ Genomic region types: Island, a CpG site located within a CpG island; Open Sea, a CpG site not in an island or annotated gene; Shelf, a CpG site located > 2 kilobases from a CpG island; Shore, a CpG site located < 2 kilobases from a CpG island;. Distance from promoter: 5'UTR, 5' untranslated region; TSS200, 200 base pairs upstream of the transcription start site; TSS1500, 200-1500 base pairs upstream of the transcription start site; 1st Exon, exon 1; 3'UTR, 3' untranslated region.

percentage of CpGs in "group" to account for biases of regions with high CpG content. (C) The ratios of Hyper-DMPs and Hypo-DMPs in genomic region types and according to their distance from the promoter.

In agreement with our PCA, a linear model comparison of the methylation patterns from the indolent and aggressive cancers using Minfi revealed 2530 significant (adjusted $P < 0.05$, Table S1) DMPs between the 2 groups. The methylation pattern of the DMPs were visualized on a heatmap and revealed that aggressive tumours were characterized by hypermethylation compared to indolent tumours (Figure 3.4A). Lastly, to explore pathways enriched by the DMPs, *gometh* and *gsameth* tools were used to interrogate the gene ontology and the KEGG pathways. Gene ontology pathways indicated that the DMPs are associated to pathways involved in cellular structure including actomyosin and I band. In addition, anatomical structure development, cell development and developmental process ontology pathways were also identified. These developmental processes suggest that the associated methylation is involved in the differentiation state of the tumours (Figure 3.4B). The KEGG pathways on the other hand in which DMPs are enriched included the prostate_cancer_DN pathway which indicates genes that have been shown to be downregulated in prostate cancer (P. Liu *et al.*, 2006) and this corroborates the hypermethylation status of the DMPs. Moreover, TP53 targets as well as methylation associated pathways (methylated in lung and colon cancer, ES with H3K27ME3) were also identified as enriched pathways (Figure 3.4C).

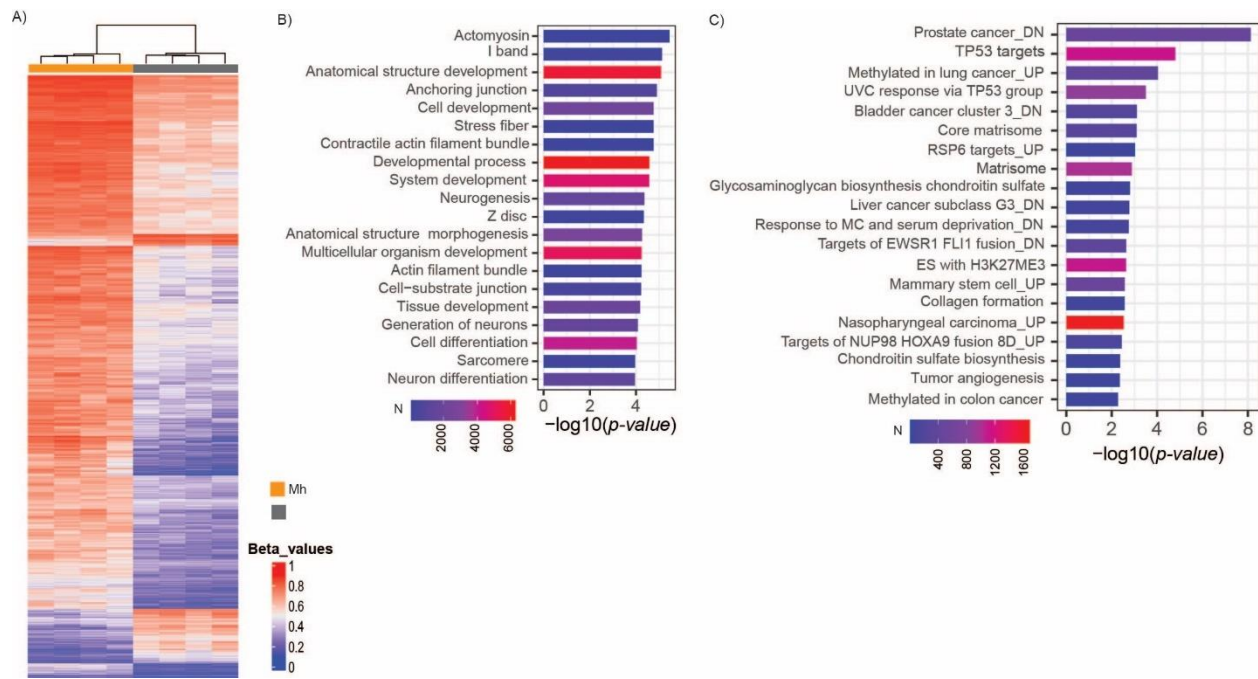


Figure 3.4 DMPs are associated with cell division related pathways. (A) Hierarchical clustering and heatmap display of the methylation levels of DMPs between the indolent (MI) and aggressive (Mh) PCa cases indicate high levels of hypermethylation in aggressive tumours. (B) DMPs enrichment analyses using gometh (left) and (C) gsmeth (right) enrichment functions in R.

3.3.4 TP53 targets expression profiles distinguish between indolent and aggressive tumours.

It was of interest to investigate further how the identified DMPs-associated TP53 targets could differentiate between high and low tumour grades using other datasets and technologies. The focus on TP53 targets stemmed from several reasons, including TP53 being a tumour suppressor gene considered to be the “gate keeper gene” and the second most mutated gene in PCa in the cBioportal database. The list of TP53 targets (1201 genes) were downloaded from the “PEREZ_TP53_TARGETS” gene set on MSigDB (https://www.gsea-msigdb.org/gsea/msigdb/cards/PEREZ_TP53_TARGETS) and the pipeline in Figure 3.5A followed. TP53 targets (70) with associated DMPs amongst the 2530 identified were selected (Figure S3.2). Thereafter, genomics data (including RNA-seq and alteration frequencies) for these

selected TP53 targets was downloaded from the TCGA Firehose study (n = 501) on cBioportal and visualized using R version 4.1.3. The data was categorized according to tumour grade (Figure 3.5B).

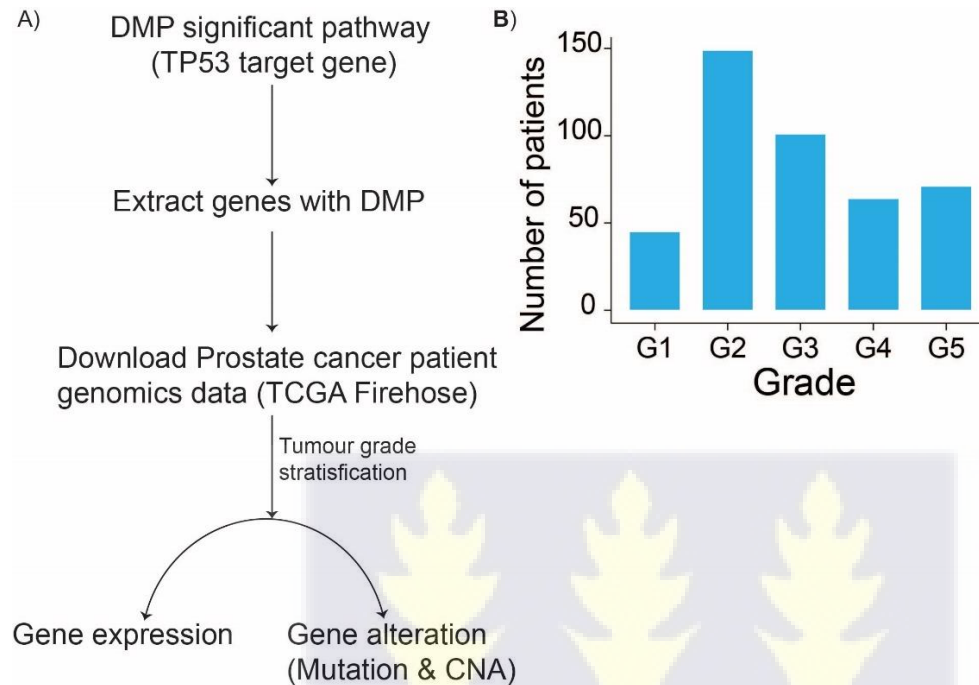


Figure 3.5 DMPs-associated TP53 targets in publicly available genomic data. (A) Flowchart depicting the assessment of alteration frequency and gene expression differences of DMP-associated TP53 targets between high and low-grade tumours. (B) Distribution of patients from the TCGA Firehose study across tumour grades.

The gene expression profiles presented as logFC (log of fold change) across the various grades was visualized on a heatmap and shows a distinct pattern between indolent and aggressive tumours in clusters 1 and 3 (Figure 3.6A). In cluster 1, the indolent tumours (G1 – G3) have a lower expression of these TP53 targets compared to the aggressive tumours (G4 and G5) while the opposite is observed in cluster 3 (Figure 3.6A). Further studies are required to investigate the

methylation dependency of each group, particularly by exploring the distinct functional attributes of genes in cluster 1 compared to those in cluster 3, even though they are both targets of TP53. Also, there is a relative increase in alteration frequency from grade 1 to grade 5 with the aggressive tumours characterized with a slightly higher proportion of gene amplifications (Figure 3.6B). Then, an oncoprint was used to visualize the mutational pattern and burden of each of the DMPs-associated TP53 target gene between the indolent and aggressive tumours (Figure 3.6C). There was no clear mutational signature for PCa aggressiveness, however, it was noticed that for most genes, there was an increased mutation frequency in aggressive tumours compared to indolent tumours.

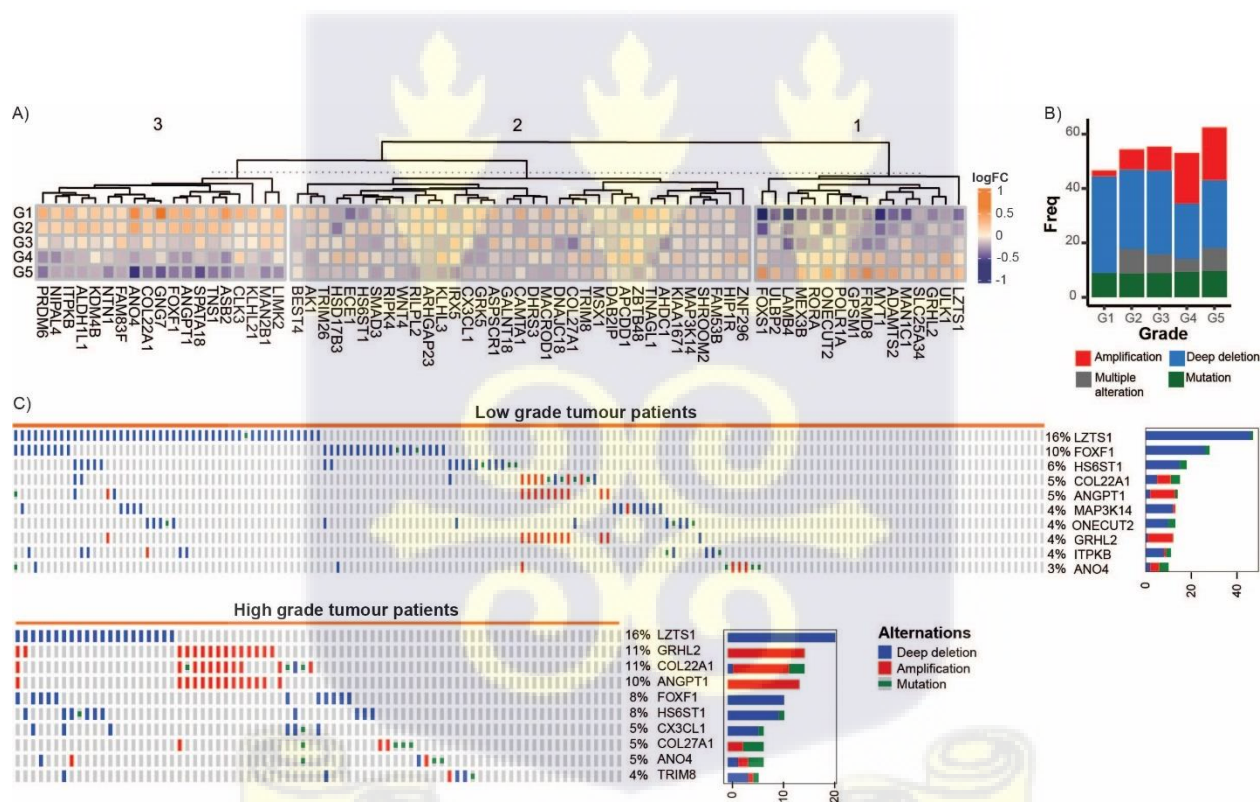


Figure 3.6 TP53 targets distinguish between indolent and aggressive tumours in the TCGA Firehose cohort. (A) RNA-seq data presented as logFC across tumour grades for the DMPs-associated TP53 targets. (B) Genetic alteration types and frequencies for DMPs-associated TP53

targets across tumour grades. (C) Oncoprint of the 10 most mutated DMPs-associated TP53 targets in indolent and aggressive tumours.

3.3.5 Novel methylation markers of PCa aggressiveness.

Since regions are thought to be more biologically meaningful than individual CpG sites (Campagna *et al.*, 2021; Korthauer *et al.*, 2019), DMRs were identified using the *bumphunter* tool in our dataset. Overall, 35 DMRs were found between aggressive and indolent PCa at FWER < 0.1. Next, to identify the genes associated with these regions, the UCSC genome browser was used based on the genomic positions to manually determine the associated genes (Table S2). Only genes at the exact DMRs positions were retained for further analyses. In total, 30 genes were detected, including the novel differential DNA methylation of *DUOX1*, *CCDC169-SOHLH2*, *AKR7A3*, *PAX9*, *ADAM8*, *SND1*, *GPR88*, *TPH1*, *FOXG1*, *DNAH10*, *AIFM2* and *GFRA3* in PCa. Other already reported DMR-associated genes were also identified including *AOX1*, *APC*, *RHCG*, *SCGB3A1*, *HLA-G*, *PON3*, *ONECUT2* (Table 3.2). Figure 3.8A shows the mean beta values of probes associated with the selected DMRs-associated genes with statistical significance between indolent and aggressive tumours in Ghanaian men. About 96% of DMRs were in the promoter regions of the genes with a hypermethylation profile observed in high grade tumours (Figure 3.7A). To understand the pathways linked to the DMR-associated genes, gene ontology enrichment analyses using the *goregion* tool of the missMethyl package v1.28.0 was performed. The top enriched pathways were, metabolism of reactive oxygen species, oxidoreductase activity, and the alpha9-beta1 integrin-ADAM8 complex (Figure 3.7B). This suggests varying oxidative stress responses between aggressive and indolent tumours to various factors including, but not limited to androgen receptor signaling and chronic inflammation of the prostate. Meanwhile, changes in the alpha9-beta1 integrin-ADAM8 complex pathway could indicate differences in cell adhesion and migration, potentially impacting the tumours' metastatic potential.

Table 3.2. DMR-associated genes

GENE	p.value	FWER
<i>DUOX1</i>	2.27E-06	0.032
<i>CCDC169-SOHLH2</i>	2.27E-06	0.032
<i>AKR7A3</i>	6.80E-06	0.032
<i>PAX9</i>	4.53E-06	0.032
<i>LEP</i>	6.80E-06	0.032
<i>AOX1</i>	6.09E-06	0.054
<i>PROM1</i>	9.91E-06	0.054
<i>SCGB3A1</i>	6.09E-06	0.054
<i>CCDC181</i>	6.09E-06	0.054
<i>ADAM8</i>	1.06E-05	0.054
<i>SND1</i>	1.97E-05	0.054
<i>LRRC4</i>	1.97E-05	0.054
<i>ZNRD1</i>	4.11E-06	0.058
<i>HLA-G</i>	4.11E-06	0.058
<i>GPR88</i>	6.37E-06	0.058
<i>CIBAR1-DT</i>	1.32E-05	0.058
<i>TPH1</i>	5.52E-06	0.078
<i>FOXG1</i>	7.93E-06	0.08
<i>PON3</i>	1.86E-05	0.08
<i>DNAH10</i>	1.25E-05	0.08
<i>AIFM2</i>	1.86E-05	0.08
<i>ONECUT2</i>	1.25E-05	0.08
<i>GFRA3</i>	1.47E-05	0.08
<i>GPX3</i>	2.15E-05	0.08
<i>HOXC10</i>	2.61E-05	0.08
<i>JDP2</i>	2.42E-05	0.08
<i>SLC34A2</i>	1.86E-05	0.08
<i>RHCG</i>	2.08E-05	0.08
<i>MIR129-2</i>	2.08E-05	0.08
<i>APC</i>	8.50E-06	0.088

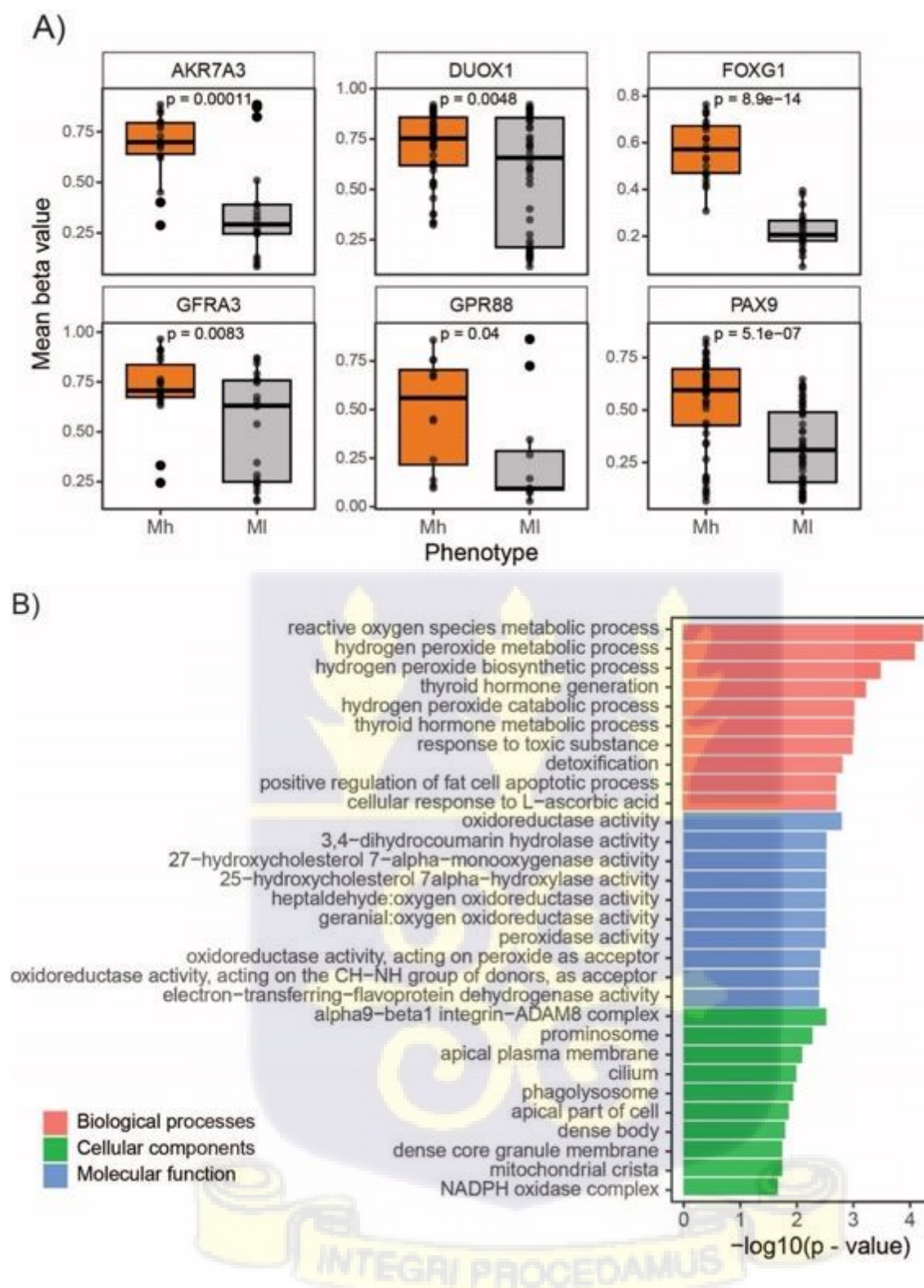
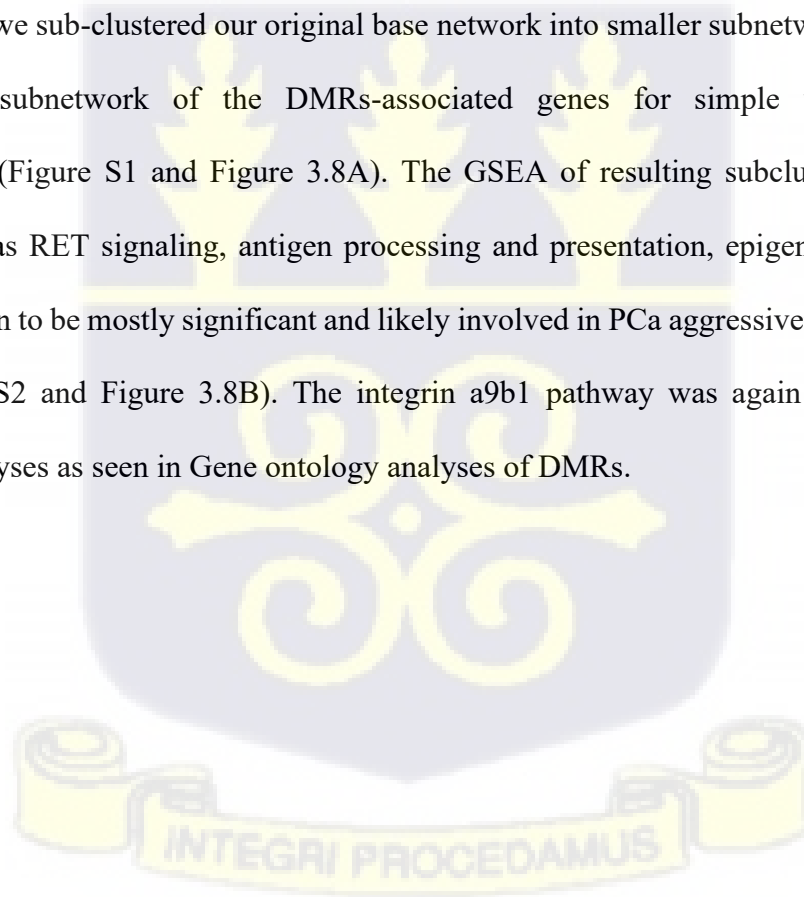


Figure 3.7 DMRs associated with PCa aggressiveness. (A) Box plot of selected DMR-associated genes between indolent and aggressive PCa. Each point on the box plot represents the mean beta

value of a specific probe associated to the respective gene. (B) Visualization of GO pathways of DMRs.

Thereafter, network propagation was used to further understand the connections between the DMRs-genes at the network level. This strategy simulates a heat diffusion process to spread a signal from genes associated with DMRs to nearby genes, which are linked based on previously established functional and/or physical relationships between proteins. Through this procedure, a group of genes is expanded into the molecular networks in which they function. To include both physical and functional interactions, we selected Pathway Commons as our base network. We chose propagation pairs with high propagation scores (Top 650 nodes with $p < 0.05$) to build the network. Then, we sub-clustered our original base network into smaller subnetworks (30 clusters) and extracted subnetwork of the DMRs-associated genes for simple visualization and comprehension (Figure S1 and Figure 3.8A). The GSEA of resulting subclusters suggest that pathways such as RET signaling, antigen processing and presentation, epigenetic regulation of rRNA expression to be mostly significant and likely involved in PCa aggressiveness in the studied cohort (Figure S2 and Figure 3.8B). The integrin $\alpha 9 \beta 1$ pathway was again identified in this enrichment analyses as seen in Gene ontology analyses of DMRs.



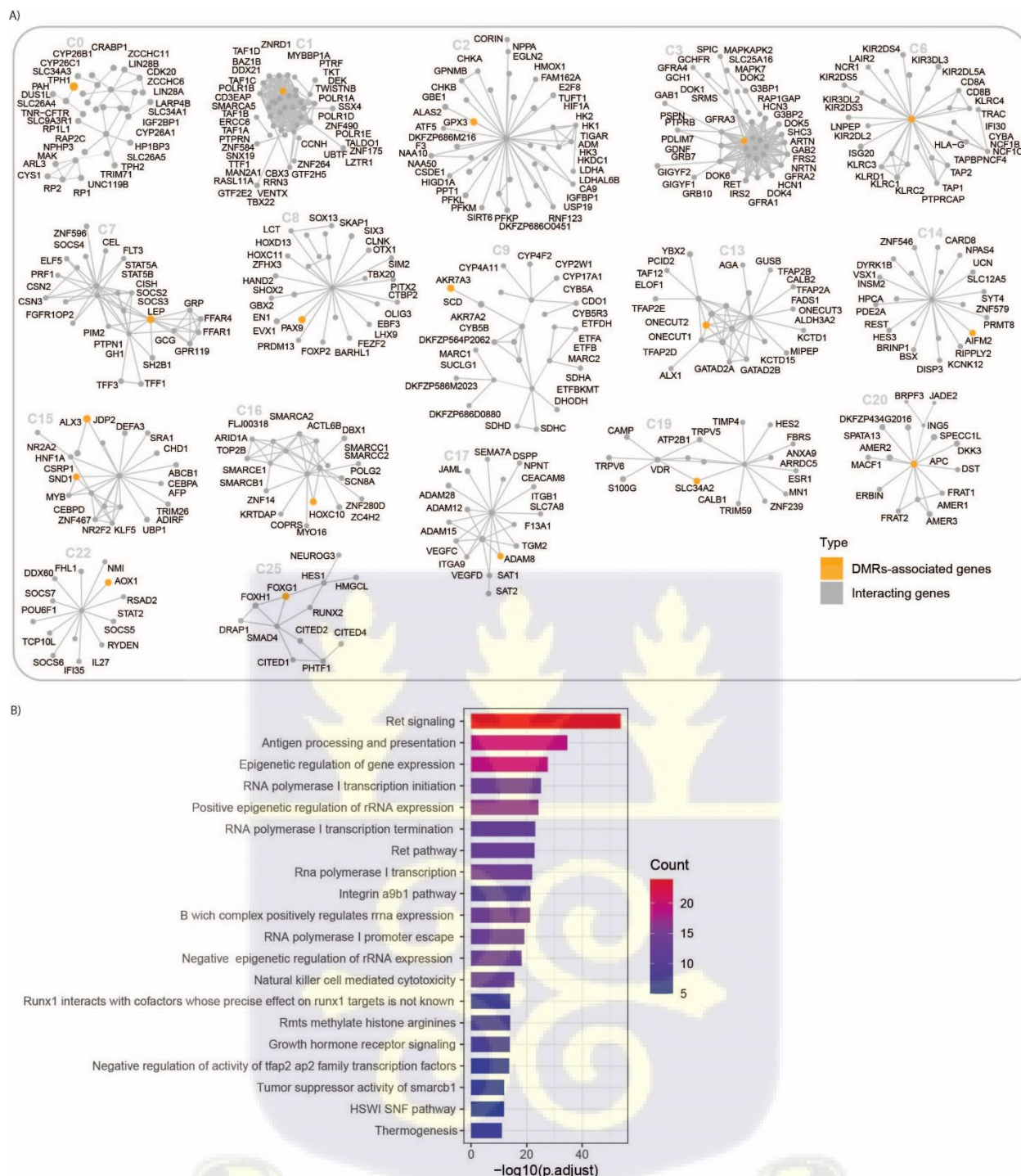


Figure 3.8. Network Propagation of DMRs-associated genes reveals functional clusters with distinct biological functions. (A) Biological subclusters from network propagation. Cluster number refers to the enrichment analysis of distinct biological processes for each cluster, displayed in Figure S3.3. The seed genes are in bold, and their associated nodes are represented by orange

circles. (B) Pathway enrichment analysis was performed on each subcluster from the network propagation. P-values were calculated by hypergeometric test, and a false-discovery rate (Benjamini-Hochberg) was used to account for multiple hypothesis testing.

3.4 Discussion

Numerous research efforts have been dedicated to identifying genetic and epigenetic indicators for diagnosing and predicting PCa. DNA methylation, a type of epigenetic alteration, can be influenced by various elements, and the unusual hypermethylation of promoter regions is linked to multiple cancer types. (L.-C. Li *et al.*, 2004). Considerable work has been directed towards evaluating the diagnostic and prognostic significance of individual hypermethylated genes. Hypermethylation of the *GSTP1* promoter is the most extensively researched epigenetic change in PCa, identified in as many as 90% of prostate tumours (Nakayama *et al.*, 2003). Also, the *RASSF1A* and *APCA* promoters are methylated in up to 53% and 30% of PCa tumours, respectively (Kang *et al.*, 2004; Kuzmin *et al.*, 2002). While these and more genes are recognized for being commonly methylated in PCa, their application as diagnostic biomarkers is constrained due to the lack of specificity of their hypermethylation to prostate tumours, which diminishes their effectiveness as markers for PCa. Nonetheless, a set of genes that are uniquely hypermethylated in prostate tumour tissues could offer a precise and sensitive method for the disease's diagnosis and prognosis. Moreover, considering the racial disparities that exist in PCa mortality in men of African descent compared to men of other ethnicities, there is a need to identify population-specific markers to improve PCa management. Despite that, there is no study so far that has attempted to profile the DNA methylome in PCa patients of Africans. This pilot study therefore represents the first effort to profile the DNA methylome of PCa in Africa.

Our study demonstrated a consistent offset as about half of the benign samples shifted to the right with malignant samples. These samples showing a consistent offset pattern also had relatively high

levels of luminal cells, which are secretory cells involved in PSA secretion. A major cause of increased PSA secretion by the prostate gland is inflammatory responses which suggest therefore that the consistent offset could be because of the effect of the microenvironment on adjacent non-neoplastic cells (Krušlin *et al.*, 2017). An inflamed microenvironment for example could result in adjacent non-neoplastic cells acquiring molecular tumour-like characteristics (Abu-Remaileh *et al.*, 2015). However, this hypothesis will need to be tested to validate the claims. It has previously been reported that, chronic inflammation could lead to a conserved epigenetic program in benign and malignant tissues in the colon (Abu-Remaileh *et al.*, 2015). Chronic inflammation has been associated with several factors including diet (W. G. Nelson *et al.*, 2002), hormones (Naslund *et al.*, 1988) or infections. It is important to note that Ghanaians and other Africans are a population living in the hub of infections. Many of these infections have the potential to cause chronic inflammation of prostate tissues (Boutayeb, 2010) and this could explain the offset observed.

It was further observed that indolent tumours clustered away from aggressive tumours. Analyses focusing on the tumour group (indolent vs aggressive) revealed 2530 DMPs (FDR < 0.05) and 35 DMRs. Gene set enrichment analyses of the DMPs revealed pathways associated with cellular structure and division. Focusing on TP53 targets, it was discovered that DMPs associated targets could differentiate between indolent and aggressive cancers, showing the relevance of these markers not only in this population. The mechanisms involved, however seem to be different in that, these differential targets were characterized by other gene variations such as copy number variations rather than DNA methylation. The identified DMRs were dominated by hypermethyations in gene promoter regions. *AOX1*, *APC*, *RHCG*, *SCGB3A1*, *HLA-G*, *PON3*, *ONECUT2*, *GPX3*, *LEP*, *PROM1*, *LRRC4*, *SLC34A2*, *CCDC181* hypermethylation previously reported to be associated with PCa biochemical recurrence as well as poor prognosis, was detected in the present study to be associated with PCa aggressiveness. Among the 35 differentially

methyated genes in the aggressive cases, this is the first report of DNA hypermethylation of *DUOX1*, *CCDC169-SOHLH2*, *AKR7A3*, *PAX9*, *ADAM8*, *SND1*, *GPR88*, *TPH1*, *FOXG1*, *DNAH10* and *AIFM2* in PCa. Some of these genes were investigated for their expression levels which corroborates with their hypermethylation status reported in the present study and/or their methylation status in other cancer types. For example, Rani *et al.*, demonstrated hypermethylation-downregulation of *PAX9* which was associated with poor prognosis in chronic lymphocytic leukemia and could serve as a potential marker (L. Rani *et al.*, 2017). Fritzsche and colleagues, showed that low expression of ADAM8 in PCa patients was associated with parameters of poor prognosis such as high Gleason scores (Fritzsche *et al.*, 2006). In 2007, Valkovskaya *et al.*, showed that ADAM8 silencing was associated with increased invasiveness and poor survival in pancreatic cancer (Valkovskaya *et al.*, 2007).

Gene ontology of the DMRs suggests that oxidoreductase activities determine the PCa aggressiveness in this population with reactive oxygen species (ROS) processes being the most significantly associated pathway. A variety of factors promote ROS generation including, infections, NADPH oxidase, cytokines and growth factors (HGF and TGF- β) (D'Autréaux & Toledano, 2007; Schieber & Chandel, 2014; Urao *et al.*, 2008). Moreover, a poor lifestyle (including alcohol consumption), hypoxia, exposure to various carcinogens and ionizing radiation, and chemotherapy drugs also account for cellular ROSs production (Rendic & Peter Guengerich, 2012). ROS formation and elimination is tightly regulated by cellular defense mechanism. However, conditions such as oxidative stress perturb ROS homeostasis leading to DNA, lipids, and protective cellular proteins damage. Damage to cellular macromolecules has been widely observed carcinogenesis (Ushio-Fukai & Nakamura, 2008).

Several studies report the role of ROS in inflammation, angiogenesis and metastasis (Coussens & Werb, 2002; Rakoff-Nahoum, 2006). Cancer cells appear to have higher basal ROS levels

compared to their normal corresponding cells (Gupta *et al.*, 2012). Moreover, studies have demonstrated that ROS can activate PI3K/Akt signaling pathway enhancing HiF-1 α expression in some cancer cell lines including PC-3 (Coussens & Werb, 2002; Han *et al.*, 2014; Mazhar *et al.*, 2006; Rakoff-Nahoum, 2006). ROS also promotes the upregulation of MMP-1, MMP-2, and MMP-9 in DU-145 prostate malignant cell line (Liou & Storz, 2010; Sauer & Wartenberg, 2005). Likewise, a study on PCa cells determined that EGD-induced epithelial-mesenchymal transition (EMT) occurs through the ROS/STAT3/HiF-1 α /TWIST1/N-cadherin signaling cascade (Cho *et al.*, 2014).

After interrogating the larger functional interacting partners of the DMRs-associated genes followed by enrichment analyses, several pathways which could be summarized into 3 main categories were observed. These categories include RET signaling, immune responses and regulation of rRNA transcription. Rearranged during Transfection (RET) is a receptor tyrosine kinase essential for the normal development and maturation of a diverse range of tissues. Alterations in RET have been associated with some malignancies including, anaplastic thyroid, urothelial, colon and breast carcinomas (Amit *et al.*, 2017; Kato *et al.*, 2017; Kunstman *et al.*, 2015; Lipson *et al.*, 2012). RET and its ligand GDNF have been reported to be upregulated in prostate tumours and surrounding stroma (Ban *et al.*, 2017). The activity of RET driven by GDNF not only encourages cell growth, invasion, and perineural invasion in both *in vitro* and preclinical animal models of PCa, but it can also be amplified when soluble variants of GFR α 1 are present. (Ban *et al.*, 2017; Gil *et al.*, 2010; H. Liu *et al.*, 2012). Increased levels of RET and GDNF correlate with an increased expression of matrix-degrading metalloproteinases, which aid in the invasion of cancer cells into the perineural area. (Baspinar *et al.*, 2017). Activation of RET has also been reported to trigger the downstream PI3K/AKT/MTOR pathways. Thereafter, the activated PI3K/AKT/MTOR pathways control the activity of RNA polymerase I (Pol I), ribosome

biogenesis and subsequently protein translation which will favour tumour progression and aggressiveness. Moreover, ROS has been suggested to also activate the PI3K/AKT/MTOR pathway (X. Lin *et al.*, 2024). This suggests that the PI3K/AKT/MTOR pathway through activation by RET and ROS drives tumour aggressiveness in Ghanaian men.

3.5 Conclusion

The preliminary analysis suggests a potential association between DNA methylation patterns and PCa aggressiveness in Ghanaian men, particularly in relation to RET signaling and oxidoreductase activities. While these findings are intriguing, they serve as an initial step and warrant further investigation. Expanding the sample size in future studies could help assess the utility of these differentially methylated genes as potential biomarkers for PCa, which could be valuable for more accurate patient stratification and addressing issues of overdiagnosis and overtreatment.

3.6 Supplementary data



Figure S3.1 Representative gel image of DNA extracted from FFPE samples. M = 1Kb marker and the numbers are codes for individual samples

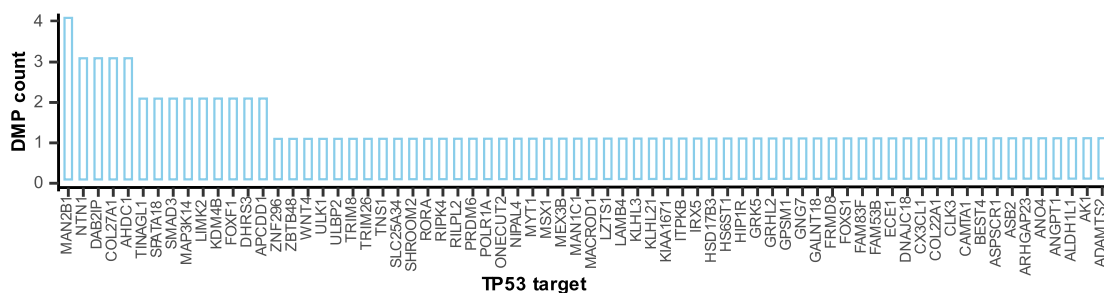


Figure S3.2. DMPs-associated TP53 targets. The total list of TP53 targets (1201) were downloaded from MSigDB from which 70 genes with associated DMPs were extracted.

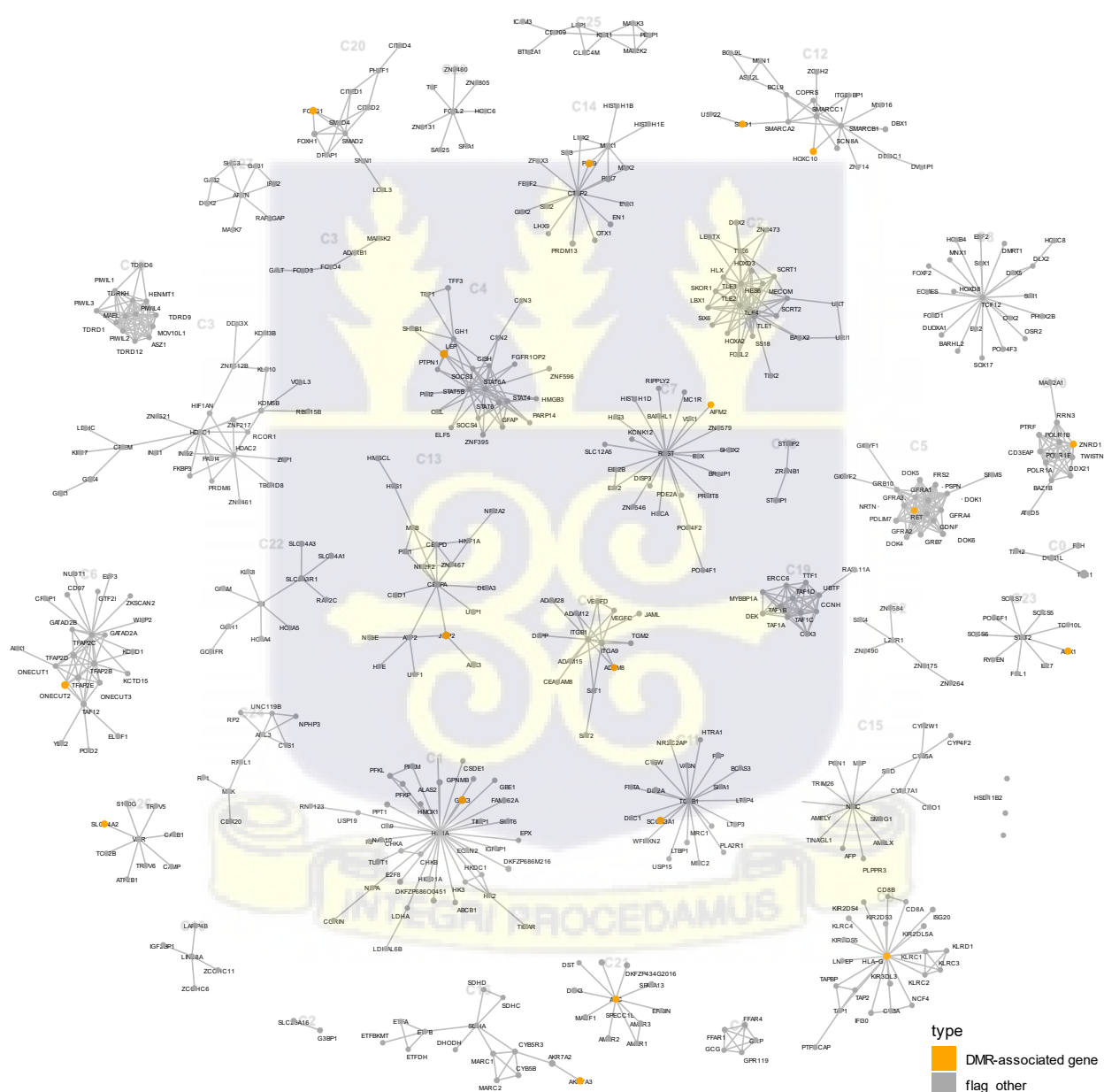


Figure S3.3 DMRs-associated network interactions

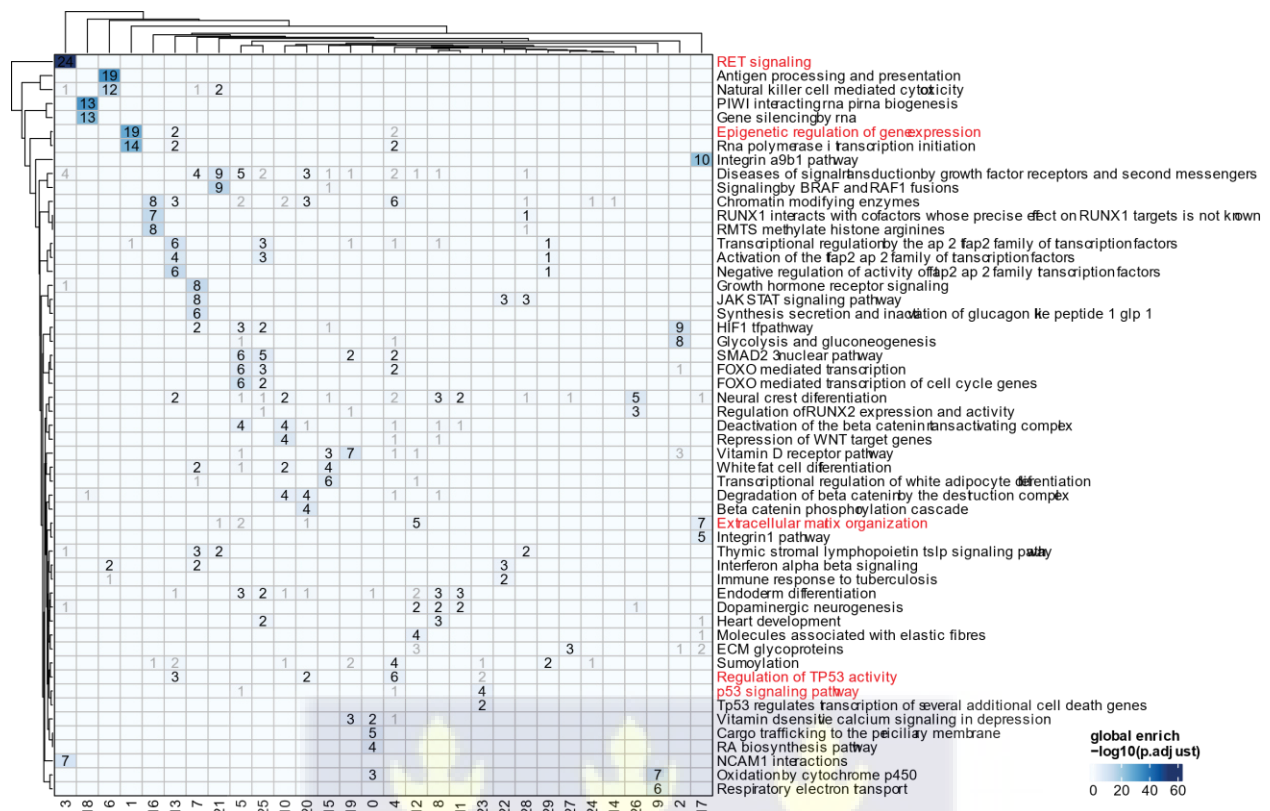


Figure S3.4 Pathways associates to DMR-associated gene network sub-clusters. An enrichment heatmap obtained after gene set enrichment analysis of the propagated DMR-associated genes subnetwork clusters. *P-values* were derived using the hypergeometric test and adjusted for multiple hypothesis testing using the FDR. Highlighted in red are the pathways closely related to the interesting pathways identified from the enrichment of DMPs.

CHAPTER FOUR

COMPUTATIONAL PREDICTION OF SCHISTOSOMA-MEDIATED PROSTATE CARCINOGENESIS PATHWAYS

4.1 Introduction

The complex interaction of infectious agents and the development of malignancies is drawing a lot of attention lately. Among these enigmatic associations, Schistosomes, a parasitic trematode endemic to various regions, has attracted some attention due to its classification as a group 1 carcinogen in bladder cancer by the IARC (International Agency for Research in Cancer, 1994). About 97% of all infections and 85% of the global population at risk of Schistosomiasis are located in Africa (Ekloh *et al.*, 2024; Steinmann *et al.*, 2006). In Ghana, the nationwide prevalence is estimated at 23.3%, with certain areas experiencing localized prevalence rates exceeding 50% (Y.-S. Lai *et al.*, 2015). Particularly prevalent within the African continent, schistosomiasis exerts a substantial burden on public health. Literature review have demonstrated incidence of prostate schistosomiasis associated with the emergence of PCa (Choto, Mduluz, Sibanda, *et al.*, 2020; Peiffer *et al.*, 2019), yet the mechanistic underpinnings of this intriguing connection remain unknown.

Schistosomiasis, a crippling neglected tropical disease caused by the parasitic flatworms *Schistosoma haematobium* and *Schistosoma mansoni*. This geographical preponderance, coupled with the rising threat of PCa, requires an in-depth exploration of the potential relationship between Schistosomes and PCa. Some studies trying to understand the effects of *S. haematobium* and *S. mansoni* on the human host focused on changes in the human transcriptome and proteome caused by schistosome infections (Dupnik *et al.*, 2018; Nacif-Pimenta *et al.*, 2019). An array of gene products (mRNA and proteins) have been shown to be differentially expressed in schistosomiasis.

It was therefore hypothesized that Schistosomes upon infection could physically or functionally interact with host proteins and hijack PCa-relevant pathways.

To test this hypothesis, an integrated approach combining network modeling, bioinformatics, and statistical analyses was employed. Herein, was identified complex molecular networks and pathways including Clathrin-mediated endocytosis (CME) and extracellular matrix (ECM) organization whose possible dysregulation by Schistosomes could affect cell proliferation, migration, and cellular microenvironment. These molecular perturbations, in turn, could ostensibly catalyze the development and progression of PCa.

In essence, this represents a pioneering endeavour to unravel the hitherto unknown interplay between schistosomiasis and PCa. This provides a preliminary understanding of a potential causal role of *S. haematobium* and *S. mansoni* which must be validated *in vitro* and *in vivo*.

4.2 Methods

4.2.1 Network propagation

Network propagation was performed using heat diffusion approach/RWR as described previously (Carlin *et al.*, 2017). The process is represented by the equation below. Briefly, two separate propagations were performed using genes products (mRNA and proteins) previously shown to be differentially expressed in RNAseq and mass spectrometry analyses ($1.5 \leq \log_2FC \leq 1.5$, p-value < 0.05) during *Schistosoma haematobium* (n=172) or *Schistosoma mansoni* (n=323) (Cosenza-Contreras *et al.*, 2019; Nacif-Pimenta *et al.*, 2019; Osakunor *et al.*, 2022) as the initial “seed nodes”. Pathway Commons subset for Reactome functional interactions (FI), physical interactions, and CORUM was used as the base network. For the first and second propagations, genes differentially expressed during *S. haematobium* or *S. mansoni* infection were labeled ‘1’ in the base network and the other genes were designated ‘0’. To account for nodes that have numerous

connections and therefore predisposed to receiving higher propagation scores because of their extensive connectivity, a permutation test was conducted. This test randomized the initial gene labels across the network 10,000 times. Then, for each gene, an empirical p-value was calculated by computing the fraction of random propagation runs that were greater than or equal to the genuine propagation run.

$$d = h * \exp(-Lt)$$

The propagated datasets (i.e., for *S. haematobium* and *S. mansoni*) were integrated by choosing the most significant pairs of genes in both propagations. The construction of a network was carried out by subgraph induction from the Pathway Commons network corresponding to the top 500 genes significant in the individual and integrative propagation outputs.

4.2.2 Clustering of propagated network

To discern the host pathways linked with *S. haematobium*, *S. mansoni* mono and coinfections, the three networks were clustered into subnetwork clusters as detailed in (Olwal *et al.*, 2023). In brief, the clustering was carried out using a strategy based on a function in the igraph package v1.4.0 called *cluster_walktrap*, (Csardi & Nepusz, 2005), and *cutreeDynamic*, a function in the dynamicTreeCut package v1.63-1. (Langfelder *et al.*, 2008). This network clustering strategy consisted of two layers. The propagation resulting network was divided into subnetworks in the first layer with 10 steps using the *cluster_walktrap* function and converted into an hclust object. The cophenetic function in R was used to determine and normalize the distance between nodes. The subnetworks that resulted were further clustered using the *cutreeDynamic* function with the arguments for minClusterSize and deepSplit set to 7 and 4, respectively.

To identify the pathways associated with each subcluster, gene set overrepresentation analysis was carried out using the clusterProfiler package v4.2.2 (Yu *et al.*, 2012). *P-values* were determined using the hypergeometric test, and adjusted for multiple testing using the false discovery rate. The

leading Gene Ontology (GO) terms for each cluster were then displayed in a heatmap (Yu *et al.*, 2012).

4.2.3 Integrating genomic data from prostate cancer patients

The freely accessible digital platform, cBioPortal (available at <https://www.cbioportal.org/>) for examining, visualizing, and interpreting multidimensional cancer genomic datasets derived from a variety of cancer patients was used (Cerami *et al.*, 2012; Gao *et al.*, 2013). The cBioPortal also contains patient clinical data. Patient data from nine prostate cancer studies provided in the cBioPortal was used: Prostate Adenocarcinoma (TCGA, PanCancer Atlas; $n = 494$) and Prostate Adenocarcinoma (TCGA, Firehose Legacy; $n = 501$), Prostate Adenocarcinoma (TCGA, Cell 2015; $n = 334$), Prostate Cancer (MSK, JCO Precis Oncol 2017; $n = 504$), Prostate Adenocarcinoma (MSK/DFCI, Nature Genetics 2018; $n = 1013$), Prostate Adenocarcinoma (MSK, Eur Urol 2020; $n = 1465$), Prostate Adenocarcinoma (MSK, Clin Cancer Res 2022; $n = 1417$), Metastatic Prostate Adenocarcinoma (SU2C/PCF Dream Team, PNAS 2019; $n = 444$) and Prostate cancer (DKFZ, Cancer cell 2018; $n = 324$).

Prostate cancer patient clinical and genomic data were extracted and analyzed to determine the haematobium and mansonii DEGs most likely involved in prostate cancer development following the steps described previously (Olwal *et al.*, 2023). In brief, clinical data from cBioPortal for the prostate cancer studies was downloaded. Following that, the prostate cancer patient identification numbers (IDs) were extracted according to each of the cancer grades, stages, and PSA category (i.e., grade 1 to grade 5, stage 1 to 4, $PSA < 10$ to $PSA > 20$). The Tumour, Node, Metastasis (TNM) staging method which is the gold standard (Balachandran *et al.*, 2015; Hu *et al.*, 2022), was used to extract patients' IDs from the studies corresponding to stage 1, stage 2, stage 3 and stage 4. Patient identifiers from the sub-stages were consolidated to correspond to the four primary stages (stages 1 to 4). These identifiers for the various grades, stages, and PSA categories of PCa were

utilized to query the cBioPortal for mutation and copy number alteration (CNA) data from studies specific to PCa. The retrieved CNA and mutation data, along with a compilation of haematobium or mansoni DEGs, were inputted into the R software for subsequent analysis. CNA or mutation frequency data, pertaining to the cataloged haematobium or mansoni DEGs, was obtained from cBioPortal and sorted by grade, stage, or PSA level. The average CNA or mutation frequencies for the haematobium or mansoni DEGs were then computed by grade, stage, or PSA. The ComplexHeatmap package v2.10.0 (Gu *et al.*, 2016) was used to create heatmaps depicting changes in mutation or CNA rates of haematobium and mansoni DEGs across prostate cancer grades, stages and PSA. The mean CNA or mutation frequencies were calculated for the grades, stages or PSA and the mean values of the heatmap rows (designating haematobium or mansoni DEGs) were used to order the DEGs (from highest to lowest). The top three mutant haematobium or mansoni DEGs per heatmap were overlaid in the relevant propagating networks (Figure 4.5 A-C, Figure 4.6 and Figures S.4.9 - S.4.10). These nodes were incorporated if they were connected to any component within the subnetwork cluster or to the most genetically altered haematobium, mansoni or combined DEGs (blue edges; Figure 4.6, Figure S4.9 and Figure S4.10) based on the Pathway Commons network.

4.2.4 Gene set over-representation analyses.

The ClusterProfiler software, version 4.2.2, was employed to conduct an over-representation analysis of gene sets to determine the pathways linked to the subnetwork clusters constituting the networks. This was a customized method developed by the Krogan lab, UCSF, USA (Hu *et al.*, 2022; Olwal *et al.*, 2023). In brief, term simplification was conducted by estimating term similarity according to gene membership, grouping the terms appropriately, and trimming the dendrogram at a specified height (0.8 for this analysis). Each clustered collection of terms was represented by

the most general phrase. Only terms with a p-adjusted value of 0.05 were chosen. The final map of enriched terms was then visualized using the ComplexHeatmap package version 2.10.0 in R.

4.2.5 Network visualization

The `graph_from_data_frame` function from the `igraph` package v1.4.0 was used to create the networks in R. The networks were then visualised in RStudio using the `create_layout` and `ggraph` functions of the `ggraph` package v2.1.0.

4.3 Results

4.3.1 Putative human targets of *Schistosoma haematobium* and *Schistosoma mansoni*.

The literature was searched for studies reporting on genes and or proteins that are differentially expressed with *S. haematobium* or *S. mansoni* infection. The hypothesis is that *Schistosoma* proteins alter the expression of these proteins (host) through a physical or functional interaction with the human host genes. These proteins were differentially expressed in urogenital and splenic murine models. From the literature search, 319 genes and or proteins were differentially expressed (in RNA-seq and mass spectrometry analyses, respectively) in *S. mansoni* infection while about 168 were differentially expressed in *S. haematobium* infection, with only 4 genes overlapping between the two species Figure 4.1B. While recent years have revealed that the prostate gland is frequently infected by schistosomes, there is minimal data regarding gene or protein expression in prostate schistosomiasis. Moreover, with the bladder being a primary site of infection and the spleen involved in the host's immune response, they represent appreciable tissue proxies for the study of prostate schistosomiasis. Initial gene over-representation analyses of these genes (“haematobium DEGs” and “mansoni DEGs”) indicated that the pathways these species target are largely distinct, with the exception of the regulation and organization of the extracellular matrix, where they overlap Figure 4.1C.

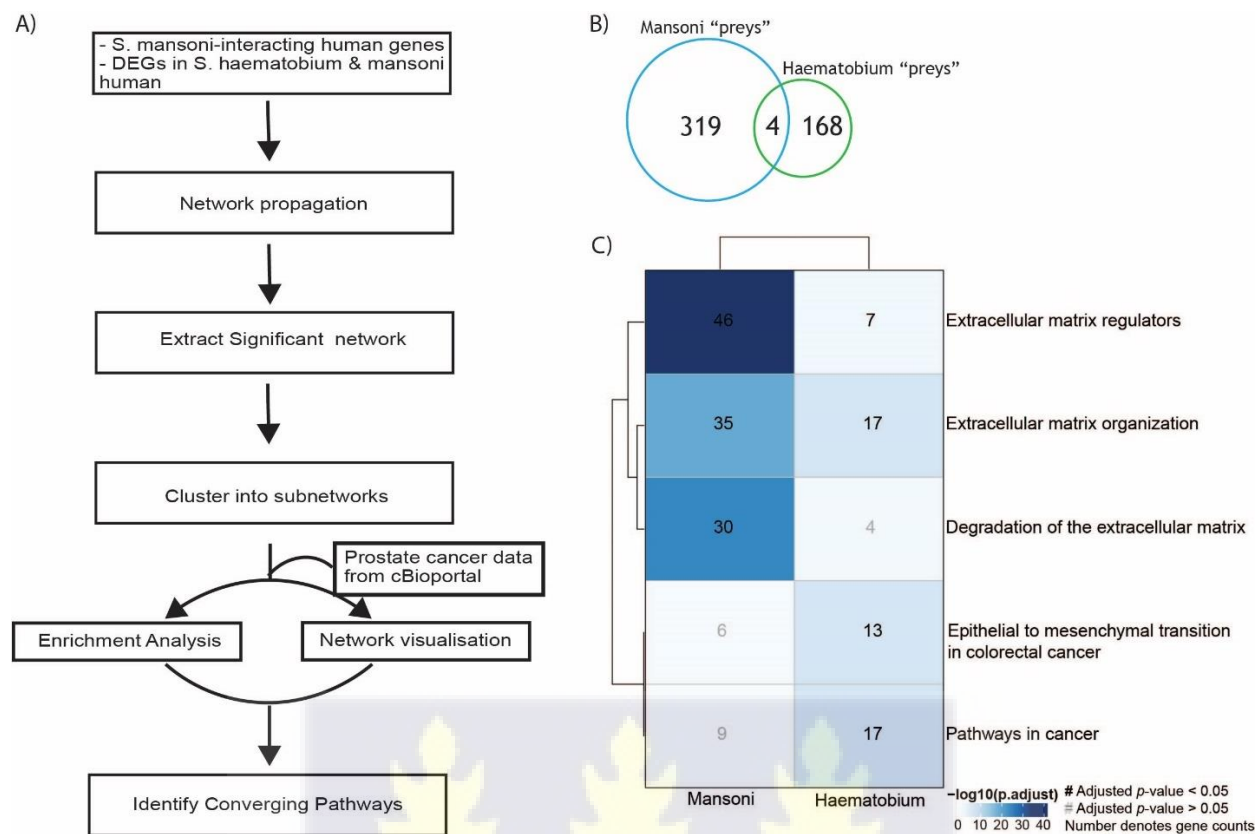


Figure 4.1. Network modeling to probe the role of *S. haematobium* and *S. mansoni* in PCa development. (A). Schematics of the overall analysis pipeline. Gene network maps were created by propagating *S. haematobium* and *S. mansoni* DEGs as “seed nodes”. This was followed by bioinformatic analyses to identify pathways and for the development of a hypothesis explaining the role of Schistosoma infections in prostate carcinogenesis. (B). A Venn diagram illustrating the shared and distinct *S. haematobium* and *S. mansoni* DEGs that were analyzed in this study. (C). A gene set enrichment analysis for *S. haematobium* and *S. mansoni* DEGs. P-values were determined using the hypergeometric test and adjusted for multiple hypothesis testing through the false discovery rate (FDR). The heatmap presents the three most significant pathway terms for each category of DEGs.

4.3.2 Network propagation analysis reveals pathways probably hijacked by *S. haematobium* and *S. mansoni* “DEGs” in PCa development.

Network propagation was used to gain a network-level perspective of how sets of “haematobium DEGs” or “mansoni DEGs” are related. This method simulates the diffusion of heat to spread a signal from selected genes ('seed nodes') to adjacent genes, connected by previously established functional and physical protein interactions. This procedure incorporates a group of genes into the broader molecular networks where they operate. Pathway Commons, consisting of a hybrid of physical and functional interacting networks was selected as the base network. Two distinct network propagations were carried out, with all 'seed' nodes assigned a value of 1 and all other nodes a value of 0, using either the (i) “haematobium DEGs” ($n = 172$), or (ii) “mansoni DEGs” ($n = 323$) as the original seed sets. A permutation test was used to determine nodes that received more "heat" than what would be predicted by random occurrence. The top 500 genes that were significant ($p < 0.05$) for both haematobium DEGs and mansoni DEGs' propagations were then selected. This was meant to favour the genes with the most significant propagation scores (smallest p-values) from each propagation, thereby discovering molecular networks in which the initial seed sets function. For a scenario of mixed infection (*S. haematobium* and *S. mansoni* combined), the top 500 intersecting genes in both propagations were selected. Next, a subnetwork consisting of key genes from our base network of original and sub-clustered it into smaller subnetwork pieces was induced. This yielded 37 (Figure 4.2), 33 (Figure S4.1) and 26 (Figure S4.2) smaller haematobium, mansoni or co-infection subnetwork clusters, respectively, to facilitate visualization and interpretation.

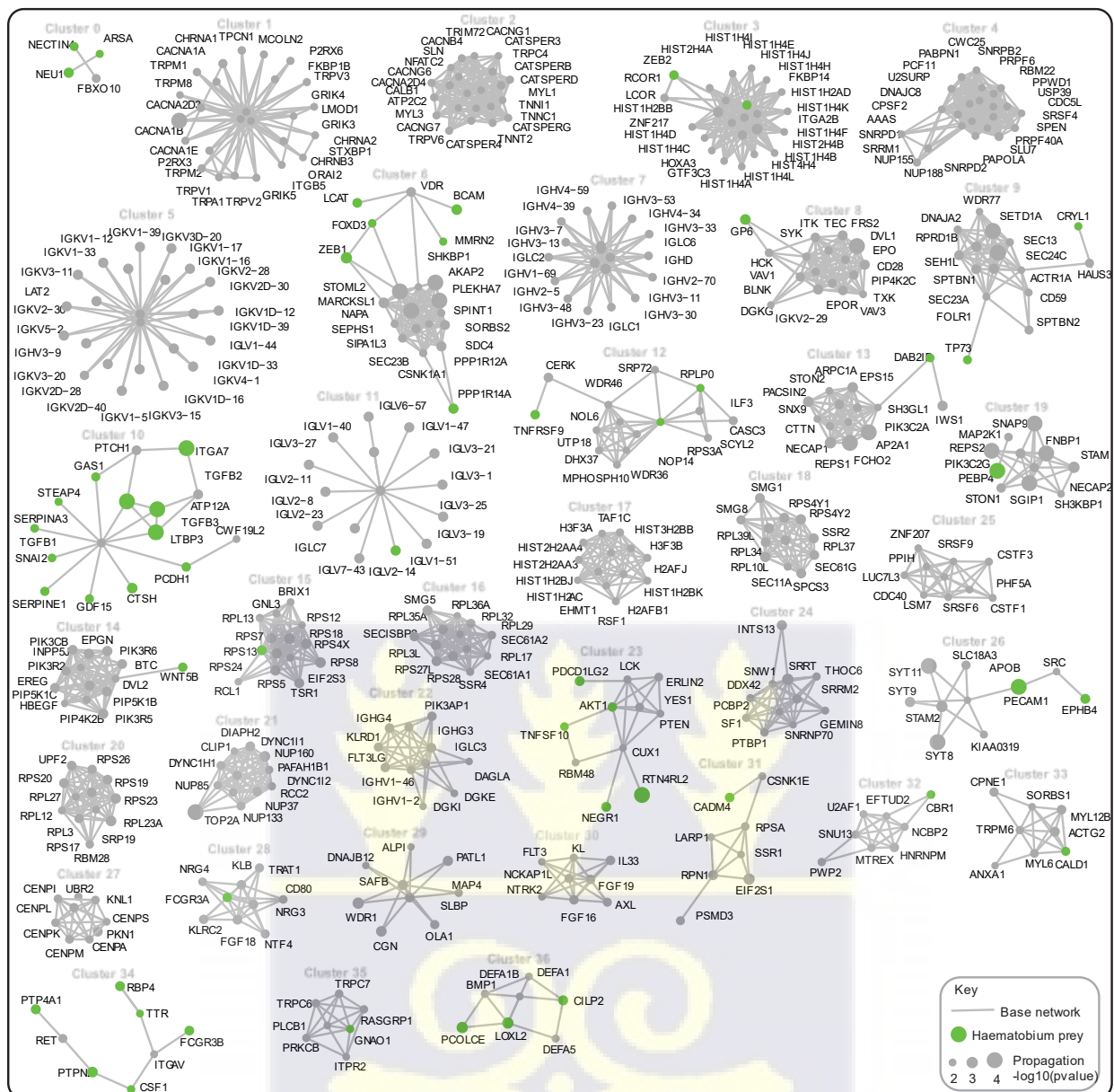


Figure 4.2 Network propagation for *S. haematobium* DEGs. Propagation analysis of biological clusters from *S. haematobium* network. Each cluster's corresponding pathway is shown in Figure S4.4. The node size in the network propagation permutation test represents the $-\log(p\text{-value})$.

4.3.3 Identification of high impact subnetworks associated with PCa based on gene alteration frequencies in cBioportal.

For the impartial selection of subnetwork clusters that are likely associated with prostate carcinogenesis, the frequency of alterations for genes in each of the haematobium, mansoni, or combined propagated subnetwork clusters were plotted. The five clusters with the highest alteration frequencies were selected (as depicted in Figure 4.3 and Supplementary Figures S4.3A-B). The five most frequently altered clusters from the haematobium propagation (clusters 36, 23, 21, 13, and 4) were found to be associated with mRNA splicing, pre-mRNA processing, clathrin-mediated endocytosis, and alpha defensins.

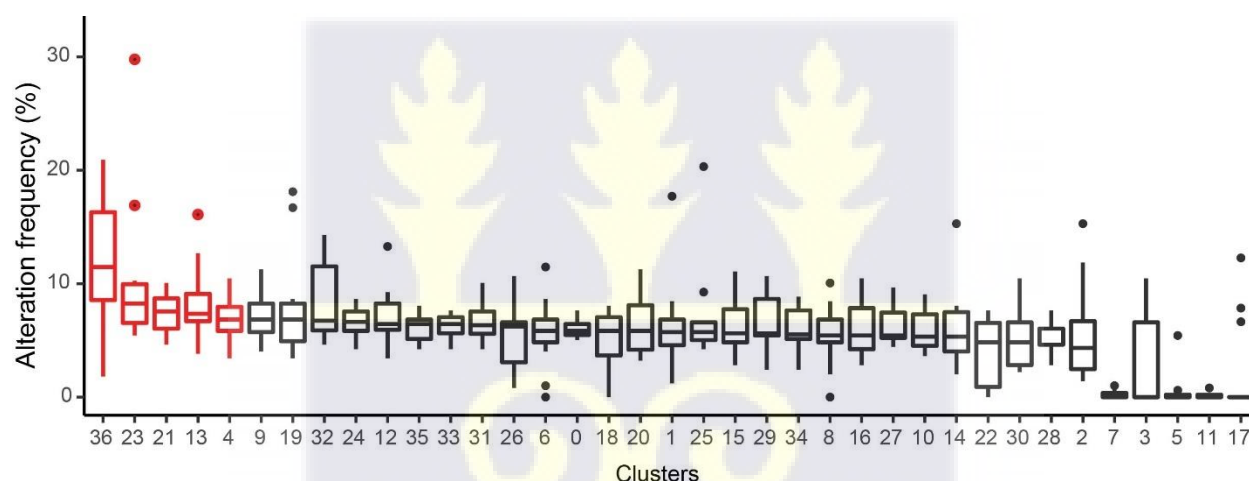


Figure 4.3 Frequency of Gene Alterations in PCa Patients of Subnetwork Clusters. A boxplot depicting the frequency of gene alterations (comprising structural variants and CNAs, point mutations and gene and protein expression) of genes in each subnetwork cluster in PCa patients from TCGA data set. The five most altered *S. haematobium* DEGs subnetwork clusters were 36, 23, 21, 13 and 4. The alteration frequency data is from cBioPortal.

4.3.4 Selected sub-networks show relevance in *Schistosoma*-associated cancers.

To determine if these pathways were altered more frequently in PCa than in other prevalent cancers in men, nodes from the top five altered haematobium, mansoni, or combined propagating clusters were extracted (Figures S4.4, S4.5 and S4.6) and their alteration frequencies across common male malignancies were annotated (Figure 4.4 and Figure S4.7A-B). It was discovered that PCa ranked second for clathrin-mediated endocytosis (CME) and Alpha defensins. These findings suggest that *S. haematobium* could mediate PCa development by hijacking CME and Alpha defensins pathways. It was noticed that genes in 4 out of the 5 most mutated clusters (clusters 4, 13, 21 and 23) had bladder cancer ranked first with the highest alteration frequencies. This is not surprising because *S. haematobium* has been recognized as a causative agent of bladder cancer (International Agency for Research in Cancer, 1994) and *S. haematobium* eggs have as primary niche the bladder.

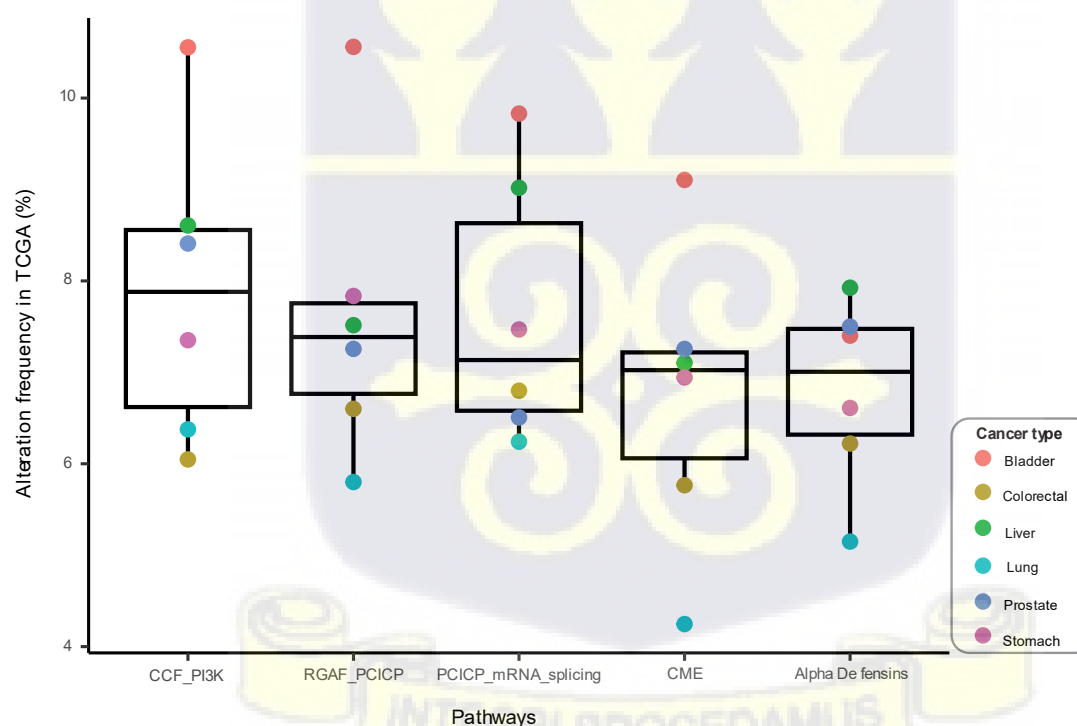


Figure 4.4 Alteration frequencies of top five clusters' nodes across the most common cancers in men. Comparing the alteration frequencies (comprising structural variants and CNAs, gene and protein expression and point mutations) of nodes in the five most altered clusters, categorized according to the pathways they are involved in, prevalent malignancies in men. The data is from cBioPortal. CCF_PI3K: Costimulation by the CD28 family and Negative regulation of PI3K AKT network, RGAF_PCICP: Rho GTPases activate Formins and Processing of capped intron containing pre-mRNA, CME: Clathrin-mediated endocytosis.

4.3.5 Top mutated haematobium and mansoni DEGs in PCa genomics data

Finding somatic mutations in Haematobium and Manson DEGs, would suggest the possibility that *S. haematobium* or *S. mansoni* could "mimic" somatic mutations that drive cancer, either via pathway regulation or by direct interaction with host proteins. For instance, a somatic mutation that disrupts the enzymatic function of a tumor suppressor gene, thereby activating a pathway that promotes cancer, could have a similar functional impact. To explore this, the mutation and CNA frequencies of Haematobium and Manson DEGs from cBioPortal across different PCa grades, stages, as well as PSA levels, were retrieved and ranked (Figure 4.5A).

PEBP4, *LOXL2*, *PTK2*, *TBC1D4*, *ATM*, *MKI67*, *DSP*, *LTBP3* and *OSCAR* were the most frequently mutated Haematobium DEGs among PCa patients (Figure 4.5B-C), while *LCPI*, *CPB2*, *SUGT1*, *LRPIB*, *FLG*, *COL7A1*, *AGRN* and *F12* were the most frequently mutated Manson DEGs during PCa development (Figure S4.8A-B).

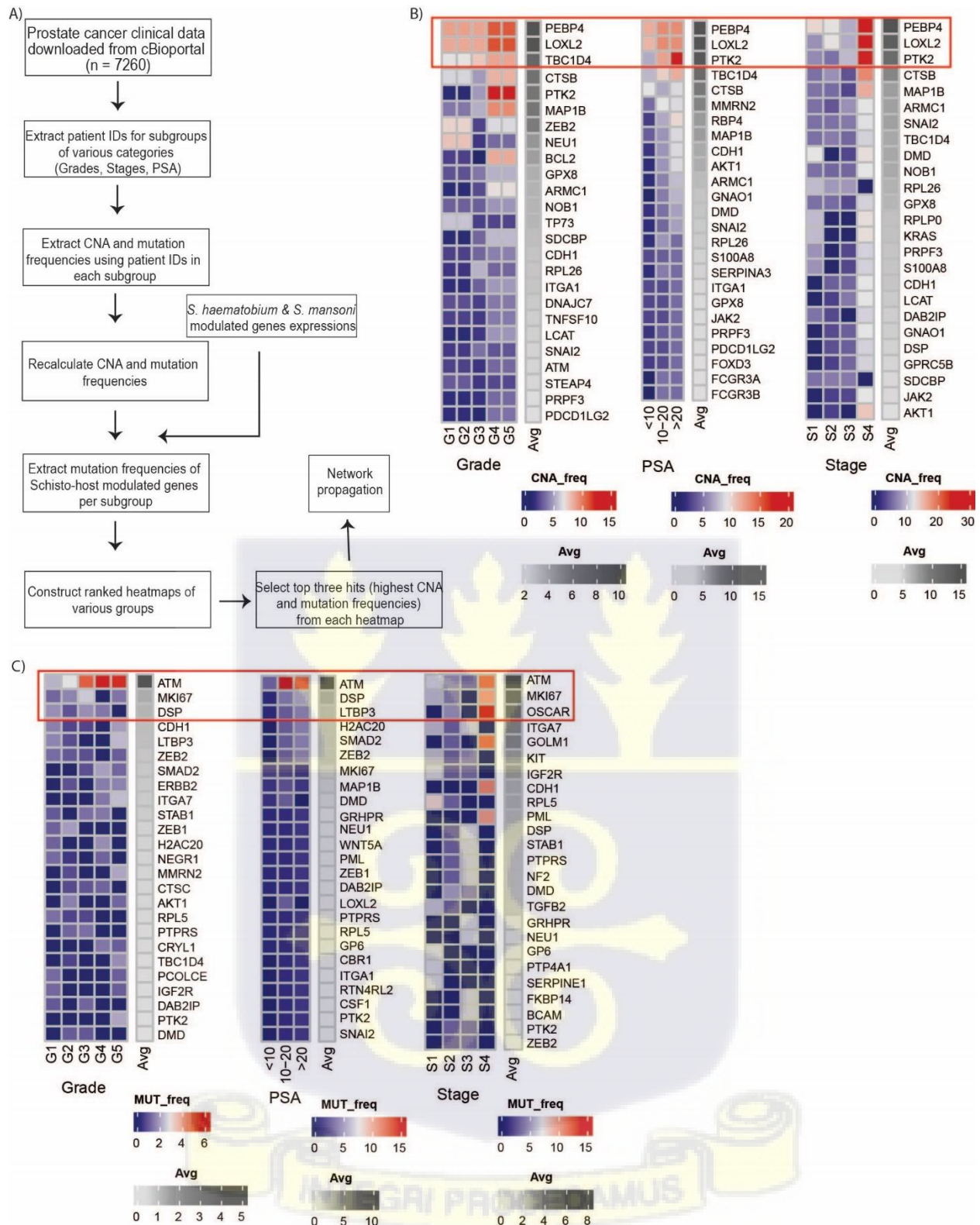
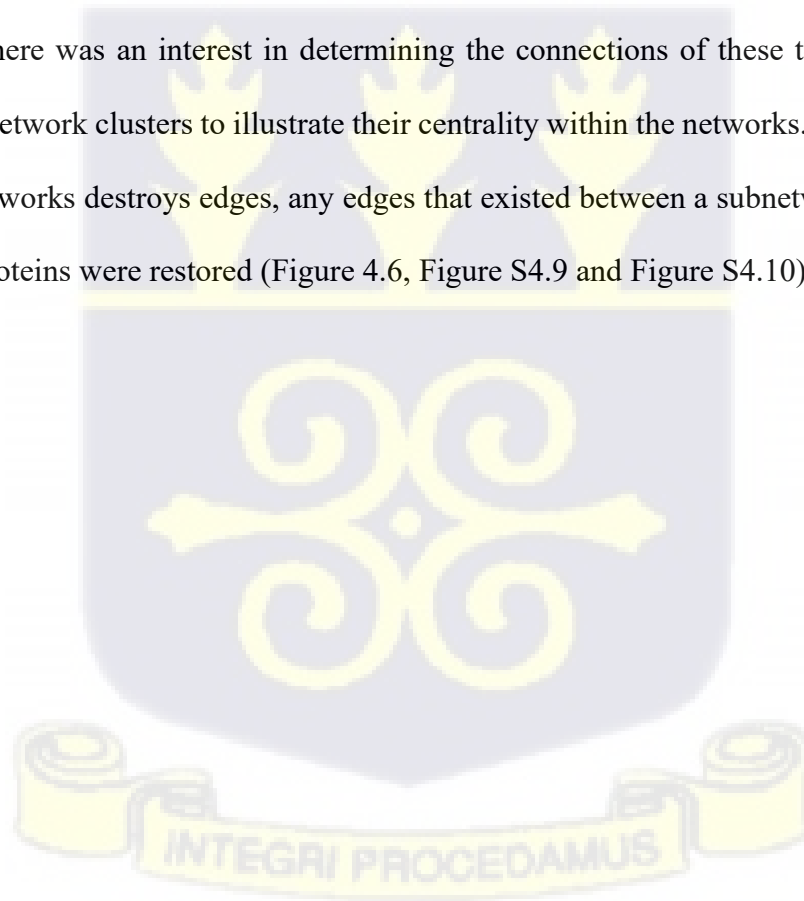


Figure 4.5 Integrating PCa genomics data into the haematobium DEG propagated Network

(A) Pipeline for integration of PCa genomics data from cBioPortal into the haematobium propagated network. PCa genomic data were extracted from the cBioPortal and analyzed to extract the top three haematobium DEGs, which were then overlaid onto the haematobium propagated networks to identify the pathways the top mutated haematobium DEGs are implicated in. (B) Ranked copy number alteration and (C) mutation frequencies of haematobium DEGs across different PCa grades, stages and PSA retrieved from cBioPortal and the three most altered DEGs selected for integration into the haematobium propagated network.

Subsequently, there was an interest in determining the connections of these top proteins to the propagated subnetwork clusters to illustrate their centrality within the networks. Since the process of clustering networks destroys edges, any edges that existed between a subnetwork cluster and a top candidate proteins were restored (Figure 4.6, Figure S4.9 and Figure S4.10).



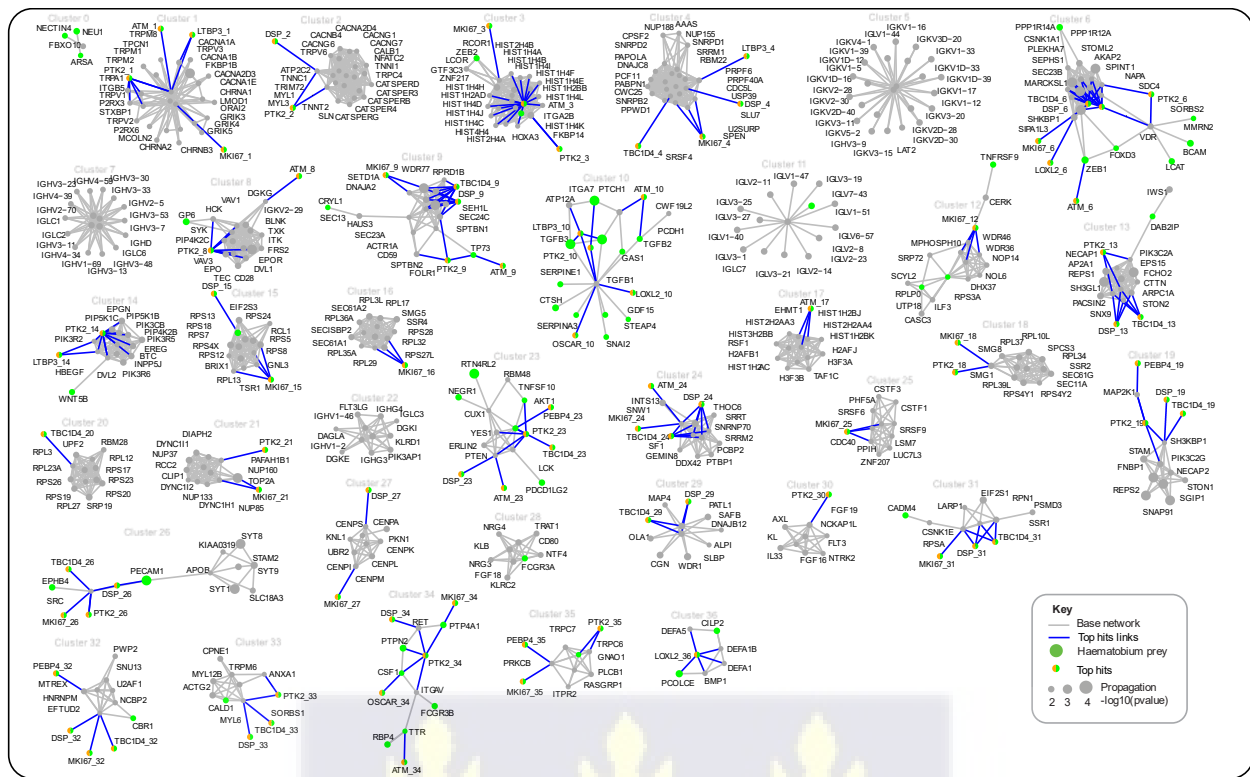


Figure 4.6 Incorporating the most mutated haematobium DEGs into the haematobium DEG propagated subnetwork clusters. Using Pathway Commons, the prominent mutated haematobium DEGs were superimposed on the haematobium-propagated network by mapping established connections between each of them and the genes within the subnetwork clusters.

The over-representation gene set analysis was conducted on propagated subnetwork clusters that had at least one link to any of the top mutated haematobium and/or mansoni DEGs. The top mutant haematobium or mansoni DEGs interconnected with haematobium, mansoni or combined propagated subnetwork clusters implicated in Clathrin mediated endocytosis, Alpha defensins and extracellular matrix degradation and organization. (Figure 4.7A-C), which is consistent with the various propagated networks subnetwork clusters (Figure S4.4, Figure S4.5 and Figure S4.6)

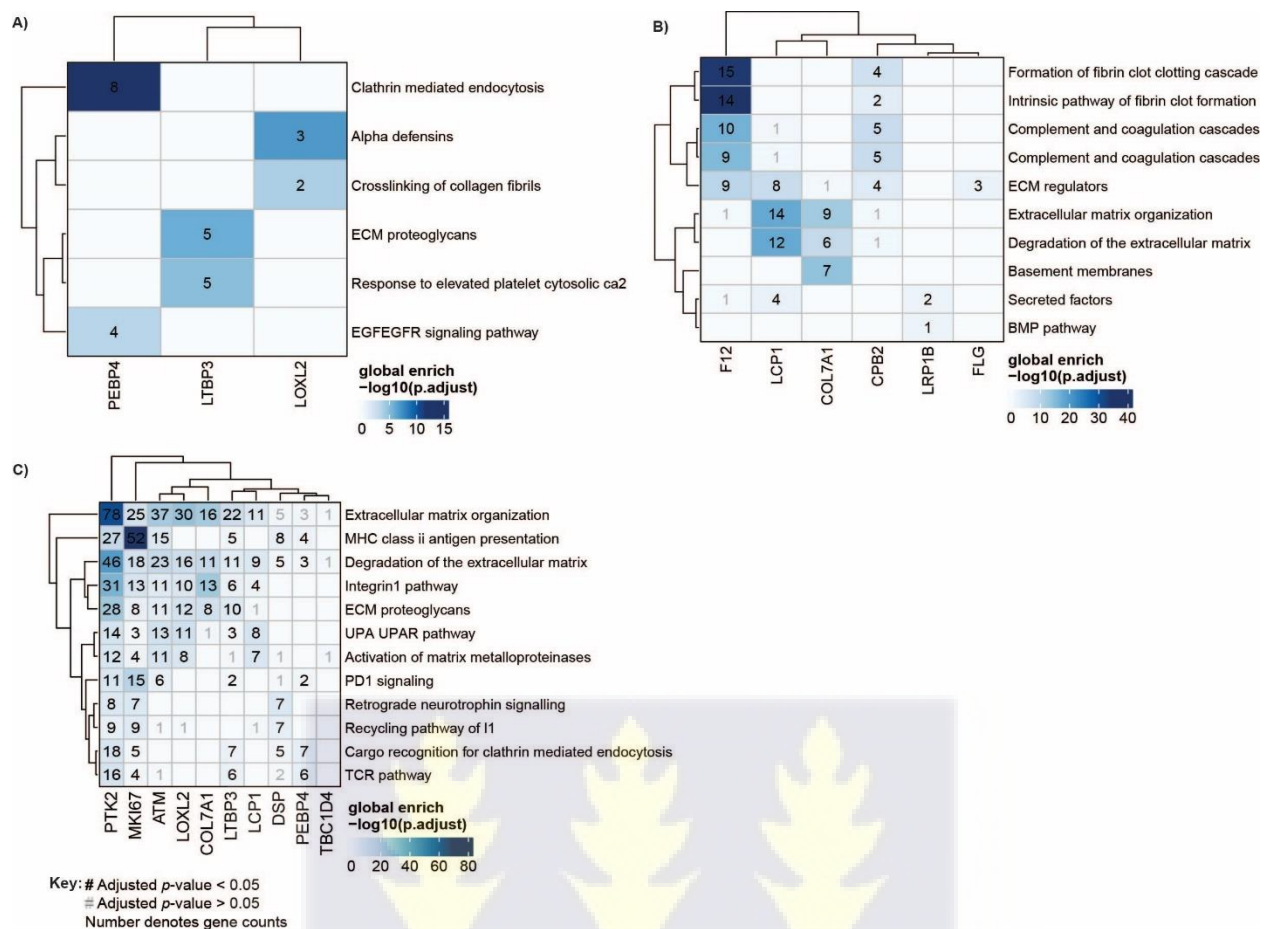


Figure 4.7 Gene set enrichment analysis for propagated haematobium, mansoni or coinfection subnetwork clusters with connections to the selected most mutated haematobium and mansoni DEGs.

4.4 Discussion

In reference to *S. haematobium*, the analyses highlighted a spectrum of pathways that could potentially be exploited by this species in prostate carcinogenesis. These pathways include clathrin-mediated endocytosis (CME), alpha defensins, costimulation mediated by the CD28

family, negative regulation of the PI3K AKT network, Rho GTPases, and mRNA splicing. These pathways have been reported to drive processes such as cell proliferation, cell motility to enable invasion, and the creation of a tumour-permissive microenvironment that promotes cancer development (Banushi et al., 2023; Chen et al., 2012; Crosas-Molist et al., 2022; Qiao et al., 2021; J. Yang et al., 2019). CME regulates epidermal growth factor receptor (EGFR) internalization, whose signaling is important in cancer development. Also, CME have been shown to recycle the EGF receptor to the surface of the cells rather than tagging it for degradation. Consequently, there is a prolonged EGFR signaling which affects downstream biological responses such as cell division (Sigismund *et al.*, 2008). CME is thought to regulate some processes that could be specifically relevant for prostate carcinogenesis and progression. More than 2 decades ago, Prescott and colleague described the involvement of CME in androgen-regulated processes, as Clathrin gene expression is regulated by androgens in the prostate (Prescott & Tindall, 1998). Additionally, clathrin-mediated internalization of Cadherin-11 has been shown to regulate its surface trafficking and migratory function in prostate cancer cells (Satcher *et al.*, 2015). Also, the α - defensins HNP1-3 has been shown to be differentially expressed in several tumours and associated with cell proliferation (Albrethsen *et al.*, 2005; Müller *et al.*, 2002). For example, an *S. haematobium* infection could lead to the production of α -defensins as part of the innate response mechanisms and these α -defensins (e.g. HNPs 1-3) have proinflammatory properties and stimulate cell proliferation (Müller *et al.*, 2002). Moreover, alpha defensins were identified as one of the proteins contained in neutrophil granules found in corpora amylacea and prostatic calculi (Sfanos *et al.*, 2009). These formations are commonly observed in radical prostatectomy specimens and are associated with inflammation. It is suggested that acute inflammation, in which alpha defensins are involved, may have a role in the biogenesis of corpora amylacea and prostatic calculi which are associated with PCa (Sfanos *et al.*, 2009). Though, the mechanism is not well understood, a

study by Kohli *et al.*, found alpha defensin genes *DEFA1*, *DEFA1B*, and *DEFA3* to be significantly higher in expression among patients who responded well to docetaxel chemotherapy for metastatic castration-resistant prostate cancer (CRPC) (Kohli *et al.*, 2015). This supposes that alpha defensin could be associated with tumour aggressiveness which is associated to effective chemotherapy response. When cell proliferation becomes excessive, CME, which is supposed to internalize growth receptors (Sigismund *et al.*, 2008), leading to lysosomal degradation may not occur if the CME-associated proteins are dysfunctional. This will lead to prolonged *EGFR* signaling and *PI3K* *AKT* and subsequently RHO GTPases activation (Soriano *et al.*, 2021). The culmination of these events may promote cellular transformation, proliferation, and migration. Moreover, these transforming cells could evade immune clearance if there was hampering of T cell activation as a result of no CD28 costimulation (Acuto & Michel, 2003).

On the other hand, it was found that *S. mansoni* potentially hijacks ECM organization and degradation, collagen biosynthesis and modifying enzymes, detoxification of ROS, amino acid metabolism and laminin interactions. ECM organization and degradation play a pivotal role in cancer invasion and metastasis (Elgundi *et al.*, 2019). The remodeling of ECM components, such as collagen and laminin, is essential for tumour cells to infiltrate adjacent tissues (Graf *et al.*, 2021). For instance, Collagen biosynthesis and modifying enzymes have been implicated in cancer progression, with alterations in collagen related-genes promoting tumour invasion and angiogenesis (Necula *et al.*, 2022). Specifically, ECM whose composition include Collagen and Laminins have been implicated in prostate tumour transmigration and metastasis (Stewart *et al.*, 2004). For instance, the laminin peptide C16 promotes the expression of the tyrosine kinase substrates Tks4 and Tks5 as well as stimulate Src. Reactive oxygen species as well as MT1- MMP (MMP14) and cortactin protein levels may be regulated by Tks molecules. All of these activities encourage the formation of invadopodia which contribute to the invasive characteristics of PCa

(Caires-dos-Santos *et al.*, 2020). Also, an increase in the collagen content of prostate tissues has been associated with PCa onset. For example, an increase in the synthesis of type I collagen was observed in activated peri-acinar fibroblasts adjacent to PIN, a precursor lesion of PCa (Tuxhorn *et al.*, 2002). Detoxification of ROS is crucial for maintaining cellular redox balance. Dysregulation of ROS detoxification mechanisms can lead to oxidative stress, contributing to DNA damage, mutation accumulation, and ultimately carcinogenesis (Martins *et al.*, 2021). Additionally, Graf and colleagues established through *in vitro* experiments that, laminins, a component of ECM induced strong migratory responses in PCa cells (Graf *et al.*, 2021). Therefore, due to the interconnection between these processes/pathways, a model of *S. mansoni* induced PCa is suggested. Inflammation during *S. mansoni* infection and subsequent production of ROS (oxidative stress) will alter the expression of collagen and laminin genes (e.g. *COL1A1* and *LAMA1* respectively) (Martins *et al.*, 2021) leading to ECM remodelling implicated in carcinogenesis and cancer progression.

Finally, because coinfections are common in some endemic communities, investigating the possible synergy of *S. mansoni* and *S. haematobium* in prostate carcinogenesis was important. The investigations highlighted probable pathways including COPI-mediated anterograde transport (COPI-MAT) and ECM organization, specifically involving integrin 1 to be likely engaged. There is not a lot of information on the function of COPI-MAT in carcinogenesis including PCa.

COPI-mediated anterograde transport is a cellular process crucial for protein trafficking within the Golgi apparatus and endoplasmic reticulum (ER). Although it is uncertain how much the Golgi can affect ECM remodeling in cancer biology, considering its crucial function in the formation of the matrix, its significance is implied. Studies of protein glycosylation, which can affect many facets of carcinogenesis, have shown a connection between the Golgi and cancer (Rodrigues *et al.*, 2018). N-acetylgalactosaminyltransferases (GALNTs) are moved from the Golgi to the ER as a

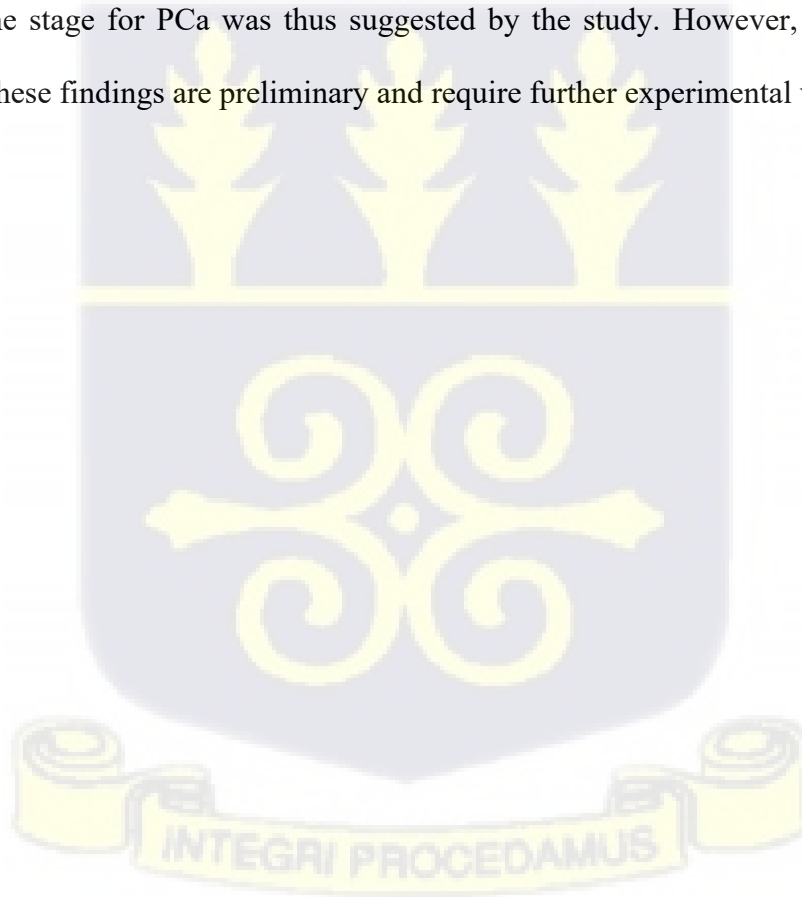
result of increased Src activity, which encourages retrograde COPI traffic and is a key component of the GALA pathway, which drives carcinogenesis (Chia et al., 2014; Gill et al., 2010). This results in increased Tn antigen synthesis, a glycoconjugate linked to cancer (Springer, 1984). Although the precise mechanisms by which the GALA pathway contributes to cancer are still unknown, the matrix metalloproteinase MMP14 is a known target (Nguyen et al., 2017). MMP14 increases localised ECM degradation, which in turn encourages metastasis, (Castro-Castro et al., 2016) facilitating cell migration and invasion. This may be strengthened even further by ER oxidoreductases' GALA-dependent export to the cell's outer layer, where they act on matrix proteins to decrease disulfide bonds and accelerate proteolytic destruction (Ros et al., 2020). On the other hand, ECM and integrin's role in carcinogenesis is supported by compelling scientific evidence, highlighting that the binding of integrins to ECM is associated with processes like cellular survival, proliferation and migration (J. Cooper & Giancotti, 2019). For instance, $\alpha 6$ integrins are often downregulated in PCa, resulting to loss of hemi-desmosomes and weak cell-cell adhesion which correlates with cancer cell invasiveness (Davis *et al.*, 2001; Schmelz *et al.*, 2002; Schmidt *et al.*, 2022). Further studies into the interaction of Integrins with ECM and the retrograde transport system are important to better understand the role of coinfection and its potential in prostate carcinogenesis.

From the PCa patient genomics data, it was noticed that the most mutated haematobium DEGs were strongly associated with high tumour grade, stage, and PSA levels. This could suggest that *S. haematobium* could be likely involved in PCa progression rather than initiation. On the other hand, the mansoni DEGs, *LCPI*, *CPB2* and *SUGT1* were most likely associated with almost all the tumour grades, stages and levels of PSA, suggesting that *S. mansoni* could be implicated in initiation and progression of PCa. *F12*, *AGRN* and *LRP1B* mansoni DEGs were mostly associated with high tumour grades, stages and levels of PSA and therefore could contribute to PCa

progression rather than initiation. These observations, however, need to be validated experimentally.

4.5 Conclusion

In this study, pathways such as clathrin-mediated endocytosis (CME) and alpha defensins were identified as potentially modulated by *S. haematobium* in prostate carcinogenesis. On the other hand, pathways related to ECM organization and reactive oxygen species (ROS) detoxification were found to be potentially manipulated by *S. mansoni*. The possibility of synergistic effects in coinfections involving both parasites was also explored, with COPI-mediated anterograde transport (COPI-MAT) and ECM organization being highlighted. A network of biological changes that could set the stage for PCa was thus suggested by the study. However, it is important to emphasize that these findings are preliminary and require further experimental validation.



4.6 Supplementary data

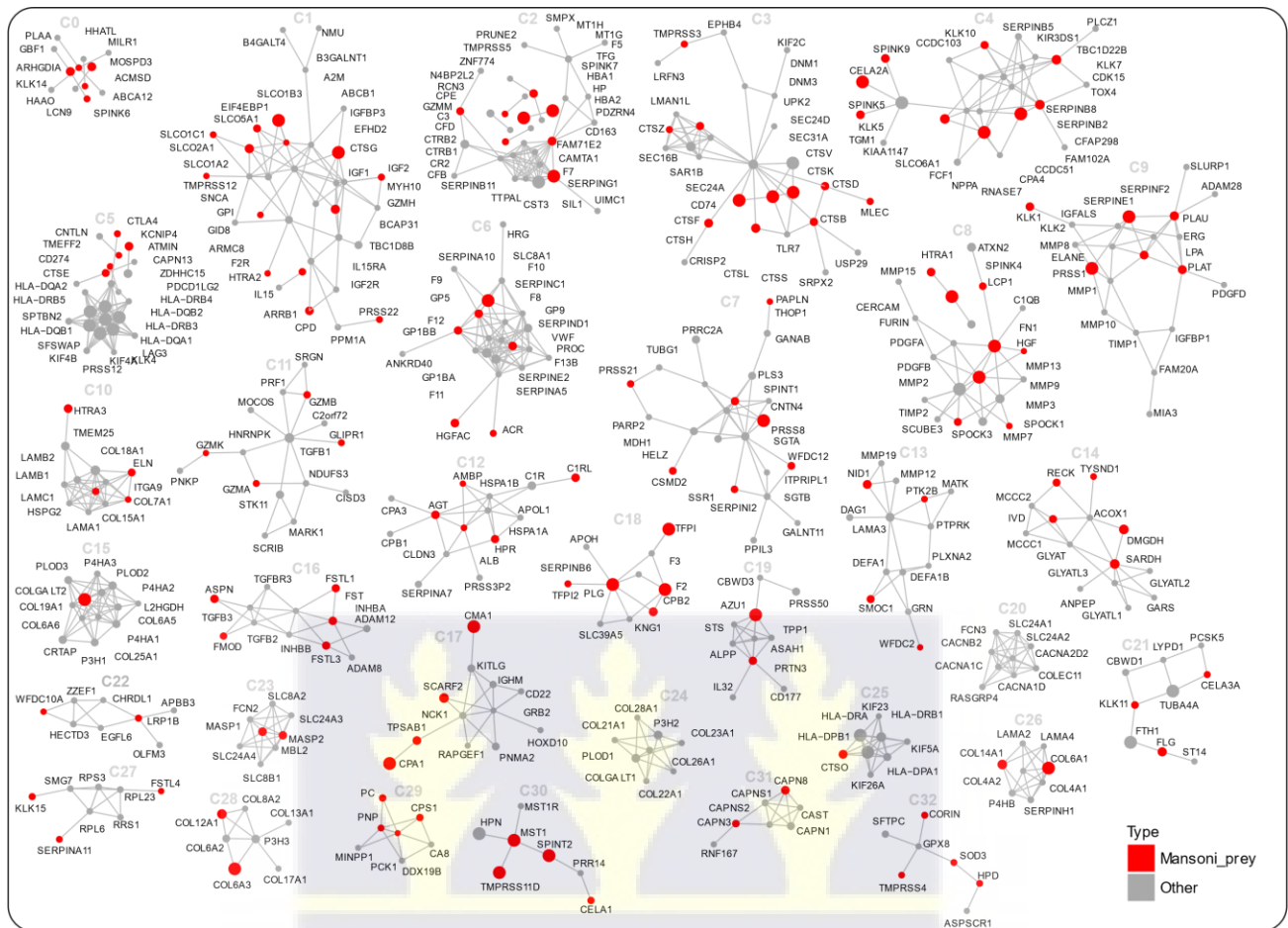


Figure S4.1 Network map for mansoni DEGs. Biological clusters from mansoni network propagation analysis. The top pathway term associated with each of the subnetwork clusters are provided in Figure S4.5. Node size depicts $-\log(p\text{-value})$ from network propagation permutation test.

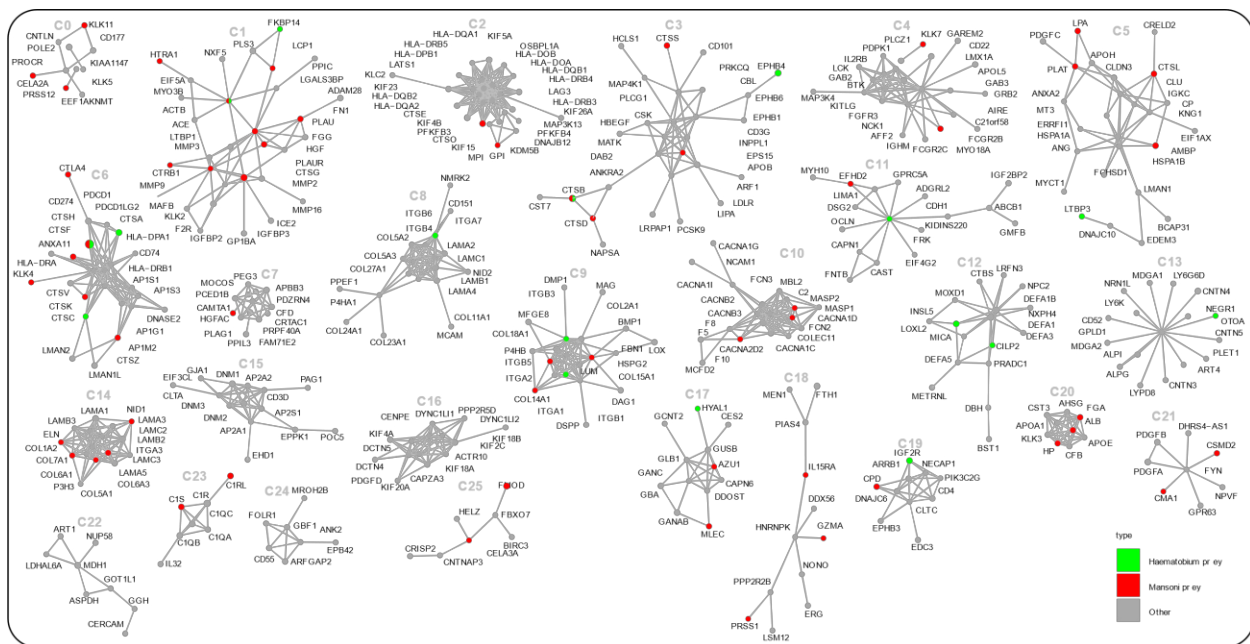


Figure S4.2 Network map for most significant haematobium and mansoni DEGs. The top pathway term associated with each of the subnetwork clusters are provided in Figure S4.6. Node size depicts $-\log(p\text{-value})$ from network propagation permutation test.

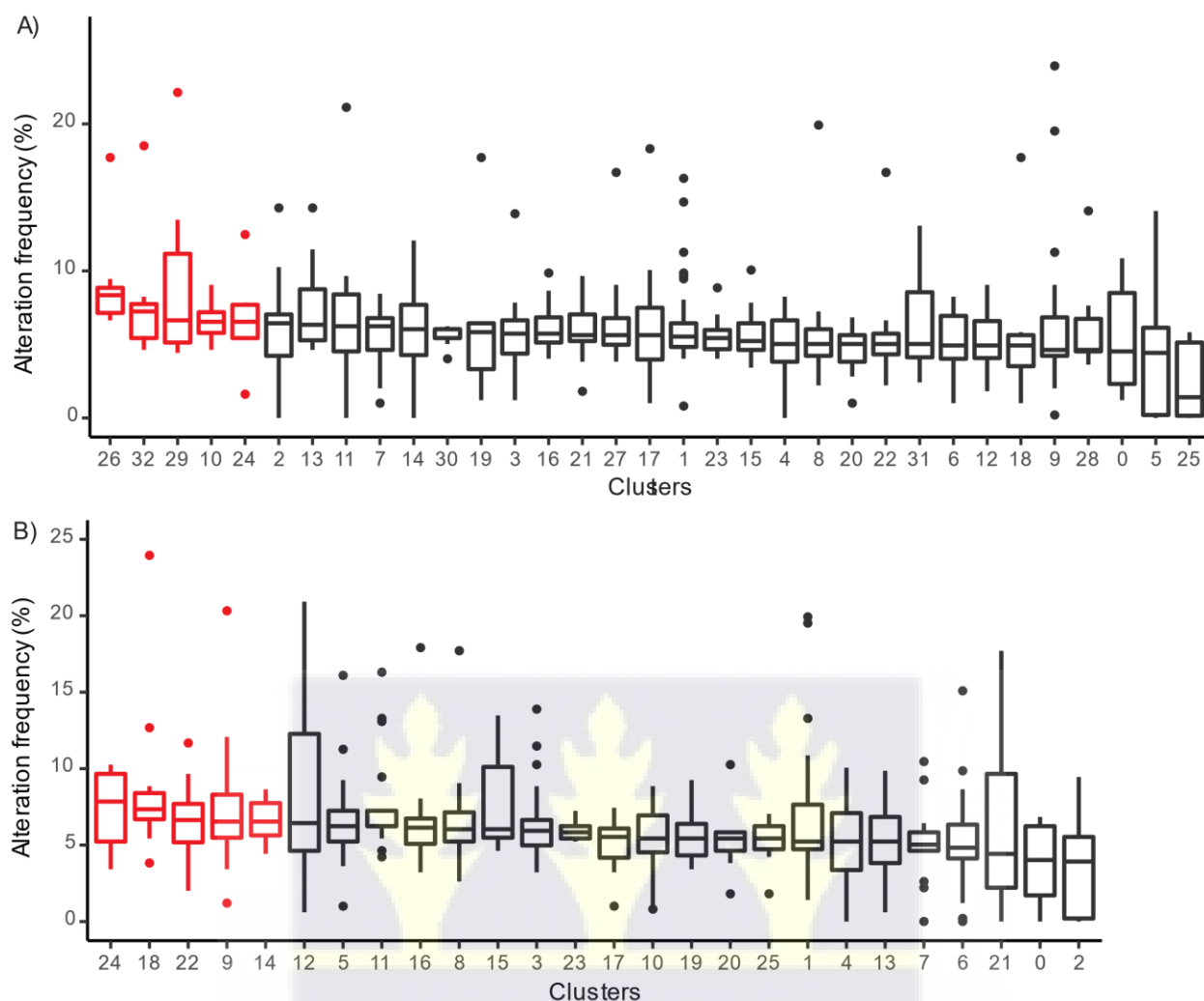
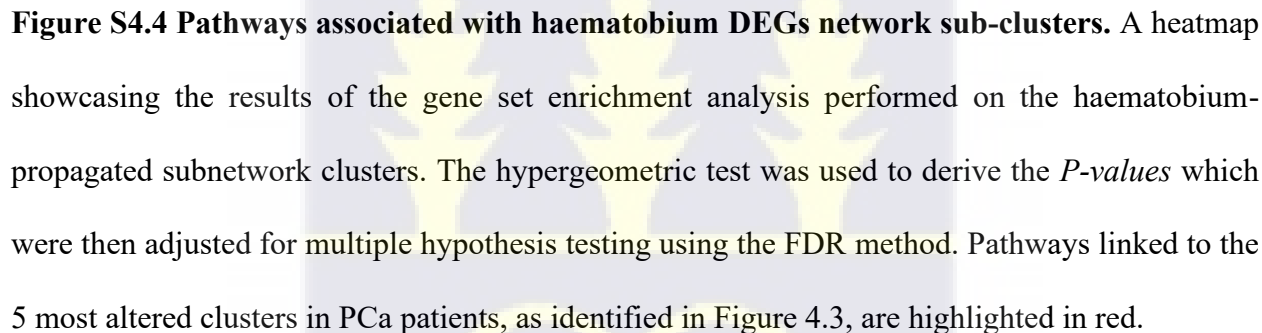


Figure S4.3 Alteration frequencies in sub-clusters. Box plots illustrating the gene alteration frequencies (consisting of structural variants and CNA, point mutations, gene and protein expression) for each subnetwork cluster. (A) for mansoni DEGs network and (B) is for a combination of top significant nodes in both mansoni and haematobium DEG network. The top five mutated subnetwork clusters (highlighted in red) were selected for unbiased selection of pathway terms in Figures S4.5 and S4.6. Data is from cBioPortal.



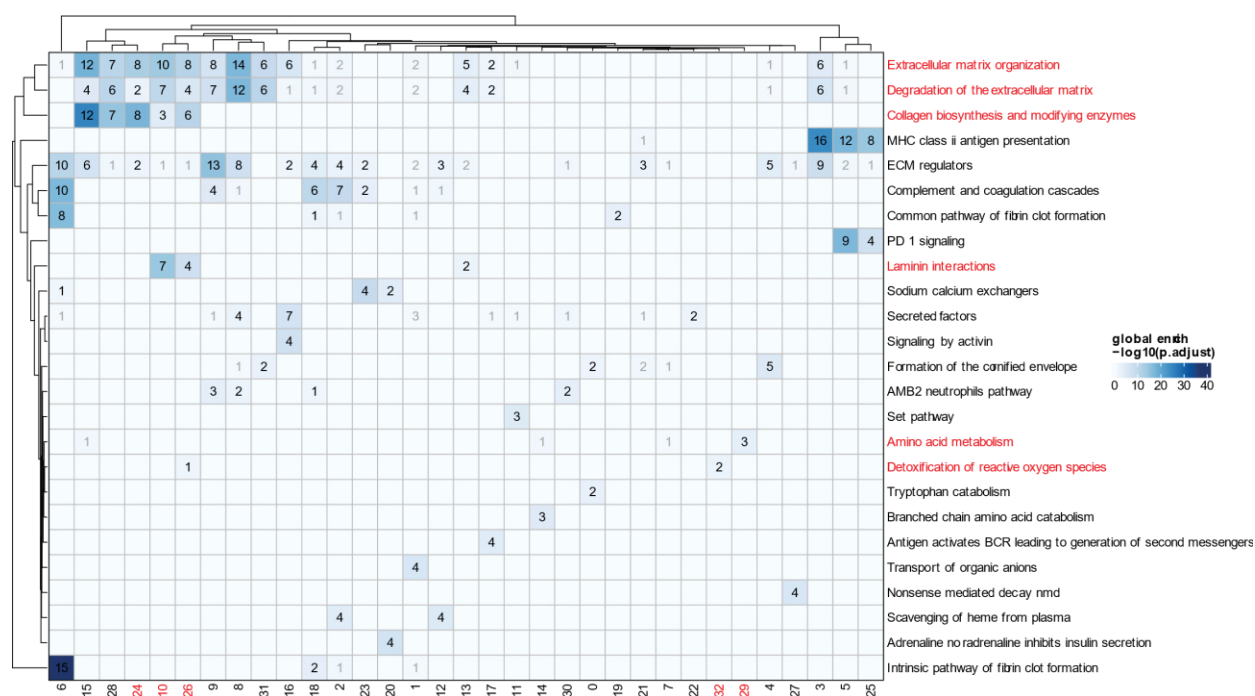


Figure S4.5 Pathways associated with mansoni DEGs network sub-clusters. A heatmap showcasing the results of the gene set enrichment analysis performed on the haematobium-propagated subnetwork clusters. The hypergeometric test was used to derive the *P-values* which were then adjusted for multiple hypothesis testing using the FDR method. Pathways linked to the 5 most altered clusters in PCa patients from Figure S4.3A are highlighted in red.

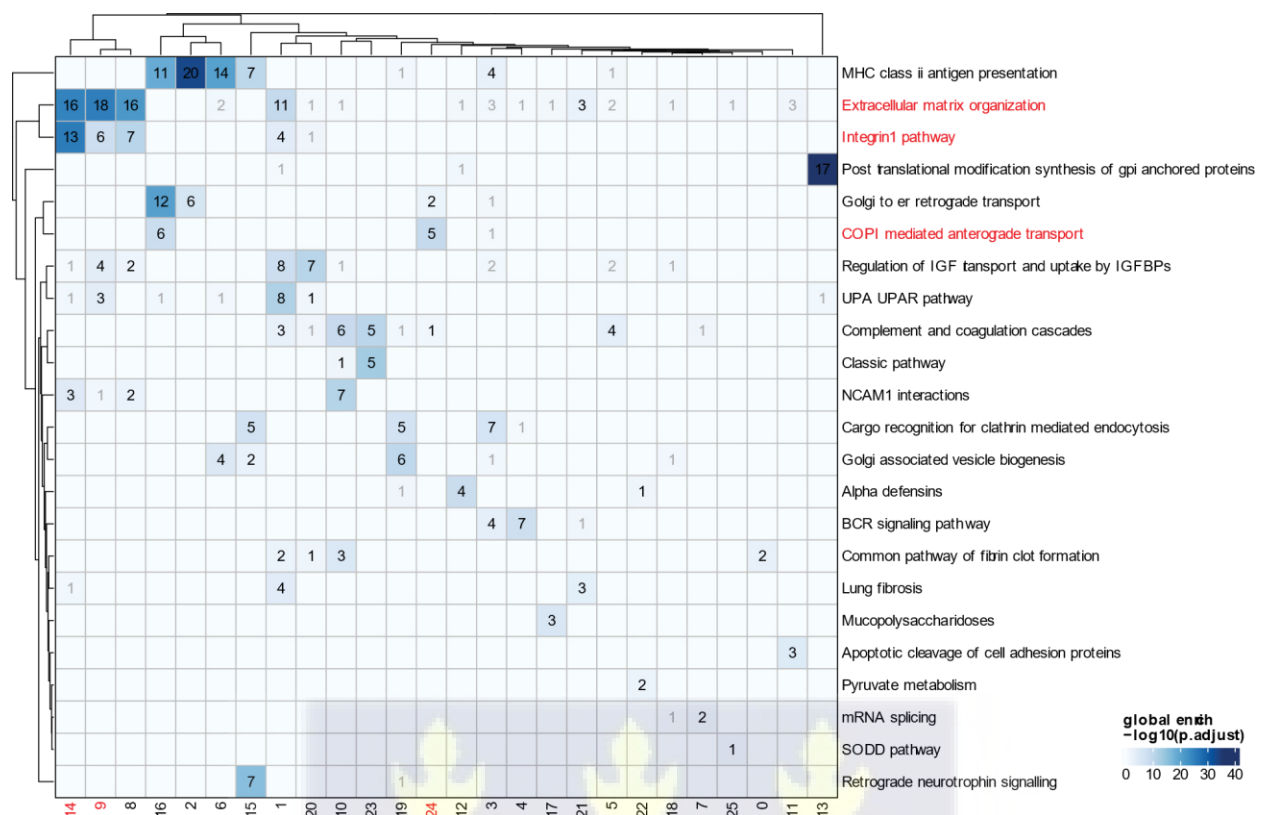


Figure S4.6 Pathways associated with top haematobium and mansoni nodes network sub-clusters. An enrichment heatmap obtained after gene set enrichment analysis of the top haematobium and mansoni nodes propagated subnetwork clusters. The hypergeometric test was used to derive the P-values which were then adjusted for multiple hypothesis testing using the FDR method. Pathways linked to the 5 most altered clusters in PCa patients from Figure 4.3 are highlighted in red.

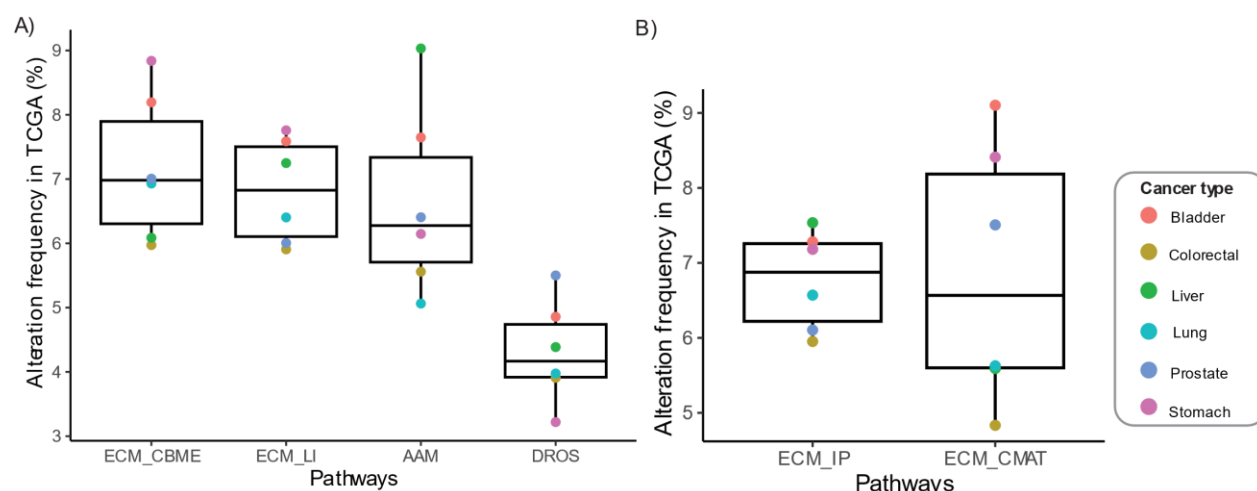


Figure S4.7 Alteration frequencies of top clusters' nodes across the most common cancers in men. Comparing the gene alteration frequencies (comprising structural variants and CNA, point mutations, gene and protein expression) of the 5 most altered clusters. These clusters are categorized by the pathways in which they operate in and are examined across malignancies that commonly affect men (A) for mansoni DEGs and (B) for both haematobium and mansoni DEGs. The data is from cBioPortal. ECM_CBME: Extracellular matrix organization & degradation and Collagen biosynthesis & modifying enzymes, ECM_LI: Extracellular matrix organization & degradation and Laminin interactions, AAM: Amino acid metabolism, DROS: Detoxification of ROS, ECM_IP: Extracellular matrix organization & degradation and Integrin 1 pathway, ECM_CMAT: Extracellular matrix organization & degradation and COPI-mediated anterograde transport.

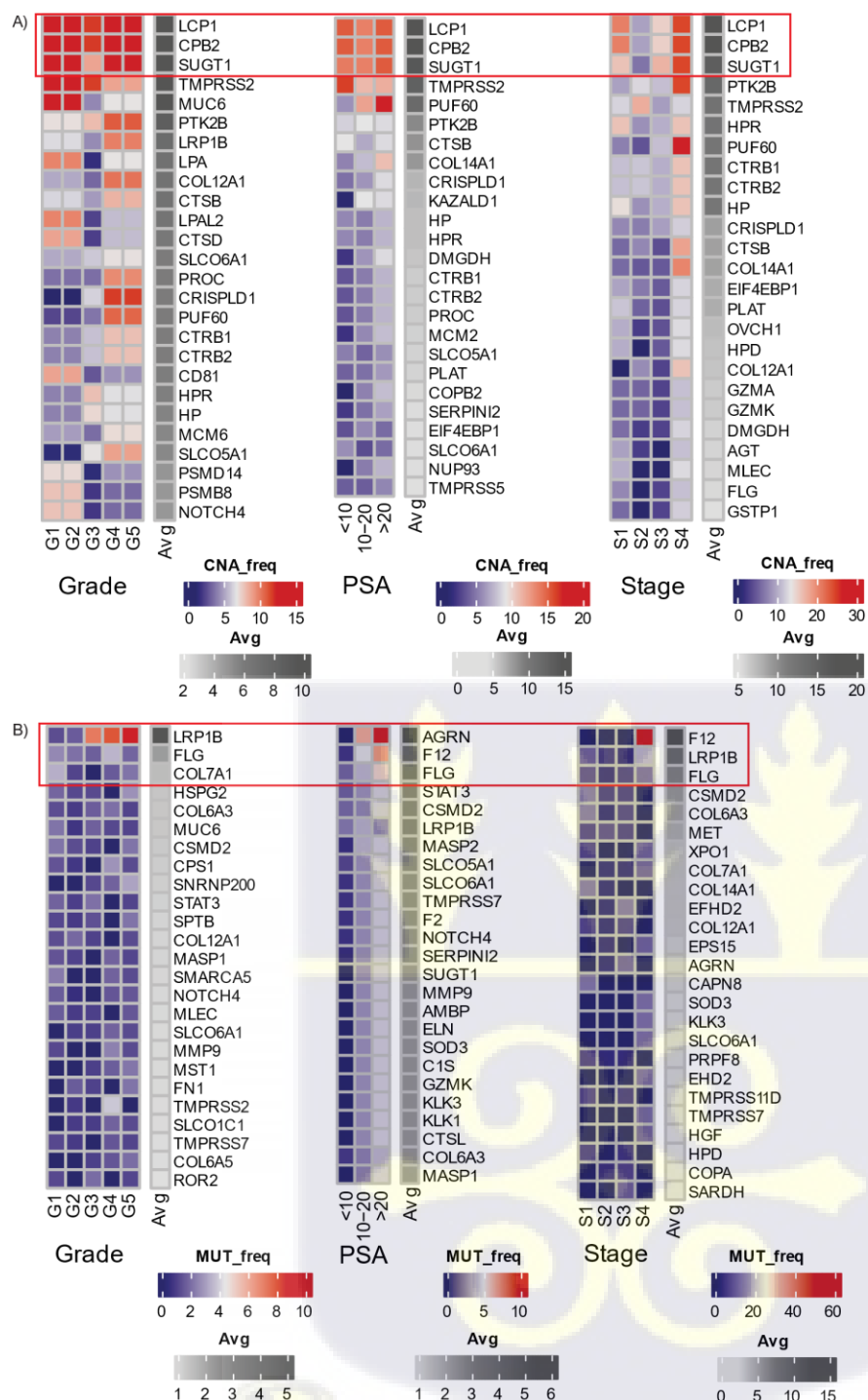


Figure S4.8 Hierarchical ranking of mansoni DEGs. (A) Ranked copy number alteration and (B) mutation frequencies of haematobium DEGs across different PCa grades, stages and PSA with the selected 3 most altered DEGs integrated into the haematobium propagated network highlighted. Data is from cBioPortal

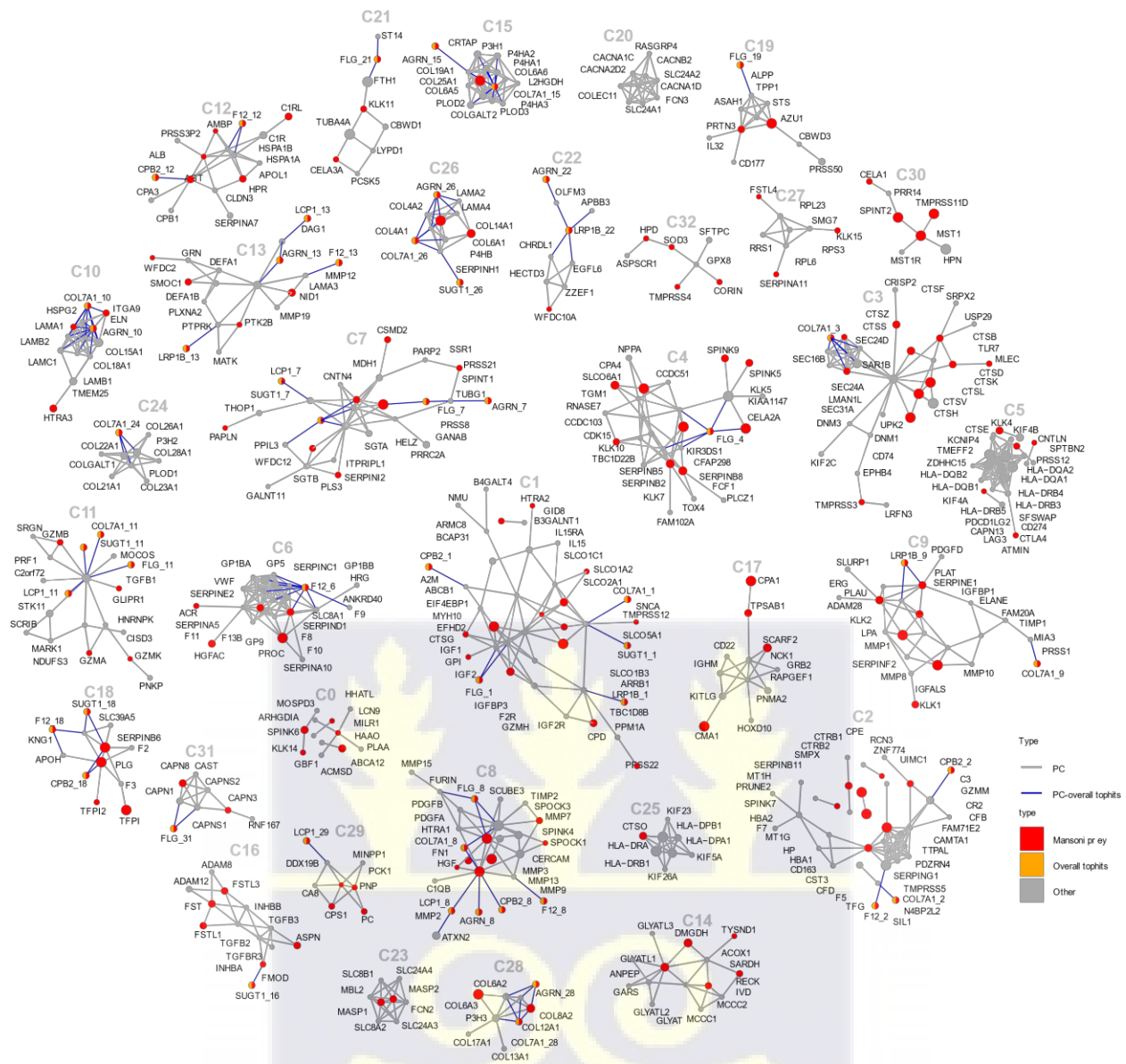


Figure S4.9 Incorporating the top mutated mansoni DEGs into the mansoni DEG propagated subnetwork clusters. Using Pathway Commons, the most mutated mansoni DEGs were integrated into the mansoni propagated network by mapping out the known interactions between these DEGs and the genes in the subnetwork clusters. The nodes' sizes in the network are indicative of their p-values; larger nodes denote more statistically significant interactions.

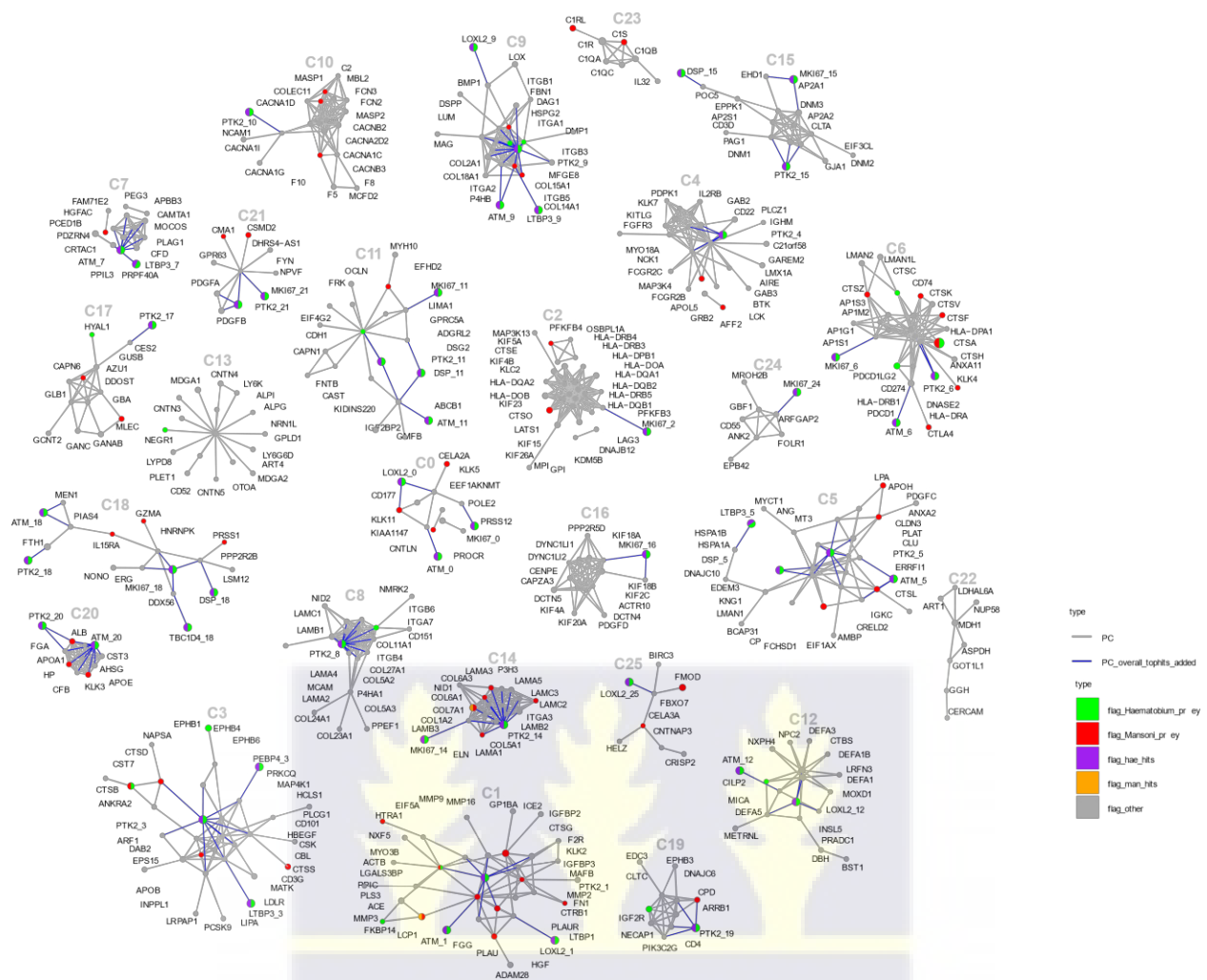


Figure S4.10. Integration of the top mutated mansoni and haematobium DEGs into the mixed infection scenario propagated subnetwork clusters. Using Pathway Commons, the top mutated mansoni and haematobium DEGs were integrated into the mixed infection propagated network by mapping out the known interactions between these DEGs and the genes in the subnetwork clusters. The nodes' sizes in the network are indicative of their p-values; larger nodes denote more statistically significant interactions.

CHAPTER FIVE

ROLE OF SCHISTOSOMA HAEMATOBIIUM AND MANSONI SOLUBLE EGG ANTIGENS IN PROSTATE CARCINOGENESIS

5.1 Introduction

Inflammation has a key role amongst other elements that influence the initiation and progression prostate cancer (PCa) (A. Rani *et al.*, 2019). The intriguing connections between chronic inflammation and the onset and development of several malignancies, including PCa, has gained a lot of attention in the past few decades. Chronic inflammation, characterized by the persistent presence of immune cells and inflammatory mediators, has emerged as a crucial factor in cancer initiation, promotion, and progression (Greten & Grivennikov, 2019; Hibino *et al.*, 2021; Muñoz-Pérez *et al.*, 2022; A. Rani *et al.*, 2019). The intricate interplay between inflammation and carcinogenesis involves various molecular pathways, often influencing the epigenetic landscape of cells (Maiuri & O'Hagan, 2016; S. Yang *et al.*, 2022). Epigenetic modifications, such as DNA methylation and histone modifications, can lead to altered gene expression patterns, ultimately contributing to oncogenesis (Pop *et al.*, 2019). Inflammation-induced oxidative stress and DNA damage can initiate DNA methylation changes, thereby promoting uncontrolled cell growth and evasion of apoptosis (García-Guede *et al.*, 2020; Sengupta *et al.*, 2022).

Schistosomiasis, a parasitic disease caused by *Schistosoma spp*, leads to the deposition of eggs in various organs, inducing an inflammatory response (Kayuni *et al.*, 2018). Recent literature has reported the presence of Schistosoma eggs in the prostate glands of infected individuals, accompanied by a pronounced inflammatory milieu (Choto, Mduluza, Mutapi, et al., 2020; Peiffer et al., 2019). The enquiry into the potential function of Schistosoma infection in prostate carcinogenesis has been spurred by this inflammatory background.

Based on this relationship, the study's central hypothesis is that soluble egg antigens derived from *S. haematobium* and *S. mansoni* would induce chronic inflammation within prostate tissues, consequently modifying the DNA methylation landscape which would be observable at the gene and protein expression levels. These could activate pathways relevant in promoting PCa development. To test this hypothesis, the aim was to investigate the effects of soluble egg antigens on normal prostate cell lines. The experimental approach incorporated a combination of cell culture techniques, multi-omic analyses, and statistical tools, allowing us to study the intricate interactions between these pathogen antigens and prostate epithelial cells.

The investigations revealed compelling insights into the probable oncogenic behavior of prostate epithelial cells exposed to *Schistosoma* soluble egg antigens. Notably, these cells exhibited relative increase in proliferation and apoptosis, both of which are hallmark features of cancer initiation and progression. Moreover, a relative increase in angiogenesis markers was also observed, although it was not statistically significant for any of the antigen treatments. Furthermore, the proteomics analyses uncovered the activation of pathways closely associated with cancer development. These findings collectively suggest that soluble egg antigens from schistosomes may indeed play a pivotal role in driving the oncogenic transformation of normal prostate epithelial cells.

As the understanding of the intricate relationship between inflammation and carcinogenesis continues to evolve, this study brings forth valuable insights into the possible mechanisms of *S. haematobium* and *S. mansoni* induced PCa. These findings underscore the importance of further exploration in this area, with potential implications for the development of novel strategies for PCa prevention and intervention, particularly in *Schistosoma*-endemic regions.

5.2 Methods

5.2.1 Cell culture

PNT2 cell lines were grown in RPMI 1640. The media was supplemented with 10% (v/v) FBS (Sigma-Aldrich, USA). Immortalized human prostate epithelial cells RWPE1(ATCC, Manassas, VA, USA) were grown in serum-free keratinocyte media, supplemented with human recombinant epidermal growth factor and bovine pituitary extract (Life Technologies, Carlsbad, CA, USA). The media were all supplemented with 100 µg/mL streptomycin and 100 IU/mL of penicillin (HyClone, Logan, UT, USA). Cells were cultivated at 37 °C and 5% CO₂.

5.2.2 Preparation of *Schistosoma haematobium*, *mansoni* and combined Soluble Egg Antigens

Pellets (obtained in collaboration with the parasitology unit of NMIMR and CSIR) containing schistosome eggs were pooled, suspended in phosphate-buffered saline (PBS), and centrifuged three times at low speed. The egg pellet obtained was then frozen for 10 minutes at -80°C. The frozen pellet was ultrasonified for 2 minutes in 5 cycles. After disrupting roughly 95% of the eggs, the lysate was centrifuged for 20 minutes at 1000 rpm at 4°C (Hitachi CF7D2, Japan). The supernatant was then collected and ultracentrifuged at 24000g for 90 minutes at 4°C. The *Schistosoma spp* soluble egg antigen (schSEA) containing supernatant, was filter-sterile and kept at -80°C until use. The protein concentration of the schSEA was determined using the Bradford method.

5.2.3 Determination of the schSEA Effect on Human Normal prostate epithelial Cells

5.2.3.1 Cell viability

The effect of schSEA on cell viability which is also used as a proxy for cell proliferation was assessed by cellTitre-Glo (Promega) and the Alamar Blue assays. Briefly, for the Alamar Blue assay, PNT2 cells were seeded at a density of 10000 cells/ well in 96 well plates. The cells were

serum-starved for 12 hours and then treated with schSEA at various concentrations (12.5, 15, 18.75, 20 and 25 mg/mL) for varying exposure times (12, 24 or 48 hours). At the end of the exposure time, the Alamar Blue working solution was added by a volume corresponding to 10% of the media (10 mL in 90 mL media). The plate was covered in aluminium foil paper and placed on a gentle shaking for 5 minutes, followed by incubation for 4 hours in the dark at 37 °C. After the incubation period, the fluorescence was measured using a Varioskan plate reader (ThermoFisher Scientific) with 560nm excitation and 590nm emission wavelengths.

Similarly, for the cellTiter-Glo assay, RWPE1 cells were seeded at a density of 10000 cells/ well in an opaque white 96 well plate. After treating the cells for 24, 48 and 72 hours, the media was changed and replaced with 50 mL of fresh media. Then 50 mL of cellTiter-Glo reagent equilibrated to room temperature was added to each well containing SEA-exposed cells, negative control cells (non-treated cells) as well as the blank (media only). The plate was then wrapped in aluminium foil and placed on a shaker for 2 minutes, followed by a 10-minute incubation in the dark. Thereafter, the luminescent signal was measured using the GloMax Discover microplate reader (Promega).

5.2.3.2 Cancer hallmarks and cytokine responses

RWPE1 cells were seeded at a density of 0.5×10^6 cells/well in 6 – well plates. The cells were then exposed to schSEA haematobium at a concentration of 20 µg/mL for various exposure times (24, 48, 72 hours). Cells were harvested and resuspended in 1 mL TRIzol (Invitrogen). RNA was isolated following a standard TRIzol extraction protocol. Briefly, 200 µL chloroform was added to the cell suspension, mixed, and then centrifuged for 15 minutes at 10000g and a temperature of 4°C. All centrifugation steps were carried out at a speed of 10000g and a temperature of 4°C except otherwise stated. The aqueous upper phase was transferred to a new tube and 500 µL of Isopropanol was added then vortexed. This was followed by another centrifugation step, for 10 minutes. The

supernatant was discarded and 1mL of 75% ethanol was added to the pellet and centrifuged for 5 minutes. Following the centrifugation, the supernatant was discarded, and the pellet was allowed to air-dry. The RNA was resuspended in 40uL of RNase free water. Subsequently the RNA was purified using the RNA clean and concentrator kit from Zymo Research according to the manufacturer's instructions. The RNA was dissolved in 40 µl RNase free water. RNA concentrations and purity were determined by absorption measurement at 260 nm and 280 nm using a Nanodrop.

The cDNA was synthesised in a 20ul total volume containing 4 l 5 first-strand buffer, 2 mM dithiothreitol (DTT), 200 U of SuperScript II (all Life Technologies, Eggenstein, Germany), 20 U of RNase inhibitor, 5 mM random hexamers, and 1 mM deoxynucleotide triphosphate (dNTP) mix (all Applied Biosystems GmbH, Weiterstadt, Germany). The reaction mixture was incubated at 24°C for 10 minutes and at 42°C for 60 minutes. After that, the reverse transcriptase was inactivated for 3 minutes at 94°C.

The Applied Biosystems® ProFlex™ PCR System thermal cycler was used for cDNA synthesis (Step 1: 25°C, 10 min; Step 2: 37°C, 120 min; Step 3: 85°C, 5 min). qRT-PCR was used to evaluate gene expression using an ABI Step One Plus and Quant Studio 5 Real-Time PCR system using the SYBR® Select Master Mix (Life Technologies, USA) and the primer pairs and PCR conditions specified in Table 4.1. The comparative cycle threshold ($2^{-\Delta\Delta C_t}$) method was used to calculate mRNA expression, which was given as the relative fold change compared to the control, normalised with GAPDH mRNA levels.

Table 5.1 RT-qPCR targeted genes and primer sequences

Gene	Forward primer	Reverse primer
<i>ANGPT1</i>	<i>ACGTGGAACCGGATTTCTCT</i>	<i>CTGCAGTCTGAGAGAGGAGG</i>
<i>BAX</i>	<i>TGACATGTTTTCTGACGGCA</i>	<i>CACCCTGGTCTTGGATCCA</i>
<i>CASP3</i>	<i>TGAGCCATGGTGAAGAAGGA</i>	<i>TCGGCCTCCACTGGTATTTT</i>
<i>MKI67</i>	<i>GGAGAAGCCCCAACC AAAAG</i>	<i>TAGGACTAGGAGCTGGAGGG</i>
<i>MYC</i>	<i>ATTCTCTGCTCTCCTCGACG</i>	<i>TGCGTAGTTGTGCTGATGTG</i>
<i>VEGFA</i>	<i>GGAGGAGGGCAGAATCATCA</i>	<i>ATCTCTCCTATGTGCTGGCC</i>
<i>IFN-g</i>	<i>AAGTGATGGCTGAACTGTCTG</i>	<i>TACTGGGATGCTCTTCGACC</i>
<i>IL-4</i>	<i>TCTCACCTCCCAACTGCTTC</i>	<i>ATATCGCACTTGTGTCCGTG</i>
<i>IL-5</i>	<i>CTGCCTACGTGTATGCCATC</i>	<i>GAAAGCAGTGCCAAGGTCTC</i>
<i>IL-6</i>	<i>AGTCCTGATCCAGTTCCTGC</i>	<i>CATTTGTGGTTGGGTCAGGG</i>
<i>IL-10</i>	<i>ATGTTCTTTGGGGAGCCAAC</i>	<i>AACAGCTAGAAAGCGTGGTCA</i>
<i>IL-13</i>	<i>AGAGGATGCTGAGCGGATTC</i>	<i>CCTCGATTTTGGTGTCTCGG</i>
<i>IL-17A</i>	<i>TACAACCGATCCACCTCACC</i>	<i>ACTTTGCCTCCCAGATCACA</i>
<i>TGFb1</i>	<i>CTACCATGCCAACTTCTGCC</i>	<i>CCGGGTTATGCTGGTTGTAC</i>
<i>TNF-a</i>	<i>GGACCTCTCTCTAATCAGCCC</i>	<i>GGTTTGCTACAACATGGGCT</i>

5.2.3.3 Signaling pathway activation and protein abundance.

PNT2 cells were seeded in 10 cm dishes at a density of 2.2×10^6 cells per dish. After 24h, the cells were treated with schSEA (18.75 μ g/mL) and incubated for 48h. As a control, cells had equivalent volumes of schSEA replaced with 1x PBS. After the incubation period, proteins were obtained from the cells using Guanidine hydrochloride lysis buffer (6M Guanidine Hydrochloride - Sigma #G3272-500G, 100mM Tris-HCl pH 8.0). Briefly, the media was discarded, and ice-cold PBS was gently dispensed onto the cells, swirled, and discarded 3 times. On the final wash, excess PBS was removed rapidly by “whacking” against a paper towel. Then, 0.6mL cold lysis buffer was added and the cells were immediately scraped, pipetted up and down to break up cells, transferred to 1.5mL microcentrifuge tubes, and placed on ice for further processing. Finally, the cell lysates were boiled for 5 minutes at 95°C to inactivate proteases and phosphatases. Quantification of protein concentration was performed using the BCA assay, the lysates were stored at -80°C and subsequently shipped to UCLA on dry ice for proteomics analyses.

The lysate was thawed on ice, followed by immediate probe-sonication of the cell lysate to ensure complete cell lysis and DNA fragmentation. Sonication was done for 15 seconds at 20% magnitude, with samples kept on ice before and after the process. Protein concentrations were reevaluated using the Bradford assay.

A mixture of Tris(2-carboxyethyl) phosphine (TCEP) and 2-chloroacetamide was used to reduce and alkylate the samples. Protein aliquots were treated with a 1:10 mixture of TCEP/IDA, followed by a 5-minute incubation at 45°C. Then the proteins were digested at a 1:100 (wt/wt) enzyme-to-substrate ratios for LysC and Trypsin, followed by an overnight incubation at 37°C. Desalting was achieved using Sep Pak tC18 columns, with careful maintenance of liquid on the column. Abundance proteomics samples were resuspended for mass spectrometry.

For data analysis, the search engine used was DIA-NN 1.8 in a library-free search mode. For protein identification, peaks were searched against the database UPR_*Homo sapiens*_9606_UP000005640 with the following parameters - deep learning-based spectra, RTs and IMs prediction, the maximum number of protease (trypsin) missed cleavages set to 1, post-translational modifications: Carbamidomethyl (C), Oxidation (M), false discovery rate (FDR) set to 1%, and match between runs was used. After obtaining the peptide counts and intensities, the search output was taken to R for analyses.

5.2.4 Bioinformatics and statistical analysis

All analyses were performed using open-source resources. As mentioned in the preceding sections, analyses and visualisation were carried out using several packages based on R v4.1.3 (R Development Core Team, Vienna, Austria) and RStudio v2023.03.1 + 446. For final visualization, all figures were refined using Adobe Illustrator 2023. Data are presented as the mean $\pm$ standard error of the mean (SEM). The threshold for statistical significance was set at a *p-value* < 0.05 unless otherwise stated.

5.3 Results

5.3.1 schSEA increases cell proliferation of exposed PNT2 and RWPE1 cells.

The evaluation of the impact of schistosome soluble egg antigens (schSEA) on prostate carcinogenesis involved multiple assays. Alamar Blue and CellTiter Glo proliferation assays were conducted, along with RT-qPCR analysis of proliferation-associated markers MKI67 and MYC. The results revealed distinct patterns of cell proliferation in response to different schSEA sources (Figure 5.1). Notably, cells treated with schSEA from *S. haematobium*, and mixed infections exhibited heightened proliferation, as demonstrated by the Alamar Blue assay (Figure 5.1A). In contrast, schSEA from *Schistosoma mansoni* triggered a mild reduction in proliferation, which persisted across various concentrations. Notably, a significant decline in cell viability occurred at

higher concentrations. Similarly, the CellTiter Glo assay underscored the time-dependent acceleration of cell proliferation following exposure to schSEA from *S. haematobium*, while *S. mansoni*-derived schSEA seemed to impede proliferation (Figure 5.1B). These findings were consistent with RT-qPCR results, indicating increased gene expression of proliferation markers *MKI67* (Figure 5.1C) and *MYC* (Figure 5.1D). Collectively, these observations suggest that schSEA from *S. haematobium* promotes cell proliferation in exposed cells in a concentration dependent manner, whereas the same effect is less pronounced in cells exposed to schSEA from *S. mansoni*.

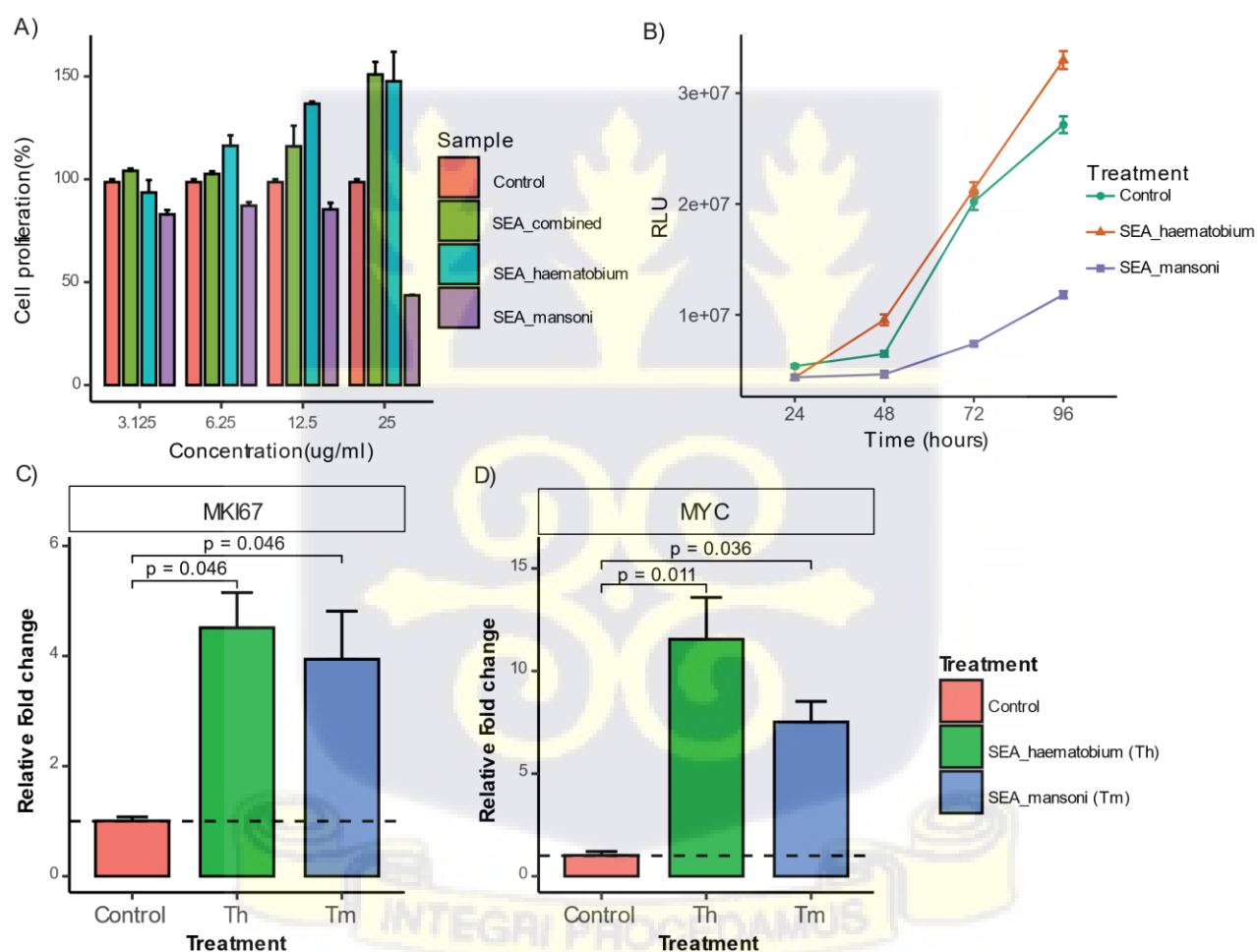


Figure 5.1 schSEA promotes cell proliferation. (A) Alamar Blue assay to assess the effect of schSEA on PNT2 cell proliferation at various schSEA concentrations. Control cells received equal

volume of PBS (vehicle) as the schSEA in treated cells. (B) The Luminescence of the RWPE1 cells treated with 20g/mL of schSEA for 24, 48 and 96h was measured using the CellTiter Glo Luminescent Cell Viability Assay kit (Promega G7570). (C) and (D) represent relative gene expression of proliferation markers MKI67 and MYC with respect to the vehicle control. GAPDH was used as the endogenous control. Fold change was calculated using the $2^{-\Delta\Delta C_t}$ method. Pairwise t-test with Holm's correction was used to assess the significance of the fold change. Each data point represents the mean of three independent experiments and the data is represented as mean $\pm$ se.

5.3.2 schSEA exposure of PNT2 cells increases the rate of programmed cell death.

In the context of our investigation into the implications of schistosome soluble egg antigens (schSEA) in prostate carcinogenesis, we sought to explore the impact on apoptotic processes, a critical facet of cancer development. Initially, we employed rt-qPCR to assess the expression of key apoptosis-related markers, BAX (Figure 5.2A) and CASP3 (Figure 5.2B). Remarkably, schSEA-exposed cells exhibited an unexpected substantial increase in the relative expression of these markers when compared to their unexposed counterparts. To validate this unexpected finding, we conducted additional assays. Employing the Caspase Glo assay, we observed an augmented activity of Caspases in the schSEA-exposed cells, with an even more pronounced increase evident in cells exposed to schSEA derived from *Schistosoma haematobium* (Figure 5.2C). Notably, the outcomes witnessed in the rt-qPCR and Caspase Glo assays were concordant with the results obtained through flow cytometry analysis, thereby reinforcing the consistency and reliability of our findings across multiple methodologies.

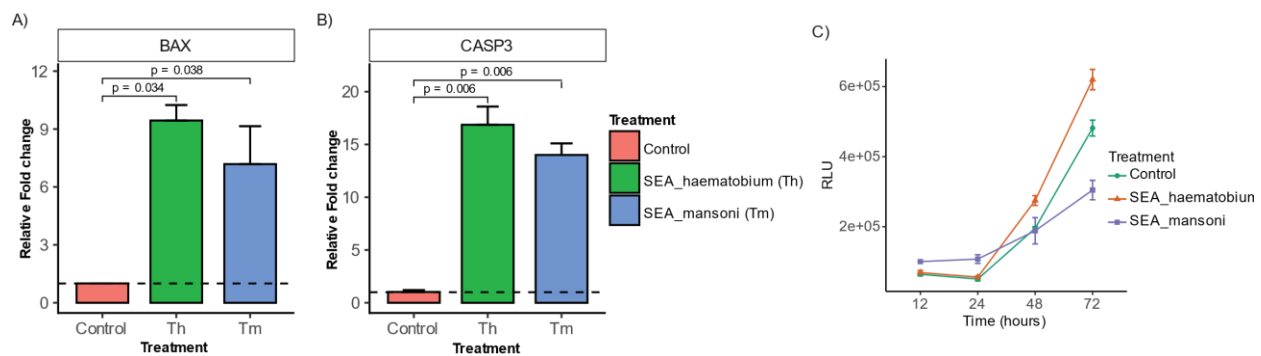


Figure 5.2 Evaluation of the effect of schSEA on Apoptosis. (A) relative gene expression of apoptosis markers (A) BAX and CASP3 compared to the vehicle control. GAPDH was used as the endogenous control. Fold change was calculated using the $2^{-\Delta\Delta C_t}$ method. Pairwise t-test with Holm's correction was used to assess the significance of the fold change. (C) The activity of caspase-3/7 was measured in schSEA-treated and control RWPE1 cells by the Caspase-Glo® 3/7 assay. schSEA haematobium showed a higher level of active caspase-3/7 compared to the control and schSEA mansoni. The caspase-3/7 activity was lowest in schSEA mansoni treated cells. Each data point represents the mean of three independent experiments. Data is represented as mean $\pm$ se.

5.3.3 Relative increased expression of the angiogenesis marker *VEGFA* in schSEA mansoni exposed cells

In the endeavor to comprehensively unravel the implications of schistosome secreted excretory/secretory antigens (schSEA), the investigations were extended to explore angiogenesis, a hallmark of cancer progression. Since schSEA is thought to trigger an inflammatory response, which has in turn a complex relationship with angiogenesis. Cytokines and growth factors released during inflammation are thought to act directly or indirectly on vascular epithelial cells. Using rt-qPCR, the relative gene expression of *ANGPT1* (Figure 5.3A) and *VEGFA* (Figure 5.3B) markers of angiogenesis of schSEA exposed cells were assessed. Strikingly, the results unveiled a discernible upregulation in the relative expression of both angiogenic markers in the schSEA-

exposed cells. Notably, the increase attained statistical significance solely in the case of the VEGFA marker (Figure 5.3B). Importantly, this augmented expression of *VEGFA* was prominently observed in cells exposed to schSEA derived from *Schistosoma mansoni*. This observation points to a potential role of schSEA from *S. mansoni* in influencing the angiogenic processes within the cellular microenvironment. These findings contribute to a more comprehensive understanding of the multifaceted impact of schSEA on key oncogenic pathways, shedding light on potential mechanisms underlying its involvement in cancer-related processes.

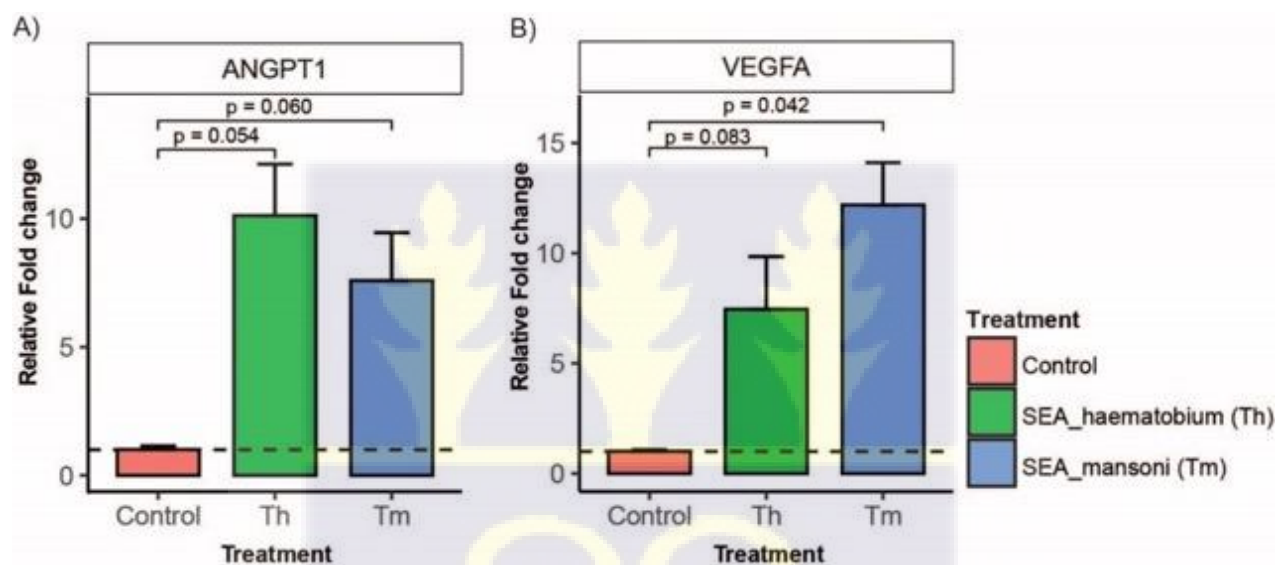


Figure 5.3 Evaluation of the effect of schSEA on Angiogenesis. Relative gene expression of apoptosis markers (A) ANGPT1 and (B) VEGFA with respect to the vehicle control. GAPDH was used as the endogenous control. Fold change was calculated using the $2^{-\Delta\Delta C_t}$ method. Pairwise t-test with Holm's correction was used to assess the significance of the fold change.

5.3.4 Dynamic gene expression of cytokines in schSEA-treated prostate epithelial cells.

In view of the well-established role of schistosoma infections in inducing chronic inflammation, it was imperative to investigate the influence of schistosome secreted excretory/secretory antigens (schSEA) on cytokine expression, considering their pivotal roles in eliciting inflammatory

responses and shaping immune dynamics. Given the association of interleukin-6 (IL-6) and interleukin-4 (IL-4) with both Th1 and Th2 cytokine pathways, respectively, and their implications in PCa, we examined the gene expression profiles of a range of cytokines encompassing the Th1 (IL-6, TNF- α , INF- γ), Th2 (IL-4, IL-5, IL-13), and Th17/Treg (IL-10, IL-17A, TGF β 1) axes. Within the Th1 context, schSEA haematobium exposure prompted a notable surge in IL-6, TNF- α , and INF- γ gene expressions during the initial 48 hours, a trend mirrored by schSEA mansoni exposure. However, the latter exhibited a more pronounced upswing at 72 hours. With regards to the Th2 and Th17 cytokines, IL-4 and IL-5 displayed a decline at 24 hours, followed by a modest increase at 48 hours which lacked statistical significance, and ultimately decreased by 72 hours in the haematobium-treated cells. Conversely, IL-13 exhibited a significant elevation in schSEA mansoni-treated cells, tapering off over time. This pattern was also mirrored in schSEA haematobium-treated cells. Divergent outcomes were observed for IL-17A: schSEA mansoni treatment yielded a steady increase in its expression, while schSEA haematobium treatment resulted in lower IL-17A expression compared to untreated cells. Shifting focus to Treg cytokines, both schSEA haematobium and mansoni treatments demonstrated similar diminishing trends in IL-10 and TGF β 1 gene expressions over time. However, schSEA mansoni exposure led to a pronounced surge in IL-10 expression at 72 hours, whereas TGF β 1 expression exhibited a decline. These findings collectively underscore the capacity of schSEA treatments to incite inflammatory immune responses, accompanied by attenuated anti-inflammatory reactions, ultimately fostering a milieu conducive to proinflammatory cytokine propagation.

The observed cytokine responses offer insights into the intricate interplay between schSEA and immune signaling. The significant increase in expression of Th1 cytokines, particularly IL-6, TNF α , and INF γ at 24 and 48 hours, underscores their potential role in the early inflammatory cascade triggered by both schSEAh and schSEAm exposure, potentially contributing to the

establishment of a proinflammatory microenvironment. The contrasting dynamics of Th2 cytokines, such as IL-4, IL-5, and IL-13, suggest a reluctance in the switch from Th1 to Th2 cytokines. Moreover, statistically significant IL-13 levels were observed at 24, 48 and 72 hours only for schSEAm exposed cells. The divergent behaviors of IL-17A in response to schSEAh and schSEAm treatments suggest that schSEA may distinctly modulate Th17-mediated pathways, which have been associated with inflammation and autoimmunity. The intricate shifts observed in Treg cytokines, IL-10 and TGF β 1, highlight the complexity of immune regulatory mechanisms triggered by schSEA exposure. Collectively, these findings elucidate the multifaceted immunomodulatory effects of schSEA, shedding light on potential mechanisms underlying their influence on inflammatory and immune processes in the context of PCa development.

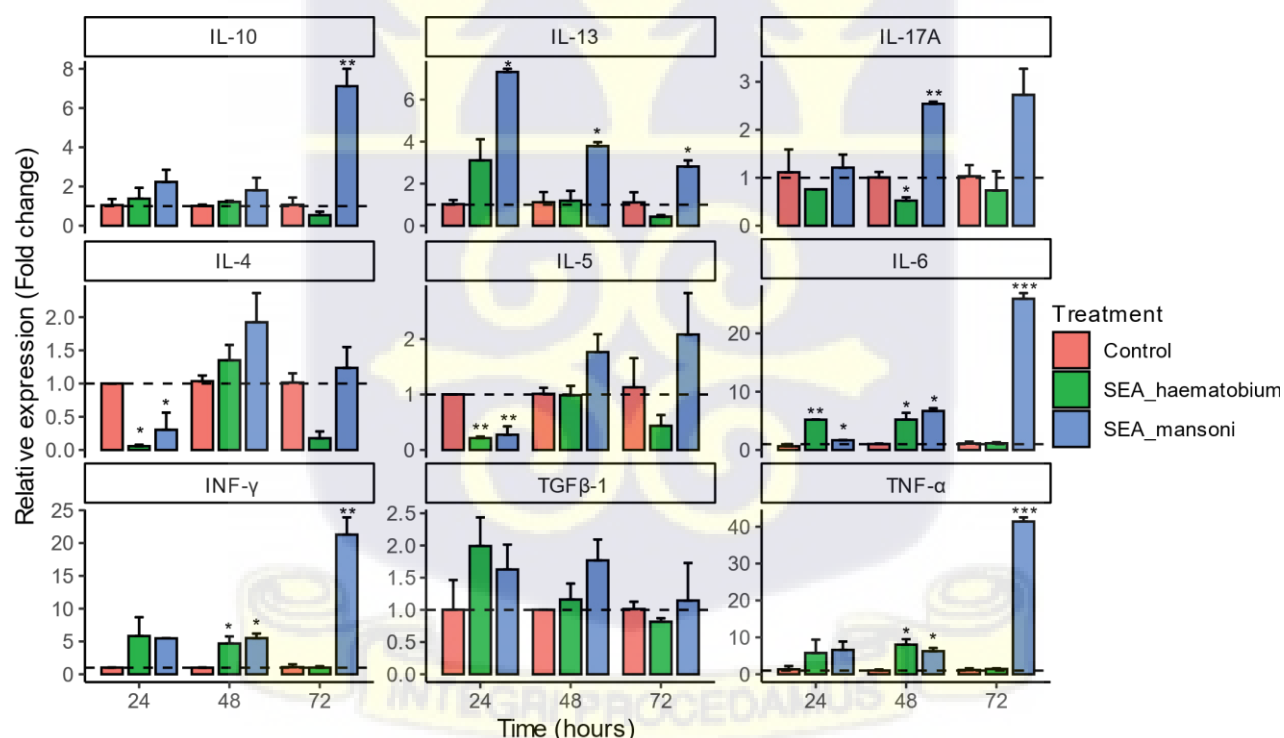
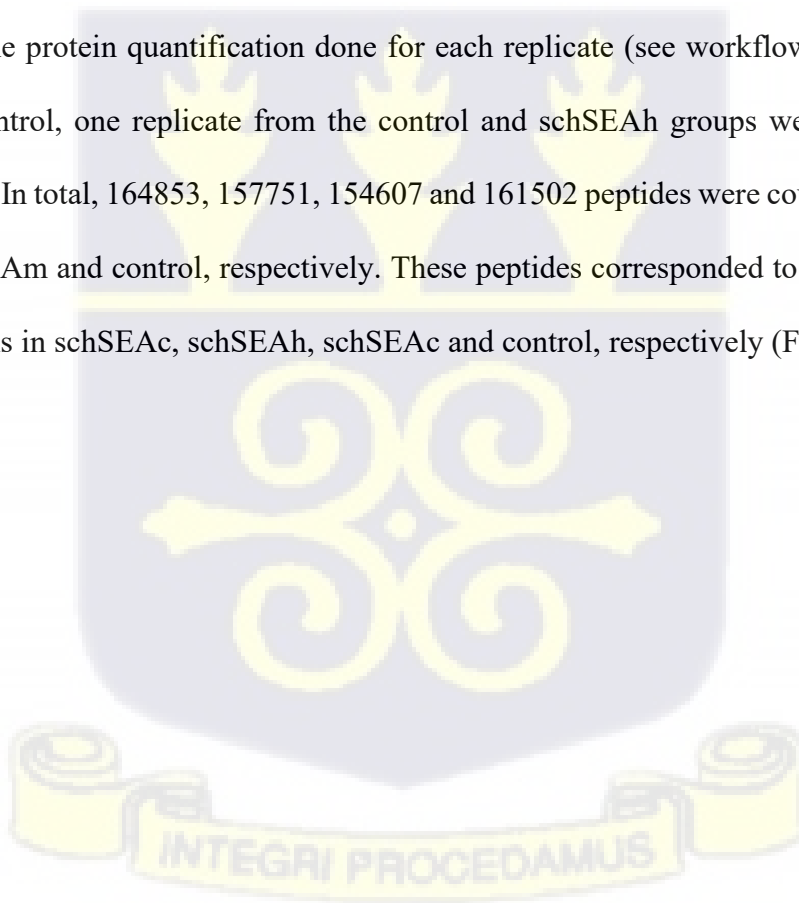


Figure 5.4 Relative cytokine gene expression of schSEA exposed RWPE1 cells. Cells treated with PBS (red bars) schSEA or SEA_haematobium (green bars), SEA_mansoni (blue bars). Error

bars represent the standard error of the mean and asterisks highlight levels of statistical significance (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). Refer to Table S5.1 for the precise p -values. Y-Axis indicates mRNA fold change compared with PBS treated control. Target gene expression was normalized to endogenous reference GAPDH.

5.3.5 Proteomic Analysis of schSEA-Induced Effects on Normal Prostate Epithelial Cells

This section sought to investigate the individual effects of schSEA isolated from *S. haematobium* or *S. mansoni* as well as the effect of schSEA from the combined species on normal prostate epithelial cells (PNT2) with a focus on protein abundance. The treatments were carried out in triplicates and the protein quantification done for each replicate (see workflow in Figure 5.5 A). After quality control, one replicate from the control and schSEAh groups were excluded from further analyses. In total, 164853, 157751, 154607 and 161502 peptides were counted in schSEAc, schSEAh, schSEAm and control, respectively. These peptides corresponded to 8130, 8034, 8013 and 8091 proteins in schSEAc, schSEAh, schSEAc and control, respectively (Figure 5.5B).



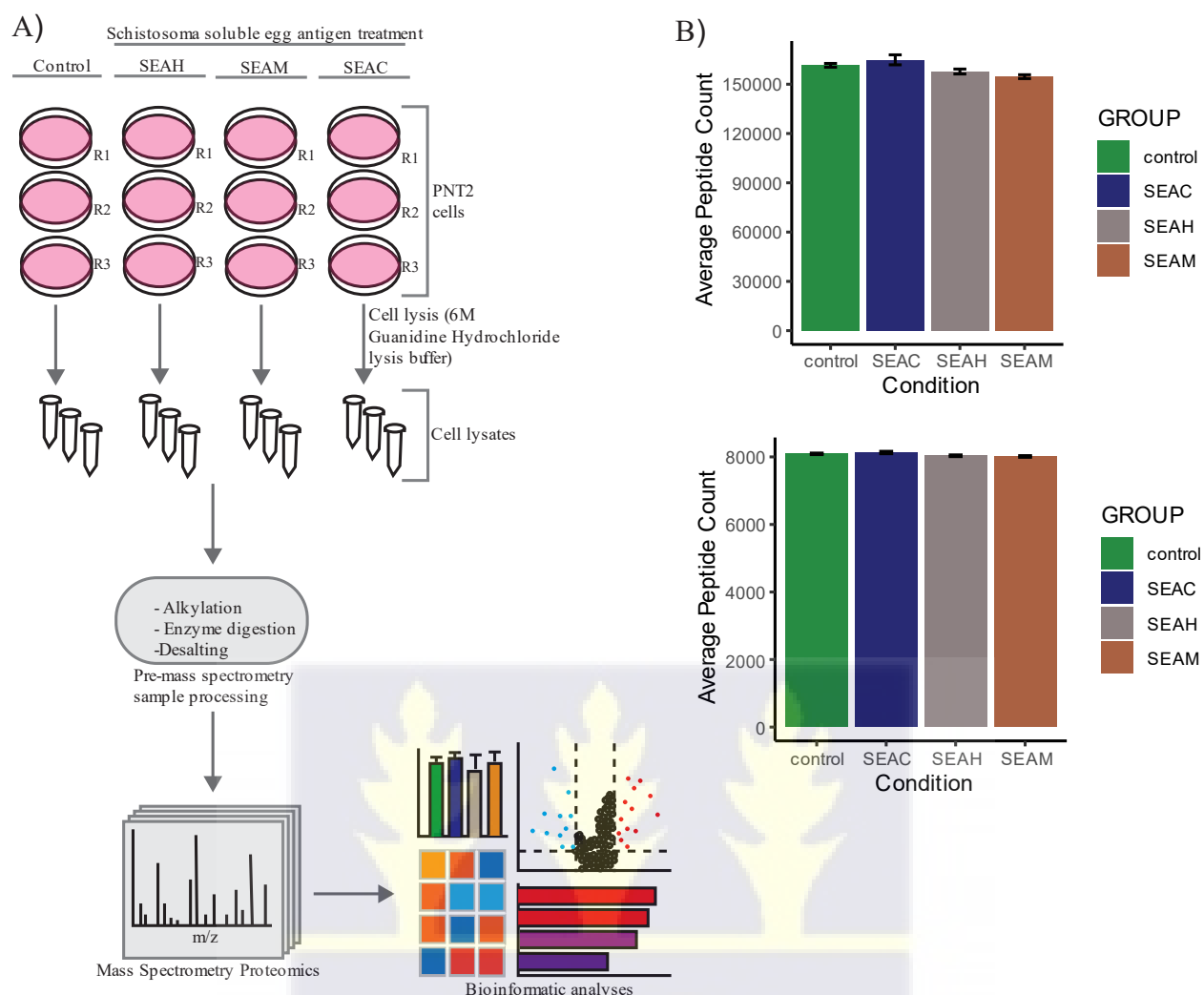


Figure 5.5 Proteomics analyses of SEA-treated PNT2 cells (A) Schematic representation of the complete workflow for the proteomics study. (B) Bar plots illustrating the counts of identified peptides and proteins across the four different conditions: Controls, SEAH treatment, SEAM treatment, and SEAC treatment.

5.3.6.1 schSEAc and schSEAh induce significant differential inflammatory responses in PNT2 cells.

At the outset, it was of interest to confirm the proinflammatory responses observed in the rt-qPCR analyses. Using a panel of 49 ISGs identified by Liu *et al.*, (2018) (including but not limited to

MX2, OAS1, STAT1, IL6 and *TRIM21*), a significant inflammatory response induced by schSEAc and schSEAh treatments was observed. These treatments showed statistically significant increases in ISG protein intensities (Figure 5.6), reinforcing the hypothesis that they provoke an inflammatory reaction as part of the host-microbe interplay, thereby validating the rt-qPCR analyses of cytokine genes. Interestingly, while schSEAm shows a slight increase in ISG protein levels compared to the control, the rise was not statistically significant. This suggests that schSEAm may not trigger the same level of inflammatory response as SEAc and SEAh.

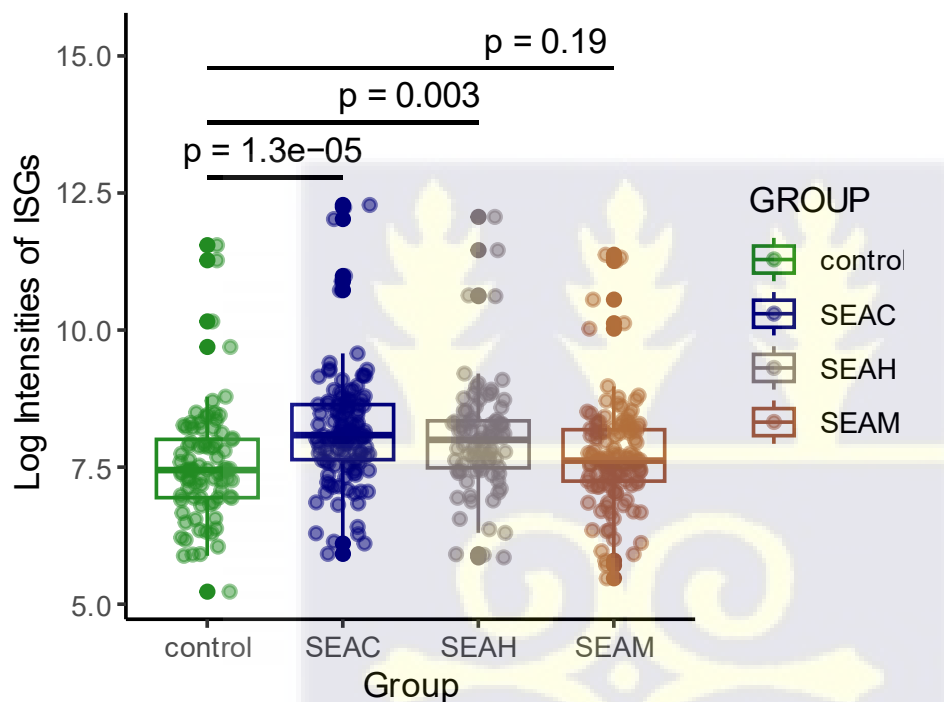


Figure 5.6 Boxplot of ISG protein intensities by treatment. Significant increases are noted in schSEAc and schSEAh treated cells compared to control, suggesting an inflammatory response. Treatment with antigen from *S. mansoni* eggs (schSEAm) shows a slight increase which is not significant.

5.3.6.2 Differential protein expression analyses in schSEA-exposed normal prostate epithelial cells

A principal component analysis (PCA) was performed to compare the proteome profiles among SEA-exposed and non-exposed PNT2 cells. A clear distinction was observed amongst the groups suggesting that there were significant differences in overall proteome expression profiles between SEA-exposed cells and non-exposed cells, as well as among the different antigens (Figure 5.7A-D).

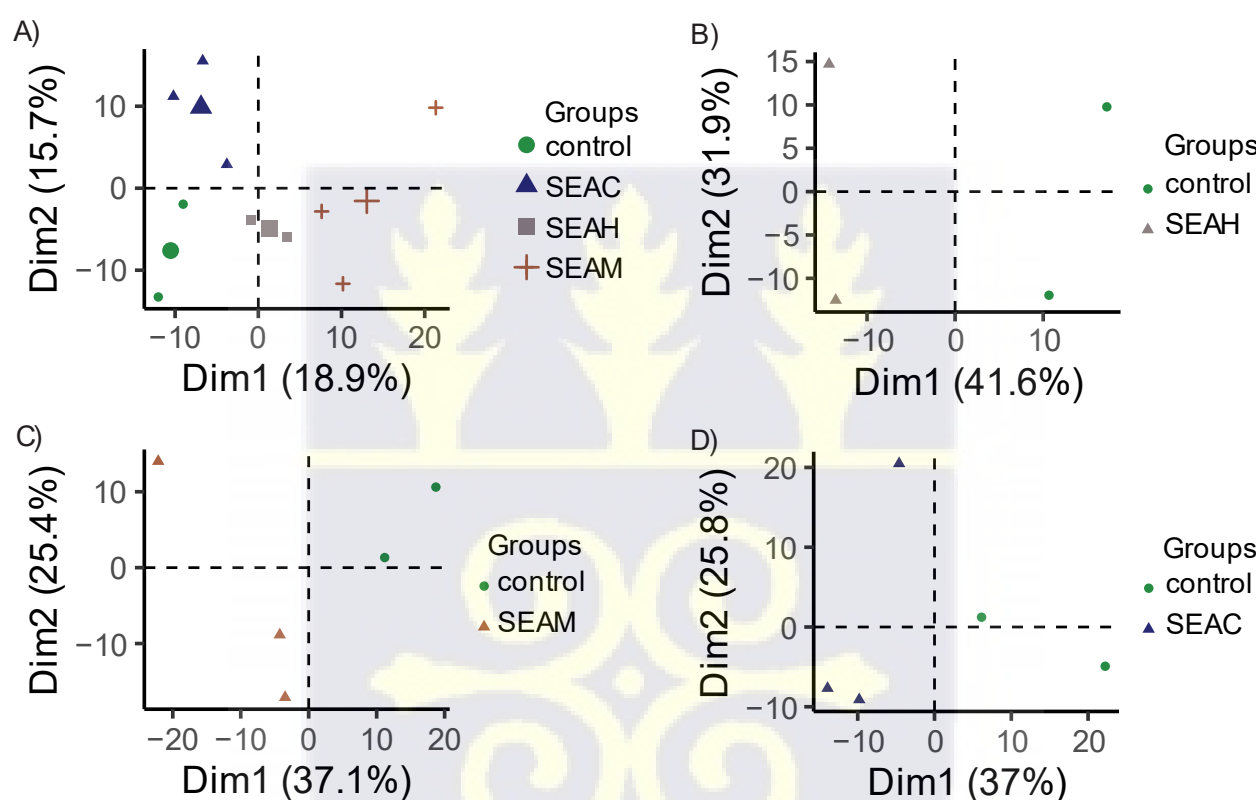


Figure 5.7 Principal component analyses of schSEA treated cells. (A) PCA plot aggregating all four conditions. Data points are color-coded and shaped with respect to the treatment groups: Controls (forest green circles), schSEAH Treatment (Grayish Rose squares), schSEAM Treatment (red-orange cross), and schSEAC Treatment (navy blue triangle). (B-D) Individual PCA plots comparing each treatment group against the control. Colors and shapes for data points adhere to

the scheme established in part A. The PCAs presented represent 7664, 7838, 7751 and 7879 proteins for the general, schSEAh, schSEAm and schSEAc PCAs, respectively.

Differentially expressed proteins (DEPs) were identified between non-exposed PNT2 cells and SEA-exposed PNT2 cells. A pair-wise comparison analysis was performed in schSEAh, schSEAm and schSEAc treated PNT2 cells compared to the non-treated PNT2 cells. T-test analysis showed that 52, 53, and 48 proteins were significantly differentially expressed with p -value < 0.05 and $\text{Log2FC} < -1$ or $\text{Log2FC} > 1$ between schSEAh, schSEAm, and schSEAc, respectively, compared to the nontreated cells. These include 24 downregulated and 28 upregulated, 32 downregulated and 21 upregulated, 14 downregulated and 34 upregulated proteins by schSEAh, schSEAm and schSEAc, respectively as visualised in the volcano plots (Figure 5.8A). These DEPs were mostly unique to each treatment with only a few upregulated and downregulated proteins shared amongst treatments (Figure 5.8 B-C). The top 20 downregulated and upregulated proteins can be visualized in the volcano plots (Figure 5.8 D-F). In schSEAh treatment, the most significant downregulated proteins were TRPC4AP, DEFA1 and TMCO4 while the most significant upregulated proteins were SAE1, RILPL2 and TNFSF10. Concerning schSEAm treated cells, the most significant downregulated proteins were TMCO4, TSPYL2 and B4GALT4 while the most significant upregulated proteins were RILPL2, MPDZ and ACE. Finally, in the schSEAc treated cells, TMCO4, MMP2 and MXRA7 were most significantly downregulated and DEFA1, RILPL2 and DNLZ were upregulated. Though most of these proteins have not been studied in the context of prostate cancer, DEFA1 an alpha defensin which was interestingly predicted in the computational analyses to be a pathway potential hijacked by schSEAh treatment is said to be a predictive biomarker of PCa therapy in castration resistant PCa. TSPYL2 was shown to inhibit the transactivation of androgen receptor (AR) on its target genes and therefore the downregulation of

this protein may promote activation of AR which might promote prostate carcinogenesis (Y. Li *et al.*, 2017). On the other hand, MXRA7 has been shown to be downregulated in PCa and deemed to potentially have diagnostic and prognostic (Jia *et al.*, 2011; S. Liu *et al.*, 2020).



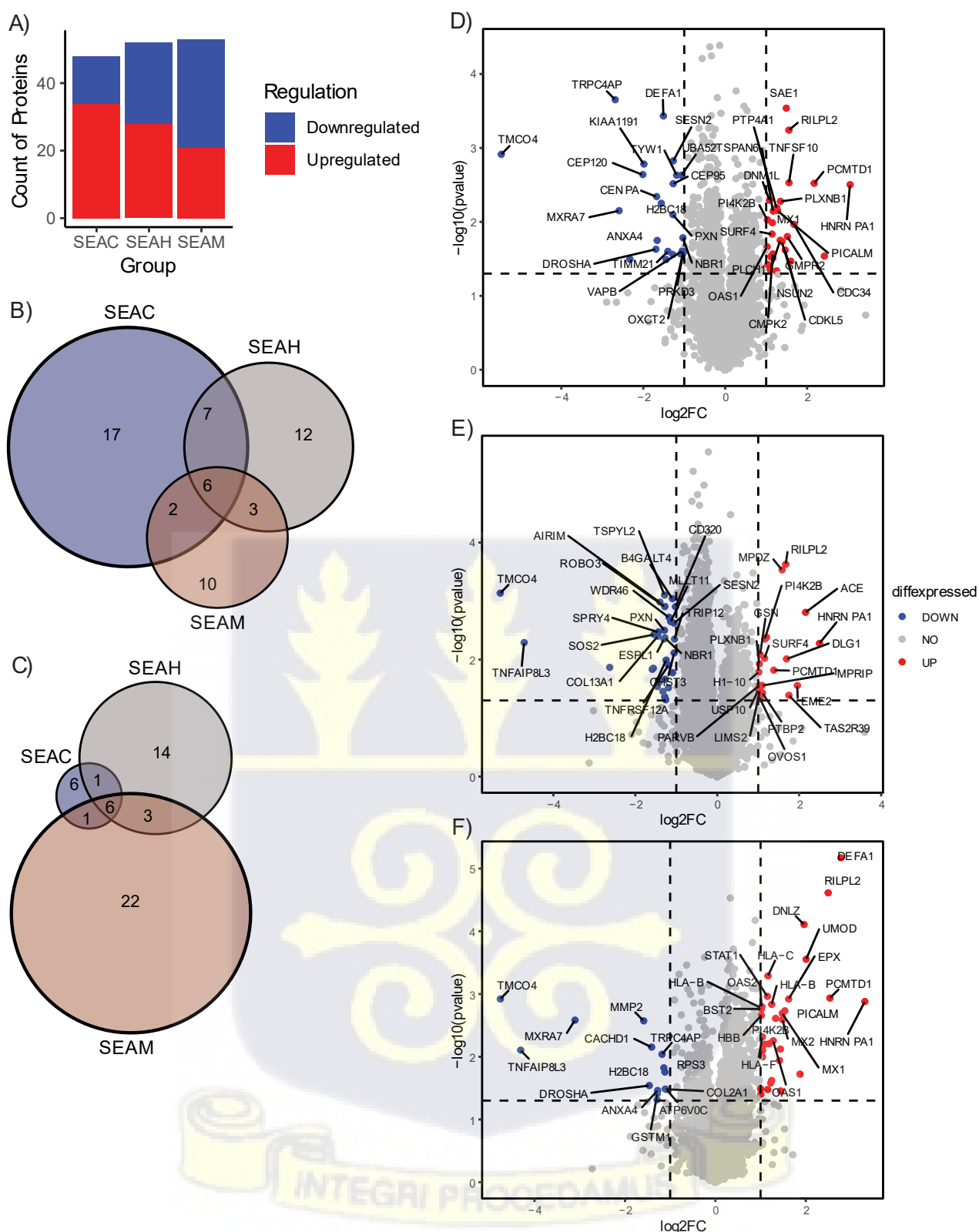


Figure 5.8 Differential protein abundance associated with various SEA treatments. (A)

Stacked bar plot representing the number of downregulated (blue) and upregulated (red) proteins

for schSEAc, schSEAh, and schSEAm treatments. (B & C) Venn diagrams illustrating the overlap and uniqueness of upregulated (B) and downregulated (C) differentially expressed proteins between treatments. Areas of overlap indicate shared proteins, while distinct areas highlight treatment-specific proteins. (D) Volcano plots for each treatment group (SEAH, SEAM, SEAC). Only data points meeting the criteria of $\text{Log}_2\text{FC} < -1$ or $\text{Log}_2\text{FC} > 1$ and $p\text{-value} < 0.05$ are highlighted as significant on the volcano plots.

5.3.6.3 Unsupervised hierarchical clustering reveals conserved and divergent protein expression across schSEA treatments in prostate epithelial cells.

To better appreciate the protein expression patterns across the various treatments, a series of unsupervised hierarchical clustering heatmaps were plotted in Figure 5.9. A global perspective of about 8200 proteins based on $\log_2$ Fold Change ($\log_2\text{FC}$) was employed to identify clusters of interest (Figure 5.9A). Cluster 1 and Cluster 4 were observed to have consistent expression trends across the schSEAc, schSEAh, and schSEAm treatments. Figures 5.9 B-D zoomed in on these clusters, showcasing log intensities in comparisons between the control and each specific treatment. Only proteins meeting the criteria; $\log_2\text{FC}$ of < -1 or > 1 and a $p\text{-value} < 0.05$ were included. Noteworthy expression patterns emerged from the data; DROSHA and SSR2, for instance, were consistently downregulated across all treatments. In contrast, CDC34 was universally upregulated. Treatment-specific upregulations were also noted; DNLZ was upregulated solely in the schSEAc treatment, while MPDZ was upregulated exclusively in the schSEAm treatment. These findings collectively revealed both conserved and divergent protein expression patterns. The observations suggest similar as well as unique effects of the various schSEA treatments on the prostate epithelial cells.

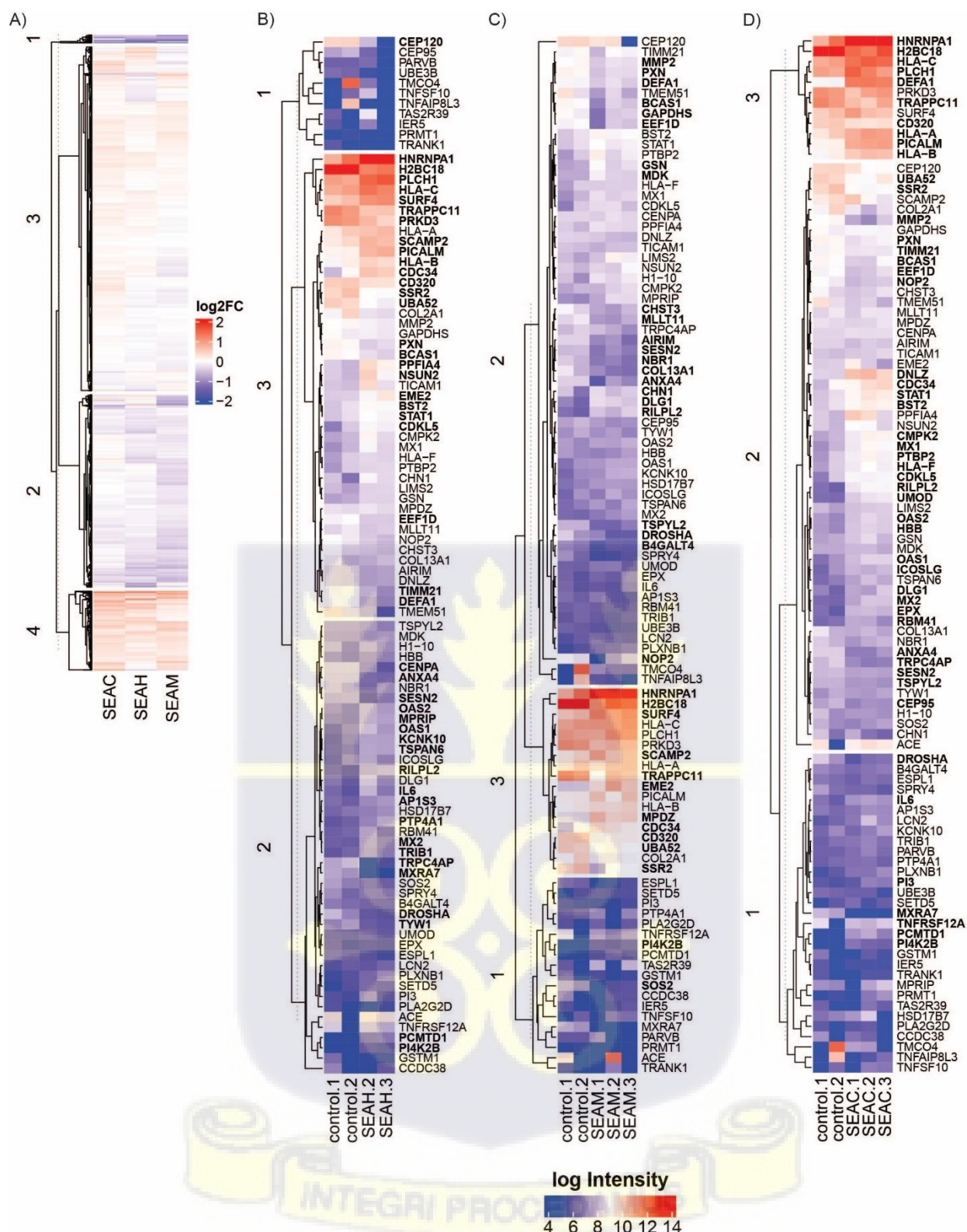
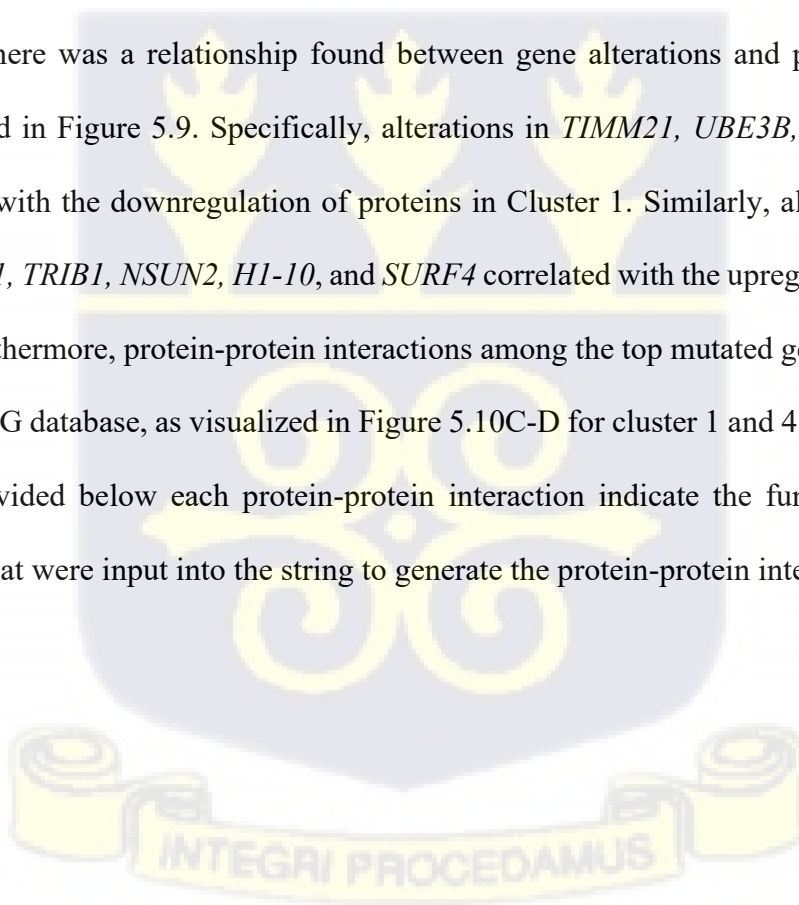
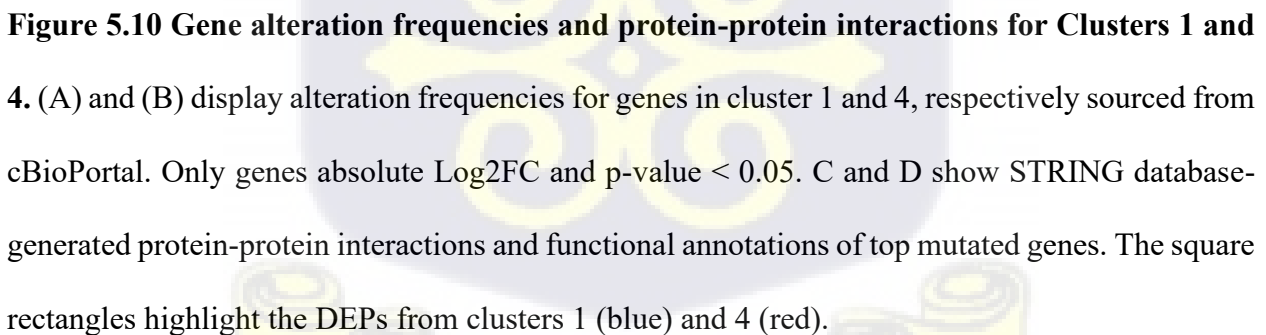


Figure 5.9 Heatmaps illustrating protein expression patterns across schSEAc, schSEAh, and schSEAm treatments. (A) shows unsupervised hierarchical clustering of ~8,200 proteins by

log₂FC. (B-D) Heatmaps display the log Intensities for proteins in Cluster 1 and 4, extracted from (A), for schSEAh (B), schSEAm (C), and schSEAc (D) treatments. Proteins demonstrating appreciable differences between control and treatment conditions are highlighted. For this focused analysis, Cluster 1 and 4 were filtered to include only proteins meeting the criteria of Log₂FC < -0.5 or Log₂FC > 0.5 and p-value < 0.05.

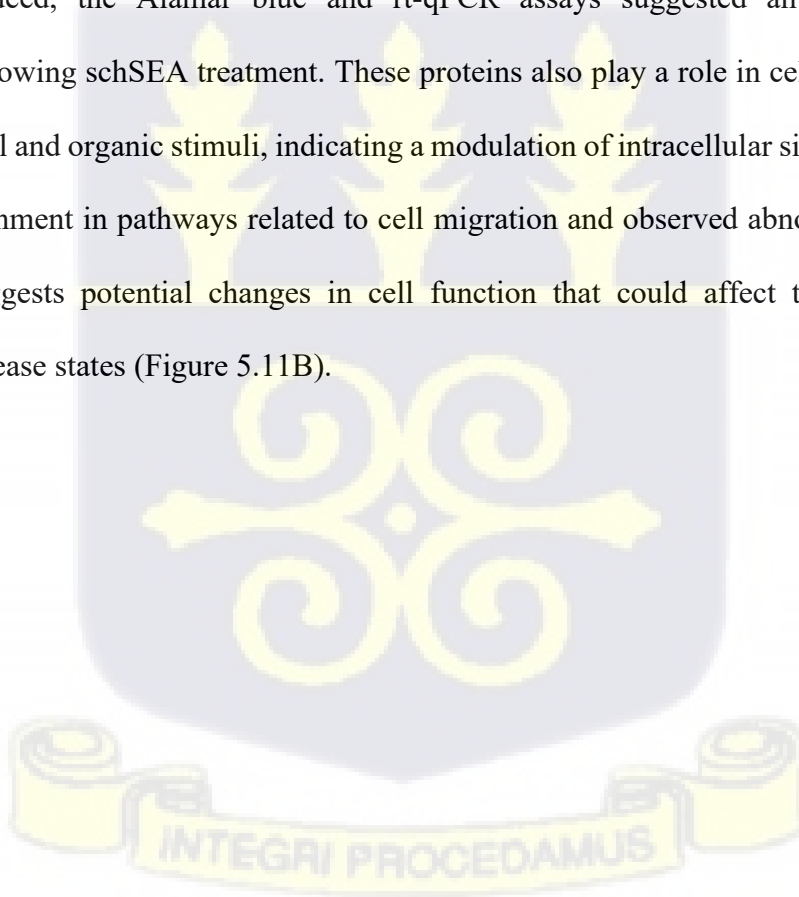
In the next analytical step, genes from cluster 1 and 4 were ranked according to their alteration frequencies using publicly available genomics data sourced from cBioPortal, as shown in Figures 5.10A-B. The focus remained on genes that met the criteria of log₂FC < -1 or log₂FC > 1 and a p-value < 0.05. There was a relationship found between gene alterations and protein expression patterns observed in Figure 5.9. Specifically, alterations in *TIMM21*, *UBE3B*, *PXN*, and *AIRIM* were consistent with the downregulation of proteins in Cluster 1. Similarly, alterations in genes such as *PCMTD1*, *TRIB1*, *NSUN2*, *H1-10*, and *SURF4* correlated with the upregulation of proteins in Cluster 4. Furthermore, protein-protein interactions among the top mutated genes were mapped using the STRING database, as visualized in Figure 5.10C-D for cluster 1 and 4, respectively. The descriptions provided below each protein-protein interaction indicate the functions of the top mutated genes that were input into the string to generate the protein-protein interactions.





5.3.6.4 Functional Enrichment of SEA-induced DEP in PNT2 cells

To explore the biological relevance of the DEPs in clusters 1 and 4, Gene Ontology analyses was conducted. For cluster 1 which were downregulated proteins, the schSEA treatments seem to affect genes important for cellular homeostasis, given their involvement in the biosynthesis and metabolism of key cellular components like glycoproteins, lipids, and carbohydrates. Additionally, their role in apoptotic pathways suggests that they could be critical for cell survival or programmed cell death, possibly serving as a regulatory mechanism under schSEA treatment (Figure 5.11A). The upregulated proteins in cluster 4 are primarily involved in cell cycle regulation and stress response, suggesting that the schSEA treatment has an impact on cell proliferation and adaptability. Indeed, the Alamar blue and rt-qPCR assays suggested an increase in cell proliferation following schSEA treatment. These proteins also play a role in cellular responses to various hormonal and organic stimuli, indicating a modulation of intracellular signaling pathways. Lastly, the enrichment in pathways related to cell migration and observed abnormal renal tubule morphology suggests potential changes in cell function that could affect tissue integrity or contribute to disease states (Figure 5.11B).



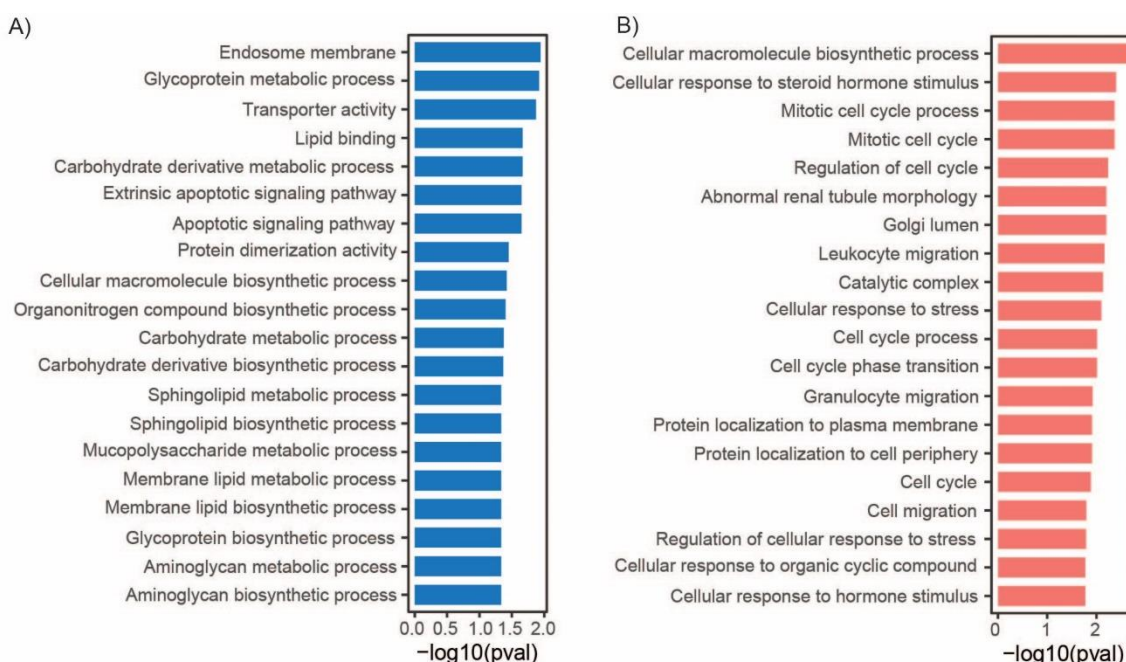


Figure 5.11 Gene ontology analyses of DEPs in cluster 1 and cluster 4. (A) GO enrichment of proteins downregulated (cluster 1) by schSEA treatment. (B) GO enrichment of proteins upregulated (cluster 4) by schSEA treatment.

5.3.6.5 Correlation of alteration frequency and log₂FC unveils key cell cycle genes in schSEA treatments.

Considering that Alamar Blue, cell titre glo, and rt-qPCR assays, along with proteomics, indicated that schSEA treatment likely promotes cell proliferation in a normal prostate epithelial cell line. To further explore this, cell cycle related genes were isolated from the enrichment analysis of cluster 4. Seven proteins were identified to be associated with the cell cycle term (Figure 5.12A). Of these, NSUN2, CDKL5, and EME2 were distinctly different between all treatments and control group (Figure 5.12B), while *DLG1* showed distinct changes in the schSEAm treatment. A subsequent analysis compared the frequency of alterations in these genes using TCGA data from cBioportal, aiming to identify potential targets for future research. This revealed that *MX2*, *DLG1*,

and *NSUN2* were frequently altered in the TCGA dataset and exhibited a relatively high fold change in response to schSEAc and schSEAh treatments. For schSEAm, only DLG1 showed an appreciable positive correlation (Figure 5.12C).

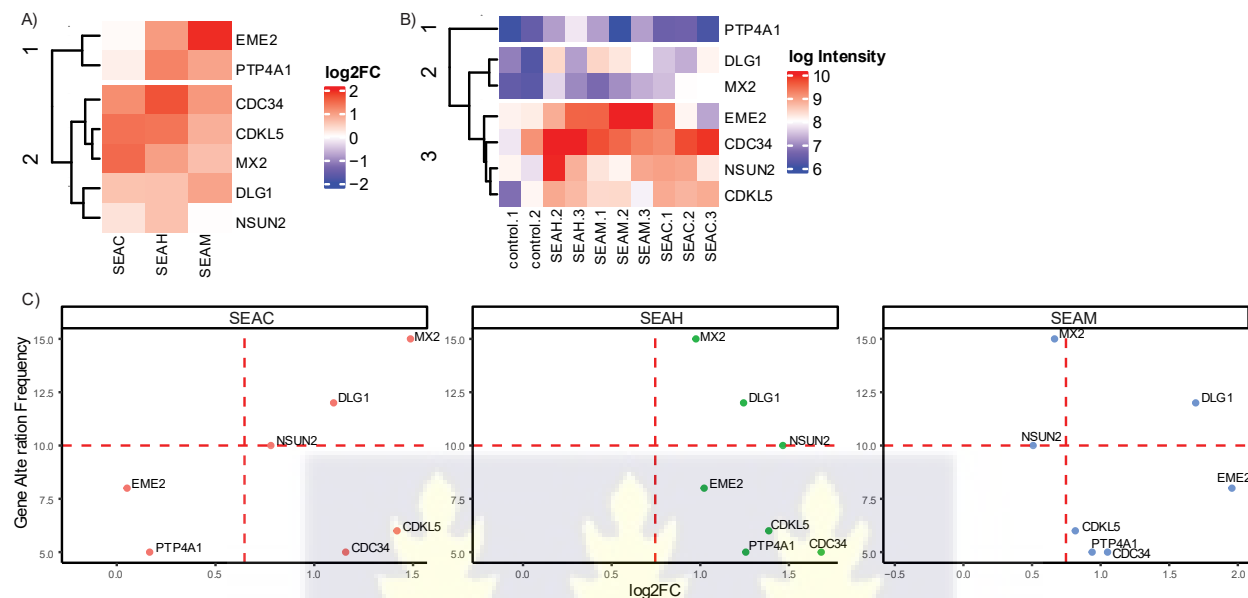


Figure 5.12 Analysis of DEPs cell cycle-related genes in schSEA-treated PNT2 cells. (A) The heatmap displays the fold changes (log2FC) of cell cycle genes across various treatments, while the heatmap in (B) shows the log intensities of these genes across treatments and control groups. (C) Correlation plots between how often each gene is altered in TCGA data (y-axis) against its fold change (log2FC) in different schSEA treatments (x-axis).

5.4 Discussion

The aim of this chapter was to evaluate the effect of schSEA treatment on a normal prostate epithelial cell line to assess its potential in driving prostate carcinogenesis. The hypothesis is that schistosomes can elicit proinflammatory stimuli in host tissues that can modify host signaling pathways that are also involved in PCa carcinogenesis.

Significant promotion of cell proliferation in prostate epithelial cells by schSEAh and schSEAc in a dose-dependent manner was observed. This finding is corroborated by a study conducted by

Tuffour *et al.*, in 2018 who also observed a dose-dependent increase in cell viability and proliferation of a schSEA exposed prostate cell line. In 2001, schSEAh was also shown to induce proliferation of urothelial cells and enhancing the expression of *BTG1* and *PCNA*, giving a correlation between cell proliferation and carcinogenesis induced by schSEA (El-Awady *et al.*, 2001).

A significant increase in expression of *MYC* and *MKI67* aligns with their function in promoting cell growth and proliferation in PCa (Qiu *et al.*, 2022). On the other hand, the significant increase in *MYC* and *MKI67* gene expressions were not reflected in the Alamar Blue and celltitre glo assays of cells exposed to schSEAm. This could be explained by various points which need to be further investigated. Firstly, schSEAm may influence the later stages of the cell cycle progression or even differentiation. Secondly, schSEAm might also be causing cell death such that the net cell number might not change, even if some cells are proliferating. To better understand the effect of schSEAm on proliferation of prostate epithelial cells, it will be important to assess the effect of the antigen at various stages of the cell cycle.

An unexpected increase in expression of the markers associated with apoptosis, namely *BAX* and *CASP3*, was observed in cells exposed to schSEAh and schSEAm. Therefore, despite the marked increase in proliferation caused by schSEAh treatment, there is also an increase in apoptosis. This phenomenon could be attributed to apoptosis-induced proliferation (AiP), a process where the proliferation of surrounding cells is stimulated by dying cells (Fuchs & Steller, 2015).

The extrinsic apoptosis pathway, mainly triggered by external signals (e.g schSEA treatment) transmitted via Natural Killer cells and cytotoxic T lymphocytes, activates caspase-8 and caspase-3 (Annibaldi & Meier, 2018; Moreno-Celis *et al.*, 2022; Nagata & Tanaka, 2017). These caspases are not only executors of cell death, but also contribute to the promotion of cell proliferation

(Feinstein-Rotkopf & Arama, 2009). The JNK signaling pathway is a key player in AiP, activated by various cytokines and involved in both cell survival and apoptosis (Moreno-Celis *et al.*, 2022). Elevated levels of reactive oxygen species (ROS) in tumours can also activate JNK and contribute to AiP (Moreno-Celis *et al.*, 2022). This complex relationship between apoptosis and proliferation could be a plausible mechanism through which schSEA contributes prostate carcinogenesis, with a particular focus on AiP's role in tumour advancement. It will therefore be important to investigate the JNK signaling in schSEA exposed prostate epithelial cells to validate this claim.

An increase in the expression of angiogenic markers, particularly VEGFA, was observed in cells exposed to schSEAm. Though this increase was also observed in schSEAh, it was not significant. The available data therefore suggest that schSEAm leads to increased vascularization, creating a microenvironment conducive to the transformation of normal prostate epithelial cells into malignant ones especially when other prooncogenic factors are met. A notable increase in proinflammatory cytokines such as IL-6, TNF α , and INF γ was observed in cells treated with schSEA. This aligns with the well-established role of schistosome infections in inducing chronic inflammation. Although the literature does not directly address the cytokine response to schSEA in prostate epithelial cells, valuable insights into the complex interplay between schSEA and immune signaling are provided by the observed cytokine profiles.

In the realm of biomedical research, proteomics plays a pivotal role in elucidating disease mechanisms and establishing causal relationships. By employing high-throughput techniques like mass spectrometry, proteomics allows for the comprehensive quantification of protein abundance, thereby shedding light on dysregulated pathways and potential therapeutic targets.

The data suggests that schSEAc and schSEAh treatments induce a significant inflammatory response in cells, as evidenced by the upregulation of Interferon-Stimulated Genes (ISGs) like

MX2, *OAS1*, *STAT1*, *IL6*, and *TRIM21*. The activation of ISGs like *MX2*, *OAS1*, and *STAT1* serves as an innate immune mechanism, providing a first line of defense against microbial infections. However, chronic activation of these pathways can lead to a state of persistent inflammation, which has been implicated in the initiation and progression of various malignancies, including prostate cancer. Chronic inflammation is known to induce oxidative stress, leading to DNA damage and genomic instability, thereby predisposing cells to neoplastic transformation. This is supported by the fact that inflammatory responses are associated to about 20% of all deaths from cancer globally, including prostate cancer (Ferrando *et al.*, 2013).

In PCa development, an inflammatory microenvironment is often an indicator of poor prognosis. ISGs like *STAT1* and *IL6* are not merely passive markers but active participants in tumorigenesis (H. Zhao *et al.*, 2021). *STAT1* modulates cell cycle progression and apoptosis (Meissl *et al.*, 2017), while *IL6* is implicated in proliferation, angiogenesis and differentiation in prostate cancer (Culig & Pühr, 2018). A population-based case-control study has also highlighted the association of circulating inflammation markers with prostate cancer, reinforcing the role of inflammation in disease initiation and progression (Ugge *et al.*, 2019). Interestingly, the data suggests that schSEAm may not elicit the same level of inflammatory response as schSEAc and schSEAh. It is necessary to test other concentrations of schSEAm and exposure time which might be different for each antigen.

Upon enrichment of the downregulated proteins across schSEA treatments, it was revealed that these proteins are implicated in cellular homeostasis. The proteins are involved in the biosynthesis and metabolism of key cellular components like glycoproteins, lipids, and carbohydrates. Downregulation of proteins associated with metabolism can lead to a shift in the metabolic landscape of the cell. This can result in metabolic reprogramming, where the prostate cells adapt to stress and sustain rapid growth and survival (Beier *et al.*, 2023). Also, glycoproteins are often

involved in cell signaling and communication (He *et al.*, 2013). Their downregulation could affect how cancer cells or damaged prostate epithelial cells would interact with their environment, potentially aiding in immune evasion (H. Lin *et al.*, 2021).

More interestingly, the upregulation of proteins in cluster 4, primarily involved in cell cycle regulation and stress response, indicates an increase in cell proliferation and adaptability. This aligns with the Alamar Blue, cell titre glo and rt-qPCR assay results for schSEAh and schSEAc treatments, which also suggested increased cell proliferation post treatment. The dysregulation of the cell cycle is a well-established mechanism in the development and progression of cancer (Malumbres & Barbacid, 2009).

In PCa, cell cycle dysregulation plays a pivotal role in its development and progression. For instance, *JunD*, a crucial regulator of cell cycle progression, has been identified as a potential target to block prostate carcinogenesis (Elliott *et al.*, 2019). Moreover, the androgenic activity, which is closely related to hormonal roles, also influences cell cycle modulators, adding complexity to cell cycle control in prostate cancer (Fernández *et al.*, 2002). In this study, seven DEPs were associated with cell cycle dysregulation, viz: CDC34, CDKL5, DLG1, EME2, MX2, NSUN2 and PTP4A1. Amongst these cell cycle-associated proteins, the fold change for MX2, NSUN2 and DLG1 correlated with high alteration frequencies TCGA data in cBioportal.

MX2 a dynamin-like GTPase2 induced by interferon, is mostly known for potent antiviral activity against human immunodeficiency virus type 1 (HIV-1). Though there is little to no evidence on the role of MX2 in the cell cycle mechanisms and in PCa biology, recent studies have shown that the malignant phenotype of clear cell renal cell carcinoma substantially correlated with *MX2* overexpression (Wei *et al.*, 2021). In addition, in 2019, Juraleviciute and colleagues identified MX2 in melanoma cells as a novel regulator of cell cycle (Juraleviciute *et al.*, 2020).

On the other hand, NSUN2 (NOP2/Sun RNA Methyltransferase 2) is a protein that has been implicated in various cellular processes, including RNA methylation and gene expression. Recent studies have shed light on its role in the cell cycle, particularly in cell growth and proliferation. *NSUN2* is thought to promote cell growth by methylating *CDK1* mRNA, thereby enhancing its translation and regulating *CDK1* expression during the cell division cycle (Xing *et al.*, 2015). *CDK1* is a crucial protein for cell cycle progression (Diril *et al.*, 2012), and its regulation by *NSUN2* suggests a direct involvement of *NSUN2* in the cell cycle. Another study focused on knocking down the *NSUN2* gene in HEK293 cells and assessing mRNA m5C modification and gene expression. The results revealed that *NSUN2* deficiency inhibited cell proliferation and migration (Z. Sun *et al.*, 2019). *NSUN2*, has also been associated to prostate carcinogenesis and progression. It is upregulated in PCa and is associated with a poorer outcome (G. Sun *et al.*, 2022). *NSUN2* plays a crucial role in stabilizing and modifying AR mRNA through cluster 5-methylcytosine (m5C) modification, particularly in the 5' region of AR mRNA, influencing various AR variants, including AR-V7 (G. Sun *et al.*, 2022; Zhu *et al.*, 2022). Additionally, a positive feedback loop exists, with AR transcriptionally regulating *NSUN2* (Zhu *et al.*, 2022). *NSUN2* therefore does not only play a significant role in cell proliferation but it's important in tumour biology.

DLG1, traditionally known for its role in maintaining cell polarity, may also be thought to have tumour-promoting functions in mammalian epithelia. The gene is frequently amplified in various cancers, suggesting that an increased expression of the gene may be involved in tumour development and progression (Saito *et al.*, 2018). Similarly, this gene was upregulated in schSEA treated prostate epithelial cells and therefore supports a possible role of schSEA in PCa development. There's however a need for functional studies of *DLG1* in the context of PCa development.

5.5 Conclusion

The findings in this chapter provide strong support for the hypothesis that schistosome infections, specifically through their soluble egg antigens (schSEA), may play a role in the development of prostate cancer. The study utilized mass spectrometry to analyze variations in protein expression and EPIC array DNA methylation profiling in a normal prostate epithelial cell line following exposure to schSEA. These findings were further validated through Gene Ontology (GO) analyses, as well as additional *in vitro* assays. All these assays revealed a potential effect of schSEA on cell proliferation and cell cycle related pathways which could lead to PCa development. Nonetheless, it is imperative to conduct functional assays (such as overexpression studies) aimed at the identified cell cycle genes to conclusively affirm these outcomes. Such experiments will enhance our understanding of the molecular mechanisms underlying the potential link between schSEA and prostate carcinogenesis.

5.6 Supplementary

Table S5.1 P-values of cytokines gene differential expressions at different times.

Gene	Time	Control vs schSEA haematobium treated (p-value)	Control vs schSEA mansoni treated (p- value)
<i>IL-4</i>	24	0.030	0.031
	48	0.476	0.075
	72	0.058	0.546
<i>IL-5</i>	24	0.006	0.006
	48	0.936	0.077

	72	0.440	0.303
<i>IL-6</i>	24	0.0014	0.0448
	48	0.027	0.025
	72	0.94	5.8e ⁻⁰⁷
<i>IL-10</i>	24	0.73	0.22
	48	0.65	0.47
	72	0.459	0.002
<i>IL-13</i>	24	0.091	0.010
	48	0.903	0.012
	72	0.152	0.017
<i>IL-17A</i>	24	0.50	0.83
	48	0.0244	0.0018
	72	0.687	0.057
<i>INF-g</i>	24	0.13	0.15
	48	0.035	0.028
	72	0.967	0.0009
<i>TGFb-1</i>	24	0.20	0.38
	48	0.72	0.12
	72	0.70	0.80
<i>TNF-a</i>	24	0.30	0.24
	48	0.034	0.036
	72	0.77	1.5e ⁻⁰⁷

CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS, LIMITATIONS AND RECOMMENDATIONS

6.1 General discussion

To reiterate, this study sought to contribute to the understanding of PCa in African men by exploring the DNA methylation profile of PCa in a ‘pure’ black population and to evaluate the potential of schistosome infections as a risk factor of PCa in the same population.

A high interindividual heterogeneity was observed in the methylation patterns across patients and similar heterogeneity was reported by Aryee and colleagues in PCa metastases (Aryee *et al.*, 2013). Moreover, there appeared to be some clustering between the tumour samples based on tumour grade (indolent vs aggressive). The DNA methylation profile associated with PCa aggressiveness suggests the involvement of cellular structure and division pathways as well as oxidoreductase activities. ROS, a key factor of oxidoreductase activities can be influenced by a variety of factors including infections, poor lifestyle, and oxidative stress (D’Autréaux & Toledano, 2007; Schieber & Chandel, 2014; Urao *et al.*, 2008). They have been implicated in inflammation, angiogenesis, and metastasis and are known to activate the PI3K/Akt signaling pathway, which plays a crucial role in cancer progression (Han *et al.*, 2014; Mazhar *et al.*, 2006).

Interestingly, using a cell deconvolution tool (*Episcore*) samples from high tumour grade patients were found to have highly significant levels of luminal cells, which are involved in PSA secretion triggered by inflammatory responses. This suggests that some inflammatory stimulus could contribute to PCa aggressiveness in this population. Some factors that can contribute to inflammatory responses in the prostate include mechanical injury, oxidative stress, and infections. Examples of infectious agents that have been associated with PCa include *Trichomonas vaginalis*, *Propionibacterium acnes* and *Schistosoma haematobium*.

Owing to the disproportionate prevalence of infectious agents, some for which Africa, some of which account for over 90% of the global burden (e.g schistosomiasis), infections could also be contributing to the observed disparity of PCa in African men. Therefore, the role of *Schistosoma* on PCa development and the potentially associated pathways were of interest. *Schistosoma haematobium* is already classified as a Group 1 carcinogen for bladder cancer and is particularly prevalent in Africa. Previous research has hinted at a link between schistosomiasis and PCa, but the molecular mechanisms behind this connection are not yet understood (Choto, Mduluz, Mutapi, *et al.*, 2020; Peiffer *et al.*, 2019). Network modeling techniques were employed to identify the molecular pathways that these parasites might exploit for prostate carcinogenesis.

For *S. haematobium*, several pathways that could be involved in PCa development including clathrin-mediated endocytosis (CME), alpha defensins, and negative regulation of the PI3K AKT network, among others were identified. CME, for instance, regulates the internalization of growth factor receptors like EGFR, which plays a significant role in cancer development (Sigismund *et al.*, 2008). Alpha defensins, part of the body's innate response mechanisms, have proinflammatory properties and stimulate cell proliferation (Müller *et al.*, 2002).

On the other hand, *S. mansoni* seems to exploit pathways related to the extracellular matrix (ECM) organization and degradation, collagen biosynthesis, and detoxification of reactive oxygen species (ROS). The remodeling of ECM components like collagen and laminin is crucial for cancer invasion and metastasis (Elgundi *et al.*, 2019). Dysregulation in ROS detoxification can lead to oxidative stress, contributing to DNA damage and ultimately, carcinogenesis (Martins *et al.*, 2021).

The study also considers the possibility of co-infections, a common occurrence especially in endemic areas. It identifies potential pathways like COPI-mediated anterograde transport (COPI-

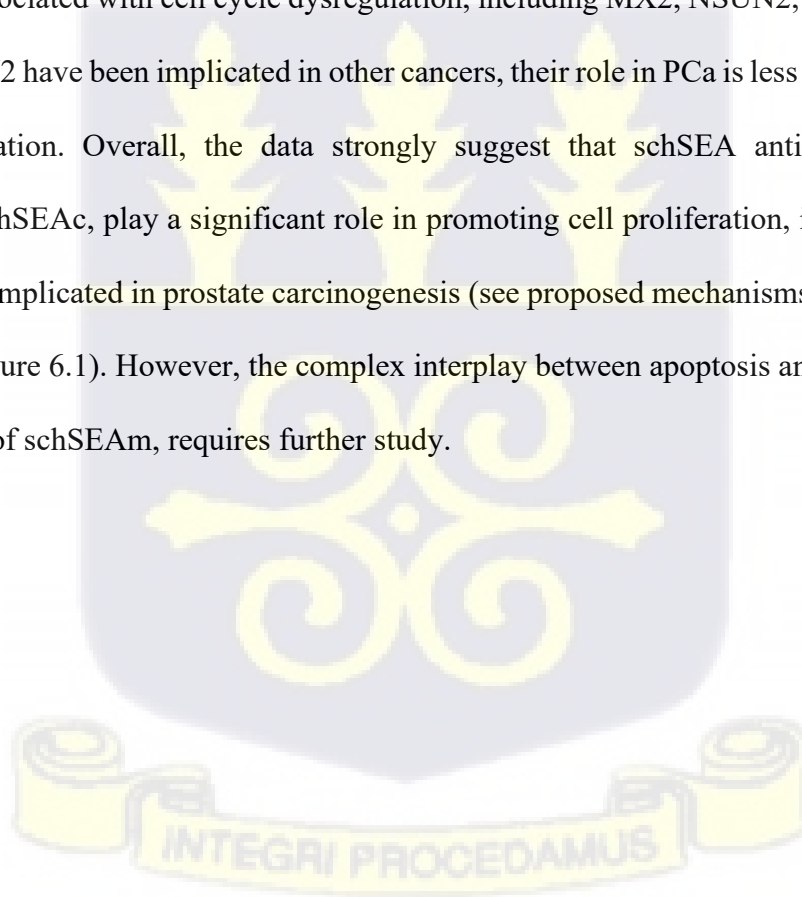
MAT) and ECM organization involving integrin 1 that could be exploited by both parasites. COPI-MAT is crucial for protein trafficking within cellular compartments like the Golgi apparatus and endoplasmic reticulum, and its role in carcinogenesis is not yet fully understood but is suggested to be significant.

Following the prediction of pathways presumably hijacked by *S. haematobium*, *S. mansoni* or both, it was important to validate using *in vitro* assays and multi omics approaches to determine the effect of schistosome exposure on a normal prostate epithelial cell line. The investigation of the role of schSEA in prostate carcinogenesis was carried out, bearing the hypothesis that schSEA can induce proinflammatory responses that alter carcinogenic signaling pathways.

Significant dose-dependent cell proliferation was observed in prostate epithelial cells treated with schSEAh and schSEAc. Additionally, increased expression of *MYC* and *MKI67* genes, known for promoting cell growth in PCa (Qiu *et al.*, 2022), was noted. However, schSEAm did not show similar effects. Interestingly, markers related to apoptosis like *BAX* and *CASP3* also increased in cells treated with schSEAh and schSEAm. This paradox could be explained by the apoptosis-induced proliferation (AiP) phenomenon, where dying cells stimulate the proliferation of surrounding cells (Fuchs & Steller, 2015). The extrinsic apoptosis pathway and JNK signaling were identified as key players in AiP (Feinstein-Rotkopf & Arama, 2009; Moreno-Celis *et al.*, 2022) and could be mechanisms through which schSEA contributes to prostate carcinogenesis. Elevated levels of proinflammatory cytokines such as IL-6, TNF α , and INF γ were also observed, aligning with schistosome's role in inducing chronic inflammation.

Increased expression of angiogenic markers, particularly VEGFA, suggests that schSEAm could promote vascularization and thus, cancer development. Protein abundance data showed a significant inflammatory response induced by schSEAc and schSEAh, as evidenced by

upregulation of Interferon-Stimulated Genes (ISGs) like *MX2*, *OAS1*, *STAT1*, *IL6*, and *TRIM21*. Chronic activation of these genes can lead to persistent inflammation, a known PCa risk. Differential expression of proteins upon schSEA treatments is testament to the fact that this treatment has a plausible effect on the physiology of normal prostate epithelial cells. Downregulation of proteins upon schSEA treatment involved in cellular metabolism suggests a metabolic reprogramming in treated cells. Interestingly, the downregulation of the alpha defensin, DEFA1 validates the pathway prediction where *S. haematobium* was predicted to hijack alpha defensin pathways amongst others. Upregulation of proteins related to cell cycle regulation indicates increased cell proliferation and adaptability. Seven differentially expressed proteins (DEPs) were associated with cell cycle dysregulation, including *MX2*, *NSUN2*, and *DLG1*. While *MX2* and *NSUN2* have been implicated in other cancers, their role in PCa is less clear and warrants further investigation. Overall, the data strongly suggest that schSEA antigens, particularly schSEAh and schSEAc, play a significant role in promoting cell proliferation, inflammation, and other pathways implicated in prostate carcinogenesis (see proposed mechanisms for Schistosoma-induced PCa Figure 6.1). However, the complex interplay between apoptosis and proliferation, as well as the role of schSEAm, requires further study.



causing prolonged inflammatory responses that favours a microenvironment for tumour development.

6.2 Conclusions

This study identified differential methylation patterns associated with PCa aggressiveness in Ghanaian men that have not been reported before. The methylation patterns are linked to pathways involved in cellular development and proliferation as well as oxidoreductase activities. Findings from this study also demonstrated that schistosome antigens induce cell proliferation, thereby attributing a potential role in the pathogenesis of PCa. Elevated levels of proinflammatory cytokines and angiogenic markers were also observed, suggesting that schistosome infections could induce chronic inflammation and promote vascularization in prostate epithelial cells, key elements in cancer development.

This study suggests that certain mechanisms, such as apoptosis-induced proliferation, and pathways like cell cycle, extracellular matrix remodeling, and alpha defensins, may be influenced by schSEA, paving the way for cancer development. This provides some evidence that schistosome infections, specifically through schSEAh and schSEAc, are likely contributing factors to PCa development in African men. However, the study also acknowledges the need for additional research to clarify the complex relationship between apoptosis and cell proliferation as well as understanding in the context of PCa, the function of DEPs identified. The study reports for the first time a DNA methylation profile associated with PCa aggressiveness in Africa. Additionally, it enhances our understanding of the role of schistosomes in PCa development in African populations, introducing potential mechanisms of Schistosoma-induced PCa. This sets the stage for future investigations into these mechanisms.

6.3 Limitations, recommendations, and prospects

Despite the different experimental approaches, the state-of-the-art platforms employed, and the insights gained through this research, it is important to acknowledge the limitations that are inherent in any study. These limitations are presented to provide the context within which the results should be interpreted. Acknowledging these limitations also paves the way for future research to build upon the present study.

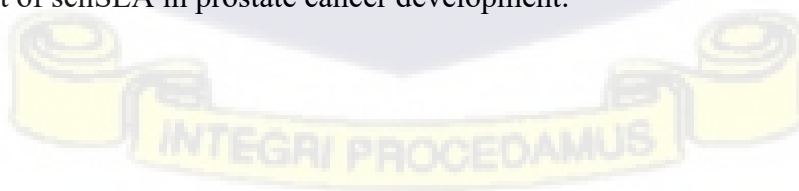
Firstly, in the DNA methylation profiling, the most evident limitation of the study is the small sample size, consisting of only 8 pairs of samples. This hampers the statistical robustness of the findings and makes it difficult to generalize to the Ghanaian population. Additionally, the lack of tissue-specific clustering in the PCA analysis suggests that the methylation differences between tumor and non-neoplastic tissues may not be as pronounced as initially hypothesized. This could potentially skew the interpretation of these results and limit their applicability. To address these limitations, first and foremost, increasing the sample size would provide a more robust dataset, enhancing the statistical power and allowing for more nuanced analyses. This would also improve the generalizability of the findings. Secondly, incorporating additional validation of the quality of the tissues (e.g. immune histochemistry) and methylation methods, such as bisulfite sequencing, could lend more credibility to the differential methylation sites identified. Finally, it would be beneficial to include other variables that would increase another layer of phenotype stratification to better understand the DNA methylation patterns.

With regards to the computational prediction of potential pathways hijacked by schistosome infections in prostate carcinogenesis, the most significant limitation of the study lies in the absence of direct experimental data concerning protein-protein interactions between *Schistosoma* and human cells, and the use of differential protein expression during schistosome infections of other tissues rather than the prostate tissue. This gap makes it challenging to draw concrete conclusions

about the specific mechanisms and these proteins may not necessarily be relevant to the prostate tissue, which has its own unique cellular and molecular characteristics. To mitigate these issues, it is recommended to incorporate prostate tissue-specific data into the analyses and possibly to conduct direct experimental assays to study Schistosoma-human protein interactions in prostate cells, which could significantly enhance the validity and applicability of the research findings.

Finally, short-term exposure of prostate cells to schSEA does not capture the chronic nature of schistosome infections, potentially missing long-term effects on cellular behaviour. Moreover, the *in vitro* setting lacks the complexity of a living organism's microenvironment, raising questions about how well these findings translate *in vivo*. Lastly, the absence of multiple time points in the various assays limits the understanding of treatment effects over time. To address these limitations, several avenues could be explored. A longitudinal study could better mimic the chronic nature of schistosome infections. Transitioning to *in vivo* models would provide a more holistic view, capturing the complexities of a living system.

Advanced techniques like RNA-seq would offer a more comprehensive view of cytokine profiling compared to the rt-qPCR technique. In addition, functional assays would be necessary to shed light on the role of differentially expressed proteins, and mechanistic studies could elucidate the pathways involved in apoptosis-induced proliferation. Assessing multiple time points post-treatment and including multiple prostate cell lines could be explored to provide a more rounded view of the effect of schSEA in prostate cancer development.



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