‘Straight Cuts’ and HIV Prevention: Translational Research on Conceptual, Methodological and Scientific Challenges in Papua New Guinea

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Declaration of Originality

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: _____ On: __02_/__02_/__2016__

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Abstract

This thesis presents data on four separate areas related to male circumcision (MC) and preventing human immunodeficiency virus (HIV) in Papua New Guinea (PNG). First, this thesis examines the literature related to MC, with a specific focus on its protective mechanisms. This systematic review of MC's different proposed protective mechanisms indicated that several mechanisms are involved in explaining the total of 60% protection against HIV demonstrated by MC, as indicated by three large randomised clinical trials.

Second, this thesis measures the proportion of surface area of the foreskin on an erect status penis, and compares this with the total exposed surface area of the penis encountering sexual fluids during sexual intercourse. The results demonstrate that the exposed surface area of the foreskin represents only 48.4% (± 9.5) of the total exposed areas of the erect penis encountering sexual secretions. These results indicate the importance of factors other than loss of surface area of the foreskin in terms of the protection offered by MC. This provides room for theories on the potential protection provided by the 'straight cut' foreskin cuttings prevalent in PNG.

Third, this thesis assesses the reliability of self-reporting MC status among men in PNG. This is measured by comparing men's self-reported MC status and investigators' classification of MC status of the participants' genital photographs. Using a three-category classification, there was 90.6% (201/222) agreement between the self-reported MC status and investigator classification (κ value: 0.805). Given the great variety of foreskin cutting practices and appearances, feasible explanations could be suggested for the two-thirds (13/21) of the discordant results. Thus, this study demonstrates a high level of agreement between self-reporting and investigator assessment of MC status in PNG, which suggests that self-reporting of MC status is highly reliable among men in PNG.

Finally, Phase 1 of the study—titled '"Straight Cuts" and HIV Prevention: The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings'—was completed at

Pacific Adventist University of Papua New Guinea. Phase 1 focused on the feasibility of conducting a complex immunohistological study in resource-limited PNG. During this study, the conceptual, methodological and scientific challenges (and opportunities) of conducting such a study were successfully assessed. In addition to generating valuable data on changes in three histological features (the keratin layer, rete pegs and supra-papillary ridges) of dorsal slit (or 'straight cut') foreskins, this study proved that conducting such a study in PNG is feasible and effective.

The scientific findings of Phase 1 of the study, while not conclusive, indicated a positive relationship between 'straight cuts' and HIV prevention. The results for keratin thickness demonstrated that the outer foreskin is thicker than the inner foreskin, which aligns with the findings of other prominent studies. For the two other histological features—the rete peg length and supra-papillary length, which were tested for the first time ever for human foreskins during this study—the results revealed that supra-papillary ridges could be an important predictor in determining the level of protection provided by dorsal slit foreskins against HIV infection. However, rete peg length needs to be measured with an improved methodology during future studies because the measurements taken during this study did not reflect the accurate effect of the rete pegs in terms of a physical barrier against HIV invasion.

In conclusion, this thesis fills gaps in knowledge in three areas related to PNG's efforts to prevent heterosexual HIV transmission using MC. Epidemiological evidence is emerging to indicate a positive correlation between 'straight cuts' and HIV prevention. The findings of this study, alongside the results of Phase 2 of the study, will provide scientific evidence to determine the importance of dorsal slit foreskin cuttings in preventing heterosexual transmission of HIV to men.

Contents

List of Tables

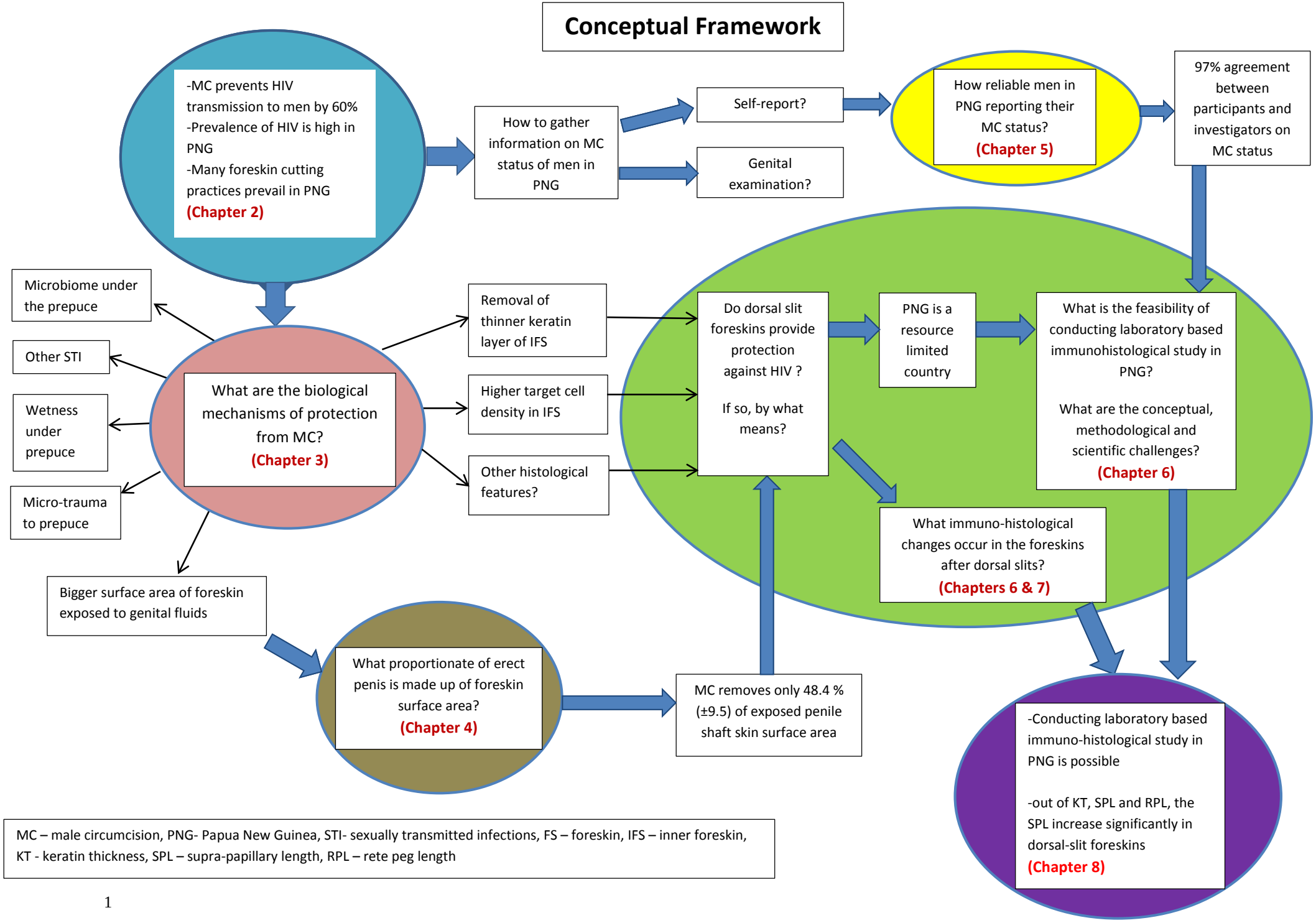
List of Figures

List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ASHM	Australian Society of HIV Medicine
AQIS	Australian Quarantine and Inspection Services
BSS	Behavioural Surveillance Survey
CIOMS	Council for International Organizations of Medical Sciences
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
GUD	Genitourinary Ulcerative Disease
H&E	Haematoxylin and Eosin
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IMR	Institute of Medical Research
IFS	Inner Foreskin
IQ	Interquartile
JCU	James Cook University
KT	Keratin Thickness
LC	Langerhans Cell
MC	Male Circumcision
MSM	Men who have Sex with Men
NBF	Neutral Buffered Formalin
NDoH	National Department of Health
OCT	Optimum Cutting Temperature
OFS	Outer Foreskin
PAU	Pacific Adventist University
PBS	Phosphate Buffered Solution
PMTCT	Prevention of Mother-to-child Transmission
PNG	Papua New Guinea
POM	Port Moresby
POMGH	Port Moresby General Hospital

RCT	Randomised Controlled Trial
RPL	Rete Peg Length
SD	Standard Deviation
SPL	Supra Papillary Length
STD	Sexually Transmitted Disease
STI	Sexually Transmitted Infection
UNAIDS	Joint United Nations Programme on HIV/AIDS
UNSW	University of New South Wales
US	United States
UTI	Urinary Tract Infection
VCT	Voluntary Counselling and Testing
VMMC	Voluntary Medical Male Circumcision
WHO	World Health Organization

Conceptual Framework



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 1: Introduction

1.1 Context

Male circumcision (MC) has been shown to reduce female to male human immunodeficiency virus (HIV) transmission by 60% (1). This protection is achieved by medical MC, in which the foreskin is removed to leave a minimal amount of tissue. Apart from the well-described medical MC, various other types of foreskin cutting are practised in Papua New Guinea (PNG), including the popular 'dorsal slit' (1). 'Dorsal slit' foreskin cutting (known as 'straight cuts' in PNG) exposes the glans penis without removing the foreskin (1). However, the level of HIV protection provided by dorsal slit foreskin cutting has not been previously established.

The National AIDS (acquired immune deficiency syndrome) Council in PNG identified MC as a priority research area in the 2008 to 2013 research agenda (2). In addition, at the National Health Policy Forum of PNG in 2011, health authorities discussed in detail the protection against HIV that may be offered to men via dorsal slit foreskin cutting (3). Such protection would be invaluable in terms of HIV prevention activities in PNG, which is a resource-limited country with moderately high HIV prevalence.

1.2 Theoretical Background

1.2.1 MC—A Useful Tool

Except in a few isolated 'special' cases, (4) a cure for HIV remains an elusive goal. Thus, prevention of HIV transmission is still the main mode of controlling HIV infections around the world. The best prevention method for many communicable diseases is an effective vaccine. However, although several vaccines have undergone clinical trials, an effective vaccine is yet to be produced for HIV. Hence, other preventative measures—such as pharmacological and biomedical methods—maintain important roles in the field of HIV/AIDS. While pharmacological modes of prevention have been proven effective, the application of this prevention on a mass scale has been problematic (5). For example, pre-exposure prophylaxis with antiretroviral drugs in

serodiscordant couples is strongly evidence based; however, adherence to the recommended regimen has been found to be lower than expected (5, 6).

Of the biomedical measures, MC is the only single-step intervention that is proven with level-one evidence to be effective against heterosexual HIV transmission—the main mode of HIV transmission around the world. Several countries have implemented mass-scale voluntary medical MC (VMMC) programs successfully after recommendations from the World Health Organization (WHO) in 2007 (7). Despite its proven effectiveness, the protection mechanism of MC is currently unclear. Research in all areas related to MC (such as epidemiological, behavioural and scientific aspects) is being conducted around the world to enable better understanding and implementation of this biomedical intervention against the HIV epidemic. The experimental area of the current study attempts to address one particular scientific aspect of MC—the immunohistological features of full MC and dorsal slit foreskin cutting—in order to gain a greater understanding of its relationship with HIV transmission.

While examining the current proposed factors and mechanisms in the literature, this study also focuses on other potential (and previously unexplored) histological factors that may relate to the level of protection. These findings contribute to understanding the implications of HIV transmission in relation to the predominant foreskin cutting practice—the 'straight cut'—in the South Pacific country of PNG. The supportive literature discussed below further demonstrates the importance of this study by identifying the gaps in the knowledge of this field.

1.2.2 MC and Heterosexual HIV Transmission

MC emerged as a potential preventative measure against HIV transmission in the late 1980s, when epidemiologists observed low prevalence of HIV in certain countries. A closer analysis of ecological data revealed that these countries practised MC routinely or in a traditional manner for religious and cultural reasons. In 2000, a meta-analysis based on 27 observational studies from Sub-Saharan Africa demonstrated a 58% protection for men with MC in the general population (8). A prospective study of serodiscordant couples conducted in the same year showed zero seroconversions

among 50 circumcised male partners of HIV-positive women, compared to the incidence of 17 per 100 person-years among 137 couples in which the male partner was uncircumcised (9). A large four-city study in Sub-Saharan Africa in 2001 further supported these correlations, with 99% of men circumcised in low HIV prevalence cities (Cotonou and Yaounde). In contrast, Kisumu had MC prevalence of 27.5% and HIV prevalence of 26.6% among the uncircumcised men. In the other city of Nodola, where MC prevalence was 9%, HIV prevalence was 26% among the uncircumcised men (10). However, potential confounding factors—such as cultural practices, sexual behaviours, personal hygiene and religion—meant that causality could not be established.

During 2005 to 2007, three large randomised controlled clinical trials were conducted in South Africa, Kenya and Uganda in order to further investigate causality between MC and HIV. With the participation of over 10,000 participants, these studies were able to clarify previous doubts on the protection offered by MC against HIV transmission (11-13). All three studies revealed that MC protects men from heterosexual HIV transmission by 50 to 60%, and all three studies were ceased prematurely because it was considered unethical to continue using men in control groups. The South African study recruited 3,274 participants and reported 61% protection, while the Kenyan study recruited 2,784 participants and demonstrated 60% reduction of transmission. The study in Uganda recruited the largest number of 4,996 participants, and showed 51% protection against heterosexual HIV transmission. The extended follow-up studies further confirmed these results, with the Kenyan study demonstrating a sustained protection of 58% at 72 months, while the Ugandan study showed 73% of protection against heterosexual transmission of HIV after five years.

Having considered this strong evidence, in 2007, the WHO recommended MC as a preventative measure against heterosexual HIV transmission in areas where there is a generalised HIV epidemic and the main mode of transmission is heterosexual contact (7). The WHO's recommendations further emphasised the need for systems to be implemented to enable counselling in order to avert any compensatory increase of risk-taking behaviour among circumcised men through developing a false sense of security and engaging in high-risk behaviours that could undermine the partial protection provided by male circumcision.(7)

Although there is no clear evidence of protection for men who have sex with men (MSM) (14, 15), some indirect protection has been demonstrated for women from MC. Mathematical modelling has shown that up to 46% of infections could be averted by women engaging in heterosexual contact with circumcised men (16). This indirect protection supposedly comes from herd immunity (which is the ultimate goal in public health) that follows substantial MCs in the local community. Considering the significant level of protection, MC is sometimes perceived as a 'surgical vaccine' that provides a behaviour-independent secure foundation upon which other forms of protection can be built (17).

1.2.3 Protective Mechanisms of MC

Despite awareness of the importance of MC as a protective measure against heterosexual HIV transmission, little is known about the mechanism of protection. Many theories have been presented, with varying strength of evidence. The main focus of attention has been on the foreskin which is the skin appendage that covers the glans penis in uncircumcised men. The most prominent hypothesis is the higher susceptibility of the inner foreskin (IFS) compared to the outer foreskin (OFS) to HIV infection (18,19). This hypothesis is founded on the distinct structural and immunological arrangement of cells and epidermis in the foreskin. Other physiological mechanisms have also been suggested, such as wetness beneath the foreskin (20) and the nature of microbiome in the preputial space (21). The increased susceptibility of the foreskin to ulcerative sexually transmitted infections (STIs) is clearly demonstrated as a significant factor in the transmission of HIV (22).

Apart from studying individual tissue components in the foreskin, studies using human explant tissues to simulate HIV infection have provided promising results to explain the protective mechanism of MC. Three such studies have shown that there is a difference in the level of HIV penetration between the IFS and OFS (19, 23, 24). Two studies of three showed that the IFS is more permeable for HIV-1 than the OFS (19, 24), while one study further demonstrated that virus-infected cells (rather than the cell-free virus) initiated genital tissue infection (19). Based on these models, researchers proposed a possible mechanism of virus entry into the male body via genital tissues. Accordingly, the cells infected with the virus come into contact with the outer

epithelium of the IFS, attach to the surface and enter through breaches on the keratin layer. These attachments lead to internalisation of virus particles into the dendritic processes, followed by transcytosis to the other target cells, such as macrophages and CD4 T-cells in the dermis. After proliferation of the virus, the local lymph nodes are invaded and the virus spreads further into the blood stream (19). These mechanisms are discussed in detail in Chapter 3 under the topic 'MC and HIV Prevention: What Do We Know? A Systematic Review of MC Protection Mechanisms'.

1.2.4 Situation in PNG

PNG has the highest HIV prevalence among the countries of the Oceania region, with 25,000 people living with HIV, and a national HIV prevalence of 15 to 49 years of 0.5% (0.4 to 0.7%) in 2012 (25). The epidemic in PNG is primarily associated with heterosexual transmission. In PNG, traditional MCs (partial foreskin cuttings) are common, while full MCs are generally uncommon unless undertaken by a medical practitioner (26, 27). Medical MC is not readily available to men in PNG, despite recent evidence showing that there is a high acceptance rate for full MC (28). This acceptance is clearly an important factor in assessing whether MC programs can be successfully implemented in PNG.

1.2.5 HIV Risk and Non-medical Forms of Foreskin Cutting

Medical practitioners in PNG and other areas have suggested that non-medical forms of foreskin cutting that expose the glans would lead to significant keratinisation, which might subsequently protect against HIV transmission (29). However, this hypothesis needs to be tested. In addition, the idea that significant keratinisation itself is a factor in protection against HIV is not totally secure. The question of whether partial circumcisions in PNG might be protective against HIV formed thebasis of the main research questions for this study.

A recently published study identified three categories of foreskin cuttings among men in PNG. The first two categories include cuts resulting in the loss of the foreskin or a change in the profile of the foreskin or penis (the dorsal slit—the most common foreskin cutting practice observed in PNG—falls into the second category). The third

category consists of incisions that do not alter the profile of either the foreskin or penis (29). Given the high prevalence of dorsal slits in PNG (about 50% of the total male population), there is a strong need for clinical and epidemiologic research to establish whether dorsal slit procedures (known as 'straight cuts' in PNG's local language) that change the profile of the penis by fully exposing the glans, without removing the foreskin, can reduce the risk of HIV acquisition among men (29).

1.3 Gaps in Knowledge

This study identified that no immunohistological study has previously been conducted in PNG or anywhere else in the world to address the issue of whether either dorsal slits or partial MCs (foreskin cuttings) are protective against HIV transmission. Several studies have investigated keratin layer thickness and target cell distribution of the foreskin in relation to the protective action of MC against heterosexual HIV transmission; however, no research has assessed the importance of other histological features of the epidermis of the foreskin, such as rete pegs and supra-papillary thickness.

1.4 Study Aims and Conceptual Framework

This thesis comprises Phase 1 of a larger two-phase study. The final objective of the overall project is to investigate the correlates of protection provided by dorsal slit foreskin cuttings against heterosexual HIV transmission. According to the original protocol of the study, there were several objectives for the complete project. This thesis, as Phase 1 of the study, aimed to achieve some, but not all, of these objectives. Essentially, this thesis examines the different mechanisms of protection provided by MC in order to gain insights to the theoretical mechanisms of any protection provided by dorsal slit foreskin cutting against heterosexual HIV transmission. A component of Phase 1 involved a study that sat outside the main study, and related to the reliability of men's self-reports of circumcision. This was seen as an important aspect of potential future translational work. As Phase 1 focused mainly on the feasibility of conducting the complete project in resource-limited PNG, this PhD thesis was titled *'Straight Cuts' and HIV Prevention: Translational Research on Conceptual, Methodological*

and Scientific Challenges in Papua New Guinea. This thesis is arranged in the following order.

Chapter 2 provides an overall background for the whole project including an introduction to HIV and mechanisms of HIV transmission through the foreskin, HIV prevention, MC and role of MC in HIV prevention, different foreskin cuttings in PNG and its potential benefit in prevention of HIV. This approach leads to the following chapters.

First, to review in detail the evidence of the mechanisms of protection afforded by MC, which provides a background to hypothesise potential protective mechanisms that could apply to dorsal slit foreskin cutting (see Chapter 3—a systematic review of MC protection mechanisms). Second, to study one of those mechanisms/factors, this thesis examines the surface area of the foreskin of the erect penis that is exposed to sexual fluids during sexual intercourse in order to determine the relationship between the amount of surface area removed during MC, and the level of protection for HIV transmission offered by MC. This was key to understanding whether it is the IFS or the total foreskin area that is responsible for HIV transmission risk reduction (see Chapter 4—a descriptive study of the foreskin surface area of the erect penis in healthy uncircumcised males). Third, it was important to determine the level of accuracy in self-reporting MC status by PNG men. This information is important for implementing HIV prevention programs (such as VMMCs) in PNG (see Chapter 5—genital examinations and the reliability of self-reporting of MC status among men in PNG).

The main objective of Phase 1 of this study was to assess the feasibility of conducting a quantitative laboratory-based immunohistological study in PNG. Phase 1 sought to identify the challenges in conducting research using freshly harvested human foreskins for the first time in the resource-limited country of PNG. In addition to assessing logistical challenges, the scientific objectives of Phase 1 comprised assessing histological changes in foreskins by measuring keratin layer thickness, rete peg lengths (RPLs), supra-papillary lengths (SPLs) and target cell distribution of the inner and outer foreskins of Melanesian men, with and without dorsal slit foreskin cutting (see Chapter 6—conceptual, methodological and scientific challenges and opportunities in immunohistological research in resource-constrained PNG). The success of Phase 1 of

the study was pivotal to proceeding to Phase 2, which consisted of broader objectives and required a longer study time and more extensive resources than were feasible during the course of a PhD thesis.

Phase 2 of the study was planned to re-examine the main scientific objectives and compare the level of protection provided by uncircumcised and dorsal slit foreskins. The latter objectives were to be achieved by measuring HIV penetration through foreskins (in vitro) removed from uncircumcised men and dorsal slit men (see Chapter 7). The author of this thesis participated in Phase 2 of the study up to the sample preparation stage, which was completed in the PC2 laboratory in Port Moresby (POM), PNG. The chapter 8 summarises all the findings of the PhD project, the gaps in knowledge and the recommendations for future research in the field.

Over the course of this PhD, literature on the mechanisms of protection provided by MC against heterosexual HIV transmission was successfully reviewed, leading to a publication titled "Male circumcision and HIV transmission: what do we know?" in *The Open AIDS Journal* (30). The study titled 'A Descriptive Study of the Foreskin Surface Area of Erect Penis in Healthy Uncircumcised Males' was completed and presented at two scientific conferences. The study titled 'Genital Examinations and Reliability of Self-reporting of MC Status among Men in PNG' was successfully completed, leading to a publication titled 'Men in Papua New Guinea Accurately Report Their Circumcision Status' in *PLOS ONE* journal (31). Phase 1 of the study was completed successfully, leading to Phase 2, with analysis of samples ongoing successfully in different laboratories located in Australia and the United States (US).

1.5 Brief Overview of Study Methods

The mechanisms of protection from MC against heterosexual HIV transmission were examined using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines to enable a systematic review. The prospective clinical study titled 'A Descriptive Study of the Foreskin Surface Area of Erect Penis in Healthy Uncircumcised Males' recruited volunteer participants to measure the surface area of the foreskins of erect penises. 'Men in Papua New Guinea Accurately Report

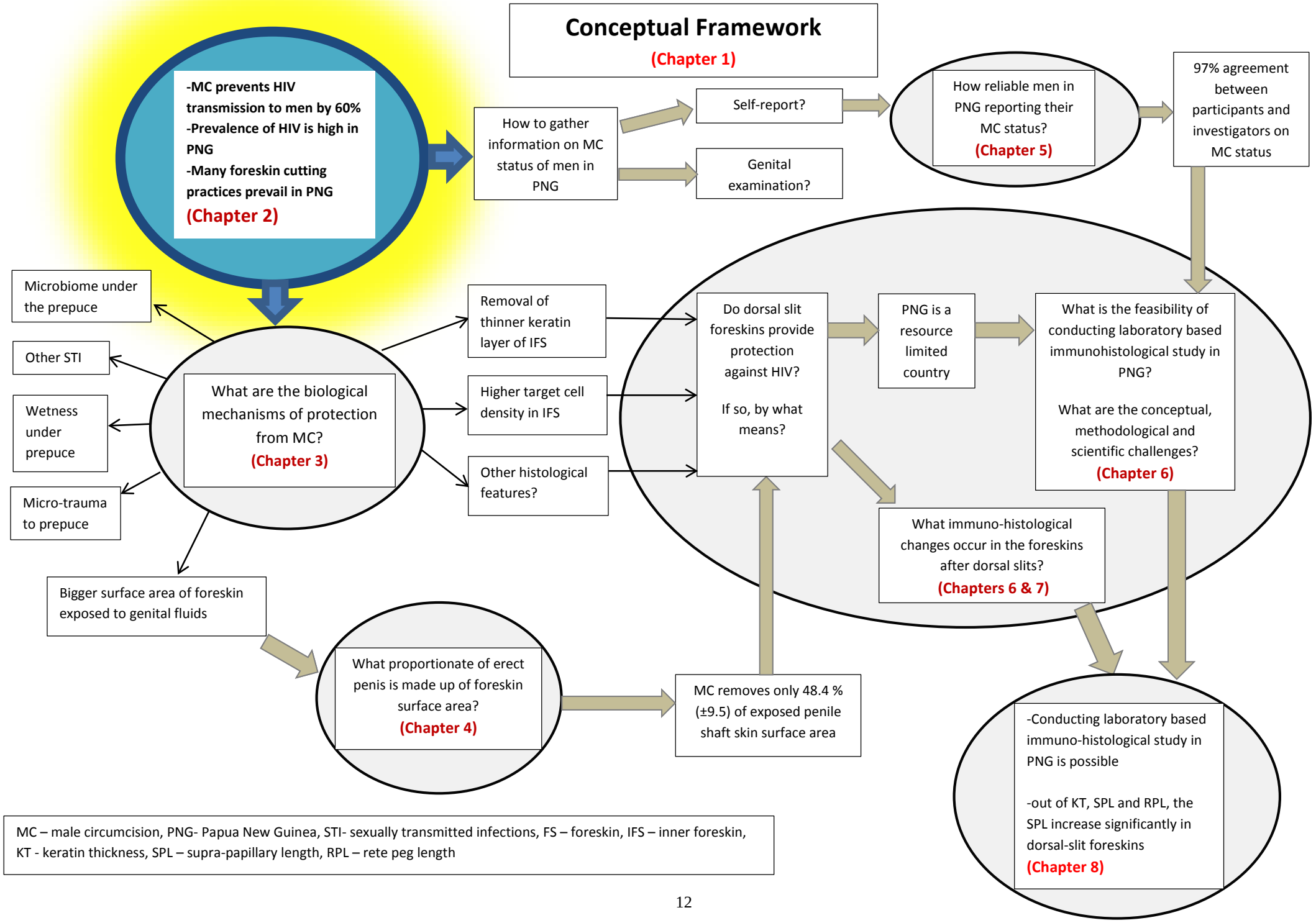
Their Circumcision Status' was a retrospective clinical study that collected data from photographs and entries from clinicians (who examined the genitals) from a previous clinical study that was designed to examine the acceptability and feasibility of MC programs in PNG. Phase 1 of the study followed standard prospective research methods for laboratory-based clinical studies.

1.6 Outline of Chapters

The thesis is organised into eight chapters, as follows:

- Chapter 1: Introduction—provides an overview of the thesis.
- Chapter 2: Literature Review—provides a review of the literature, with critical appraisal of the current research on MC, HIV prevention and the relevance of these in the context of PNG.
- Chapter 3: MC and HIV Prevention: What Do We Know? A Systematic Review of MC Protection Mechanisms—examines the mechanisms of protection demonstrated by MC against heterosexual HIV transmission.
- Chapter 4: Exposed Foreskin Surface Area and Protection from HIV—examines the foreskin surface area of erect penises in healthy uncircumcised males to provide insight to how the surface area of the foreskin relates to the level of protection against heterosexual HIV transmission.
- Chapter 5: Genital Examinations and the Reliability of Self-reporting MC Status among Men in PNG—examines the validity of self-reported circumcision status by men in PNG.
- Chapter 6: 'Straight Cuts' and HIV Prevention: The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings—Phase 1 of the study, which provides an overview of the methods applied and lessons learnt in the pilot study conducted to assess the study's feasibility, with specific emphasis on logistic issues. The methods, results and discussions for objectives on immunohistological correlates and challenges and opportunities in immunohistological research in resource-constrained PNG are presented distinctively for clarity.

- Chapter 7: Phase 2 of the Study—analyses the level of protection provided by dorsal slit foreskin cuttings, and summarises the steps and progress of the main study leading to achieve the final objectives of the study.
- Chapter 8: Conclusions and Recommendations.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 2: Literature Review

2.1 Preface

This chapter examines the literature relevant to the entire thesis. It begins by providing an introduction to the HIV epidemic, including HIV modes of transmission and the behavioural and biomedical approaches to HIV prevention. Of the biomedical interventions, MC is described in detail, focusing on its historical evolution, level of protection against HIV, and current global situation as a preventative measure for heterosexually-driven HIV epidemics. Further, this chapter explores the specific form of MC prevalent in PNG: dorsal slit foreskin cutting. It emphasises the potential immunohistological features and mechanisms of dorsal slit foreskin cutting that provide protection against heterosexual HIV transmission.

2.2 What is HIV?

HIV is a ribonucleic acid retrovirus that is the aetiological agent of AIDS (32). The two variants of the virus are known as HIV-1 and HIV-2, of which the HIV-1 variant is considered more virulent, infective and responsible for the majority of HIV infections globally (33).

M-tropic and T-tropic are the two main strains of HIV-1, and are classified according to their tropism for target cells. M-tropic (macrophage) strains of HIV-1 (or R5 viruses) use the β-chemokine receptor (CCR5) for entry, and replicate in macrophages and $CD4^+$ T-cells (34). T-tropic or syncytium-inducing (also named X4 viruses (34)) isolates replicate in primary $CD4^+$ T-cells and macrophages, and use the α-chemokine receptor (CXCR4) for entry (35, 36). Dual-tropic HIV-1 strains are the transitional strains of HIV-1, that use both CCR5 and CXCR4 co-receptors for entry into cells (37). Three groups of HIV-1 have been identified on the basis of differences in the envelope (*env*) region: M, N, and O. Group M is the most prevalent and is subdivided into eight geographically distinct subtypes based on the whole genome (38).

2.3 Global Situation of HIV

AIDS is one of the top five lethal infectious diseases of the twentieth century, having claimed about 34 million lives around the world since beginning of the epidemic (39). By the end of 2012, an estimated 35.3 (32.2 to 38.8) million people were living with HIV (40). However, with the implementation of efficient preventative measures and treatment strategies, the rate of new infections has been falling consistently. There were only 2.3 million new HIV infections globally in 2012—a 33% decline compared to the 3.4 million new infections in 2001 (40). Similarly, the number of AIDS-related deaths are declining, with 1.6 million deaths reported in 2012 compared to 2.3 million deaths in 2005 (40).

2.3.1 WHO Goal: Reduce Sexual Transmission of HIV by 50% by 2015

Between 2001 and 2012, a 50% or greater reduction in the number of new annual HIV infections among adults and adolescents was reported in 26 countries, which met the WHO/Joint United Nations Programme on HIV/AIDS (UNAIDS) goal of reducing sexual transmission of HIV by 50% by 2015 (40). However, many countries are yet to achieve this target, while an overall favourable trend towards less risky sexual behaviour was observed in high-prevalence countries during the last decade. However, in several countries in Sub-Saharan Africa, a decrease in condom use and/or an increase in the number of sexual partners have been observed, meaning that a behavioural approach for HIV prevention will be challenging in these countries (40). This has led to some authorities considering biomedical interventions as a means of prevention.

Several biomedical methods (such as Pre-exposure Prophylaxis (PreP) and microbicides) for the prevention of HIV transmission are now supported by solid scientific evidence (40). One such intervention is MC, which was shown to have a protective effect of around 60% for heterosexual HIV transmission (the main mode of HIV transmission globally), with level-one evidence. In 2012, several countries scaled up VMMC programs as a measure to prevent heterosexual HIV transmission (40).

2.4 HIV Transmission

Several transmission modes have been described for HIV. Sexual transmission in genital or colonic mucosa is the most common mode, while exposure to infected fluids (such as blood or blood products) and transmission from mothers to infants are responsible for a significant number of infections. Apart from these, occupational exposure through accidental needle stick injuries or mucosal splashes with contaminated blood have been reported occasionally (41).

Although the main mode of HIV transmission differs from region to region, heterosexual transmission is the predominant mode of transmission globally. The main mode of HIV transmission in PNG is considered as heterosexual (1). During the initial stages of heterosexual HIV transmission to men, the virus penetrates the penile tissue from the infected genital fluids of the woman. Unprotected heterosexual intercourse accounts for more than 85% of new HIV infections in developing countries, whereas, in developed countries, HIV is predominantly transmitted among MSM, injecting drug users and sex workers (42). For the purposes of this study, this thesis focuses mainly on heterosexual HIV transmission from women to men—the transmission direction demonstrated to be significantly prevented by MC.

2.4.1 Factors that Enhance HIV Transmission

The HIV crosses the mucosal barriers of both male and female genital tracts (41). This process is enhanced by micro-trauma or mucosal disruptions of the genital tissues (41). Further, concurrent infections with sexually transmitted infections (STIs) are known to increase the risk of HIV infections. A study conducted to identify risk factors for HIV infection found that current or recent (within the past 12 months) infection with herpes simplex virus (HSV-2), syphilis or genital ulceration increased the prevalence of HIV infection in men (43). In women, infection with HSV-2, syphilis, gonorrhoea or trichomoniasis increased the likelihood of acquiring HIV infection. A systematic review of 68 studies conducted between 1986 to 2006 found that the number of sexual partners, a history of paid sex, and infection with HSV-2 or other STDs each showed

significant associations with HIV infection, and are equally important for HIV transmission during advanced and early HIV epidemics (44).

In another study, a lack of MC was shown to be one of the main biological factors increasing the probability of HIV transmission from an infected woman to an uninfected man (45). Among other biological factors are high viral load (observed in primary infection or advanced-stage disease), the presence of concurrent STDs, anal intercourse, genital trauma and menstruation (45). A larger foreskin surface area, wetness beneath the foreskin and the microbiome of the foreskin are anatomical and physiological factors that increase HIV transmission to men. These factors are described in detail in the next chapter on the mechanisms of HIV transmission.

2.4.2 HIV Prevention

There is no single strategy to provide complete protection against HIV transmission, although combined methods have shown additive or synergistic effects by reducing HIV incidence at the population level (46-49). According to the UNAIDS definition, a combination of HIV prevention measures involves:

> the strategic, simultaneous use of different classes of prevention activities (biomedical, behavioural, social/structural) that operate on multiple levels (individual, relationship, community, societal) to respond to the specific needs of particular audiences and modes of HIV transmission, and to make efficient use of resources through prioritizing, partnership, and engagement of affected communities (50).

This approach needs to be supported with evidence-based biomedical, behavioural and structural interventions that are appropriate, acceptable and deliverable to populations with high levels of coverage and adherence. Fortunately, the efficacy of several biomedical and behavioural HIV prevention strategies have been strongly supported by randomised controlled trials (RCTs) that have been successfully implemented in multiple settings around the world (51). Most countries tend to adopt a combination method for HIV prevention today, which is the best way of tackling the epidemic.

2.4.2.1 Behavioural and Structural Approaches to HIV Prevention

Behavioural change has been demonstrated to have a significant effect on HIV prevention, yet strategies to modify risk behaviours remain a major challenge in HIV

prevention. According to U.S Census and UNAIDS estimates, a 67% decrease in HIV prevalence was seen in Uganda between 1991 and 2001, and linked to a delay in age of onset of first intercourse, lesser frequency of multiple partners, fewer non-regular partners, a narrowing of the age gap between girls and their male partners and increase in condom use (52). In the Mbeya region of Tanzania, a decrease of 7% in HIV prevalence (20 to 13%) was demonstrated over 10 years, and attributed to an approach involving rolling out antiretroviral treatment programs, voluntary counselling and testing programs, treatment of STIs, and condom and health promotion. This was implemented through a regional plan of enhanced surveillance for planning and assessment (53).

These studies demonstrated that radical behavioural changes are needed in a large number of people to reduce HIV transmission. They also indicated that simple and clear messages about risk reduction and treatment options should be disseminated through a variety of communication channels. In addition, participation of the most affected members of the local community to motivate behavioural change is an essential step for the success of behavioural preventative programs (54). In summary, multilevel approaches need to be adapted to effect behavioural change, targeting both HIV-uninfected and -infected populations. For the best results, behavioural strategy programs should be directed at different societal groups (couples, families, social networks, sexual networks, institutions and entire communities) and applied in the appropriate context.

2.4.2.2 Biomedical Approaches to HIV Prevention

In the absence of an effective vaccine in the foreseeable future, current preventative strategies have an important role in reducing the risk of acquiring HIV. The development of a HIV vaccine has been extremely difficult due to the extent of the genetic diversity of HIV and its capacity to escape the effects of neutralising antibodies (55). Some researchers are optimistic that a vaccine may be developed that induces broadly neutralising antibodies, as produced by some HIV-infected individuals, and which has demonstrated protection against HIV infection in non-human primates. However, it is predicted that such a vaccine is at least 20 years away (56).

Preventative measures have been developed for almost all modes of HIV transmission, such as sexual, blood-borne (including injecting drug use and accidental infections in healthcare settings) and mother-to-child transmission, with variable, but sometimes substantial, success. Antiretroviral prophylaxis has played a significant role in the prevention of mother-to-child transmission (PMTCT) by lowering maternal viral load, and this can be used as both pre- and post-exposure prophylaxis in the infant. Local application of topical prophylactic antimicrobial products is another approach that has indicated success as a preventative measure (57). With a high level of adherence, tenofovir gel applied intra-vaginally before and after sexual intercourse has reduced HIV acquisition among urban and rural KwaZulu-Natal women by 54% (57).

Early initiation of antiretroviral therapy is another biomedical approach that has shown significant results in reducing sexual transmission of HIV-1 rates (58). A study by Cohen et al. demonstrated a relative reduction of 96% in the number of HIV-1 transmissions through the early initiation of antiretroviral therapy, compared with delayed therapy (58). Such therapy produces both personal and public health benefits in the field of HIV prevention (58). Male condoms remain the gold standard for preventing sexually transmitted HIV prevention. Their effectiveness for preventing HIV transmission is 85%, and, when used consistently, effectiveness can be as high as 95% (59). Nevertheless, condom use has been limited among certain populations for several reasons related to sociocultural and economic factors, female subordination and gender inequality, which affect women's ability to promote and enforce condom use. In this context, MC has gained remarkable momentum in some parts of the world as a measure to prevent heterosexual HIV transmission. The WHO's recommendation for MC as an important preventative measure for HIV risk reduction in men in 2007 (7) is considered a historic landmark in the field of HIV prevention.

Any biomedical intervention must be implemented, sustained and scaled-up, alongside other behavioural and structural prevention strategies. This is important to maintain adherence and avoid compensatory behaviours that could offset the positive effects caused by the intervention.

2.4.2.3 MC as a Biomedical Intervention with Level-one Evidence

MC is a biomedical intervention that has achieved level-one scientific evidence in terms of effectiveness (60) (level 1 evidence- Evidence from a systematic review of all relevant randomized controlled trials (RCT's)). MC is traditionally practised for religious, cultural and medical reasons, and its prevalence in different populations varies from less than 5% to more than 80%. Prevalence is affected by factors such as geographical location, religious affiliation and socioeconomic classification (61).

Despite the recent evidence of MC's benefits, there remain some concerns regarding MC and related activities. Although surgical complications are rare and relatively minor if undertaken in safe medical settings, relatively high rates of complications are seen following unsterile forms of circumcisions (62). The other major concern is the potential risk of compensatory behaviour in the form of increased risk taking after MC (63). Early resumption of sexual activity after MC before wound healing could potentially increase HIV transmission risk several times, compared to men who are not circumcised. Therefore, it is very important to substitute traditional circumcisions with surgically safe MCs, and accompany the surgical procedure with intensive counselling on the need for risk-reduction behaviours (such as condom use) and abstention until complete wound healing is achieved (61).

The protection by MC provided to women in heterosexual HIV transmission remains questionable. As elaborated in the next chapter, different studies have shown different levels of evidence of protection for women from MC, which suggests that further research is needed prior to reaching conclusions. Further, studies conducted to assess the protective efficacy for MSM have shown that there is no protection for homosexual men from HIV through MC. This is thought to be due to the presence of the micro-trauma that occurs during anal sex, and higher viral loads in rectal and anal secretions (61).

2.4.2.4 Combination HIV Prevention: The Way Forward

Although HIV prevalence has declined in a number of countries during the last decade, the level of HIV prevention and treatment is recognised as inadequate (64). It is

emphasised that the sustainability of HIV prevention programs depends on structural approaches that support, promote and facilitate behavioural and biomedical prevention strategies (65). Combination prevention (behavioural, structural and biomedical) programs that effectively reach people at high risk of contracting HIV provide the best hope for successful control of the disease. This requires locally contextualised approaches and an appreciation of the fact that the HIV/AIDS epidemic is highly dynamic (65). As 85% of worldwide HIV transmission is attributable to heterosexual intercourse, priority should be given to implementing preventative activities that target heterosexual transmission.

2.4.3 MC Significantly Reduces the Risk of Heterosexual HIV Acquisition

Researchers hypothesised a link between the lack of circumcision and AIDS as early as 1986—the time when HIV was named officially as the causative agent for AIDS (66). During the early stages of the epidemic, researchers examined ecological data and found that some populations that had a low prevalence of circumcision concurrently had higher HIV prevalence (67, 68). For instance, countries in West Africa where MC rates were high reported very low HIV prevalence, compared to eastern and southern African countries where MC rates were lower (69).

In 1998, through a prospective study of male clients of female sex workers in Nairobi, Kenya, Cameron et al. reported a more than eight-fold increased risk for uncircumcised men to acquire HIV (70). A meta-analysis published in 2000 of 27 observational studies from Sub-Saharan Africa observed a 58% lower prevalence of HIV among circumcised men, and the association was stronger among men at high risk of HIV (71). That same year, a prospective study of discordant couples conducted in Rakai, Uganda, showed zero seroconversions among 50 circumcised male partners of HIV-positive women, compared with an incidence of 17 per 100 person-years among 137 couples in which the male partner was uncircumcised (9). These observations were further supported by findings published in 2001 from a study of four cities in Sub-Saharan Africa that showed that MC was the strongest predictor of HIV prevalence (72).

In 2001, Auvert *et al*. identified factors that could be responsible for the differences in the rate of spread of HIV between different regions in Sub-Saharan Africa (43). The study analysed data from two cities with a relatively low HIV prevalence (Cotonou, Benin, and Yaounde, Cameroon) and another two cities with a high HIV prevalence (Kisumu, Kenya, and Ndola, Zambia). They found that concurrent STIs (especially syphilis and HSV-2), MC, condom use and certain sexual practices (such as anal intercourse, 'dry sex' and the rate of partner change) positively influenced the transmission of HIV (43). They also concluded that the efficiency of HIV transmission, mediated by biological factors (especially concurrent STIs and MC status), were stronger predictors than sexual behaviour in explaining variation in the rate of spread of HIV among the four cities. Auvert *et al*, further suggested that substantial reductions in HIV incidence and prevalence may be achieved by reducing the prevalence of HSV-2 infection, and by circumcising all men in cities with high HIV prevalence (43).

Subsequently, level-two evidence emerged from three RCTs that demonstrated 50 to 70% protection against heterosexual HIV transmission by MC. The study conducted in Orange Farm, South Africa, with the participation of 3,274 uncircumcised men, demonstrated a 60% protection for circumcised men, compared to men with intact foreskins (11). When the results controlled for behavioural factors, condom use and health-seeking behaviour, the level of protection remained at 61%. The significant protection achieved by the intervention group forced the investigators to stop the trial prematurely at 18 months (11). The second study was conducted with the participation of 2,784 men in Kisumu, Kenya, and also demonstrated a significant 53% protection/reduction in the risk of acquiring a HIV infection. This study was followed by a larger study with 4,996 participants in Rakai, Uganda, which reported a protection of 51% for circumcised men against heterosexual HIV transmission The summary ratio for these three trials was 0.42 (95%, CI 0.31 to 0.57), which was similar to the results obtained from a meta-analysis of observational studies (which also reported an adjusted relative risk of 0.42) (73). Together, these studies provided level-one scientific evidence supporting MC's use as one of the major biomedical prevention interventions against HIV transmission today (73).

2.4.4 International Response

Based on the results of the three RCTs, UNAIDS and the WHO recommended that MC be implemented as an additional HIV risk-reduction strategy in areas with hyper-endemic and generalised HIV epidemics and low prevalence of MC (7). The endorsements comprised 11 conclusions with 43 recommendations for VMMC in HIV prevention (7). These recommendations emphasised the importance of conducting country-specific situation analyses for policy and human rights considerations, cultural sensitivities and gender issues (74)67]. The recommendations further emphasised the need for a 'comprehensive HIV prevention strategies' to complement circumcision (74)[67]. The identified essential services were the removal of the foreskin, HIV voluntary counselling and testing (VCT), screening and treatment of STIs, preoperative and postoperative education, risk-reduction counselling, and promotion and provision of condoms (7).

2.4.5 Public Health Benefits of MC

MC has become one of the highest HIV prevention priorities due to some of its unique characteristics, including the fact that it is a one-time, highly effective, relatively quick and cost-saving intervention (60). Although MC provides only partial protection against HIV, the protection is considered substantial and requires no repeated treatments to maintain the effect. All other currently available interventions—such as microbicides, pre-exposure prophylaxis and HIV treatment—lack this permanency and demand user compliance (60). PMTCT programs have proven to be highly effective; however, protection is only during pregnancy, parturition and breast feeding, which leaves lifelong exposure risk unchanged. In addition, like antiretroviral therapy, PMTCT programs require indefinite maintenance (75).

MC indirectly reduces the risk for women, uncircumcised men and eventually infants. This is achieved through a reduction in HIV prevalence among circumcised men due to the reduced rate of new HIV infections. This subsequently reduces the likelihood of a woman encountering a HIV-infected male sexual partner, thereby reducing the chance of contracting the disease (75). Mathematical models suggest a substantial indirect protection from MC for women. One such model demonstrates an almost

equal degree of protection for women and men if the prevalence of MC is increased to 80% equally across males aged 15 to 49 in high-prevalence countries (76). A lower HIV incidence and prevalence among women of reproductive age eventually translates into fewer infants being at risk of vertical transmission. In time, even uncircumcised men will face a lower risk of acquiring HIV due to reduced HIV prevalence in the community (75).

A further advantage of MC is that it provides a unique opportunity to reach men and adolescent boys with an array of behavioural and clinical health programs that might not otherwise be possible. HIV testing rates among males can be significantly improved through MC programs. Current testing rates are very low (less than 25%) in half of high HIV prevalent countries (75). As HIV testing is routine before MC, this would further increase awareness among boys and men of HIV risks and their HIV status. Identification of HIV-positive men through testing is also an advantage because these men can be directed to HIV care and treatment services (75).

2.5 MC: A Closer Look

2.5.1 History of Medical MC

MC is one of the world's oldest surgical procedures. This has been performed globally for hundreds of years for cultural and religious reasons (77). According to the anthropologists, the MC has originated about 15,000 years ago in ancient Egypt. Evidence has been found of MC in old Egyptian mummies (as old as 6,000 years) and wall carvings (77). MC has been traditionally practised for nontherapeutic reasons—namely, religious and cultural reasons—as well as therapeutic purposes, including medical conditions of the prepuce. It is believed that elite classes of Egyptians and Aztecs performed MC for hygienic reasons (78). Historically, one of the reasons for circumcision was as a cure for masturbation (77). MC was also considered a treatment for various (mostly unrelated) medical conditions, such as epilepsy, paralysis, insanity, headaches and hernias (77). MC was performed as a rite of passage to puberty in some African and Melanesian countries (79). In other cultures, it was practised as a ceremonial sacrifice to the gods or a mark of cultural identity (80).

During the nineteenth century, the British royalty started circumcising male heirs, thereby creating a social status and a trend (79). As a result, majority of upper class men (80%) were circumcised compared to 50% of working class men in the United Kingdom at the time of World War II. However, these rates dropped dramatically when the national healthcare system was introduced in 1948, which omitted publicly funded nontherapeutic MC (79). Similarly, Australian and Canadian MC rates dropped after the recommendation of Australian and Canadian paediatric societies against routine MC in the early 1970s (77). The *Genital Mutilation Act* (2007) in the US is a recent example of advocacy against MC that cited physical and psychological evidence of damage caused by MC (79). Thus, MC has been a widely practised procedure in the past, and is currently a subject of controversy due to its social, religious, political and health implications. Accordingly, the prevalence of MC varies significantly across different populations depending on geographical location, religious affiliation, political situation and socioeconomic status (81).

Currently, medical MC is performed mainly for phimosis, which is defined as an unretractable foreskin due to a narrowed opening (82). It is also performed for paraphimosis (a condition in which the foreskin is trapped behind the corona, forming a tight band that leads to swelling of the glans penis and foreskin), balanoposthitis (an inflammatory condition of the IFS) and for the precancerous sclerosis of the glans and foreskin (balanitis xerotica obliterans) (82). Apart from its discussed role in HIV prevention, MC can be performed as a preventative measure for some STIs, such as syphilis, chancroid and recurrent urinary tract infections (UTIs) in children (83). Another added benefit is a reduction in the risk of cervical cancer for the female partners of circumcised men (84).

The ethnicity, knowledge on health and sexual benefits of MC, and the willingness to adapt to social norms are considered as main factors influencing MC (82). It is estimated that about 30% of men globally are circumcised, out of which two-thirds are Muslims living in Asia, the Middle East and North Africa (82). A 0.8% of circumcised men are Jewish (82). Despite religious reasons, the rapid fluctuation in the prevalence of non-religious MCs in some countries is an indication for changing perceptions of its health and sexual benefits, and cultural mixing (82).

2.5.2 Traditional Foreskin Cuttings

The practice of MC differs significantly in both the indication and method of circumcision. As a result, apart from the well-known medical MC, men may have different forms of foreskin cuttings and other penile modifications, such as injections and insertions of various materials inside the penile skin (85). These variations in practice have geographical and cultural determinants, and are closely bound with local cultures and their religious beliefs (85). Non-medical MCs and other penile modifications are common in some African and Melanesian countries. For example, a 2006 PNG behavioural surveillance survey (BSS) showed that 50% of men in PNG have some form of foreskin cutting, with a dorsal slit cut being the most prevalent (86).

2.5.2.1 Foreskin Cutting and Other Practices in PNG

Full MC (complete removal of the foreskin) is generally uncommon in PNG, unless undertaken by a medical practitioner (87, 88). Instead, the practice of non-traditional foreskin cuttings (Figure 2.1), penile modifications such as penile inserts (e.g. ball bearings, beads, toothpaste handles), injection of silicon into penile shaft are widely seen among men in Southeast Asia and Melanesia (86, 88-90). Of 1,358 working adult men participated in the 2006 PNG National HIV/AIDS BSS, 26% of truck drivers, 45% of Ramu sugar workers, 67% of military personnel and 70% of port workers reported either some form of foreskin cutting or medical circumcision (86). The reasons for foreskin cutting were cultural, health related, sexual, biblically influenced, peer or partner influenced, or the result of a parental decision (28).

Figure 2.1: Examples of Different Foreskin Cutting Practices Found in PNG (Photo courtesy Dr David MacLaren)

2.5.3 HIV Risk and Non-medical Forms of Foreskin Cutting

It is believed that non-medical forms of foreskin cutting that result in complete or near complete exposure of the glans and retraction of the remaining foreskin could lead to significant keratinisation of the IFS and glans (29). A recently published study identified three categories of foreskin cutting (29). The first two categories result in the loss of foreskin, or change in the profile of the foreskin or penis (the second category includes the dorsal slit—the most common foreskin cutting practice observed in PNG). The third category consists of incisions that do not alter the profile of either the foreskin or penis (29). This previous study emphasised the need for clinical and epidemiological research to ascertain whether dorsal slit procedures that fully expose the glans, without removing the foreskin, could reduce the risk of HIV acquisition in men (29).

2.5.4 MC from the Histopathological Aspect

Foreskin at the level of the coronal sulcus is removed during medical MC, thereby exposing the glans penis and potential preputial cavity to the outside. Analysing the histological structure of the foreskin is important to understand the mechanisms of HIV transmission through the foreskin into the male body.

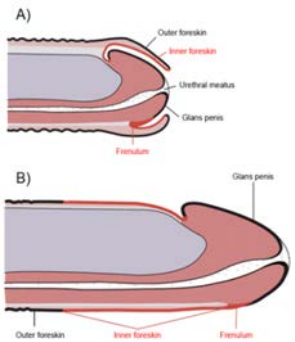


Figure 2.2: Uncircumcised Penis Anatomy

(A) A flaccid uncircumcised penis. (B) An erect uncircumcised penis demonstrating a retracted foreskin that exposes the inner aspect of the foreskin directly to vaginal secretions (91). (Reproduced with permission (91).

In the flaccid state, the foreskin covers the major area of the glans; however, once erect, the foreskin is stretched over the penile shaft, which exposes the glans completely (Figure 2.2). Thus, the erect penis exposes both the glans and IFS to genital fluids during sexual intercourse. The prepuce is the forward extended skin of penile shaft that forms a fold to continue the IFS in the form of mucosa, before attaching to the coronal sulcus of the glans penis. Hence, the prepuce is a specialised, junctional mucocutaneous tissue that marks the boundary between the mucosa and skin that divides the IFS and OFS (92). The IFS consists of a smooth and light coloured mucous membrane, while the OFS comprises rough and darker skin. The histological structure is also different between these two membranes in terms of the outer keratin layer and distribution of target cells.

The male foreskin is composed of several layers (Figure 2.3). The IFS epithelium is similar to the squamous mucosal epithelium covering the glans. The lamina propria beneath the epithelium consists of loose collagen and many blood vessels. The underlying loose connective tissue forms dermis, where numerous blood vessels, nerve trunks, glands and connective tissue are found (92). The abundant elastic tissue and the dartos muscles in the dermis help the foreskin return to its original position after an erection. The OFS epithelium consists of stratified squamous cells, and contains more melanocytes than does the IFS within the basal layers (92).

The arterial supply for the penis is mainly through the dorsal artery of the penis, while the deep dorsal vein carries blood back from the penis. Lymphatic drainage from the penile skin is through superficial inguinal nodes, while the glans drains into deep inguinal nodes. There is a unique innervation into the prepuce, making it an erogenous tissue (92).

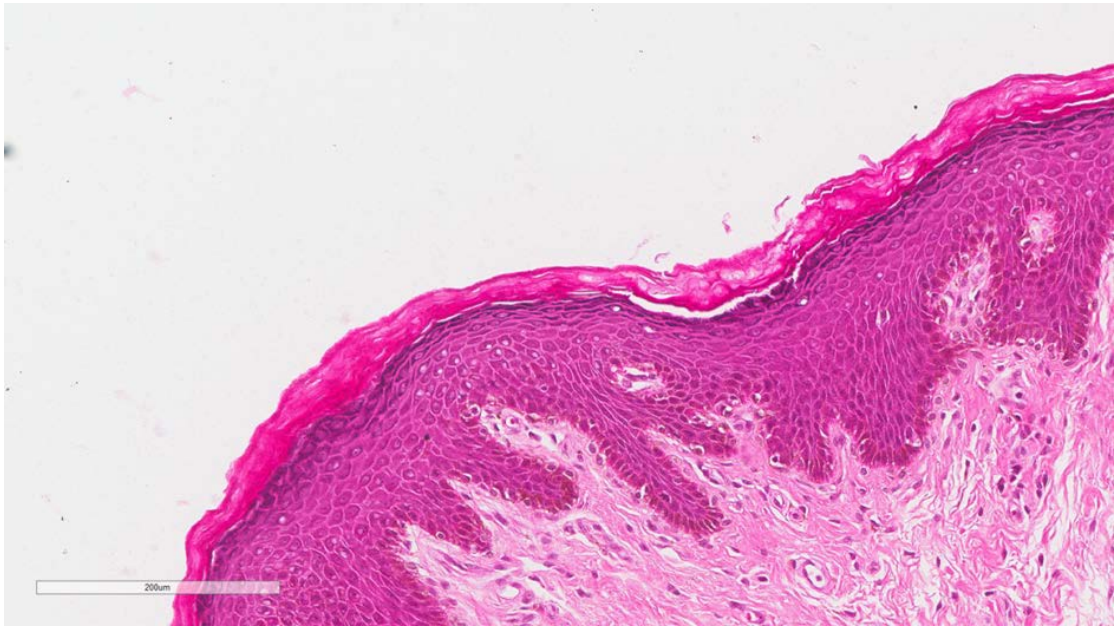


Figure 2.3: An Example of a Haematoxylin and Eosin (H&E)–Stained Paraffin Section of the OFS (Light Microscope)

2.5.4.1 Keratin Layer

The keratin layer plays an important role as a primary barrier in innate immunity. Several studies have been conducted to measure the keratin thickness (KT) in different parts of the foreskin. Interestingly, these studies have reported contrasting results about the thickness of the keratin layer in the IFS and OFS.

The keratin layer of the IFS and OFS is believed to be the first-line defence against the entry of HIV to the penile tissues. The few studies conducted on keratin layer thickness have produced contrasting results. Studies by Patterson et al., McCoombe and Short, and Ganor and Bomsel demonstrated that the IFS is significantly less keratinised than the OFS (24, 91, 93). In contrast, a study by Qin et al. showed that the IFS is thicker than the OFS (94). Two studies by Dinh et al. demonstrated a different outcome to the above results, in which the thickness of the keratin layer was equal in the inner and outer layers of the foreskin (95, 96). These results are discussed in detail in the next chapter on the mechanisms of protection offered by MC.

2.5.4.2 Rete Pegs and Supra-papillary Ridges

The epidermis forms an undulating appearance, with intermittent regular protrusions of the epidermis layer entering the underlying connective tissue (dermis). These epithelial extensions in skin and mucous membranes are known as ‘rete pegs’ (also known as ‘rete processes’ or ‘rete ridges’). The rete pegs are less pronounced in some areas of the body, such as the palms and soles. The pillars of the dermis next to the rete pegs form the rete ridges. The small areas of epidermis between the rete pegs are called ‘supra-papillary plates’ or ‘supra-papillary ridges’ (97). The most consistent structural abnormality found in ageing skin is flattening of the dermo-epidermal interface and effacement of the dermal rete pegs. A study conducted in Japan reported that the number of rete pegs per unit surface area decreased from 18.5 at ages 21 to 30, to 8.9 at ages 61 to 70 among Japanese women, and from 18.1 at ages 21 to 30, to 10.1 at ages 61 to 70 among Japanese men. Clinically, this morphologic change is proposed to predispose older people to shear-type injuries and bullae formation (98).

2.5.4.3 Target Cells

There are number of cells bearing receptors to which HIV can attach. The primary receptor for HIV, CD4, is found on T-helper cells and macrophages (99). The second docking area on the CD4 cell surface is a chemokine receptor, and the two forms of the receptor are CCR5 and CXCR4. The viral preference for using one co-receptor versus another is called ‘viral tropism’. Chemokine receptor 5 (CCR5) is used by macrophage-tropic (M-tropic) HIV, and around 90% of all HIV infections involve the M-tropic HIV strain. CXCR4, also called ‘fusin’, is a glycoprotein-linked chemokine receptor used by T-tropic HIV (the ones that preferentially infect CD4 T-cells) to attach to the host cell (99).

The target cells are located in both the epidermis and dermis of the foreskin. Langerhans cells (LCs) comprise the first line of the body’s cellular immune defence system, and are required for normal immune function. Dendritic cells (DCs) are also prime targets for HIV-1, expressing CCR5, dendritic cell-specific ICAM-3-grabbing non-integrin (DC-SIGN) and other C-type lectin receptors.

2.5.5 Mechanisms of HIV Transmission through the Foreskin

Different mechanisms have been suggested for the initial steps of HIV transmission through human foreskin tissue. According to one of the prominent mechanisms presented by Ganor et al., the virus enters the male body exclusively through the IFS (Figure 2.4). Ganor et al. demonstrated that, when the virus comes into contact with the epithelium, it forms synapses with foreskin keratinocytes, resulting in HIV-1 budding and rapid capture, internalisation and transcytosis by epidermal LCs (19). Accordingly, LCs play an active role in sampling HIV-1 at the foreskin, and transferring to T-cells. Those infected T-cells then initiate the local expansion of a small population of HIV-1 infected cells in the dermis of the foreskin, which is a prerequisite for HIV-1 dissemination and systemic infection (19) (Figure 2.4). HIV-1 transmission through the OFS was not observed when cell-free viruses for infection were used (19).

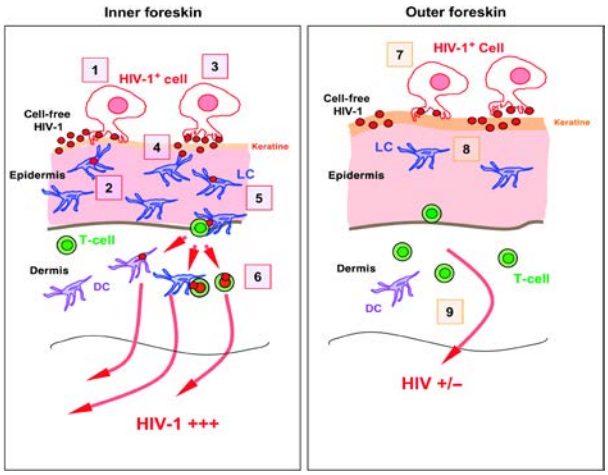


Figure 2.4: Schematic Diagram Showing the Suggested Initial Events Occurring in the Foreskin during HIV (Type-1) Transmission

(Reproduced with permission (19)).

(Left) The IFS: While cell-free HIV-1 and lightly HIV-1 infected cells are unlikely to initiate and disseminate infection through the IFS, cells infected heavily with the virus easily infect the target LCs in the epidermis, and disseminate infection at the dermis through DCs and T-cells (1 to 6). **(Right) The OFS:** Both newly budded HIV-1 particles and cell-free HIV-1 virions remain trapped in the thick layer of keratin at the OFS, thereby limiting virus entry to the epidermis and preventing infection being disseminated (7 to 9).

2.5.6 How Does MC Reduce Heterosexual HIV Transmission?

There are numerous explanations for the potential mechanism of MC protection against HIV transmission. While each mechanism explains unique ways of protection, none explain the total protection provided by MC. It is believed that there could be several mechanisms working together to create the demonstrated 50 to 70% protection. These mechanisms are described extensively in Chapter 3 of this thesis.

2.5.7 Non-medical MCs and Protection from HIV

While medical MC has a proven protective effect against heterosexual HIV transmission, it is important to determine whether other (non-medical) foreskin cuttings also provide protection against HIV transmission.

2.6 Does Dorsal Slit Foreskin Cutting Protect against Heterosexual HIV Transmission?

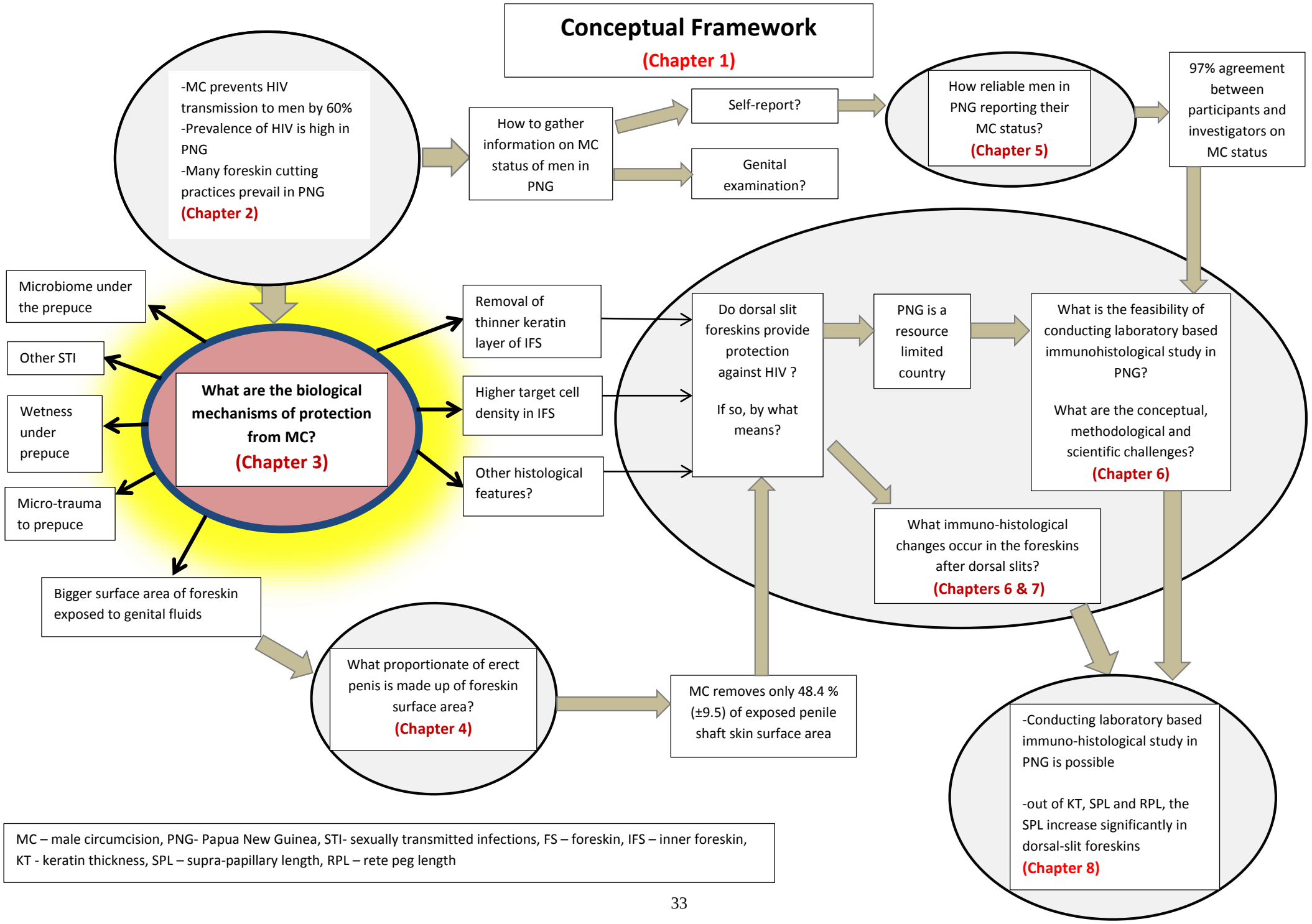
In 2011, a health policy meeting on Male circumcision for HIV prevention in Papua New Guinea, held in POM extensively discussed the use of mass MC as a preventative measure for the high prevalence rate of HIV in PNG. In addition to discussing MC and its advantages and implications, this meeting also explored the effect of widespread non-medical circumcisions/foreskin cuttings in the context of PNG. The high rates of foreskin cuttings already present in PNG were a major concern, and the level of protection provided by these alternative forms of foreskin cuttings against HIV transmission was questioned. Further, the need for research in the area was highlighted to provide scientific evidence for the level of protection (if any) provided by other forms of foreskin cuttings (specifically, dorsal slits) against heterosexual HIV transmission.

Hill and Tynan highlighted the need for clinical and epidemiologic research to establish whether dorsal slit procedures that fully expose the glans without removing the foreskin can reduce the risk of HIV acquisition in men (29). This need was further emphasised by Gray et al. in their recent publication, ‘Impact of Male Circumcision

on the HIV Epidemic in Papua New Guinea'. While admitting that MC is a potential measure for HIV control in PNG, they stressed the importance of understanding the protective efficacy of longitudinal foreskin cuttings for successful preventative MC programs in PNG (100).

2.7 Conclusion

The studies undertaken in this thesis were an attempt to find answers to the questions posed above. While the epidemiological studies are important to understand the eventual public health benefits of dorsal slit foreskin cutting, this research also attempted to understand the level of protection from a biomedical perspective. The studies in this thesis were planned to investigate biomedical aspects through observing the immunological and histological structural changes of the foreskin, and examining the changes in viral penetration through the foreskin after dorsal slit foreskin cutting. The next chapter provides an initial approach to this by closely examining the mechanisms suggested thus far on MC, and the protection provided by MC from heterosexual HIV transmission.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 3: Male Circumcision and HIV Prevention: What Do We Know? A Systematic Review of Protection Mechanisms of Male Circumcision

3.1 Preface

As discussed in the previous chapter, MC is the only biomedical preventative mechanism for HIV transmission proven with level-one evidence. In the absence of a cure for HIV, proof of 60% protection provided by MC against heterosexual HIV transmission was an important finding in the field of preventative medicine to reduce HIV transmission. Based on strong evidence, the WHO promptly accepted MC in 2007 and recommended it as an effective preventative measure against heterosexual HIV transmission. Since the association between MC and HIV protection was established, various mechanisms have been proposed to explain this association.

Most of these proposed mechanisms have focused on the special histological arrangement of the IFS, which was thought to make it more vulnerable to HIV infection. Apart from histo-anatomical features—such as KT, target cell density and weak intercellular bonds—other mechanisms have been proposed regarding physiological factors (such as the wetness of the preputial space and changes in microbiome) as contributing towards the protection. Some of these mechanisms have been associated with substantial evidence, while others remain debatable. Considering all the mechanisms of protection proposed thus far, it is clear that the total protection provided by MC cannot be explained with a single factor or mechanism. Instead, several factors or mechanisms are presumed to be working together to accomplish the epidemiologically demonstrated protection of MC.

This chapter examines the mechanisms of protection from MC proposed thus far, under their respective headings. The literature search was performed as a systematic review that used several prominent current databases. Data filtering was performed using PRISMA guidelines. A total of 61 articles were examined for this systematic review, of which 29 articles were original research papers. This chapter begins with

an introduction, followed by discussing the methods used for the systematic review. It then discusses the mechanisms of protection, with critical analysis of the methods used in some previous studies. After discussing the mechanisms, it presents a general discussion that summarises all the previously proposed mechanisms, with authors' views incorporated. The chapter ends by identifying the gaps in knowledge and providing suggestions for future studies.

This chapter was published in *The Open AIDS Journal*, as detailed below.

Details of the publication on which the chapter is based	**Nature and extent of the intellectual input of each author, including the candidate**
Jayathunge PHM, McBride WJH, MacLaren D, Kaldor J, Vallely A, Turville S. Male circumcision and HIV transmission: what do we know? The Open AIDS Journal. 2014 Sep 30; 8:31.	The authors co-developed the research question, while PHM Jayathunge collected the data, performed the systematic review and wrote the first draft of the paper, which was revised with editorial input from William JH McBride, David MacLaren, John Kaldor, Andrew Vallely and Stuart Turville. PHM Jayathunge also developed all the figures and tables.

3.2 Introduction

UNAIDS estimated that there were 35.3 (32.2 to 38.8) million people living with HIV by the end of 2012 (101). Since peaking in 1999, the annual number of new HIV infections has been in steady decline; however, despite antiretroviral therapy and the subsequent reduction in AIDS-related deaths, HIV is still increasing in prevalence (101). This means that HIV remains a significant public health issue on a global scale.

Three landmark phase-three clinical trials of MC conducted in South Africa, Kenya and Uganda demonstrated that MC has an efficacy of around 60% in preventing heterosexual HIV acquisition in men (11, 13, 102). These findings confirmed earlier observational and ecological studies, and prompted the WHO recommendations that MC be considered an important component of comprehensive HIV prevention packages for locations in which HIV prevalence is high, most infections are transmitted through heterosexual sex, there is low prevalence of existing MC, and sexual health counselling can be conducted (7).

The three intervention studies that demonstrated a reduction in HIV acquisition in men who had been circumcised proposed a number of mechanisms to explain these findings. All three proposed the removal of IFS (which has a higher number of cellular receptors for HIV) as a mechanism for reduced HIV transmission. Beyond the number of receptors, three groups proposed different mechanisms to explain their results, including increased KT of the glans penis when protection from the foreskin is absent, more rapid genital drying after intercourse in circumcised men, and reduced surface area of the penis after circumcision (11, 13, 102). One study highlighted the heightened susceptibility of cellular receptors to HIV infection (102), while another study highlighted differential KT between the IFS and OFS, plus micro-trauma (13) (Figure 3.1). Given the varied mechanisms proposed for the protection afforded by MC, it is timely to review the current evidence of how MC protects men from HIV infection.

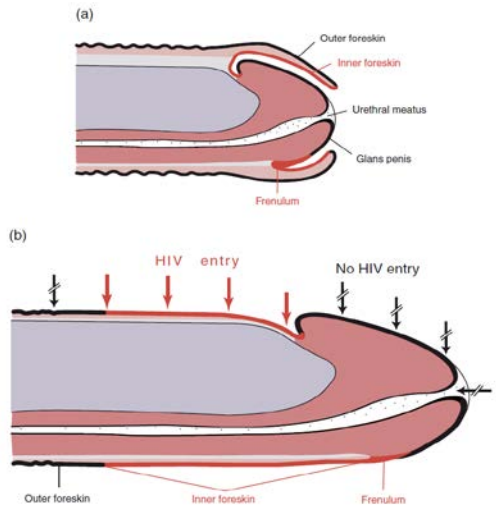


Figure 3.1: (a) Flaccid Uncircumcised Penis. (b) Erect Uncircumcised Penis with Foreskin Retracted, Showing Likely Sites of HIV-1 Entry (Reproduced with permission (91).

3.3 Methods

This study searched MEDLINE, Web of Science, Scopus and Cochrane electronic databases using the paired keywords ‘male circumcision’ and ‘HIV transmission’ as both text words and medical subject heading terms. Only the articles published in English during the period of 1981 to 2013 were searched. In addition, articles identified by citations in discovered articles were retrieved, and duplicate records were removed. The eligibility of articles was assessed based on titles and abstracts, and the full texts of eligible articles were reviewed for content. Only original research was included in the systematic review of mechanisms.

For the purpose of this review, anatomical and histological terms were standardised, as per Figure 3.2. The histological term ‘epithelium’ used in some literature is referred to here as the ‘OFS’ or ‘epidermis’, according to context, while the term ‘mucosa’ is referred to as the ‘IFS’, and ‘submucosa’ as the ‘dermis’. Studies are collated under eight main headings, which cover the main areas of research into the biological mechanisms of HIV transmission across the foreskin and the protection afforded by removing the foreskin.

3.3.1 Literature Review

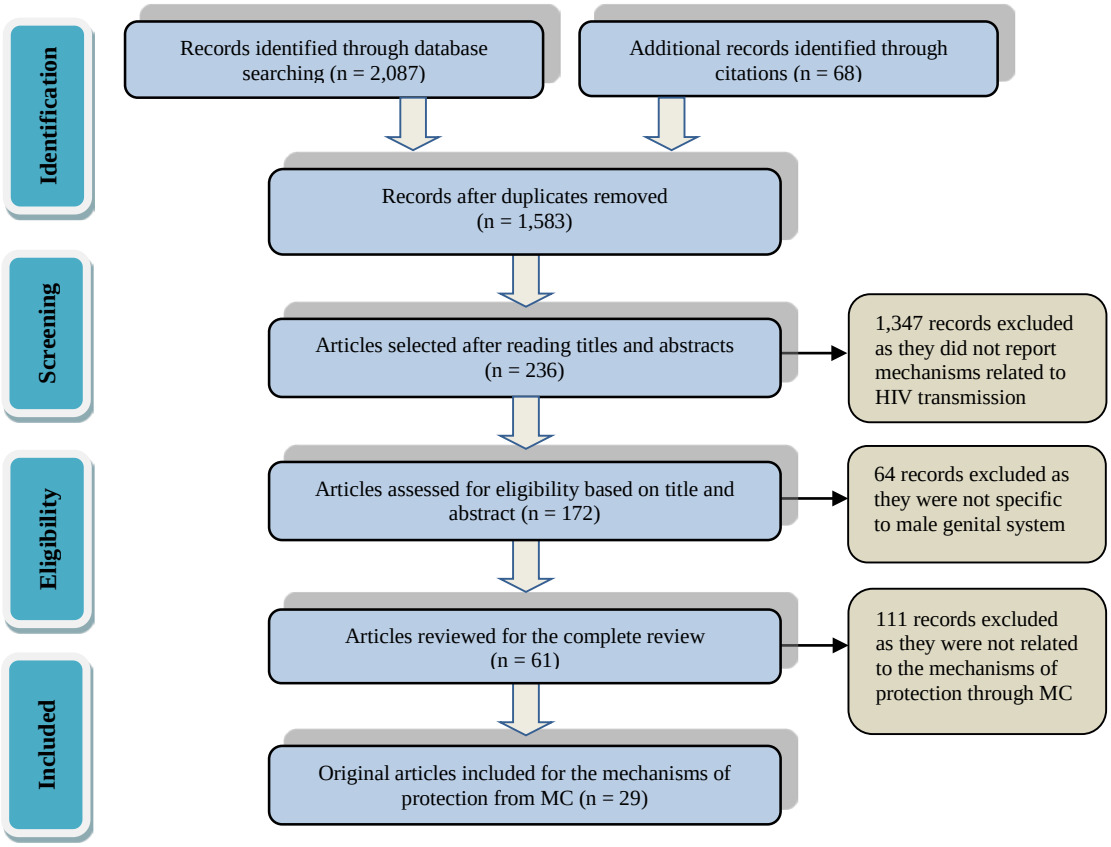


Figure 3.2: PRISMA Diagram Illustrating the Method of Selecting Articles

3.4 Results: Mechanisms Suggested for Protection

3.4.1 KT Differences of Foreskin Epithelium (Table 3.1)

Several studies have been published on the keratin layer thickness of the IFS and OFS, which is believed to be the first-line defence against the entry of HIV to the penile tissues. McCoombe and Short's study showed that the IFS (mean thickness: 1.8 units; SE: 0.1) was significantly less keratinised than the OFS (mean thickness: 3.3 units; SE: 0.1) or glans penis (mean thickness: 3.3 units; SE: 0.2; $P < 0.05$) using cadaveric samples of HIV sero-negative individuals obtained within 18 hours of death (91). In this study, KT was subjectively assessed on a scale of zero to five arbitrary units, where zero corresponded to no keratin, and five to maximum keratinisation (KT $\geq$ 20 µm). There was no difference in keratinisation between the OFS and glans penis. The frenulum and urethral meatus were less keratinised, and there was no keratin observed

in the penile urethra (91). Similarly, Patterson et al. reported that the extent of keratinisation was much greater in the OFS surface than the IFS surface, although actual data on KT were not provided (24). In a later review, Ganor and Bomsel reported a higher number of apical keratin layers in the OFS that made the OFS thicker and denser than the IFS at the ultra-structural level (93).

In contrast, Dinh et al. found no clear difference in KT between the IFS and OFS in their study that included 16 consenting male participants undergoing circumcision for medical conditions—mainly phimosis (96). Keratin of the IFS was thicker than that of the OFS in seven of their donors, while keratin of the OFS was thicker than the IFS for three donors, and no significant difference was seen between the OFS and IFS for six donors. Intra-individual variability in the measured KTs of some donors was also noted, while a significant heterogeneity between the donors was observed. The authors attributed this to genetic differences in skin composition and exposure to environmental stimuli over an individual's lifespan that could induce changes in the expression of epidermal proteins, such as filaggrin or keratin (96).

Dinh et al. published a similar study in 2012 with foreskins from 19 healthy men who underwent prophylactic circumcision in Uganda (95). This study was conducted to avoid the possible shortcomings of their previous study—that is, using foreskin samples taken from men circumcised for foreskin pathologies, and the probable inadequate representation of different sites of foreskin in the analysis. However, the results of this study were consistent with their previous results, and showed no difference in the thickness of the keratin layer between the IFS and OFS (95) (see Table 3.1).

Qin et al.'s study examined preschool boys (some with a history of UTIs) and adults (all without a history of UTI), and reported that the KT was much greater in the IFS than in the OFS in the adults and the boys with a history of infection (94). There was no significant difference in the KT of the IFS and OFS tissues in boys without a previous history of UTI. This study demonstrated a close relationship between the thinner keratin layer of the OFS and desquamation in adults and boys. Further, it reported the keratin layer as thinner in boys' foreskins compared to those of adults. Qin et al. attributed these differences to the anatomical and functional characteristics

of ethnic groups (foreskins of men of Asian origin are reported to have a thinner stratum corneum layer compared to foreskins of men of other origins (103, 104)), as well as socioeconomic, hygienic and nutritional factors (94).

In addition to keratin layer thickness, some studies have suggested that the skin's barrier function also relies on components such as intercellular junctions. These junctions serve to regulate cell and epidermal growth, and to protect the integrity of the epidermis (105). In their review, Dinh et al. stated that expression of these proteins (intercellular junctions) can vary between the epithelial tissues in different areas of the body, which explains the difference in level of protection in certain areas of the body compared to others (18). Through their earlier studies, Dinh et al. further demonstrated the subtle differences in protein expression patterns of foreskin and cervical tissues, which may contribute to differences in the movement of HIV between the female and male genital tract (18).

Table 3.1: Studies Examining KT

Study	Subjects	Indication for Circumcision	STI Status	Specimen Processing and Microscopy	Results and Conclusions
Patterson, 2002 **(24)**	14 subjects Aged 10 months to 69 years From Chicago, US	Phimosis, balanitis, adhesions (three subjects) Religious, cultural or cosmetic (11 subjects)	Not specified	Processed within: three hours Fixed with: Streck Tissue Fixative Embedded in: paraffin or OCT Section size: 5 µm Stained with: immunohistochemistry Examined with: MetaMorph software V4.5	**IFS:** data not shown **OFS:** data not shown Extent of keratinisation was much greater in the OFS surface than the IFS **Concluded that keratin layer of OFS is thicker than IFS (OFS > IFS)**
McCoombe, 2006 **(91)**	Nine cadavers† (mean age 77.4 years) 21 subjects† (mean age 28.9 years) Eight penile necropsy specimens† (mean age 30.9 years) From Melbourne, Australia	Not specified	HIV negative	Processed within: necropsy specimens obtained within 18 hours of death Fixed with: formalin Embedded in: paraffin Section size: 8 µm Stained with: H&E stains and Ayoub-Shklar stains Examined with: light microscopy 200–400 magnification	**IFS:** 1.8 units **OFS:** 3.3 units (units: 0 = no keratin; 5 = max keratin > 20 µm assessed subjectively) **Concluded that IFS and frenulum are protected by a much thinner layer of keratin than those in the glans penis or OFS (OFS > IFS)**
Qi Qin, 2009 **(94)**	80 subjects 60 were aged two to seven years 20 were aged 20 to 29 years From Hangzhou, China	Phimosis or redundant foreskin with UTI (30 boys) Cultural or cosmetic (30 boys) Not specified for adults who were otherwise healthy	Not specified	Processed within: not specified Fixed with: 4% para formaldehyde Embedded in: paraffin Section size: 4 µm Stained with: H&E stains Examined with: light microscopy 100–400 magnification	**IFS:** 12.1 ± 4.1 µm **OFS:** 9.3 ± 2.0 µm **Concluded that OFS is less keratinised than the IFS in healthy adults and in boys with infectious history (IFS > OFS)**

Dinh, 2010 **(96)**	16 subjects† From Chicago, US	Phimosis	Not specified	Processed within: not specified Fixed with: 3.7% formaldehyde, 0.1mol/l PIPES buffer Embedded in: paraffin, OCT Section size: 10 µm (cryosections) Stained with: immunofluorescence and H&E Examined with: DeltaVision RT systems using Softworx software	**IFS:** data not shown **OFS:** data not shown KT of seven subjects: IFS > OFS KT of three subjects: IFS < OFS KT of six subjects: no difference **Concluded no difference between the IFS and OFS keratin layers (IFS = OFS)**
Dinh, 2012 **(95)**	19 subjects Aged 21 to 41 years From Rakai, Uganda	As a prophylactic measure for HIV acquisition	HIV negative No evidence of current STIs	Processed within: not specified Fixed with: 3.7% formaldehyde, 0.1mol/l PIPES buffer Embedded in: OCT Section size: 10 µm Stained with: immunofluorescence and H&E Examined with: DeltaVision RT systems using Softworx software	**IFS:** 14.676 ± 7.48 µm **OFS:** 13.306 ± 8.49 µm Frenar band: 16.916 ± 12.42 µm **Concluded no difference between the IFS and OFS keratin layers (IFS = OFS)**

Note: † = age range not specified. OCT = optimum cutting temperature. PIPES = piperazine-N,N′-bis(2-ethanesulfonic acid).

3.4.2 Distribution of Cellular Receptors for HIV in the Foreskin (Table 3.2)

Researchers have generally focused on the foreskin epithelia as a likely site of entry for HIV. They have followed basic immunohistochemical methods using antibodies specific to unique antigens to visualise and enumerate the target cells for HIV in the penile epithelia and adjacent tissues. Accordingly, the cellular receptors for HIV in the foreskin are most likely the CD4 (principal receptor for HIV-1) and CCR5 (co-receptor) bearing T-cells and CD1a+ LCs. LCs are found among the epithelial cells in the squamous epithelium/epidermis (106). DCs, T-cells and C-type lectin receptor-expressing CD68+ macrophages lie deeper in the dermis; however, nonetheless, they are also likely cellular receptors for HIV (106, 107). The role of the urethral mucosa as a site of HIV entry has received less attention (108).

Although these studies used similar methods of visualisation of target cells, they adopted different methodologies to enumerate the target cells in the foreskin tissues. While some of the studies counted cells in the IFS and OFS, other studies compared two histological strata—the epithelium (epidermis, including inner and outer) and submucosa (dermis). Further, the counting method of cells also differed—some studies provided actual numbers, while others provided cell counts as a percentage or a proportion of all staining cells. This made comparison of results difficult, although the current study standardised the results into similar units as much as possible.

One of the earliest studies published on the cell distribution of foreskin tissues was undertaken with the participation of 10 subjects, and revealed no significant difference in LC concentration between the IFS and OFS (109). In contrast, McCoombe et al. found the highest density of LCs in the OFS, which was followed (in descending order) by the IFS, frenulum, glans penis and urethral meatus (91). Qi Qin et al. also reported that the majority of LCs were located in the outer superficial layers of the foreskin, and that the OFS contained significantly higher number of LCs than did the IFS in healthy boys and adults (there was no significant difference in LC density in the OFS and IFS of boys with a history of UTIs). The study also found an increased density of LCs in the IFS of boys with a history of UTIs, compared with the IFS of healthy boys (94). Qi Qin et al. further demonstrated much higher LC density in boys' foreskins

than in adults' foreskins (94). Farbach et al. similarly demonstrated higher LC density in the OFS than the IFS through their studies (110).

In contrast to all the above results, Patterson et al. observed that LCs and CD4+ T-cells (previously activated memory CD4+ T-cells) are present in high densities in the IFS epithelium (24). They identified CCR5 as the predominant co-receptor expressed on HIV-1 target cells, and reported the dermis as the site for cells expressing the highest level of CCR5 (24). In 2009, from penile explant tissue studies, Fischetti et al. demonstrated that LCs were highest in the glans, followed by the IFS and OFS at the early stages of the foreskin being removed from the body (23). They observed changes in LC densities after culturing tissues for few days. Ganor et al. also used penile explant tissues for their studies, and demonstrated higher number of LCs and CD4 cells in the IFS than the OFS (19). Another study of young Kenyan males at high risk of HIV exposure revealed that intra-epidermal LCs were localised as close as 24 µm to the outer surface of the IFS (107). This was close enough for their dendrites to reach out and sample HIV particles from genital secretions of a sexual partner (107). Abundant CD4+ T-cells were also reported in the dermis of the IFS in this study (107).

Fahrbach et al. attempted to understand the dynamics of the immunologic environment in the male genital tract by examining target cell activity in the IFS and OFS in response to inflammatory cytokines (110). They found a significantly higher responsiveness to some cytokines among the LCs and CD4+ T-cells in the IFS compared to the OFS. They further demonstrated that the LCs maintained dynamic responsiveness to external stimuli, and that intact IFS had greater access to materials in its external environment than did the OFS (110).

Table 3.2: Studies Examining Target Cell Distribution in Foreskin Tissues

Study	Subjects	Indication for Circumcision	STI Status	Results (LCs, CD4 T-cells and Macrophages) and Conclusions
Hussain, 1995 **(109)**	10 subjects Seven subjects aged three to seven weeks Three subjects aged seven to 36 years From London, United Kingdom	Not specified	Not specified	**LCs:** Infants: 41.9 ± 4.6 cells/mm^2 in IFS. Density in OFS not enumerated. Older: 26.0 ± 5.7 cells/mm^2 in IFS. Density in OFS not enumerated. **No significant difference between IFS and OFS in terms of LC density (IFS = OFS)** **CD4+T-cells:** Large number of CD4+ T-cells found in dermis, and a small number found in epidermis **Macrophages:** Found in dermis
Patterson, 2002 **(24)**	14 subjects Aged 10 months to 69 years From Chicago, US	Phimosis, balanitis, adhesions and redundant foreskins	Not specified	**LCs**: Significantly greater in IFS compared to OFS$^{\gamma}$ (**IFS > OFS)**. The majority of LCs found in epidermis (inner and outer) **CD4+ T-cells**: Significantly greater in IFS compared to OFS$^{\gamma}$ (**IFS > OFS**). The majority of CD4 T-cells found in dermis **Macrophages:** percentage* of macrophages was similar in IFS and OFS **Proportion of all three target cell types increased with age** **History of balanitis/STIs significantly increased the number of target cells**
McCoombe, 2006 **(91)**	30 subjects Nine cadavers$^{\phi}$ (mean age 77.4 years) 21 subjects$^{\phi}$ (mean age 28.9 years) Eight penile necropsy specimens† From Melbourne, Australia	Nine penile cadaveric specimens were obtained within 18 hours of death 21 men were healthy and undergoing elective circumcision	HIV negative	**LCs:** 61.3 cells/mm^2 in IFS; 85.5 cells/mm^2 in OFS; 56.0 cells/mm^2 in frenulum; 41.0 cells/mm^2 in glans **OFS > IFS** > **frenulum > glans > urethral meatus** **LC dendritic processes in IFS came within 4.8 µm of epithelial surface, compared to 20 µm in OFS** **CD4+ T-cells:** Found in epidermis, but predominant in dermis$^{\gamma}$ **Macrophages:** Found in epidermis, but predominant in dermis$^{\gamma}$

Donoval, 2006 **(111)**	39 subjects Aged 18 to 24 years From Kisumu, Kenya	Foreskins obtained from a RCT in Kenya (102)	20 men without a history of STIs 19 men with a history of treated STIs	Epidermis † / Dermis † LC cells 1.23% / 0.30 % CD4 T-cells 0.08% / 0.075% Macrophages 0.02% / 0.04% **LC:** Mainly found in epidermis[γ] **CD4+ T-cells:** No difference in the median percentages between epidermis and dermis[γ] **Macrophages:** Found predominantly in dermis[γ]
Qi Qin, 2009 **(94)**	80 subjects 60 subjects aged two to seven years 20 subjects aged 20 to 29 years From Hangzhou, China	Group 1—circumcised for medical reasons related to UTIs Group 2—circumcised for cultural and cosmetic reasons Adult men had no history of UTIs	Not specified	**LCs in children:** UTI related: 132.2 cells/mm^2 in IFS; 131.7 cells/mm^2 in OFS Non-UTI related: 87.5 cells/mm^2 in IFS; 123.7 cells/mm^2 in OFS LCs in adults: 53.7 cells/mm^2 in IFS; 88.3 cells/mm^2 in OFS **OFS > IFS of adults and healthy boys** **OFS = IFS in boys with a history of infection** **CD4+ T-cells:** not specified **Macrophages:** not specified
Fischetti, 2009 **(23)**	Subjects[ϕψ] (number of subjects or age range not specified) From London, United Kingdom	Elective circumcisions after gender reassignment (after six weeks off hormonal therapy)	Not specified	**LCs**: 230 cells/mm^2 in IFS; 170 cells/mm^2 in OFS Exclusively reside in epidermis **The distance to dendritic projections of LCs from the epidermis surface glans > IFS = OFS** **Average number of LCs greatest for glans> IFS > OFS** **CD4+ T-cells:** 260 cells/mm^2 in IFS; 2.6 times higher than OFS Typically present in dermis, infiltrate epidermis under inflammatory conditions **Average number of CD4+ T-cells greatest for glans > IFS > OFS** **Macrophages:** not specified
Hirbod, 2010 **(107)**	33 subjects Aged 18 to 24 years From Kisumu, Kenya	Foreskins obtained from a RCT in Kenya (102)	All men were negative for STIs for a period of three months prior circumcision	**LCs**: 150 cells/mm^2 in both epidermis and dermis (60% in epidermis and 40% in dermis) **Intraepithelial LCs localised as close as 24 µm to the outer surface of IFS** **CD4+ T-cells:** abundant CD4+ cells present in dermis[γ] under the epidermis of foreskin function as early sites of viral replication **Macrophages:** exclusively found in dermis[γ]

Ganor, 2010 **(19)**	Subjects$^{\psi}$ (number of subjects not specified) Aged 17 to 87 years From Paris, France	Personal reasons or phimosis	Foreskins with history of infectious pathologies were not used	**LCs:** IFS ≈ 580 cells/mm^2; OFS ≈ 220 cells/mm^2; and LC cells only found in epidermis of IFS **IFS > OFS** **CD4+ T-cells:** mainly in dermis, and about 2 × concentration in IFS v. OFS **IFS > OFS** IFS OFS % CCR5 expressing LCs out of total LCs 20.9 ± 1.6 6.1± 3.1 % CCR5 expressing T-cells out of total T-cells 35.5 ± 11.4 5.3± 3.6 **Macrophages:** Exclusively found in dermis and the density in IFS = OFS$^{\gamma}$

Note: ϕ = age range not specified. ψ = number of subjects not specified. * = percentages were based on the number of brown staining cells indicating a specific immune-phenotypic marker, divided by the total number of nucleated cells. † = the mean cell percentages were derived from the amount of cellular area staining positive per total cellular area in five fields covering the IFS, containing 50% epithelium and 50% dermis in each field. $^{\gamma}$ = primary data not given.

3.4.3 In Vitro Experiments of Human Explants Cultures for the Permeability for HIV (Table 3.3)

Three studies have been published on human genital explants tissue to study the initial steps of HIV-1 transmission (19, 23, 24). Of these, two studies identified the IFS as the predominant portal of HIV entry, as opposed to the OFS (19, 24), while the other study proved no enhanced susceptibility of one surface to the other (23).

Two studies of three investigated the mechanism of HIV-1 entry to the foreskin using high doses of cell-free HIV-1 at periods of more than 24 hours. The first study used agarose sealed foreskin and cervical tissue explants, and showed evidence of different levels of permeability for different strains of HIV at different genital tissue sites (24). In this study, after adjusting for the differences in target cell proportions, the infectivity for HIV-1 of the adult IFS was several times greater than cervical tissue. Notably, there was no HIV-1 infiltration in the tissue taken from the OFS (24). The researchers further observed an extensive infection of both the CD4 T-cells and LCs through CCR5, when HIV-1BaL isolate was introduced to the foreskin tissue explant cultures. An ineffective infection was noted with HIV-1Lai isolate in all tissue samples (95). However, this study was questioned later for its sealing efficiency and polarisation of the system (93). In 2009, Fischetti et al. designed their study using non-polarised foreskin tissue explants, in which HIV-1 gained access to both apical and basal sides of the tissue surfaces. They conducted research on different tissue explants taken from glans, urethral meatus, urethra and foreskin (inner and outer), and found no significant difference in terms of susceptibility to HIV infection (23). The OFS and IFS were infected to a similar degree with CCR5 (but not CCR4) with cell-free HIV (23).

In contrast, Ganor et al. (2010) used two novel models of human adult foreskin epithelium (19). One model consisted of an ex vivo foreskin (inner or outer) explant placed on a permeable membrane in a two-chamber system. In this model, a polarised epidermal surface was created by gluing hollow plastic cloning ring cylinders tightly onto the epidermal surface. The other model consisted of an in vitro reconstructed immuno-competent foreskin constructed by seeding primary IFS and OFS fibroblasts and keratinocytes together with immature LCs and DCs in the apical compartment of the two-chamber system. By optimising culture conditions, they managed to create in

vitro models that resembled natural structural and morphological characteristics of foreskins.

They used both cells infected with virus and cell-free virus in high and low concentrations for infection of explant tissues, and measured the viral penetration through tissues in shorter time points. Using these models, Ganor et al. demonstrated an efficient HIV-1 transmission after one hour of polarised exposure of the IFS to mononuclear cells (such as macrophages and CD4 cells) highly infected with HIV-1 (19) (Figure 3.3). Highlighting the next steps in the mechanism of infection, they showed how such contact leads to viral synapse formation with foreskin keratinocytes, resulting in HIV-1 budding and rapid capture, internalisation and transcytosis by epidermal LCs (19). Accordingly, LCs play an active role in sampling HIV-1 at the foreskin and transferring to T-cells. Those infected T-cells then initiate the local expansion of a small population of HIV-1 infected cells in the dermis of the foreskin, which is a prerequisite for HIV-1 dissemination and systemic infection (19) (Figure 3.3). The researchers did not observe HIV-1 transmission through the OFS when they used the cell-free virus for infection (19).

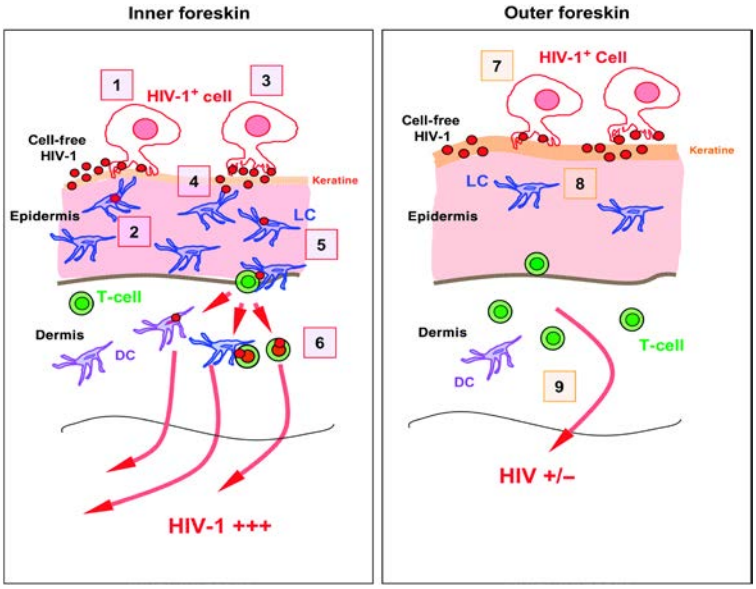


Figure 3.3: Schematic Diagram Showing the Suggested Initial Events Occurring in the Foreskin during HIV (Type-1) Transmission

(Reproduced with permission (19)).

(Left) The IFS: While cell-free HIV-1 and lightly HIV-1 infected cells are unlikely to initiate and disseminate infection through the IFS, cells infected heavily with the

virus easily infect the target LCs in the epidermis, and disseminate infection at the dermis through DCs and T-cells (1 to 6). **(Right) The OFS:** Both newly budded HIV-1 particles and cell-free HIV-1 virions remain trapped in the thick layer of keratin at the OFS, thereby limiting virus entry to the epidermis and preventing infection being disseminated (7 to 9).

Table 3.3: Studies on Tissue Explants to Examine the Early Stages of HIV-1 Transmission

Study	Tissue Source and Methods	Results and Conclusions
Patterson, 2002 **(24)**	**Tissue**: foreskins circumcised for phimosis, balanitis, adhesions and redundant foreskins. Cervical tissue samples from removed uteri for benign conditions **Culture:** three 4.0 mm biopsies from the IFS and three from the OFS, cultured for one day **Infection**: foreskins were infected with CCR5-using (R5) HIV-1Bal or the CXCR4-using (X4) HIV-1Lai for one day **Measuring infectivity**: infectivity was quantified using real-time quantitative polymerase chain reaction for HIV-1 pol deoxyribonucleic acid (DNA)	**Susceptibility of human foreskin to HIV-1:** Infection was predominant in CD4 T-cells and LCs of the IFS Infection was below the level of detection in the OFS Adult foreskin infection was higher than in an infant (22 months) foreskin Adult foreskin from those without past history of STIs was nine times more susceptible than cervical tissue
Fischetti, 2009 **(23)**	**Tissue:** penile tissues (glans, urethra and foreskin tissues) from gender reassignment patients **Culture**: tissues sized 2 to 3 mm^2 were cultured for 10 days **Infection**: cultured cells were exposed to HIV-1BaL or HIV-1Lai for two hours for infection. After being resuspended in Polyhydroxyalkanoates (PHA) medium overnight, the tissue explants were removed and cultured for three days **Measuring infectivity**: assayed for HIV-1 P24 antigen level using the enzyme-linked immunosorbent assay (ELISA) from cultured supernatants harvested every two to three days	**Susceptibility of human foreskin to HIV-1:** No significant differences in the level of HIV-1 infection between different tissue sites (glans, urethra and foreskin) No evidence to support enhanced susceptibility of the IFS relative to the OFS or glans HIV-1BaL productively infected all tissue sites, but no infection was detected with HIV-1Lai
Ganor, 2010 **(19)**	**Tissue**: normal healthy foreskin tissues circumcised for personal reasons or phimosis **Culture**: 8 mm diameter round foreskin tissues used as novel ex vivo explant model, with restricted access of virus to the non-epidermal side of either the IFS or OFS **Infection**: (1) with low and high cell-free HIV-1 loads; (2) with peripheral blood mononuclear cell (PBMC)s weakly or highly infected with HIV-1 **Measuring infectivity**: quantified HIV-1 by p24 ELISA	**Susceptibility of human foreskin to HIV-1:** HIV-1 enters IFS explants, but is trapped in (thick) keratin layer of OFS. HIV-1 infected cells form viral synapses with apical keratinocytes HIV-1 transmission is more effective when foreskin is inoculated with HIV-infected cells, not the cell-free virus LC–T-cell conjugates permit transfer of HIV-1 from LCs to T-cells Exposure of IFS to HIV-1 infected cells results in efficient translocation of HIV-1 Seminal plasma mixed with cervical-vaginal secretions decreases HIV-1 translocation through IFS

3.4.4 Foreskin Surface Area and HIV Transmission

Kigozi et al. studied the effect of foreskin surface area (measured after circumcision) in HIV transmission. They examined 965 foreskins for surface area, excised from men enrolled in the Rakai Community Cohort Study in Kenya (112). They found that the mean foreskin surface area among men who seroconverted to HIV (43.3 cm^2) was significantly larger than that among men who remained uninfected (36.8 cm^2) (P = 0.01). The risk of HIV acquisition was significantly increased among men with foreskins in the upper quartile of surface area (45.6 to 99.8 cm^2) compared to men in the lowest quartile (adjusted initial rate of return: 2.37; 95% CI 1.05–5.31) (112).

3.4.5 'Wetness' beneath the Foreskin

A study was conducted in 2006 among men at an STI clinic in Durban, South Africa, to examine the association between sub-preputial penile wetness and HIV transmission. This study used direct clinical examination and HIV prevalence as tools to measure the association (20). Via genital examinations performed by three physicians, the degree of wetness of the glans observed after foreskin retraction was classified into four grades—dry, slight wetness, wet or very wet—with or without smegma. The results showed that, among the men assessed as having any level of penile wetness, HIV prevalence was 66.3%, compared to 45.9% among men with no penile wetness. After adjusting for other predictors of HIV and confounders, the Odds Ratio (OR) was 2.38 (95% CI 1.42–3.97; P = 0.001). Interestingly, the study included a number of circumcised men, and the prevalence of HIV infection among them (42.9%) was similar to that among uncircumcised men with a dry penis (45.9%) (20).

3.4.6 Microbiomes of the IFS

Price et al. highlighted the idea that the anoxic microenvironment of the sub-preputial space may support pro-inflammatory anaerobes that can activate LCs to present HIV to CD4 cells in draining lymph nodes (21). They identified 42 unique bacterial families, of which *Pseudomonadaceae* and *Oxalobacteriaceae* were the most abundant, irrespective of circumcision status. They further demonstrated in 12 HIV-negative uncircumcised Ugandan men that circumcision was associated with a

significant change in the overall microbiota (p = 0.007). There was a significant decrease in putative anaerobic bacterial families 12 months after circumcision (p = 0.014)—mainly Clostridiales Family XI and Prevotellaceae, which were uniquely abundant before circumcision. Within these families, they identified a number of anaerobic genera previously associated with bacterial vaginosis, such as Anaerococcus spp., Finegoldia spp., Peptoniphilus spp. and Prevotella spp. Thus, the reduction in putative anaerobic bacteria after circumcision may play a role in protection from HIV and other STIs (21).

3.4.7 HIV and Other STIs

The idea that mucosal disruption associated with ulcerative STI facilitates HIV transmission drew attention from researchers to investigate a possible interaction between HIV and other STIs. Cameron et al. showed that the acquisition of HIV was highly associated with having genitourinary ulcerative disease (GUD), being uncircumcised, and having frequent contact with sex workers (113). In their study, men who reported a single contact with sex workers, and who had seroconverted, all had genital ulcers. In their extensive review, Galvin and Cohen demonstrated that people with STIs that cause ulcers and inflammation are more vulnerable to HIV than are healthy individuals (22). According to Fleming et al., the adjusted risk ratio for HIV acquisition for a person with GUD ranges from 2.2 to 11.3, whereas, with non-ulcerative STIs, it ranges from three to four (114). Dickerson et al. reported that such associations persist in most cases, even after adjusting for sexual behaviour and other confounding factors (115).

Weiss et al.'s systematic review of MC and ulcerative STI strongly indicated that circumcised men were at lower risk of chancroid and syphilis. However, the review reported only a borderline statistical significance for HSV-2 (116). In 2009, Metha et al. demonstrated in their study in Kisumu, Kenya, that MC did not reduce the risk of acquiring non-ulcerative STIs (*N. gonorrhoeae, C. trachomatis* and *T. vaginalis*) (117). The same authors later showed that circumcised men had fewer *M. genitalium* infections (118). In their review, Anderson et al. showed that *Treponema pallidum, Haemophilus ducreyi* and *Neisseria gonorrhoeae* infections did enhance HIV transmission, and that all those infections were less frequent after circumcision (108).

In an MC trial in Rakai, Uganda, the researchers observed a 46% reduction in GUD (13) and 28% reduction in HSV-2 acquisition from MC (119). Similarly, an MC trial in Orange Farm, South Africa, demonstrated a 30% reduction in HSV-2 incidence among participants (120). In contrast to the findings from the South African and Ugandan trials, Metha et al. demonstrated in 2012 that the protective effect of MC against HIV was independent of GUD and HSV-2, and that MC had no effect on HSV-2 incidence (121). According to Dinh et al., the reason for the disparity seen between the effect of MC on viral and bacterial pathogens is not entirely clear, but likely relates to differences in the routes taken during transmission (that is, the squamous epithelia found in the foreskin, glans and shaft tissue versus the columnar epithelium of the urethra) (18).

3.4.8 Genital Mucosal Disruptions

Penile cuts, abrasions and tears during sexual intercourse are presumed to be another potential mechanism that places uncircumcised men at increased risk of acquiring HIV through the disruption of epithelial and mucosal barriers. O'Farell et al. suggested that such abrasions are more common, and elaborated mucosal discontinuity as a constant finding with poor standards of genital hygiene. They further suggested that rapid healing of those abrasions is delayed due to the moisture beneath the foreskin, which provides an excellent niche for other STI pathogens (122). Szabo et al. highlighted that the frenulum, which is a highly vascular area of penile skin, is more susceptible during sexual intercourse and a common site on the penis of ulcerative lesions from STIs. Thus, they suggested that MC reduces HIV–STI synergy by removing foreskins (123).

In contrast, in 2009, Mehta et al. reported that early sex after MC is not associated with increased HIV risk (124). In a later publication based on a RCT in Kenya, Metha et al. demonstrated that self-reported penile coital injuries were common among healthy 18- to 24-year-old men. Nevertheless, circumcised men were at lower risk of coital injuries (0.62, 95% CI 0.56–0.70) than were uncircumcised men (125). The exact mechanisms by which such injuries may increase risk for STIs and HIV infection are yet to be investigated.

3.5 Discussion

There is strong evidence that MC can reduce HIV acquisition in heterosexual men by around 60%; however, the exact mechanisms of protection remain unclear. This review has summarised the existing research that is advancing knowledge about the mechanisms of protection from HIV afforded by MC.

The first natural barrier found on the foreskin is the outermost keratin layer (stratum corneum), which consists of dead keratin and a basal layer of live keratinocytes. Different studies have shown different measurements of the keratin layer of the IFS and OFS, making the evidence inconclusive. Some evidence supports the concept that the IFS is less keratinised than the OFS, and consequentially provides easy access for the virus to enter the submucosa. However, other evidence shows that the OFS is equally or more thinly keratinised, and offers no favourability for transmission of the virus. Moreover, there is evidence of inter-individual and intra-individual differences in foreskin KT, as well as differences in KT among different races.

One explanation for the contrasting results of KT is the different methodologies used to measure KT in different studies. For example, Qi Qin et al. did not measure the thickness of the most superficial dead keratin layer when they measured keratin layer thickness (94). Different tissue sampling (such as different distances to the tissue sample from the coronal sulcus), different processing methods, and possible underlying pathologies (such as STIs among the tissue donors) may also have contributed to the different results. In addition, the keratin layer is easily swollen through exposure to water and can easily detach from the stratum granulosum during fixation, which could be another factor responsible for the difference in measurements. Finally, cadaver samples could also have different KT. It is important to remember that variation in KT among races, between individuals and within individuals suggests that factors other than race work together to create KT differences, and that further laboratory studies need to be performed to understand these factors using tissue samples from men in vulnerable populations.

There are different schools of thought regarding the protection provided by the keratin layer against HIV acquisition. One argument is that the superficial keratin layer is easily sloughed off; therefore, an intact layer is unlikely to be found after sexual intercourse to provide any protection against HIV infection. Another argument is that HIV transmission through the oral mucosa, with a very thin keratin layer, is very inefficient (18).

There is a general agreement about the types of target cells present in the foreskin—such as LCs, DCs, macrophages and T4 cells—that bear cellular receptors of CD4, CCR4 and CCR5, although contrasting results have been published about the distribution of these cellular HIV targets in the IFS and OFS. Differences are not only regarding the cell location, but also the number of cells in different sites of penile tissues (Table 3.2). A plausible explanation for these differences would be the presence of possible underlying pathologies in tissue donors that could result in changes in the distribution of immunological cells in and around an inflammatory process. These different results could also be due to the difference in site of origin of the tissue from the intact foreskin used in the analyses. Fischetti et al. also suggested the possibility of surgical trauma–induced cellular redistribution, with alteration of target cell densities in penile tissues after removal, as an explanation for the different results achieved in previous studies (23). After reviewing these previous studies, the current study can conclude that there is general consensus on the types of HIV target cells present in foreskin tissues, and that there are more cellular targets found in the IFS than in the OFS.

Studies that undertook infection of HIV into tissue explants to simulate the actual biological process of infection in vitro have provided the best evidence to date for the tissue sites involved with the acquisition of HIV. The IFS was demonstrated to be the main area for viral entry in two out of three studies done on human explant tissues. While Patterson's and Ganor's studies demonstrated the IFS as the only susceptible tissue of the two foreskin surfaces, Fishchetti et al. demonstrated no significant difference between tissues in terms of susceptibility to HIV among all the exposed areas of the penis (the glans, IFS and OFS). However, Fischetti et al. concluded that circumcision removes two out of three susceptible exposed areas of the penis, thereby reducing the chance of the virus coming into contact with susceptible target cells (23).

Patterson et al. considered the OFS as equivalent to the penile shaft for comparison with the IFS of the penis, and concluded that penile shaft tissue is also impermeable for infiltration by HIV-1 (24), on the basis that the penile shaft is covered by a keratinised stratified squamous epithelium, similar to the OFS. Although this finding rejected a previous hypothesis that the penile shaft may act as an entry point for HIV in circumcised men, further research is needed to enhance evidence about HIV entry along the penile shaft (24). Some studies have speculated that the route for HIV-1 infection in uncircumcised males could be through the epithelium of the glans, as it is protected by the foreskin and is thus likely to be less keratinised in adults than the glans of the circumcised penis. Szabo and Short examined the glans of seven circumcised and six uncircumcised men, and found that the epithelia of both groups were equally keratinised (123). They demonstrated that, in circumcised males, only the distal penile urethra is lined with a mucosal epithelium, and speculated that it is unlikely to be a common site of infection because it contains comparatively few LCs. Instead, they suggested that infection could occur through disruptions of the penile shaft epithelia caused by GUD or trauma (123).

Patterson et al.'s and Fischetti et al.'s explant models had only a few hours of functional integrity, contained migratory immune cells activated by surgical procedures on explant tissues, and had inefficient sealing of the edges of explant tissues to ensure the polarisation of HIV infection. Therefore, those models are assumed to have failed to maintain the stratified architecture or actual numbers of immune cells to simulate the natural situation in foreskins (93). Ganor et al. managed to overcome those problems with their novel models, and demonstrated more solid evidence of HIV transmission through the male genital tissues, as described above. Ganor et al. further demonstrated that interaction of HIV-infected mononuclear cells in a partner's sexual fluids with the IFS is the key to initiating infection, and further speculated in a later publication that this rapid process could be impeded by as-yet ill-defined components activated during the mixing of genital fluids (19). Thus, according to Ganor et al., removal of the foreskin—especially the inner aspect—with circumcision eliminates a mucosal surface rich in HIV-1 target cells that serves as an efficient HIV-1 entry portal in men (19).

The observations in Kigozi et al.'s study strongly suggest that larger foreskin size is a risk factor for HIV acquisition in uncircumcised men (112). These findings support Fischetti et al.'s explant tissue findings, which, in addition to the observational studies and RCTs, again contribute plausibility to the hypothesis that the foreskin is the main tissue of the penis that is vulnerable to HIV acquisition. The increased risk of HIV acquisition among men with a larger foreskin surface area may be due to the presence of a larger number of HIV target cells in the IFS that are exposed to infected vaginal fluids during sexual intercourse. Men with a larger foreskin surface area may also be more vulnerable to trauma of the foreskin during sexual intercourse, thereby increasing the risk of HIV acquisition. Kigozi et al. emphasised the implications of these findings for the surgical procedure of circumcision, and suggested the need to minimise residual foreskin tissue after MC (112).

O'Farrel et al.'s study on sub-preputial wetness provides better insight to another important factor that could contribute to HIV transmission through foreskin tissues (20). According to this study, two-thirds (66.3%) of the men who were infected with HIV had some amount of penile wetness, as opposed to 45.9% who were infected with HIV but had no penile wetness ($p = 0.001$). The authors suggested several possible explanations responsible for their findings

- impaired healing of sexually acquired ulcers due to wetness in the prepuce
- micro-ulcerations caused by sub-preputial balanitis due to wetness
- enhanced adherence of infective HIV virions to the HIV target cells of the IFS in the presence of wetness
- recruitment of more HIV target cells due to enhanced immune response by the wetness of the IFS (20).

However, this study did not adequately assess the level of penile wetness of circumcised men, and the number of circumcised participants were few ($n = 55$) compared to the number of uncircumcised participants ($n = 589$).

Price et al. extended their study on the level of anaerobic bacteria in the preputial space to determine the bacterial diversity in male genital mucosa (21). They revealed a decrease in anaerobic bacteria after circumcision, which may have been related to the elimination of anoxic microenvironments under the foreskin (21). Detection of these

anaerobic genera in other human infectious and inflammatory pathologies suggests that they may mediate genital mucosal inflammation or co-infections in the uncircumcised state. Hence, according to the researchers, the decrease in anaerobic bacteria after circumcision may complement the loss of the IFS to reduce the number of activated LCs near the mucosal surface and the risk of HIV acquisition in circumcised men (21).

As well as the mechanisms discussed above, there is a body of evidence indicating that STIs that cause mucosal inflammation and ulcers contribute to the spread of HIV by increasing infectiousness, susceptibility or both (22). Therefore, MC, which has been shown to be protective against such inflammatory and ulcerative STI acquisition (as in most of the studies mentioned above), could have a protective effect on HIV transmission.

Genital ulcers caused by STIs (on the glans and especially in the area of the frenulum) and their associated inflammation are expected to increase the number of HIV-susceptible/target cells locally. With the observation that the IFS is rich in CD4+ T-cells, macrophages and LCs, the presence of STIs affecting the IFS further exacerbates the risk of HIV transmission through the IFS by migrating immune cells (36). With regard to non-ulcerative STIs, Mayer et al. held a similar view to Ganor et al., who suggested enhanced activity of CD4 cells after STIs (such as *N. gonorrhoeae*)(126). Mayer et al. further emphasised the association between HSV-2 and HIV, whereby HSV-2 induces persistent expression of CCR5, which is a main co-receptor for HIV-1, making genital tissue vulnerable for HIV-1 even after treating for HSV-2 (126). Apart from biological mechanisms, Mayer and Venketesh highlighted an epidemiological link between the high HIV susceptibility of patients with STIs (126). They proposed that STIs could be a marker of increased sexual risk behaviour and contact with a HIV-infected partner.

Another area of concern in the area of HIV prevention is the level of protection provided by MC for women. An RCT with participation of 922 uncircumcised men in Rakai, Uganda (which was stopped early due to futility), demonstrated that circumcision of HIV-infected men did not reduce HIV transmission to female partners over 24 months, although the study did not assess longer-term effects (127, 128). A

meta-analysis of data from six longitudinal studies and one RCT also did not provide sufficient evidence for a direct effect of MC in reducing HIV risk among women (summary relative risk: 0.80; 95% CI 0.53–1.36) (129).

Beaten et al. demonstrated a ~40% (statistically insignificant) reduction in the risk of acquisition of HIV by women from a circumcised male partner (hazard ratio: 0.62; 5% CI 0.35–1.10; p = 0.10). They reported no increased risk for women in serodiscordant couples in which the male partner was seropositive and circumcised; however, they suggested a 'potential' decreased risk from MC for male-to-female transmission of HIV-1 (130). Hallete et al. undertook mathematical modelling of data generated from two independent observational cohorts on the long-term effect of MC for male-to-female HIV transmission. They estimated that there would be an effective 46% reduction in HIV transmission rates from two years after the MC operation (16).

A presentation at the 2014 Conference on Retroviruses and Opportunistic Infections revealed a statistically significant (p = 0.004) 15% reduction in risk for women who had sex with only circumcised men compared to women who reported that some or all of their partners were uncircumcised (131). This study was conducted in Orange Farm, South Africa—the place that hosted the first RCT of MC for HIV prevention. Nevertheless, it was unclear in this study whether this protection was due to the direct effect of MC or the low HIV prevalence among circumcised men. Based on all the evidence, there could be direct protection from HIV for women who have sex exclusively with circumcised men, and indirect 'herd immunity' from HIV infection for women as a result of MC. However, this important relationship needs to be supported with further epidemiological and clinical research.

Numerous studies have reported on HIV risk modification by MC for MSM. As early as 1993, it was shown that uncircumcised MSM had a two-fold increased risk of HIV infection (adjusted OR: 2; 95% CI 1.0, 4.0) (132). Later, a study by Buchbinder et al. demonstrated a similar two-fold increase in risk of HIV acquisition for MSM associated with lack of circumcision (133), although the population attributable ratio in their study population was relatively low (10.2).

Grulich et al. demonstrated no difference in MC status of men infected by receptive or insertive unprotected anal sex (134). However, this study did not control for the behavioural risk of participants. Based on cross-sectional data, Millet et al. found no evidence to support MC as a protective measure against HIV infection among black or Latino MSM (135). Findings from a study in Seattle also suggested that MC did not have a significant effect on HIV or STI acquisition among MSM (136). A meta-analysis of observational studies of MSM in 2008 further demonstrated a lack of evidence for the protection provided by MC against HIV infection or other STIs (137). The 2008 National HIV Behavioural Surveillance System cross-sectional survey conducted in 21 US cities among 5,183 MSM not previously diagnosed with HIV infection demonstrated that incarceration history, circumcision status and sexual networks were not independently associated with HIV infection (138). Gust et al. reanalysed a phase-three HIV vaccine clinical trial and reported that being uncircumcised did not confer a statistically significant increase in HIV infection risk among men who reported unprotected insertive anal sex with HIV-positive partners (139).

According to Sanchez et al., circumcision did not have a significant protective effect against HIV acquisition among MSM from Peru and US; however, they suggested a possible reduced risk for men who were primarily insertive with their male partners (140). Similarly, Templeton et al. demonstrated a significant reduction in HIV incidence among Australian participants who preferred the insertive role during anal intercourse (141). Later, in a systematic review, Templeton et al. further demonstrated that circumcised MSM who predominantly take the insertive role during anal intercourse could be at a lower risk of HIV infection (142). According to the Cochrane review in 2011, MC 'may be' protective among MSM who practice primarily insertive anal sex; however, the role of MC in the overall prevention of HIV and other STIs among MSM remains to be determined (143).

3.5.1 Research Priorities based on the Literature

After reviewing the available literature, the current study identified several research priorities in defining the MC mechanism/s that reduce the risk of HIV acquisition. One priority was to analyse the KT of the IFS and OFS in live, healthy men, using physical

properties inherent to the keratin layer, such as optical absorbance. This could be done using non-invasive methods, such as stratum corneum infrared densitometry (144). Non-invasive measurements can improve measurements due to structural changes that occur in tissues once they are removed from the body or once the person is deceased. The researchers believed this would shed new light on measuring KT differences between the IFS and OFS.

Another priority was to assess the physical properties of resistance for viral entry, using non-invasive methods—such as trans-epithelial water loss, moisture content of the stratum corneum and skin surface pH—in order to provide perspectives beyond KT alone as a principal mechanism of protection. In addition, quantitative research could be helpful to measure the exact relationship between HIV transmission and the level of 'wetness' of the preputial area, which could be measured with newer methods, such as moisture meters. Similarly, the effects of the microbiome of the penis in HIV transmission through male genital tissues need to be quantified. The limited research done to date requires strengthening to validate its observations.

Finally, a more nuanced understanding of the mechanism of protection provided by MC against HIV transmission should be applied in order to understand how modified forms of circumcision affect HIV transmission. For example, men in PNG commonly practice traditional forms of penile cuttings that involve a longitudinal cut of the foreskin without foreskin removal (1, 145). It is important to determine whether these modified forms of MC (which may transform the properties of the IFS) can affect HIV transmission. This can be achieved by studying the changes in foreskin tissue components—such as KT, target cell density and penile wetness—in order to further understand HIV transmission via male genital tissues, and the mechanism of protection afforded by MC.

3.6 Conclusion

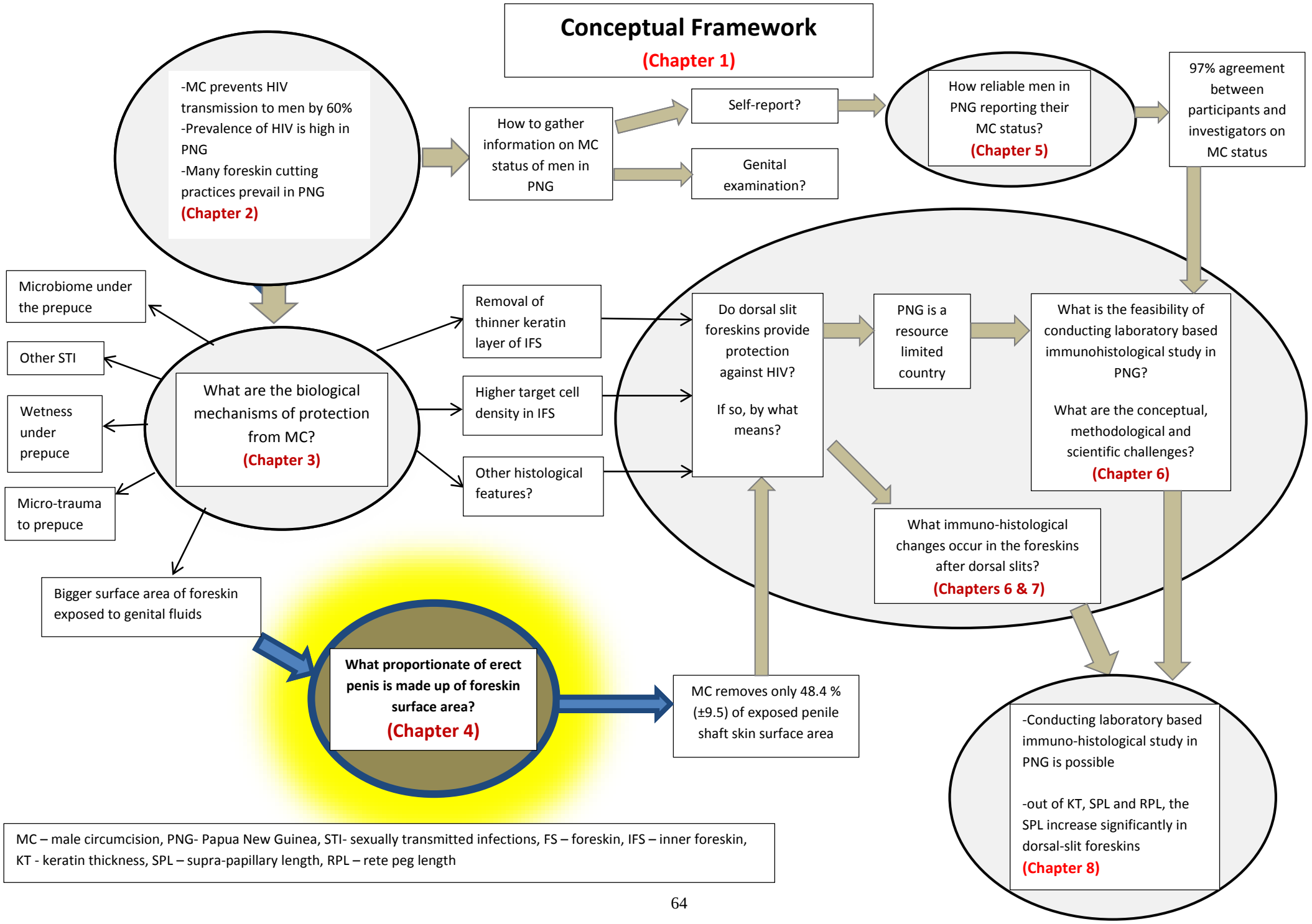
This review has summarised previous research on the bio-physical mechanisms for protection provided to men by MC from heterosexual HIV transmission. Although there is a substantial body of knowledge on this topic, there remain unresolved areas

regarding the exact mechanism of protection. Based on all the evidence summarised above, this mechanism is undoubtedly complex, with numerous factors working together to facilitate this well-documented protection from HIV.

Based on the evidence from the summarised studies, the mechanism of HIV transmission through the penile tissues occurs as follows. When HIV-1-infected cells come into contact with the foreskin, especially the IFS, they make synapses with the epithelial cells. This results in HIV-1 budding, and subsequent capture by epidermal LCs through dendrites. This is followed by transfer of the virus to T-cells to initiate local expansion, HIV dissemination and systemic infection. The large surface area of the foreskin increases the chances of synapse formation by increasing the number of contacts, while sub-preputial wetness can facilitate the process by keeping the virus alive.

This process is facilitated by the abundant HIV target cells found in the IFS, and their higher responsiveness through altered cellular protein expression in response to external stimuli. Chemokines present in genital fluids further change the spatial distribution of HIV target cells (especially LCs), thereby favouring connections with HIV-infected cells. Further, the presence of concurrent STIs and microbiomes under the prepuce and induced inflammation therein amass the target cells into dermal and epidermal tissues to facilitate the process. The closeness of target cells and their dendrites reaching closer to the apical surface further enhance the HIV infection process.

Physiological factors that cause micro-trauma—such as mechanical friction during sexual intercourse—can also provide easy access for the virus. Removal of the foreskin by MC disrupts most of these mechanisms, and helps achieve protection for men from sexual HIV transmission. Investing in research to increase understanding of the mechanisms of HIV transmission and protection against heterosexual HIV acquisition in men should be a priority that supplements the judicious implementation of MC programs in settings with high HIV prevalence.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 4: Exposed Foreskin Surface Area and Protection from HIV—A Descriptive Study of the Foreskin Surface Area of the Erect Penis in Healthy Uncircumcised Males

4.1 Preface

Dorsal slits are the most frequently used foreskin cutting of the many different foreskin cuttings observed in PNG (1). This prompted the current study to answer the question as to whether dorsal slit foreskin cutting, that leaves the foreskin intact and exposes the glans penis, provides any protection against HIV transmission. The fundamental difference between medical MC and dorsal slit foreskin cutting is that medical MC removes the total foreskin at the root of the prepuce, leaving no foreskin tissue, while the dorsal slit makes a longitudinal cut along the dorsal aspect of the penis, leaving the foreskin in place (146).

With evidence that removal of the foreskin is associated with 60% protection against HIV transmission (11, 13, 102), it is reasonable to propose that the intact foreskin associated with a dorsal slit would not be associated with the same amount of protection. As discussed in the previous chapter on the mechanisms of protection from MC, a study in Africa demonstrated that the heterosexual HIV transmission rate was proportional to the size (surface area) of the foreskin. However, these studies were not designed to assess whether total skin area reduction in medical MC accounts for the demonstrated 60% of protection. In other words, does MC remove 60% of the surface area of the penis exposed to sexual secretions during sexual intercourse? If not, it is reasonable to assume that factors other than the removal of surface area play a role in providing the demonstrated protection. If so, it is important to determine whether foreskin modifications other than total removal of the foreskin offer any protection from HIV.

Anecdotally, some medical practitioners in PNG speculate that partial circumcisions/dorsal slits could provide protection from HIV due to changes that occur

in the foreskin (especially by increased keratinisation) to improve the physical barrier, after a period of time has elapsed since the dorsal slit foreskin cutting (29). Thus, this study is an attempt to measure the surface area of the foreskin of the erect penis that is exposed to sexual fluids during sexual intercourse. This measurement will help determine the relationship between the amount of surface area removed during MC, and subsequently the level of protection in HIV transmission offered by MC. This is central to understanding the importance of having the IFS exposed to the environment, versus total removal of the foreskin.

This study first examines the literature regarding the mechanisms of protection from MC, with a focus on the surface area factor. This is followed by a presentation of the methods used in this study, and the results and discussion of those results in the context of dorsal slit foreskin cuttings.

Given that this study dealt with a culturally sensitive issue (taking genital photographs), the researchers encountered a number of challenges in recruiting participants to the project. Hence, this study dealt with important considerations concerning participant recruitment, research methodology and data dissemination. This study was presented as a poster presentation at the 2012 Australian Society of HIV Medicine (ASHM) Conference in Melbourne, Australia.

Details of the publication on which the chapter is based	**Nature and extent of the intellectual input of each author, including the candidate**
Jayathunge PHM, McBride WJH, MacLaren D. What is the measure of a man? Poster presented at: ASHM; 17-19, October, 2012.	PHM Jayathunge and WJH McBride co-developed the research question. PHM Jayathunge collected the data, performed the analysis and wrote the first draft of the paper, which was revised with editorial input from WJH McBride and D MacLaren. PHM Jayathunge also developed the figures and tables.

4.2 Introduction

Three large-scale phase-three clinical trials conducted in Johannesburg, South Africa; Kisumu, Kenya; and Rakai, Uganda demonstrated a protective efficacy of around 60% for MC in preventing heterosexual HIV acquisition among men (11-13, 147). These findings, in addition to the prior observational studies, added plausibility to the hypothesis of the foreskin being the main site of the male genital tissues vulnerable to HIV acquisition and transmission of the virus. MC is also associated with lower rates of other STIs (45).

Various theories have been proposed for MC's mechanism of protection from heterosexual HIV transmission. Some attribute this protection to the difference in thickness of the keratin layer on the IFS and OFS (30). Another theory suggests the presence of an increased number of target cells in the IFS that capture HIV (30). Other mechanisms include minute trauma (especially to the IFS during sexual intercourse) and the retention of genital fluids under the foreskin, supporting viral survival after sexual intercourse (30). Overall, this protection is thought to be due to a combination of several of the above mechanisms; however, the exact mechanism remains unknown.

Kigozi et al. studied the effect of foreskin surface area in HIV transmission, and reported that the mean foreskin surface area among men who seroconverted to HIV was significantly larger than that among men who remained uninfected (112). They demonstrated that the risk of HIV acquisition was significantly higher among men with foreskin surface areas in the upper quartile of surface area, compared with men with foreskin surface areas in the lowest quartile. They concluded that there was a significant trend of increasing HIV incidence with increasing size of foreskin area (112). These observations strongly suggested that larger foreskin size is a risk factor for HIV acquisition in uncircumcised men. Methodologically, in Kigozi et al.'s study, the foreskin area was measured after it had been removed, and the amount of tension applied to the removed foreskin before measuring the surface area was unclear. Further, the study did not relate the foreskin area to the total area of the penile skin exposed. Thus, the current study hypothesised that this method of measurement may not have accurately reflected the area of the foreskin prior to the circumcision.

The increase in risk of HIV acquisition among men with a larger foreskin surface area may be due to the presence of a higher number of HIV target cells in the IFS epithelium, which is exposed to infected vaginal fluids during sexual intercourse (112). It is also possible that men with a larger foreskin surface area are more vulnerable to trauma of the foreskin mucosa during sexual intercourse, thereby increasing the risk of HIV acquisition (112). These findings provide an important reflection on the surgical procedure of circumcision, and suggest the need to minimise the residual foreskin tissue during MC, particularly in the forceps-guided procedure that leaves 0.5 to 1.0 cm of the IFS proximal to the corona (112). Remnant foreskin is less of a problem with the dorsal slit and sleeve procedures (148). However, this is only a theoretical concern, given that Kigozi et al. did not observe a significant increased risk of HIV acquisition among men with smaller foreskin surface areas, which were substantially larger than residual tissue retained after a circumcision (112).

As many as 50% of men in PNG have a dorsal slit—a form of penile cutting that is sometimes identified as a 'V cut' that exposes the glans penis, but does not remove any of the foreskin (1). It is still unclear whether this form of circumcision confers any protection against HIV. If foreskin surface area is shown to be a significant risk factor for HIV acquisition, men with the 'dorsal slit' may elect to have their redundant foreskin removed. If other factors discussed were found be responsible for protection, then the case for further surgical intervention would be diminished. Thus, the main idea behind the principle research question for this study was: can the degree of protection afforded by MC against HIV be accounted for exclusively by the reduced surface area of the penis in the erect state?

4.3 Methodology

4.3.1 Research Hypothesis

The main hypothesis of this study was that the proportion of the surface area occupied by the foreskin of an erect penis is less than the degree of protection offered by MC against heterosexual transmission of HIV.

4.3.2 Research Questions

The main research question for the study was: what is the proportion of the surface area occupied by the foreskin on an erect penis?

4.3.3 Objectives of the Study

The objective for this study was to determine the proportion of surface area occupied by the foreskin on an erect penis. This would enable determination of whether the surface area of the foreskin in the erect state is sufficient to account for the level of protection demonstrated in clinical studies of HIV prevention by MC. The end point of the study was to measure the surface area of the erect penis covered by both the IFS and OFS in uncircumcised men, as a proportion of the total surface area of skin over the shaft of the penis.

4.3.4 Study Design

4.3.4.1 Study Site and Population

The study was conducted on volunteer participants from students and staff of James Cook University (JCU), from September 2011, until August 2014. The study site was the Cairns campus of JCU: 14–88 McGregor Road, Smithfield, Queensland, Australia, 4878.

4.3.4.2 Eligibility Criteria

Uncircumcised men aged 18 years or older were recruited in the study. The study's objectives, potential benefits and inconveniences associated with participation were explained prior to obtaining informed consent. Other essential criteria for participation in this study required the participants to be able to operate a digital camera and be willing to photograph their own erect penises.

Anyone with a history of operation or trauma to the penis, or any other condition that affected the morphology of the penis was excluded from the study. In addition, those with a history of STIs or symptoms suggestive of an STI were excluded. Those with

abdominal obesity that would preclude adequate visualisation of the penis for self-photography were also ineligible, and were not recruited in this study.

4.3.4.3 Ethical Considerations and Informed Consent

Ethics approval to conduct this research was obtained from the Human Research Ethics Committee at JCU, Townsville, Queensland, Australia (approval number: H4339). Participants who met the eligibility criteria completed informed consent forms and received a participant information sheet that included the key messages of the study (Annex 4).

4.3.4.4 Study Procedure

Participation in the study was voluntary. Information on the study was distributed among male students via flyers and group emails at the Cairns campus of JCU. Once informed consent was provided, the participants underwent a short interview with the principal investigator to check their eligibility for the study. The eligible subjects were assigned a specific identification number and were identified using that number throughout the study. Names and contact details were also collected, but were not linked with the study identification number. The details were only used to keep track of the study camera equipment provided to the participants during the study.

Participants were provided with a ruler and non-permanent marker pen, and, using a plastic model of male genitalia, were given a demonstration about the study procedure, including marking the penis. Participants were asked to undertake the following: while the penis was in a flaccid state, to make one marking at the edge of the foreskin at the junction of the IFS and OFS, and one on the OFS corresponding to the palpable coronal sulcus, where the foreskin is incised in a circumcision operation (Figure 4.1). They were also given instructions on the method for taking photographs of the erect penis under optimal conditions.

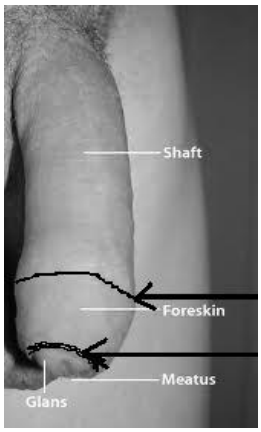
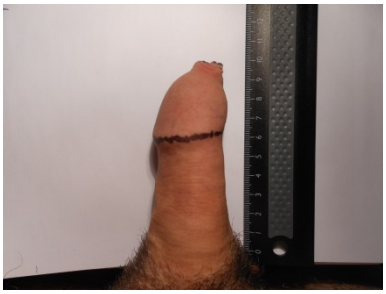
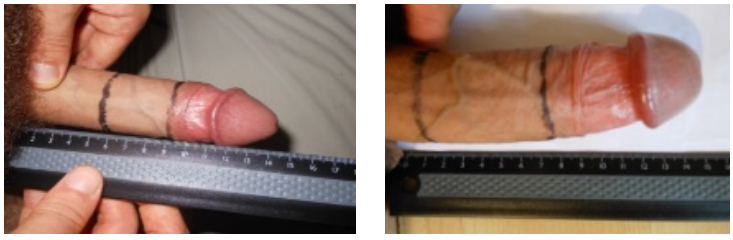


Figure 4.1: Surface Marking of Penis before Photograph

A digital camera was provided to the participants, and they were requested to take four photographs of their penis in the flaccid state (two photographs) and erect state (two photographs) (Figure 4.2). The participants were able to complete the process in a location convenient to them. Once the photographs were taken, they were retrieved and stored in a secure research folder in the principal investigator's digital storage. The memory card of the camera was reformatted before the camera was given to the next participant.



(a)



(b)

Figure 4.2: Sample Pictures from the Study Participants. (a) Erect Penis with Markings. (b) Measuring the Erect Penis

The photographs were analysed using standard photographic software (Adobe Photoshop) and several measurements were taken for calculations (see Figure 4.3).

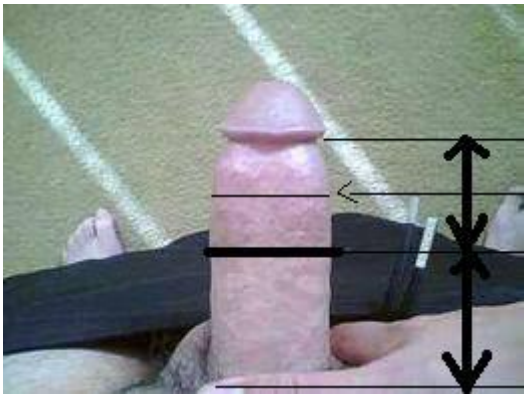


Figure 4.3: Measurements of the Penis

Calculating the proportions:

$$x = \frac{a + b}{a + b + c} \times 100$$

x = percentage of exposed foreskin in erect penis

a = length from corona to IFS–OFS junction

b = length from IFS–OFS to the OFS–shaft junction

c = length from the OFS–shaft junction to the base of the penis

Assumptions: an erect penis is a near-uniform cylinder; hence, the length is proportionate to the surface area.

4.3.5 Confidentiality and Data Security

Participants' study folders containing clinical data were securely filed by identification number in a designated locked filing cabinet located at the Cairns Clinical School of JCU. Only the investigators were able to access these records and were confirmed their role and responsibility to maintain subject confidentiality. Digital pictures were stored electronically on a hard drive that was held by the principle investigator. The study folder was password protected.

4.3.6 Data Analysis and Reporting

The digital photographs were standardised and the measurements were taken to the nearest millimetre. The mean proportions were calculated with a one-sample t-test using the SPSS 18 statistical package. The study hypotheses were as follows:

- H_0 = the exposed surface area of the foreskin during sexual contact equals the 60% demonstrated protection from MC
- H_a = the exposed surface area of the foreskin during sexual contact is less than the 60% demonstrated protection from MC.

The significance level was set at 0.05.

4.4 Results

All participants in the study were university students. Of a total of 11 participants, eight were Caucasian and three were Mongolian nationals. The mean age of the participants was 26.4 years. Table 4.1 demonstrates the measurements taken from the genital pictures of the 11 participants. The mean proportion of surface area of the foreskin (both inner and outer) per total erect penile surface area was 0.484 (standard deviation [SD] ± 0.095). The t-statistics were: $t(10) = 4.026$, $p = 0.002$.

Table 4.1: Measurements of Genital Pictures

Participant	IFS Length (a) (cm)	OFS Length (b) (cm)	Full Foreskin Length (a + b) (cm)	Shaft Length (Erect) (a + b + c) (cm)	IFS – Length /Shaft	Total – Foreskin /Shaft (a + b/a + b + c) (Calculated)
SAP01	3	3.5	6.5	17	0.18	0.38
SAP02	1	5	6	14	0.07	0.42
SAP03	1	4	5	14	0.07	0.35
SAP04	2.5	3.5	6	15	0.17	0.40
SAP05	4	6	10	15	0.27	0.66
SAP06	4	5	9	15	0.27	0.60
SAP07	4	6	10	18	0.22	0.55
SAP08	2	3.5	5.5	16	0.12	0.34
SAP09	2.5	5	7.5	17	0.15	0.44
SAP10	3	3.5	6.5	13	0.23	0.50
SAP11	1.5	5.5	7	13	0.12	0.54
Mean (SD)						0.484 (± 0.095)

4.5 Discussion

According to the results of the study, removal of the complete foreskin would account for a 48.4% (± 9.5) reduction of the penile shaft skin surface area that is exposed to sexual secretions from a partner. The IFS is postulated to be the main component of the foreskin through which HIV enters the penile tissue during sexual intercourse. This is postulated to be due to the number of inherent properties of the IFS, such as the presence of a higher number of target cells, the thinner keratin layer and the higher susceptibility to micro-trauma during sexual intercourse (149). Circumcision removes this susceptible tissue and subsequently provides protection against heterosexual HIV transmission.

This study's findings disprove the hypothesis that removal of the surface area (of the exposed penile surface area during sexual intercourse) is solely responsible for the reported 60% of protection in interventional studies. Removal of both the IFS and OFS (the total prepuce) would only provide around 48% of protection. The relatively large SD (± 0.095) is a result of the limited number of participants in the study; hence, studies enrolling a higher number of participants may increase the precision of the estimate. Recruitment into this study was understandably challenging, and this study needed to extend the recruitment period considerably. Thus, these study results cannot contribute to the debate on whether reduction of the penile skin surface area plays a contributory role in HIV transmission risk reduction.

The popular form of circumcision in PNG is the longitudinal cut (also known as the 'dorsal slit'), which does not result in the removal of any foreskin, but exposes the IFS significantly to the outside environment. This exposure is believed to alter the characteristics of the IFS, such as the keratin layer thickness and distribution of target cells. If foreskin removal was the sole factor responsible for the reported HIV risk reduction/protection, it could be postulated that the longitudinal cut would provide no or minimal protection against HIV infection. The current study does provide supportive evidence that the characteristics of the IFS (either removal or exposure) are indeed important in reducing HIV risk. However, this study does not provide any insight as to what changes occurring in the IFS after substantial exposure following the longitudinal cut could contribute to protection against HIV transmission. Ecological studies and histopathological correlates discussed later in this thesis are more likely to provide insight to whether and how incomplete circumcisions reduce HIV risk.

To the best of the researchers' knowledge, this is the first study conducted to assess the foreskin surface area of uncircumcised men. The results provide a better perspective on the challenges of and limitations to conducting such a study. The main limitation and challenge encountered during the study was recruiting a sufficient number of participants. Despite extensive local publicity, participation remained low and the total number recruited to the study during the allocated time was less than the minimum required number of participants (power analysis: when $\alpha = 0.05$, $1 - \beta = 0.95$; effect size: 0.8, total sample size: 19). Understandably, this study involved culturally sensitive issues, such as exposing participants' genitalia for photographs, which deterred many potential participants.

In addition, there were a few technical problems experienced during the study. The clarity and orientation of the photographs was suboptimal in a few of the photographs taken by the participants. It was also difficult to take some measurements on the photographs because the ruler was not placed accurately alongside the penis and, in some photographs, there was a reflective glare from the flash of the camera on the ruler. Further, in some photographs, 'adequate tension' had not been applied to remove all wrinkles of the foreskin from the surface of the erect penis. Eight unclear pictures from six individuals (a maximum of two from an individual) were removed from the

analysis, thereby minimising the effect on the final result (according to study protocol, two photographs were required for each measurement, which gave the opportunity to select the best of two for analysis). The researchers believe that these technical issues could have been prevented by a clearer demonstration using pictures of the correct technique. Having photographs taken by an investigator or another party would have further diminished the level of participation.

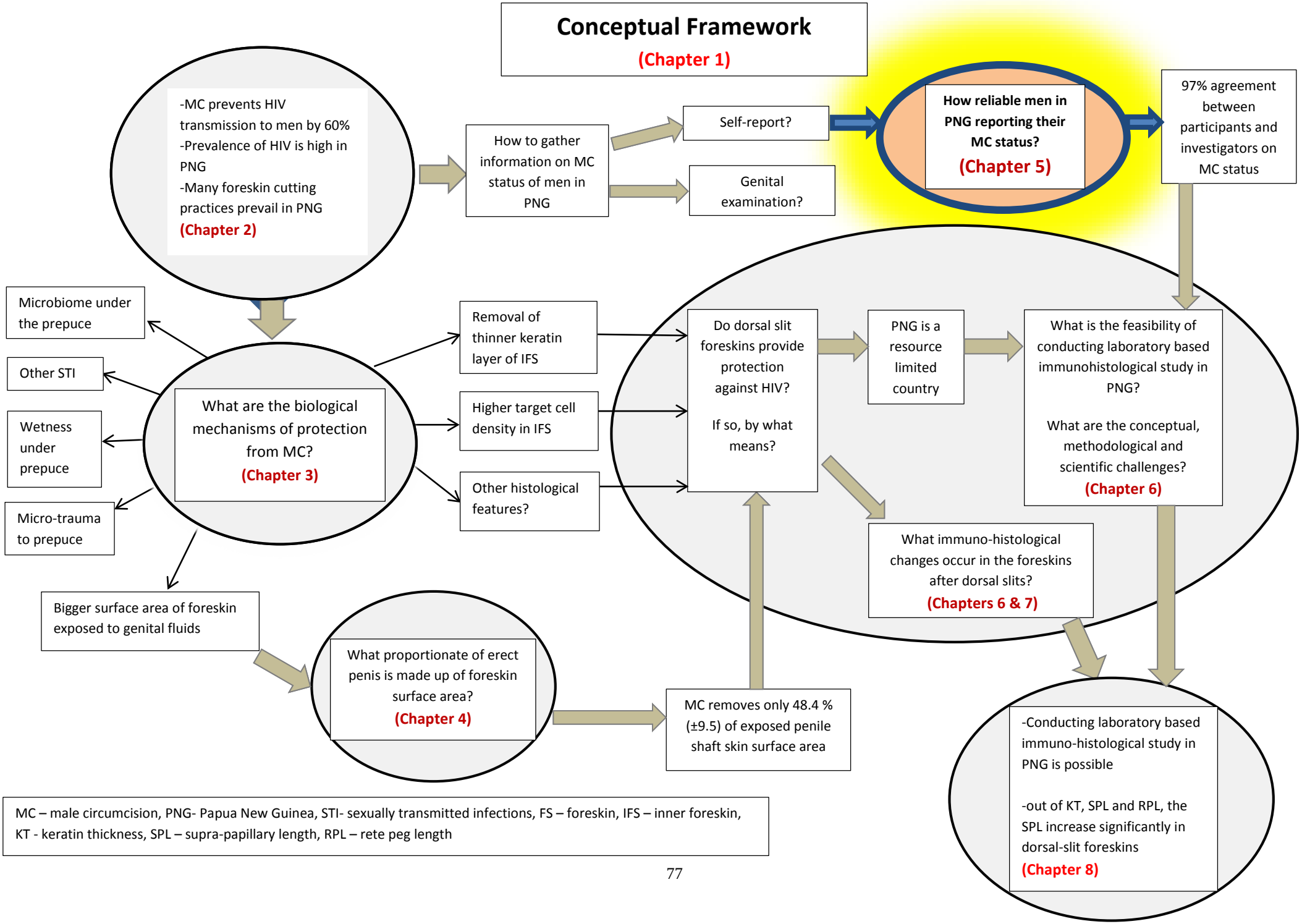
Despite the limited size of this study, the findings are valuable. The result of this study rationalises and prompts further scientific research to establish the efficacy of the other proposed mechanisms (other than removal of surface area) for the prevention provided by MC against HIV transmission. Well-planned RCTs are advisable to examine MC's other proposed mechanisms of protection.

4.6 Conclusion

The results of this study indicated that 47% of the total surface area of the erect penis is covered by the foreskin. This highlights that factors apart from surface area are responsible for the protection offered by MC against heterosexual HIV transmission. Further studies are recommended with wider participation to support these findings.

4.7 Research Communication and Dissemination

The findings of this study were disseminated via poster presentations during research week at JCU in Townsville and at the 2012 ASHM Conference in Melbourne. Further, the output of this research will lead to a publication in a peer-reviewed scientific journal.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 5: Genital Examinations and the Reliability of Self-reporting Circumcision Status among Men in PNG

5.1 Preface

The previous chapter demonstrated that surface area alone is not responsible for the total protection provided by MC. Given this finding, the hypothesis that dorsal slit foreskin cutting may be associated with protection against HIV transmission should be explored. In the design of larger ecological studies, a question arose relating to the accuracy of self-reporting circumcision status, and whether it was necessary to incorporate physical examination in studies that evaluate MC status for HIV seroconversion or prevalence. A research question arose as to how reliably PNG men were reporting their MC status. High reliability in self-reported MC status would lessen the necessity of having a separate genital examination to determine the MC status of men participating in future research.

VMMC programs are becoming increasingly important preventative measures for heterosexual HIV transmission, especially in countries with moderate to high HIV prevalence. However, the success of VMMC programs depends on accurate information of the MC status of the men in a particular geographical area. Since most MC data are based on self-reported MC status, the accuracy of self-reported MC status is an important factor to ensure the success of preventative programs, including VMMC.

VMMC programs were part of the proposals of the National AIDS Council of PNG to address the increasing number of HIV infections related to heterosexual HIV transmission. In preparation for these programs, it is important to assess the reliability of PNG's men in reporting their MC status. Thus, the outcomes of the current research will provide policymakers with scientific evidence to consider whether to include a separate genital examination as part of surveys ahead of any VMMC programs, or whether they can rely on self-reported MC status by men in PNG.

This study examined this question retrospectively using genital photographs taken and information gathered by clinical investigators during a broader study titled ‘Is Male Circumcision an Acceptable and Feasible Intervention to Reduce HIV Transmission in Papua New Guinea?’ (National Health and Medical Research Council project grant no. 60103). The results of the following study are significant and contribute a substantial piece of new knowledge to epidemiology in the field of HIV prevention in PNG.

This study first examines the available literature on the reliability of self-reporting of MC status in other countries, followed by a description of the methods used to evaluate the study participants’ genital photographs. Following this, this study compares the study participants’ photographs and investigators’ classification of these photographs in order to analyse the reliability of self-reporting using statistical methods. This study was published as a journal article in *PLOS ONE* journal.

Details of the publication on which the chapter is based	**Nature and extent of the intellectual input of each author, including the candidate**
Jayathunge PHM, McBride WJH, MacLaren D, Browne K. Men in Papua New Guinea accurately report their circumcision status. PLOS ONE. 2015; 10(4).	The authors co-developed the research question. PHM Jayathunge and K Browne collected the data and performed the analysis. PHM Jayathunge wrote the first draft of the paper, which was revised with editorial input from WJH McBride, D MacLaren and K Browne. PHM Jayathunge also developed the figures and tables.

5.2 Introduction

The HIV epidemic in PNG is the largest in the Oceania region. In 2012, the national adult HIV prevalence in PNG (age 15+) was estimated at 0.5% (0.4 to 0.7%), with around 22,000 (18,000 to 28,000) people living with HIV (40). The HIV epidemic in

PNG is primarily linked to heterosexual transmission, and exhibits substantial heterogeneity across the country (150). Innovative strategies for HIV prevention, treatment and care are needed to address this complex public health issue in PNG, where extreme geographical, linguistic and cultural diversity is present (2). MC was identified as a prioritised research area for preventing HIV in the 2008 to 2013 PNG National AIDS Council research agenda (2).

MC is defined as the surgical removal of all or part of the prepuce (foreskin) covering the glans penis (151). In PNG, multiple forms of circumcision and foreskin cutting have been described. Some forms involve circumferential cuts that result in complete removal of the foreskin, while others involve longitudinal cuts in which the foreskin is cut, yet not removed (87, 88, 90, 152, 153). The most common foreskin cut in PNG is the longitudinal cut, which most often involves a single dorsal slit without removing the prepuce, but exposing the inner aspect of the foreskin and the glans (100). In some places in PNG, longitudinal cuts are described as a 'V cut' because of the resultant appearance of the modified foreskin (85). Moreover, there are non-traditional types of penile modification, such as penile inserts (ball bearings, beads, plastics and so forth) into the skin of the penile shaft (86, 88, 90, 154). A multi-site study conducted in PNG in 2010 reported that 47% of men had some form of longitudinal foreskin incision and 10% had full circumcision (1). The 2006 PNG National HIV/AIDS BSS reported some form of foreskin cutting or medical circumcision among 26% of truck drivers, 45% of farm factory workers, 67% of military personnel and 70% of port workers (86). A BSS conducted in rural development enclaves in PNG between 2008 and 2010 reported that more than one-third of the men (34.3%) had longitudinal cuts of their foreskins (155).

VMMC is a component of comprehensive HIV prevention services in some countries with high HIV prevalence, following recommendations of the WHO in 2007 (156). Among the priority countries identified by WHO, Lesotho and Malawi were initially hesitant to scale up VMMC procedures because their own national survey results seemingly demonstrated higher HIV prevalence among those who reported being circumcised (156). MC data were mainly based on studies in which men self-reported MC status. Both countries performed relatively few VMMC procedures during 2008 to 2012 (2.8% and 1.7% of the WHO targets, respectively); however, this is now increasing (157, 158).

MC status in previous studies has been recorded by either: (i) self-reporting of MC status, (ii) genital examination by an expert/clinician, or (iii) a combination of both. Several studies that used a combination of self-reporting and genital examination in different settings have shown large discrepancies in the results between these two methods (156). For example, self-report was shown to be a valid measure of MC status in homosexual men in Australia (159), while data from five population-based studies in north-western Tanzania showed accuracy ($p < 0.001$) in self-reported data, although the researchers described some tendency for MC to be over-reported among those populations (160). In contrast, Lagarde et al. observed that 14% of self-reported MC statuses were discordant from clinical examination in their community-based cross-sectional study conducted in Westonaria district, South Africa (161). A 2002 study conducted on adolescent boys in Houston, Texas, demonstrated that self-reported MC status was discordant by 31% and 35% among circumcised and uncircumcised groups, respectively (162). The researchers highlighted that self-reported circumcision status was questionable because one-quarter (27%) of their participants did not know their circumcision status (162).

A study conducted during Lesotho Defence Force applicant screening in 2009 used a self-administered MC questionnaire, followed by a physician-performed genital examination (156). This study documented that 27% of men self-reported as being circumcised. Of these, physical examination showed that only half (50%) were fully circumcised, while about one-quarter (27%) had a partial circumcision and the remaining quarter (23%) were not circumcised. However, according to the researchers, the questionnaire did not allow participants to report 'partial' circumcision status—it only offered a 'yes/no' categorisation, which may have confused participants in reporting their circumcision status. The researchers recommended adding partial MC categories and graphics depicting forms of MC to improve the quality of the study (156). Studies conducted in Zambia and Swaziland in 2009 demonstrated improved self-reporting of MC status among illiterate participants when they were provided with an illustration of circumcised and uncircumcised status (misreporting reduced from 13% without an illustration to 10% with an illustration) (163). Similarly, Scholossberger et al.'s 1992 publication on 'Early Adolescent Knowledge and Attitudes about Circumcision' reported improved accuracy of self-reporting from 68%

to 92% when using visual aids to report circumcision status among adolescents in the US (164).

The study presented in this paper was conducted with the objective of assessing the reliability of self-reporting of MC status among men in PNG. Reliability of self-reported MC data is vital in planning and delivering health services and HIV prevention programs for the general public in PNG and other populations.

5.3 Methods

This study was undertaken as a sub-study of a large multi-site study titled ‘Is Male Circumcision an Acceptable and Feasible Intervention to Reduce HIV Transmission in Papua New Guinea?’ This was conducted in 2010 in four sites of PNG: Pacific Adventist University (PAU) (National Capital District), Porgera Joint Venture (Enga Province), Divine Word University (Madang Province) and Higaturu Oil Palms (Oro Province) (1, 28). These sites represent places of work or study for men from all over PNG. All participants were aged 18 or older, and provided informed written consent before participating in the study. The research was conducted in compliance with human research ethics approvals from the PNG National AIDS Council (approval no. RES10 0011) and JCU in Australia (approval no. H3757). Two methods were used to assess MC status in this study, as follows.

5.3.1 Questionnaire

The study participants were given a self-administered questionnaire that recorded data on demographics (age, education, province of origin, religious affiliation and employment); foreskin cutting status; and MC-related information, such as the method of foreskin cutting, logistics of foreskin cutting (setting and provider) and beliefs associated with foreskin cutting. The participants, some with limited literacy had a trained interviewer administer the questionnaire, some others who were fully illiterate requested to have an interviewer read them questions from the questionnaire, and then they verbally responded to each question.

The question that required participants to record their MC status employed a series of seven photographs of the most common foreskin cutting practices in PNG. The participants were requested to mark the most relevant photograph 'that most looks like your own foreskin'. There was an eighth option for 'other', with a request for participants to draw a picture. The full questionnaire, including the series of seven photographs can be accessed through:
http://www.biomedcentral.com/content/supplementary/1471-2458-13-818-s1.pdf.

5.3.2 Clinical Examination and Photographs

Following the questionnaire, the participants were invited to an optional clinical examination by a health professional. During these optional clinical examinations, the participants were asked if the clinician could record their circumcision status using digital photography. The photography was optional and not a condition of the clinical examination.

At the two university sites, the study participants were residents (students and staff) on the university campus. The participants completed the questionnaire in their homes or dormitories, and returned the completed questionnaires (in sealed envelopes). The invitation for optional clinical examination involved an extra step to travel to a nearby clinic or hospital at designated times.

At the two rural sites, the study participants were residents from the area who were visiting the local health centre (or a family member of someone visiting the health centre). The invitation to the optional clinical examination involved an immediate examination in the health centre, in which photography was requested, but not a condition of the examination. Clinical advice, treatment and/or referral were offered for any condition the participant presented with, not just sexual health conditions.

Genital photographs were taken from 266 (31%) of the 861 participants in the study. Prior to analysis, the photographs were assessed for clarity, and poor-quality photographs (n = 32) were discarded from the analysis. Similarly, any photographs that could not be matched with the participants due to unclear numbers (n = 5) were removed from the analysis. Accordingly, genital photographs from 229 participants

from four sites were included in the analysis. Given that clinical examination was a logistic challenge and not a requirement of participating in the study, nor was photographing the genitals a requirement of the clinical examination, the overall 31% of study participants participating in photographing their genitals did not indicate a 69% refusal rate.

The photographs were analysed separately by two investigators with clinical backgrounds. They classified the MC status of the photographs into eight categories, as shown in the questionnaire (the investigators were blinded to the participants' own classifications during the process). After the photographs were independently classified, the two investigators discussed the differences in photograph classification between them, and reached a consensus. To avoid complicated comparison across closely related categories, the investigators summarised the eight categories into three major categories of 'no cut' (Category 1), 'straight cut' (Categories 2 to 6 and 8) and 'round cut' (Category 7), before analysing the agreement level between the participants and investigators.

5.3.3 Data Analysis

All the data were coded and initially entered into Microsoft Excel and transferred to SPSS v. 20 statistical software, before analysis using SPSS. Agreement between the participants' reporting and investigators' classifications of MC status was analysed using Cohen's kappa statistical method. The confounding factors for accord and discord were analysed using binary logistic regression.

5.4 Results

Table 5.1 presents the demographic characteristics of the men in the study whose photographs were analysed. The median age of the men was 27 years (interquartile [IQ] range 22 to 33). The majority of photographs (93.5%) were from participants at the two rural sites (Porgera and Popondetta), with 64% from Popondetta. Almost 98% of participants identified as Christian and 54.3% were married. Primary school was the highest education for 58.6% of participants, while 24.9% had attended secondary or

high school. Manual/agricultural workers comprised 67.6% of participants, while 24.3% were employed in trade or technical work.

Table 5.1: Demographic Characteristic of Participants

Demographic Characteristics	Study Group	General Population
Age in years (median)	27 (IQ range 22–33)	NA
Site		
Divine Word University	6 (2.6%)	NA
PAU	9 (3.9%)	
Porgera	67 (29.3%)	
Popondetta	147 (64.2%)	
Marital status		
Single	96 (41.9%)	NA
Married and living together	125 (54.6%)	
Married, but not in union	8 (3.5%)	
Education		
Non/elementary/primary	133 (58.6%)	64%
Secondary/high	57 (24.9%)	12%
Vocational/tech/ college	26 (11.4%)	Not available
University	11 (4.8%)	< 1%
Employment		
Unemployed	6 (2.3%)	1.8%
Agriculture	156 (67.6%)	72.7%
Industry	56 (24.3%)	3.6%
Services	11 (4.6%)	22.7%

Note: NA = not applicable.

Of the 229 participants with both questionnaire data and genital photos, 19 (8%) reported that they had inserts or attachments, while 39 (17%) had injected some kind of substance into the penis. Seven were excluded from the analysis because the photographs showed such grossly dysmorphic features (after being injected with oils or potions) that it was difficult to classify the foreskin cutting status. Table 5.2 displays the data from the participants and investigators on the three-category foreskin cutting classification.

Table 5.2: MC Status Classification from Reports of the Participants and Investigators

MC Status	Participant N (%)	Investigators' Consensus N (%)
No foreskin cut	83 (37.4)	82 (36.5)
Straight cut	129 (58.1)	134 (60.8)
Round cut	10 (4.5)	6 (2.7)
Total	222 (100)	222 (100)

Table 5.3 displays the participants' self-assessment and investigators' assessment of photographs using the three-category classification. Agreement on MC status between participants and investigators (consensus) was present in 90.6% of all cases (kappa 0.805).

Table 5.3: Comparison of Participants' Self-assessment of MC Status and Investigators' (Consensus) Assessment of Photographs

Investigators / Participants	No Cut	Straight Cut	Round Cut
No cut	74 (33.3%)	6 (2.8%)	3 (1.4%)
Straight cut	5 (2.2%)	124 (55.9%)	0 (0%)
Round cut	3 (1.4%)	4 (1.8%)	3 (1.4%)

Of the 222 participants in the final analysis, 21 (9.4%) had a difference between self-classification and investigator (consensus) classification:

- eight of 222 (3.6%) self-classified as having a cut foreskin (five straight cut and three round cut), while investigators classified them as having no cut
- nine of 222 (4.1%) self-classified as having no cut, while the investigators classified them as having had a cut (six straight cut and three round cut)
- four of 222 (1.8%) self-classified as having a round cut, while the investigators classified them as having a straight cut (see Table 5.3).

A regression analysis of socioeconomic status/employment, education level and the potential confounding factor of age of the participants revealed that there was no significant association between these factors and accord/discord.

5.5 Discussion

This study provides the first evidence on the level of agreement for MC status between self-assessment and clinical assessment using photographs in PNG. The results demonstrate a high degree of agreement using a three-category classification, and suggest that self-assessment of MC status by men in PNG is highly reliable. High reliability in self-reported MC status has important implications for planning VMMC programs because data can be used to accurately estimate the volume of surgical intervention and resources required, and identify the individuals for whom the intervention is indicated.

Following further investigation, this study suggests several important factors that may explain the different responses from participants and investigators for two-thirds (13/21) of the discordant results (comprising 6.2% of the overall participants). Disagreements may be because:

1. investigators classified photographs as ‘round cut’, but participants reported ‘no cut’ in instances in which participants had naturally short foreskins or wore the foreskin retracted behind the glans penis (n = 3)
2. investigators classified photographs as ‘no cut’, but participants reported ‘straight cut’ in instances in which there were such small cuts that it caused minimal change in the morphology of the foreskin (n = 5)
3. investigators classified photographs as ‘straight cut’, but participants reported ‘round cut’ in instances in which remnant foreskin was obvious in the photograph, but the glans may have resembled the ‘round cut’ photograph in the questionnaire (n = 4)
4. investigators classified a photograph as a ‘straight cut’, but the participant reported ‘no cut’ in an instance in which the participant may have had his foreskin retracted (n = 1).

For the remaining eight of 222 of discordant classifications (comprising 3% of the overall participants), this study can offer no explanation. In these discordant cases:

- five participants self-reported ‘no cut’ when there was clear evidence from the photograph of a scar and/or foreskin remnant behind the glans
- three participants self-reported ‘total removal of foreskin’ when there was clear evidence from the photograph that the glans penis was completely covered with foreskin.

Therefore, overall, this study demonstrated direct agreement in 91% of cases and a plausible explanation for a further 6%. This left only 3% discordance between the self-assessment and clinical assessment of the photographs. This result of 97% agreement between self-assessment and investigator assessment is in direct contrast to studies conducted in Africa, where agreement can be as low as 50% (156). The researchers believe that the high level of agreement between self-reports and investigator recordings in this study could be due to the higher awareness among PNG men of MC and foreskin cutting practices. It is possible that men in PNG discuss MC and foreskin cutting because these practices have a long cultural tradition in some regions. This may be due to the awareness programs in PNG on HIV prevention by various health programs. However, further research is needed to support these hypotheses.

This study also analysed the level of agreement of self-reporting with the individual investigators' classifications across the eight different types of foreskin cuts. The level of agreement with anything other than a three-category classification (no cut, straight cut or round cut) remained low (data not displayed). The researchers believe that this could be explained by the complexity of the eight-level classification system, where each category contained only subtle differences. Classification levels two to six were all sub-variations of the longitudinal 'straight cut'. Classifying each participant required a subjective decision based on appearance and interpretation of the participant's penis and the photographs in the questionnaire. When classification levels two to six were grouped into the broader category of 'straight cut', the level of agreement between self-assessment and investigator assessment dramatically improved, thereby indicating the three-level system to be a more practical method to assess MC in this population.

This study made some important observations on MC status reporting. At the time of the initial photograph classification by investigators, there were variable assessments of photographs from men who permanently wore the foreskin retracted behind the glans penis, which is not uncommon in some parts of PNG. In such cases, final classification was agreed to via consensus between the investigators. This study also noted that the participants reported slightly more 'no cut' and 'round cut' than did the investigators. Social desirability bias could be considered a probable explanation for this discrepancy, although, with such small numbers, it may simply be due to random errors and/or variable literacy levels. Further, there is great diversity in social, cultural and spiritual practices across PNG's 800 distinctly different language groups. Celebrating conformity within these groups and highlighting differences between these groups is an enduring feature of life in PNG. Therefore, this may explain a context in which men are confident to report their own MC status, even if it is different to other men who participated in the study (who may reside at the study site, but originate from any one of the other 800 language groups).

MC and other associated sexual practices are informed by local cultural practices, with MC/foreskin cutting a historically important initiation process to adulthood in some cultural groups (90). Local culture also plays a role in determining the type of foreskin cutting, as different cultural groups practice different forms of foreskin cutting modes

(165). Apart from foreskin cutting, injections and inserts to the foreskin and penile shaft were also common among this population. In such cases, the penis appeared to be scarred and hardened, and sometimes formed a sclerosing lipogranuloma—a fibrous tissue development due to mineral oil injection (165). Some participants reported both a longitudinal foreskin cut and injecting substances into the remnant foreskin. Photographs from such participants were so difficult to classify because of their dysmorphic appearance that they were excluded from this study; however, there is no reason to believe that these exclusions affected the overall results of this study.

Due to PNG's great diversity of social, cultural and spiritual practices, with associated MC and sexual practices, the results of this study cannot be generalised across the entire PNG population. However, they do provide strong evidence that there is a high level of agreement between MC self-reporting and clinician assessment of photographs in PNG. It further demonstrates that this method is feasible in PNG; however, anything beyond a three-level MC category may prove to be impractical to assess levels of agreement. The results of this study emphasise that the accuracy of self-reporting of MC status could vary across different countries and different populations, and highlight the need for location-specific studies that reflect culturally specific practices.

One of the novelties of this study was the analysis of photographs of participants to measure agreement between self-reporting and investigator classification. An advantage was that the investigator could analyse the photographs at any time and seek a second opinion from another investigator to reach a consensus. In addition, this study was also able to use real photographs of the most common foreskin cutting practices in PNG in the questionnaire, instead of descriptions using words, sketches or diagrams. Having a large number of photographs for the analysis was another strength of this study that provided freedom for investigators to exclude unclear photographs, while still having sufficient numbers for statistical analysis. Several photographs taken of the penis from different angles provided additional information and further facilitated assessment of MC status.

A disadvantage of this study was that the photographs could only offer a two-dimensional image of the penis, and the investigator was unable to perform a clinical

examination of the actual participants to assess MC status. A further limitation was that the recruitment of participants occurred from only four sites, and the results are not generalisable, although the sites were chosen because of migration from across PNG, which serves to improve the applicability and transferability of results.

Given that the WHO has recommended that nations with high or moderate HIV prevalence implement VMMC programs, the need for reliable MC prevalence data has become critical (156). Studies conducted in different parts of the world have demonstrated different levels of agreement between self-reported MC status and investigator-assessed MC status. The results of the current study show that there is a high level of agreement between self-assessment and investigator assessment in PNG, which suggests that self-reporting of MC status is highly reliable among men in PNG.

5.6 Summary

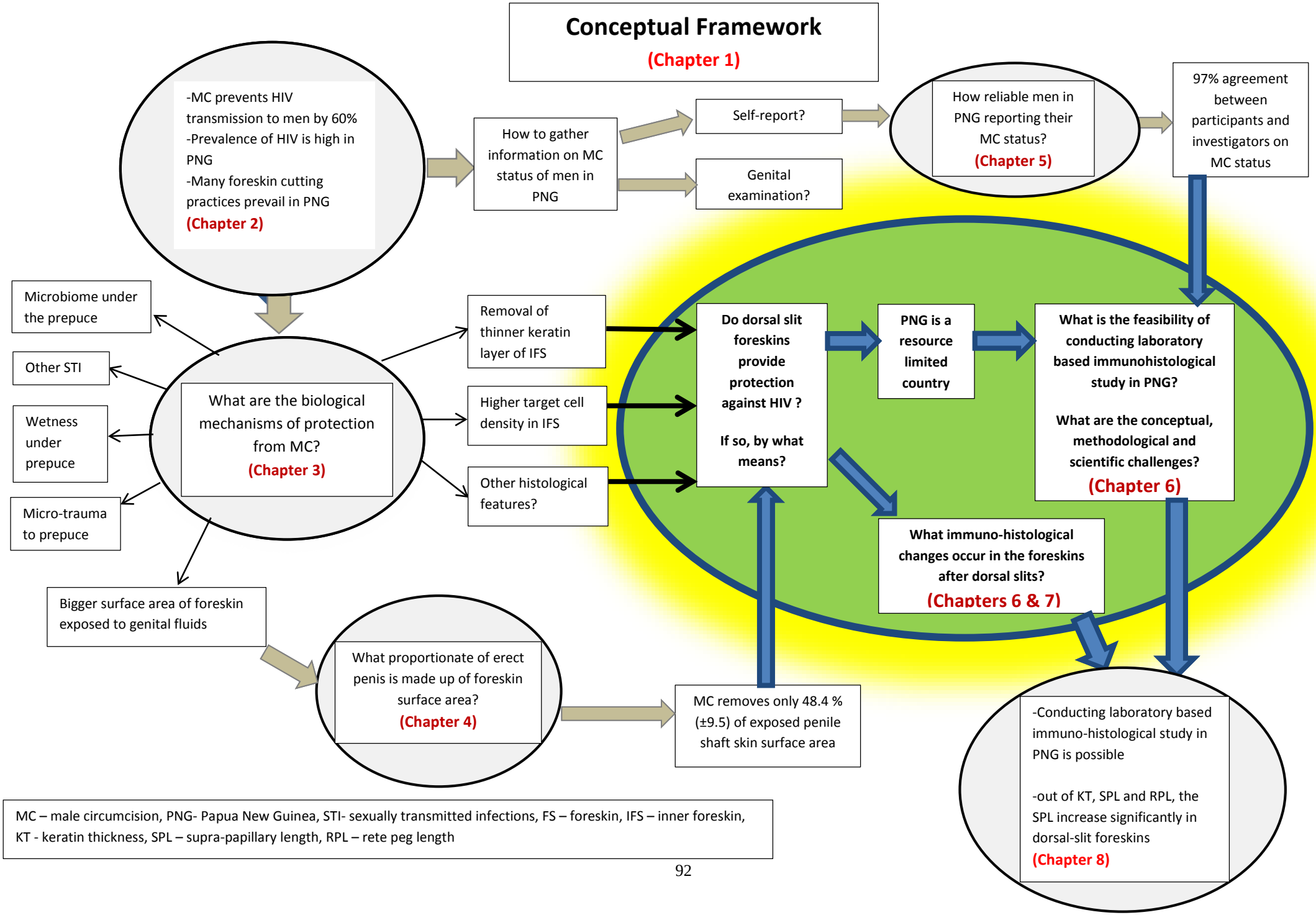
MC is a well-established component of HIV prevention in countries with high HIV prevalence and heterosexually-driven epidemics. However, delivery and monitoring of MC programs are reliant on good quality MC data. Such data are often generated through self-reported MC status surveys. Thus, this study examined self-reported MC status in comparison with genital photographs from men in PNG.

This retrospective, non-interventional study collated self-reported MC status data from the ‘acceptability and feasibility of MC’ study at four sites in PNG during 2010 to 2011. The participants reported their MC status based on an eight-category photographic classification, covering the range of foreskin cutting practices in PNG. Genital photographs of 222 participants from this study were independently classified by two investigators. The eight-category photographic classification was simplified into a three-category classification of ‘no cut’, ‘straight cut’ and ‘round cut’ before comparing for agreement between the self-reporting and investigator assessment using Cohen’s kappa measure.

Using the three-category classification, there was 90.6% (201/222) agreement between the self-assessment and investigator classification (κ value 0.805). Of the discordant 9.4% (21/222):

- 3.6% (8/222) self-classified as having a cut foreskin (five straight cut and three round cut), while the investigators classified them as having no cut
- 4.1% (9/222) self-classified as having no cut, while the investigators classified them as having had a cut (six straight cut and three round cut)
- 1.8% (4/222) self-classified as having a round cut, while the investigators classified them as having a straight cut.

Given the great variety of foreskin cutting practices and appearances, feasible explanations were suggested for two-thirds (13/21) of these discordant results. Thus, overall, this study demonstrated a high level of agreement between self-reporting and investigator assessment of MC status in PNG, which suggests that self-reporting of MC status is highly reliable among men in PNG.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 6: ‘Straight Cuts’ and HIV Prevention—The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings

Part 1: Methodology

6.1 Preface

This chapter presents Phase 1 of the larger study undertaken. It examines the conceptual, methodological and scientific challenges (and opportunities) in immunohistological research in resource-constrained PNG. The ‘“Straight Cuts” and HIV Prevention: The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings’ study was planned to fill the gap in knowledge to address an important health issue in PNG. The final objective of the overall project was to test the hypothesis that dorsal slit foreskin cutting of men in PNG could possess protective efficacy against sexual transmission of HIV. This objective was to be achieved in two phases. Phase 1 was this PhD study, which was based on a number of objectives that led to the main objectives of Phase 2 of the study, and the overall project.

Phase 1 of this study described in this chapter was intended to investigate the conceptual, methodological and scientific challenges of conducting a study on immunohistological changes of the foreskin (that may be related to protection against HIV transmission) after a ‘dorsal slit’ foreskin cut (dorsal slit foreskin cutting is the most common form of foreskin modification observed in PNG (1)). Thus, the overall objective of this study was to assess the feasibility of conducting such a complex study in resource-limited PNG.

The two phases of the original project emphasised different objectives, as follows:

- Phase 1: a feasibility study with emphasis on conducting a small study and assessing challenges and solutions for Phase 2
- Phase 2: the main study, building on the lessons learnt in Phase 1, with practical and logistical solutions that emphasise the scientific objectives.

A detailed background and literature review for this study is provided in Chapter 2. The current chapter describes the methods, results and discussion for Phase 1 of the study.

6.2 Methods

6.2.1 Study Design

This prospective pilot clinical study (Phase 1) was conducted from September 2012 to December 2013 in parallel with a VMMC service provided at the PAU, Kioari Park campus, POM, PNG. The VMMC service started at the PAU in 2010, when it was recognised by the university that there was a need for such a service in order to prevent unsafe foreskin cutting practices that were occurring informally among the male university students.

6.2.1.1 Research Hypotheses

This study was based on the following research hypotheses:

1. the keratin layer is thicker in the OFS than in the IFS of Melanesian men
2. the keratin layer thickness of the IFS (and glans) increases on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting
3. the RPL increases in the IFS on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting
4. the SPL increases in the IFS on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting
5. the target cell density of the IFS and OFS changes after a period has elapsed after dorsal slit foreskin cutting
6. an immunohistological study to test the above hypotheses can be conducted successfully in PNG.

6.2.1.2 Research Questions

This study was based on the following research questions:

1. Is the keratin layer of the OFS thicker than the keratin layer of the IFS in Melanesian men?
2. What are the histological changes that occur in the foreskin after a dorsal slit foreskin cut?
    a. Does the keratin layer thickness increase in the IFS on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting?
    b. Do RPLs increase in the IFS on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting?
    c. Do SPLs increase in the IFS on exposure to the outside after a period has elapsed after dorsal slit foreskin cutting?
3. What is the distribution of the target cells (CD4+ and LCs) in the IFS and OFS of healthy men with and without dorsal slit foreskin cuts? Does the target cell density of the IFS and OFS change after a period has elapsed after dorsal slit foreskin cutting?
4. What are the conceptual, methodological and scientific challenges of conducting a biomedical study involving clinico-laboratory components for immunohistological analysis in PNG?
5. What measures need to be taken to overcome the discovered logistical challenges to successfully complete such a research study?

6.2.1.3 Research Objectives

The two main objectives for this study were as follows:

1. scientific objectives—to determine the:
    a. histological changes (KT, RPL and SPL) in the epidermis of the foreskin in men who had undergone dorsal slit foreskin cutting
    b. changes in the target cell density of the foreskin in men who had undergone dorsal slit foreskin cutting
2. non-scientific objectives—to assess the challenges of and find solutions to conducting a laboratory-based clinical study in resource-limited PNG.

6.2.2 Methods Used to Address the Scientific Objectives (1a and b)

6.2.2.1 Study Preparation: Preliminary Visit and Study Site

A preliminary visit was undertaken in August 2011 to identify the logistical issues and state of resources at the study site and local laboratory. Discussions were held with other research partners (a surgeon and histopathologist at the POM General Hospital [POMGH] and vice chancellor of the PAU) during a visit to discuss the importance, logistics and conduct of the research project. Discussions were held with the senior surgeon of the POMGH, which focused on discussing his needs in order to conduct MCs, and with the chief pathologist at the same hospital for permission to use the histopathology laboratory and -80°C freezer to process and store (paraffin and cryopreserved) tissue samples.

Following the preliminary visit, the study was organised in parallel with a planned MC service being provided on campus for students enrolled at the PAU, National Capital District, PNG. Tentatively, the dates were fixed for the study along with the MC clinic during the semester break from 12 to 15 June 2012. The histopathology laboratory of the POMGH—17 kilometres from the PAU campus—was to be the facility for tissue processing.

6.2.2.2 Ethics Clearance for the Study

Ethics approval from the JCU (H4424), PAU, Medical Research Advisory Committee (MRAC 12.26) and National AIDS Council Secretariat (RES 11-031) was obtained for Phase 1 of the study (Appendix 7). Due to delays in the ethics approval process, the study was postponed from the scheduled date in June 2012 to September 2012. Biosafety approval for the project was obtained from the JCU, Australia. Further, accreditation for the PAU clinic to conduct circumcisions, as advised by the surgeon, was obtained by PAU researchers from the Medical Board of PNG.

6.2.2.3 Implementation of Phase 1 of the Study at PAU

Eight weeks prior to the study commencing, all male students at the PAU were informed of the circumcision clinic and the study via an email and flyer (Annex 1). A

total of 38 students registered for the MC clinic with a (male) local investigator two weeks prior to the start of the clinic. Once all the ethics approvals were received one week prior to the scheduled date of the MC clinic, another email was sent to all male students at the PAU to inform them of the opportunity to participate in the MC clinic and the study.

A meeting with students and student leaders was held three days prior to the MC clinic at the PAU. This meeting provided general information on the MC clinic, details of the circumcision procedure (Annex 2) and basic information regarding the study to educate the participants on what to expect during the clinic and study. Participant information sheets were distributed for the students to read at their leisure before giving informed consent. Participation in the study was voluntary and the participants had the opportunity to withdraw their participation at any stage without giving reasons. Following the meeting, 32 males expressed interest in participating in the MC study concurrently with participating in the MC clinic.

All the study equipment was transported to the site five days prior to the study, including the optimum cutting temperature (OCT) compound media, moulds for cryosections, sample containers, paraffin tissue moulds and stationery. All necessary Case Report Form (CRF) s (Annex 3) prepared according to protocol were transported to the study site on the same day. Permission to use the POMGH histology laboratory was obtained from the chief executive officer of the POMGH (Annex 4) three days prior to the study. Immediately prior to the start of the study, the chief executive officer, surgeon, histopathologist and laboratory staff of the POMGH were again visited and informed of the study steps, time periods and resource usage at the hospital for the tissue processing.

6.2.2.4 Eligibility Criteria

Men aged 18 years or over at the time of enrolment were eligible to participate in the study. They could either be uncircumcised or have received a dorsal slit foreskin cutting a minimum of six months prior to the time of recruitment. The participants were also required to have the ability to understand the objectives and potential benefits and inconveniences associated with study participation. Similarly, the

participants were required to complete the study's informed consent procedures. The participants' willingness to undergo a clinical examination and VCT for HIV and to provide foreskin samples for immunohistological analysis was also essential, and included as eligibility criteria.

6.2.2.5 Exclusion Criteria

Subjects with clinical signs suggestive of an STI (such as dysuria or urethral discharge) and/or genital symptoms at enrolment, or those diagnosed to have an STI/s were excluded from the study (according to the protocol, such participants were to be directed to relevant medical authorities). Further, if the research staff decided for any reason that it was not be in the participant's best interest to participate in the study (for example, a person who appeared agitated or uneasy in the initial interview), they were also excluded. Declining genital examination or VCT upon screening was another criterion to disqualify an individual from participating in the study.

6.2.2.6 Screening Visit for the Study Participants

Given that the study was conducted alongside the MC clinic organised by the PAU, measures were taken to avoid interference with the conduct of the MC clinic during the study activities. The study investigators worked closely alongside the surgeon, nursing staff and other clinic staff engaged in the MC clinic activities to complete the study steps.

Prospective participants for the study attended screening prior to the MC clinic. A short interview was conducted during the screening to collect demographic, clinical and sexual behaviour data (Annex 5) and check eligibility. Questions about sexual history and genital symptoms were included to assess the current STI status of the participants. Informed consent was obtained for genital examination, testing for HIV and collection of foreskins for analysis of the immunohistological aspects. All participants were capable of understanding the content of the information sheet and demography forms, and had the opportunity to ask questions.

Once all forms were filled, the study participants underwent VCT for HIV as part of the study. Participants who consented for VCT were also asked to provide a venepuncture specimen for onsite rapid HIV testing using the Determine (Abbott, Inverness) simple rapid assay. The confirmatory testing was to be undertaken at the Department of Pathology, POMGH, according to the standard national HIV test algorithms. Currently, confirmatory testing by Serodia (Fujirebio, Japan), Determine (Abbott, Inverness) and Immunocomb (Orgenics, Israel) is performed if the screening test is positive.

6.2.2.7 Circumcision Clinic and the First Steps of the Study

6.2.2.7.1 MC Clinic of the PAU

Informed consent for the surgical procedure (circumcision) was obtained separately from all participants in the MC clinic by clinical staff immediately prior to the operations. Those who consented were prepared for the operation according to their registration sequence in the clinic. All circumcision operations were performed under local anaesthesia by administering a ring block at the root of the penis. A dorsal slit method was used to perform the MCs, and the bleeding during circumcisions was arrested using surgical ligations. Male nursing students from the School of Nursing at the PAU assisted the surgeon (Dr Limo from the Department of Health, PNG) during the operations (see Figure 6.1). Once the operation was finished, the patients were observed for three hours in an adjacent observation room for any operation-related complications. The patients were discharged from the clinic with prophylactic antibiotics once the surgeon confirmed that they were free of complications.

Figure 6.1: A Participant Undergoing a Circumcision

6.2.2.7.2 Study Steps in Parallel to the Clinic

Among the participants for the clinic, those who consented for the study and genital examination received a genital examination performed by the principle investigator for clinically obvious STIs before the circumcision procedure began (while the surgeon was preparing for the operation). The surgeon was informed about the study requirements prior to beginning the operations, especially regarding minimal tissue handling and tissue orientation when the foreskin was removed. The surgeon was also provided with a colour permanent marker pen to mark the outer surface of the foreskin in order to aid tissue orientation during tissue preparations.

6.2.2.7.3 Surgical Specimen Collection and Tissue Preparation

Once the foreskin tissue was removed from the body, the orientation of the tissue sample was confirmed with the surgeon and marked with a tiny metal pin at the edge of the IFS, before it was cut into strips measuring 0.5 cm × 2 cm. Two samples from the dorsal side for paraffin and frozen sections were prepared from the foreskins of participants who had had previous dorsal slits and were previously uncircumcised. The IFS and OFS were separated so that each tissue section had the IFS and OFS lying towards each end of the sample (Figure 6.2). The tissue pieces were immersed in 10% neutral buffered formalin (NBF) for paraffin sections and in normal saline for frozen sections before being transported to the laboratory.

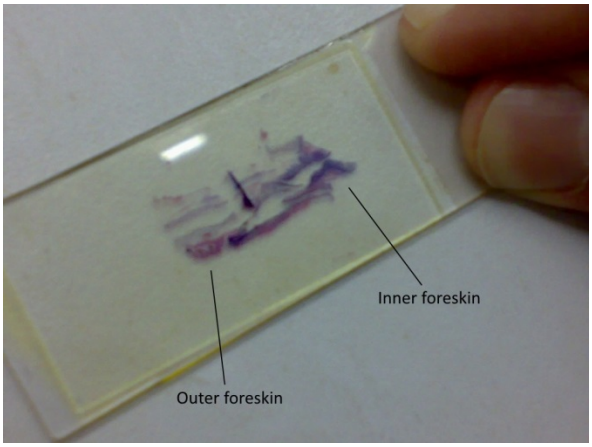
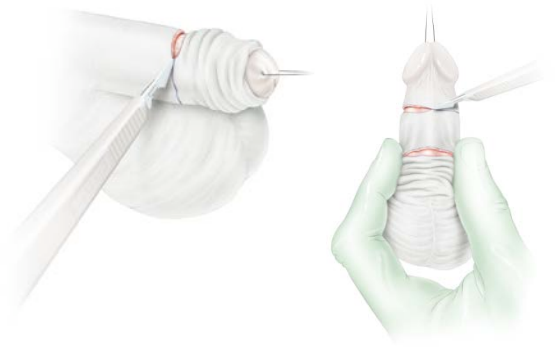


Figure 6.2: Tissue Orientation on Glass Slide

6.2.2.7.4 Foreskin Tissue Separation

In an uncircumcised male, the relaxed state of the penis allows for the formation of an inner and outer aspect of the foreskin (see Figure 6.3). MC in adults is usually performed with two circumferential cuts in the foreskin in the relaxed state (Figure 6.3a) and retracted state (Figure 6.3b).



(a) (b)

Figure 6.3: Adult MC (Reproduced from Elder JS. *BJU International.* 2007. 99 (6): 1553-1564)(166)

The foreskin is marked at the base in the relaxed state, and circumferentially incised. It is then retracted and circumferentially incised again distally, and removed from the penis by dissecting away the underlying fascia (166). With an indelible marker pen, the surgeon marked the outer aspect of the foreskin (the external surface) prior to removal (as mentioned above). If this was not feasible, the outer aspect of the foreskin could also be distinguished from the inner aspect after removal via three gross characteristics, as outlined in Table 6.1.

Table 6.1: Gross Characteristics of the Adult Male Foreskin

Characteristic	IFS	OFS
Colour/pigmentation	Lighter	Darker
Rugae	Smoother, less rugae	Rougher, more rugae
Area	Less (1/2–1/3 less than OFS)	Greater (most of the tissue)
	There may also be a faint fold to help distinguish the transition between the OFS and IFS, though this is not as reliable due to a natural fold located on the ventral surface or underside of the foreskin (overlying the urethra).	

Fresh tissues destined for frozen samples were immersed immediately in sterile specimen containers labelled with the participant's study identification numbers. These tissue samples were temporarily stored in the freezer compartment of the refrigerator at the clinic, until being transported on ice to the POMGH for processing at the end of the two clinics over two days.

6.2.2.7.5 Sample Processing

Tissues were prepared in two batches as frozen and paraffin sections at the POMGH laboratory. Several steps of frozen tissue processing took place at the University of New South Wales (UNSW), Sydney, Australia (as shown in Figures 6.4 and 6.5) due to limited facilities at the POMGH.

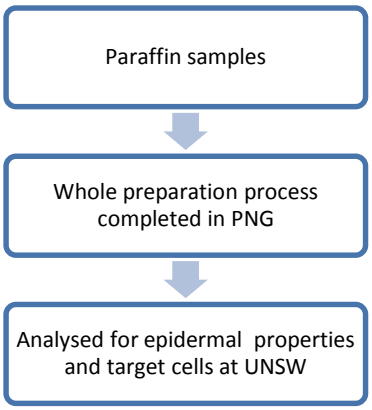


Figure 6.4: Preparation of Paraffin Samples

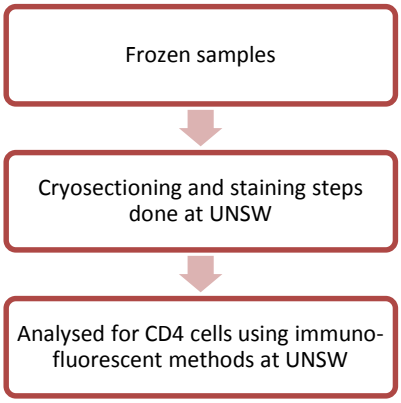


Figure 6.5: Preparation of Frozen Samples

6.2.2.7.6 Paraffin Sample Processing

Paraffin tissue processing involved five main stages:

1. fixation—to stabilise and harden tissues with minimal distortion of cells
2. dehydration—to remove water and fixatives from the tissue
3. clearing—to remove dehydrating solutions, making the tissue components receptive to the infiltrating medium
4. infiltrating—to permeate the tissue with a support medium
5. embedding—to orientate the tissue sample in a support medium and allow it to solidify.

Figure 6.6: Paraffin Tissue Preparation Equipment

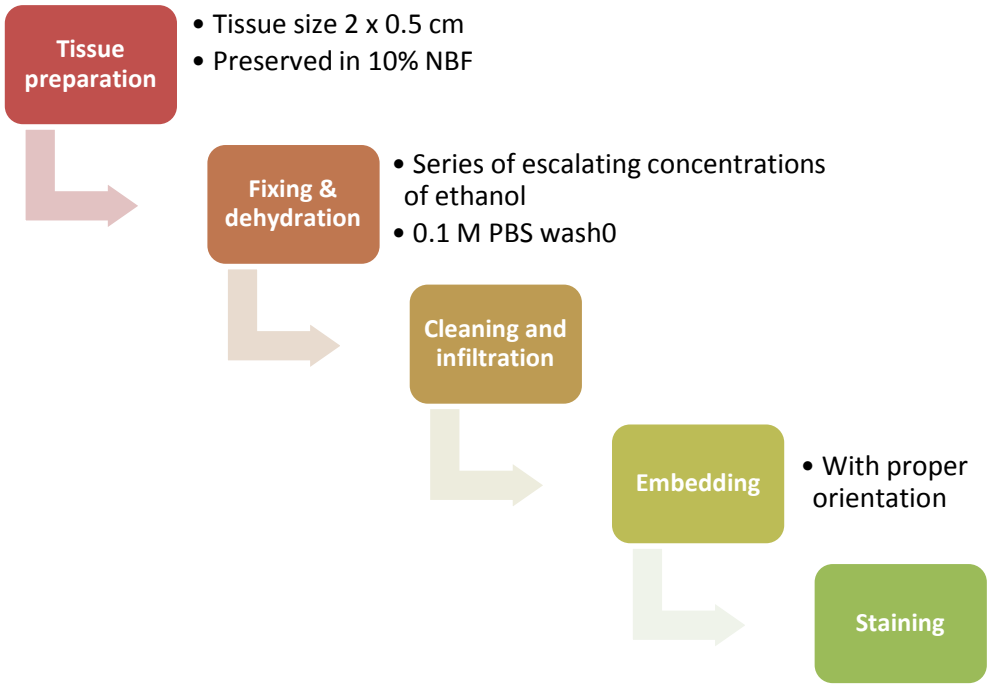


Figure 6.7: Summary of Paraffin Tissue Preparation Process

Once the tissues were removed from the body, they were preserved in 10% NBF. Subsequently, the tissue samples were fixed at room temperature for six hours inside histology cassettes (small sealable containers) using 70% ethanol (the cassettes were labelled before being placed in the container filled with ethanol). This process was completed using an automated fixing machine (LEICA ASP 200, see Figure 6.8) at the histopathology laboratory at the POMGH, which performs dehydration, clearing and infiltration with paraffin using its pre-set program (fixed samples were washed with 0.1 M phosphate buffered solution [PBS] several times in the clearing step process) (see Table 6.2).

Table 6.2: Overnight Tissue-processing Schedule

Station	Reagents	Time	Temp.
1	10% formalin	1 h	38°C
2	10% formalin	1 h	38°C
3	50% alcohol/formalin	1 h	38°C
4	70% alcohol	1 h	38°C
5	95% alcohol	1 h	38°C
6	95% alcohol	40 min	38°C
7	100% alcohol	1 h	38°C
8	100% alcohol	40 min	38°C
9	Xylene	1 h	38°C
10	Xylene	30 min	38°C
11	Paraffin	30 min	60°C
12	Paraffin	30 min	60°C
13	Paraffin	30 min	60°C
14	Paraffin	30 min	60°C

Figure 6.8: Automated Tissue Fixing Machine

The tissues were subsequently embedded in paraffin blocks and stored in a cool, dry place until sectioned.

6.2.2.7.7 Microtomy of Paraffin Section

The paraffin-embedded samples were sectioned using the microtome (Figure 6.10) into 3 to 5 µm sections at the histopathology laboratory of the POMGH by the principal researcher and laboratory assistant, before being stained with H&E. The following tools were used for microtomy:

- flotation (water) bath
- slide drying oven
- fine-pointed and curved forceps
- sable brush
- scalpel
- slide rack
- clean slides
- teasing needle
- ice tray
- chemical-resistant pencil.

6.2.2.7.8 Staining of Paraffin Sections

The tissues were stained with H&E using an automated machine at the histopathology laboratory at the POMGH (Figure 6.9). The samples were later transported to the histopathology laboratory at the Cairns Base Hospital for quality assurance.

Figure 6.9: Sample Staining Machine

Figure 6.10: Microtome

6.2.2.7.9 Frozen Sample Processing

Figure 6.11 demonstrates a summary of the steps taken during frozen tissue preparation.

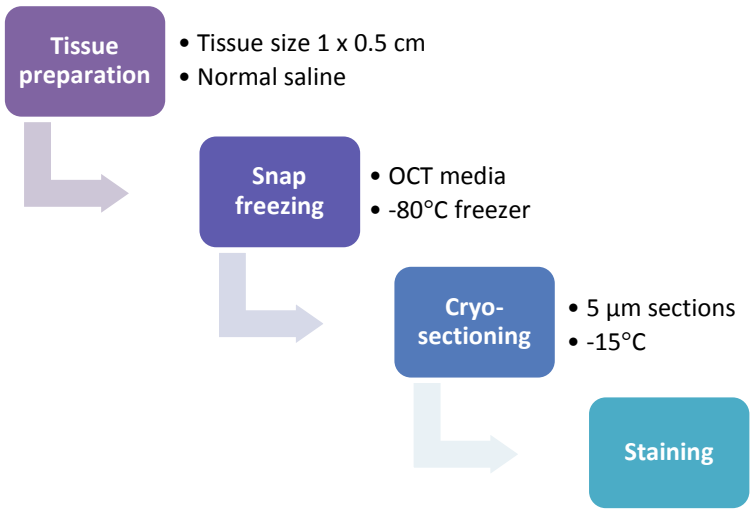


Figure 6.11: Summary of the Process of Frozen Tissue Preparation

6.2.2.7.10 Freezing of Fresh Unfixed Tissue

The frozen samples were prepared using an OCT embedding compound (SAKURA) and plastic tissue moulds (Fisher) held in the -80°C freezer (Figure 6.12). Once the moulds were labelled with tissue identification, they were filled partly with the OCT compound. The moulds were then kept inside the -80°C freezer for a few seconds until the OCT media turned into a semisolid state. The foreskin tissues were dabbed onto a towel to remove any fluid and blood, and then immediately placed in a tissue mould

with OCT in the best orientation for the tissue to be frozen and settle to the bottom. More OCT media was added on top of the tissue to cover it completely and fill the mould before putting it back into the freezer. Once the OCT media with tissue sample was completely frozen, the samples were wrapped in foil, labelled and stored immediately back in the -80°C freezer.

6.2.2.7.11 Transportation of Samples

The frozen samples were kept stored in a -80°C refrigerator until dry ice was transported to POM to maintain a cold chain for frozen sample transport. In June 2013, dry ice was shipped from Cairns and samples were transported to the UNSW, Sydney, maintaining a cold chain for processing (cryosectioning, fixation and staining) and detailed immunohistochemical and histological analysis.

The immunohistochemical analysis was conducted at the UNSW, where the special microscope—the Aperio image analyser—was located. The analysis involved complex technology, and needed to be completed under the supervision of experts in immunohistological methods at the UNSW. The H&E-stained tissue slides made at the POMGH laboratory were transported separately to the UNSW for further analysis, as described above.

Quarantine clearance for the sample transport was obtained from both the quarantine office at the PNG custom service, and from the Australian Quarantine and Inspection Services (AQIS) prior to transporting the samples. All human tissue samples were collected, transported, handled and disposed according to the internationally accepted *Human Tissue Act 2004*.

6.2.2.7.12 Cryosectioning and Fixation at UNSW

Once the frozen sections arrived at the immunohistology laboratory at the UNSW, Sydney, in June 2013, they were stored in a -80°C freezer. The sections were then cut by a cryostat between 5 to 10 µm at -15 to 23°C, and the tissues were mounted on gelatine-coated histological slides (LabScientific Inc.) by the histology core at the laboratory. First, the sections were air dried for 30 minutes at room temperature to

prevent them from falling off the slides during antibody incubations. Then, 50 µL of ice-cold fixation buffer was added immediately to each tissue section upon removal from the freezer, followed by fixing of tissues for eight minutes at -20°C for 20 minutes.

Figure 6.12: -80°C Freezer at the POMGH

6.2.2.7.13 Fluorescent Staining of Cryostat Sections

The laboratory protocol of the UNSW immunohistopathology laboratory for fluorescent staining was followed (Annex 6) to stain the frozen tissues. Cryostat sections stored in the freezer were thawed at room temperature for 10 to 20 minutes. The slides were then rehydrated in wash buffer for 10 minutes, and the excess wash buffer was drained. After surrounding the tissue with a hydrophobic barrier using a barrier pen, non-specific staining was blocked between the primary antibodies and tissue by incubating in a blocking buffer (1% horse serum in PBS) for 30 minutes at room temperature. Primary antibodies diluted in incubation buffer were applied according to the manufacturer's instructions. The slides were then washed three times for 15 minutes each in a wash buffer, and incubated with the Northern Lights secondary antibody diluted in incubation buffer. Once the slides were washed again three times for 15 minutes each in a wash buffer, 300 µL of diluted 4',6-diamidino-2-phenylindole (DAPI) solution was added to each well, and incubated for two to five minutes at room temperature (DAPI binds to DNA and is a convenient nuclear counter

stain). Finally, the slides were mounted with an anti-fade mounting media and visualised using a fluorescence microscope.

6.2.2.8 Image Analysis

Image analysis was conducted at the Histology and Microscopy Unit in the histopathology laboratory of the Faculty of Medicine at the UNSW, Sydney. Once the slides for scanning were delivered to the Histology and Microscopy Unit (HMU) in labelled boxes with all the necessary information, each slide was provided with a HMU reference number. The slides were then scanned with an Aperio ScanScope XT Slide Scanner (Figure 6.13)—a microscope that is able to construct a complete high-resolution electronic image of glass histology slides, and permanently archives the images one by one. The time taken for analysis of a slide was based on the complexity of the tissue. The average time for scanning of one slide of this set of samples was around 30 minutes. Once the slides were scanned, a digital version of the slides could be accessed online and the physical slides could be collected.

Figure 6.13: Aperio ScanScope XT Slide Scanner (Reproduced from http://www.leicabiosystems.com/digital-pathology/aperio-digital-pathology-slide-scanners/)

The high-resolution, internet-accessible digital slides were analysed using ImageScope software, which was downloaded from the manufacturer's website. Digital images were password protected and could only be accessed by logging in with a username and password.

6.2.2.9 Clearing of Paraffin and Frozen Tissue Samples for Analysis

Prior to measuring the histological features, the quality of samples was checked using the following parameters:

1. tissue orientation confirmation (for example, an issue could arise from neutral orientations in which the direction of the tissue was difficult to determine)
2. tissue artefacts (such as vacuolisation in frozen sections)
3. defects in staining (such as diffuse staining of all tissue structures or overstraining)
4. shattered, cracked or folded sections where measurements could not be taken.

Any tissue sample with any of the above defects was removed from analysis.

6.2.2.10 Measuring KT, SPLs and RPLs

High-resolution digital slides (H&E) were taken on a wide monitor to analyse using tools in the ImageScope software. Ten 300 µm squares from both the IFS and OFS were randomly selected to measure the KT, SPL and RPL (see Figure 6.14). However, any convoluted, damaged or difficult to measure areas were excluded, and new squares were randomly selected. While the measurements were obtained under the maximum achievable magnification, as shown in Figure 6.15, the finer details were examined using a zooming rectangle.

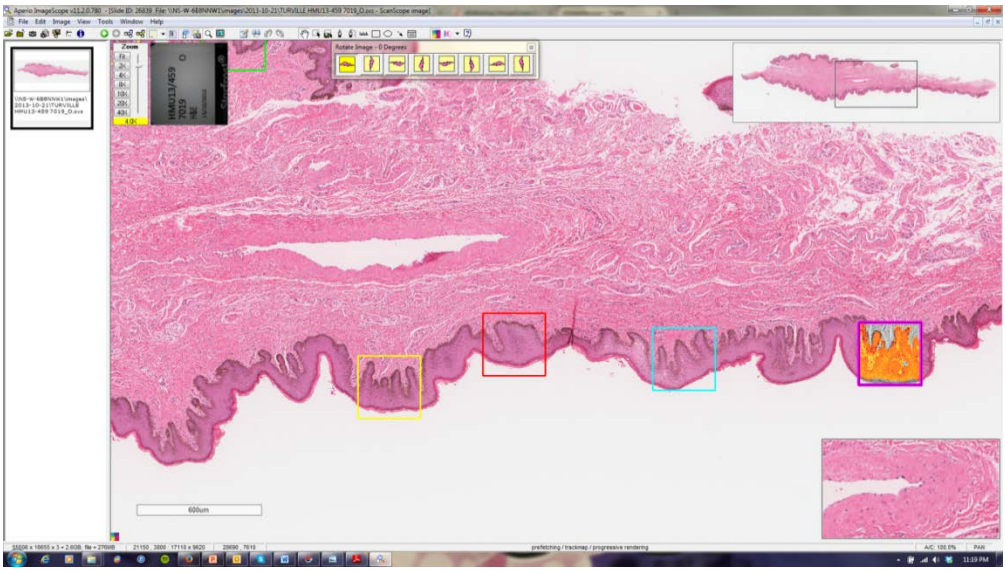


Figure 6.14: Marking 300 µm Squares

6.2.2.10.1 KT Measurement

From each square of 300 x 300 µm, 10 KT measurements were taken in equal (30 µm) distances. Each measurement was taken from the edge of the stratum granulosum to the outer edge of the dead keratin layer.

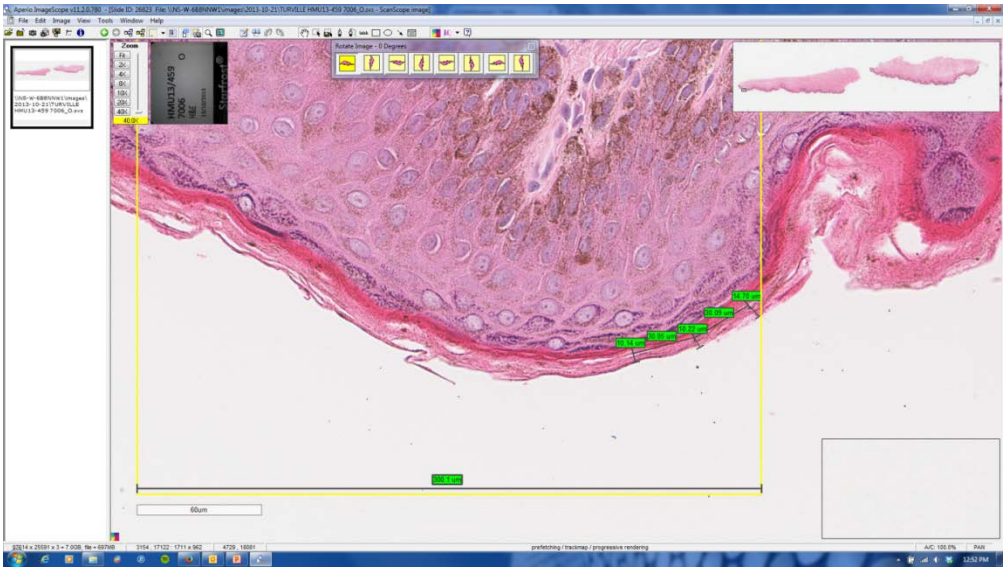


Figure 6.15: Taking KT Measurements

6.2.2.10.2 Rete Pegs Measurement (Figure 6.16)

Similar to the KT, the RPLs and SPLs were measured within 300 µm squares. The RPLs were taken from the tip of the rete peg to the outer edge of the stratum granulosum, through a line drawn perpendicular to the base of the rete peg tip.

6.2.2.10.3 SPL Measurements (Figure 6.16)

The SPL was measured as the shortest distance from the papillary tip to the edge of the stratum granulosum. All measurements were completed under the maximum zoom available (×40).

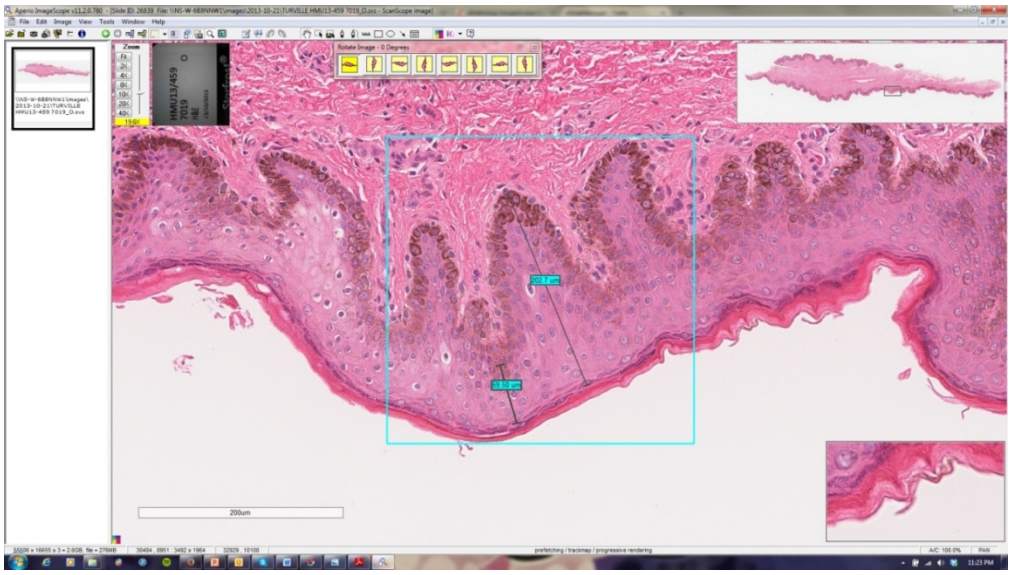


Figure 6.16: Taking RPL and SPL Measurements

6.2.2.11 Analysis of Target Cells

Analysis of the target cells was done in stages. While both the paraffin-embedded and frozen foreskin samples were used to stain for target cells, the paraffin-embedded samples were initially stained mainly for quality assessment purposes. The CD4 cells were selected as the first target cell to be analysed. Three different antibodies were tested one after the other in order to select the best antibody for CD4 cells to be visualised on immunofluorescence. The tested antibodies were:

- rabbit anti-human CD4 monoclonal antibody (Clone SP35) (cat # M3350)
- STEMCELL Technologies (Clone OKT4) (cat # 60016)
- CD4 AIDS reagent antibody.

These antibodies were tested to select the best antibody to demonstrate the CD4 cells with immunohistochemistry. The stained tissues with immunohistochemistry were intended to be subjected for analysis of target cells; however, this was not completed due to time constraints. However, the work is being continued by another PhD student as an approach to Phase 2 of the study, leading to another PhD thesis arising from this project.

6.2.3 Methods Used to Address the Non-scientific Objectives

6.2.3.1 Methods for Collecting Data on Feasibility and Logistics

Data on logistical challenges were collected via several methods:

1. log book recordings: entries were made during each step of the study from the researcher's direct interactions with various parties involved, from preparation to the end of the study
2. personal reflections: some observations mentioned here were the researcher's personal reflections on incidents and interactions during different stages of the study
3. discussion with other study investigators: the personal experiences of the other study investigators who had previously undertaken collaborative research with PNG investigators were gathered and used
4. discussions with local investigators in PNG: the knowledge and experience of local investigators with study projects and local communities were collected and used to analyse some situations.

6.2.4 Safety Assessment

No harm to the participants was expected to arise during the study process. However, contact information were provided to all participants in participant information sheet to report any form of adverse events, either physical or psychological, to relevant authorities. Furthermore, the principal researcher was at the study site at all times to oversee any unusual responses or incidents during the study. A suitably qualified surgeon from the POMGH undertook all circumcisions, with the support of nursing staff from the School of Nursing, PAU. They were available to answer any queries or concerns of the participants regarding the surgical procedure, before and after the operation. Further, the participants were given the opportunity to seek advice if they felt any distress when discussing a sensitive topic, such as HIV, or were upset or distressed in any other way due to the study. They could seek advice from any of the investigators or from Dr Leeroy Elisha, Director of Counselling Services at the PAU.

6.2.5 Data Storage and Disposal

Participant study folders with study identification numbers were securely filed by number in a designated locked filing cabinet in one of the PAU investigator's offices. Forms that contained subject identifiers (such as name, age and address), such as the informed consent forms and study register, were stored in a separate locked cabinet. Only research staff and other investigators who required access to these records were allowed to do so after confirming their responsibility to maintain subject confidentiality. Data analysis used de-identified data, from which it was impossible to identify individuals in project summary reports, scientific conference presentations, papers submitted for publication in scientific journals or other study outputs.

At the end of the study, the participant information that was stored in the locked filing cabinets at the PAU was moved into long-term storage in a secure facility at the JCU. Only research staff considered by the principal investigator and senior investigators to require access to the archive were allowed to do so, and confirmed their responsibility to maintain subject confidentiality. The data archive will be maintained for a minimum of seven years, prior to disposal by incineration.

Similarly, all the laboratory research data were kept in a password-protected electronic database on a password-protected computer with a coded subject number. This subject number was only linked with the participant's name on the study folder kept in the locked cabinet in a locked office. All these measures were taken to ensure participant confidentiality during the study.

6.2.6 Laboratory Data Management and Reporting Arrangements

The principal investigator was responsible for specimen collection, processing, storage and transport, as well as timely and accurate data entry of the laboratory results into a confidential, secure database.

6.2.7 Data Analysis

The KTs, SPLs and RPLs were analysed using the student's t-test (SPSS v. 20). The mean measures of the above histological features (KTs, SPLs and RPLs) from different histological sites were compared. Altogether, 20 tissue samples were included in the analysis, which was undertaken using paired and independent sample t-tests with a two-tailed significance level set at 0.05 (see Table 6.3).

Table 6.3: Statistical Analysis of Histological Features

	Aim	Hypothesis	Statistical Test
KTs	Dorsal slit, IFS v. OFS	H_0 = KT of IFS equals KT of OFS	Paired sample t-test
	No cut, IFS v. OFS	H_0 = KT of IFS equals KT of OFS	Paired sample t-test
	Dorsal slit IFS v. no cut IFS	H_0 = KT of IFS is equal in dorsal slit and no cut men	Independent sample t-test
	Dorsal slit OFS v. no cut OFS	H_0 = KT of OFS is equal in dorsal slit and no cut men	Independent sample t-test
RPLs	Dorsal slit, IFS v. OFS	H_0 = RPL of IFS equals KT of OFS	Paired sample t-test
	No cut, IFS v. OFS	H_0 = RPL of IFS equals KT of OFS	Paired sample t-test
	Dorsal slit IFS v. no cut IFS	H_0 = RPL of IFS is equal in dorsal slit and no cut men	Independent sample t-test
	Dorsal slit OFS v. no cut OFS	H_0 = RPL of OFS is equal in dorsal slit and no cut men	Independent sample t-test
SPLs	Dorsal slit, IFS v. OFS	H_0 = SPL of IFS equals KT of OFS	Paired sample t-test
	No cut, IFS v. OFS	H_0 = SPL of IFS equals KT of OFS	Paired sample t-test
	Dorsal slit IFS v. no cut IFS	H_0 = SPL of IFS is equal in dorsal slit and no cut men	Independent sample t-test
	Dorsal slit OFS v. no cut OFS	H_0 = SPL of OFS is equal in dorsal slit and no cut men	Independent sample t-test

The logistical challenges and solutions were analysed in each step of the research procedure in order to answer the last two research questions.

Part 2: Results of Phase 1 of the Study

6.3 Preface

This section presents the results of Phase 1 of the study. First, Part 2.1 presents the results of the experimental (scientific objectives) aspects of the study. Second, Part 2.2 presents the results of the non-scientific objectives—that is, the conceptual, methodological and scientific challenges (particularly the logistical, resource and administrative challenges) and opportunities identified during Phase 1 in order to ensure the smooth operation of Phase 2.

6.3.1 Part 2.1: Results of the Experimental (Scientific) Objectives of Phase 1

This section presents the results of the scientific objectives of Phase 1 of the study. The results consist principally of the findings for the KTs, RPLs and SPLs of the paraffin-embedded foreskin samples from the participants with a dorsal slit and the participants who were uncircumcised. Table 6.4 presents the demographic characteristics of the study participants. The participants' mean age was 22.84 years, with median age of 23 years (IQ range 20.5 to 25.0). All participants were single. Of the 27 participants, 83% had no paid work. Almost 96% of participants were Seventh Day Adventists.

Table 6.4: Demographics of Participants

Demographic Characteristic		Study Group
Age in years	Mean Median	22.84 23 (IQ range 20.5–25)
Marital status	Married Single	0 27 (100%)
Employment	Had paid job No paid job	5 22 (83%)
Religion	Catholic Seventh Day Adventist	1 26 (96%)

In the pre-study questionnaire, of the 27 participants, 15 reported having had previous foreskin cuts. Upon clinical examination, these were all identified as dorsal slits. Fourteen participants had their foreskin cuts performed by friends, while one participant performed the operation on himself. All dorsal slits had been performed more than one year before the MC clinic.

6.3.1.1 Analysis of KT

Foreskin samples that had tissue-processing artefacts and tissue-orientation uncertainty were excluded, which resulted in 14 participants' sample results being discarded. Analysis was performed on the data from samples of 13 participants (seven uncircumcised and six with dorsal slits). Table 6.5 presents the KT measures stratified according to the MC status of the participants. The results indicated that the KT measurements of the dorsal slit foreskins showed substantial inter-individual variation.

Table 6.5: KT Measures According to the Type of Cut

Sample	Sample Identification	IFS	OFS
NC 1	24807	12.65	11.92
NC 2	24815	10.01	10.36
NC 3	24816	10.57	12.94
NC 4	24831	10.28	13.61
NC 5	24833	11.88	13.79
NC 6	24835	10.57	17.59
NC 7	24825	10.05	16.63
DS 1	24817	21.62	23.32
DS 2	24819	15.35	25.79
DS 3	24826	7.86	11.25
DS 4	24837	9.18	12.71
DS 5	24846	13.74	22.55
DS 6	24845	8.11	8.83

Note: NC = no cut. DS = dorsal slit.

The following images (Figure 6.17) are from some of the paraffin-embedded tissues from Phase 1, taken at the histopathology laboratory, Cairns Hospital, using a high-resolution light microscope.

(a) (b) (c)

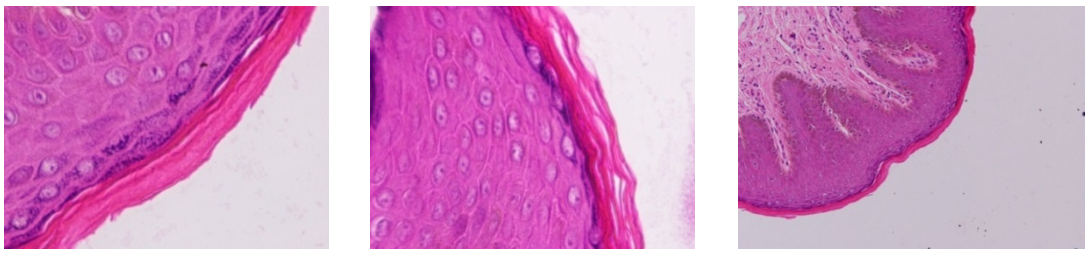


Figure 6.17: Examples of Paraffin Sections under a Light Microscope (magnification (a) x400, (b) x 200, (c) x 40)

6.3.1.2 KT: Analysis of Pooled Data across Dorsal Slit and Uncircumcised Men

Figure 6.18 demonstrates the analysis of pooled data for seven uncircumcised men (no cut) for the KT between the IFS and OFS.

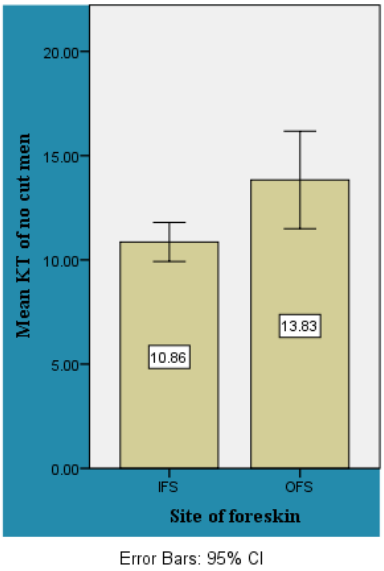


Figure 6.18: KT Measurements of Uncircumcised Men

Similarly Figure 6.19 presents pooled data of six men with a dorsal slit, demonstrating that the KT of the OFS was significantly thicker than that of the IFS (P = 0.03) among men with a dorsal slit (Table 6.6).

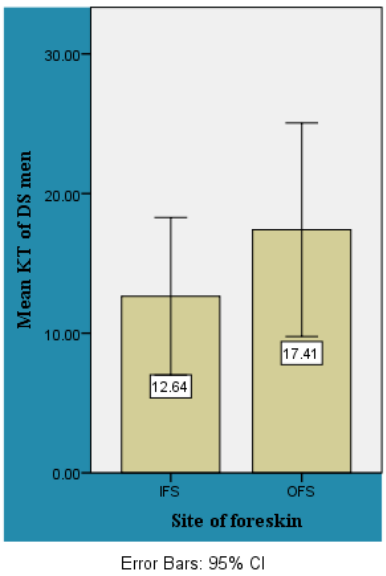


Figure 6.19: KT Measurements of Men with a Dorsal Slit

Table 6.6 presents the analysis of the pooled KT measures. The analysis of the KT measurements demonstrated that the KT of the OFS was significantly higher than that of the IFS in both uncircumcised men (no cut) and men with a dorsal slit.

Table 6.6: Inter-tissue Site Analysis of KT Measures

Type of Cut	Tissue Site	Mean KT Measure	SD	Significance
No cut	IFS	10.86	1.01	P = 0.04
	OFS	13.83	2.53	
Dorsal slit	IFS	12.64	5.37	P = 0.03
	OFS	17.41	7.28	

6.3.1.3 KT: Inter-MC Status (Dorsal Slits v. Uncircumcised) Analysis

Table 6.7 presents the data for the analysis of the inter-MC status for the KT measurements. The inter-MC status analysis showed that there was no significant difference in the KT of the IFS or OFS between men with a dorsal slit and uncircumcised men, although the mean KT measurements of both the inner and outer aspects of the dorsal slit foreskins were higher.

Table 6.7: Inter-MC Status Analysis of KT Measures

Site of Foreskin	MC Status	Mean KT Measure	SD	Significance
IFS	No cut	10.85	1.01	P = 0.40
	Dorsal slit	12.64	5.37	
OFS	No cut	13.83	2.53	P = 0.25
	Dorsal slit	17.40	7.28	

6.3.1.4 Analysis of Rete Peg Measurements for Dorsal Slit and Uncircumcised Men

According to the pooled inter-individual analysis of the RPLs (as shown in Table 6.8), the mean RPL was higher in the OFS than in the IFS in both the dorsal slit and uncircumcised (no cut) men. However, the mean RPL in the OFS of men with a dorsal slit was significantly higher than that of the IFS. The difference was not significant between the IFS and OFS of the uncircumcised men.

Table 6.8: Inter-tissue Analysis of RPLs

Site of foreskin	RPL µm (mean)		Significance
	IFS	OFS	
Men with a dorsal slit	143.9	206.1	$p < 0.001$
No cut men	164.6	164.0	$p = 0.95$

6.3.1.5 Rete Pegs Inter-MC Status (Dorsal Slits v. Uncircumcised) Analysis

Figure 6.20 presents the inter-MC status RPL measurements of the IFS. The mean RPL of the IFS of the uncircumcised men was higher than that of the men with a dorsal slit.

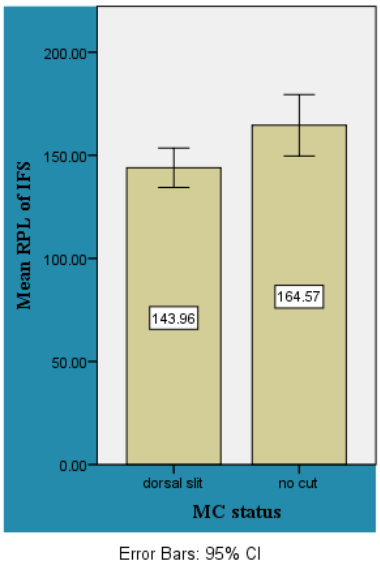


Figure 6.20: Inter-MC Status RPL Measurements of the IFS

Figure 6.21 presents the RPL measurements of the OFS between the dorsal slit and uncircumcised (no cut) men. The results showed a significantly longer RPL in the OFS of the men with a dorsal slit compared to that of the uncircumcised men.

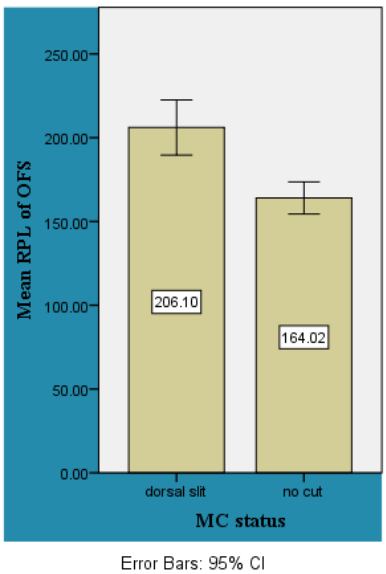


Figure 6.21: Inter-MC Status RPL Measurements of the OFS

Table 6.9 presents the inter-MC status mean RPL measures of the IFS and OFS. Accordingly, the RPL in the IFS of the uncircumcised men was significantly larger than the RPL of the IFS of the men with a dorsal slit. In contrast, the RPL of the OFS of the men with a dorsal slit was significantly higher than that of the uncircumcised men.

Table 6.9: Inter-MC Status Analysis of Rete Pegs

Site of FS	MC Status	RPL (µm) (mean)	Significance
IFS	Dorsal slit	143.9	p = 0.02
	No cut	164.6	
OFS	Dorsal slit	206.1	p < 0.001
	No cut	164.0	

6.3.1.6 Analysis of SPL Measurements for Dorsal Slit and Uncircumcised Men

Table 6.10 presents the inter-tissue pooled SPL data analysis. There was no difference in mean SPL between the IFS and OFS of men with a dorsal slit. However, the mean SPL between the two foreskin surfaces of the uncircumcised (no cut) men was significantly different (OFS > IFS).

Table 6.10: Inter-tissue Analysis of SPLs

Type of Cut	SPL (µm) (mean)		Significance
	IFS	OFS	
Men with a dorsal slit	55.1	51.1	p = 0.20
No cut men	46.1	52.7	p = 0.01

6.3.1.7 SPLs Inter-MC Status Analysis

The distribution of the inter-MC status mean SPL measurements of the OFS showed that the mean SPL of the uncircumcised (no cut) men was slightly longer than the mean SPL of the men with a dorsal slit.

Figure 6.22 presents the distributions of the inter-MC status mean SPL measurements of the IFS. The mean SPL of the IFS of the men with a dorsal slit was longer than the mean SPL of the IFS of the no cut men.

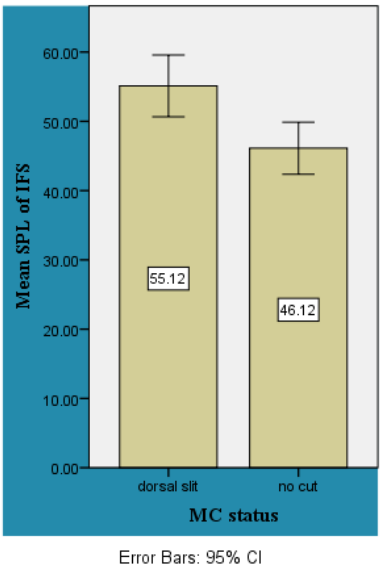


Figure 6.22: SPL Measurements of IFS

Table 6.11 presents the inter-MC status mean SPL analysis. A significant difference in SPL was observed in the IFS between the men with a dorsal slit and the uncircumcised (no cut) men. However, the difference was not significant in the OFS of the two groups.

Table 6.11: Inter-MC Status Analysis of SPLs

Site of FS	Comparison	SPL (µm) (mean)	Significance
IFS	Dorsal slit	55.1	P = 0.002
	No cut	46.1	
OFS	Dorsal slit	51.4	P =0.65
	No cut	52.7	

6.3.1.8 Analysis of Target Cells (CD4 Cells)

The analysis of target cells was incomplete (see chapter 6.5.2.4); hence, no data are presented here. Nevertheless, the pictures below demonstrate several of the samples processed with immunohistochemical methods. The CD4 cells were stained in blue in the epidermis and dermis (predominantly). Figures 6.23 and 6.24 present examples of paraffin sections in which the CD4 cells were attached to Clone SP35 and OKT4 antibodies respectively.

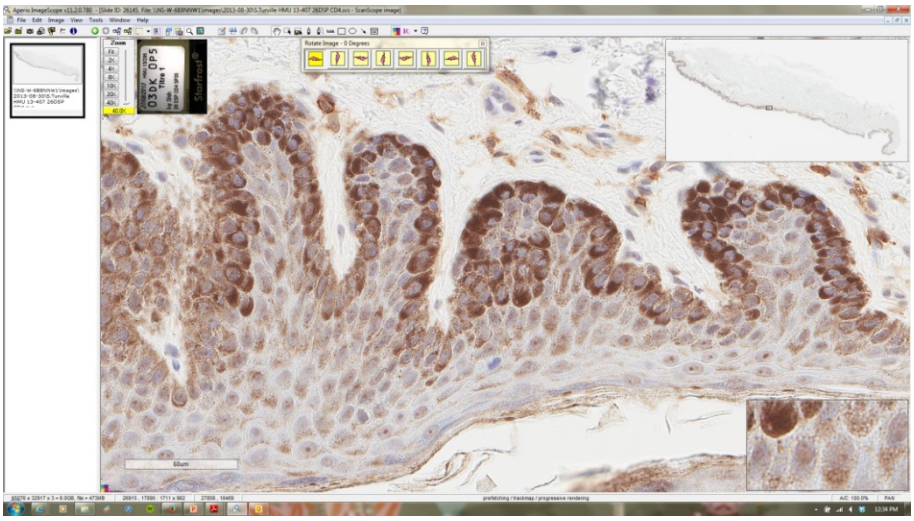


Figure 6.23: OFS Stained with Antibody Clone SP35

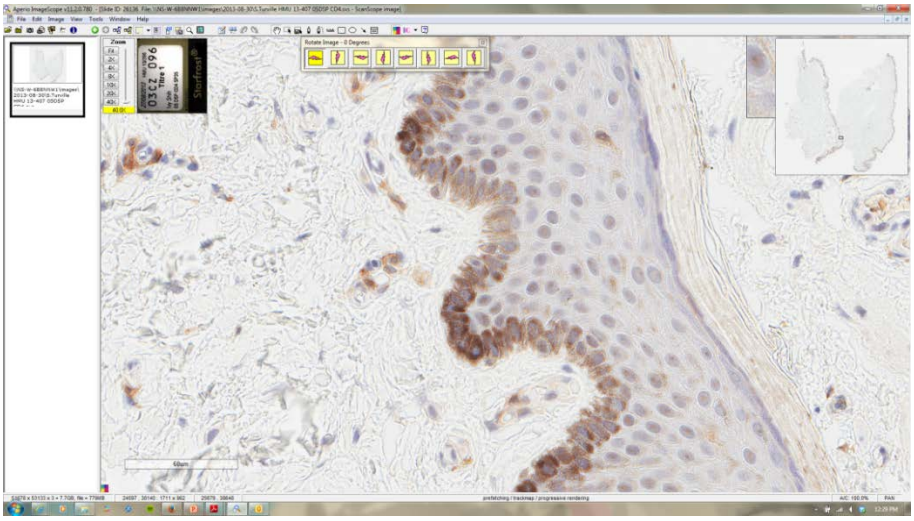


Figure 6.24: IFS Stained with Antibody Clone OKT4

6.3.1.9 Problems Identified in Tissue Samples

Defects in samples were identified in both the paraffin-embedded tissues and frozen sections during quality assurance processes, before analysing for the histological features of interest. The samples discarded from 14 participants had one or a few of the following problems in the tissues.

6.3.1.9.1 Paraffin-embedded Tissue Samples

The paraffin-embedded tissue samples encountered the following issues:

- Tissue orientation issues: orientation of the tissue was key to identifying the site of the tissue (IFS or OFS) in order to compare the histological changes in each tissue site. The main orientation issues identified were the tissues not being maintained in parallel with the longitudinal aspect of the slide, according to the original protocol. This led to uncertainty in the orientation because the markings on the tissues at the time of removal from the body were not sufficiently resistant to processing, and did not appear on the glass slides.
- Excessive dehydration causing desquamation of outer keratin layers: delay in transporting samples to the POMGH laboratory from the PAU clinic meant that some tissue samples remained in the preservative longer than the expected time duration, leading to dehydration and desquamation.

6.3.1.9.2 Frozen Samples

Some of the frozen samples encountered the following issues:

- vacuolisation: formation of vacuoles inside the cytoplasm of cells due to inconsistent freezing of samples is identified as vacuolisation
- shattering/freezing artefacts: shattering occurs when ice crystals are formed in intercellular spaces due to improper freezing of tissue.

Figure 6.25 presents an example of shattering and vacuolisation of frozen samples.

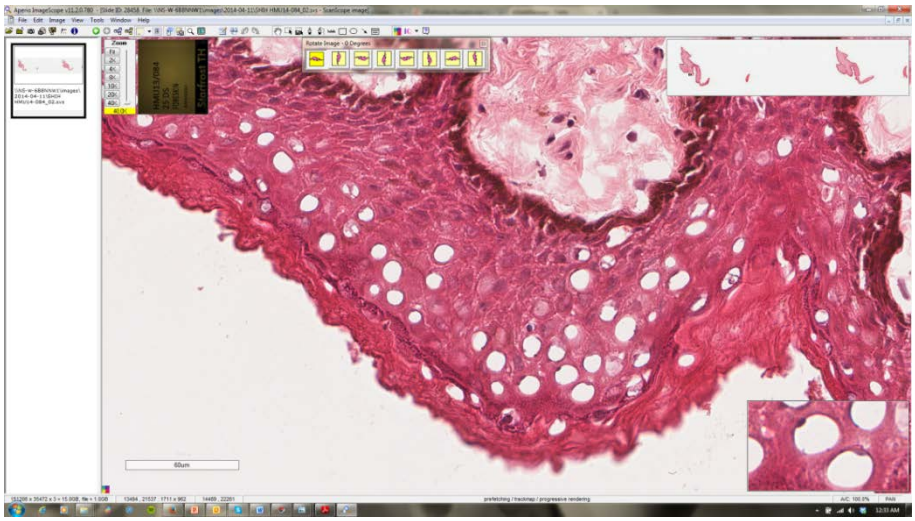


Figure 6.25: Vacuolisation in Frozen Sections

6.3.2 Part 2: Results for Non-scientific Objectives (Conceptual, Methodological and Scientific Challenges and Opportunities)

6.3.2.1 Preliminary Work for the Study

A preliminary visit was made to the study site in August 2011 in order to view the clinic set up and identify any logistical issues, such as human resources and transportation of samples involved with the study.

6.3.2.2 Identification of Resources

The preliminary visit also identified the available resources that were important for the conduct of the study, such as a well-equipped histopathology laboratory at the POMGH. Further, the visit identified limitations in resources at the primary study site (the PAU clinic), such as the lack of surgical instruments, VCT kits and good light sources for the surgical operations. The minor procedure room of the clinic was identified as the location for the MC operations by the PAU authorities, with a few improvements, as described in Part 1 of this chapter.

6.3.2.3 Logistical Challenges

Some logistical issues had to be overcome before starting the study. Among these were assessing the level of participation of students in the study, gathering essential research equipment and other consumables (such as the chemicals needed to process samples) and arranging ways to store and transport samples from the PAU to the POMGH and then to Australia.

6.3.2.4 Arranging Human Resources

The visit further provided the opportunity to meet all the research partners, such as the surgeon and histopathologist of the POMGH and the vice chancellor of the PAU in order to discuss the importance, logistics and conduct of the research project. Input from the discussions with local investigators was helpful in preparing the study protocol.

6.3.2.5 Study Preparation

6.3.2.5.1 Permission from Ethics Committees

Ethics approval from four ethics committees was requested for this study. While the ethics approval process with some institutions was quicker than others, the total process took nine months to complete. The main challenge regarding ethics approvals was obtaining all approvals before the planned date of the MC clinic.

6.3.2.5.2 Permission to Use Premises as Study Site

Permission to use the university clinic for the study project was obtained without much difficulty due to the strong research culture established at the PAU. Permission to use the POMGH histopathology laboratory was also forthcoming because of the enthusiasm of the chief histopathologist (Dr Morawaya) for the research. Obtaining permission from the chief executive officer of the POMGH to use the laboratory and hospital site for the research was delayed administratively.

6.3.2.5.3 Import and Export Permits

Obtaining import and export permits for transporting biological samples was a challenge in both PNG and Australia; however, the challenges took different forms in the two countries. Identifying sources of information and the responsible institutions with which to coordinate was a problem in PNG, while administrative delays were more prominent for the AQIS in Australia in terms of granting the approval.

6.3.2.5.4 Biosafety Requirements

Obtaining biosafety approval was a well-structured process at the JCU, in which the investigators had to fulfil certain criteria in terms of having the knowledge required to safely handle biological materials in research. This knowledge was gained through a one-day workshop, and then reviewed online before the researcher was assessed and certified.

6.3.2.5.5 Skills Training

The basic skills identified as essential for Phase 1 of the study were preparing and processing paraffin and frozen sections until slides were made from them, prior to analysis using microscopes. The skills for the paraffin sample processing for the principal investigator were obtained through training from senior staff at the histopathology unit at Cairns Base Hospital, Cairns, Australia. Basic training in the processes for frozen sample preparation was provided by an experienced researcher at the UNSW, Sydney.

6.3.2.5.6 Preparing Protocols, Procedures and CRFs According to the Resources Available

Study protocols—particularly some study steps—had to be changed to suit the identified limitations in resources. For example, preparing frozen sections using liquid nitrogen and dry ice was changed because of the lack of availability of dry ice. Alternatively, a method that uses a -80°C freezer to freeze tissue embedding material was used to make frozen sections.

6.3.2.5.7 Preparing Funding for the Study

This study was conducted using the limited budget allocated for a PhD project. Nevertheless, the study was able to be completed with the available funds—mainly due to the support given by the PAU and local investigators. Overhead costs were minimal because all the local research assistants volunteered to help in the clinic and during the study.

6.3.2.5.8 Collection and Preparation of Resources

The main challenge in terms of resources was locating dry ice in PNG. Local sources informed the researcher that the few industries that had previously produced dry ice in POM had closed, which meant it was difficult to attain dry ice within a short period. No commercial institutions or outlets were able to supply dry ice. University laboratories and the suppliers of chemicals to laboratories in POM were also unable to

provide dry ice. An attempt to transport dry ice from a manufacturing plant in Lae, PNG, also failed due to manufacturing machinery issues.

Essential surgical instruments (such as disposable MC kits) and study equipment (such as onsite tissue-processing tools) were transported from Australia because there was a delay in the supply chains in PNG.

6.3.2.6 Recruitment of Participants

6.3.2.6.1 Educating and Advertising

Educating the male student community of the PAU on the MC clinic and study was facilitated by the male student leaders of the PAU. The message was spread around the campus by word of mouth, emails, flyers and posters. An information session was arranged, which successfully addressed the issues and questions the students had regarding the MC clinic, circumcision procedure and associated study.

6.3.2.6.2 Registering for the Study

Registration of the participants (male students of the PAU) was undertaken exclusively by a male investigator due to cultural issues because, in PNG culture, this is considered ‘men’s business’. This was successful because the male participants were more open to male investigators to communicate their concerns and discuss confidential issues relating to MC.

6.3.2.6.3 Informed Consent

Obtaining informed consent was relatively quick and easy due to the high comprehension level of the cohort of participants in this study, and because the study procedures were understood easily enough to facilitate the participants’ decision making.

6.3.2.6.4 Study Participation

Participation for the screening visit remained low due to the students' preparations for PNG Independence Day celebrations on the following day (15 September 2012); hence, the screening of participants was postponed to the morning of the day of the MC clinic. In addition to this logistic issue, the participants also preferred having one session for the screening, study and MC clinic, rather than two separate sessions, for reasons of convenience.

6.3.2.7 Conduct of the Study

6.3.2.7.1 Genital Examination

Having a separate session for genital examination was difficult because intimate genital examination is a culturally sensitive issue and the participants did not feel comfortable with it. This issue was resolved by combining the genital examination with the MC procedure—that is, conducting the genital examination immediately prior to the MC operation, while the patient was lying on the operation table and the surgeon was preparing for the operation.

6.3.2.7.2 Sample Collection (Tissue Orientation)

The IFS side was initially orientated using a permanent marker pen. However, this was identified as a poor technique during tissue processing because the markings on an initial set of the tissues were erased during the fixation process. An improved technique to mark tissue orientation was used on the second day, in which a piece of cork sheet and tiny steel pins were used to pin the tissue for orientation. This also helped avoid curling of the tissues, which was observed after fixation of the first set of samples. Laminated labels that could withstand the fixation process were made by a label maker, and used to tag the tissue moulds. The tissues (in containers) prepared for the frozen sections were stored in an esky filled with ice packs until being transported to the laboratory.

6.3.2.7.3 Sample Transport

Arranging transport of samples with a limited budget was another challenge in PNG. Discussions with the PAU authorities enabled access to university vehicles to transport the samples from the study site to the laboratory. Nevertheless, there were limitations and difficulties in the process, mainly due to limited resources.

6.3.2.7.4 Frozen Sample Preparation

Despite the resource and logistical barriers, the researchers devised a reliable method to produce frozen samples during this study. There are several documented methods to achieve subzero temperatures to freeze fresh tissue samples. Some of these methods use liquefied nitrogen (-190°C), isopentane (2-methylbutane) cooled by liquid nitrogen (-150°C), dry ice (-70°C), carbon dioxide gas (-70°C) and aerosol sprays (-50°C). The best frozen sections are achieved when the tissue is frozen very quickly using isopentane and liquid nitrogen).

In this study, liquid nitrogen or dry ice was not available at the time of sample processing. Further, due to the logistical challenges, it was difficult to arrange to freeze the samples soon after the tissues were removed from the body. Therefore, tissue samples were kept preserved in normal saline until they were frozen a few hours later at the PNG histopathology laboratory using a -80°C freezer, as previously described.

6.3.2.7.5 Paraffin Sample Preparation

The main challenge during paraffin tissue preparation was maintaining the time periods required to fix the samples according to the study protocol. This was mainly due to problems in transport arrangements. Alternative transport means were used to keep to the time requirements. Paraffin sample preparation is regularly undertaken at the histopathology laboratory at POMGH; however, sample preparation for research is different because it needs to follow research protocols. Unfortunately, the laboratory technician allocated for the study appeared disinterested and did not follow the exact research protocol resulting in dis-orientating some of the tissue samples on the slides.

6.3.2.7.6 Sample Storage

The nearest -80°C freezer was at the POMGH; therefore, the samples had to be transported from the PAU clinic to the POMGH to process and store the frozen samples, until they were transported to the immunohistopathology laboratory in Sydney. The frozen samples were stored in a -80°C freezer at the POMGH histopathology laboratory from September 2012 to June 2013 (nine months) until logistics were arranged for international transport of samples.

6.3.2.7.7 Prevention of Complications

Although not a direct responsibility of the study investigators, a few essential requirements for the conduct of the MC clinic were identified by the investigators, and attempts were made to rectify them. One such arrangement was to hire a diathermy machine from Cairns and transport it to the PAU for the MCs during the preparation stage for the study and MC clinic. However, this arrangement failed and the MC clinic had to continue without a diathermy machine. Haemostasis during MC procedures was achieved using surgical ligations. One major bleeding complication (in which a significant amount of blood was lost and the surgeon had to re-open the surgical wound to arrest the bleeding) and two minor bleeding episodes (which were easily managed by pressure bandages) were observed as surgical complications during the two days of the MC clinic (see chapter 6.12.5). These complications were reported by participants to the study team, and the study team arranged for the surgical team to attend to the incidents promptly.

6.3.2.8 Sample Analysis

6.3.2.8.1 Transport of Samples from the POMGH Laboratory to the UNSW

There were a few challenges encountered during the international transport of samples from PNG to Sydney. First, it took eight months—from August 2012 to April 2013—to get permission from the AQIS to transport the samples. Second, several options had to be considered to arrange the logistics for the international transport of samples. Of those options, the high cost prevented the use of a courier service to transport the samples from PNG to Australia, and the regulations of airlines restricted the transport

of samples in personal luggage. Attempts to find a dry shipper (a substitute for dry ice for frozen sample transport in the absence of dry ice) also failed. Finally, the samples were transported to UNSW, Sydney, using dry ice transported from Cairns, as described in the methodology section.

6.3.2.8.2 Finding a Proper Antibody for Immunofluorescence (Issues During Antibody Preparation)

Finding the correct antibody for proper immunofluorescent images was a challenge. This was mainly a trial-and-error process, in which several antibodies were trialled to determine the best antibody that provided all the necessary information on the identification and distribution of target cells in a given tissue sample. Both the paraffin-embedded and frozen samples were useful in testing the antibodies for the target cells and the methods used to stain the samples (see also 6.5.2.4).

Part 3: Discussion of Scientific Output of Phase 1 of the Study

6.4 Preface

Part 2.1 of the previous section presented the scientific outcomes of Phase 1 of the study, answering the first three research questions. It presented the results for the analysis of keratin layer thickness, RPL and SPL, including an incomplete analysis of the CD4 target cells of the dorsal slit and uncircumcised Melanesian men. This section, Part 3, discusses in detail the importance of those results in the context of MC and HIV prevention. The results will be compared to similar studies conducted elsewhere. First, this section analyses the demographic characteristics of the participants, followed by discussing the results of the histological features. Finally, this section summarises the importance of this study and its implications in the context of PNG.

6.5 Discussion

The specific scientific objectives of this study comprised analysing the likely immunohistological changes that were expected to occur in the foreskin after traditional dorsal slit foreskin cuttings among men in PNG. The main histological changes focused on were KT, RPL and SPL. In addition, immunological changes were expected in the distribution of the target cells in the IFS and OFS. While the measures of three histological features in the epidermis could be completed, the analysis of the target cells and their densities in respective tissue locations were not completed for two reasons:

1. There was limited time allocated for Phase 1 of the study and it was likely to take a considerable time to analyse the target cells, thereby exceeding the allocated time for the completion of the PhD.
2. After establishing that frozen tissues could be successfully transported from the clinic to a laboratory where the immunohistochemistry could be completed, and identifying where improvements could be effected in this process, it was better to concentrate on the foreskins collected in Phase 2 for this important work.

Nevertheless, important knowledge was gained (in relation to measuring the target cell density of the foreskins) from the work done with regard to preparing the frozen

sections for immunofluorescent staining and the process of selecting appropriate antibodies against the CD4 cells. Analysis of the results of three histological features in Phase 1 of the study enabled some important observations, as discussed below.

6.5.1 Demographic Characteristics of Participants

The participants' characteristics were diverse because the participants comprised university students at the PAU from different backgrounds and geographical areas of PNG. Hence, this study considered the sample to be generally representative of the general male population in PNG with regard to culture and geography. However, the epidemiological characteristics of the participants had little relevance to the scientific objectives of the study, as the main outcome was based on an adequate number of samples from dorsal slit and uncircumcised (no cut) participants in order to compare the difference in histological changes that occurred after a dorsal slit.

6.5.2 Analysis of Histological Characteristics

The research findings of this study supported some of the study hypotheses, while other hypotheses were supported partially. There was one inconclusive finding (for RPL) due to technical issues in obtaining appropriate measurements. Some of the changes observed in the foreskins provided clues of a scientific basis for the hypothesis that partial MC (exposing the glans without removing the foreskin) could protect men from sexual transmission of HIV. The remainder of this chapter will discuss the results of the analysis of KT, RPL and SPL, followed by the partially completed analysis of the target cells.

6.5.2.1 Analysis of Keratin Layer Thickness

The keratin layer is the first line of defence against the entry of potential pathogens into the foreskin. The thickness of the keratin layer (alternatively, the number of keratin layers in the stratum corneum) is important for the strength of the physical barrier providing resistance to the entry of organisms such as HIV. This study demonstrated that the keratin layer of the OFS is significantly thicker than the keratin layer of the IFS in both uncircumcised (no cut) (13.83 Vs 10.86, $p = 0.036$) and dorsal

slit foreskins of Melanesian men (17.41 Vs 12.64, p = 0.032). The fact that the keratin layer is thicker in the OFS than in the IFS has been a commonly held view, although several studies have reported contrasting results. The KT was equal in the IFS and OFS in some studies, while the KT of the IFS was thicker than that of the OFS in a few other studies. Further, studies conducted using different races have produced different results for the IFS and OFS keratin layer thickness (24, 91, 94-96). Accordingly, the results of this study contrast to the results of the studies that found no difference in KT (95, 96), or that the KT of the IFS is thicker than that of the OFS (94). The results of this study agree with the results of the studies conducted by Patterson and McCoomb that demonstrated a significantly thicker OFS keratin layer compared to the IFS (24, 91).

The study by Qi Qin et al. reported a thicker keratin layer of the IFS compared to the OFS, yet was later criticised for methodological errors, such as desquamation of keratin layers during sample processing. Dinh et al. reported equal thickness for the keratin layer in the IFS and OFS. However, they used foreskins from cadavers as well for their analysis, leaving uncertainties about the reliability of the results due to post-mortem biological degradation of tissues. Ganor and Bomsel attributed these discrepancies in KT results to the lack of a standardised method to evaluate KT across studies. They further suggested that age and genetic factors can play a part in the degree of foreskin keratinisation (93). They repeatedly demonstrated in their studies that, at the ultra-structural level, there are a higher number of apical keratin layers in the OFS, making it thicker and denser than the IFS (93). One of the objectives of Phase 2 of the current study is analysing a number of apical keratin layers in the foreskin epidermis, which is currently underway.

Inter-individual variability was noted in the measured KT of the foreskins of this study's participants. This significant heterogeneity among the participants may be attributed to: (i) genetic differences in the skin composition of the participants and/or (ii) different levels of exposure of the IFS to environmental stimuli over the period since the foreskin cut in the dorsal slit men. According to Dinh et al., exposure could also induce changes in the expression of epidermal proteins, such as filaggrin or keratin (96). A similar hypothesis of epidermal protein expression was proposed by Ganor and Bomsel in their review of HIV transmission in the male genital tract (93).

The findings of the current study demonstrated an increase in the thickness of the keratin layer in both the IFS and OFS of the men with a dorsal slit, compared to the foreskins of the uncircumcised men, although the differences were not statistically significant. The foreskins of men with a dorsal slit in this study had been exposed to the external environment for a considerable period (> one year). Accordingly, the results of this study showed that exposure of the IFS (but not removing the foreskin) leads to increased keratinisation, and may provide protection against HIV transmission, thereby favouring the second hypothesis of the study. A thicker keratin layer would improve innate immunity by making it difficult for external organisms to enter the male genital tissues and interact with the underlying target cells. Further studies, including Phase 2 of this project, should reveal further information by measuring the number of keratin layers in the epidermis, and particularly by measuring HIV transmission through uncircumcised and dorsal slit foreskins using in vitro HIV transmission experiments.

This study is significant because it is the first to measure the KT of both uncircumcised men and men with any form of foreskin cutting in a Melanesian population. The small numbers did not enable examination of the KT results according to the duration of exposure, although all the foreskin cuts (dorsal slits) were more than one year old in the participants of this study. There were also some challenges in tissue preparation during this study. One such issue was fixation of the foreskin tissue samples for a few hours more than recommended, which could have dehydrated the outer layers of the keratin and affected the keratin layer thickness. This occurred due to delay in transporting samples from the study site to the POM laboratory. The need for dedicated transportation was one of the identified logistical challenges during this study. The delay resulted in seven of the tissue samples from day one being fixed for 10 to 15 hours (instead of eight to 10 hours, as per the original protocol) in 10% formalin.

A commonly accepted concept is that the IFS is poorly keratinised, and that this structural difference could enable HIV-1 to interact more frequently with the underlying abundant target cells of the IFS, thereby facilitating HIV-1 transmission. This postulate (that the IFS could be the primary site of HIV-1 infection) is supported by the findings of this study. In summary, the findings of Phase 1 of this study support the first two hypotheses that: (i) the keratin layer is thicker in the OFS than in the IFS

in Melanesian men, and (ii) the keratin layer becomes thicker in the IFS following exposure to the outside for a period after the dorsal slit foreskin cutting. However, further studies are needed to confirm these findings.

6.5.2.2 Analysis of Rete Pegs

This study's hypothesis was that the RPL of the IFS should increase after exposure to the outside environment for a certain period after dorsal slit foreskin cutting. The findings of this study demonstrated a significant difference in the RPL (OFS > IFS) in dorsal slit foreskins. This might be explained by the level of exposure of the foreskin to the environment, and possibly the effects of extra friction on the outer side of the hanging foreskin among dorsal slit men. This is in contrast to the almost equal RPL of the IFS and OFS of the uncircumcised men.

On the other hand, the RPL of the IFS of the dorsal slit men was significantly smaller than the RPL of the IFS of the uncircumcised men. While there was no obvious explanation for this, further research is warranted to clarify this discrepancy. Overall, this study found a reduction in the RPL of the IFS and an increase in the RPL of the OFS in the men with a dorsal slit, compared to the respective foreskins of the uncircumcised men.

The rete peg is a feature of the epidermis that is important to strengthen the skin against shearing forces and micro-trauma. According to Fenske and Lober, during ageing in a human, rete pegs flatten out, become effaced and lose their strength, which leads to more wear and tear injuries (98). Further, they demonstrated that a more undulating rete peg pattern in the epidermis is a sign of younger skin (98). To the best of the current researchers' knowledge, this is the first study conducted to measure RPL to assess the importance of the rete pegs of the epidermis as a potential protective histological feature of the foreskin against HIV infection.

However, during this study, it was realised that length alone may not reflect the significance of rete pegs because of the significant variability in the morphology of the rete pegs within a small area. This variable morphology of rete pegs may provide inaccurate measurements when taking perpendicular measures from the tip of the rete

pegs to the stratum granulosum, and give an inaccurate measure of the effect of rete pegs as a physical barrier in the foreskin. As a result, measuring the area covered by rete pegs may be more accurate and unbiased than measuring the RPL. This fact has been taken into consideration and corrected during Phase 2 of the study.

6.5.2.3 Analysis of Dermal Papillae

Dermal papillae and supra-dermal papillary plates (supra-papillary ridges) are also considered an important feature of innate immunity in mucosal surfaces. The supra-papillary ridge is the narrowest area in the epidermal keratin layer of the epidermis. These narrow areas are postulated to provide the closest access point to the dermis for external pathogens, such as HIV in a sexual partner's genital secretions. These supra-papillary areas could be more susceptible to micro-trauma during sexual intercourse, thereby facilitating viral entry further into the inner epidermis, where the target cells for HIV binding are located.

According to Hladick et al., shearing forces during sexual intercourse could lead to physical abrasions of the epithelium, particularly in micro-anatomical regions where the dermal papillae are enriched with dermal DCs that reach close to the epithelium (106). Further, it is emphasised that these micro-abrasions of the mucosal surface may allow HIV to directly access to target cells, such as DCs, T-cells and macrophages, at the basal epithelium and underlying dermis (167). According to Yeaman et al., areas above the dermal papillae—where the epithelium is relatively thin and where LCs on the epithelial-cell side and T-cells and macrophages on the dermal side congregate—appear particularly vulnerable to viral invasion (168).

The current study's findings revealed that the IFS consists of significantly thinner SPL than does the OFS, which could make the IFS more vulnerable to infections. This further strengthens the idea that the IFS is generally 'weaker' than the OFS, making the IFS more vulnerable to environmental changes, physical damage and external infections. The significantly higher SPL observed in the IFS of men with a dorsal slit is considered an important new finding of this study. This correlate indicates improved protection for men with a dorsal slit, compared to uncircumcised men, from external infections. However, this needs to be confirmed with in-vitro viral penetration studies.

This is an important initial step given that no previous study has compared SPL in the foreskins of uncircumcised or partially circumcised men. Further, this provides clues for the existence of histological features other than KT that could play a vital role in the innate immunity of the foreskin against infections.

In contrast to the significant difference of SPL between the IFS and OFS of the uncircumcised men, the difference in SPL between the two foreskin surfaces was non-significant among the dorsal slit men. In the men with a dorsal slit, SPL was relatively longer in the IFS, making the difference between the IFS and OFS non-significant. The mean SPL of the IFS of men with a dorsal slit significantly exceeded the mean SPL of the IFS of uncircumcised (no cut) men. This provides an important clue for potential protection provided by enhanced resistance against shear forces due to the changes in the SPL of the exposed IFS of dorsal slit men.

The RPL and SPL of the epidermis can be considered more reliable and accurate histological features than the KT because rete pegs and supra-papillary ridges are not thought to be affected by sample processing, since they lie inside the keratin layer of the epidermis. These two histological features internal to the keratin layer are less exposed to external factors, such as the heat and chemicals that are used for dehydration, preservation and fixing of the tissues during sample preparation.

In summary, the results of Phase 1 of the study support the initial hypothesis that the SPL of the IFS increases upon exposure to the outside environment after a certain period after dorsal slit foreskin cutting. Further research is required to explore this histological feature as an important feature of the innate immune protection of foreskins against HIV and other STIs.

6.5.2.4 Analysis of Target Cells

The next main objective of the study was analysing target cell distribution/densities in the IFS and OFS in the uncircumcised men and men with dorsal slit in PNG, as well as the changes in those distributions in the dorsal slit foreskins after exposure to the external environment. The primary measure for the target cells was to analyse the change in the density of CD4 cells, which are the main target cells for HIV. Both the

paraffin-embedded and frozen samples were used for this purpose in order to compare results.

Previous sections have discussed the logistical and technical issues encountered during the processing of samples. The researchers also identified histological defects of the samples (especially the frozen samples) while preparing to analyse the tissues for the target cells. Two prominent issues identified in the frozen histological samples of this study were shattering and vacuolisation, which are commonly found in frozen samples. Shattering is described as parallel marks on the frozen sections, while vacuoles can be found anywhere on the tissue sample. These defects in frozen samples are likely to be related to suboptimal tissue processing. According to Bancroft and Gamble, shattering is a phenomenon known to occur when freezing and cryosectioning tissue samples (169). 'Shutters' are formed when the sample is too cold to cut, and the sample shatters when bent by the knife bevel during cryosectioning. Hence, the visible symptom of cutting too cold is 'shutters' or shatter marks parallel to the knife blade. The mechanism described for the formation of vacuoles by Bancroft and Gamble indicates initial ice crystal formation in tissues in subzero temperatures, followed by the formation of vacuoles when the ice crystals are dissolved (169). The presence of shutters and vacuoles, with the consequent spatial derangement of anatomical structure, disrupted the analysis of target cell densities when using frozen samples in Phase 1 of the study.

Identification of the best antibody to visualise the CD4 target cells was another technical aim for the study. Immunological staining of the target cells (CD4 cells) was a long trial-and-error process, in which several antibodies were tested to select the best antibody to demonstrate the CD4 cells with immunohistochemistry. The three initial antibodies tested were:

- rabbit anti-human CD4 monoclonal antibody (Clone SP35) (cat # M3350)
- STEMCELL Technologies (Clone OKT4) (cat # 60016)
- CD4 AIDS reagent antibody.

While some good immunofluorescence pictures were produced with these antibodies, there were still some issues to be resolved, such as background staining of the samples. Despite the lack of time to complete the optimisation and analysis, the preliminary

work was useful as a learning experience to incorporate into Phase 2 of the study at the laboratory at the UNSW in Sydney.

6.5.3 Problems Identified in Tissue Samples

The two major issues identified for the two types of samples in this study were: (i) inaccurate tissue orientation in the paraffin samples and (ii) the artefacts (shattering and vacuolisation) formed in frozen tissue samples (as mentioned above). Uncertain tissue orientation can be prevented during standard tissue preparation via several methods. First, the tissue can be marked using permanent tissue marker ink on a predetermined tissue site at the time of tissue separation from the body (the marked side of the tissue with ink is embedded facing up). These tissue markers can be used as guides to embed the tissue sample in a preferred direction when embedding in paraffin blocks. However, a problem identified during this stage of the study was the use of a permanent marker pen, instead of a tissue marker, to mark the orientation for the first few samples. After realising that those markings wore off when dissecting tissues into adequate sample sizes, the tissue directions were marked using tiny metal pins. Using metal pins was also helpful to avoid curling of the tissues that was observed in tissues immersed in normal saline prior to dehydration. This was achieved by restraining the tissues on corkboards until being encased into cassettes, before putting them into the automated machine for dehydration.

Preserving orientation during the tissue embedding stage is also important. This was achieved by applying firm pressure to the entire specimen during orientation and the initial solidification of paraffin blocks to obtain flat tissue and avoid rotation. Although most technical errors could be prevented by using these methods, human error did complicate the process in some instances. As a result, a few of the samples had to be discarded from the analysis due to the uncertain orientation of the tissues.

The tissue artefacts identified on the frozen tissues could have been prevented by improving a few study steps. Two such important preventable actions were snap-freezing samples soon after the tissue was separated from the body (without delaying or slow freezing) and avoiding tissues being immersed in saline as much as possible before freezing. Maintaining tissue samples in a frozen state for immunohistochemistry was difficult during transportation, and there was one such

incident at the POM international airport in the course of sample transport from PNG to Australia.

6.5.4 Using the Phase 1 Samples during Phase 2

The samples produced in Phase 1 were useful to provide a basis for Phase 2 of the study in several aspects. During Phase 1, the samples were produced in difficult conditions with minimal resources. Nevertheless, the quality assessment revealed that the samples, especially the paraffin-embedded samples, were of good quality and high standard, despite the obstacles encountered during the sample preparation process. This provided the opportunity to conduct a valid analysis on good quality samples and produce some important results in line with the study objectives, while advancing to the start of Phase 2. This was an additional, yet important, outcome of this study because Phase 1 was considered mainly a feasibility study, according to the initial objectives.

The samples of Phase 1 enabled examination of quality assurance and effectiveness of the methods to be used in Phase 2. That is, any defects in samples would enable refinements in methods. These samples also provided an opportunity to study the optimum conditions for processing, storing, transporting and staining samples. Providing extra samples to test reagents and refine research methods for Phase 2 was an additional advantage of the samples produced, and, most importantly, they provided essential controls for some of the experiments in Phase 2.

6.5.5 Summary: The Study Objectives, Findings and Implications for PNG

The next section of this chapter, Part 4, discusses the conceptual and methodological challenges encountered, as well as the opportunities and lessons learnt while planning and conducting the study, and after the study. The current section has discussed the scientific contributions made by Phase 1 of the study to the field of HIV prevention.

Medical MC reduces heterosexual HIV transmission by 60%, as proven by three large randomised clinical trials (11, 13, 102). The results provided the scientific basis for the implementation of VMMC programs in some African countries ever since its

recommendation in 2007 by the WHO (7). The WHO recommended MC as an effective preventative measure against heterosexual HIV transmission in settings where HIV prevalence is high, most infections are transmitted through heterosexual sex, prevalence of existing MC is low, and appropriate sexual health counselling is available (7). However, it remains unclear what level of protection (if any) is provided by non-medical (traditional) forms of circumcision and foreskin cuttings that are prevalent in some parts of the world, such as PNG.

The situation in Oceania, especially in PNG, is special because one type of traditional foreskin cutting—the dorsal slit—predominates all other traditional circumcisions. Dorsal slit foreskin cutting is reported among nearly 50% of adult men in PNG (1). Anecdotal evidence shows that complete or near complete exposure of the glans and retraction of the remaining foreskin leads to significant keratinisation in many of those undergoing traditional or contemporary forms of penile cutting (146). These findings inspired this research to investigate whether there was any protection provided by dorsal slit foreskin cuttings against HIV transmission. This was important in order to answer the subsequent question: do dorsal slit foreskin cuttings need to be considered for remnant excision in any MC program that is proposed in PNG to prevent heterosexual HIV transmission?

Few epidemiological studies have investigated the efficacy of traditional MC in preventing HIV transmission (170-172). The epidemiological studies that have been undertaken have produced mixed results on the protective efficacy of traditional circumcisions against HIV transmission. A study in South Africa found greater HIV risk for partially circumcised men than fully circumcised men, but participant numbers were small. The researchers recommended early circumcisions for boys, and emphasised the importance of working with traditional surgeons to reduce the number of partial circumcisions (170). There were no details of traditional/partial circumcisions, nor did they state the level of exposure of the glans penis or IFS in those traditional circumcisions.

A recent study conducted by Maclaren et al. demonstrated a strong association between MC and dorsal slit foreskin cuts and HIV prevalence in PNG (172). In this study, 99% of the observed geographical variability of HIV prevalence in PNG was

explained by MC and dorsal slit foreskin cuttings ($p < 0.01$) (172). Although this provides indirect evidence for a protective role of the predominant dorsal slit foreskin cutting in PNG, further research is needed to demonstrate a direct relationship between dorsal slit foreskin cutting and HIV prevention.

No biomedical research has been conducted thus far to assess the relationship between traditional forms of foreskin cutting and HIV transmission. Therefore, it is imperative to find answers to the questions: Do alternative forms of foreskin cuttings provide protection against HIV transmission? If so, what is the level of protection? Could these forms of foreskin cutting be considered an alternative to medical MC in areas where foreskin cutting practices dominate medical MC? Given that medical MC is the only proven biomechanical protective measure against HIV acquisition, it is important to assess whether partial circumcisions (such as dorsal slit foreskin cuts) also provide protective efficacy against HIV transmission. This study attempted to fulfil this gap in research regarding traditional forms of circumcision and prevention of HIV.

PNG is a developing country in which there can be substantial challenges to conducting complicated laboratory-based clinical research, relating to limited resources and logistics. Taking this into consideration, the current researchers decided to initially conduct a feasibility study (Phase 1) in order to assess the possibility of conducting such research in PNG, before beginning the main study (Phase 2) comprising broader objectives. The field work of Phase 1 was completed successfully in September 2012.

As well as assessing the challenges of the study's conceptual, methodological (logistical) and scientific aspects, Phase 1 was able to contribute important knowledge to the field of HIV prevention. However, the main research questions are yet to be fully answered, and laboratory studies to achieve this target will be conducted during Phase 2.

The scientific objective of this study was to characterise the foreskins of Melanesian men who were uncircumcised or had dorsal slit foreskin cuttings, in order to investigate the level of protection that dorsal slit foreskins may provide against HIV transmission. To achieve this target, it was necessary to identify a few histological

features of the foreskin, and their role in HIV transmission. First, the researchers decided to determine the KT of the foreskin of Melanesian men as a baseline. The results indicated that the KT of the IFS of Melanesian men was thinner than that of the OFS. Further, they demonstrated an increase in the keratinisation in both the IFS and OFS of dorsal slit men after exposure to the outside for a period.

Two other histological features considered important for innate immunity (the SPL and RPL) were also investigated during the study. The results showed significant changes in the SPL in favour of dorsal slit foreskin cuttings improving innate immunity. However, the RPL results were inconclusive and require further investigation (especially focusing on the area covered by rete pegs, rather than a single measure of length) to evaluate their importance in the innate immunity of the foreskin. Apart from these findings' relevance to dorsal slit foreskin cuttings, it is also important to assess how they fit into the context of medical MC and its mechanism of preventing heterosexual HIV transmission.

The following implications can be drawn from the scientific findings of this study:

1. It is important to educate young men in PNG regarding partial circumcisions, and encourage them to seek safe medical MC until further research demonstrates the benefits and safety of dorsal slits.
2. There is a need for further research to finalise the findings of this study.
3. Future researchers planning laboratory-based clinical research in PNG should be informed of the various challenges and ways to negotiate these challenges to succeed in their research.
4. Future researchers should be informed of the importance of collaborating with local investigators in each step of the study.

Although some of this study's scientific findings were inconclusive, the concepts generated and experience gathered from this research can be considered important not only for Phase 2 of this study, but also for future research in this field.

Part 4: Discussion of the Challenges and Opportunities of Immunohistological Research in Resource-constrained PNG

6.6 Preface

Having presented all the challenges encountered during Phase 1 of the study in the previous sections, in order to answer Research Question 4, part 4 seeks to answer Research Question 5 on the measures needed to overcome such logistical challenges. Part 4 elaborates more on the conceptual, methodological and scientific challenges of conducting an immunohistological laboratory-based clinical study in resource-limited PNG, and the ways to find solutions to these challenges. Hence, this part discusses the various challenges and opportunities identified, and how those challenges were managed during Phase 1 of the study titled '"Straight Cuts" and HIV Prevention: The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings'.

This study, as one of the first immunohistological studies of this nature in PNG, provided a wealth of experience and imparted valuable lessons regarding the challenges of conducting such clinical research in PNG. The lessons learnt in Phase 1 (the pilot study) were instrumental for the success of Phase 2 (the main study), conducted at the same site one year later. The successful completion of the initial stages of Phase 2 is proof of the successful achievement of the main objective of this study. It is clear that this knowledge will hold important implications for future studies of this nature.

Prior to discussing the main challenges and opportunities, this part first provides background information to discuss the ethical and legal aspects of conducting research in resource-limited settings. Next, it discusses the challenges and solutions found to these challenges during the study, highlighting some of the similarities and differences found between the challenges of this study and other similar studies. The final section of the part is dedicated to discuss the researcher's interpretations of these challenges in view of their implications for similar studies in the future.

6.7 Clinical Research in Resource-limited Countries

Conducting research in a resource-limited setting is a challenge. This challenge is greater for clinical research that involves human subjects, and highest when a clinical study comprises a component of sophisticated bench work that involves a high degree of complexity. PNG is a culturally diverse country in Oceania where more than 800 different languages are spoken. It has been a source of numerous health-related research publications, including Nobel Prize–winning studies (173). However, quantitative studies that comprise complex immunohistological analysis of human tissue samples using modern immunohistochemical methods in PNG are scarce. The researchers of this study were aware of the challenge, although they were not completely aware of the degree and type of challenges of conducting this study, prior to its commencement in 2012.

Clinical researchers working in resource-limited settings must deal with similar issues encountered by researchers from resource-replete developed countries. However, there are additional issues that require special attention when conducting research in resource-limited settings (174, 175). Resource-limited countries are occasionally selected for clinical research. Compared to developed countries, the cost of running a trial is relatively low, mainly due to low salaries and perceived low overhead costs (176, 177). However, according to the experience of some of the researchers in the current research team, the validity of these beliefs is questionable in the PNG setting, where there are large overhead costs in conducting some clinical research. An important positive factor is the smaller amount of time required to enrol participants in resource-limited settings due to the high prevalence of some of the clinical conditions of concern (178). Although it was not involved with a clinical condition, this study in PNG provided the opportunity to collect all the necessary samples within a short period from a single location (through the MC clinic at the PAU). Nevertheless, the recruitment process was undoubtedly facilitated by the three-year partnership built between the PAU and JCU researchers.

Certain guidelines must be followed when conducting clinical research in a resource-limited developing country. A major set of guidelines was authored by the Council for

International Organizations of Medical Sciences (CIOMS), which replaced several other previous sets of guidelines. The CIOMS presented its *International Ethical Guidelines for Biomedical Research Involving Human Subjects* in 1982 (179). Revised in 2002, these guidelines were intended to guide resource-limited lower-income countries to apply the ethical principles documented in the Declaration of Helsinki (174). According to CIOMS Guideline 8, people in resource-limited developing countries should not ordinarily be involved in research that could be undertaken in developed countries. Further, research should be responsive to the health needs and priorities of the community in which it is conducted (180).

This particular biomedical laboratory-based clinical study justified the above guidelines in two respects. First, this was a study to explore a unique and important scientific problem in this particular developing country, PNG. Second, this study was an attempt to use an opportunity offered by the MC clinic at the PAU to collect foreskin tissues from a single location in a short period. (This would otherwise have involved a time-consuming process waiting for a few surgical clinics to collect foreskin samples from eligible participants.) Thus, this study can be considered one that addressed a locally important issue in the right time at the right place, giving researchers a unique opportunity to study different forms of foreskin cuttings and their level of protection against heterosexual HIV transmission.

Measures were taken to the fullest extent possible to conduct this study according to the international guidelines of biomedical research. It is important to note that this study experienced most of the challenges identified in the literature as arising when conducting research in a resource-limited country. Nonetheless, there were some unique, PNG-specific infrastructural, organisational, cultural and ethical challenges that needed to be overcome for the successful completion of this study project.

6.8 Infrastructural Challenges

The two main obstacles for conducting research in resource-limited developing countries are identified as scarcer resources and difficult working conditions (181). Of these, the availability of sufficient resources for investigators to maintain high

standards in the research and monitor safety standards during the study is important (181). It was observed in PNG that the trial capacity was limited in the laboratory-based clinical research mainly because of limited resources, rather than difficult working conditions. During preliminary visits, limited resources were recognised in areas such as infrastructure, human resources and research equipment at selected sites for this particular study in PNG.

6.8.1 Limited Infrastructural Facilities at Study Site

Inadequate infrastructure facilities for the research study at the primary study site were a major concern from the start of the study. The facility selected for MC operations was a basic clinic room at the PAU health centre that served the health requirements of university students and patients from a number of surrounding villages. The clinic was not designed (and hence not adequately equipped) for surgical operations, although it 'facilitated' some minor surgical procedures and initial treatment for major medical issues before transferring patients to the nearby POMGH.

The lack of several basic requirements was identified (as mentioned below) in this minor procedure room that was proposed to function as the 'operating theatre' for circumcisions during the MC clinic. Several infrastructure items at the PAU clinic needed to be amended prior to starting the MC clinic and study. To make the room a workable area, non-functioning air conditioners were replaced with a portable air conditioner for the surgical procedures. The lack of a proper operating light source was a challenge that could not be corrected, and the surgical team used a normal light source in the room for MC operations.

6.8.2 Research Equipment

According to Mbuagbaw, the difficulties and cost of importing scientific equipment and spare parts are a principle limiting factor for scientific research. They emphasised that this problem is seldom recognised by governments in most developing countries (171). During preliminary visits, limitations of both surgical instruments and research equipment were noted at the study site. For instance, the basic surgical instruments necessary for small procedures, such as MC, were not available at the PAU clinic.

Therefore, new surgical instruments and study equipment (such as onsite tissue-processing tools) had to be transported from Australia to PNG prior to beginning the project because they were not readily available for purchase in PNG.[1]

The tissue-processing facilities at the histopathology laboratory at the POMGH were satisfactory. New and up-to-date equipment used for histopathological procedures had been installed at the facility, which was reassuring for the study requirements for safe and successful processing of paraffin samples.

6.8.3 Consumables: Dry Ice

Searching for dry ice was the most time-consuming process of all preparation activities in this study. Dry ice was an essential consumable for frozen sample preparation and transport, without which the study could not begin and continue. Dry ice was preferred over liquid nitrogen in the methodology of this study because dry ice affords more flexibility in terms of logistics, as opposed to the relatively hard to find and difficult to handle liquid nitrogen. Previous studies conducted in resource-limited settings, such as African countries, have used dry ice successfully for sample processing and transport (95, 107). Therefore, using dry ice for frozen sample processing and transporting to Australia emerged as a possible alternative during the planning stage of the study.

6.8.3.1 Dry Ice in PNG

From early 2012, an exhaustive search was conducted using all possible means to explore the availability of dry ice in PNG. First, all major chemical importers in PNG (such as Beltek, Chemica, ORICA, Origin, BOC and Coca-Cola) were contacted and personally visited to check the availability of dry ice (or liquid nitrogen). Of these, BOC indicated that dry ice was available to purchase; however, it stated that there was a requirement to ship the required amount of dry ice from its manufacturing plant in

[1] As a courtesy, to help with the MC clinic, arrangements were made to hire a diathermy machine from Cairns and transport it to the PAU for the MCs during the preparation stage of the study and MC clinic. However, a change in the MC clinic dates due to ethical approval delays disrupted this plan, and the diathermy machine could not be rented and transported at short notice.

Lae (a province in the north) to POM—for which there is no road connection. After four months of follow up, BOC indicated their inability to supply dry ice due to unresolved technical issues in the production line.

Meanwhile, the search was extended to other possible institutions, such as universities. The histopathologist and laboratory manager of the POMGH suggested contacting the suppliers of consumables to the hospital laboratory and medical faculty facilities at the University of PNG. However, all these attempts failed, and dry ice became a difficult to find item for this research study in PNG.

6.8.3.2 Other Options for Dry Ice

Alternative options were considered when the local search for dry ice failed. As a first alternative, shipping dry ice from Australia to PNG was discussed, but had to be abandoned because the estimated cost exceeded the allocated budget. The second option was to travel with dry ice in personal luggage from Cairns to POM. According to the regulations of most commercial airlines, the dry ice allowance is limited to 2.5 kg per person, should be stored in a container that permits the release of CO_2, and should be labelled according to the International Air Transport Association regulations.

These guidelines were useful, yet transporting dry ice to PNG from Australia was no easier than searching for dry ice in PNG. The process involved liaising with several stakeholders, such as Qantas and Air Niugini airlines, AQIS and Cairns airport. The main challenge in transporting dry ice was the relatively fast sublimation period of dry ice, which is 2.0 ± 0.3%/h when packaged in commonly used quantities (182). This was a significant challenge, since the dry ice needed to be kept for several days at the study site to collect and process all the frozen samples before storing in a -80°C freezer. The third option was to consider manufacturing dry ice using CO_2 at the study site. However, this option also had to be abandoned because it required a costly dry ice producing machine, and therefore a substantial amount of capital investment.

With the failure to locate dry ice, the only option to proceed with the study was to revise the research methodology for frozen sample preparation. Instead of using dry

ice or liquid nitrogen to bring the temperature down to -80°C for frozen samples at the study site, samples were transported to the histopathology laboratory at the POMGH, where a -80°C freezer was located. The frozen samples were prepared close to the -80°C freezer, where tissue samples in OCT media could be snap-frozen by placing samples temporarily inside the freezer. This was one of the most difficult methods for snap-freezing samples, as the researcher had to expose his/her hands to subzero temperatures on multiple occasions, and subsequently risk frostbite damage to the fingers. Further, this method had the disadvantage of increasing the temperature of the freezer due to the frequent door opening, thereby causing damage to other samples already stored inside.

6.8.3.3 Dry Ice—Still Needed for Sample Transport

Although the sample preparation stage was accomplished using the -80°C freezer, dry ice was still a necessary item to transport the samples from PNG to Sydney. Despite previous unsuccessful attempts, the search for dry ice continued for another six months, with expectation of a change in conditions to enable access to dry ice in PNG. Meanwhile, the use of a dry shipper was considered as an alternative for sample transport because this device does not require dry ice or liquid nitrogen to be carried. Again, the high cost of dry shippers was a concern, given the limited budget for the project. Several attempts to find a dry shipper for lease from the JCU and a few other universities in Australia also failed because they were not available or had already been loaned to other research projects. The delay caused by all these failed efforts meant the frozen samples had to be kept locked in the freezer at the POMGH for eight months following collection.

With no other option available, the researchers decided to fly to POM with a small amount of dry ice—enough to bring samples from Phase 1 of the study back to Sydney from POM in June 2013. Eventually, the expensive option of sending a large dry ice shipment from Sydney to POM was selected for Phase 2 of the study to prepare and transport samples back to Australia securely and efficiently, without compromising sample quality and integrity.

6.9 Challenging Working Conditions (Organisational Issues)

According to Van den Broeck et al., difficult working conditions are another considerable impediment to the conduct of studies (181). Two main factors—poor organisational structure and function—are responsible for difficult working conditions for study projects in developing countries (181). A number of challenging organisational issues were encountered during this project, in different forms at different stages, as discussed below. However, on a positive note, it was also clear that recently developed research partnerships with international institutions have improved research capacity, and hence working conditions, in some fields in PNG.

6.10 Ethics Approval

This study was granted ethics approval from several institutions, including JCU (Australia), PAU (PNG), National AIDS Council (PNG) and Medical Research Advisory Committee (PNG). Ethics approvals took nine months from submission, and required five letters clarifying questions for the ethics committees. Lengthy ethics approval process from some institutions meant that the study had to be postponed from the scheduled start date in June 2012 to September 2012.

The first ethics applications for the project were submitted to the ethics committees of two universities on 18 November 2011, for which one university granted its approval on 22 of December 2011, with two amendments. After receiving the amendments, the other university granted its final approval on 10 February 2012. The ethics application to one PNG institution was lodged on 11 November 2011. This institution requested: (i) evidence of ethics approval granted from the two universities and (ii) lodgement of a fresh ethics application to the PNG Institute of Medical Research (IMR) because the original study protocol involved analysing serum and urine samples at the PNG IMR for STI screening of participants. The institution also requested a separate ethics approval from another institution dealing with medical research in PNG before the start of the study. At the end of this multi-stage process, the approval was granted for the study on 29 May 2012.

Obtaining ethics approval from the second PNG institution posed different challenges. First, new methods to communicate with this institution were needed because conventional methods (such as emails, letters and telephone calls) were not efficient. Second, continuous follow up and vigilance through regular reminders were required to prevent delays in responses in order to accelerate the ethics process, so that the study could be conducted at the scheduled date for the MC clinic. The ethics approval from the second PNG institution was granted six months after submitting the application, on 5 September 2012. Due to the delays in the ethics processes, discussions were held with PNG investigators and PAU authorities to postpone the MC clinic from June 2012 until the next semester break in September 2012.

6.10.1 Sample Transport

Once an import permit was obtained from the AQIS, the investigators flew to POM with dry ice one morning in order to transport the frozen samples back to Australia on the same day (the total travel period was well within the sublimation period of 2.5 kg of dry ice in a polystyrene cool box). The samples were loaded into a box filled with dry ice at the POMGH, and brought to the POM airport to be transported through a major airline in PNG. The airline officials at the terminal checked all documentation, including certification for transporting non-infectious human tissue samples, and obtained approval for the consignment from the head of the airline cargo department. The sample box was then accepted to be sent to Sydney in the cargo compartment of the flight, and the researcher embarked onto the flight.

However, just before the flight departed, the airline authorities decided to retain the sample box at the POM airport. Upon realisation of this situation a few hours later when the flight arrived in Sydney, speedy recovery of the samples stranded at POM airport was required to prevent damage to the samples due to changes in temperature. By the time the airline officials at POM were contacted and a local research investigator in PNG had arranged to recover the samples from the airport, the box containing tissue samples and dry ice had been exposed to ambient temperatures for more than 24 hours. This delay risked the sample integrity due to sublimation of the dry ice, and the damage was evident when the frozen samples were later stained at the

histopathology laboratory at the UNSW, which identified artefacts from ice formation due to temperature changes.

6.10.2 Permission to Use Hospital Laboratory Facilities

During the initial feasibility visit, successful discussions were held with the head of the pathology department and laboratory managers to obtain permission to use the laboratory facilities at the POMGH for paraffin tissue sample processing. Accordingly, official permission to use the laboratory facilities was submitted in writing to the hospital authorities five months before the start of the study (including a three-month delay due to postponement of the study). With constant follow-ups and a visit to the premises, the approval was obtained three days prior to the study beginning.

6.10.3 Use of Human Resources

Human resources are another challenge when conducting studies, especially studies with technical components. Expertise in the required fields was difficult to attain in PNG and, even when a technician was available, his/her enthusiasm for the research-related activities was low. The first difficult task encountered was identifying a histopathology technician with knowledge of and enthusiasm for research, which was needed according to the governing rules of the POM histopathology laboratory. Thus, the tissue embedding had to be shared between the main laboratory technician and principal investigator because the study investigators were not allowed to function alone in the laboratory to process the samples.

This restricted the final number of paraffin slides from 27 to 13 for the study because 14 of the samples embedded by the laboratory technician had orientation issues during embedding in the paraffin, making them unusable. (The training of a technician was deemed unnecessary because the laboratory manager reassured the researchers, and expressed confidence in the supporting technician’s technical expertise to support the particular study step.)

6.10.4 Import and Export Permits

Import and export permits are necessary to transport human tissue samples from one country to another. Obtaining import and export permits to transport samples from POM to Australia was another challenge that involved a complex, time-consuming process requiring back and forth communication over several months. The delay was significant, especially at the PNG end, in the absence of a separate institution for quarantine purposes to deal with the process.

The recommendation from the officers at the customs office at POM airport was communicated to us after their discussions of the issue with other parties over seven weeks. The message was that, as long as the study was approved by research ethics committees, transporting samples was not a concern for the quarantine services of PNG. According to the customs officers, there was no separate institution (such as the Therapeutic Goods Administration institution in Australia) to seek permission for export permits for human tissue samples. Alternatively, an import permit was obtained from the AQIS, but this was also a lengthy process that delayed sample transport significantly, and resulted in samples being stored in the freezer at the POMGH for eight months.

6.10.5 Logistical Challenges: Ground Transport in PNG

Finding a reliable and reasonable transport service is a challenge in POM. While there is a comprehensive public transport service in PNG upon which the majority of PNG people rely, it was difficult to find an appropriate alternative mode of transport to transport study samples. The majority of taxi services in POM have no fee regulation (fees are mostly dependent on the personality and bargaining power of the customer) and these taxis can have questionable security, especially for non-local or foreign passengers. Renting a car is expensive (the minimum charge for a rental car is approximately A$250 per day, which could not be borne by the limited budget of this project) and also carried risks, such as security issues for a foreigner. Therefore, the researchers used the option of transporting samples from the study site to the histopathology laboratory using the transport system provided to students of the PAU.

Samples were prepared throughout the day and needed to be transported to the laboratory on the same day, which was 17 kilometres from the study site.

During initial discussions, the PAU authorities kindly agreed to provide university transport facilities to transport the collected samples from the university to the POMGH, despite their limited facilities. However, when the study began and the samples were ready to be transported, the transport department found it difficult to arrange the bus due to their limited resources and unexpected university requirements on the day. This unanticipated situation affected the original project protocol. For example, some of the samples collected on day one (which needed to be frozen and stored in the -80°C freezer) could not be transported and had to be stored in the freezer compartment of a regular refrigerator, before being transported on the afternoon of day two. The day two samples encountered similar transport challenges, although they were ultimately transported using a PNG investigator's private vehicle, in the middle of the night on the same day.

6.10.6 Funding the MC Clinic and Study Project

According to Israel et al., conflicts frequently arise over funding in research projects. Common questions in these situations focus on who is the fiduciary of the funds, and how the funds are distributed (183). Funding was another challenge identified in this study, as it was an international study requiring high costs for logistics. The project was allocated a limited budget, although it was sufficient to complete the study successfully. Early discussions held with PAU authorities cleared potential funding issues that could arise during the conduct of the study, which prevented unexpected expenses.

It was important to clarify the sources of funds for the MC clinic and study project with the PAU authorities before starting the study. The MC clinic was considered by the PAU authorities to be a service for the students in order to prevent untoward complications among the students from traditional non-medical forms of foreskin cuttings. The MC clinic program at the PAU had started two years earlier and had been successfully conducted each year on the same premises. On both occasions, the clinic had been organised and sponsored by the university; similarly, the MC clinic in 2012

was sponsored by the PAU. However, the investigators supplied most of the essential basic surgical items for the clinic as a courtesy, despite funding the research study in total.

6.10.7 Security of the Researchers

Considering the unsafe situation in POM, assuring the security of the researchers was vital during the period of the project. The security threat for the investigators was imminent when the samples were transported to the POMGH during the first night of Phase 1 of the study. However, the risk was mitigated by using one of the PNG researchers' vehicles, and by the investigators being accompanied by a colleague with a strong physique when transporting the samples. The fact that the histopathology laboratory was situated inside the hospital protected the researchers from any security threats during sample processing.

6.11 Ethical Conduct of Research

Apart from the regulations of research, ethical conduct of the study was particularly important in PNG because it is a developing country with vast cultural diversity. Developing a culturally meaningful approach to informed consent, communicating the results to the stakeholders (PAU staff and students) and respecting the cultural values of the participants were important and valuable considerations to ensure the ethical conduct of this study.

6.11.1 Culturally Meaningful Approach to Informed Consent

Obtaining informed consent was facilitated by the local investigators during Phase 1 of the study in PNG. Discussions with local PNG investigators gave initial thoughts on how participants should be approached and educated in a culturally appropriate manner, prior to obtaining informed consent. During the recruitment stage, the local investigators approached male student leaders of the university who had a close relationship with other students to disseminate information on the MC clinics and associated study. The information from these student leaders and local study partners

guided investigators to maintain cultural sensitivity on several occasions throughout the project, as follows.

One of the proposals at the planning stage of the study was to conduct circumcisions at a different clinic site due to limited resources at the PAU campus. However, this idea was not welcomed by the students of the PAU due to privacy and cultural sensitivity issues. They believed that conducting clinics at a different site might compromise their privacy. Therefore, the researchers withdrew their initial plan to shift the clinic to conduct circumcisions at a different location with more facilities. Similarly, the study team was careful to recruit male investigators to interact with the participants to respect cultural sensitivity. Hence, information sessions, recruitment, gaining informed consent and most other activities at the study site during the MC clinics were conducted by male investigators. Special attention was given by the PAU authorities to recruiting male student nurses with whom the participants were familiar to work as surgical assistants during the operations.

The language barrier during the informed consent process was minimal in this study because all the participants were university students who were able to communicate in English. This made the informed consent process easier.

6.11.2 Communicating Results to the Stakeholders

Communicating the study results to the study participants is a fundamental requirement of any research. The research colloquia held at the PAU in September 2013 provided an opportunity for the investigators of this study to present the updated feedback of the results of Phase 1 to the university staff and study participants. This move was welcomed by the university, and provided the opportunity to inform the university of Phase 2 of the study.

6.11.3 Respect for Culture and Human Values

One of frequently mentioned challenges in conducting effective research is a lack of trust and perceived respect, particularly between researchers and participants (183). It was crucial in this clinical research to maintain trust, especially with the local

investigators and participants, during and after the study. As stated earlier, collaborating with local investigators, involvement in capacity building activities (prior to the project) and respecting local cultural and human values were crucial to building trust between foreign investigators, participants and all other stakeholders.

6.11.3.1 Post-MC Rituals

Responding to cultural rituals is a component of being sensitive to the local culture. After an important life event, men in PNG generally come together to celebrate. Participating in the MC clinic signified one of these significant occasions. Thus, the local investigators from the PAU responded to this by arranging a celebration for all the participants of the clinic at one of the male investigators' residences.

6.11.4 Adverse Effects on Participants

Adverse effects on participants are not uncommon during clinical research studies (181). There were no adverse effects reported during Phase 1 of the histopathology study; however, three adverse effects were reported during the MC clinic. This was brought to the investigators' attention during Phase 1, since the study and MC clinic had a close association. MC is a minor surgical operation during which minimal complications are expected (184). However, even minor surgical procedures in resource-limited settings can result in unexpected complications (185). One such incident was bleeding from the surgical wound of one of the participants, which occurred on day one of the MC clinic. The surgical team reflected that this adverse effect was mainly due to the lack of a diathermy machine among the surgical instruments to stop bleeding during operations. Two other minor bleedings were also observed as surgical complications during the two days of the MC clinic. These complications were reported by the participants to the study team, and the study team arranged for the surgical team to attend the incidents promptly. Although the surgeon managed to achieve haemostasis with ligations during the MCs, the need for and importance of having a diathermy machine was strongly emphasised in order to avoid the risk of such bleeding complications during operations.

6.12 Discussion—Lessons Learnt and Recommendations

Every research has its own challenges; nonetheless, this research study was all about challenges in conducting immunohistological research in resource-limited PNG. These challenges and the manner in which they were managed strengthened and paved the way for the successful conduct of Phase 2 of the study.

6.12.1 Infrastructure and Resources

The lack of infrastructure and resources posed the most difficult challenges during this study. Locating dry ice in PNG was one such difficult challenge, which compelled the investigators to plan Phase 2 of the study appropriately by ordering a shipment of dry ice from Australia immediately. Apart from dry ice, all other resource-related issues could be managed successfully during Phase 1, which built a solid platform for the start and conduct of Phase 2 one year later at the same study site.

It is important to understand that scientific research is becoming increasingly expensive (186). While contemporary research demands a range of sophisticated equipment, the cost of equipment and its maintenance is continuously increasing (186). Accordingly, maintaining scientific equipment can be a real problem, and unserviceable equipment is often found in laboratories in developing countries because there are insufficient human resources to undertake even minor repairs (186). The POMGH histopathology laboratory used in this study had similar issues, as evidenced by several abandoned unusable tissue-processing machines. However, the basic study equipment required for paraffin tissue processing was in a good, workable condition.

Resources can typically be underestimated by investigators who draft initial budget plans (181). Further resources and planning may be required for studies in developing countries, compared to similar-sized trials in developed countries (187). Added costs are likely to be required to cover issues of transport, communication and information technology, and the training of local investigators. It was observed in this study that, with proper budgeting and preparation, the resource and logistical problems in PNG could be easily overcome.

6.12.2 Organisational Issues

It is notable that this study was influenced by a few administrative issues of some institutions in PNG at different stages. These influences forced the study to be postponed by several months at one stage. This illustrates the presence of challenges apart from resource and logistic issues that can significantly hinder the successful conduct of a clinical study in a resource-limited setting. However, on reflection, as the investigator team from the JCU in Australia, the researchers had unrealistic expectations of how some procedures should occur in a disorganised research environment in resource-limited PNG.

Israel et al. described the two fundamental moral commitments of human clinical research. The first is to improve human welfare by advancing scientific knowledge of the condition in question. The second is to protect the dignity and health interests of the research participants (183). The current study clearly fulfilled both of these requirements, although one PNG ethics committee questioned the genuineness of the investigators of the project with regard to its objectives during the ethics application process. It was clear that these reactions could be a result of a long history of research from which there has been no direct benefit (and sometimes actual harm) to the community, and/or no feedback of the results to the community, which has developed into anger and suspicion (183). According to Israel et al., ‘once established, trust cannot be taken for granted; researchers must continually prove their trustworthiness’ (183).

Ethical review of research provides a safeguard for the participants. Brunet-Jailly described that the mechanisms and procedures for local ethical review in some developing countries are under-developed, probably due to a lack of appropriately trained people and resources, which leads to problems in establishing ethics committees (180). The situation was different in PNG, where there were several institutions established to review ethics applications for scientific research. Further, according to Brunet-Jailly, ethics committees can occasionally be physically, socially, economically or culturally removed from the population or community that is to be studied. As a result, it can be very difficult to obtain appropriate ethical review of clinical research proposals in some areas (180). However, the investigators’ experience

with the ethics committee members in the PNG institutions was that they were closely associated (socially and culturally) with all stakeholders of the clinical research conducted in PNG.

Brunet-Jailly further demonstrated that, even when there is recognition of the need and value of the ethical review process, and suitably trained and experienced people to participate, lack of finances can act as a major constraint (180). This was more relevant to one of the ethics committees in PNG, as the ethical review was given a low weighting relative to other pressing budgetary issues in the institution, which disrupted efficient granting of ethics approval for this project. Thus, according to the experience of investigators, ethics committees in PNG can be described as in a transition stage, moving towards the standards of ethics committees in developed countries.

In addition, the people in PNG are very sensitive and (possibly) vulnerable to exploitation due to their low socioeconomic status. This would explain why some of the ethics institutions expressed strong views towards foreign investigators. However, those misapprehensions were overcome and trust was built with the local stakeholders and other relevant institutions, mainly through working closely with local investigators, providing timely feedback and conducting the project in a culturally sensitive manner with the support of local investigators. In summary, investigators willing to work on research projects in PNG should be aware of these challenges and responsibilities, and be prepared well in advance by allocating sufficient time for areas such as planning the project and obtaining ethics applications.

The sample transport incident at the POM airport that led to disintegration of some of the frozen samples indicated that the unexpected action of a single person or institution can jeopardise the effort invested by investigators in a project. Although these incidents are difficult to predict, it is important to carefully go through all research steps with an open mind, rather than making negative assumptions, in order to clear any obstacles that could arise in the process of the research.

Generally, policymakers' reluctance to incorporate research findings into policy formation is considered one of the fundamental problems of policymaking in developing countries (175). This is supposedly because policymakers in these

countries give low priority and do not value research findings during the policymaking process. Stephenson and Hennink highlighted that the poor communication between researchers and policymakers is one of the main reason for this problem (175). The situation was not much different in PNG. For example, the National Department of Health (NDoH) in PNG has demonstrated limited willingness to receive the results of previous studies in order to make evidence-based policies in some areas, such as in the field of HIV and AIDS.

A national policy forum for HIV/AIDS was organised in 2011, in which researchers working on research projects of HIV/AIDS in PNG were invited to participate and provide their expert views on combating the HIV epidemic in PNG, with special emphasis on MC and its applicability in the PNG context. Many participants and institutions showed great enthusiasm towards this research project and its outcomes during the meeting, and assured their support for the study. The forum finally emphasised the importance to the NDoH of implementing and supporting some of these projects, and considering research outcomes during policymaking. The NDoH indicated a positive response to the proposals and future research on preventing HIV in PNG, which demonstrated a successful completion of the policy forum. This was not only encouraging for current and future researchers of projects in PNG, but a good sign for people in PNG, who would enjoy the benefits of quality research.

6.12.3 Administrative Bottlenecks

According to Mbuagbaw's analysis, administrative bottlenecks can seriously impede or delay studies, potentially rendering studies meaningless or outdated by the time they are able to start. This is a serious issue that should not be overlooked in discussions regarding research impediments (178). It was evident that slow administrative processes equally hindered the progress of this study at several stages, and created challenges to overcome.

6.12.3.1 Deadlines

Some PNG institutions made it difficult to meet the necessary research deadlines. This could be due to their limited experience and knowledge in research, and the absence of a research culture in those institutions.

6.12.3.2 Limitations in Information and Expertise

Some officers in a few institutions in PNG seemed to have unclear duties and roles. This was clear when no one at the customs/quarantine services at POM airport knew the exact procedure involved in importing and exporting biological samples to and from PNG, or where to seek further information. The situation sometimes ended by providing inaccurate information regarding quarantine permits for transporting samples overseas. Further, there was no documented or online information available on the process. This common administrative pitfall in some institutions in PNG is a significant barrier to the administrative requirements of research, and should be considered closely.

6.12.3.3 Lack of Guidelines

Some of the institutions with which the investigators interacted during Phase 1 (especially administrative interactions) had neither guidelines to follow nor responsible and/or knowledgeable personnel to guide their clients in the relevant fields.

6.12.3.4 Recommendations

From reflecting on the experience of this study, it is recommended that having an administrative officer dedicated to handling administrative issues should be considered in future research studies. This would release the burden of administrative activities and allow the investigators to devote more time and focus to the scientific aspects of the study.

6.12.4 Ethical Conduct of the Study

The ethical conduct of clinical research does not end when informed consent is obtained from the participants (188). Researchers are obligated to the participants and host community to develop and implement procedures to maintain the confidentiality of the information collected, respect participants' rights to withdraw, inform participants about the progress of the study and manage any complications arising from participating in the study (188). This study fulfilled all the above requirements.

Confidentiality was a fundamental requirement, both ethically and culturally, because this study collected sensitive information from the participants. This process was greatly supported by the local PNG investigators, who were knowledgeable on sensitive issues of local cultures in PNG. Delivering updates to the host institution and participants was both a moral commitment and a fulfilment of ethical duties. This was warmly welcomed by the host institution (PAU), and further strengthened the trust of participants and local community in the study investigators.

Misunderstandings and miscommunication about scientific research are likely when investigators and participants speak different languages; hence, informed consent documents need to be translated in the absence of equal expressions for local languages, and when the notion of informed consent is unfamiliar (189). Both the process of informed consent and the delivery and distribution of information to a wide range of participants were easier when local investigators in the study team were involved in the process. Therefore, having partnerships is a key to succeeding in good ethical conduct of studies in culturally diverse PNG.

Another important aspect for successful study projects in PNG is adapting to cultural differences and responding appropriately to respect those cultural differences. Again, local investigators can play a significant and an important role by participating in planning the study to ensure that the study protocol is culturally sensitive.

6.12.5 Other Important Issues

In a research project, adverse medical, social and psychological events are not predictable, but must always be managed (188). Although it was not part of the research project (Phase 1), an adverse situation was experienced when three participants in the 2012 MC clinic presented with bleeding complications following their MC operations. The adverse event rate in this study was 9% compared to 3.8 %, 1.7% and 8% in 3 major RCT s conducted in Africa (11, 13, 102). This situation was managed successfully by the surgical team, yet it alerted the study investigators to act and prepare protocols to deal with such situations in future studies (when and if the adverse reaction is related to the research study). It is recommended to have standard operating procedures to deal with unexpected situations in future clinical research studies. According to Lang et al., operational tools (such as standard operating procedures) need to be easy to follow, culturally sensitive and a guide for both inexperienced and skilled staff (174).

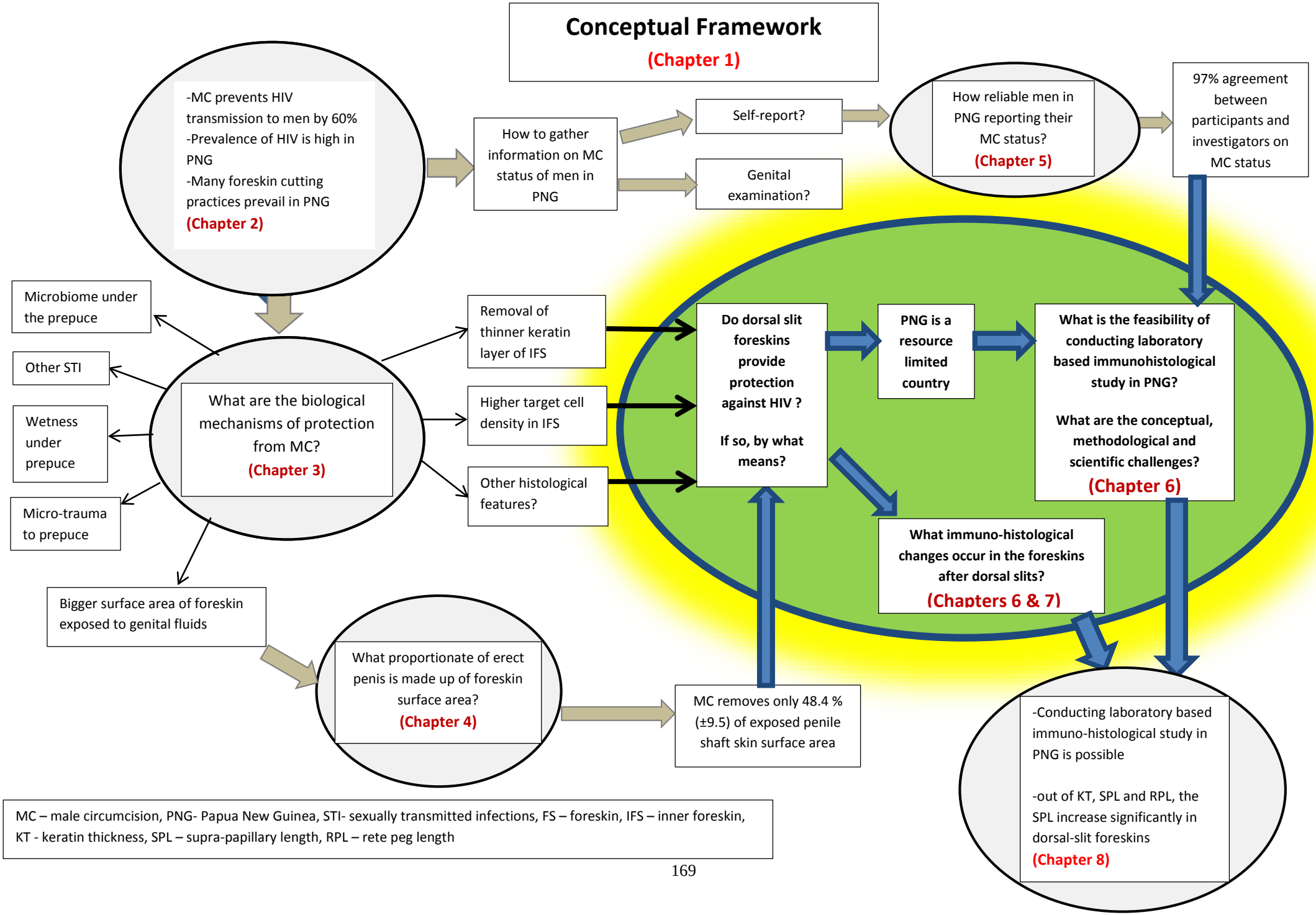
Even if all other requirements are fulfilled, financial bottlenecks can still disrupt the smooth conduct of a study due to unexpected expenses, especially when the research is conducted in a resource-limited setting, such as PNG. Yet again, including local investigators in the planning stage of the study can help reduce unforeseen expenses. It is recommended to draw budgets for research projects in PNG after extensive groundwork in order to avoid unexpected situations that could significantly affect the progress of the study. Apart from this, during the planning stage of the study, special attention should be given to ensure the safety and security of researchers during their stay in PNG.

6.13 Conclusion

Prior to this study, there was uncertainty among the study investigators on the possibility of conducting and completing this complex immunohistopathological study successfully in resource-limited PNG. Examples in the international literature of such research studies in resource-limited settings are scarce. However, it was necessary to

be proactive to find answers to the important research question of whether common forms of foreskin cutting in PNG are protective against heterosexual transmission of HIV. Thus, the challenge was recognised, a feasibility study was planned and the study (Phase 1) was implemented successfully. A multitude of different challenges were encountered throughout the study, for which solutions were found effectively, thereby ensuring the success of Phase 2 of the study that followed. The experience gathered was invaluable, in addition to the set of good quality paraffin samples (which was a bonus of Phase 1) that provided useful material for high-quality laboratory work at the UNSW in Sydney and Hope's Laboratory in Chicago, US (the details of these studies are yet to be published).

Internationally, the experience of this study further endorses that researchers from developed countries who conduct studies in developing countries have a great responsibility beyond the successful completion of their research projects. Building partnerships, exchanging research skills, being aware of the presence of administrative bottlenecks and understanding and respecting the local culture must be essential components of foreign investigators' research agendas.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 7: Phase 2 of the Study

7.1 Main Objectives of Phase 2

The success of Phase 1 (the pilot study) confirmed the feasibility and successful conduct of Phase 2 (the main study). Phase 2 was conducted with broader objectives at the same premises one year later. In addition to the investigators of Phase 1, several new investigators participated in Phase 2, representing different fields of HIV prevention. With expansion of the objectives and other developments, such as new international collaborations in the project, the researcher's participation in Phase 2 was limited to the role of a co-investigator, while a senior researcher from the UNSW took over as the principal investigator.

All the scientific objectives of the original project '"Straight Cuts" and HIV Prevention: The Immunohistological Correlates of Dorsal Slit Foreskin Cuttings' were to be tested during Phase 2. This included an additional objective apart from the scientific objectives of Phase 1. The objectives of Phase 2 were to determine:

1. the histological changes (KT, RPL and SPL) in the epidermis of the foreskin after dorsal slit foreskin cutting
2. the changes occurring in the target cell density of the IFS and OFS in a period after dorsal slit foreskin cutting
3. the efficiency of viral penetration through the dorsal slit foreskin, compared to the uncircumcised foreskin.

As expected, Phase 2, which was conducted one year later in 2013 at the same study site, was significantly supported and guided by the experience gained and lessons learnt during Phase 1.

7.2 Positives Outcomes from Phase 1 for Phase 2

By definition, a pilot study is a version of the main study that is run in miniature in order to test the ability of all the components of the main study to work together (190). Phase 1 of this project can be considered a miniature version of Phase 2 in terms of all

the important stages of a research study. Phase 1 mainly focused on factors such as processes, resources, management and scientific aspects, which were vital aspects of the main research study.

Process (the study planning) assessed the feasibility of the steps that needed to occur as part of Phase 2. One important step was determining participant recruitment and retention rates. The recruitment process was relatively easy during Phase 2 because of the experience gained by investigators in recruiting participants during Phase 1. Most participants were aware of Phase 2 prior to participating in the information session. A number of participants attending the information session were students who did not get the opportunity for circumcision during the previous MC clinic in 2012 due to the limited available spaces. The participant information sessions in Phase 2 were conducted in a similar manner to the sessions in Phase 1 to inform the participants of Phase 2 of the study and MC clinic.

The next focus of Phase 1 was to assess how resources could be managed during Phase 2. Phase 1 dealt mainly with issues such as assessing the total time required for each step of the project, any budget problems that could arise during Phase 2, and the minimum resources needed for the MC procedure. Any time management or budgetary issues could efficiently be managed based on the experience from Phase 1. For example, the preparation of the university health clinic for the MCs was well planned for Phase 2 by arranging surgical instruments and research equipment well in advance. The problem of dry ice, which was one of the main challenges during Phase 1, was also managed successfully by transporting it in adequate amounts before beginning Phase 2.

From the management perspective, Phase 1 initially helped to set the infrastructure at the study site in all required aspects, including human resource management. Phase 1 informed and educated the university population about the close relationship between the MC clinic and histopathological study conducted on their premises. It was mandatory to update on the progress and results of Phase 1 to the PAU staff and study participants. This was important to convince them of the usefulness of conducting Phase 2 to achieve the final objectives of the project. This further helped investigators of Phase 2 prepare the study site in advance by obtaining ethical approval from the

institution in a timely manner, and gaining the support of other human resources at the university for Phase 2. The ability to streamline data management in Phase 2 was another advantage gained from the experience of Phase 1.

Substantial progress towards the scientific objectives was also achieved through the work of Phase 1. Sample preparation, processing and analysis were major areas of focus during Phase 1, and the lessons learnt in each of those areas helped immensely to improve the comparable procedures during Phase 2. The knowledge acquired regarding choosing appropriate sites for tissue sampling from the entire foreskin, the minimum size of the required tissue samples, and the orientation of tissues during embedding were useful to prepare better quality samples with minimal errors during Phase 2. Further, knowing how to package the samples for storage and which storage conditions to use until the samples were transported to the laboratory were helpful for sample preparation in general. Paraffin sample preparation was especially supported by the knowledge gathered during Phase 1 regarding the tissue dehydration process, embedding, microtomy and staining. Similarly, for the preparation of frozen sections, the sample freezing methods, transport of samples and immunofluorescent staining methods (especially in terms of selecting the optimum antibodies for immunofluorescence) were important.

7.3 Challenges Encountered during Phase 2

However, the main study was not completely free of challenges and problems. Two such prominent issues during the sample collection stage of Phase 2 included: (i) a security threat to the investigators during the laboratory work in POM and (ii) issues encountered in getting the dry ice consignment released during transport of the dry ice from Sydney to POM.

7.3.1 Security Threat

A PC2 laboratory facility was needed for Phase 2 because it involved in vitro HIV penetration studies through human foreskin tissues. Phase 2 conducted its initial laboratory work at a PC2 laboratory facility at PNG's IMR in POM, which was

situated in a different location to the histopathology laboratory of the POMGH (this facility was not available during Phase 1). This was associated with a new challenge in which the security of the investigators had to be ensured, particularly during the night, as some of the work needed to be finished on the same day as sample collection. Although there was no incident reported, one researcher from the UNSW was under stress and anxious about his security in the absence of security personnel at the premises when working at night, as the area was known for previous security breaches and criminal activities.

7.3.2 Release of Dry Ice at Customs

Despite all the difficulties, challenges and lessons learnt during Phase 1 related to dry ice, new challenges were encountered for dry ice transport during Phase 2. This issue was encountered at the international air cargo terminal in POM during Phase 2 in 2013. According to the plan, a large consignment (25 kg) of dry ice was sent from Sydney to POM, which was enough to complete Phase 2, including transporting samples back to Sydney at the end of the study. This was sent to POM through air cargo two days prior to starting the MC clinic. During the process, there was a significant delay in the loading process in Sydney. Similarly, the release of the shipment was difficult at the air cargo department in POM because the shipment was flown into POM on a public holiday. Since a delay in the release of the dry ice could have disrupted both the dry ice and study plans, measures were taken immediately to have the dry ice consignment released via the personal contacts of the PNG researchers.

7.4 Overall Success and Results of Phase 1

According to Arain et al., if a pilot study shows significant results, researchers may find it unnecessary to conduct the main trial (191). They further elaborated that some pilot studies can be considered substantive, and that the data from those pilot studies can contribute to the final analysis. These types of studies are referred to as ‘internal pilot studies’ (191). Phase 1 of this study can be considered a substantial study that was able to produce important results on its own. The majority of pilot studies are complete studies conducted with smaller sample sizes to test a number of

methodological components and clinical outcomes simultaneously. However, since pilot studies tend to be small, the results should be interpreted with caution (192). In contrast to most pilot studies, Phase 1 of this study was completed with a similar number of participants to Phase 2, thereby improving the validity of the methodological outcomes and results of Phase 1.

The feasibility and logistical assessment, as well as the experience gained can be considered the most important outcomes of Phase 1, as opposed to the results. Alternatively, if the researchers identified faulty procedures or the results were unfavourable during Phase 1, Phase 2 was less likely to be continued or considered useful. According to Thabane et al., 'no study is a complete failure; it can always be used as a bad example!' (190).

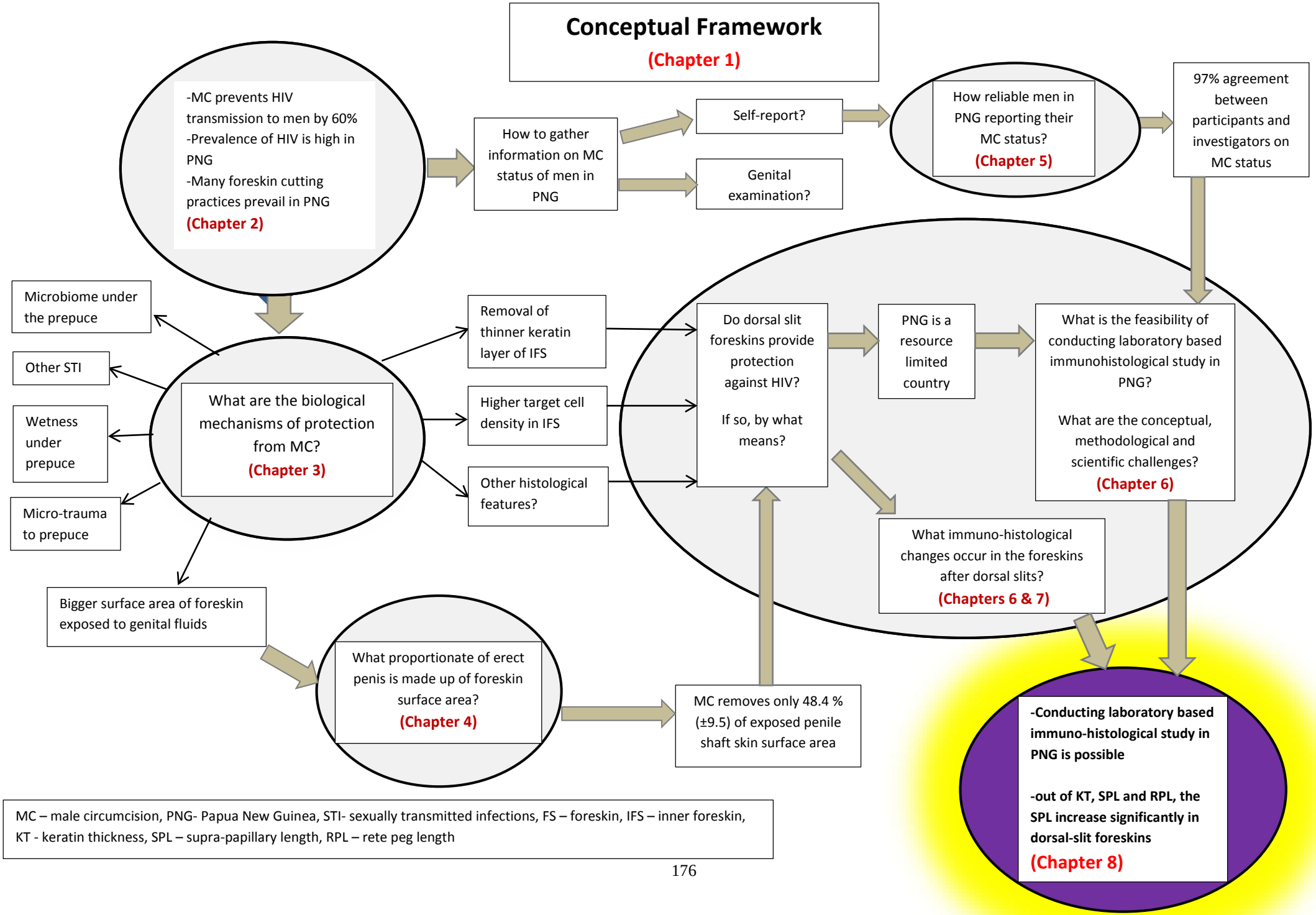
According to the criteria of a successful pilot study, based on primary feasibility objectives (190), Phase 1 was a certain success. This study not only enabled the researchers to interpret the results of Phase 1, but also enabled them to determine the feasibility of proceeding to Phase 2. Therefore, the recommendation at the end of Phase 1 was to continue the study into phase 2 with a slightly modified protocol—and the successful completion of phase 2 was expected to be feasible with the modifications.

7.5 Phase 2 to Finalise the Work

The smooth operation of Phase 2 was important to achieve the main objectives of the research. With leading researchers in the field joining the team, Phase 2 was intended to attain breakthrough findings in the field of dorsal slit foreskin cutting related HIV prevention. The objective of conducting viral penetration studies was a new addition to Phase 2, and the researchers planned to conduct the majority of the work in PC3 laboratories with the necessary facilities.

A substantial amount of work has been completed in Phase 2 to date. Paraffin and frozen samples have been prepared and are being analysed following similar protocols to Phase 1. Additionally, at the study site, the initial step in this viral penetration study

to assess the level of viral penetration through the exposed foreskins of dorsal slit men, compared to the foreskins of uncircumcised men, has been conducted. Some of those samples are being analysed at the UNSW, and the others were transported to the Tom Hope Laboratory at Northwestern University, Chicago, US, for viral penetration studies. It is believed that several important, unanswered questions regarding dorsal slit foreskin cutting in relation to HIV prevention will be answered at the conclusion of this project. The results from Phase 2 are, as yet, unpublished.



MC – male circumcision, PNG- Papua New Guinea, STI- sexually transmitted infections, FS – foreskin, IFS – inner foreskin, KT - keratin thickness, SPL – supra-papillary length, RPL – rete peg length

Chapter 8: Conclusions and Recommendations

Over the duration of this study, the importance of MC as a tool that could contribute to an AIDS-free generation has gained momentum and been joined by other highly effective measures. A combination of preventative methods is the main strategy currently identified in the global move towards an AIDS-free generation (193). The three core measures identified in this strategy are antiretroviral therapy for people living with HIV, PMTCT and VMMC (193). Of these three, MC is unique because it is a once-in-a-lifetime preventative measure against HIV for individuals. Further, this biomedical intervention has efficacy closer to a vaccine, and may be the most cost-effective intervention of the three at present (194).

The attention focused on VMMC increased after three randomised clinical trials that demonstrated the protective efficacy of 60% for MC in reducing heterosexual HIV transmission. These studies further demonstrated the susceptibility of the male foreskin to HIV by proving decreased male acquisition of HIV infection after circumcision (11, 13, 102). When HIV prevalence decreases among circumcised men, HIV prevalence among women also decreases indirectly, and hence also decreases among those women's uncircumcised male sexual partners. Ultimately, this could reduce HIV prevalence in the total population over a period of one to two decades (193).

The protective efficacy of partial circumcisions is yet to be evaluated. Almost 50% of the male population in PNG possess a distinctive partial foreskin cutting—a dorsal slit, which is performed as a cultural practice (1). With moderately high HIV prevalence in PNG, the level of protection from HIV by dorsal slit foreskin cutting emerged as an important question for HIV prevention programs launched in PNG. Epidemiological evidence is being gathered on the possible protection offered by dorsal slit foreskin cuttings. A recent study demonstrated the strong association between MC and dorsal slit foreskin cuts with HIV prevalence in PNG (172). In this study, 99% of the observed geographical variability of HIV prevalence in PNG was explained by MC and dorsal slit foreskin cuttings ($p < 0.01$) (172).

All the research documented in this thesis focused on one main objective: to assess the effectiveness of dorsal slit foreskin cuttings compared to medical MC in the context of heterosexual HIV transmission. This thesis considered other questions in order to answer the main research question. One such question was to determine the relationship between the surface area of the foreskin and HIV transmission risk reduction in order to better understand whether a procedure that does not remove any foreskin (such as dorsal slit foreskin cuttings) can provide any degree of protection against HIV. The researchers felt that it was unlikely that HIV risk reduction due to MC was simply a function of reducing the penile skin surface area. However, no previous studies had investigated this factor. A finding that the degree of HIV risk reduction was not explained by a reduced penile skin area would support the need to identify other factors and biomedical changes that occur after dorsal slit foreskin cutting.

It was also important to assess the reliability of PNG men in reporting their MC status. The accuracy of self-reported MC data could be important in the event of launching a VMMC program in PNG, in which collecting MC data by clinical examination would be a challenge. Prior to conducting the immunohistological study, it was also essential to assess the feasibility of conducting such a study in resource-limited PNG. Although laboratory-based scientific research has been conducted successfully in PNG previously, this project encountered some unique challenges because it involved international collaborative work and complicated laboratory procedures.

The study titled ‘Exposed Foreskin Surface Area and Protection from HIV’ addressed the relationship between the surface area of exposed foreskin (for sexual fluids during sexual intercourse) and the level of protection provided by MC. The study recruited 11 men among whom the total foreskin area was calculated using photographs of their erect penises. The results demonstrated that the surface area of the removed foreskin only resulted in a 47% reduction in penile skin area, compared to the 60% protection demonstrated in large randomised clinical trials. Thus, these results indicated that there are factors other than the simple removal of foreskin surface area determining the protection conferred by MC. Thus, it could be assumed that dorsal slits (which open the glans penis, but leave the entire foreskin intact) might also provide protection against HIV through other changes, such as keratinisation of the IFS, changes in

microbiomes of the preputial space, and maintenance of a dry environment in the preputial space.

These results rationalised the need to conduct research to determine whether there is any protection provided by dorsal slits, and, if there is, the level of protection provided. If there was sufficient biological plausibility for the hypothesis that a circumcision involving a dorsal slit provides protection against HIV, this would have significant implications in the context of PNG, where evidence-based advice needs to be provided to nearly 50% of the male adult population with these dorsal slits. Given that this was a simple and small study recruiting a small number of participants, studies with a larger number of participants with improved methodology to measure the surface area of the foreskin would strengthen the results of this study.

The reliability of men in PNG in reporting their MC status was also investigated during this study. Due to the background of various reliability levels being reported in studies from the African continent, it was important to conduct this study to assess the level of reliability in reporting MC status among men in PNG. This study used genital photographs of men who participated in the study titled 'Foreskin Cutting Beliefs and Practices and the Acceptability of MC for HIV prevention in Papua New Guinea', which started in 2010 in four sites of PNG. The clinician's assessment of those photographs was used to determine the agreement between the participants' self-reporting and the clinician's classification of MC status. The results of the study demonstrated high agreement (97%) in self-reporting MC status with clinician assessment of genital photographs of men in PNG, thereby indicating a high reliability of self-reported MC status. The findings of this study were important not only for this project, but also for any future project related to MC and HIV prevention in PNG, in which data on the MC status of men in PNG can be reliably obtained from a well-constructed questionnaire. High reliability in self-reported MC status can reduce the cost of mass-scale projects, such as VMMC programs, by means of cutting the additional costs, resources and time required to conduct physical examinations to clarify the participants' MC status.

The next step was to work towards the main objectives of the study. These objectives were to assess the possible correlates of the protection provided by dorsal slit foreskin

cuttings against heterosexual HIV transmission. This was to be achieved via several steps:

1. establishing the normal foreskin tissue structure of Melanesian men
2. examining the histological changes occurring in foreskins after dorsal slit foreskin cutting
3. analysing the difference in tissue penetration by HIV between uncircumcised and dorsal slit foreskins.

Prior to starting this sophisticated histopathological study, several issues had to be managed to assess the feasibility of conducting such a study in PNG.

It is well known that conducting clinical research in a resource-limited setting is a challenge to any investigator. A similar situation was expected in PNG, which is a resource-limited setting, especially when conducting a laboratory-based immunohistological study. Hence, the researchers decided to conduct a pilot study (Phase 1) that limited its scientific objectives to examining the histological changes occurring in foreskins after dorsal slit foreskin cutting. The idea was to assess the feasibility of conducting such a study in PNG prior to conducting the main (Phase 2) study with broader objectives.

Phase 1 was conducted in 2012, with the participation of 27 men from the PAU. Conceptual, methodological (resource and logistic issues inherent to a resource-limited setting) and scientific challenges were encountered and dealt with during Phase 1, providing valuable lessons in conducting such a study in PNG. This clarified most of the uncertain areas in the process, resources, management and scientific aspects of conducting a laboratory-based immunohistological study in PNG. Apart from assessing the feasibility, the study also produced good quality samples and therefore provided some important results that strengthened the entire project. At the end of Phase 1, the investigators had ‘road tested’ a proven methodology in conducting laboratory-based immunohistological research in PNG by identifying the associated limitations and challenges.

Phase 1 also demonstrated how to work with the people of PNG during a research project by means of building partnerships. It demonstrated the importance of capacity building (which occurred mainly prior to the study) as another essential aspect in

conducting research in such a resource-limited country. Cultural sensitivity and adaptability to local conditions were other important lessons learnt from Phase 1. All these lessons on feasibility and logistics, as well as the new scientific knowledge added to the field, made this research study a successful translational laboratory-based research in resource-limited PNG.

Through the scientific output, Phase 1 demonstrated that the keratin layer thickness of the IFS is thinner than that of the OFS in uncircumcised men in PNG. These results align with other research conducted internationally (24, 91). Further, it demonstrated that SPLs are an important measure, and are subject to increase in length on exposure to the outside environment for a substantial period (more than six months in this study) after the dorsal slit procedure. The change was mainly found in the IFS of men who had a dorsal slit, where the SPLs were increased significantly, compared to what was found in the uncircumcised men. This could possibly confer a decreased risk by means of an improved physical barrier to outside invasion of pathogens, including HIV. This finding is important for any research trying to measure the protective efficacy of dorsal slit foreskin cuttings against HIV transmission. This correlation between SPL and HIV transmission needs to be further examined with future research using immunohistological methods, along with the in vitro viral penetration studies planned in Phase 2, in order to determine whether this is a significant factor for protection against HIV.

The assessment of RPLs and their significance was inconclusive, partly because of doubts (by not covering the variability in the morphology of the rete pegs –see section 6.5.2.2) regarding the validity of the rete peg measurements. As identified during the study, the rete pegs showed significant polymorphism naturally in the anatomical structure, making simple measures of lengths from the basement membrane to the tip of the rete peg useless. It was realised that this drawback could be avoided by measuring the surface area covered by the rete pegs, instead of the lengths.

With help from the experience gained and lessons learnt during Phase 1, the main study was conducted successfully in 2013. Essential tissue slides (both paraffin and frozen) were produced and processed for the analysis. The initial steps of viral penetration studies—the most complicated aspect of the entire project—were also undertaken

successfully at the study site, and further analysis of samples is currently being performed in two prestigious laboratories in Australia and the US.

Several gaps in knowledge were identified during Phase 1. These need to be addressed to further understand the level of protection offered by MC against HIV transmission, and the (potential) protection offered by dorsal slit foreskin cuttings against HIV transmission. The identified knowledge gaps and research questions from this PhD project are as follows:

1. What are the external physical factors that could change the keratin layer thickness in the foreskins of dorsal slit men?
2. How much of the internal physical properties (such as moisture, pH and number of cell lines in the keratin layer) affects the resistance/innate immunity of the foreskin against HIV transmission?
3. What histological changes occur in the glans penis and distal urethra after dorsal slit foreskin cutting?
4. What changes are brought to the composition of microbiomes of the preputial space after dorsal slit foreskin cutting?
5. What is the rate of acquiring mucosal disruptions on the foreskin during sexual intercourse after dorsal slit foreskin cutting, compared to uncircumcised men?
6. What level of protection is offered against ulcerative STIs by dorsal slit foreskin cutting?
7. What is the significance of the volume of rete pegs in preventing HIV entering the foreskin?
8. Does the SPL of the foreskin have a role to play in preventing HIV or any other infective agent transmission into the foreskin?

The answers to these questions would not only improve knowledge on the potential mechanisms of protection offered by dorsal slit foreskin cuttings, but also broaden knowledge on the mechanisms of protection offered by MC against heterosexual HIV transmission, which are still unclear.

The following recommendations are made for future research in relation to dorsal slit foreskin cuttings for HIV prevention in PNG:

1. undertake epidemiological studies to establish the protection provided by dorsal slit foreskin cuttings against HIV transmission

2. confirm the findings for the rete pegs in this study with different methodology to measure the surface area/volume covered by rete pegs, rather than the length of the rete peg from the base of the stratum granulosum
3. undertake research on other physiological and histological factors that may be related to the protection offered by MC
4. continue to foster further partnerships with local investigators in PNG to enhance research capacity and thereby further research in the field of MC and HIV prevention.

The work initiated in this study continues through Phase 2 of the research in order to achieve the main objectives of the project. The final outcome of the entire project will help answer an important question regarding the most common traditional MC in PNG, which will be a small, yet important, step forward to achieve the ultimate goal of an AIDS-free generation.

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Appendix 1. Information Flyer for participants

Appendix 2. Information on Medical Circumcision for Participants

Pacific Adventist University
EDUCATE TO SERVE

Circumcision; All you need to know

What is a circumcision?

Circumcision is the surgical removal of the foreskin of the penis.

What does the surgery involve?

The procedure can be carried out either under a general anaesthetic (when you are asleep) or local anaesthetic (where the area is numbed and you remain awake). The foreskin covering the head of the penis is gently cut away and the remaining skin is then stitched back using dissolvable stitches. The procedure usually takes about 20-30 minutes.

What are the risks?

Circumcision is a commonly performed procedure. However, as with any surgery, complications do occasionally occur and may include:

- Post-operative bleeding, requiring further corrective treatment
- Unpleasing cosmetic results.
- An infection that may need to be treated with antibiotics.
- On rare occasions, a change in sensation of the penis, either during sexual intercourse or, an awareness of increased sensitivity at the tip ('head' or 'glans') of the penis, when not having sexual intercourse. This increased awareness may actually be felt as discomfort or pain in some cases.
- On rare occasions, narrowing of the urinary opening (meatal stenosis) may occur. This may impede urination and require corrective treatment.

How long will I be in hospital/clinic?

Circumcision is a day case procedure and you will normally be able to go home as soon as you feel comfortable. If you have a general anaesthetic, you will need to stay in the unit for a minimum of two hours after your surgery.

What happens before the operation?

Prior to surgery, you will need a pre-operative assessment. This may be performed using a health questionnaire at the pre-assessment clinic.

You should bath or shower before coming for surgery. In preparation for your discharge it is advisable to wear comfortable, loose fitting trousers.

You will be asked to change into a theatre gown. The surgeon will answer any questions that you have.

What happens after the operation?

Your blood pressure, pulse and wound will be checked. You will be offered light refreshments as soon as you feel ready. The nurses will let you know when you can get up.

Some pain is to be expected around the wound site. The nurse will check how you are feeling and give you painkillers, if necessary. A small amount of bleeding from the wound may also occur.

Nurses will monitor the wound site and apply further dressings, if necessary.

You can usually go home when you are comfortable and the effects of any anaesthetic have worn off.

After discharge, you are advised to see your clinic nurse for any follow up requirements. If the surgeon does wish to see you again, you will be notified of this prior to discharge and an appointment card will be posted to you.

What activities will I be able to do after my surgery?

You can return to normal physical activities when you feel comfortable, although it is wise to avoid heavy or strenuous activity for the first couple of weeks. It is important to avoid sexual activity for at least six weeks as this may cause pain and bleeding.

How much pain can I expect?

It is normal to experience some pain and soreness around the wound site, particularly over the first few days. It is therefore important for you to take painkillers regularly over the first two to three days (but remember that you should not exceed the stated maximum daily dose). If the level of pain is still not acceptable to you, your clinic nurse should be able to offer you advice. You may notice some discomfort for several weeks after the operation.

How do I care for my wound?

You can remove the wound dressings the day after your surgery. You should then shower or bathe daily. Over the first week or so, you may notice a sticky discharge around the wound that should be gently washed away. Soap and water is entirely adequate and you may wash as often as you wish. Between washes it is important to keep the area clean and dry until healed. Most stitches are self-dissolving and will dissolve after a few weeks. Marked swelling and bruising of the penis is common after surgery and should subside over a couple of weeks. If your wound should become increasingly painful, swollen or mucky you should see your clinic nurse. Rarely, a bleed can occur from the wound several days after surgery. If this should occur, apply gentle pressure to the wound using a clean cloth. Any bleeding should subside after a few minutes. If bleeding continues or becomes very profuse, you should attend the nearest casualty department.

When can I return to work?

You can return to work as soon as you feel well enough - usually after a few days. If your work is heavy or strenuous, you may need about two weeks off work.

When should I seek help?

- If you develop a fever above 38.5° C (101° F) or shivering and chills (rigors).
- Increasing pain, redness, swelling or discharge of the wound site.
- Bleeding that soaks through dressings and onto clothes or requires several changes of dressings in the first few days.
- Difficulties in passing urine.

Appendix 3. CRFs for Histology Study

Histopathology Study – Eligibility Checklist v0.2 (14.06.2010)

Study ID: **HIS-**☐☐☐☐-☐	Initials: ☐☐	Date: ☐☐/☐☐/☐☐☐☐

Eligibility criteria		
Interviewer: check items 1-2 BEFORE starting Informed Consent procedures	**Yes**	**No**
1. Aged 18-years or over at the time of clinic visit	☐	☐
2. Willing to undergo a clinical examination and to provide laboratory specimens	☐	☐
Interviewer: check items 3-4 AFTER Informed Consent procedures		
3. Able to understand why the study is being carried out, the potential benefits and inconveniences associated with study participation	☐	☐
4. Able to complete study informed consent procedures	☐	☐
Additional exclusion criteria		
• Where staff feel for whatever reason it would not be in a client's best interests to participate in the study, that client will be excluded from enrolment (e.g. risk of social harm) ***Does this situation apply for this client?***	☐	☐

Interviewer: Are you satisfied that the eligibility criteria above have been satisfied?

Yes ☐ **No** ☐

If 'No' then explain to the client why this is the case and provide advice about the possibility of future participation

Form completed by (Name)	Signature	Date

Histopathology Study – Clinical Form v0.2 (14.06.2012)

Study ID: **HIS-**☐☐☐☐-☐	Initials: ☐☐	Date: ☐☐/☐☐/☐☐☐☐

Q1	Current symptoms	*(Interviewer: tick all that apply)*
1.1	**Abdominal pain**	Yes ☐ No ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.2	**Lumps in groin**	Yes ☐ No ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.3	**Pain on passing urine**	Yes ☐ No ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.4	**Penile discharge**	Yes ☐ No ☐ N/a ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.5	**Genital sore(s)**	Yes ☐ No ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.6	**Scrotal itching / irritation / soreness**	Yes ☐ No ☐ N/a ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.7	**Anal itching / irritation / soreness**	Yes ☐ No ☐
	If yes, how long have you had these symptoms?	1-2 days ☐ 1-4 weeks ☐ 3-7 days ☐ More than 1 month ☐
1.8	**Other symptoms / reason for coming to clinic**	
	Specify:	

_____

_____

_____

_____

Histopathology Study – Demographic Form v0.2 (14.06.2012)

Study ID: **HIS-**☐☐☐☐-☐	Initials: ☐☐	Date: ☐☐/☐☐/☐☐☐☐

Section 1: Participant Details

Q1 Date of birth: ☐☐/☐☐/☐☐☐☐ (dd / mm / yyyy) Estimated Age (*if DOB unknown*): ☐☐ years

Q2 Marital status — Married ☐ Single ☐ Separated ☐ Divorced ☐ Widowed ☐

Q3 What do you consider your home province?

- Eastern Highlands ☐
- Western Highlands ☐
- Jiwaka ☐
- Southern Highlands ☐
- Hela ☐
- Simbu ☐
- Enga ☐
- Central ☐
- Gulf ☐
- East Sepik ☐
- West Sepik (Sandaun) ☐
- Madang ☐
- Milne Bay ☐
- East New Britain ☐
- West New Britain ☐
- New Ireland ☐
- Oro (Northern) ☐
- AR Bougainville ☐
- Manus ☐
- Western (Fly) ☐
- National Capital District ☐
- Other (*specify*) ☐ _____

Q4 Employment prior to coming to University

- I had a paid job ☐
- No paid job ☐

Specify:

- Teacher ☐
- Security guard ☐
- House duties ☐
- Gardening / marketing ☐
- Other formal employment (*specify*) ☐ _____
- Other (*specify*) ☐ _____

Q5 What religion do you belong to?

- Catholic ☐
- Lutheran ☐
- United Church ☐
- Seventh Day Adventist ☐
- Revival Church ☐
- Pentecostal ☐
- Anglican ☐
- Evangelical Alliance ☐
- No religion ☐
- Other (*specify*) ☐ _____

Section 2: Penile practices

Q6 Do you have a cut in the skin of your penis? Yes ☐ No ☐

6a If yes, what kind of cut?

- Round cut ☐
- Straight cut ☐
- 'V' cut ☐
- Other kind of cut (*specify*) ☐ _____

Histopathology Study – Examination Form v0.2 (14.06.2012)

Study ID: HIS-☐☐☐☐-☐	Initials: ☐☐	Date: ☐☐/☐☐/☐☐☐☐	Lab No: ZZZ☐☐☐☐☐

Q1	Abdominal pain on palpation?		Yes ☐	No ☐
Q2	Enlarged lymph nodes in groin?		Yes ☐	No ☐
	If yes, are nodes tender?		Yes ☐	No ☐
Q3	Scrotum tender or swollen on palpation?		Yes ☐	No ☐
Q4	Sore(s) on external genitalia?		Yes ☐	No ☐
	If yes, describe type of ulcer:	Single ☐	Painful ☐	Blister ☐
		Multiple ☐	Painless ☐	Indurated ☐
	If yes, indicate location of ulcer	Foreskin ☐	Glans penis ☐	Peri anal ☐
			Penile shaft ☐	Scrotum ☐
Q5	Urethral discharge?		Yes ☐	No ☐
Q6	Genital warts on external genitalia?		Yes ☐	No ☐
	If yes, indicate location	Foreskin ☐	Glans penis ☐	Peri anal ☐
			Penile shaft ☐	Scrotum ☐
Q7	Evidence of secondary syphilis?		Yes ☐	No ☐
	If yes, specify		Rash ☐	Condylomata ☐
Q8	**Evidence of penile cutting on examination?**		Yes ☐	No ☐
	If 'Yes', type of procedure?	Round cut ☐	Straight cut ☐	V cut ☐
		Other *(specify)* ☐	_____	
	Foreskin remnant on examination?		Yes ☐	No ☐
Q9	**Evidence of penile inserts or injection on examination?**		Yes ☐	No ☐
	If yes, specify	_____		
		_____		

Appendix 4. Approval to use Histopathology Laboratory of Port Moresby General Hospital

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Appendix 5. Participant Information and Consent Form

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Appendix 6. SOP for Histological Methods

Standard Operating Procedures

Male Circumcision (MC) Histopathology Study

TISSUE PROCESSING FOR PARAFFIN AND OCT EMBEDDED SAMPLES

Name & Title of Author: PHM Jayathunge **Signature of Author:** _ _

_ _ _ _

Name & Title of Approver: Dr Stuart Turville **Signature of Approver:** _ _ _ _ _ _ _ _

Effective Date: **September 2013**

Revision History:

Version	Author	Date	Reason for Revision
1	PHM Jayathunge	26.08.2013	Initial Draft
2	Stuart Turville	30.08.2013	Draft after reviewed by Dr Turville

Measurement of infection of foreskin tissues by HIV

Primary endpoints;

- Keratin thickness, number of corneocyte layers in epidermis of inner and outer foreskins
- Target cell (CD4 cells, LH cells, DC and Macrophages) densities of inner and outer foreskins.
- Permeability of inner and outer foreskin to HIV particles
- Intercellular protein expression of remaining penile epithelia after circumcision and dorsal slit procedure

Tissues will be prepared in three batches as paraffin sections, frozen infected and frozen uninfected.

Specimen Collection

1. Specimen collection for paraffin blocks

1. Tissue samples will be collected from the surgical suite at the clinic at PAU campus.
2. Surgeon will be asked to mark the outer foreskin with an indelible marker or stitch.
3. Foreskin will be separated into inner surface and outer (external) surface.
4. 2 foreskin tissue samples measuring 0.5cm x 2cm from inner and outer foreskins will be prepared using a scalpel and forceps. Frenar end of each tissue sample will be coloured with TMD-R tissue marking dye for orientation.
5. Fresh tissues will be dabbed on a towel (Kimwipes) to remove any residual fluid/blood before they are immersed immediately in 10% formalin contained specimen containers labeled with the participant's Study ID Numbers.
6. The samples will be transported to the laboratory (IMR affiliated) set up at UPNG, Port Moresby. After 12 hours, the tissues will be changed into 70% alcohol and store in the lab until transported to UNSW, Sydney. Paraffin embedding, cutting, H/E staining and slide scanning will be performed at the UNSW histology core.

2. Specimen collection for frozen Sections

Materials needed;

Scalpel, scalpel blades ,Forceps, Standard 24-well plates, 1X PBS, Plastic cryomolds (Sakura), OCT compound (Sakura), HIV-1 (kept at -80^{0}C).

Methods:

a. Preparing infected frozen samples

1. Tissue taken from surgical suite will be placed immediately into normal saline or 1X PBS. These tissues covered with ice (at 4^{o}C) will be transported to the laboratory (IMR affiliated) set up at UPNG, Port Moresby.

2. In the lab, foreskin tissue will be separated into inner surface and outer (external) surface*. Approximately 0.5 cm^2 sections of inner and outer foreskin will be obtained for each experiment (see below). All experiments will be performed in duplicate.
3. Using a 24-well plate, each tissue section will be placed into a well with 1X PBS until ready for infection.

The following is ideally done in a BSL-2+ laboratory containing tissue culture/fume hood, tissue culture incubator 37°C+5% CO_2:

4. When viral supernatant has thawed, under the hood, saline will be removed from each well, taking care not to disturb the tissue sections.
5. As the virus is infectious, the following precautions will be taken when working and disposing of this material. **A.** Firstly, those manipulating the samples will used use appropriate PPE, including disposable plastic gowns, two sets of acetonitrile gloves and protective eye wear.

   B. All liquid waste will be disposed of in plastic sealable containers with a final concentration of sodium hyperchlorite of 2% (w/v). This will remain with the biosafety hood for a minimum of 30 minutes prior to disposal with other liquid wastes.

C. All surfaces (Tissue culture plastics etc) that have come into contact with virus must be exposed to sodium hyperchlorite of 2% (w/v) for a minimum of 30 minutes.

After thorough chemical decontamination in B and C, the waste products can enter the clinical waste streams or equivalent waste disposal.

After use of the biosafety cabinet, surfaces must be decontaminated with 1% hyperchlorite, followed by water and 70% ethanol.

Experiments are usually set up as follows:

Control, no virus (x 1-2 for inner/outer foreskin)

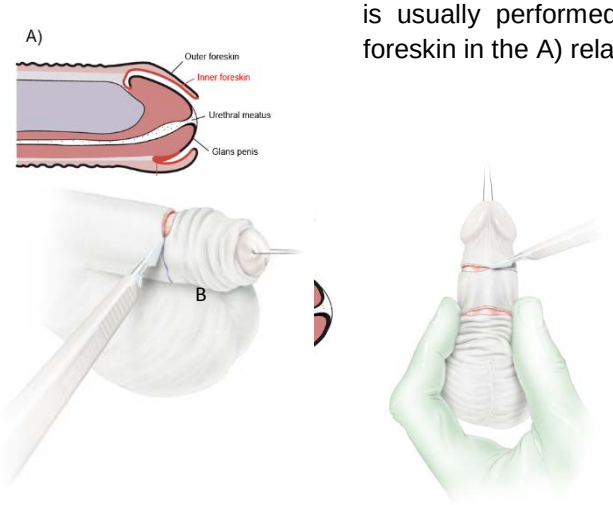
(+) virus Delta Envelope psmorange, AD8NL43 psmorange, WITO psmorange, WITO psmorange/infra-red 1x three hour time point (x 2)

6. Each tissue section will be incubated with approximately 500ul of viral supernatant at 37°C for 3 hours. With sterile forceps, it will be assured that each tissue section is placed epithelium-side-down to maximize epithelial exposure to HIV.
7. **After HIV exposure, tissue will be washed twice in PBS and then cut into small sections using surgical forceps.** ***Note: care must be taken during this procedure as there is the risk of being cut with the surgical instrument. Scalpel blades can also be used, but with extreme caution, as the risk of being cut is deemed greater. IT MUST BE NOTED THE RISK OF HIV INFECTION INCREASES SIGNIFICANTLY ONCE THE SKIN IS CUT AND/OR DAMAGED. All surgical items that are in contact with the tissue must be eventually chemically decontaminated by exposure to*** **sodium hyperchlorite of 2% (w/v) and then preferably being autoclaved. Surgical items MUST NOT be recirculated into general use for surgery etc.**
8. At each time point, plastic cryomolds will be set up with labels for each tissue section type (inner/outer), date tissue obtained, virus used, time incubated with virus, and duplicate #1 or #2. Fill each cryomold with OCT compound. **TISSUE EXPOSURED**

TO VIRUS AND FROZEN IN THIS MANNER IS STILL DEEMED INFECTIOUS AND SHOULD BE TREATED AS SUCH.

9. Tissue sections will be carefully removed from individual wells with sterile forceps and place into appropriately labeled cryomold. With forceps, it will be assured that tissue is placed laterally in OCT. The tissue, when on edge, will also tend to curl inward, so it will make sure tissue is as un-curled as possible in OCT.
10. Cryomold will be snap frozen with tissue section in OCT using dry ice and isopentane, or liquid nitrogen. If the latter is not available, a -80 freezer will be used. During the freezing process, it will be assured that the tissue sections remain "standing" and un-curled.
11. Tissue sections will then be kept at -80^{o}C until ready for further analysis (cryosectioning onto glass slides, staining with specific antibodies, and imaging with deconvolution microscopy). (Analysis will be performed further at UNSW and potentially the Hope lab).

(*Foreskin tissue separation: In an uncircumcised male, the relaxed state of the penis allows for the formation of an inner and outer aspect of the foreskin (fig 1). Male circumcision in adults is usually performed by two circumferential cuts in the foreskin in the A) relaxed and B) retracted state (fig 2).



A

uncircumcised penis with epithelia hypothesized to be more (red) or less (black) susceptible to HIV-1. **B)** An erect uncircumcised penis exhibiting retracted foreskin which exposes the inner aspect directly to

Figure 2: Adult Male Circumcision. The foreskin is marked at the base in the relaxed state and circumferentially incised. It is then retracted and circumferentially incised again distally, and removed from the penis by dissecting away the underlying fascia.
Elder JS. *BJU International.* 2007. 99 (6): 1553-1564.

Ideally, the surgeon could mark the outer aspect of the foreskin (the external surface) prior to removal with an indelible marker or stitch. If this is not feasible, the outer aspect of the foreskin can also be distinguished from the inner aspect after removal via certain gross characteristics, outlined in table 1.

Table 1. Gross Characteristics of the Adult Male Foreskin

Characteristic	**Inner Foreskin**	**Outer Foreskin**
Color/Pigmentation	Lighter	Darker
Rugae	Smoother, less rugae	Rougher, more rugae
Area	Less (1/2-1/3 less than Outer)	Greater (most of the tissue)

There may also be a faint fold to help distinguish the transition between Outer and Inner Foreskin, though this is not as reliable due to a natural fold located on the ventral surface or underside of the foreskin (overlying the urethra).

The foreskin should be separated into the inner and outer aspects prior to further separation into smaller components for further studies, as the inner and outer aspects may contain structural and immunologic differences responsible for increased infection in the uncircumcised male.)

Analysing for permeability:

Frozen sections will be analyzed using a DeltaVision RT system and SoftWorx software (Applied Precision Instruments, Issaquah, WA, USA), psmorange will be photo-activated within the viral particles and each tissue section surveyed for. As an additional variable, we have hybrid virions that fluoresce with psmorange and infra-red. The use of the latter is to determine whether infra red particles can be used instead of photoactivatable variants.

1) Total number of viral particles on the epithelial surface
2) Number of particles that have entered the epithelial surface, and
3) The depth of each viral penetrator into the tissue.

b. Preparing non-infected frozen samples

Frozen sections will be prepared using O.C.T. embedding compound (SAKURA), plastic tissue molds (Fisher), 2-methylbutane (Fisher), dry ice, a forceps or other clamping device, and a metal or plastic 1L container.

Procedure;

1. Tissue taken from surgical suite will be placed immediately into normal saline or 1X PBS. These tissues will be transported to the laboratory (IMR affiliated) set up at UPNG, Port Moresby covered with ice (at $4^{o}C$).
2. In the lab, foreskin will be separated into inner surface and outer (external) surface. Approximately 0.5 cm^2 sections of inner and outer foreskin are obtained and dabbed on a towel to remove any residual fluid/blood.
3. Molds will be bar coded with proper tissue identification and fill partly with O.C.T.
4. The dissected tissue will then be immediately placed in a tissue mold with O.C.T. During the process, it will be assured that the tissue sections remain “standing” and un-curled in best orientation for the tissue to be frozen in, and let it settle to the bottom.
5. More O.C.T. will be added on top of tissue to cover it completely and fill the mold.
6. Then the plastic container will be filled half way with 2-methylbutane (Isopentane).Several small pieces of dry ice will be added to it and wait few moments for the temperature to drop (-40C).
7. The edge of the mold will be grasped with the forceps and dip into 2-methylbutane. (The O.C.T. will begin to turn white as it freezes).
8. When all of the O.C.T. is frozen, the mold will be dropped into the cold 2-methylbutane to freeze thoroughly (~5min).

9. Finally, the mold will be removed from the freezing liquid, wrap in foil, and immediately store at -80C.
10. These surgical samples will be transported in batches to the respective laboratories of investigators Turville (UNSW, Australia) and potentially Hope and Dinh (Northwestern, USA) for collaborative immunohistochemical and histological analysis.

The transportation of samples will be done by a courier service after packing the tissue samples into a specialized box to transport human tissue samples. Dry ice will be used to maintain cold chain.

Cryo-sectioning and Fixation of frozen samples

1. The sections will be cut by cryostat at 5-10 µm and mount on gelatin-coated histological slides. (The cryostat temperature will be between -15 and -23 °C).
2. First the sections will be air dried for 30 minutes at room temperature to prevent sections from falling off the slides during antibody incubations.
3. Then, 50 µL of ice-cold Fixation Buffer will be added immediately to each tissue section upon removal from the freezer followed by fixing for 8 minutes at 2-8 °C or, optimally, at -20 °C for 20 minutes.

Fluorescent Staining of Cryostat Sections

Reagents to be used; Primary Antibodies, Northern Lights Secondary Reagents (or equivalent) ,Wash Buffer: 1X PBS (0.137 M NaCl, 0.05 M NaH_2PO_4, pH 7.4) ,Incubation Buffer: 1% bovine serum albumin, 1% normal donkey serum, 0.3% Triton® X-100, and 0.01% sodium azide in PBS ,Blocking buffer: 1% horse serum in PBS, DAPI (4',6-diamidino-2-phenylindole) , solution: Add 1 µL of 14.3 mM stock for every 5 mL of PBS, Anti-Fade Mounting Medium ,Antigen Retrieval Reagents (if required; Protocol for Heat-induced Epitope Retrieval (HEIR))

Procedure;

1. Cryostat sections stored in a freezer will be thawed at room temperature for 10-20 minutes.
2. Then the slides will be rehydrated in wash buffer for 10 minutes and drain the excess wash buffer.
3. After surrounding the tissue with a hydrophobic barrier using a barrier pen, non-specific staining will be blocked between the primary antibodies and the tissue, by incubating in blocking buffer (1% horse serum in PBS) for 30 minutes at room temperature.
4. Primary antibodies diluted in incubation buffer will be applied according to manufacturer's instructions.
5. Then the slides will be washed 3 times for fifteen minutes each in wash buffer and incubate with the Northern Lights secondary antibody diluted in incubation buffer according to the manufacturer's instructions.
6. Again, the slides will be washed 3 times for fifteen minutes each in Wash Buffer. 300 µL of the diluted DAPI solution will be added to each well, and incubate 2-5 minutes at room temperature. (DAPI binds to DNA and is a convenient nuclear counter stain).
7. Mount with an anti-fade mounting media and visualise using a high resolution fluorescence microscope (Deltavision, Applied Precision) in the laboratories of Hope/Dinh and Turville.

Paraffin Tissue Preparation:

1. 10% neutral buffered formalin and 4% para-formaldehyde fixatives will be used.
2. Tissues will be fixed at room temperature for 24 hours with the use of histology cassettes, a small sealable container, and 70% ethanol. (The cassettes will be labeled before placed in the container filled with 70% Ethanol).
3. After fixation they will be washed with 0.1M PBS several times.
4. Then the tissues will be embedded with paraffin blocks and stored in a cool dry place to be transported.
5. Processed tissues will be sectioned by microtome into 3-5 µm sections and stained with H&E.

Microscopy:

A system of combined high resolution microscopy and imaging (Aperio ScanScope) will be used to analyze the paraffin and frozen sections and the samples will be analyzed using several parameters;

1. Sample integrity by nuclear staining,
2. Target cells;

   Cells of immune origin by staining CD45 antigen

   CD4 positive T cell compartment (the primary receptor for HIV) by staining CD4 antigen

   LH cells, DC cells and macrophages by staining Langerin (CD207), DC-SIGN (CD209), and Mannose Receptor (CD206) respectively
3. The keratin layer by staining keratin using Filaggrin.
4. The degree of cornification of inner and outer foreskins will be determined by a method of alkaline reconstitution (this increases corneocyte volume for layers to be counted) followed by Nile Red staining (can detect the corneocyte layer versus the live keratin layer).
5. Tissues embedded in paraffin blocks will be analyzed for keratin thickness of inner and outer foreskins.

Appendix 7. Ethics Approvals

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