Evaluation of long-term immunological responses following reduced-dose quadrivalent human papillomavirus vaccine schedules in Fiji

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Abstract

Human papillomavirus (HPV) vaccines when administered as a three-dose schedules have >96% vaccine efficacies against HPV infection and cervical precancerous lesions. At the end of 2014, the World Health Organization (WHO) revised their recommendation for HPV vaccination from three to two doses for girls <15 years old, provided the doses are administered six months apart. This recommendation was made based on clinical studies that demonstrated non-inferior antibody responses between girls <15 years old who received two doses (six months apart) and women aged 16-26 years old who received three doses, in which vaccine efficacy against cervical precancerous lesions have been previously established. This recommendation has significant implications for low-middle income countries planning to introduce HPV vaccines in terms of affordability and the logistical relief on delivering multiple doses. The longest follow-up study of antibody responses following three-dose schedule was nine years, while for two-dose schedules this was four and five years for quadrivalent (4vHPV) and bivalent HPV vaccine (2vHPV), respectively. The question of whether a two-dose HPV schedule provides comparable long-term immunity as the three-dose schedule remains unknown.

This thesis describes a prospective cohort study that was undertaken in Fiji to investigate the antibody and cellular immune responses in Fijian girls who received zero, one, two or three doses of 4vHPV six years previously and their responses to a ‘booster’ dose of 2vHPV. The primary aim was to compare the antibody and cellular immunity of girls who received two doses of 4vHPV with girls who received three doses. Analysis of HPV immune responses including neutralising antibodies (using the HPV pseudovirion neutralisation assay) and cellular immunity (including IFNγ ELISPOT assay, flow cytometry and multiplex cytokine array) was used to compare immunogenicity between the zero, one, two or three 4vHPV dosage groups.

Six years following the last dose of 4vHPV, girls who received two doses had neutralising antibody titres that were not significantly different to girls who received three doses for all four HPV vaccine types. Although girls who received one dose had significantly lower antibody titres than girls who received three doses, their titres were still at least five-fold higher than unvaccinated girls. Following a booster dose of 2vHPV, girls who received...
one, two or three doses of 4vHPV six years previously had antibody levels that were not significantly different, suggesting that similar immune memory responses were induced for these dosage groups. In addition, a subset of study samples from each dosage groups were randomly selected for analysis of cellular immune responses (T cells and cytokines) to HPV16 and 18, and significantly lower HPV18 responses were observed in girls who received one or two doses compared with girls who received three doses, although the clinical significance of this is unknown. No adverse events were observed in this study, demonstrating the safety of mixed HPV vaccine schedules.

In summary, this is the first study to document the induction of immune memory and antibody persistence up to six years following just one dose of 4vHPV. In addition, this study also represents the longest follow-up study for a two-dose 4vHPV schedule. The antibody results support the current WHO recommendation of a two-dose HPV schedule, and provides evidence to support the assessment of the vaccine efficacy against HPV infection following a single-dose HPV schedule. Further studies in larger cohorts to confirm the lower HPV18 cellular immune responses following reduced-dose schedules observed in this study will also be important to determine the clinical relevance of these findings. Longer-term studies (greater than six years) of reduced-dose HPV schedules are needed to ensure that immunogenicity and protection is maintained over the period of greatest risk for HPV infection in girls and young women.
**Declaration**

This is to certify that

1) The thesis comprises only my original work towards the PhD except where indicated in the preface,

2) Due acknowledgement has been made in the text to all other materials used,

3) The thesis is less than 100,000 words in length, exclusive of tables, maps, bibliographies, and appendices

Name: Zheng Quan Toh

Signature: 

Date: 14\(^{th}\) July, 2017
Preface

Under the guidance of my supervisors Associate Professor Fiona Russell, Associate Professor Paul Licciardi and Professor Kim Mulholland, I was responsible for obtaining ethics approval (including drafting the study protocol, participant information statement, informed consent form and questionnaires) from the Fiji National Research Ethics Review Committee and the Royal Children’s Hospital Human Research Ethics Committee, Melbourne, Australia, processing of study samples, all experimental procedures and the analysis and presentation of all data in this thesis unless otherwise indicated below:

Study design: My supervisors secured the funding and designed the clinical aspects of the study in consultation with the collaborators in Melbourne (Prof Suzanne Garland and A/Prof Sepehr Tabrizi) and Fiji (Dr James Fong, Dr Rachel Devi, Dr Mike Kama, Dr Eric Rafai, and Dr Lisi Tikoduadua).

Fieldwork: Rita Reyburn, Kathryn Bright, Tupou Ratu, Dr Evelyn Tuivaga and Mere Delai were responsible for contacting and recruiting the study participants, collection of blood samples as well as maintaining the study database. The fieldwork standard operating procedures were written by Rita Reyburn and Kathryn Bright under the guidance of A/Prof Fiona Russell. The processing and storage of study samples (e.g. peripheral blood mononuclear cells and serum) were performed by myself with the help of A/Prof Paul Licciardi and Silivia Matanitobua in Fiji. Rita Reyburn performed the analysis on the social economic status of the study participants.

Experimental procedures: The setup of the HPV pseudovirion neutralisation assay at the Murdoch Children’s Research Institute was facilitated by Rohit Sinha and Prof Ian Frazer (Translational Research Institute, Queensland, Australia). A/Prof Paul Licciardi and Dr Edwin Hoe assisted with the setup of IFNγ ELISPOT assay and flow cytometry staining protocol. The experiments in Chapter 4 and 5 were performed with the help of an Honours student, Kathleen Cheow.
Publications

The following publications were written with inputs from the co-authors:


Posters and Conference Presentations

Oral Presentations

1. 15th Public Health Association of Australia (PHAA) National Immunisation Conference, June 2016, Brisbane, Australia. Session 1B vaccine preventable diseases

2. European Research Organisation on Genital Infection and Neoplasia (EUROGIN) June 2016, Salzburg, Austria. Session OC6 New developments in HPV prophylactic vaccines, OC6-10

3. International Congress of Immunology, August 2016, Melbourne, Australia. Session Immunity to Virus 1

4. European Research Organisation on Genital Infection and Neoplasia (EUROGIN) Oct 2017 (Accepted), Amsterdam, Netherlands.
Poster Presentations

1. Australasian Society for Infectious Diseases (ASID), April 2016, Launceston, Australia

2. International Papillomavirus (IPV) Conference, February 2017, Cape Town, South Africa

Funding

This study was supported by the Department of Foreign Affairs and Trade of the Australian government and the Fiji Health Sector Support Program (FHSSP). FHSSP is implemented by ABT Associates on behalf of the Australian government. I was fortunate to be a recipient of a Melbourne Research Scholarship from the University of Melbourne and a top-up scholarship from the Murdoch Children’s Research Institute (MCRI). During the course of my PhD, I have also received student conference support from MCRI, a conference travel award from EUROGIN and a Department of Paediatrics Travelling Scholarship from the University of Melbourne to present my work at the EUROGIN conference in 2016.
Acknowledgements

Supervisors
I wish to thank A/Prof Fiona Russell for her support and encouragement throughout my PhD. I am extremely grateful for her prompt feedback and guidance on all my writings, which have challenged me to think and write with clarity. I would not have done my PhD without A/Prof Paul Licciardi. He has been a constant support throughout my PhD and has provided guidance in every aspects of my work. The sample processing with him till early morning in Fiji was an experience that is hard to forget. I am grateful for all the opportunities to work on various different projects with him, and the chance to attend multiple conferences throughout my PhD. I have learnt a lot from him as a person and scientist. He has been a great boss, colleague and friend. I am indebted to him for what I have achieved. I wish to thank Prof Kim Mulholland for his valuable feedback on all my work, his support for me to attend various international conferences, his belief in me and the opportunities to be involved in various large clinical trial studies, for which I am extremely grateful. His ability to manage multiple large clinical trials from across the world on multiple infectious disease agents is inspiring. I am extremely lucky, thankful and grateful to be supervised by each of these supervisors.

Study participants
I wish to thank all the study participants and their families for their involvement in this study. This study would not have been possible without them and I am truly grateful.

Study staff
This study would not have been possible without the tireless effort of the Fiji HPV study staff. I wish to thank all of them, including all the Fijian collaborators for all their hard work in making this study a success. Specifically, I wish to thank the following individuals of the study team: Kathryn Bright who is the study coordinator of this study, and also Rita Reyburn who is the epidemiologist, both have helped to facilitate the whole study process. For Evelyn Tuivaga, Tupou Ratu and Mere Delai who is the study doctor and nurses involved in recruitment, blood collection and maintained the study databases, their commitment and positive attitude to the study despite the long working hours. For Silivia Matanitobua, who helped to process the blood samples in Fiji. For Rohit Sinha,
who taught me the HPV neutralisation assay, a technique that has led to many collaborations, and for Prof Ian Frazer, for allowing the technology transfer of the HPV neutralisation assay and his valuable feedback in the area of HPV research.

**Pneumococcal Immunology Research Group**
I am extremely fortunate and grateful to be part of the Pneumococcal Immunology research group. I wish to thank every past and present members of the group who have made my PhD experience easier and made me look forward to coming to work on most days. This group is like a second family to me and I specifically give thanks to the following individuals of the group: for Anne Balloch, who is the most senior member of the group, her care and concern for everyone in the team, her guidance on my laboratory work and her sudden outburst of laughter, as well as for hiring me as a part time research assistant so that I can make ends meet. For Paul Licciardi, who also happen to be my supervisor, it has been handy that he sits in the same office as the rest of the team, which meant that he is always a ‘shout out’ away whenever I needed help. For Edwin Hoe, who was a postdoc in the group, his constant positive energy and attitude that has brought many laughter and ‘pearls of wisdom’ to the group. For Rachel Marimla and Jordan Nathanielsz, who are research assistants, their help in my part-time work, and lending their listening ears and complaints that I might have on- and off-work. For Lien Anh Ha Do, who is a postdoc in the laboratory, her positive attitude and engaging discussion. For Kuang-Chin Hsiao, who is a PhD student, his encouragement, support and strategies discussed together to tackle PhD. For Kathleen Cheow, who was an Honours student in the group, her help on the cellular immunity experiments of this study.

**Family and friends**
This PhD would not have been possible without my family support. I am grateful to my parents for the opportunity to pursue my studies, their understanding and encouragement throughout my PhD. I am grateful to my sister for her understanding and encouragement, and for shouldering up the responsibility of taking care of our parents back home. I am also fortunate to have a close group of friends back home who did not give up on me despite having been abroad for so many years, and also my friends in Melbourne who I can always count upon.
Most importantly, I wish to thank my wife, Melissa for her ongoing support in all possible ways, including housework, dishes and financially, so that I can concentrate on my PhD. Her care and concern, and her understanding on the time away from home due to work commitments. I am grateful for what she has done and appreciate the work that goes into keeping my life in check.
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<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2vHPV</td>
<td>Cervarix®</td>
</tr>
<tr>
<td>4vHPV</td>
<td>Gardasil®</td>
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<tr>
<td>9vHPV</td>
<td>Gardasil®9</td>
</tr>
<tr>
<td>AE</td>
<td>Adverse event</td>
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<tr>
<td>AIS</td>
<td>Adenocarcinoma in situ</td>
</tr>
<tr>
<td>ASR</td>
<td>Age-standardised rate</td>
</tr>
<tr>
<td>ATP</td>
<td>According to protocol</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CIN</td>
<td>Cervical intraepithelial neoplasia</td>
</tr>
<tr>
<td>CRF</td>
<td>Case report form</td>
</tr>
<tr>
<td>CVT</td>
<td>Costa Rica Vaccine Trial</td>
</tr>
<tr>
<td>CWMH</td>
<td>Colonial War Memorial Hospital</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked ImmunoSPOT assay</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FID</td>
<td>Fijian of Indian Descent</td>
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<tr>
<td>FNRERC</td>
<td>Fiji National Research Ethics Review Committee</td>
</tr>
<tr>
<td>GAVI</td>
<td>Global Alliance for Vaccines and Immunisation</td>
</tr>
<tr>
<td>GMC</td>
<td>Geometric mean concentration</td>
</tr>
<tr>
<td>GMT</td>
<td>Geometric mean titre</td>
</tr>
<tr>
<td>GSK</td>
<td>GlaxoSmithKline</td>
</tr>
<tr>
<td>GSUA</td>
<td>Greater Suva Urban Area</td>
</tr>
<tr>
<td>GW</td>
<td>Genital warts</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
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<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>HREC</td>
<td>Human Research Ethics Committee</td>
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<tr>
<td>HSIL</td>
<td>High-grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IRR</td>
<td>Incidence risk ratio</td>
</tr>
<tr>
<td>iTaukei</td>
<td>Indigenous Fijian</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>ITT</td>
<td>Intention to treat</td>
</tr>
<tr>
<td>LCR</td>
<td>Long control region</td>
</tr>
<tr>
<td>LMIC</td>
<td>Low-middle income country</td>
</tr>
<tr>
<td>LSIL</td>
<td>Low-grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td>MCRI</td>
<td>Murdoch Children’s Research Institute</td>
</tr>
<tr>
<td>MITT</td>
<td>Modified intention to treat</td>
</tr>
<tr>
<td>mL</td>
<td>millilitre</td>
</tr>
<tr>
<td>MoH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MSM</td>
<td>Men who have sex with men</td>
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<tr>
<td>NAb</td>
<td>Neutralising antibody</td>
</tr>
<tr>
<td>NHRC</td>
<td>Fiji National Health Research Committee</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OPC</td>
<td>Oropharyngeal cancer</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
</tr>
<tr>
<td>PsV</td>
<td>Pseudovirion</td>
</tr>
<tr>
<td>r</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RRP</td>
<td>Recurrent respiratory papillomatosis</td>
</tr>
<tr>
<td>SAE</td>
<td>Serious adverse event</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SEAP</td>
<td>Secreted alkaline phosphatase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
</tr>
<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>TT</td>
<td>Tetanus toxoid</td>
</tr>
<tr>
<td>TVC</td>
<td>Total vaccine cohort</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particle</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
General Introduction

This thesis describes the immune responses of Fijian girls who previously received zero, one, two or three doses of 4vHPV, and their responses to a single ‘booster’ dose of 2vHPV. The first chapter describes the current literature and overview of the field of HPV research, with a particular focus on reduced-dose HPV vaccine schedules. This chapter has led to two publications: a perspective article and a review article on reduced-dose HPV vaccine schedule (Appendix one and two). Chapter two describes the methodology of this study, including the study design, study procedures and laboratory procedures. Chapters three to five are the result chapters of this thesis: Chapter three describes the neutralising antibody responses of Fijian girls following reduced-dose 4vHPV schedules, and are presented in manuscript form that was published in Clinical Infectious Diseases in 2017. Chapter four and five describes the cellular immune responses in a subset of Fijian girls from each dosage groups in the form of T cell and cytokine responses, respectively; these two chapters are presented in standard thesis chapter format. The last chapter is the General Discussion chapter, highlighting the significant findings of this thesis, and discussed the potential implications that these results might have on the optimal number of doses required for protection against HPV infection. In addition, insights into future work on this study cohort and recommendations on immunological measurements for future studies examining reduced-dose schedules are also included the last chapter.
1. Literature Review

1.1. Introduction

The link between human papillomavirus (HPV) and cervical cancer was first reported by Harold zur Hausen and his team in the early 1980s, when they successfully isolated the virus (HPV type 16, and subsequently HPV type 18) from cervical cancer biopsies. This discovery and subsequent technological advances (e.g. polymerase chain reaction) led to the identification of various HPV genes and their role in causing cervical cancer (Schwarz, Freese et al. 1985, Munger, Phelps et al. 1989). By the early 1990s, a number of epidemiological studies had established HPV as the causal agent of cervical cancer (Munoz, Bosch et al. 1992, Bosch, Manos et al. 1995).

HPV infection is the most common sexually transmitted infection (World Health Organization 2015). The worldwide prevalence of HPV infection in women without cervical abnormalities is estimated to be 11-12%, and these rates vary between geographical regions with the highest rates in sub-Saharan Africa (24%), Eastern Europe (21%) and Latin America (16%) (Forman, de Martel et al. 2012). In addition, the HPV infection rates have been found to peak in women under the age of 25 (Forman, de Martel et al. 2012).

There are currently three licensed virus-like particle (VLP)-based HPV vaccines; Gardasil® (quadrivalent; 4vHPV), Cervarix® (bivalent; 2vHPV) and Gardasil®9 (nonavalent; 9vHPV). All three vaccines are licensed as a three-dose schedule administered over six months. Despite the availability and high efficacy of HPV vaccines, cervical cancer incidences still remain high. Issues such as limited access to HPV vaccine due to their high cost, limited resources for vaccine introduction and delivery, and poor cervical cancer screening programs are some of the reasons why rates of cervical cancer remain high in low-middle income countries (LMICs). These issues have led to the exploration of alternative HPV schedules which could potentially alleviate the issues of vaccine cost and delivery.
1.2. Human Papillomavirus

Human papillomavirus is a non-enveloped, double stranded DNA virus that belongs to the virus family known as Papillomaviridae. The HPV genome is approximately 8kb in size, and can be classified into three regions consisting of eight coding genes and a control region: (1) ‘early’ genes coding for six non-structural proteins (E1-E4, E6 and E7) that are associated with cellular transformation; and (2) ‘late’ genes coding for two structural proteins (L1 and L2) that forms the viral capsid, and a long control region (LCR), which regulates replication and gene function (Figure 1.1). The HPV genome is surrounded by 72 capsid proteins, comprising mainly of protein L1 and a small percentage of protein L2, which gives the virus an icosahedral symmetry structure. These capsid proteins play a major role in the infection process, and their ability to self-assemble into VLPs form the basis of current HPV vaccines. The L1 protein is also important for the classification of HPV genotypes. There are currently more than 170 HPV genotypes identified with approximately 40 genotypes causing genital infections (Munoz, Bosch et al. 2003, Bernard, Burk et al. 2010). These can be classified into low-risk types (e.g. HPV6 and 11), which are commonly known to cause anogenital warts and benign/low-grade abnormalities in the genital areas, and high-risk types (e.g. HPV16 and 18), which are often associated with persistent infections which could lead to cancer of the anogenital areas such as cervix, vulva, vagina, penis and anus, as well as some oropharyngeal cancers.

Figure 1.1: Genome organisation of HPV16. The HPV genome is approximately 8kb in size, and typically consists of six early genes (E1-E2, E4-E7) and two late genes (L1 and L2), as well as a long control region (LCR) that regulates DNA replication. This figure is obtained from Doorbar et al. (Doorbar, Quint et al. 2012).
1.3. HPV life-cycle and pathogenesis

The HPV life-cycle is closely associated with the biology of human epithelial cells. It is postulated that HPV infects the basement membrane of epithelial cells (i.e. cervix epithelial cells in the case of cervical cancer) through a microwound that exposes these cells (Schiller, Day et al. 2010). As these basal layer cells consist of stem cells and transient-amplifying cells that are constantly dividing to provide a reservoir of suprabasal cells (the outer layer of the epithelium), this could facilitate the spread of the virus as part of the wound healing process (Valencia, Bonilla-Delgado et al. 2008). After the initial infection, the virus is maintained at low copy numbers (10-200 copies/cell) with limited virus gene expression in the host cell, which are poorly recognised by the host immune system (Stanley, Pett et al. 2007). The infected cell then undergoes cell division and differentiation, leading to amplification of the viral genome. This results in amplification of viral copy numbers (at least 1,000 copies/cell), high expression of E6 and E7 genes, and initiation of late gene expression (L1 and L2 proteins) (Stanley, Pett et al. 2007). As these cells progress towards terminal differentiated suprabasal cells, the infectious virions are assembled into capsid proteins and the mature virions are released from the epithelial surface to infect other tissues (Doorbar 2005). The roles of each viral protein to maintain the HPV’s life cycle are summarised in Table 1.1.
### Table 1.1: HPV proteins and their roles in HPV’s life-cycle

<table>
<thead>
<tr>
<th>HPV proteins</th>
<th>Functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>DNA helicase protein; initiation of viral DNA replication.</td>
<td>(Hughes and Romanos 1993)</td>
</tr>
<tr>
<td>E2</td>
<td>DNA binding protein; recruits E1 to viral origin of replication, and host cell factors for viral transcription, replication and mitotic segregation; important regulator of viral transcription.</td>
<td>(Bellanger, Tan et al. 2011, Muller and Demeret 2012)</td>
</tr>
<tr>
<td>E4</td>
<td>Genome amplification in differentiated cells; and expression of capsid proteins (only for high-risk types).</td>
<td>(Wilson, Fehrmann et al. 2005, Wilson, Ryan et al. 2007)</td>
</tr>
<tr>
<td>E5</td>
<td>Genome amplification and late gene expression in differentiated cells; may be involved in cell cycle progression/transformation</td>
<td>(Straight, Hinkle et al. 1993, Fehrmann, Klumpp et al. 2003)</td>
</tr>
<tr>
<td>E6</td>
<td>Oncogene; degrades p53 (tumour suppressor protein) with or without E6AP (cellular ubiquitin ligase); degrades BAK (apoptotic effector protein); activates telomerase (hTERT); resists cell apoptosis and immortalises cells (only for high-risk types).</td>
<td>(Huibregtse, Scheffner et al. 1991, Jackson, Harwood et al. 2000, Oh, Kyo et al. 2001, Camus, Menendez et al. 2007)</td>
</tr>
<tr>
<td>E7</td>
<td>Oncogene; degrades Rb (retinoblastoma; tumour suppressor protein) family proteins (pRb, p107 and p130); induces chromosome abnormalities; inhibit cyclin-dependent kinase inhibitors (p21 and 27); interacts with histone deacetylase (HDAC); promotes cell cycle progression (only for high risk types)</td>
<td>(Boyer, Wazer et al. 1996, Zerfass-Thome, Zwerschke et al. 1996, Funk, Waga et al. 1997, Duensing, Lee et al. 2000, Zhang, Laribee et al. 2004, Longworth, Wilson et al. 2005, Barrow-Laing, Chen et al. 2010)</td>
</tr>
</tbody>
</table>
L1 Major capsid protein; required for binding to cell receptors (heparan sulphate proteoglycans and laminin) for cell invasion (Joyce, Tung et al. 1999, Giroglou, Florin et al. 2001, Culp, Budgeon et al. 2006, Johnson, Kines et al. 2009)

L2 Minor capsid protein; facilitates binding to cells and transport to the nucleus (Richards, Lowy et al. 2006)

The initial entry into the basal layer cells is thought to involve the interaction of the capsid protein, L1 with heparan sulphate proteoglycans and laminin as part of the extracellular matrix of the basal membrane (Joyce, Tung et al. 1999, Giroglou, Florin et al. 2001, Culp, Budgeon et al. 2006, Johnson, Kines et al. 2009). This interaction results in structural changes to the capsid proteins that exposes the minor capsid protein L2 to cleavage by cellular furin protease, and facilitates binding to a secondary receptor on the basal cell (Richards, Lowy et al. 2006). This is necessary for virus internalisation and subsequent nuclear entry. Other cell surface receptors such as Alpha 6 Integrin and growth factor receptors may also be involved in this process but their roles require further clarification (Doorbar, Quint et al. 2012). Once the virions are internalised, they undergo endosomal transport, which separates L1 from the L2-viral genome particles. The L1 protein is left in the endosomes, and ultimately subject to lysosomal degradation, while the L2-viral genome particles are transferred to the Golgi complex prior to entry into the nucleus (Doorbar, Quint et al. 2012).

The HPV genome replicates in the nucleus and establishes itself as low-copy number episomes using host cell DNA machinery (Doorbar 2005, Moody and Laimins 2010). The viral proteins E1 and E2 are important for the initiation and maintenance of the viral genome; both E1 and E2 proteins form a complex that binds the origin of replication at the viral genome and recruits cellular polymerases and accessory proteins to mediate replication. The E1 protein is a helicase protein that separates the viral DNA to allow viral replication (Hughes and Romanos 1993), while E2 is a site-specific DNA binding protein that regulates transcription. The binding sites for E2 are located adjacent to sites for
cellular transcription factors that activate the early promoter (p97 for HPV16 and 31, p105 for HPV18), which makes E2 protein an important regulator for viral replication. At low levels, E2 binds its recognition sequences and activates the early promoter to express E1 and E2, while at high concentrations, it represses by blocking the binding of cellular transcription factors (Bellanger, Tan et al. 2011). In addition, the E2 protein may also have a repressive role on E6 and E7 gene expression, as both are expressed by the early promoter (Goodwin and DiMaio 2000, Bellanger, Tan et al. 2011).

In normal epithelial cells, the basal cells divide and differentiate into para-basal cells, which migrate upwards, forming the squamous layers; this process usually results in cell cycle exit and loss of nuclei (Doorbar 2005, Hamid, Brown et al. 2009). However, HPV-infected cells are usually still active in the cell cycle upon differentiation due to the various activities of E6 and E7 proteins that promote cell cycle progression. The basal cell differentiation is thought to activate the late promoter (p670 for HPV16) of the viral genome in HPV-infected cells and results in amplification of early and late proteins, resulting in increased viral copy numbers (>1,000 copies), although the exact signal for activation of the late promoter is still not clear (Stanley 2008, Hamid, Brown et al. 2009), and proteins E4 and E5 seem to be important for this process.

E6 and E7 proteins are important for cellular transformation, mainly through interaction with cell cycle and tumour suppressor proteins. The protein E6 interacts and causes degradation of p53 (tumour suppressor protein that promote cell apoptosis following DNA damage) with or without E6AP (cellular ubiquitin ligase) (Huibregtse, Scheffner et al. 1991, Camus, Menendez et al. 2007). The degradation of p53 and also pro-apoptotic protein BAK by E6 resists apoptosis and increases chromosomal instability (Jackson, Harwood et al. 2000). In addition, E6 is also found to activate telomerase (multi-subunit complex that synthesises hexamer repeats to the ends of chromosome), which is an enzyme often associated with tumour growth (Oh, Kyo et al. 2001). The interaction and activation of these cellular proteins with E6 are linked to promoting cellular growth. The E7 protein interacts with another set of cellular proteins to immortalise cells. The protein E7 degrades Retinoblastoma protein (Rb), a protein involved in cell cycle regulation, and allows transcription factor E2F to express constitutively, leading to uncontrolled cell
cycle progression (Boyer, Wazer et al. 1996). This interaction with Rb protein family is also found to cause genomic instability through the induction of aneuploidy (Duensing, Lee et al. 2000). In addition, E7 is also found to be associated with cyclin-dependent kinase inhibitors (p21 and p27), as well as histone deacetylases (HDACs), which activate cellular promoters. These events promote cell cycle progression (Zerfass-Thome, Zwierschke et al. 1996, Funk, Waga et al. 1997, Zhang, Laribee et al. 2004, Longworth, Wilson et al. 2005). Although high-risk E6 and E7 can independently cause cellular hyper-proliferation (Hawley-Nelson, Vousden et al. 1989, Halbert, Demers et al. 1991, Song, Pitot et al. 1999), the effects of both proteins significantly increase the frequency of cell immortalisation and tumour growth (Hawley-Nelson, Vousden et al. 1989, Hudson, Bedell et al. 1990). However, the E6 and E7 proteins from low- or high-risk types seem to differ in their ability to immortalise the cells; the E6 and E7 of low-risk HPV types have weaker binding or no degradation of cell cycle proteins as compared to high-risk types, thus no cell cycle progression (Munger, Phelps et al. 1989, Halbert, Demers et al. 1992, Lechner and Laimins 1994). This might explain the high frequency of high-risk types found in cervical and anogenital cancers, but not low-risk types.

HPV infection is necessary but not sufficient to cause cervical cancer. In most cases of cervical cancer, the HPV genome, particularly the high-risk types, is found integrated into the host cell genome (Klaes, Woerner et al. 1999, Pett and Coleman 2007). This integration often leads to the deletion of part of the viral genome that includes E1, E2, E4, and E5 open reading frames (ORFs); E6 and E7 genes, and the upstream regulatory region are retained (Pett and Coleman 2007). The loss of part of the viral genome, particularly E2, which has a regulatory role in genome regulation, accounts for the increased expression of proteins E6 and E7. These events are associated with cell cycle progression, and increased genomic instability of the host cell, resulting in carcinogenesis.

1.4. Burden of HPV-associated diseases
HPV is known to cause a range of diseases. In addition to causing anogenital warts and benign/low-grade abnormalities in the genital areas, the low-risk HPV types can also cause recurrent respiratory papillomatosis (RRP), a medical condition in which tumours grow in the respiratory tract, causing breathing difficulties in infected individuals. As for
high-risk HPV types, other than their association with anogenital cancers, they are also known to cause a small but increasing number of cases of oropharyngeal cancers (D'Souza, Kreimer et al. 2007, Marur, D'Souza et al. 2010). Among these diseases, cervical cancer is the most common and serious HPV-related disease due to the fact that virtually all cervical cancers are caused by HPV.

Although HPV infection is necessary to cause cervical cancer, most individuals infected with HPV are asymptomatic and are able to clear the infection within six months. Approximately 90% of HPV-infected individuals clear the infection within two years (Molano, Van den Brule et al. 2003). However, persistent infection can lead to pre-cervical cancer lesions and invasive cervical cancers (Ho, Einstein et al. 2011), which are more commonly seen in immunocompromised individuals such as individuals infected with human immunodeficiency virus (HIV) (Frisch, Biggar et al. 2000).

1.4.1. Anogenital warts
Anogenital warts (AW), also known as Condylomata acuminata is a common sexually transmitted infection that are benign growths on the skin and mucous membrane of the genital region (vagina, vulva, cervix, anus and penis). Anogenital warts are attributed to HPV infection, in which 90% of the cases are caused by HPV6 and 11. The median annual combined incidence for both men and women in high-income countries is 194.5 per 100,000. The incidence of AW peaked before 24 years of age in women and between 25 and 29 years of age among men (Patel, Wagner et al. 2013). The prevalence of AW in LMICs is less clear, as it is believed that health seeking behaviour for treatment is likely to be low due to the high treatment costs and limited resources. Some data on AW incidence rates have been reported for LMIC in Africa. The overall prevalence of high-risk groups (i.e. female sex workers) range from 2.4% - 14%, depending on the region, and the rates were higher among HIV individuals (Banura, Mirembe et al. 2013). Although non-life threatening, AW are associated with a lower quality of life, including psychosocial effects and has a significant economic burden. This includes shame and embarrassment related to the diagnosis, domestic violence, the inconvenience and discomfort of treatment, as well as the costs related to treatment and management of AW (Bhatia, Lynde et al. 2013, Qi, Wang et al. 2014, Tan, Chio et al. 2014).
1.4.2. Recurrent respiratory papillomatosis

Recurrent respiratory papillomatosis (RRP) is a rare condition in which benign abnormal cell growth in the mucosal layers of the respiratory tract, causing difficulty in breathing. Depending on the age of disease presentation, RRP can be classified into juvenile (<12 years old) or adult (≥12 years old) onset. Juvenile-onset RRP is most commonly diagnosed between two and four years of age, while adult-onset RRP peak incidence is between 20-40 years of age (Larson and Derkay 2010). Similar to GW, RRP is caused mainly by HPV types 6 and 11. Despite the low prevalence rate of 0.75-4 per 100,000 in the United States, it has a very high recurrence rate and potentially has severe complications (i.e. airway obstruction, malignant degeneration, and chronic lung disease or anaesthetic complications) that could be life-threatening (Wilcox, Hull et al. 2014).

1.4.3. Oropharyngeal cancers

Oropharyngeal cancer (OPC) is often associated with alcohol and tobacco use, poor oral hygiene and genetic factors. However, it has been recognised that HPV infection (in particular by HPV type 16) causes a subset of OPC, with the incidence rising (D'Souza, Kreimer et al. 2007, Marur, D'Souza et al. 2010). Approximately 32–60% of OPC are caused by HPV, although these vary between regions (Mehanna, Beech et al. 2013). The distinct features of OPC caused by HPV and not by external (i.e. alcohol and tobacco) or genetic factors are that the cancer site occurs deep in the tonsils, contain HPV-DNA and have fewer tumour suppressor gene (p53) mutations in biopsies as compared with external- or genetic-related OPC. In addition, individuals with OPC caused by HPV also have a higher survival rate after treatment as compared with individuals with external- or genetic-related OPC (Gillison, Koch et al. 2000).

1.4.4. Anogenital cancers

Cervical cancer is the fourth most common cancer in women worldwide, with approximately 530,000 new cases and 266,000 deaths every year, with 85% of these cases occurring in LMICs (GLOBOCAN2012 2012). Countries in sub-Saharan Africa, Latin America and Melanesia (i.e. Papua New Guinea, Fiji and Vanuatu) have the highest cervical cancer rates (age-standardised ratio; ASR of >33 per 100,000) and mortality
(ASR >20 per 100,000) (GLOBOCAN2012 2012). These incidences and mortality rates are approximately six to ten-fold higher than countries such as Australia and New Zealand prior to HPV vaccine introduction. The reasons for such high incidence rates are likely due to a combination of different factors, mainly the lack of comprehensive cervical cancer screening program and HPV vaccine introduction. The majority of cervical cancer cases are squamous cell carcinomas (SCCs), and less commonly, adenocarcinomas. Globally, HPV16 and 18 are responsible for 70% of cervical cancer (de Sanjose, Quint et al. 2010). Other HPV types that are known to cause cervical cancer include HPV31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 66.

The incidence rates for vaginal, vulvar, anal and penile cancers related to HPV infection are lower than cervical cancer. Unlike cervical cancer, not all cases are related to HPV infection. Penile cancers are rare and represent 0.5% of cancers in men (Parkin and Bray 2006). The majority of these cases occur predominately in older men (mean age 60 years old) (Bleeker, Heideman et al. 2009). The incidence can vary significantly between different populations and countries, with LMICs having the highest incidence. In Western countries such as Australia, United States, United Kingdom, the ASR is less than 1 per 100,000, whereas in Uganda and Brazil, the ASR is close to 4 per 100,000 (D.M. Parkin, S.L. Whelan et al. 2002). This variation is likely due to socio-economic and religious practices (e.g. lack of circumcision is a risk factor for penile cancer) (Barnholtz-Sloan, Maldonado et al. 2007). About 40-50% of all penile cancers are positive for HPV-DNA and are mostly attributed to HPV16 and 18 infections (Dillner, von Krogh et al. 2000, Parkin and Bray 2006).

The ASRs for vulvar cancer obtained from cancer registries range between 0.5 and 1.5 per 100,000 with higher rates observed in several European populations (i.e. United Kingdom, North America, Denmark), and lower incidence rates observed in Asia and Latin America (Parkin and Bray 2006, Sankaranarayanan and Ferlay 2006). This variation could be attributed to the prevalence of HPV infection, and other risks factors such as smoking, immune deficiency, high number of sexual partners, differences in health seeking behaviour, diagnosis and reporting (Brinton, Nasca et al. 1990). The peak incidence is observed in women between 60 and 74 years of age, with the majority (88%)
of the tumours identified as SCCs, and the rest made up of melanomas, adenocarcinomas, multiple entities and other tumours (Dittmer, Katalinic et al. 2011). In the United States, about 70% of vulvar cancers are caused by HPV, and almost half are caused by HPV16 (InSinga, Liaw et al. 2008, Gargano, Wilkinson et al. 2012).

Cancers of the vagina are less frequent than vulvar cancers, with the ASR ranging between 0.3–0.7 per 100,000 in most countries based on available data from cancer registries (D.M. Parkin, S.L. Whelan et al. 2002). Vaginal cancer does not seem to have a clear association with the incidence of vulvar or cervical cancer but is influenced by the incidence of HPV infection (Dittmer, Katalinic et al. 2011). The peak incidence of vaginal cancer occurs around 50 years of age based on epidemiological data in a German population (Dittmer, Katalinic et al. 2011). Similar to vulvar cancer, almost 80% of primary vaginal cancers are SCCs (Beller, Sideri et al. 2001), with the remainder made up of adenocarcinomas, and to a lesser extent, verrucous carcinoma, vaginal melanomas and sarcomas (Creasman, Phillips et al. 1998). HPV-DNA, particularly for HPV16, is detected in more than half of the vaginal cancer cases (InSinga, Liaw et al. 2008).

Cancers of the anus are tumours arising in the anal canal, which are predominantly SCCs, adenocarcinomas, or basaloid and cloacogenic carcinomas. Anal cancer is rare in the general population with an ASR of less than 2 per 100,000 in almost all countries that have data reported (D.M. Parkin, S.L. Whelan et al. 2002, Hartwig, Syrjanen et al. 2012). Despite anal SCCs being twice as common in women compared with men, the incidence of anal cancer is particularly high amongst men who have sex with men (MSM) (Parkin and Bray 2006, Steinau, Unger et al. 2013). MSM is associated with high rates of HPV infection, and risk (relative risk: 31.2) of acquiring anal cancer (Frisch, Smith et al. 2003). The incidence of anal cancer is even higher in MSM who are HIV-positive: 81% of HIV-infected individuals have had some grade of anal intraepithelial neoplasia (Palefsky, Holly et al. 2005). Other risk factors include cigarette smoking, anal intercourse, and the number of lifetime sexual partners (Daling, Madeleine et al. 2004). Both HPV16 and 18 causes 87% of HPV-related anal cancers (Hartwig, Syrjanen et al. 2012). Although anal cancers can occur in individuals aged between 30 and 35 years old, the incidence increases exponentially with age in both men and women, with almost half of the cases
diagnosed in people over 65 years of age (Jin, Stein et al. 2011, Hartwig, Syrjanen et al. 2012).

1.5. Immune response to HPV

1.5.1. Natural HPV infection

The immune response to natural infection with HPV is weak and very slow (up to 18 months), as important signals for the induction of immune responses are absent due to the ability of the virus to evade the host immune system. Replication of HPV is exclusively intra-epithelial, and there is minimal or no exposure of viral proteins on the infected cells, preventing activation of an immune response (Stanley 2008). Furthermore, HPV infection is not blood-borne, and does not induce cell death, resulting in very weak or no inflammatory response (Stanley 2008).

Although HPVs are very successful infective agents, the majority of HPV-infected individuals are able to clear the infections within two years, without displaying any clinical symptoms (Molano, Van den Brule et al. 2003). Women who develop benign cervical lesions usually mount a late, but successful cell-mediated immune response, which causes the lesion to regress (Stanley 2009). The role of cell-mediated immunity in the clearance of HPV infection is evident in immunosuppressed individuals (i.e. HIV-infected individuals), who have multiple HPV infections and higher risk of persistence, a higher incidence of genital warts, and an increased risk of progression from sub-clinical to clinical HPV-related disease (Wright, Ellerbrock et al. 1994, Fennema, van Ameijden et al. 1995, Fruchter, Maiman et al. 1996). Serum neutralising antibodies (NAb) against L1 capsid are generated in approximately 50-70% of infected individuals (Carter, Koutsky et al. 2000). However, the peak level of antibodies generated from natural infection is low, reflecting the ability of the virus to cause an exclusive intra-epithelial infection (Kirnbauer, Hubbert et al. 1994, Carter, Koutsky et al. 2000). The long-term significance of immunity induced by natural infection is still uncertain, with some clinical studies suggesting that antibodies elicited by natural infection from the virus may not provide complete protection in the long-term (World Health Organization 2009). Hence, it is recognised that antibody levels detected following natural infection may be less reliable in predicting protection from subsequent infection by HPV.
1.5.2. HPV vaccination

There are currently three licensed HPV vaccines (Table 1.2): Gardasil® (Merck & Co., Inc.; 4vHPV) a quadrivalent vaccine with aluminium-based adjuvant that protects against four genotypes (HPV6, 11, 16 and 18); Cervarix® (GlaxoSmithKline; 2vHPV), a bivalent vaccine with the novel adjuvant AS04 (made up of an aluminium salt and monophosphoryl lipid A) that activates innate immunity (Garcon, Wettendorff et al. 2011), and protects against infection with HPV16 and 18; and a new nonavalent HPV vaccine, Gardasil® 9 (Merck & Co., Inc.; 9vHPV), which contains an additional five cancer-causing HPV types (HPV31, 33, 45, 52, and 58) in addition to the four types in Gardasil®. The 9vHPV vaccine was recently approved by The U.S. Food and Drug Administration (FDA) (December 2014) and Canada Health (February 2015). This vaccine may potentially prevent 90% of cervical cancers. Both 4vHPV and 2vHPV are licensed for females and males aged 9-26 years old, while 9vHPV is licensed for females aged 9-26 years old and males aged 9-15 years old. All HPV vaccines are licensed as a three-dose schedule, delivered intramuscularly at 0, 2, and 6 months (4vHPV and 9vHPV) or 0, 1, and 6 months (2vHPV). The prophylactic vaccines are virus-like particle (VLP)-based vaccines, which does not contain the viral genome, and so are not infectious.

Antibody responses following vaccination peak at month 7 (one month after the third dose) at titres between 10 and 100 fold higher than following natural infection, depending on the HPV type and vaccine (Harper, Franco et al. 2004, Villa, Ault et al. 2006, Villa, Costa et al. 2006). Following an initial decline, they appear to plateau at 18 to 24 months, remaining stable for at least five years at levels above or at least equivalent to those seen with natural infection (Harper, Franco et al. 2006, Villa, Ault et al. 2006, Villa, Costa et al. 2006, Rowhani-Rahbar, Alvarez et al. 2012). The longest duration of antibody response measured following HPV vaccination for 2vHPV and 4vHPV are 9.4 and 8 years, respectively (Ferris, Samakoses et al. 2014, Naud, Roteli-Martins et al. 2014). The new 9vHPV was only licensed in 2014 and studies evaluating the duration of antibody response are currently ongoing. Overall, seroconversion occurs in 99 to 100% of those vaccinated in randomised double-blind placebo-controlled trials (Villa, Costa et al. 2005, Harper, Franco et al. 2006, Joura, Giuliano et al. 2015).
**Table 1.2:** Characteristics of HPV VLP vaccines

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>GlaxoSmithKline™ (Cervarix®)</th>
<th>Merck™ (Gardasil® 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Licensed</td>
<td>2006</td>
<td>2014</td>
</tr>
<tr>
<td>L1 VLP types</td>
<td>6, 11, 16 and 18</td>
<td>6, 11, 16, 18, 31, 33, 45, 52 and 58</td>
</tr>
<tr>
<td>Dose</td>
<td>20/40/40/20 µg</td>
<td>20/20 µg</td>
</tr>
<tr>
<td>Producer cells</td>
<td>Saccharomyces cerevisiae (baker’s yeast) expressing L1</td>
<td>Trichoplusia ni (Hi 5) insect cell line infected with L1 recombinant baculovirus</td>
</tr>
<tr>
<td></td>
<td>225 µg aluminium hydroxyphosphate sulfate</td>
<td>500 µg aluminium hydroxyphosphate, 50 µg 3-O-deacylated-4’-monophosphoryl lipid A</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>500 µg aluminium hydroxyphosphate sulfate</td>
<td>500 µg aluminium hydroxyphosphate sulfate</td>
</tr>
<tr>
<td>Vaccination schedule</td>
<td>0, 2 and 6 months</td>
<td>0, 1 and 6 months</td>
</tr>
</tbody>
</table>

HPV: Human papillomavirus; VLP: Virus-like particle

The administration of the HPV vaccine intramuscularly helps to circumnavigate the virus’s strategy of intra-epithelial evasion of the immune system. Unlike HPV infection, the administered VLPs from the vaccine are able to enter the lymphatics immediately and encounter dendritic cells, which are antigen-presenting cells that go on to activate helper T cell responses and naive B cells in the lymph node (Stanley 2008, Stanley 2010). Activated B cells proliferate and differentiate into plasma cells and secrete HPV-specific antibodies into the lymph and blood that reach the site of infection through transudation. A proportion of activated B cells differentiate and migrate to the bone marrow and survive as long-lived memory B cells that differentiate into plasma cells when reactivated (Olsson, Villa et al. 2007, Stanley 2010). These are depicted in Figure 1.2.
Figure 1.2: Adaptive immune response induced by HPV vaccination. The HPV VLPs are taken up by dendritic cells (DCs) near the injection site, and migrate to the secondary lymphoid organ to activate CD4+ T cells. Following that, CD4+ T cells undergo proliferation and differentiation into effector T cells that interact with naive B cells and produce cytokines to assist in the maturation of B cell responses. Naive B cells upon activation will differentiate into plasma cells which secrete HPV-specific neutralising antibodies into circulation. A portion of naive B and T cells differentiate into HPV-specific memory B and T cells. A majority of these memory cells will migrate to the bone marrow to survive as long-lived memory cells that differentiate into plasma cells (B cell) or activated T cells (T cell) upon re-encounter of the same HPV genotype. It is still unclear if HPV vaccination induces CD8+ T cells. In vivo mouse models have demonstrated that naive B cells can be activated by HPV VLPs in a T cell independent mechanism (Yang, Murillo et al. 2005), but it is not known whether this also occurs in humans.
Neutralising antibodies are believed to be the main mechanism of protection against HPV infection. Vaccine-induced T cells (cytotoxic T cells) are less likely to play a significant role in prophylactic protection. This theory is based on evidence that the L1 component of the HPV vaccine is a structural component of HPV that is expressed only in the later stages of infection and that T cells only recognise their target antigen in the context of major histocompatibility complex (MHC)-based antigen presentation which occurs only after cells have become infected. However, T helper cells have been shown to be critical for the generation and maintenance of B cell memory, as well as antibody class switching (Siegrist and Aspinall 2009). HPV vaccination is a relatively new area of research and the immune mechanisms are still not fully understood (Figure 1.2).

1.6. Efficacy of HPV vaccines

1.6.1. HPV vaccine efficacy in women (15-26 years old)

Table 1.3 summarises the Phase 2 and 3 clinical studies that have evaluated the efficacy of the three HPV vaccines against HPV infection and cervical lesions. Both 4vHPV and 2vHPV have greater than 90% efficacy against grades 1 to 3 cervical intraepithelial neoplasia (CIN), adenocarcinoma in situ and invasive cervical carcinoma due to HPV16/18 in women aged 15-26 years old (Future II Study Group 2007, Garland, Hernandez-Avila et al. 2007, Future I/II Study Group, Dillner et al. 2010, Lehtinen, Paavonen et al. 2012). The administration of 4vHPV also has 96-100% effectiveness in the prevention of HPV16- and HPV18-related vulvar intraepithelial neoplasia, vulvar cancer and vaginal cancer, as well as HPV6- and HPV11-related genital warts and cervical, vulval, vaginal, and anal intra-epithelial neoplasia as precursors to respective cancers (Future II Study Group 2007, Future I/II Study Group, Dillner et al. 2010). Both 4vHPV and 2vHPV when administered as a three-dose schedule have also been shown to be cross-protective against persistent infection and CIN1 or worse caused by high-risk types (i.e. HPV31, 33, 45, 52 and 58) that are phylogenetically-related to the vaccine types (Harper, Franco et al. 2006, Paavonen, Jenkins et al. 2007, Brown, Kjaer et al. 2009, Paavonen, Naud et al. 2009, Wheeler, Castellsague et al. 2012, Konno, Yoshikawa et al. 2014). In accordance with this, cross-NAb to HPV31, and 45 were also generated following 4vHPV or 2vHPV vaccination (Smith, Brownlow et al. 2007, Barzon, Squarzon et al. 2014). The 2vHPV seems to generate greater cross-protection against
certain types (i.e. HPV45) than 4vHPV, which may be due to the adjuvant in 2vHPV (Herrero 2009). However, it is still not known if these antibodies function and persist similarly to the vaccine-type antibodies (Smith, Brownlow et al. 2007). The new 9vHPV is likely to confer protection against these additional high-risk types.

Due to ethical issues, the 9vHPV was compared with 4vHPV instead of a placebo in a multi-centre double-blind, randomised, controlled trial in 16-26 years old women. The per-protocol vaccine efficacy was greater than 96% against cervical, vulval and vaginal pre-cancer and cancer caused by HPV31, 33, 45, 52, and 58 (Merck & Co. 2014, Joura, Giuliano et al. 2015). The antibodies against the common genotypes (HPV6, 11, 16 and 18) between 9vHPV and 4vHPV were also compared, and similar antibody responses were observed, one month post dose three, suggesting that the addition of five new types to 9vHPV did not alter the antibody response (HPV6, 11, 16 and 18) and should be just as effective as the 4vHPV (Joura, Giuliano et al. 2015). More clinical studies on 9vHPV are expected to be completed in the next few years.

Despite HPV vaccines having high efficacy against pre-cancer lesions and cervical cancers, they have limited efficacy (approximately 40%) against pre-existing HPV lesions, and they do not accelerate viral clearance (Hildesheim, Herrero et al. 2007). This is why HPV vaccination is recommended to be administered before exposure to HPV infection (i.e. before sexual debut) to have the greatest benefit.
<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Study</th>
<th>Endpoints</th>
<th>Cohort</th>
<th>Number of subjects</th>
<th>Efficacy, % (95%CI)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gardasil®</td>
<td>Merck 007</td>
<td>HPV persistence (&gt;4 months)</td>
<td>ATP</td>
<td>235</td>
<td>89 (70 – 97)</td>
<td>(Villa, Costa et al. 2005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cervical/external genital disease (7 months)</td>
<td>ATP</td>
<td>233</td>
<td>100 (16 – 100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV persistence (5 years)</td>
<td>ATP</td>
<td>2,241</td>
<td>95.6 (83.3–99.5)</td>
<td>(Villa, Costa et al. 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIN1+ (5 years)</td>
<td>ATP</td>
<td>2,258</td>
<td>100.0 (&lt;0.0 – 100.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FUTURE I</td>
<td>CIN 1+, AIS</td>
<td>ATP</td>
<td>2,261</td>
<td>100 (94 – 100)</td>
<td>(Garland, Hernandez-Avila et al. 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITT</td>
<td>2,723</td>
<td>55 (40 – 66)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>External genital lesions</td>
<td>ATP</td>
<td>2,279</td>
<td>100 (94 – 100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITT</td>
<td>2,684</td>
<td>73 (58 – 83)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FUTURE II</td>
<td>CIN 2+, AIS</td>
<td>ATP</td>
<td>366</td>
<td>98 (86 – 100)</td>
<td>(Future II Study Group 2007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITT</td>
<td>5,260</td>
<td>92.9 (70.0 – 98.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV persistence (6 months)</td>
<td>ATP</td>
<td>355</td>
<td>100 (76.8 – 100)</td>
<td>(Harper, Franco et al. 2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TVC</td>
<td>560</td>
<td>87.5 (64.6–95.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIN 1+</td>
<td>ATP</td>
<td>353</td>
<td>93.5 (51.3–99.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TVC</td>
<td>553</td>
<td>92.9 (70.0–98.3)</td>
<td></td>
</tr>
<tr>
<td>Cervarix®</td>
<td>HPV-001</td>
<td>HPV persistence (6 months)</td>
<td>ATP</td>
<td>414</td>
<td>96.0 (75.2–99.9)</td>
<td>(Harper, Franco et al. 2006)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TVC</td>
<td>481</td>
<td>94.4 (78.2–99.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV persistence (12 months)</td>
<td>ATP</td>
<td>385</td>
<td>100.0 (52.2-100.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TVC</td>
<td>481</td>
<td>94.0 (61.1-99.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIN 1+</td>
<td>ATP</td>
<td>470</td>
<td>100.0 (42.4-100.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CIN 2+</td>
<td>ATP</td>
<td>470</td>
<td>100.0 (-7.7-100.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HPV-007</td>
<td>HPV persistence (6 months)</td>
<td>ATP</td>
<td>401</td>
<td>100.0 (90.0-100.0)</td>
<td>(GlaxoSmithKline Vaccine, Romanowski et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV persistence (12 months)</td>
<td>ATP</td>
<td>372</td>
<td>100.0 (81.8-100.0)</td>
<td></td>
</tr>
<tr>
<td>(6.4 years following vaccination)</td>
<td>CIN 1+</td>
<td>TVC</td>
<td>100.0 (73.4-100)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>CIN 2+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>7,344</td>
<td>7,312</td>
<td>92.9 (79.9-98.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC</td>
<td>8,040</td>
<td>8,080</td>
<td>94.5 (86.2-98.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CIN 3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>7,344</td>
<td>7,312</td>
<td>80.0 (63.3-98.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TVC</td>
<td>8,040</td>
<td>8,080</td>
<td>90.9 (60.8-99.1)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*PATRICIA

| CIN 2+                          |        |     |                  |
| ATP                             | 7,344  | 7,312 | 92.9 (79.9-98.3) |
| TVC                             | 8,040  | 8,080 | 94.5 (86.2-98.4) |
| CIN 3+                          |        |     |                  |
| ATP                             | 7,344  | 7,312 | 80.0 (63.3-98.1) |
| TVC                             | 8,040  | 8,080 | 90.9 (60.8-99.1) |

PATRICIA-43.7 months

| CIN1+                          |        |     |                  |
| ATP                             | 7,344  | 7,312 | 92.9 (79.9-98.3) |
| TVC                             | 8,040  | 8,080 | 94.5 (86.2-98.4) |
| CIN2+                          |        |     |                  |
| ATP                             | 7,344  | 7,312 | 80.0 (63.3-98.1) |
| TVC                             | 8,040  | 8,080 | 90.9 (60.8-99.1) |
| CIN3+                          |        |     |                  |
| ATP                             | 7,344  | 7,312 | 80.0 (63.3-98.1) |
| TVC                             | 8,040  | 8,080 | 90.9 (60.8-99.1) |

CVT

| HPV persistence (12 months)    | ATP    | 2,635 | 2,677 | 90.9 (82.0-95.9) |
|                                | ITT    | 3,727 | 3,739 | 49.0 (38.1-58.1) |
| CIN2+                          |        |     |      |                  |
| ATP                             | 2,635  | 2,677 | 89.8 (39.5-95.9) |
| ITT                             | 3,727  | 3,739 | 49.0 (38.1-58.1) |

Japan study

| HPV persistence (12 months)    | ATP    | 382   | 383   | 100.0 (74.8-100.0) |
|                                | ITT    | 413   | 409   | 100.0 (79.5-100.0) |
| CIN1+                          |        |     |      |                  |
| ATP                             | 406    | 404   | 100.0 (42.2-100.0) |
| ITT                             | 438    | 431   | 100.0 (56.6-100.0) |
| CIN2+                          |        |     |      |                  |
| ATP                             | 406    | 404   | 100.0 (-8.0-100.0) |
| ITT                             | 438    | 431   | 100.0 (32.3-100.0) |

9vHPV study

<table>
<thead>
<tr>
<th>Gardasil®9 9vHPV study</th>
<th>96.1% confidence intervals used in PATRICIA instead of 95%; acaused by HPV31, 33, 45, 52 and 58</th>
</tr>
</thead>
<tbody>
<tr>
<td>9vHPV group 4vHPV group</td>
<td>9vHPV study</td>
</tr>
<tr>
<td>9vHPV group</td>
<td>4vHPV group</td>
</tr>
<tr>
<td>CIN 1+, AIS</td>
<td>96.3 (79.5–99.8)</td>
</tr>
<tr>
<td>HPV persistence (6 months)</td>
<td>96.0 (94.4 – 97.2)</td>
</tr>
</tbody>
</table>

CI: Confidence intervals; AIS: Adenocarcinoma in situ; ATP: According to protocol; CIN: Cervical intraepithelial neoplasia; CIN1+: CIN grade 1 or worse; CIN2+: CIN grade 2 or worse; FUTURE: Females united to unilaterally reduce endo/ectocervical disease; GSK: GlaxoSmithKline; ITT: Intention to treat; MITT: Modified intention to treat; TVC: Total vaccine cohort; PATRICIA: Papilloma trial against cancer in young adults. Modified from (Schiller, Castellsague et al. 2008).
1.6.2. HPV vaccine efficacy in adolescents (below 15 years of age)
Due to ethical issues of evaluating clinical outcomes in adolescents below the age of 15 years, safety and immunogenicity bridging studies of HPV vaccination are conducted instead, so that the licensing of the vaccines (licensed for 15-26 years old women) can be extended for use in young adolescent girls or boys before the onset of sexual activity.

In immunobridging studies evaluating either 4vHPV or 2vHPV, the type-specific antibodies induced by HPV vaccination were non-inferior or at least one to two fold higher in adolescent girls and boys when compared with women, following a three-dose schedule. These observations were consistent across all time points (one month post dose one, two or three) (Block, Nolan et al. 2006, Pedersen, Petaja et al. 2007, Petaja, Keranen et al. 2009). For 9vHPV, higher (one to two fold) HPV vaccine type antibodies were also observed in young adolescent boys and girls (9-15 years old) as compared with young women (16-26 years old) one month post-dose three (Van Damme, Olsson et al. 2015). These observations (lower antibody responses in individuals >15 years of age) could be due to prior or pre-existing HPV infection. However, for those who are HPV DNA or seronegative, it is unclear why there is a difference in antibody response in different age groups.

1.7. Effectiveness of HPV vaccines
In 2014, approximately 64 countries worldwide have introduced HPV vaccination (either 4vHPV or 2vHPV) into their national immunisation programmes, although the specific target age group and catch up programmes, as well as uptake rates vary between countries (Markowitz, Tsu et al. 2012, Garland, Kjaer et al. 2016). Within a few years after HPV vaccine introduction, positive impacts against HPV-associated diseases have been reported by some countries. For example, within three to four years of 4vHPV introduction in Australia, a significant decrease in HPV prevalence, genital warts and CIN grade 2 or worse were observed in girls below the age of 25 years (Brotherton, Fridman et al. 2011, Donovan, Franklin et al. 2011, Tabrizi, Brotherton et al. 2012). Similar observations were also observed in the Danish and Czech Republic populations (Baandrup, Blomberg et al. 2013, Baldur-Felskov, Dehlendorff et al. 2014, Petras and Adamkova 2015). The impact and effectiveness of 4vHPV against HPV infection, genital
warts and cervical abnormalities over ten years since the vaccine was introduced were summarised in a systematic review (Garland, Kjaer et al. 2016). In the United Kingdom, a significant decline in the prevalence of HPV infection was also seen following 2vHPV introduction in 2008 (Mesher, Soldan et al. 2013). Interestingly, the number of genital warts cases has also decreased following 2vHPV introduction (Howell-Jones, Soldan et al. 2013). This is unexpected since 2vHPV does not protect against HPV6 and 11, which accounts for 90% of genital warts cases. Some possible explanations were the decrease in genital warts cases caused by other low-risk types, the use of 4vHPV in the private sector and possible cross-protection against HPV6 and 11 by 2vHPV (although evidence is scarce on this and more studies are needed) (Howell-Jones, Soldan et al. 2013). Since 2012, the UK HPV immunisation program has changed to 4vHPV.

The impact of HPV vaccination programmes is expected to vary between countries based on the vaccine used, implementation strategies, vaccination coverage and infection/disease rates. School-based vaccination programmes have shown the greatest success, which can be attributed to the high vaccine coverage rates (> 70%) demonstrated in the United Kingdom and Australia (Markowitz, Tsu et al. 2012, Kavanagh, Pollock et al. 2014). In contrast, HPV vaccination provided through primary care providers were found to have low completion rates for the full three doses (38% in 2013) in the United States (Stokley, Jeyarajah et al. 2014). Despite low completion rates, there was still a substantial reduction in the prevalence of vaccine HPV types among those vaccinated with at least one dose of 4vHPV (Kahn, Brown et al. 2012). A systematic review and meta-analysis of the population effects of HPV vaccination by Drolet et al. highlighted a significant reduction in HPV16 and 18 infections, as well as HPV31, 33 and 45 infections and genital warts after HPV vaccine introduction in countries with more than 50% coverage, irrespective of whether 4vHPV or 2vHPV was used (Drolet, Benard et al. 2015). These reductions in anogenital warts and HPV infection by vaccine types were not only seen in girls/women, but also in boys/men, indicating that the vaccines confer substantial herd immunity. In countries with less than 50% coverage, there was only a significant reduction in HPV16 and 18 infections and anogenital warts in girls below 20 years old (no cross-protection or herd immunity, or HPV-related outcomes were observed in boys/men or older women). These studies demonstrated that HPV vaccines are
effective in preventing HPV infection, genital warts and cervical lesions, and at coverage rates greater than 50% confer herd immunity, at least in populations of developed nations. However, impact studies at a population level following HPV vaccine introduction in LMICs are lacking, which is important since these countries have the greatest burden of HPV-associated diseases.

1.8. Alternative HPV vaccination schedules

Despite the high effectiveness of HPV vaccines, there is still a large burden of HPV-associated diseases, in particular cervical cancers and genital warts in LMICs. The main challenges in LMICs are poor cervical cancer screening programs, barriers to implement HPV vaccines including high cost and the logistical difficulty of administering vaccines to adolescents or preteens, attaining high vaccination coverage, and poor access to medical services. These issues have prompted the exploration of alternative vaccination schedules, including reduced doses of HPV vaccines.

1.8.1. Extended dosing schedule

Several studies have investigated the possibility of delaying the second and third dose of the HPV vaccine to extend the duration of protection against HPV, and also provide more flexibility in a three-dose schedule (Table 1.4). These studies demonstrated that the administration of the third dose of the vaccine could be given between six and twelve months after the second dose and still produce similar antibody responses as the standard three-dose schedules.
**Table 1.4: Clinical trials examining alternative schedules for three doses of HPV vaccines**

<table>
<thead>
<tr>
<th>Study location</th>
<th>Trial design</th>
<th>Vaccine</th>
<th>Gender/ Age</th>
<th>Dose/ Timeline</th>
<th>Results</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Romania, Slovakia and Italy</td>
<td>Multi-centre, Randomised, open-label</td>
<td>2vHPV</td>
<td>Female 15-25 years</td>
<td>(0, 1, 12) vs (0, 1, 6) months</td>
<td>Similar seroconversion rates and non-inferior antibody GMTs</td>
<td>(Esposito, Birlutiu et al. 2011)</td>
</tr>
<tr>
<td>USA</td>
<td>Randomised, open-label</td>
<td>4vHPV</td>
<td>Female 18-23 years</td>
<td>(0, 2, 12) vs (0, 2, 6) months</td>
<td>Non-inferior and superior antibody GMTs for HPV6, 11 and 16, one month after 3rd dose for alternate schedule</td>
<td>(Zimmerman, Nowalk et al. 2010)</td>
</tr>
<tr>
<td>USA</td>
<td>Randomised, open-label</td>
<td>4vHPV</td>
<td>Male 18-24 years</td>
<td>(0, 2, 12) vs (0, 2, 6) months</td>
<td>Non-inferior and superior antibody GMTs for all types one month after 3rd dose for alternate schedule</td>
<td>(Lin, Zimmerman et al. 2014)</td>
</tr>
<tr>
<td>Vietnam</td>
<td>Cluster randomised, open-label</td>
<td>4vHPV</td>
<td>Female 11-13 years</td>
<td>(0, 3, 9) or (0, 6, 12) or (0, 12, 24) months</td>
<td>Non-inferior antibody GMTs for 0, 3, 9 and 0, 6, 12 months for all four HPV types; inferior antibody GMTs for 0, 12, 24 months for all four HPV types, when compared to standard dosing</td>
<td>(Neuzil, Canh do et al. 2011)</td>
</tr>
</tbody>
</table>

**GMT: Geometric Mean Titres**

2vHPV: Cervarix® (HPV genotypes 16 and 18)

4vHPV: Gardasil® (HPV genotypes 6, 11, 16 and 18)

Source of table from (Stanley, Sudenga et al. 2014)
1.8.2. Reduced-dose HPV vaccination schedules

The use of reduced-dose schedules in vaccinology is not unusual as other vaccines such as pneumococcal (as a primary schedule for children less than one year old) and hepatitis A dosing schedules have been revised from three to two doses, following clinical studies that have demonstrated non-inferiority in immunogenicity and safety (Russell, Carapetis et al. 2011, Raczniaik, Thomas et al. 2013). As described above, the immunisation of adolescent girls yielded a much higher antibody response than women, which raises an interesting question as to whether a two-dose HPV schedule in adolescents is similarly immunogenic and produces long-lasting antibody responses compared to a three-dose schedule in women. However, since there have been no breakthrough infections from vaccinated individuals in clinical trials, and no identified correlates of protection, it is difficult to establish a protective threshold level of antibody. Therefore, studies based on non-inferiority of antibody responses have been used in clinical trials for comparison.

1.8.2.1. Antibody responses

There are a number of clinical studies that have evaluated antibody responses following either 2vHPV or 4vHPV in the context of reduced-dose HPV vaccination. These studies have demonstrated non-inferior antibody responses in healthy girls (aged 9 to 14 years) who received two doses (0 and 6 months), compared with women (range from 15-25 years old) who received three doses (2vHPV-0, 1 and 6 months; 4vHPV- 0, 2 and 6 months) (Kreimer, Rodriguez et al. 2011, Romanowski, Schwarz et al. 2011, Dobson, McNeil et al. 2013, Puthanakit, Schwarz et al. 2013, Romanowski, Schwarz et al. 2014) (summarised in Table 1.5). Follow-up studies of these girls to date have found that these non-inferior antibody responses against vaccine types persist for at least three and four years after the last vaccine dose of 4vHPV and 2vHPV, respectively, (Dobson, McNeil et al. 2013, Romanowski, Schwarz et al. 2014). Studies of reduced-dose 9vHPV schedules are ongoing and similar/higher antibody responses were demonstrated one month after the third dose in girls who received two doses compared with women who received three doses (Iversen, Miranda et al. 2016).
However, the antibody response seems to wane earlier in the two-dose schedule when compared with the standard three-dose schedule for certain HPV types. Girls aged 9 to 13 years old who received two doses of 4vHPV had a lower HPV18 and 6 antibody levels 24 and 36 months after the last vaccine dose, respectively, when compared with girls of the same age who received three doses (Dobson, McNeil et al. 2013). This was no longer the case for HPV6 at 60 months (Dobson 2017). In another study, girls (10 year old) who received either one or two doses of 2vHPV had lower HPV16 and 18 antibody levels, 24 months following the last dose of the vaccine when compared with girls who received three doses (LaMontagne, Mugisha et al. 2014). Despite this, reduced antibody levels does not necessarily reflect a possible increase in the risk of HPV infection since there are no established correlates of protection. Furthermore, differences in serological assays measuring HPV-specific antibodies makes comparison of the results between studies using different HPV vaccines difficult (Brown, Garland et al. 2011). Whether these inferior responses impact on long-term protection in girls receiving reduced-dose HPV schedules remains to be determined.
Table 1.5: Clinical studies on antibody responses following reduced-dose HPV schedules

<table>
<thead>
<tr>
<th>Study</th>
<th>Vaccine</th>
<th>Primary endpoints</th>
<th>No. of participants and vaccine dose</th>
<th>Non-inferiority criteria</th>
<th>Major findings</th>
</tr>
</thead>
</table>
| **NCT00128661  
Costa Rica** | 2vHPV   | Vaccine efficacy between reduced dose Cervarix® vaccine and control (Hep A vaccine). | HPV vaccine vs control vaccine (18-25yo women); 3 doses (n= 2,965 vs 3021); 2 doses (n= 488 vs 441); 1 dose (n= 277 vs 274) | lower limit of the 95% CI of the GMT ratio >0.5                                           | No difference in vaccine efficacy between different dosage group compared to control group                                                     |
|                |         |                                                                                  | *3 doses (n= 120); 2 doses (1m apart) (n= 140); 2 doses (6m apart) (n= 52); 1 dose (n= 78) ^Natural infection (n= 113)   |                                                                                             | Cross protection against 1 year persistent infection with HPV31, 33 and 45 for three dose but not two dose group; one dose group too small for analysis |
|                |         |                                                                                  |                                                                                                     |                                                                                             | Non-inferiority Ab levels between 2-dose groups (0,1m and 0,6m) and 3-dose group; 2-dose group (0,1m borderline non-inferiority).              |
|                |         |                                                                                  |                                                                                                     |                                                                                             | 1-dose group HPV16 and 18 Ab levels 9 and 5 times higher than natural infection, respectively                                                  |
|                |         |                                                                                  |                                                                                                     |                                                                                             | Strong correlation between HPV16 ELISA and neutralisation titres regardless of different doses; significantly lower cross-NAb for HPV31 in 1-dose group than 3-dose group |
| **NCT00541970  
Germany/Canada** | 2vHPV   | Non-inferior ab response between 2-dose schedules (with or without alternative formulations) and 3-dose schedule. | Healthy girls/women (9-14, 15-19, 20-25yo) 2 doses (20/20ug)(0,6m)(n=240); (40/40ug)(0,6m)(n=241); (40/40ug)(0,2m)(n=240); compared with 3 doses (20/20ug)(0,1,6m) (n=239) | lower limit of the 95% CI of the GMT ratio >0.5; upper limit of the 95% CI of the GMT ratio <2 | Non-inferior HPV16 and 18 Ab titres between 2-dose and 3-dose group except for HPV16 in 2 dose group (40/40ug) (0,2m).                   |
|                |         |                                                                                  |                                                                                                     |                                                                                             | Compared to women (15-25yo) (3-dose group) 1 m after last dose, non-inferior HPV16 and 18 in:                                          |
|                |         |                                                                                  |                                                                                                     |                                                                                             | - 9-14yo girls in all 2-dose groups;                                                                                                           |
|                |         |                                                                                  |                                                                                                     |                                                                                             | - 15-19yo in all 2-dose groups except HPV16 in the 2-dose group (40/40ug)(0,2m);                                                           |
|                |         |                                                                                  |                                                                                                     |                                                                                             | - 20-25yo women in 2-dose group (40/40ug) (0,6m) and only HPV18 in 2-dose group (20/20ug) (0,6m)                                    |
|                |         |                                                                                  |                                                                                                     |                                                                                             | Non-inferiority maintained up to 24m for 9-14yo girls except for 2-dose group (40/40ug)(0,2m) when compared with women (15-25yo) (3-dose group); non-inferiority maintained up to 48m for a subset of girls and women |

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<table>
<thead>
<tr>
<th>Study ID</th>
<th>HPV Type</th>
<th>HPV Schedule</th>
<th>Ab Response</th>
<th>Lower Limit of 95% CI of GMT Ratio</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT01717118</td>
<td>2vHPV</td>
<td>Non-inferior Ab response between standard schedule (0.1,6m) and extended schedule (0.6,60m)</td>
<td>Standard schedule: Girls (9-10yo) (n=474) Women (18-24yo) (n=500) Extended schedule: Girls (9-10yo) (n=1026)</td>
<td>Upper limit of the 95% CI of the GMT ratio &lt;2.</td>
<td>Non-inferior HPV16 and 18 Ab titres between 2- and 3-dose group at 7 and 21m. Higher but non-inferior Ab titres in 3 dose (girls) than 2 dose (girls). Higher but non-inferior Ab titers in 2 dose (girls) than 3 dose (women)</td>
</tr>
<tr>
<td>NCT00501137</td>
<td>4vHPV</td>
<td>Non-inferior Ab response between 2-dose and 3-dose schedules in girls (9-13yo) and women (16-26yo).</td>
<td>Girls: 3 doses: n=261 2 doses: n=259 Women: 3 doses: n=310</td>
<td>Lower limit of the 95% CI of the GMT ratio &gt;0.5</td>
<td>Non-inferior HPV6, 11, 16 and 18 Ab titres between 2 dose (girls) and 3 dose (women) at 7m, and remained up to 36m. Non-inferiority for HPV18 and 6 did not persist by 24m, and by 36m, respectively in 2 dose (girls) compared to 3 doses (girls).</td>
</tr>
<tr>
<td>Uganda</td>
<td>2vHPV</td>
<td>Ab response between girls (10yo) who received 1, 2 or 3 doses, more than 24m after last dose</td>
<td>Girls: 3 doses: n=195 2 doses: n=145 1 dose: n=36</td>
<td>Inferior HPV16 and 18 Ab titres in girls who received 1 or 2 doses when compared with 3 doses</td>
<td>Higher HPV16 and 18 Ab titres than Ab titres induced by natural infection from other studies. Higher HPV16 and 18 Ab responses in girls than women (from CVT study) who received the same number of doses.</td>
</tr>
<tr>
<td>Germany, Canada, Italy, Taiwan and Thailand</td>
<td>2vHPV</td>
<td>Ab and cell-mediated responses between girls (9-14yo) receiving 2 (0.6 or 0.12m) and 3 doses (0.1,6m)</td>
<td>Girls/Women: 3 doses: n=472 2 doses (0,6m): n=548 2 doses (0,12m): n=408</td>
<td>Non-inferior HPV16 and 18 Ab response in girls in the 2-dose groups (0,6m and 0,12m) compared with women in the 3 dose group (0,1,6m), 1m after last dose</td>
<td>Similar HPV16, 18, 31, 45 Ab and CMI responses between 2-dose and 3-dose groups.</td>
</tr>
</tbody>
</table>
India 4vHPV Immunogenicity between 1, 2 (0,6m; 0,2m) and 3 (0,2,6m) doses Girls: 10-18yo; 3 doses: n=4348 2 doses (0,6m): n=4979 2 doses (0,2m): n=3452 1 doses: n=4950 Lower limit of the 95% CI of the GMT ratio >0.5 Non-inferior Ab response between 2-dose (0,6m) and 3-dose group across all 4 HPV types 1m post-vaccine and over 48m period Inferior Ab response for 2-dose (0,2m) and 1-dose group compared to 3 dose group at 18m and 36m Non-inferior avidity index between 2-dose (0,6m and 0,2m), 1-dose and 3-dose group at 18m No difference in HPV16/18 persistent infection rates between dosage groups

^HPV16 or HPV18 L1 seropositive women prevaccination serum; simulating the “natural infection” group; yo: years old; Ab: antibody; CI: confidence interval; GMT: geometric mean titres; m: month; NR: not reported; w/o: without; CVT: Costa Rica Vaccine Trial
1.8.2.2. Vaccine efficacies against HPV infection, genital warts and cervical cancer precursor lesions

Table 1.6 summarises the studies on reduced-dose HPV vaccine efficacies against HPV infection and abnormal cervical lesions. A study in Costa Rica reported no significant difference against newly acquired HPV16/18 infection in 7,153 women aged 18-25 years, who received either one, two or three doses of 2vHPV when compared with the control group who received modified Hepatitis A vaccine post-vaccination. However, the analysis for the group who received one dose should be interpreted with caution due to small number of events in the control group (N=10 and N=15 for greater than 12 and 6-month persistent infection, respectively) and limited follow-up (Kreimer, Rodriguez et al. 2011). A combined analysis of data from this study and the PATRICIA studies suggest that even one dose of 2vHPV can be protective (Kreimer, Struyf et al. 2015). However, more studies evaluating a single dose of HPV vaccine are needed, especially for long-term protection. The effectiveness against abnormal cervical lesions in terms of reduced-dose HPV schedules have also been evaluated in the Australian population. Women immunised with either one, two or three doses of 4vHPV had a reduced risk (hazard ratio of 0.76; 95% CI 0.72 to 0.80) of developing cytological-confirmed low-grade cervical disease (low-grade squamous intraepithelial lesions (LSIL) and/or atypical endocervical cells of uncertain significance) when compared with unvaccinated women (Gertig, Brotherton et al. 2013). The reduced risk in cytological-confirmed low-grade cervical disease in those vaccinated with one or two doses could also be attributed to herd immunity from HPV vaccine introduction in the country (Tabrizi, Brotherton et al. 2014). However, only those women who received three doses of 4vHPV had a significant reduction in histological- and cytological-confirmed high-grade cervical diseases (CIN2, CIN3, and/or AIS; high-grade squamous intraepithelial lesion (HSIL) and/or adenocarcinoma) (Gertig, Brotherton et al. 2013), suggesting that a reduced-dose schedule may have limited protection against high-grade cervical diseases. Although the other dosage group (one- and two-dose groups) did not have any significant reduction in high-grade cervical diseases, the sample size was insufficient to make firm conclusion. A similar observation was also seen in a case-control study in Australia: individuals vaccinated with three doses of 4vHPV had a lower risk of acquiring HPV infection, and
the risk increased as the number of doses decreased when compared with unvaccinated individuals (Crowe, Pandeya et al. 2014).

The effectiveness of a reduced-dose HPV vaccine schedule on genital warts seems to have a dose effect (decreased risk with increased vaccine dose). In Sweden, the incidence risk ratio (IRR) of genital warts in girls (10-16 years old) who received three, two or one dose of the vaccine was dose dependent: 0.18, 0.29 and 0.31 respectively (Herweijer, Leval et al. 2014). In addition, the genital wart IRR was higher in older women (17-19 years old); 0.23, 0.35 and 0.71 for those who received three, two and one dose of the 4vHPV, respectively. This is not surprising given that higher immunogenicity against HPV vaccine types have been reported in young adolescents (9-15 years old) when compared with women (>16 years old) following a two- (0, 6 months) or three-dose schedule (0, 1 or 2, 6 months) (Block, Nolan et al. 2006, Petaja, Keranen et al. 2009, Romanowski, Schwarz et al. 2011, Dobson, McNeil et al. 2013, Lazcano-Ponce, Stanley et al. 2014). It is postulated that an additional 59 genital warts cases per 100,000 person-years will be prevented with three doses of 4vHPV as compared with two doses. However, whether this observation relates to more serious diseases, such as cervical cancer, is not known. Nevertheless, some of these studies suggest that there may be some protection against HPV vaccine types after having one dose of HPV vaccine, particularly in adolescents, and when given before HPV exposure.

These studies, including those that reported non-inferior antibody responses for reduced-dose schedules, have provided evidence for the World Health Organization (WHO) to change its recommendations to include a two-dose HPV vaccine schedule for girls <15 years old, provided the interval between each dose is at least six months (World Health Organization 2014). However, it is important to note that the recommendations of three doses of the HPV vaccine remained unchanged for girls >15 years old and immunocompromised individuals (i.e. HIV-infected) (World Health Organization 2014). In addition, individuals whose first and second doses of the HPV vaccine are less than six months are also recommended to receive a third dose.
**Table 1.6: Efficacy of reduced-dose HPV vaccination schedules**

<table>
<thead>
<tr>
<th>Country of Study</th>
<th>Vaccine</th>
<th>Primary endpoints</th>
<th>No. of participants and vaccine dose</th>
<th>Major Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costa Rica</td>
<td>2vHPV vs control vaccine (Hep A)</td>
<td>Newly detected HPV16 or HPV18 infection that persisted for at least 10 months</td>
<td>3 dose (2vHPV: 2,957; control: 3,010) 2 dose (2vHPV: 422; control: 380) 1 dose (2vHPV:196; control: 188)</td>
<td>No significant difference between dosage group VE for: 3 dose- 80.9% (95% CI = 71.1% to 87.7%) 2 dose- 84.1% (95% CI = 50.2% to 96.3%) 1 dose- 100% (95% CI = 66.5% to 100%)  Cross-protection against HPV31, HPV33, and HPV45 for 3-dose group (41.3%; 95% CI: 18.9%–57.9%), but not for 2-dose group (~25.9%; 95% CI: −334%–61.1%); number of events too small for analysis for 1-dose group</td>
</tr>
<tr>
<td>Australia</td>
<td>4vHPV</td>
<td>Effectiveness against cervical abnormalities in a screening population of women eligible for vaccination in the school-based cohorts (aged 17 or younger in 2007)</td>
<td>3 dose- 21,199 2 dose- 3,412 1 dose- 2,568 0 dose- 15,192</td>
<td>Hazard ratio 3 dose- 0.72 (95% CI: 0.58–0.91) 2 dose- 1.02 (95% CI: 0.68–1.53) 1 dose- 1.47 (95% CI: 0.97–2.23)  - No significant difference in risk ratio for women who received less than 3 doses - High grade cytological abnormalities - 3 dose: 0.71; 95% CI: 0.61–0.83 - 2 dose: 0.95; 95% CI: 0.73–1.23 - 1 dose: 0.85; 95% CI 0.62–1.17 - Low grade cytological abnormalities - 3 dose: 0.79; 95% CI: 0.75–0.84 - 2 dose: 0.64; 95% CI 0.57–0.72 - 1 dose: 0.67; 95% CI 0.59–0.76 - Reduced risk of low-grade cytological abnormalities for women who received one or two doses of vaccine compared with unvaccinated women.</td>
</tr>
<tr>
<td>Country</td>
<td>Study Type</td>
<td>HPV Vaccine</td>
<td>Analysis</td>
<td>Odds Ratio for Exposure to HPV Vaccine Compared with No Vaccine for CIN2+/AIS</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>Australia</td>
<td>4vHPV Case-control analysis</td>
<td>Effectiveness of 4vHPV against cervical abnormalities four years following vaccine introduction</td>
<td>- 3 dose- 0.54 (0.43- 0.67) - 2 dose- 0.79 (0.64- 0.98) - 1 dose- 0.95 (0.77- 1.16)</td>
<td></td>
</tr>
<tr>
<td>(Queensland)</td>
<td>(Crowe, Pandeya et al. 2014)</td>
<td>3 dose- 25,119 2 dose- 12,073 1 dose- 10,879 0 dose- 60,282</td>
<td>- Odds ratio for exposure to HPV vaccine compared with no vaccine for low grade abnormalities or abnormal cytology - 3 dose- 0.66 (0.62- 0.70) - 2 dose- 0.79 (0.74- 0.85) - 1 dose- 0.95 (0.89- 1.02)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>- Decreased odds ratio in older women (0.95; 0.63- 1.45)</td>
<td></td>
</tr>
<tr>
<td>Denmark</td>
<td>4vHPV Retrospective-cohort study</td>
<td>HPV vaccination status and the effect on risk of GWs in Denmark population</td>
<td>Reduced risk of GWs in vaccinated girls (at least one dose): 0.12-0.62 (depending on age; lower risks in younger birth cohorts)</td>
<td></td>
</tr>
<tr>
<td>(Blomberg, Dehlendorff et al. 2013)</td>
<td></td>
<td>Vaccinated- 248, 403 Unvaccinated-151, 367</td>
<td>- No dose stratification results</td>
<td></td>
</tr>
<tr>
<td>Sweden</td>
<td>4vHPV Retrospective-cohort study</td>
<td>Estimation of incidence rates per 100,000 person-years of GWs in 5 different age groups</td>
<td>-IRR associated with HPV vaccine compared with unvaccinated for GWs - 3 dose- 0.20 (0.17- 0.23) - 2 dose- 0.32 (0.26- 0.40) - 1 dose- 0.54 (0.43- 0.68)</td>
<td></td>
</tr>
<tr>
<td>(Herweijer, Leval et al. 2014)</td>
<td></td>
<td>3 dose- 89, 836 2 dose- 107, 338 1 dose- 115, 197 0 dose- 1, 045, 157</td>
<td>-IRD for GWs - 3 vs 2 dose- 66 (23-109) - 3 vs 1 dose- 191 (121-262) - 3 vs 0 dose- 449 (430-467)</td>
<td></td>
</tr>
</tbody>
</table>

CIN1+/CIN2+/AIS: Cervical Intraepithelial Neoplasia grade 1 or worst/ Cervical Intraepithelial Neoplasia grade 2 or worse/ Adenocarcinoma in-situ; IRR: Incidence rate ratios, GWs: Genital warts, IRD: Incidence rate difference per 100,000 person-years
1.9. Gaps in the knowledge of reduced-dose HPV schedules

Despite the recommendation of two-dose HPV schedules by the WHO, further research is required to assess the clinical effectiveness of this schedule, particularly LMICs, where disease burden is greatest (World Health Organization 2014). The evidence for non-inferior immunogenicity of two- versus three-dose HPV schedules in adolescents was inconclusive according to a recent systematic review and meta-analysis of the literature (Donken, Knol et al. 2015). As current vaccines are not licensed as a two-dose schedule, countries planning to adopt this schedule need to ensure adequate surveillance programs are in place to monitor the duration of protection of such schedules against cervical cancer and other HPV-associated diseases (Stanley M and Wheeler C. 2014).

Whether the duration of antibody response generated by reduced-dose schedules is similar to three doses is still not known and follow-up studies are ongoing. A study by Dobson et al. reported lower HPV18 and HPV6 antibody levels in girls aged 9-13 years old 24 and 36 months after receiving two doses of 4vHPV when compared with girls who received three doses, respectively (Dobson, McNeil et al. 2013). Furthermore, several clinical studies have reported the generation of cross-NAb by 4vHPV or 2vHPV (i.e. HPV31 and 45) when administered in a three-dose schedule, but not with two doses (Kreimer, Rodriguez et al. 2011, Safaeian, Kemp et al. 2013, Barzon, Squarzon et al. 2014). A cross-sectional study in Australia demonstrated some cross-protection only on fully vaccinated women six years following the introduction of 4vHPV (Tabrizi, Brotherton et al. 2014). So, the question of whether reduced-dose HPV schedules generates a similar level and function of cross-NAb as a three-dose schedule is still unknown and needs to be determined. This is important as both 4vHPV and 2vHPV does not protect against all oncogenic HPV types, and a reduced-dose schedule may not offer the additional protection against related non-vaccine high-risk HPV types (i.e. HPV31 and 45). One study postulated an approximate 5% increase in cervical cancer incidence, assuming no cross-protection from a two-dose schedule, and at least 30 years protection in countries with high vaccine coverage (Jit, Choi et al. 2014). The new 9vHPV may potentially address the issue of cross-protection, but are expected to be more expensive than 2vHPV or 4vHPV, therefore unlikely to be adopted by LMICs in the near future. Although HPV genotype replacement is possible following HPV vaccination, the data on this is insufficient to draw any meaningful conclusions (Pons-Salort, Thiebaut et al. 2013). Moreover, as the virus is relatively stable, it is
unlikely that type replacement will be seen, but surveillance on the prevalence of non-vaccine types causing diseases still needs to be maintained.

Other issues regarding reduced-dose HPV schedules include whether the two-dose vaccination schedule can be extended to individuals >15 years old, and whether the implementation of two different dose schedules for different age groups is feasible, especially in LMICs (Kahn and Bernstein 2013).

It is believed that HPV infection may be prevented by vaccination with only one dose of HPV vaccine. Preliminary evidence of similar vaccine efficacy between those vaccinated with one dose compared with those vaccinated with two or three doses were reported in a combined analysis of two Phase 3 clinical trials and a prospective cohort study in India (Kreimer, Rodriguez et al. 2011, Kreimer, Struyf et al. 2015, Sankaranarayanan, Prabhu et al. 2016). In addition, although a lower antibody level against the vaccine HPV types were reported following one dose of HPV vaccine as compared with those vaccinated with two or three doses, the level of antibodies seven years after vaccination were still higher than antibodies generated by natural infection (Safaeian, Porras et al. 2013, Kreimer 2017). However, the numbers included in these analyses were small and more studies of single dose HPV vaccination are warranted to support these observations.

1.10. Background to Fiji HPV Vaccination Study
In Fiji, cervical cancer rates (42.4 per 100,000) and mortality (26.5 per 100,000) among women aged 20-69 years old are one of the highest in the world (Law, Fong et al. 2013). Cervical cancer is ranked first for cancer mortality in Fijian women, with a higher burden in iTaukei (Indigenous Fijian) women compared with Fijians of Indian descent (FID) (Kuehn, Fong et al. 2012, Law, Fong et al. 2013). It is reported that 98% of cervical cancer cases in iTaukei women and almost half of that for FID women are fatal due to delayed presentation (Kuehn, Fong et al. 2012). HPV infection in Fiji is most prevalent in women aged between 16-24 years old, while the peak cervical cancer incidence is between 45-54 years old, and mortality rates are the highest between 65-74 years old (Kuehn, Fong et al. 2012).

In 2008/9, the Ministry of Health (MoH) in Fiji accepted a one-off donation of 110,000 doses of 4vHPV based on the high cervical cancer disease burden. There was enough vaccine to
vaccinate four birth cohorts of girls (30,338 girls aged 9-12 years old) with a three-dose schedule via a school-based program. However, not all the girls received the three-dose schedule, mainly due to absence from school on the day the school health team were visiting. The 4vHPV coverage following the initial and the subsequent mop-up campaign was: 62%, 56%, and 55% for doses one, two, and three respectively.

With the support from Australian Aid, the Fiji MoH introduced 2vHPV for all girls in their last year of primary school in 2013 as part of their national immunisation program. This provided a unique opportunity to rigorously investigate the question of whether reduced-dose schedules induce long-term immunity compared to the standard three-dose schedule.

Previous studies outlined above comparing two and three doses of HPV vaccines have demonstrated promising results on the potential utility of reduced-dose HPV schedules in Fiji and other LMICs. The challenges of cost, coverage rates and vaccine delivery would be alleviated by schedules requiring the administration of fewer doses, and facilitate the implementation of national HPV vaccination programs in LMICs. It may also allow the additional advantage of vaccinating boys with these cost savings. However, it is still unclear whether reduced-dose HPV schedules can generate long-lasting immune responses similar to the standard three-dose schedule to reduce disease outcomes. Therefore, this PhD thesis aimed to address some of these important questions in the Fiji HPV study and will contribute to the body of evidence on the value of a two-dose schedule.

1.11. Rationale for the immunological measurements
Memory responses are important as they indicate whether the immune system has been adequately primed to remember the organism and respond rapidly to clear the infection upon second encounter. As memory cells only circulate in the bloodstream at very low levels, the only way to demonstrate whether the immune system has been adequately primed to respond to infection is by stimulating the memory cells induced by prior immunisation. This is routinely done in a clinical trial by giving a “booster” dose of vaccine (in this study, 2vHPV is given) and measuring the antibody response. An indication of the induction of immune memory can be demonstrated by an anamnestic antibody response one month after the ‘booster’ dose. In addition, cellular immune markers (i.e. memory B- and/or T-cells, cytokines) are also of great interest and are thought to be a more reliable predictor of long-term immunity and/or
protection. Although the memory B cell response is an important surrogate for predicting long-term immunity and will be important in the case of reduced-dose schedule, there were issues with obtaining the VLPs required to examine these responses, and was not measured in this study. Therefore, this study will compare these immune responses (neutralising antibodies, IFNγ-producing cells as a surrogate for T cell responses and cytokine production as a surrogate for the immune cell subsets) in girls that have received zero, one, two or three doses of the 4vHPV.

1.12. Aims and Hypotheses
The primary hypothesis is that NAb responses to HPV 6, 11, 16 and 18 in the group that had received two doses of 4vHPV in 2008/9 are not significantly different to the group who received three doses of 4vHPV, six years following the last vaccine dose.

Secondary hypotheses are that those girls who received two doses of 4vHPV will have an immunological profile that is not significantly different to those who received three doses of 4vHPV in terms of:
1. NAbs one month post 2vHPV administration;
2. HPV-specific IFNγ-producing cells both pre- and one month post 2vHPV; and
3. Cytokine responses both pre- and one month post 2vHPV.

The primary aim of my study is to determine whether two doses of 4vHPV provide similar immunological evidence of long-term protection to the standard three-dose 4vHPV schedule in terms of NAbs to the HPV genotypes present in the 4vHPV (HPV6, 11, 16 and 18), six years following the last vaccine dose.

Secondary aims are to compare the following parameters between the groups who previously received zero, one, two or three doses of 4vHPV in 2008/9:
1. NAb responses to HPV6, 11, 16 and 18, one month post 2vHPV;
2. HPV-specific T cell to HPV16 and 18, both pre- and one month post 2vHPV; and
3. Cytokine responses to HPV16 and 18, both pre- and one month post 2vHPV.
2: Materials and Methods

2.1 Setting
Fiji is a country in the Melanesian region of the South Pacific Ocean. The country is comprised of two main islands, Viti Levu and Vanua Levu and more than 300 islands, of which 100 islands are inhabited (Commonwealth 2017). Fiji has an estimated population of 869,458 with over 70% of the population residing on Viti Levu (Fiji Bureau of Statistics 2007). The two main ethnic groups are indigenous Fijians (I-Taukei) (57%), and Fijians of Indian descent (FID) (37%); the remaining population are made up of European, Chinese and other Pacific Islander minorities (Fiji Bureau of Statistics 2007). Suva is the capital of Fiji in the Viti Levu Island. Together with its neighbouring suburbs (Nausori, Nasinu and Lami), they are known as the Greater Suva Urban Area (GSUA), and holds approximately 57% of the urban population (244,000 people) (Fiji Bureau of Statistics 2007).

2.1.1 Study Locations
The study visits were held at the maternal and child health clinics (Nausori Health Centre and Valelevu Health Centre), and Colonial War Memorial Hospital (CWMH) in the GSUA. Immunological experiments were undertaken at the Ministry of Health Public Health Surveillance Laboratory, Mataika House (Tamavua, Fiji), as well as at the Murdoch Children’s Research Institute (Melbourne, Australia).

2.1.2 Ethical procedures
Ethical approval (2014.FNRERC.5.SU) was provided by the Fiji National Research Ethics Review Committee (FNRERC) and the Fiji National Health Research Committee (NHRC). In addition, the study was separately reviewed and approved by the Human Research Ethics Committee (HREC) at the Royal Children’s Hospital, Melbourne, Australia, to allow laboratory analyses to be done on-site at the Murdoch Children’s Research Institute, Melbourne, Australia.

2.2 Study Design
This was a prospective cohort study of 200 healthy girls who were unvaccinated or have previously vaccinated with one, two or three doses of 4vHPV in 2008/9 in the GSUA. All participants in the study were vaccinated with one dose of 2vHPV, Cervarix®, and 25 ml of blood was collected before and 28 days after 2vHPV.
2.2.1 Selection of study participants
School vaccination lists were sought from the Fiji Ministry of Health and Fiji Ministry of Education to identify potential participants who previously received one, two or three doses of 4vHPV. Girls who had previously received 4vHPV and were contactable for this study were 676 (three doses), 204 (two doses) and 116 (one dose) in the GSUA. However, due to difficulties locating the girls from the school vaccination lists because of relocation and/or change of phone number, girls recommended by their recruited friends were also recruited once the school vaccination lists were exhausted. Girls who did not receive any HPV vaccine previously were recruited via recommendations of friends of the participants and by informal networks (i.e. word of mouth). The study nurse contacted the parent/guardian of the girls via phone to inform them about the study, and invited them to participate. After a few days, another phone call was made to the parent/guardian to ascertain their willingness to participate, and an appointment was made for them to attend the closest health centre (Nausori, Valelevu or CWMH) to take part in the study. They were asked to bring in any of the girl’s immunisation records they may have to verify their vaccination status (including girls who were recommended by their recruited friends). Girls who wished to participate but did not wish to receive the vaccine were not enrolled in the study. They were advised of the risks of acquiring HPV and its association with cervical cancer, and contact details to get the vaccination were given to them should they change their mind in future.

2.2.2 Informed consent
Written informed consent or assent (Appendix 3) was obtained from all girls aged ≥ or <18 years old, respectively. This was undertaken in private (without her parent/guardian) so that the participant do not feel pressured by their parents to participate. For girls aged <18 years of age, written informed consent was also obtained from the girl’s parent/guardian (Appendix 4). The rationale for the study, study process and the choice to withdraw from the study at any time without any interference to their normal health care were fully explained by the study nurse to the girl and their parent/guardian.

2.2.3 Eligibility criteria
After informed consent was obtained, the girl’s eligibility was checked, including completion of a questionnaire, which was done in private without the parent/guardian nearby, as some of these questions were sensitive. The girl was reassured that her answers will remain confidential.
These questions included: the well-being of the participant, and whether she is pregnant (contra-indication to HPV vaccination) (Appendix 5). The parent/guardian were also asked the first part of the eligibility questionnaire e.g. if there was any adverse event after previous vaccinations. Another set of questions which included the family demographic details, the date and number of HPV vaccines received previously were also completed by the parent/guardian and the study staff (Appendix 5).

2.2.3.1 Subject inclusion criteria
Girls who lived in the GSUA and were previously vaccinated with one, two or three 4vHPV doses in the 2008/9 campaign were eligible for the study. Girls who did not receive 4vHPV were also eligible for the study.

2.2.3.2 Subject exclusion criteria
Any girl who had anaphylaxis following a previous dose of the vaccine, anaphylaxis to any vaccine component, or possible pregnancy was excluded from the study. In addition, any girl whose dates of previous 4vHPV vaccination were uncertain, or who had received 2vHPV previously, or who had an axillary temperature greater than 38°C was excluded from the study.

2.2.4 Enrolment and masking procedures
Once the informed consents were obtained from both the parent/guardian and the girl, and the girl met all eligibility criteria, the girl was enrolled in the study and a unique study number was allocated. This number was recorded on the consent form, data collection forms, blood tubes and blood collection form.

2.2.5 Study vaccine, vaccine storage and administration
2.2.5.1 Vaccine formulation
Cervarix® (2vHPV) is a recombinant protein particulate (VLP) vaccine containing the major capsid (L1) protein of HPV types 16 and 18. Each 0.5 mL monodose pre-filled syringe or vial contains 20 μg each of HPV types 16 and 18 adjuvanted with AS04 (AS04 is comprised of 500 μg aluminium hydroxide and 50 μg of 3-O-desacyl-4’-monophosphoryl lipid A [MPL]); 4.4 mg sodium chloride; 624 μg sodium dihydrogen phosphate dihydrate. This vaccine (2vHPV) has completed Phase 3 clinical trials and is licensed for use in over 100 countries around the world including United States, Australia and countries in the European Union (EU) for girls
aged 9-26 years. Fiji currently uses this vaccine to vaccinate school-aged girls as part of their national immunisation schedule.

2.2.5.2 Storage and product accountability of study vaccine

The vaccines were stored in the project vaccine fridge at +2°C to +8°C located at CWMH. There was a vaccine temperature log to ensure that the vaccine was maintained at the right temperature. The vaccines were transported to the health centres each day in a cool box with ice bricks at +2°C to +8°C. A temperature log was maintained throughout the study. The HPV vaccine was administered according to product information by the manufacturer, GSK. The study staff maintained a record of the number of vaccines stored in the fridge and given out during each study visit.

2.2.5.3 Dosage and Route of Administration

A 0.5ml dose of the licensed 2vHPV (Cervarix®) were given to all the participants in the deltoid muscle as per the manufacturer’s instructions at the first study visit. As most of the participants (150 out of 200) in this study was either unvaccinated, or have received one or two doses of 4vHPV during the 2008/9 campaign, they were followed up to receive additional dose(s) of the 2vHPV as part of an ethical requirement of the study, so that all participants were fully vaccinated with three doses of HPV vaccine; off study procedures. Three doses is the recommended number of doses to be fully protected against HPV genotypes 16 and 18 according to the vaccine manufacturer, and was consistent with the number of doses which school aged girls received in the Fiji MoH school based-HPV national program at the time of the study.

2.3 Study visits and procedures

The study visits are summarised in Table 2.1. On the first study visit (Day 0), informed consent and checking the participant’s eligibility, including recording information of the participant’s family demographics and number of HPV vaccine doses received previously were described in Section 2.2.2 to 2.2.3. If preferred, local anaesthetic cream was applied on to the venepuncture site of the study participant for at least 40 minutes before the blood was taken. Twenty-five mL of blood in total was drawn from each participant by the trained study nurse or study doctor; i.e. two 10 ml sodium heparin tubes, and one 5 ml gel tube.
Following the blood collection, a dose of 0.5mL of 2vHPV was administered by intramuscular injection to the left deltoid muscle by the study nurse. In the case of any adverse events, the participant was immediately referred to the health centre’s doctor or CWMH for assessment and treatment. A second appointment was also made for another blood test to determine the vaccine response 28 days later.

On the day 28 study visit, the study nurse documented the participant’s health status and any adverse reaction that had occurred following the previous vaccination. Another 25 ml of blood was drawn from the participant to assess the vaccine response to 2vHPV in the same way as described previously. To ensure all participants received three doses of HPV vaccine by the end of the study, off-study appointments were made for study participants to receive off-study vaccine for those who did not receive any HPV vaccine previously or received only one dose of 4vHPV in 2008/9 campaign. The number of 2vHPV doses given to each dosage group is summarised in Table 2.1.

Table 2.1: Time line for study visits and off-study procedures

<table>
<thead>
<tr>
<th>4vHPV Dosage groups in 2008/9</th>
<th>*Current study vaccination</th>
<th>Off-study vaccination (from day 0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 28</td>
</tr>
<tr>
<td>0</td>
<td>2vHPV</td>
<td>2vHPV</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
</tr>
<tr>
<td>1</td>
<td>2vHPV</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
</tr>
<tr>
<td>2</td>
<td>2vHPV</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
</tr>
<tr>
<td>3</td>
<td>2vHPV</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Blood</td>
<td>Blood</td>
</tr>
</tbody>
</table>

*25 mL of blood samples were collected from all participants at day 0 and day 28
2.3.1 Participant withdrawal from the study

The participants or their parent/guardian were able to withdraw their children at any time during the study. They were reassured that their withdrawal from the study would not affect any health care provided to them. Participants that experienced any serious adverse events (observed/reported to the Study Co-ordinator) or vaccine reaction were withdrawn from the study. The reason for withdrawal or any loss-to-follow-up was documented on the participants’ completion/withdrawal section of the case report form. No additional participants were recruited to compensate for those who have withdrawn from the study.

2.3.2 Adverse event reporting

An adverse event (AE) was any untoward medical occurrence in a participant having the blood test or Cervarix® vaccination. An AE was any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with having the procedures taken, whether or not related to it. Any medical condition that was present at the time of screening or just prior to the receipt of treatment were recorded and considered as baseline and not reported as an AE. However, the condition was recorded as an AE if it deteriorated within the treatment period.

AEs were graded for intensity and relationship to the procedure taken as follows:

- Mild: events require minimal or no treatment and do not interfere with the patient’s daily activities.
- Moderate: events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe: events interrupt a patient’s usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually incapacitating.
- Life threatening: Any adverse experience that places the patient or subject, in the view of the investigator, at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that had it occurred in a more severe form, might have caused death.
A Serious Adverse Event (SAE) was defined as an AE meeting one of the following conditions:

1. Death during the period of protocol defined surveillance
2. Life Threatening Event (defined as a subject at immediate risk of death at the time of the event)
3. An event requiring inpatient hospitalisation or prolongation of existing hospitalisation during the period of protocol defined surveillance
4. Results in a persistent or significant disability/incapacity.

Any other important medical event that did not result in death, be life threatening, or require hospitalisation, were considered a serious adverse experience when, based upon appropriate medical judgment, the event jeopardised the subject and required medical or surgical intervention to prevent one of the outcomes listed above. Changes in the severity of an AE were documented to allow an assessment of the duration of the event at each level of intensity to be performed. AEs characterised as intermittent required documentation of onset and duration of each episode.

2.3.3 Assessment of adverse events
The study nurse observed the participant for 30 minutes following vaccination. For any signs of anaphylaxis, adrenaline was available for the treatment. Parents/participants were phoned two to three days following the first vaccination to record any potential adverse events: fever, local injection site reactions, nausea, headache, vomiting, dizziness, changes in appetite, and any medical attendances (Appendix 5). For any adverse event, the girl was immediately recommended to go to her nearest health centre or CWMH for assessment and treatment.

2.3.4 Documentation of adverse reactions
Any adverse events were recorded on the case reporting form (CRF). For any untoward event that occurred during blood collection or vaccine administration, the study co-ordinator was contacted, and the event was documented in the workbook, which was then discussed with the principal investigator.

2.3.5 Eliciting Adverse Event Information
Parents/participants were phoned two to three days following the first vaccination to record any potential adverse events (Appendix 5). In particular, participants and/or their
parents/guardians were asked to report on any illness or febrile episodes, the duration of these episodes, and if they had used any new medication.

2.3.6 Serious Adverse Event Reporting
Parents/guardians and participants were given a study card with contact details of the study nurse and were encouraged to maintain close contact as well as report any AE or discuss concerns that they had regarding the intervention during the course of the study. In particular, parents/guardians were asked to contact the study team for any SAE. For any SAEs, the girl was immediately referred to the health centre’s doctor or CWMH for assessment and treatment. Clinical trials insurance was provided by MCRI.

Any SAE occurred in a study participant were reported to the Fiji HREC within 24-72 hours of notification of occurrence, in accordance with the safety reporting policy of the HREC.

For any participant who experienced an unexpected, serious adverse event not related to the vaccine, the participant was withdrawn from the study and reported to the relevant HREC according to the standard procedure.

2.3.7 Sample collection, Processing and Transport
All laboratory procedures were undertaken in a blinded manner and each individual sample was identified according to a unique study number.

For both study visits, the blood samples were collected as described previously in the Section 2.3-Study Visits and Procedures, and were transported in a clinical specimen bag to Mataika House, Tamavua, Fiji within 2 hours after collection. Blood samples were processed immediately upon arriving at Mataika House. The gel tube was centrifuged at 700 x g for 10 min and serum was collected and stored at -80°C. For blood collected in heparinised tubes, plasma and peripheral blood mononuclear cells (PBMCs) were separated from whole blood by centrifugation (700 x g, 10 min). Plasma was collected and aliquoted to 2 ml aliquots and stored at -80°C. PBMCs were then separated from the red blood cells by density gradient centrifugation (400 x g, 30 min), and were frozen in liquid nitrogen (for cellular immune response analysis). All samples (plasma and serum stored at -80°C and PBMCs in liquid nitrogen) were shipped on dry ice to MCRI, Melbourne, Australia where experimental procedures were undertaken.
2.3.8 Storage and removal of samples
Following participant’s consent, the biological samples collected were used for laboratory analysis as described in Section 2.4 and the remaining biological samples (PBMCs and plasma/serum) were stored at MCRI for future use. Additional ethics approval is required for the use of these samples for any HPV related studies. Samples (frozen PBMCs and plasma/serum) without consent for future use and storage were disposed of in biosafety bags and incinerated 15 years following the completion of the study as per Good Clinical Practice guidelines.

2.4 Laboratory analysis

2.4.1 Neutralising antibody assays
Neutralising antibody titres to all four HPV-vaccine genotypes (HPV6, 11, 16 and 18) present in 4vHPV were performed using the WHO recognised pseudovirion (PsV) neutralisation assay (Buck, Pastrana et al. 2005), adapted from Prof Ian Frazer’s laboratory at The University of Queensland, Australia.

2.4.1.1 Maintenance of 293TT cells
The 293TT cells (human embryonal kidney cell line transformed with sheared adenovirus type 5 DNA and simian virus-40) were kindly provided by Prof Ian Frazer’s laboratory under a Materials Transfer Agreement. The cells were grown in DMEM with phenol red [Gibco, New York, USA], 10% heat inactivated FBS [Hyclone, Utah, USA], 1% 100x Penicillin/Streptomycin/L-glutamine [Gibco, New York, USA], 1% Glutamax [Gibco, New York, USA], 1% non-essential amino acid [Gibco, New York, USA]; this media is known as D10.

The cells were maintained every three to four days by removing the culture media and washing the cells with 5 mL of PBS [Gibco, New York, USA]. The cells were then detached by the addition of 1-2 mL trypsin [Gibco, New York, USA] and incubation at 37°C, 5% CO₂ for 1-2 min. This was followed by resuspension in 4-5 mL of D10 and the cells were split at 1:6 (90-100%) or 1:12 (70-80%) into a new T-75 cm² flask [Corning, New York, USA] based on level of confluence. The cells were then incubated at 37°C, 5% CO₂.
2.4.1.2 HPV pseudovirion production

The HPV plasmid DNAs (Table 2.2) were obtained from Addgene in bacterial stab (Escherichia coli). The bacteria containing the plasmid of interest were grown on Luria-Bertani (LB) agar plates or broth [Becton Dickinson, Missouri, USA] with antibiotics (plasmid containing the specific antibiotic-resistant gene). Plasmid DNAs were extracted using the Qiagen Maxiprep kit [Qiagen, Hilden, Germany] and quantitated using the Nanodrop machine 2000 spectrophotometer [Thermo Scientific, Australia]. The plasmid DNAs were then verified through restriction enzyme digestion and electrophoresis to make sure they are the correct plasmids.

**Table 2.2:** Plasmid DNAs for PsV production

<table>
<thead>
<tr>
<th>PsV type</th>
<th>Plasmids</th>
<th>Antibiotic-resistant gene</th>
<th>Restriction enzyme</th>
<th>Fragment sizes (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPV6</td>
<td>p6sheLLr</td>
<td>Ampicillin</td>
<td>SacI</td>
<td>5.3, 3.9, 1.7</td>
</tr>
<tr>
<td>HPV11</td>
<td>p11L1w</td>
<td>Kanamycin</td>
<td>BamHI</td>
<td>4.6, 1.6</td>
</tr>
<tr>
<td></td>
<td>p11L2w</td>
<td>Kanamycin</td>
<td>EcoRI</td>
<td>2.0, 1.6, 1.3, 1.0</td>
</tr>
<tr>
<td>HPV16</td>
<td>p16sheLL</td>
<td>Ampicillin</td>
<td>BamHI</td>
<td>6.1, 2.2, 1.7, 0.9</td>
</tr>
<tr>
<td>HPV18</td>
<td>p18sheLL</td>
<td>Ampicillin</td>
<td>SalI</td>
<td>9, 1.5, 0.3</td>
</tr>
<tr>
<td>Reporter</td>
<td>pYSEAP</td>
<td>Blasticidin</td>
<td>BamHI</td>
<td>1.6, 3.7</td>
</tr>
</tbody>
</table>

The HPV PsV were produced through transfection of 293TT cells, and each HPV type was produced independently. The 293TT cells were first seeded at 1 x 10^6 cells per 15 mL in a T-75 cm² flask for four days at 37°C, 5% CO₂. At day four, the plasmids (19 µg of HPV6, 16 or 18 plasmid shell and 19 µg of secreted alkaline phosphatase (SEAP) plasmid; 12 µg HPV11L1 and 12 µg HPV11L1 and 12 µg SEAP) were diluted accordingly in 2 mL of Opti-MEM® [Gibco, New York, USA] (termed: DNA dilutions) and were incubated for 20 min at room temperature (RT). At the same time, 2 mL of Opti-MEM® was also mixed with 85 µL of Lipofectamine [Invitrogen, California, USA] (termed: mastermix) for 20 min at RT. After 20 min, the DNA dilutions and mastermix (termed: DNA-mastermix) were combined and incubated further for 20 min at RT. The DNA-mastermix was then added to the pre-plated flask for 5 h at 37°C, 5% CO₂. After 5 h, media containing DNA-mastermix was removed, and replaced with 15 mL of pre-warmed D10 for 48 h at 37°C, 5% CO₂.
After 48 h, the cell supernatant was collected. The ‘transfected’ cells were detached by adding 2 mL of trypsin and incubating for 2 min at 37°C, 5% CO₂. The collected supernatant (5 mL) was added to stop the trypsinisation and cells were collected in a 15 mL tube. The flask was then rinsed with 3 mL of PBS to collect most of the cells. The cells were then washed with cold PBS twice by centrifugation and resuspension (5 min, 300 x g 4°C). The cells were then resuspended with 0.5 mL PBS with 9.5 mM MgCl₂ [Merck, Victoria, Australia] and transferred to a low-binding microfuge tube [Sarstedt, Hildesheim, Germany]. The total cell volume was estimated based on comparison with dummy tubes of various volumes, and 10% Brij 58 [Sigma-Aldrich, Missouri, USA] as well as RNase cocktail [Ambion, USA] were added to the cells at 1:25 and 1:1000 of the total cell volume, respectively. The cell lysate containing the PsV were then left to mature overnight at 37°C, 5% CO₂. After the incubation, the PsV were chilled on ice for 5 min then aliquoted, snap-frozen and stored at -80°C until use in the neutralisation assay.

### 2.4.1.3 HPV PsV titration assay

Before the use of the PsV for the neutralisation assay, each HPV type PsV were titrated to determine the optimal concentration for use. Firstly, 293TT cells were seeded at a concentration of 3 x 10⁵ cells/mL in neutralisation buffer (D10 without phenol red; NB) onto a 96-well flat-bottom tissue culture plate [Greiner Bio-One, Germany] and incubated for 4-6 h at 37°C and 5% CO₂. The PsV were then diluted from 1:1,000 to 1:64,000 in NB, and added in triplicate to the pre-seeded 293TT cells. For each of the PsV dilution, 25 µL of heparin was added as a ‘positive control’ to block PsV infection in one of the three wells, while 25 µL of NB was added to the remaining two wells. The 96-well culture plate was then incubated for four days at 37°C and 5% CO₂. After four days, the plate was checked under the microscope to make sure there was no cytopathic effect. The plate was lightly shaken to obtain a homogenised cultured supernatant, and 120 µL of this supernatant was collected into a corresponding 96-well round-bottom plate [Greiner Bio-One, Germany]. This plate was then centrifuged at 1000 x g for 5 min at 4°C. At the same time, 20 µL of 0.05% CHAPS solution [Sigma-Aldrich, Missouri, USA] was added onto another 96-well flat-bottom tissue culture plate. After centrifugation, 40 µL of the supernatant was transferred to the 96-well flat-bottom tissue culture plate containing the CHAPS solution. The plate was then heat-inactivated at 65°C for 30 min. During this time, the substrate solution was made by combining 0.5mM zinc chloride [Sigma-Aldrich, Missouri, USA], 1 mM magnesium chloride, and p-nitrophenyl phosphate tablets [Sigma-Aldrich, Missouri, USA] in 2 M diethanolamine [Sigma-Aldrich, Missouri,
USA]. After heat-inactivation, the plate was cooled on ice to RT, and 200 µL of the substrate solution was added to each well. The plate was left to develop for 2-4 h, and then read on an ELISA plate reader at 405 nm [Biotek instruments, Vermont, USA].

2.4.1.4 HPV PsV neutralisation assay
For the neutralisation assay, the 293TT cells were seeded as per Section 2.5.1.1- HPV PsV titration assay. Serum samples were serially diluted two-fold in the range of 1:20 to 1:6400 (for some samples, the serum was diluted up to 1: 81,920 to determine the effective dose 50; ED50; highest serum dilution that yield 50% reduction in SEAP activity). This is followed by mixing 25 µL of serum dilution with 100 µL of PsV (diluted accordingly based on previously determined concentration from 2.5.1.1) pre-plated in a 96-well low-binding plate [Corning, New York, USA]. The diluted serum and PsV was then incubated for 1 h on ice. After incubation, 100 µL of serum-PsV was added to their respective wells on the pre-plated 293TT cells. The 96-well culture plate containing serum-PSV-293TT cells was incubated for four days at 37°C and 5% CO2. After four days, the plate was checked under the microscope to make sure there was no cytopathic effect. The cultured supernatant was harvested, assayed and developed as per Section 2.5.1.1. To standardise each assay, the plate was read when the negative control wells (PsV and cells only) reaches an OD of 1.0 and the ratio between the negative and positive control was within 5-15 according to pre-determined specifications. The ED50 was then determined for each sample serum.

2.4.2 HPV-specific T-cell analysis
Specific T-cell responses characterised by IFNγ-producing cells were measured to HPV16 and 18 in a subset of participants (N=15 per group) by Enzyme-Linked ImmunoSpot (ELISPOT) assay based on a previous publication (Smolen, Gelinas et al. 2012). Specific T cell subsets (i.e. CD4+ or CD8+) producing IFNγ were also identified through flow cytometry. Specific HPV peptides of HPV16 and 18 L1 proteins (15-mer peptides with 4 amino acids overlap), corresponding to 124 and 139 peptides, respectively were generated and quality control assessed by Sigma-Aldrich [Sigma-Aldrich, Missouri, USA]. The peptides were dissolved in 80% DMSO-20%PBS to 0.5 mg/mL and were used at a concentration of 1 µg/mL for PBMC stimulation. ELISPOT assay were performed according to the BD manufacturer’s instructions [BD Bioscience; San Diego, CA] using the following antibodies and reagents: Human IFNγ ELISPOT pair [BD Bioscience; San Diego, CA], AEC substrate set [BD Bioscience; San Diego, CA] and Streptavidin-HRP ELISPOT [BD Bioscience; San Diego, CA]. Specific T cell
subsets were identified through staining with monoclonal antibodies: CD4-PE [BD Bioscience; San Diego, CA], CD8-FITC [BD Bioscience; San Diego, CA], CD45RO-APC [BD Bioscience; San Diego, CA] and IFNγ-BUV737 [BD Bioscience; San Diego, CA]. Unstained PBMCs were used as a control and a minimum of 20,000 events were analysed per sample using the BD LSRII flow cytometer. The analyses of the flow cytometry data were performed using the BD FACSDiva 8.0.1 software.

2.4.2.1 Cell culture and stimulation
Cryopreserved PBMCs were thawed at 37°C in a water bath, and R10 media (RPMI 1640, supplemented with 10% heat inactivated FBS [Hyclone, Utah, USA], and 1% 100x Penicillin/Streptomycin/L-glutamine [Gibco, New York, USA]) was added dropwise slowly to avoid temperature shock to the cells, up to a final volume of 15 mL. The PBMCs were then centrifuged at 500 x g for 10 min and resuspended with 1 mL of R10 media for cell counting. Following that, the PBMCs were diluted to 1 x 10^6 cells/mL in R10 media.

Specific antigens and controls were used to determine HPV-specific T-cell responses: (1) negative control: 80% dimethylsulfoxide (DMSO)-20% PBS (diluent for HPV peptides), (2) positive control: Phytohaemagglutinin (PHA; Remel, Kansas, USA; 5 µg/mL), (3) antigenic control: Tetanus Toxoid (Statens Serum Institut, Denmark; 10 µg/mL), (4) HPV16 peptides (1 µg/mL) and (5) HPV18 peptides (1 µg/mL). The PBMCs (1 x 10^6 cells/mL) were stimulated for 48 h at 37°C and 5% CO₂.

2.4.2.2 IFNγ ELISPOT assay
The number of IFNγ producing cells was determined using an ELISPOT according to the manufacturer’s protocol. Anti-Human IFNγ capture antibody was diluted 1:200 (5 µg/mL final concentration) in sterile PBS, and added to a high protein binding 96-well Multiscreen® filter plate (Merck Millipore, Ireland) at 100 µL/well. The coated plate was sealed and stored at 4°C overnight. The capture antibody was then discarded and the plate washed once and blocked with R10 medium (200 µL/well), for 2 h at RT.

Following the 48 h culture, PBMCs samples were centrifuged at 500 x g for 10 min at RT, and supernatants for each of the five conditions were collected in Eppendorf tubes, and stored at -80°C until use for cytokine analyses. The PBMC samples were washed once with 2 mL of 2mM ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich, St. Louis, USA)-PBS buffer.
supplemented with 0.5% (v/v) FBS and centrifuged at 500 x g for 10 min at RT. The supernatant was discarded and PBMCs resuspended in 100 μL of R10 medium to determine the cell viability and concentration using Trypan Blue exclusion dye on a haemocytometer. PBMCs were adjusted to 1 x 10^6 cells/mL and then plated in at least duplicate for each condition at 100μL/well on the ELISPOT plate (after 2 h blocking with R10), followed by an overnight incubation at 37°C and 5% CO₂. After overnight incubation, the cell suspension was discarded and the ELISPOT plate was washed twice with deionised water (200 μL/well) and then three times with 200 μL PBS containing 0.05% (v/v) Tween-20 (PBS/T; MP Biomedicals, France). Biotinylated anti-human IFNγ detection antibody was diluted 1:250 (2 μg/mL final concentration) in PBS containing 10% (v/v) FBS (dilution buffer) and then added to the ELISPOT plate at 100 μL/well, followed by a 2 h incubation at RT. Detection antibody was then discarded and the ELISPOT plate washed three times with PBS/T. Streptavidin-horseradish peroxidase (HRP; BD Bioscience, San Diego, USA) was diluted 1:100 in dilution buffer and then added to the ELISPOT plate at 100μL/well, followed by 1 h incubation in the dark at RT. The Streptavidin-HRP was then discarded and the ELISPOT plate washed four times with PBS/T then twice with PBS before adding 100 μL/well of the 3-Amino-9-ethylcarbazole (AEC) final substrate solution (BD Biosciences, San Diego, USA). Spot development was monitored for approximately 15-30 min and the substrate reaction was subsequently stopped with 200 μL/well of deionised water followed by a single wash with 200 μL/well of deionised water. The ELISPOT plate was left to air-dry overnight in the dark at RT and then read and analysed by an automated ELISPOT Reader and software version 6.0 (AID GmbH, Strassberg, Germany).

2.4.2.3 Flow cytometry
A separate set of PBMC cultures (excluding the TT condition) were set up and stimulated as described above to determine the different T cell subsets producing IFNγ. Five-six hours before harvesting, protein inhibitor cocktail [eBioscience Inc, San Diego, USA] was added at a concentration recommended by the manufacturer (2 μL/mL) to each culture condition to block IFNγ from secreting out of the cells. The stimulated PBMCs were then washed with 2 mL of FACs buffer (2% FBS-PBS with 0.1% sodium azide) by centrifugation at 500 x g for 10 min at RT and decanting the supernatant, followed by staining with extracellular surface markers; CD4-PE, CD8-FITC, and CD45RO-APC. The PBMCs were then washed with 2 mL of FACs buffer to remove excess/unbound staining antibodies. Following that, the PBMCs were fixed with fixation buffer [eBioscience Inc, San Diego, USA] for 30 min, and washed with 2 mL of
permeabilisation buffer [eBioscience Inc, San Diego, USA] twice before performing intracellular staining with IFNγ-BUV737. The PBMCs were then washed with 2 mL of permeabilisation buffer, and then analysed immediately or stored overnight in fridge before analysing the next day. Compensation were performed for each antibody using the compensation beads [BD Bioscience; San Diego, CA]. Unstained PBMCs were used as a control and a minimum of 20,000 events per sample were analysed using the BD LSRII flow cytometer. The analyses of the flow cytometry data were performed using the BD FACSDiva 8.0.1 software.

2.4.3 Cytokine analysis
A panel of Th1 (IFNγ, TNFα, IL-2) and Th2 (IL-5 and IL-10) cytokines were selected based on previous publications and their roles in vaccine immunology (Pinto, Castle et al. 2005, Garcia-Pinieres, Hildesheim et al. 2007, Siegrist 2008). The concentrations of IFNγ, TNF-α, IL-2, IL-10, and IL-5 in the cultured PBMCs supernatants were determined using the Milliplex® MAP kit [EMD Millipore, Massachusetts, USA] according to the manufacturer’s protocol. The kit was stored at 4°C and brought to RT before use. A low protein binding Durapore® membrane 96-well Multiscreen® filter plate (Merck Millipore, Ireland) was prepared with wash buffer supplied in the kit, and sealed and incubated on a plate shaker at 500 rpm for 10 min at RT. The plate was kept on a plate holder and wrapped in foil at all times. Two vials of Quality Controls and the Human Cytokine Standard (10,000 pg/mL) were reconstituted with 250 μL of deionised water and left on a roller for 10 mins to mix. The Cytokine Standard supplied by the kit was serially diluted five-fold using the assay buffer in the range of 2,000 pg/mL, 400 pg/mL, 80 pg/mL, 16 pg/mL, and 3.2 pg/mL. The Standards, Quality controls and background (Assay buffer) were added to the plate. The R10 medium (25 μL/well) was added to all standard and control wells and Assay Buffer (25 μL) was added to all sample wells. Twenty-five microliters of supernatant was then added to the respective wells on the plate in singlicate. Each antibody-bead vial supplied by the kit was sonicated for 30 sec then vortexed for 1 min. Sixty microliters of each antibody-bead vial and 2.7 mL of bead diluent were added to the mixing bottle supplied in the kit to a volume of 3 mL, and the bottle was vortexed for 1 min. Twenty-five μL of the mixed beads were then added to all wells and the plate was sealed and incubated on a plate shaker for 2 h at RT. The plate was then washed twice with wash buffer (200 μL/well) via vacuum filtration, followed by the addition of 25 μL/well of detection antibodies and a 1 h incubation on a plate shaker at RT. Twenty-five microliters/well of Streptavidin-Phycoerythrin was added to the wells and incubated at RT for
30 min. The plate was washed twice followed by the addition of 150 μL/well of sheath fluid and incubated on shaker for 5 min. The plate was then run on the Luminex 200 and analysed using the Luminex xPONENT software version 3.1 [Luminex Corporation, Texas, USA].

2.5 Statistical Methods

2.5.1 Sample size calculation
The primary outcome is the comparison of the geometric mean titres (GMTs) of HPV-specific NAb responses against HPV6, 11, 16 and 18, between the groups that previously received zero, one, two or three doses of 4vHPV. Based on published data from a five year follow-up studies (Olsson, Villa et al. 2007), the sample size required to provide 80% power to detect a 30% difference in HPV antibody levels with a two-sided 5% significance level was determined to be in a range of 26 and 47 per dosage group for HPV16 and HPV18, respectively. Therefore, the sample size was rounded to 50 per dosage group, with a total of 200 girls for the primary outcome. For the secondary outcome, based on a previous publication (Smolen, Gelinas et al. 2012), the mean number of HPV16 and HPV18 T cells (mean ± SD) were $50 \times 10^5 \pm 10 \times 10^5$ and $40 \times 10^5 \pm 10 \times 10^5$. A sample size of 15 per dosage group were used, which gives us 60% power to detect a 20% difference in number of HPV16/18-specific T cells with a two-sided 5% significance level.

2.5.2 Statistical Analysis Plan
All the participants’ characteristics were double entered into a database using Epidata. The characteristics of the study participants were then summarised by calculating the median and inter-quartile range for each of the continuous variables and tabulating the total numbers and frequencies for each dosage group for each categorical variable. The characteristics of the different dosage groups were compared using the one-way ANOVA test for continuous variables or compared using the Chi-Square test for categorical variables. To determine social-economic status (SES), a short list (i.e. vehicle, fridge, computer, electricity, washing machine and television) was made based on the household assets that were considered more affluent and less affluent households by local Fijian staff. Data on these were collected and possession of each asset was scored (1 or 0). This score was weighted for consistency with other assets using principal components analysis as described by Filmer et al. (Filmer and Pritchett 2001), and the median was divided into high and low scores, which represents the richer and poorer groups, respectively.
The primary objective was to determine the antibody responses in girls who previously received zero, one, two or three doses of 4vHPV, and subsequent responses to a dose of 2vHPV. The primary analysis was comparison of the geometric mean titres (GMTs) of HPV-NAb specific for HPV6, 11, 16 and 18 in girls who previously in 2008/9 campaign received zero, one or two doses of 4vHPV with girls who received three doses.

The secondary analyses were the comparison of the NAb GMTs one month post-2vHPV between girls who received zero, one or two doses with girls who received three doses. In addition, girls were stratified in each dosage group by ethnicity, and their NAb GMTs were compared before and one month post-2vHPV within each dosage groups, and between the one- or two-dose group and the three-dose group. Within the two-dose group, the girls were stratified into those who received two doses less than six or six or more months apart, and compared their NAb GMTs before and one month post-2vHPV. For these analyses, the NAb titres were log-transformed and compared using the Student’s t-test or Mann-Whitney test (for comparison of NAb GMTs between iTaukei and FID within the zero-dose group only).

As part of the secondary analyses, the number of IFNγ producing cells as surrogate for T cells and the cytokine concentrations (IFNγ, IL-2, TNFα, IL-10 and IL-5) were also compared between girls who received zero, one or two doses of 4vHPV and girls who received three doses of 4vHPV previously, and their responses one month post 2vHPV. In addition, the girls in each dosage groups were also stratified by ethnicity, and the number of IFNγ producing cells and their cytokine concentrations (IFNγ, IL-2, TNFα, IL-10 and IL-5) were compared before and one month post-2vHPV within each dosage groups, and between the zero-, one- or two-dose group and the three dose group. These analyses were performed using the Mann-Whitney U test. The HPV16/18 IFNγ producing cells (ELISPOT) were correlated with the NAb of the respective HPV types. Each cytokine were correlated with HPV16/18 IFNγ producing cells (ELISPOT) and NAb. All correlation analyses were performed using Spearman’s rank correlation test. All statistical analyses were performed using GraphPad Prism 5.0. A p-value <0.05 was considered statistically significant for all analyses.
3. Results 1: HPV antibody responses six years following reduced dose quadrivalent HPV vaccine schedules (published manuscript)

This chapter is presented in the form of a manuscript titled: Sustained Antibody Responses six years following one, two or three doses of quadrivalent human papillomavirus (HPV) vaccine in adolescent Fijian girls, and subsequent responses to a single dose of bivalent HPV vaccine: a prospective cohort study, published in Clinical Infectious Diseases journal, in April 2017, URL: Clin Infect Dis. 2017 Apr 1;64(7):852-859. doi: 10.1093/cid/ciw865

Hypothesis: Girls who received two doses of 4vHPV six years previously have similar HPV vaccine-type specific antibody responses compared with girls who received three doses.

Primary aim: The primary aim of this chapter was to compare the HPV6, 11, 16 and 18 neutralising antibody (NAb) titres in girls who previously received zero, one or two doses of 4vHPV with girls who received three doses six years previously using the HPV pseudovirion neutralisation assay.

The secondary aims of this chapter were to compare:
1. the HPV6, 11, 16 and 18 NAb titres one month after a dose of 2vHPV between girls who received zero, one or two doses six years previously with girls who received three doses.
2. the HPV6, 11, 16 and 18 NAb titres between the two ethnic groups (indigenous Fijians; iTaukei, and Fijians of Indian Descent; FID) within each dosage group pre- (six years after last dose of 4vHPV) and post 2vHPV
3. the HPV6, 11, 16 and 18 NAb titres between girls who received their second dose less than six months, or six or more months apart within the two-dose group
Sustained Antibody Responses 6 Years Following 1, 2, or 3 Doses of Quadrivalent Human Papillomavirus (HPV) Vaccine in Adolescent Fijian Girls, and Subsequent Responses to a Single Dose of Bivalent HPV Vaccine: A Prospective Cohort Study

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Background. The duration of antibody response following reduced human papillomavirus (HPV) vaccine doses has not been determined. We compared the antibody responses in girls previously vaccinated with zero, 1, 2, or 3 doses of quadrivalent HPV vaccine (4vHPV; Gardasil, Merck) 6 years previously.

Methods. A prospective cohort study was undertaken in 200 Fijian girls 15–19 years of age. Approximately equal numbers of girls from 2 main ethnic groups (Fijians of Indian descent [FIT] and Indigenous Fijians [I Tuakel]) in Fiji were recruited for each dosage group. Blood was drawn before and 28 days following a single dose of bivalent HPV vaccine (2vHPV; Cervarix, GlaxoSmithKline). We measured neutralizing antibodies (NAB) against HPV-6, -11, -16, and -18 using the pseudovirus-based neutralization assay.

Results. After 6 years (before a dose of 2vHPV was given), the geometric mean NAB titers for all 4 HPV types were not statistically different between 2-dose (2D) and 3-dose (3D) recipients: HPV-6 (3D: 2216 [95% confidence interval (CI), 1695–2896]; 2D: 1476 [95% CI, 1010–2137], P = 0.07), HPV-11 (3D: 4431 [95% CI, 3396–5781]; 2D: 2951 [95% CI, 1984–4360], P = 0.09), HPV-16 (3D: 3277 [95% CI, 2511–4480]; 3D: 3277 [95% CI, 2432–4373], P = 0.89), and HPV-18 (3D: 628 [95% CI, 445–888]; 3D: 660 [95% CI, 462–862], P = 0.89), and were higher in FIT than I Tuakel girls. Although 1-dose recipients had significantly lower NAB titers than 2/3-dose recipients, their NAB titers were 5- to 30-fold higher than unvaccinated girls. Post-2vHPV NAB titers against HPV-16 and -18 were not statistically different between girls who received 1, 2, or 3 doses of 4vHPV previously.

Conclusions. Two doses of 4vHPV provide similar NAB titers as 3 doses for 6 years, although the clinical significance is unknown. A single dose of 4vHPV elicits antibodies that persisted for at least 6 years, and induced immune memory, suggesting possible protection against HPV vaccine types after a single dose of 4vHPV.

Clinical Trials Registration. NCT02276521.

Keywords: human papillomavirus; reduced doses; neutralizing antibodies; immune memory; adolescents.

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who received 2 doses (at zero and 6 months) and women who received 3 doses, of 4 and 5 years, respectively [10–12]. One dose of 2× HPV induced antibodies that persisted for at least 4 years, and also demonstrated high vaccine efficacy against incident HPV infection [13, 14]. This suggests that even a single dose of HPV vaccine may be sufficient for protection against HPV infection, although further research is needed to confirm this finding.

In 2008–2009, the Fiji Ministry of Health and Medical Services received a donation of 4×HPV based on the high burden of cervical cancer [15]. All girls aged 9–12 years were eligible to receive the 3-dose schedule recommended at that time. However, due to logistical challenges, not all girls received 3 doses of the vaccine, with some receiving only 1 or 2 doses. In 2013, the Fijian government introduced 2×HPV as a 2-dose schedule into its national immunization program, providing an opportunity to examine the immune response in girls who had received <3 doses of 4×HPV during the 2008–2009 campaign, and to ensure these girls completed 3 doses of HPV vaccine.

We aimed to determine whether girls previously vaccinated with 1 or 2 doses of 4×HPV in 2008–2009 had similar immune responses to girls who received 3 doses. Our secondary aim was to evaluate the effect of a single dose of 2×HPV in these same groups of girls. A group of girls who did not receive any HPV vaccine previously (zero-dose group) were recruited as a comparison group.

METHODS

Study Design and Participants

We conducted a prospective cohort study in Fiji between February and March 2015. A total of 200 Fijian girls (now aged 15–19 years) were recruited. Each of the 4×HPV dosage groups comprised similar numbers of girls from the 2 main ethnic groups: Indigenous Fijians (Tavuisci) and Fijians of Indian descent (FID).

We obtained the immunization records of the 4×HPV campaign of 2008–2009 from the Fiji Ministry of Health and Medical Services and recruited girls from these lists who had previously received 1, 2, or 3 doses of 4×HPV and lived in the Greater Suva area (Suva, Nasau, and Valelevu). The vaccination lists were ordered by the number of 4×HPV doses received previously. For each dosage group, the girls were sequentially contacted by the study nurses to participate in the study, but due to difficulties in locating girls on the vaccine list, friends of recruited girls were also approached to participate. Girls who had not received any prior 4×HPV vaccine were recruited by the recommendation of friends of recruited girls and by informal network (ie, word of mouth).

Procedures

Written informed consent or assent was obtained from all girls aged ≥18 or <18 years old, respectively. Additional written informed consent was obtained from the girl’s parent/guardian for girls <18 years of age. We excluded any participant who had anaphylaxis following a previous dose of the vaccine or any vaccine component, had received 2×HPV previously, had an axillary temperature >38°C, or could possibly be pregnant.

The vaccination status of the girls was verified from the school vaccination list. A questionnaire, administered by the study staff, was completed, which included participant demographics and potential confounders. Despite being an important potential confounder, the number of sexual partners was not asked, as local investigators thought this would not be acceptable to ask in this age group. Participants were observed for any adverse events (ie, faintness; anaphylaxis or allergies, wheezing/rash) for 30 minutes following vaccination. In addition, the parents/participants were telephoned 2–3 days following the first study visit and asked on day 28 to record any potential adverse events, including whether the participant experienced any of the following symptoms: headache, faintness, fever, fatigue, nausea, vomiting, diarrhea, loss of appetite, muscle or joint pain, redness, swelling or pain at injection site, convulsion, rash, and hospitalization, or required any sort of medical attention. Any serious adverse events (defined as any adverse event that met one of the following: death, life-threatening complication, hospitalization, or persistent/significant disability) were also documented.

Blood samples were collected to determine the immunogenicity at day 0 and 28 days following a single dose of 2×HPV (Cervarix, GlaxoSmithKline, Belgium). 0.5 mL monoclonal vial containing 20 μg each of HPV virus-like particle types 16 and 18 adjuvanted with AS04, given at day 0 to all participants to demonstrate immunological memory.

Laboratory Methods

The blood samples were separated into serum and peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation. The serum samples were frozen at –80°C and the PBMCs were cryopreserved in liquid nitrogen. Both serum and PBMCs were shipped on dry ice to Murdoch Children’s Research Institute, Melbourne, Australia, for laboratory analysis.

The neutralizing antibodies (NAbs) against HPV types 6, 11, 16, and 18 were measured using the pseudovirus-based neutralization assay previously described by Burrow et al [16]. The neutralizing titer (ED50) is defined as the highest serum dilution that reduces the secreted alkaline phosphatase activity by at least 50% in comparison to a control (pseudovirons without serum). A sample with an ED50 value of ≥100 was considered HPV seropositive, seronegative samples were given a value of 50. All laboratory staff were blinded to the vaccination status of each participant, and each sample was identified according to a unique study number.

Statistical Analysis

All the participants’ characteristics were double entered into a database using EpiData. The characteristics of the different dosage groups were compared using the 1-way analysis of variance.
test for continuous variables or compared using the $\chi^2$ test for categorical variables. To determine socioeconomic status, a short list was made of household assets (ie, vehicle, refrigerator, computer, electricity, washing machine, and television) that were considered to be present in more affluent and less affluent households by local Fijian staff. Data on these assets were collected and possession of each asset was scored (1 or 0). This score was weighted for consistency with other assets using principal components analysis as described by Filner et al [17], and the median was divided into high and low scores, which represent the richer and poorer groups, respectively.

The primary analysis was the comparison of the geometric mean titers (GMTs) of the HPV-specific NAb titers against HPV-6, -11, -16, and -18 in girls who previously received 1 or 2 doses of 4vHPV with girls who received 3 doses. The secondary analyses were the comparison of the NAb GMTs of 1-month post-2vHPV between girls who received 1 or 2 doses with girls who received 3 doses. In addition, we stratified the girls in each dosage group by ethnicity, and compared their NAb GMTs before and 1 month after 2vHPV within each dosage group, and between the 1- or 2-dose group and the 3-dose group. Within the 2-dose group, we stratified the girls into those who received 2 doses <6 months or 6 months apart, and compared their NAb GMTs before and 1 month after 2vHPV. For these analyses, we log-transformed the NAb titers and compared them using the Student t test or Mann-Whitney test (for comparison of NAb titers between iTaiteki and FID within the zero-dose group only). All statistical analyses were performed using GraphPad Prism software, version 5.0. A $P$ value < .05 was considered statistically significant for all analyses. To provide 80% power to detect a 30% difference in HPV antibodies with a 2-sided 5% significance level, the sample size was determined to be in a range of 26 and 47 per dosage group for HPV-16 and HPV-18, respectively, based on published data from a 5-year follow-up study [18].

Ethics Approval

The study was approved by the Fiji National Research Ethics Review Committee, Fiji National Research Committee (2014.5.FNREC.R.5.SU), as well as the Royal Children’s Hospital Human Research Ethics Committee, Melbourne, Australia (34239A). The study was registered with ClinicalTrials.gov (identifier NCT02276521).

RESULTS

Of the 284 girls approached for this study, 204 were eligible (80 girls were ineligible), and among these girls, 4 girls did not have consent to participate in the study. A total of 200 girls were recruited: 66 (3-dose group); 80 (2-dose group); 40 (1-dose group); 34 (zero-dose group) (Figure 1). Three girls (2 from the zero-dose group and 1 girl from the 2-dose group) were lost to follow-up for the second visit. The characteristics of the study participants did not differ by dosage group, except for the girls in the zero-dose group, who were slightly older compared with girls from the 1-dose group; the time difference since last dose of 4vHPV in the 1-dose group compared with the 2- or 3-dose groups; and the difference in timing of doses 1 and 2 in the 3- and 2-dose groups (Table 1). In addition, a higher percentage of participants in the zero-dose group attended university compared with other groups. No other variables between the groups were significantly different except for lava consumption, which was lower in the 1-dose group compared with other dosage groups. No serious or any other adverse events, including local injection site reactions listed above, were observed following 2vHPV.

The number of girls from each dosage group who were seropositive for each HPV type 6 years since the last dose of 4vHPV are shown in Table 1. A dose-response in NAb titers (highest in the 3-dose group) was observed across all 4 HPV types (Figure 2). No significant differences in the GMTs across all 4 HPV types were found between girls who previously received 2 or 3 doses of 4vHPV (HPV-6, $P = .074$; HPV-11, $P = .686$; HPV-16, $P = .887$; HPV-18, $P = .885$). Girls who had received 1 dose of 4vHPV had significantly lower GMTs for all 4 HPV types compared with girls who received 2 or 3 doses, but their NAb titers for all 4 HPV types were still 5- to 30-fold higher than unvaccinated girls.

To determine whether there is a difference in immunological memory following reduced-dose HPV schedules, a dose of 2vHPV was given to all girls in the study. The NAb titers for HPV-16 and -18 in the 1-dose group increased 46- and 84-fold following 2vHPV, respectively, to a level that was not significantly different between the 2- and 3-dose groups (Figure 3). The NAb levels for HPV-6 and -11 were not significantly different after a dose of 2vHPV for all groups.

To determine the impact of the spacing between the first and second 4vHPV dose on NAb titers, we stratified girls in the 2-dose group into those who received their second dose <6 months or 6 months apart. No significant differences were seen for all 4 HPV types before or after 4 dose of 2vHPV (Supplementary Figure 1). To determine whether there is a difference in NAb responses between ethnic groups, we stratified girls by ethnicity. Significantly higher NAb titers for all 4 HPV types were seen in FID compared with iTaiteki in the 2- or 3-dose groups, 6 years following 4vHPV vaccination (Supplementary Table 1). This observation was consistent following 1 dose of 2vHPV, except for HPV-18 or -11 in the 2-dose group. The NAb titers among iTaiteki in the 3-dose group for HPV-16 and -18 were not significantly different compared with iTaiteki in the 1-dose group (HPV-16, $P = .279$; HPV-18, $P = .959$) 6 years after 4vHPV vaccination, whereas FID had significantly higher NAb titers in the 3-dose group compared with FID in the 1-dose group (HPV-16 and -18, $P < .0001$). In addition, FID in the 2-dose group had significantly higher NAb titers compared with iTaiteki in the 3-dose group before (HPV-16, $P = .0063$ and HPV-18, $P = .002$) and after (HPV-16, $P = .02$ and HPV-18, $P = .002$) 2vHPV.
Figure 1. Recruitment flowchart and study design. Two girls from the zero-dose group were excluded from the analysis as we found high levels of neutralizing antibody titers against all 4 human papillomavirus (HPV) types in visit 1, and subsequently learned that they had lived in overseas countries where HPV was on the national schedule; we assumed they received HPV vaccine previously and therefore their names were not on the school vaccination lists when they were recruited. Abbreviations: 2vHPV, bivalent human papillomavirus vaccine; 4vHPV, human papillomavirus; IT, Indigenous Fijian (Faisali).

DISCUSSION

To our knowledge, this is the first study to show evidence of persistent antibody levels 6 years after 1 dose of 4vHPV, and the generation of a robust memory response following only 1 dose of 4vHPV. Two doses of 4vHPV provided similar NAb titers against HPV-6, -11, -16, and -18 as 3 doses. Although girls in the 1-dose group had lower NAb titers than girls in the 2- or 3-dose groups, their levels were still higher than unvaccinated girls. Moreover, 1 month following a dose of 2vHPV, girls vaccinated with 1 dose of 4vHPV 6 years previously mounted an anamnestic antibody response to a level that was similar to girls who received 2 or 3 doses, suggesting that sufficient immune responses had been induced after 1 dose of 4vHPV. It has been suggested that 1 dose of HPV vaccine might be sufficient to protect against vaccine-type HPV infection, although the evidence is limited. A multicenter prospective cohort study in India examined the immunogenicity and HPV infection rates in girls following 1, 2, or 3 doses of 4vHPV up to 4.7 years, and found similar vaccine efficacies against persistent infection with HPV-6, -11, -16, and -18 regardless of the number of 4vHPV doses given despite lower antibody levels in girls given 1 dose of 4vHPV compared with girls who received 2 or 3 doses [11]. One dose of 2vHPV was also shown to induce antibody responses that persisted for
Table 1. Baseline Characteristics of Study Participants

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Dosage Group</th>
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<tr>
<td></td>
<td>3 Doses (n = 66)</td>
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<td>Age at recruitment, y, median (IQR)</td>
<td>170 (160–180)</td>
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<td>Time in months between:</td>
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<td>*Doses 1 and 2, median (IQR)</td>
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<tr>
<td>*Doses 1 and 3, median (IQR)</td>
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<tr>
<td>Time, y, since last dose of 4vHPV, median (IQR)</td>
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<tr>
<td>Weight, cm, median (IQR)</td>
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<tr>
<td>BMI, kg/m², median (IQR)</td>
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<tr>
<td>Partner status at enrollment</td>
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<tr>
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<tr>
<td>Never consumed</td>
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<tr>
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<td>Parental education</td>
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<tr>
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</tr>
<tr>
<td>Rich</td>
<td>33 (50)</td>
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<tr>
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<tr>
<td>No. of alcohol drinkers</td>
<td>22 (33)</td>
</tr>
<tr>
<td>No. of kava drinkers</td>
<td>30 (45)</td>
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at least 4 years, and were at least 9 and 5 times higher for HPV-16 and -18, respectively, than naturally infected individuals [13]. A combined post hoc analysis of two phase 3 clinical trials (Costa Rica Vaccine Trial and PATRICIA; Pāpiłomə Triał against Cancer in young Adults trial) also found similar vaccine efficacy against incident HPV-16/18 infection between individuals who were vaccinated with 1, 2, or 3 doses of 2vHPV [14]. These studies, and our data, provide the basis for assessing vaccine efficacy against HPV infection following a 1-dose HPV vaccine schedule.

Noninferiority antibody responses that persist for up to 4 and 5 years have been documented for both 4vHPV and 2vHPV, respectively, in girls <15 years of age who received 2 doses administered 6 months apart, compared with women aged 15–26 years who received 3 doses [8, 10–12, 19–23]. Our data extend these findings, showing that similar immunogenicity between 2 and 3 doses may persist for at least 6 years. A randomized, phase 3, noninferiority study by Dobson et al found inferior antibody responses for HPV-18 and HPV-6 after 24 and 36 months, respectively, in girls aged 9–13 years receiving 2 doses of 4vHPV 6 months apart compared with girls who received the standard 3-dose 4vHPV schedule [12]. Although our study was not powered for noninferiority analysis, we did observe inferior NAb titers against HPV-6 and -11, but not HPV-16 and -18 in the 2-dose group compared with the 3-dose group, using the noninferiority criteria (the lower bound of the 95% confidence interval of the GMT ratio between 2 vs 3 doses was >0.5). The clinical significance of this is unknown, and confirmation of this effect will be needed in studies with larger sample sizes. A cohort study in Sweden examined the occurrence of

**Figure 2.** Neutralizing antibody (NAb) titers against human papillomavirus (HPV) types 16, 18, 6, and 11, six years after last dose of quadrivalent HPV vaccine (4vHPV). Dose-response was seen across all dosage groups for all 4 HPV types. There was no significant difference between the 2- and 3-dose groups. The 1-dose group had significantly lower NAb titers than the 2- and 3-dose groups, but significantly higher than the zero-dose group. Error bars represent geometric mean titers with 60% confidence interval. *P < 0.05; **P < 0.01; ***P < 0.001. Abbreviations: (E50), effective dose 50; PSV, pseudovirus.
genital warts (typically caused by HPV-6 and -11) and vaccination with varying doses of 4vHPV in women aged 10–24 years, and found that girls (10–19 years old, mean follow-up of 3.8 years) vaccinated with fewer than three 4vHPV doses had an increased incidence rate compared with girls vaccinated with 3 doses [24]. Whether reduced-dose HPV vaccination is effective against genital warts in the long term remains to be elucidated.

Ethnicity has been shown to affect the immune response to other vaccines (i.e., influenza, hepatitis B, and rubella) [25–27]. In Fiji, the 2 main ethnic groups are the iTaukei and FID. We found that, in general, FID had higher NAB titers than iTaukei 6 years following 4vHPV vaccination, and also after a “booster” dose of 2vHPV. The precise mechanism for this is unknown, and could be due to genetic factors or differing prevalence of HPV infection. Another possible suggestion could be the body mass index (BMI) of the girls in the ethnic groups, as studies on hepatitis B vaccination have found that higher BMI is associated with poorer immune responses [28]. Although we did not observe a significant difference in the BMI between dosage groups, significantly higher BMI was observed in iTaukei than in FID within the 2- and 3-dose groups, as well as the whole cohort (data not shown), which may explain some of the ethnic differences we found in the immune responses.

We did not detect any significant differences in NAB titers when the second dose was given <6 months or ≥6 months apart in the 2-dose group, although our study was underpowered for this analysis. Higher immunogenicity is usually observed when the timing of the second dose is >6 months [11, 14, 19, 20]; however, vaccine efficacy against HPV-16/18 incident infections has not been shown to be different in this context.

A unique aspect of our study was the ability to examine the safety and immunogenicity of mixed HPV vaccine schedules comprising both 4vHPV and 2vHPV. The fact that we did not observe any increase in HPV-6 and -11 NABs following 2vHPV is not unexpected, as these 2 types differ phylogenetically from HPV-16 and -18 [29]. No adverse reactions were documented in our study, with only minor adverse events not requiring medical intervention observed in a previous study of mixed HPV vaccine schedules [30], providing confidence on the safety and possible use of mixed HPV vaccine schedules. Given that the Gardasil 9 vaccine (9vHPV) is now being licensed in more countries globally, the safety of mixed HPV vaccine schedules is therefore relevant for countries transitioning from either 4vHPV or 2vHPV schedules to 9vHPV [31]. It is worth noting that girls who received an initial series of 2 or 3 doses are not recommended to be boosted routinely, rather that this was done for the purpose of this study.

The major limitations of our study were the small sample size and the study design, which includes potential selection bias as reflected in the differences in participants’ age and education at enrollment. Another limitation in our study was the lack of HPV infection data, including information on participants’ sexual behaviors (i.e., whether they have had sexual relationships and the number of sexual partners), which are potential confounders that may affect the antibody response.

In conclusion, our results lend support to the WHO recommendation of 2 doses of HPV vaccine for young girls, and contribute to the growing evidence of the possibility of long-term protection against HPV following 1 dose of HPV vaccine. Furthermore, it would seem safe to use both vaccines within an individual. Further studies that specifically address the issue
of single-dose HPV schedules and long-term protection are warranted. In terms of how these immunogenicity findings translate into preventing HPV infection, we currently have a follow-up study in young pregnant women to determine HPV detection rates by HPV dosage group. Fiji has since moved to a 2-dose zHPV schedule, with a gap of 6 months between doses.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyrighted and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. F. M. R. has received grants from ARTB Associates. S. M. G. has received grants from Merck, GlaxoSmithKline, CSL, the Commonwealth Department of Health, and Merck; has received nonfinancial support from Merck; and has delivered lectures and received speaking fees from MSD and Sandi Pasteur Med for work performed in her personal time. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References


Supplementary Figure 1: NAb titres against HPV16, 18, 6 and 11 in two dose group, stratified by timing of 2\textsuperscript{nd} dose (<6 months, n=22; ≥6 months, n=38). No significant difference between girl’s whose 2\textsuperscript{nd} dose were given less than six months apart, and those six or more months apart. Error bars represents GMT±95%CI. ED50: effective dose 50
**Supplementary Table 1: NAb titres against HPV 6, 11 16 and 18 stratified by ethnicity for each dosage groups**

<table>
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<td>16 (GMT) 95% CI</td>
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<tr>
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</tbody>
</table>

GMT: geometric mean titre, CI: confidence interval, iTaukei: Indigenous Fijians, FIDs: Fijians of Indian Descent

Visit 1: six years after last 4vHPV dose, Visit 2: one month after a “booster” dose of 2vHPV

p-value < 0.05 considered significant (highlighted in bold)
4. Results 2: HPV-specific T cell responses six years following reduced dose quadrivalent HPV vaccine schedules

4.1. Abstract
Cellular immune responses are required for the production of robust antibodies. Previous studies have documented the induction of T cell responses following HPV vaccination in individuals fully vaccinated with three doses of either 2vHPV or 4vHPV, one month after their last dose. We examined the HPV-specific T cell response (characterised by IFNγ producing cells as T cell is a major producer of IFNγ) in girls who were previously vaccinated with zero, one, two or three doses of 4vHPV six years ago, using IFNγ ELISPOT assay, and their responses following a dose of 2vHPV. A subset of the study cohort (n=15) was randomly selected from each dosage group. Each dosage group comprised of approximately equal number of girls from the two main ethnic groups (iTaukei and FID). Six years following the last dose of 4vHPV, girls who received two doses, of 4vHPV had significantly lower HPV18-specific IFNγ producing cells compared with girls who received three doses (p=0.004). These differences were no longer significant following a dose of 2vHPV. There was no significant difference in HPV16-specific IFNγ producing cells between girls who received one or two doses of 4vHPV and girls who received three doses six years previously, and one month after a dose of 2vHPV. Although the clinical significance is unknown, the number of HPV-specific IFNγ producing cells between the two and three-dose groups varied by HPV types after six years.
4.2. Introduction

Immune memory is generated following previous encounter with the pathogen (i.e. natural infection or vaccination), and is critical to protect against reinfection of the same pathogen. The effectiveness of vaccination is based on generation of immune memory, and this is characterised by the induction of specific memory T and B cells that act in specific ways to support antibody production. In order to produce highly specific antibodies and immune memory, the “help” provided by T cells are essential. This family of T cells known as T helper cells can be classified into Th1 or Th2 based on the cytokines they produce: Th1 family are commonly associated with cell mediated immunity, and memory responses which activates B cells, and/or cytotoxic T cells known to eliminate intracellular pathogens (Siegrist 2008). In contrast, the Th2 family are associated with humoral responses, which involves highly specific antibody production (Siegrist 2008).

The HPV vaccines are subunit vaccines made using recombinant technology based on the self-assembly of the L1 viral protein into virus-like particles that resembles the virus, but do not contain the viral genome, and thus non-infectious. The primary mechanism of protection for HPV vaccines is thought to be type-specific neutralising antibodies (Stanley 2010), which were found to persist for at least 8.5 years following three doses of HPV vaccine (Rowhani-Rahbar, Alvarez et al. 2012). The generation of memory B and/or T cells have also been observed in individuals fully vaccinated with three doses of either 2vHPV or 4vHPV, one month after the last vaccine dose (Einstein, Baron et al. 2009, Einstein, Baron et al. 2011, Smolen, Gelinas et al. 2012). The 2vHPV was found to induce higher T cell and antibody responses than 4vHPV (Einstein, Baron et al. 2009, Einstein, Baron et al. 2011), an observation attributed to the AS04 adjuvant in 2vHPV through its ability to activate innate immunity, and induce higher numbers of memory B cells than the standard aluminium formulations in 4vHPV (Giannini, Hanon et al. 2006). Despite higher immune responses induced by 2vHPV than 4vHPV, both vaccines have demonstrated to be effective against cervical pre-cancer lesions (Future II Study Group 2007, Paavonen, Naud et al. 2009, Future I/II Study Group, Dillner et al. 2010). Whether the immunity induced by 2vHPV persists for longer than 4vHPV remains to be determined. Immune memory responses following HPV vaccination have also been demonstrated by the induction of anamnestic antibody responses following a booster dose several years later (four and seven years for 2vHPV and 4vHPV, respectively) (Olsson, Villa et al. 2007, Moscicki, Wheeler et al. 2012). This was characterised by a rapid increase in antibody responses one
week post-booster to a level that was similar to the one observed one month after the completion of the three-dose primary series (Olsson, Villa et al. 2007). This antibody response was even higher at one month post booster than those achieved one month after the three-dose primary series (Olsson, Villa et al. 2007).

HPV vaccines are prophylactic vaccines, and do not clear or accelerate clearing of existing lesions (Hildesheim, Herrero et al. 2007). Therefore, it is likely that these vaccines work by predominately activating helper T cells rather than cytotoxic T cells, although this has not been studied extensively. In addition, the long-term cellular immune responses following HPV vaccination is unknown. Therefore, the question of whether HPV vaccines stimulate effective cellular immunity and the relationship to NAb responses remains to be determined.

There was a unique opportunity to determine cellular immunity in a group of Fijian girls who were previously vaccinated with zero, one, two or three doses of 4vHPV, six years ago, and correlate with the NAb titres obtained from Chapter 3 of this thesis. The most relevant immunological cell populations to measure following reduced-dose schedules are memory B and T cells. However, since there were issues in obtaining the necessary VLPs required for the memory B cell assays, the cellular immunity analyses focused on T cell responses to HPV16/18 as a surrogate for long-term immunity following reduced-dose schedules. In addition, the cellular immune responses differed by ethnicity were also examined.

Hypothesis: Girls who received two doses of 4vHPV six years ago have similar HPV-specific T cell responses compared with girls who received three doses.

Primary aim: The primary aim of this chapter was to compare the HPV-specific T cell responses (characterised by IFNγ producing cells) in girls who previously received zero, one or two doses of 4vHPV with girls who received three doses six years previously.

The secondary aims of this chapter were to:

1. compare the HPV-specific T cell responses in girls who previously received zero, one or two doses of 4vHPV with girls who received three doses one month after a dose of 2vHPV, using IFNγ-ELISPOT assay.
2. compare the HPV-specific T cell responses between the two ethnic groups (iTaukei, and FID) within each dosage group pre- (six years after last dose of 4vHPV) and post 2vHPV
3. phenotype the HPV-specific T cell subsets between each dosage groups using flow cytometry and correlate with IFNγ producing cells examined using ELISPOT
4. correlate the HPV-specific T cell responses with neutralising antibody titres

4.3. Methods

4.3.1. Study design and participants
Details of the selection criteria have been reported earlier and published (Toh, Russell et al. 2017). In brief, a subset (n=15) of study samples from each dosage group were randomly selected for the HPV-specific T cell analyses, with each dosage group consisting of approximately equal numbers of the two main ethnic groups (iTaukei and FID).

4.3.2. Study procedures
The study procedures were previously described in Toh et al. (Toh, Russell et al. 2017). Blood samples were collected on day 0 (before) and 28 days following a single dose of 2vHPV (Cervarix®, GlaxoSmithKline: 0.5mL monodose pre-filled vial containing 20μg each of HPV VLP types 16 and 18 adjuvanted with AS04, Rixensart, Belgium) to determine immunological memory.

4.3.3. Laboratory procedures

4.3.3.1. Cell culture and stimulation
Cryopreserved PBMCs were thawed at 37°C in a water bath, and R10 media was added dropwise to the cells, up to a final volume of 15 mL. The PBMCs were then centrifuged at 500 x g for 10 mins and resuspended with 1 mL of R10 media for cell counting. Following that, PBMCs were diluted to 1 x 10^6 cells/ mL in R10 media, and then were aliquoted to five culture tubes, with each stimulated with the following antigens and controls for 48 h at 37°C and 5% CO2 : (1) negative control: DMSO-PBS (diluent for HPV peptides), (2) positive control: PHA (5 μg/mL), (3) antigenic control: Tetanus Toxoid (TT; 10 μg/mL), (4) HPV16 pooled peptides (1 μg/mL) and (5) HPV18 pooled peptides (1 μg/mL); the generation of HPV pooled peptides were previously described in Chapter 2. The stimulation and ELISPOT assay (described below) were modified from previous publication (Smolen, Gelines et al. 2012) following optimisation
of cell culture conditions: (1) dose; HPV16 (1 µg/mL, 2.5 µg/mL, 5 µg/mL) and (2) duration; two time points (two and six days) of antigen stimulation based on previous publications (Steel, Roberts et al. 2002, Smolen, Gelinas et al. 2012, Kim, Jin et al. 2014) (Appendix 6). Both pre- and post- PBMCs samples of each individual were assayed on the same day to prevent inter-assay variation.

4.3.3.2. **IFNγ ELISPOT assay**

The number of IFNγ producing cells was determined using an ELISPOT according to manufacturer’s protocol. High protein binding 96-well Multiscreen® filter plate was coated with anti-human IFNγ capture antibody (5 µg/mL final concentration) diluted in sterile PBS, and stored at 4°C overnight (up to one week). The capture antibody was then discarded and the plate (known as ELISPOT plate) was washed once with R10 medium and blocked with 200 µL of R10 medium for 2 h at RT. Following the 48 h culture, PBMCs samples were harvested, and supernatants for each of the five conditions were collected in Eppendorf tubes, and stored at -80°C until use for cytokine analyses. The PBMC samples were washed once with EDTA-PBS buffer supplemented with 0.5% (v/v) FBS, and then resuspended in 100 µL of R10 medium for cell counting. The PBMCs were adjusted to 1 x 10^6 cells/mL and then plated on the ELISPOT plate. Each culture conditions were assayed in quadruplicate, depending on sample availability (at least duplicate). The ELISPOT plate was then incubated overnight at 37°C and 5% CO₂. Following overnight incubation, the cell suspension was discarded and the ELISPOT plate was washed with deionised water twice and then PBS containing 0.05% Tween-20 (PBS/T) three times. Biotinylated Anti-Human IFNγ detection antibody (2 µg/mL final concentration) was then diluted in PBS containing 10% (v/v) FBS (dilution buffer) and added to the ELISPOT plate, followed by a 2 h incubation at RT. Detection antibody was then discarded and the ELISPOT plate was washed three times with PBS/T. Streptavidin-Horseradish Peroxidase was diluted in dilution buffer and then added to the ELISPOT plate, followed by a 1 h incubation in the dark at RT. The Streptavidin-HRP was then discarded and the ELISPOT plate washed four times with PBS/T and two times with PBS. The substrate solution was then added to the plate. Spot development was monitored for approximately 15-30 mins and the substrate reaction was subsequently stopped by deionised water followed by a single wash. The ELISPOT plate was left to air-dry overnight in the dark at RT and then read and analysed by an automated EliSpot Reader and software version 6.0 (AID GmbH, Strassberg, Germany).
4.3.3.3. Flow cytometry
A separate set of PBMC cultures were set up and stimulated as described above (excluding TT condition) to determine the different T cell subsets that expressed IFNγ through intracellular staining using flow cytometry. Five-six hours before the end of the 48 h incubation, protein inhibitor cocktail (eBioscience Inc, San Diego, USA) was added to each culture condition to block IFNγ from secreting out of the cells. The stimulated PBMCs were then washed with FACs buffer (2% FBS-PBS with 0.1% sodium azide) before staining with the surface markers CD4-PE, CD8-FITC, and CD45RO-APC to identify various T cell subsets; CD4+ and CD8+ markers represent helper and cytotoxic T cells, respectively, and CD45RO+ cells represent the activated/memory T cell population, while CD45RO- cells represent naïve T cells. Following that, the PBMCs were fixed and permeabilised using the fixation and permeabilisation solutions (eBioscience Inc, San Diego, USA) before intracellular staining with IFNγ-BUV737 to stain for IFNγ producing cells. Unstained PBMCs were used as a control and a minimum of 20,000 events per sample were analysed using the BD LSRII flow cytometer. Compensation of spectral overlap was performed for each experiment on the individual antibodies used in the experiment using BD Bioscience CompBeads. Analyses of flow cytometry data were performed using the BD FACSDiva 8.0.1 software (BD Bioscience, San Diego, USA), and the gating strategy for each of the cell populations are shown in Appendix 7.

4.3.3.4. Statistical analysis
All statistical analyses were performed using GraphPad Prism 7.0 software. The primary analysis for this chapter is to compare the corrected HPV-specific IFNγ producing cells (after the spots in the negative control was subtracted from the antigenic conditions) from IFNγ ELISPOT assay between the three-dose group and zero-, one- or two-dose group using the Mann-Whitney U test. The secondary analyses for this chapter were to compare the HPV-specific IFNγ producing cells post-2vHPV from ELISPOT assay between the three-dose group and zero-, one- or two-dose group using the Mann-Whitney U test. In addition, the proportion of HPV-specific IFNγ producing T cells from flow cytometry (CD4+IFNγ+, CD8+IFNγ+, CD4+45RO+IFNγ+ or CD8+45RO+IFNγ+) pre- and post-2vHPV were also compared between the three-dose group and two, one or zero-dose group using the Mann-Whitney U test. As part of the secondary analyses, the HPV-specific IFNγ producing cells derived from ELISPOT assay from each dosage groups were stratified into their ethnic groups (iTaukei and FIDs) and compared within each dosage group using the Mann-Whitney U test. Comparison
of HPV-specific IFNγ producing cells derived from ELISPOT assay within each dosage groups pre- and post-2vHPV were performed using the Wilcoxon matched-pairs signed rank test. Correlation test between the HPV genotype-specific NAb titres and HPV-specific IFNγ producing cells (from ELISPOT), and between HPV-specific IFNγ producing cells (from ELISPOT) and HPV-specific IFNγ producing cells (from flow cytometry; total IFNγ producing cells or CD4+ IFNγ producing cells including CD4+45RO+ IFNγ cells; CD8+ IFNγ producing cells were omitted for this analysis due to low cell populations) were done using the Spearman’s rank test. For all statistical analyses, p value <0.05 was considered significant.

4.4. Results
A subset of 60 girls (n=15 with approximately equal numbers from both ethnic groups of each dosage group) were randomly selected for T cell analysis.

4.4.1. HPV-specific T cell response following reduced dose 4vHPV schedules and subsequent responses to a dose of 2vHPV
Six years after receiving the last dose of 4vHPV, HPV18-specific IFNγ producing cells in the two-dose (p=0.004) but not the one-dose group were significantly lower than the three-dose group (Figure 4.1). This was no longer significant following a dose of 2vHPV for the two-dose group. In addition, although higher number of HPV16- and 18-specific IFNγ producing cells were observed following a dose of 2vHPV, this was only statistically significant in the two-dose group for HPV18 (p=0.008). Both HPV16 and 18-IFNγ producing cells were significantly higher in the three-dose group compared with the zero-dose group pre- (HPV16, p=0.001; HPV18, p=0.001) and post-2vHPV (HPV16, p=0.004; HPV18, p=0.014). Overall, higher HPV16-specific IFNγ producing cells were observed compared with HPV18-specific IFNγ producing cells pre- and post-2vHPV. All PHA-stimulated cells (positive control) produced approximately 10-fold higher IFNγ producing cells when compared with HPV16 or HPV18 stimulated cells (Appendix 8).
Figure 4.1: Number of HPV-specific IFNγ producing cells per 10^6 PBMCs to HPV16 (A) and HPV18 (B) six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and IFNγ producing cells were measured by ELISPOT. Red bars represent three-dose group (n=15); blue bars represent two-dose group (n=14); purple bars represent one-dose group (n=15) and green bars represent zero-dose group (n=15). Bars represent mean ± standard error of mean (SEM).
**4.4.2. HPV-specific T cell response between ethnic groups following reduced-dose 4vHPV schedules**

Generally, higher HPV-specific IFNγ producing cells were observed in FID girls than in iTaukei girls for all dosage groups, although most comparisons within dosage groups were not significantly different. Prior to 2vHPV administration, iTaukei girls in the one-dose group had significantly higher HPV16-specific IFNγ producing cell responses than FID girls (p=0.020), but this was not observed following a dose of 2vHPV. After a dose of 2vHPV, only FID girls in the zero-dose group had higher HPV18-specific IFNγ producing cell responses compared with iTaukei girls (p=0.038) (Figure 4.2).

**Figure 4.2:** Number of HPV-specific IFNγ producing cells per 106 PBMCs stratified by ethnicity to HPV16 (A) and HPV18 (B) six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and IFNγ producing cells were measured by ELISPOT. Red bars represent iTaukei girls (n=7) for all dosage groups; blue bars represent FID girls (n=8) for all dosage groups except two-dose group (n=7). Bars represent mean ± SEM.
4.4.3. Phenotypic analysis of HPV-specific T cell responses

Before a dose of 2vHPV was given, the two-dose group had significantly lower HPV18-specific CD4+IFNγ+ cells (p=0.012), while the one-dose group had significantly lower HPV16- (p=0.009) and HPV18- (p=0.004) specific CD4+IFNγ+ cells, when compared with the three-dose group (Figure 4.3). No significant differences were observed between the zero- and three-dose groups for both HPV16 and 18-specific CD4+IFNγ+ cells. Following a dose of 2vHPV, a significantly higher proportion of CD4+IFNγ+ cells were observed in the three-dose group compared with each of the other dosage groups for both HPV16 (two-dose group, p=0.021; one-dose group, p=0.004; zero-dose group, p=0.012) and 18 (two-dose group, p=0.011; one-dose group, p=0.002; zero-dose group, p=0.004). Only the zero-dose group had significantly lower CD4+IFNγ+ cells against HPV16 (p=0.010) and 18 (p=0.010) after a dose of 2vHPV when compared with pre-2vHPV levels. There were no significant differences in CD8+IFNγ+ cells population between the three-dose group and the other dosage groups, except for HPV18 in the one- (p=0.029) and zero-dose group (p=0.044) pre-2vHPV, and HPV16 in the zero-dose group post-2vHPV (p=0.021).

A higher proportion of CD4+45RO+IFNγ+ cells were detected compared with CD8+45RO+IFNγ+ cells in all dosage groups pre- and post-2vHPV (Figure 4.4). Although the three-dose group had a higher proportion of CD4+45RO+IFNγ+ cells, their levels were not significantly different from the other dosage groups, pre- and post-2vHPV. In addition, there were no significant differences in CD4+45RO+IFNγ+ or CD8+45RO+IFNγ+ cells between pre- and post-2vHPV, except for the zero-dose group which had a smaller proportion of CD4+45RO+IFNγ+ cells against HPV18 post-2vHPV (p=0.037). No significant correlations were observed between any population of IFNγ producing cells (total IFNγ+, CD4+IFNγ+ or CD4+45RO+IFNγ+) obtained by flow cytometry and ELISPOT (Figure 4.5).
**Figure 4.3:** Proportion of IFNγ+ producing cells stratified into CD4+ cells (CD4+IFNγ+) cells to HPV16 (A) and HPV18 (B) or CD8+ cells (CD8+IFNγ+) to HPV16 (C) and HPV18 (D), six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and IFNγ producing cells were measured by flow cytometry. Red bars represent three-dose group, n=7 (pre), n=8 (post); blue bars represent two-dose group, n=9 (both pre and post); purple bars represent one-dose group, n=8 (pre), n=7 (post) and green bars represent zero-dose group, n=11 (pre), n=10 (post). Bars represent mean ± SEM.
Figure 4.4: Proportion of memory helper T cells (CD4+45RO+IFNγ+) pre- (six years after last vaccine dose) and post-2vHPV to HPV16 (A) and HPV18 (B), and memory cytotoxic T cells (CD8+45RO+IFNγ+) to HPV16 (C) and HPV18 (D) six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and IFNγ producing cells were measured by flow cytometry. Red bars represent three-dose group, n= 7 (pre), n= 8 (post); blue bars represent two-dose group, n= 9 (both pre and post); purple bars represent one-dose group, n= 8 (pre), n= 7 (post) and green bars represent zero-dose group, n=11 (pre), n= 10 (post). Bars represent mean ± SEM.
Figure 4.5: Correlation between IFNγ producing cells (IFNγ+, CD4+45RO+IFNγ+ and CD4+IFNγ+) obtained by flow cytometry and ELISPOT for HPV16 (A, B, C) and 18 (D, E, F) pre- and post-2vHPV. The number of IFNγ producing cells in the ELISPOT assay are presented as per 10^6 PBMCs, and the number of IFNγ producing by flow cytometry are presented as per 10^6 lymphocytes.
4.4.4. Correlation of HPV-specific T cell responses and neutralising antibodies

There was a moderate correlation between HPV16-specific IFNγ producing cells and HPV16 NAb (r=0.48) and between HPV18-specific IFNγ producing cells and HPV18 NAb (r=0.42). For both HPV types, these correlations were highly significant (p<0.0001) (Figure 4.6).

![Figure 4.6](image)

**Figure 4.6:** Correlation between IFNγ producing cells (from ELISPOT) and neutralising antibody titres (NAb) specific for HPV16 (A) and HPV18 (B) regardless of dosage groups (n=59).
When the HPV-specific T cell responses and NAb titres were stratified into their respective dosage groups, only the two-dose group had a moderate but significant correlation for both HPV16 ($r=0.40$, $p=0.04$) and 18 ($r=0.44$, $p=0.02$) (Figures 4.7 and 4.8). This was also observed in the zero-dose group for HPV18 ($r=0.51$, $p=0.004$) (Figure 4.8).

**Figure 4.7:** Correlation between IFNγ producing cells (from ELISPOT) and neutralising antibody titres (NAb) specific for HPV16 stratified by dosage group pre- and post-2vHPV. A: three-dose group (n=15), B: two-dose group (n=14), C: one-dose group (n=15) and D: zero-dose group (n=15).
Figure 4.8: Correlation between IFNγ producing cells (from ELISPOT) and neutralising antibody titres (NAb) specific for HPV18 stratified by dosage group pre- and post-2vHPV. A: three-dose group (n=15), B: two-dose group (n=14), C: one-dose group (n=15) and D: zero-dose group (n=15).
4.5. Discussion

The results from this Chapter demonstrated that the T cell responses (characterised by the production of IFNγ cells) to HPV18 in the two-dose group were significantly lower than the three-dose group six years after the last dose of 4vHPV. Interestingly, the HPV18-specific T cell responses in the one-dose group were higher than the two-dose group, and were not significantly different from girls in the three-dose group. One explanation for this observation could be due to previous/current HPV18 infection among girls in the one-dose group which may have boosted this response, however no HPV prevalence data was collected from these groups of girls to confirm this.

Consistent with the NAb data, higher HPV-specific T cell responses for HPV16 than HPV18 pre- and one month post-2vHPV were observed. The HPV-specific T cell responses were examined to HPV16 and 18, as these two types are the most common oncogenic types, and are included in all HPV vaccines. Furthermore, since no cross-reactive antibodies against HPV6 and 11 following a dose 2vHPV were observed in this study, the T cell analyses were focussed on HPV16 and 18. Our results were somewhat similar to a previous study by Smolen et al., in which they found both HPV16 and 18-specific T cells (characterised by IFNγ producing cells using ELISPOT assay) were lower in the two-dose group when compared with the three-dose group, one month after the last dose of 4vHPV (Smolen, Gelinas et al. 2012). These observations suggest that IFNγ-secreting cells produced following reduced doses of 4vHPV vary between HPV types, and may be lower than the three-dose schedule one month after the last dose, and remains lower after six years. However, since there is no defined correlate of protection, and similar antibody responses were observed between the two- and three-dose groups (in chapter 3; (Toh, Russell et al. 2017)), whether lower HPV-specific T cell response following reduced-dose schedule have an impact on protection against HPV remains to be determined. Therefore, longer term studies following reduced-dose schedules are needed.

As described in the previous chapter, ethnicity was shown to affect the immune response to other vaccines (i.e. influenza, hepatitis B, and rubella) (Hsu, Lin et al. 1996, Haralambieva, Salk et al. 2014, Avnir, Watson et al. 2016), and differences in NAb titres were also demonstrated between the iTaukei and FID girls from this study. Although higher T cell responses were observed in FID girls compared with iTaukei girls in most cases, and this was
consistent with the NAb response reported previously (Toh, Russell et al. 2017), this was not statistically significant. Furthermore, the sample size for this analysis was insufficient to draw any conclusions.

There was a positive correlation between the NAb and T cell responses, but this was less convincing when the responses were stratified by dosage groups, which could be due to the small numbers in each dosage group. This correlation analysis between T cells and NAb responses has not been previously demonstrated in HPV clinical studies. Only correlations between cytokines (i.e. IFNγ, IL-5 and IL-10) as a surrogate of T cell responses and antibody responses have been performed, and the outcomes were inconclusive (Pinto, Edwards et al. 2003, Pinto, Viscidi et al. 2006, Garcia-Pineres, Hildesheim et al. 2007).

There have been limited studies on T cell responses in the context of reduced-dose HPV schedules. Moreover, different assays have also been used in these studies, making comparison of results difficult. The IFNγ ELISPOT assay was used as it is a sensitive assay, and was similar to the one used by Smolen et al to examine memory T cell responses following two or three doses of 4vHPV (Smolen, Gelinas et al. 2012). However, there were some differences in methodologies - firstly, Smolen et al measured T cell responses in study participants one month after the last dose of 4vHPV, whereas the T cell responses in this study were measured six years after the last dose, so the results were not directly comparable. Secondly, the PBMCs of the participants were stimulated with the HPV peptides for two days in the present study instead of six days (as per Smolen et al.), as previous optimisation time-course experiments found no difference in this response over this time, with higher cell viability observed at day two (Appendix 5). Besides that, the addition of IL-2 into the culture medium may artificially increase T cell responses (Besser, Schallmach et al. 2009), potentially masking any real differences and so was omitted from our PBMC cultures.

Flow cytometry has also been used to quantitate HPV-specific T cell responses (Einstein, Baron et al. 2011). In the study, T cell responses were compared between individuals who were vaccinated with either 2vHPV or 4vHPV at different time points post-vaccination, and a positive T cell response was defined as ≥500 HPV-specific CD4+ T-cells per million CD4+ T-cells expressing either two out of four immune markers (CD40L, IL-2, TNFα and IFNγ) (Einstein, Baron et al. 2011). This could be another method to evaluate T cell responses in future HPV studies. The advantage of performing the IFNγ ELISPOT assay over flow
cytometry is that the former is a more sensitive technique to quantitate T cell responses (Saade, Gorski et al. 2012), although all IFNγ producing cells (i.e. CD4+, CD8+, NK cells and macrophages) can be potentially detected. One solution around this is to identify a particular cell population and then perform ELISPOT, or set up a separate culture and perform intracellular staining using flow cytometry to phenotype the IFNγ cell populations. This was done in this chapter as a secondary analysis.

Flow cytometry was performed to phenotype whether the IFNγ producing cells were CD4+ or CD8+, since T cells are the major producer of IFNγ (Schoenborn and Wilson 2007), and whether they expressed naïve (CD45RO-) or memory (CD45RO+) markers (Machura, Mazur et al. 2008). In addition, the cytokine IL-4 were also examined as a surrogate for Th2 cells using intracellular staining in an attempt to differentiate Th1 (IFNγ) and Th2 (IL-4) cells. However, the level of IL-4+ Th cells were too low for detection, and this approach was not pursued further. The frequency of memory cells (CD4+CD45RO+IFNγ+ or CD8+CD45RO+IFNγ+) were found to be low overall, and a higher proportion of IFNγ+ cells were CD4+ rather than CD8+ cells, as would be expected. Although significantly higher CD4+IFNγ+ cell populations (both naïve and memory) were observed in the three-dose group compared to other dosage groups one month after a dose of 2vHPV, when the results were analysed by fold-change (after background correction), there was no significant difference between the three-dose group and other dosage groups (Appendix 9). This suggests that unstimulated PBMCs in the three-dose group had a higher background IFNγ secretion than other dosage groups, possibly reflecting their in vivo activation status. These results suggest that the population of interest may be too low to detect any significant differences using this panel of markers. One study in tuberculosis patients that used the same approach similarly detected ~1% of CD4+IFNγ+ cells in PBMCs, although a different stimuli (Mycobacterium leprae purified protein derivative) and timing of stimulation (16 h) was used compared to our study (Antas, Sales et al. 2004). There is only one other study (Einstein, Baron et al. 2011) that examined CD4+ T cell responses using flow cytometry following 4vHPV or 2vHPV vaccination. A panel of markers (CD40L, IL-2, TNFα and IFNγ) was used and the actual proportion of cells were not reported (i.e. positive response: ≥0.05% CD4+ T-cells expressing either two out of four immune markers; CD40L, IL-2, TNFα and IFNγ), thus the results of this study were not comparable. The panel used in this study were selected on the basis that any change in the memory population would be more readily detected by changes in these markers. The CD45RO+ marker is a memory cell marker, and this panel allow direct comparison of
IFN\(\gamma\) producing cell results obtained from ELISPOT and flow cytometry. It would be interesting to see if there is a difference in T cell responses in this study cohort using the approach by Einstein et al. (Einstein, Baron et al. 2011).

It is not surprising that the frequency of CD8+IFN\(\gamma\)+ cells was low since HPV vaccines are prophylactic and do not clear lesions (Hildesheim, Herrero et al. 2007). It is expected that the majority of effector/memory cells induced by the vaccines would be mainly CD4+ cells. However, there are other studies that suggest a role of CD8+ cells following vaccination with 4vHPV. One study examined the immunity of HIV-infected children one month following three doses of 4vHPV demonstrated that the depletion of CD8+ cells resulted in reduced number of IFN\(\gamma\) producing cells from IFN\(\gamma\) ELISPOT assay compared with whole PBMCs (Weinberg, Song et al. 2012). This suggest that CD8+ cells were induced following 4vHPV vaccination, although their role in vaccine-induced protection remains unclear. In addition, several case reports have reported the regression/clearance of cutaneous warts following three doses of 4vHPV, suggesting that CD8+ cells can be induced by the HPV vaccine and may be involved in warts regression (Kreuter, Waterboer et al. 2010, Venugopal and Murrell 2010, Silling, Wieland et al. 2014). However, CD8+ cells were not measured in some of these studies, and one study found no increase in the CD4/CD8 ratio in a splenectomised patient before- and after 4vHPV vaccination (Silling, Wieland et al. 2014). More studies are needed to determine the role of CD8+ cells following HPV vaccination, and their association in patients with skin warts.

There are some limitations to the work in this chapter. Firstly, as these studies were secondary analyses, the sample size in each dosage group was small, contributing to the lack of precision around the estimates and power, especially when stratifying by ethnicity. Therefore, these results should be interpreted with caution. Nevertheless, significant differences were observed and the confidence intervals were relatively narrow in most cases, providing some confidence in the results. Secondly, there is no defined HPV epitope for stimulation, and no standardised stimulation protocols to assess cellular immunity following HPV vaccination which may have led to reduced/missed T cell responses. The timing of the sample collection may also have contributed to reduced responses as some studies have demonstrated peak cellular immune responses at 7 to 10 days post-vaccination (Pichichero, Voloshen et al. 1999, Speiser, Lienard et al. 2005, Arifuzzaman, Rashu et al. 2012), whereas the blood sample in our study was collected 28 days post-vaccination for analysis of the primary outcome. Collection of multiple
bloods is one way around this but can be ethically challenging to achieve in studies based in LMICs.

In summary, the HPV-specific T cell responses against HPV16 but not 18 were not significantly different between girls who received one, two or three doses of 4vHPV six years ago, although a larger sample size is needed to confirm this observation. These findings suggest that the HPV18-specific T cell responses may be lower following reduced-dose HPV schedules, and is consistent with the NAb data described in Chapter 3 of this thesis. Longer term studies are needed to extend these findings, which might have important implications for the use of reduced-dose HPV schedules.
5. Results 3: HPV-specific cytokine responses six years following reduced dose quadrivalent HPV vaccine schedules

5.1. Abstract

The role of cytokines induced by HPV vaccination is unclear and has not been evaluated in the context of reduced-dose schedules. This chapter examined cytokine responses (IFNγ, IL-2, TNFα, IL-10 and IL-5) in a randomly selected subset of Fijian girls (n=15/dosage group, made up of approximately equal numbers of the two main ethnic groups). Six years after the last dose of 4vHPV, the cytokine responses (except IL-2) to HPV16 in girls vaccinated with one or two doses were not significantly different to girls vaccinated with three doses. Significantly lower HPV18 cytokine responses (except IL5) were observed in girls vaccinated with one or two doses compared with girls vaccinated with three doses. Following the administration of a single dose of 2vHPV, the cytokine responses were not significantly different between the two-dose group and the three-dose group for HPV16 and 18. No significant difference in cytokine responses were observed between the two ethnic groups, although a higher proportion of Th1 compared to Th2 cytokines were found. The cytokine responses showed moderate to strong correlations with the HPV-specific T cell response, and weak to moderate correlations with the HPV-specific antibody response when all individuals were pooled, irrespective of dosage group. Taken together, these results suggest that the cytokine response vary between HPV types, and HPV18 cytokine responses following reduced-dose schedules may be weaker after six years, although the clinical significance of this is unknown. These findings warrant further studies to examine the immunogenicity and efficacy against reduced-dose schedules in the long-term. Future studies examining cytokine responses following HPV vaccination may help elucidate important immune mechanisms associated with HPV vaccines.
5.2. Introduction

Cytokines are important proteins produced mainly by immune cells (i.e. dendritic cells, B- and T cells, Natural Killer cells) to ‘communicate’ and regulate immune responses. Generally, cytokines can be classified into pro- or anti-inflammatory based on their respective roles in the initiation of immune responses to clear/contain pathogens and resolution of inflammation to maintain homeostasis (Berger 2000). Unregulated pro-inflammatory cytokine responses due to the lack of immune regulation to return to a ‘normal state’ can lead to autoimmune diseases (i.e. rheumatoid arthritis, psoriasis, and lupus erythematosus) (Kunz and Ibrahim 2009). On the other hand, the lack of pro-inflammatory responses may lead to an inability to initiate immune responses to clear an infection (Wakeham, Wang et al. 1998, Boelen, Kwakkel et al. 2002). With respect to activation of the adaptive immune system, cytokines play an important role in recruitment, activation and differentiation of naïve T- and B-cells in the secondary lymphoid organs into effector or memory cells (Chabalgoity, Baz et al. 2007).

The differentiation of naïve T cells into various T cell subsets [i.e. helper T cell (Th); Th1, Th2, Th17 and regulatory T cell (Treg)] are dependent on the activation signals delivered by antigen presenting cells (Morel and Turner 2010). These T cell subsets can be identified based on the cytokines they produce, and determine the type of immune responses elicited. For example, Th1 cells produce cytokines such as IFNγ, TNFα and IL-2 that activate cytotoxic T cells to clear viruses or intracellular pathogens, and are also associated with memory responses, while Th2 cells produce cytokines including IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 that are associated with robust antibody production, and also initiate clearance of extracellular parasites (Zhu and Paul 2008). Examples of the cytokines produced by the Th family and their major roles are shown in Table 5.1.
Table 5.1: Role of cytokines in the immune response

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Major roles</th>
<th>Th family</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ, TNFα and IL-2</td>
<td>Pro-inflammatory cytokines; activate cells (i.e. cytotoxic T cells, NK cells, macrophages) to target intracellular pathogens (virus, bacteria, protozoa), and tumour cells                                                                ----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>Th1</td>
</tr>
<tr>
<td>IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13</td>
<td>Anti-inflammatory cytokines; activate cells (i.e. mast cells and eosinophils) to target extracellular pathogens and parasites; helps in production of highly specific antibodies</td>
<td>Th2</td>
</tr>
<tr>
<td>IL-17</td>
<td>Pro-inflammatory cytokine; recruit cells (i.e. neutrophil) to eliminate extracellular bacteria (i.e. <em>Streptococcus pneumoniae</em> and <em>Klebsiella pneumoniae</em>)</td>
<td>Th17</td>
</tr>
<tr>
<td>TGFβ, IL-10</td>
<td>Regulate immune responses; maintenance of peripheral tolerance and homeostasis; suppress persisting Th1 responses; prevent infection-induced immunopathology</td>
<td>Treg</td>
</tr>
</tbody>
</table>

Adapted from (Chabalgoity, Baz et al. 2007)

Treg: regulatory T cells, Th: helper T cells, NK: natural killer cells, IL: interleukin, IFNγ: interferon gamma, TNFα: tumour necrosis factor alpha, TGFβ: transforming growth factor beta

In recent years, cytokine measurement has become an important marker for predicting and understanding vaccine-induced immune responses, including memory responses. For example, a study examined the immune response in adolescents previously vaccinated with Bacillus Calmette–Guérin (BCG) vaccine (13-14 years ago) found significantly higher cytokines and chemokines (IP-10, IL-1α, IFN-γ, GM-CSF and sIL-2Ra) in supernatants of whole blood stimulated cultures of previously vaccinated adolescents compared with unvaccinated adolescents, suggesting the induction of long-term immunity (Smith, Lecher et al. 2012). In addition, these cytokines also predicted the immune cells (i.e. Th1, Th2 and Treg) involved in the immune response following BCG vaccination. Another study of the yellow fever vaccine (YF-17D) found a significant increase in IP-10 (chemo-attractant for various immune cells such as monocytes, macrophages, T cells, NK cells and dendritic cells) and IL-1α (induces pro-inflammatory cytokine release and proliferation of epithelial cells) following vaccination of healthy adult volunteers, which was postulated to be important for protection since they were also found to be upregulated during other flavivirus infections (Querec, Akondy et al. 2009). Different types of vaccines (e.g. live-attenuated, inactivated and subunit vaccines) and their
adjuvants are likely to induce different immune cells and cytokine profiles (Morel and Turner 2010).

The role of cytokines induced by HPV vaccination is unclear and has not been evaluated for its clinical significance. Previous studies have demonstrated a significant increase in a range of Th1 and Th2 cytokines one month following one or three doses of the first generation HPV16 VLP vaccine, or three doses of the 2vHPV when compared with unvaccinated individuals in whole blood and *ex vivo* cultures (Pinto, Castle et al. 2005, Garcia-Pineres, Hildesheim et al. 2007, Goncalves, Giraldo et al. 2015), suggesting the involvement of various immune cells in the immune response induced by HPV vaccination. However, the cytokine profiles induced by the licensed HPV vaccines (2vHPV, 4vHPV and 9vHPV) have not been evaluated following reduced-dose schedules or beyond one month post-three dose primary series. This is not surprising since the NAb response is thought to be the primary mechanism of protection. The question of whether reduced-dose HPV vaccine schedules generate a similar cytokine profile as three-dose schedules, and their role in vaccine-induced protection against HPV infection remains unknown.

As part of the secondary analyses to evaluate cellular immunity in the Fiji HPV prospective cohort study, cytokine analysis using multiplex-bead array was performed on the supernatants of *ex vivo* PBMCs cultures stimulated with HPV16 or 18 peptides from a subset of girls, previously described in Chapter 4. A panel of Th1 and Th2 cytokines were selected for this purpose. As significant differences in NAb titres were observed between the two ethnic groups (shown in Chapter 3), and to further evaluate ethnic differences in cytokine responses, comparison of cytokine profiles between the two ethnic groups were also performed. In order to better understand the cell-mediated immune response following HPV vaccination, the cytokine profiles and Th1/Th2 ratios were also examined, as well as correlations analyses performed between cytokine responses and NAb titres, as well as with HPV-specific T cell responses.

Hypothesis: We hypothesised that girls who received two doses of 4vHPV six years ago have similar cytokine profiles when compared with girls who received three doses.
Primary aim: The primary aim of this chapter was to compare HPV-specific cytokine responses (IFNγ, IL-2, TNFα, IL-10, IL5) in girls who received zero, one or two doses of 4vHPV with girls who received three-doses of 4vHPV six years ago, and their subsequent responses to a dose of 2vHPV.

The secondary aims of this chapter were to:

1. compare the cytokine responses (IFNγ, IL-2, TNFα, IL-10, IL5) between the two ethnic groups (indigenous Fijians; iTaukei, and Fijians of Indian Descent; FID) within each dosage group pre- (six years after the last dose of 4vHPV) and one month post 2vHPV;
2. compare the cytokine profiles in the form of Th1:Th2 ratios between each dosage group;
3. correlate cytokine responses with NAb titres to HPV16 and 18; and
4. correlate cytokine responses with HPV-specific T cell responses

5.3.Methods

5.3.1. Study design, participants and procedures
The study design, recruitment and selection criteria, as well as the study procedures were previously described in Chapter 3 and published in Toh et al. (Toh, Russell et al. 2017). The details of the study samples were previously described in Chapter 4.

5.3.2. Laboratory procedures

5.3.2.1. Cell culture and stimulation
The PBMC cell culture procedures were previously described in Chapter 4. Following the 48 h culture, the supernatants of the PBMCs cultures were collected and stored at -80°C until cytokine analysis.

5.3.2.2. Multiplex-bead array assay
A panel of Th1 (IFNγ, TNFα, IL-2) and Th2 (IL-5 and IL-10) cytokines were selected based on previous publications and their roles in vaccine immunology (Pinto, Castle et al. 2005, Garcia-Pinieres, Hildesheim et al. 2007, Siegrist 2008). The concentrations of IFN-γ, TNF-α, IL-2, IL-10, and IL-5 in the supernatants of the PBMC cultures were determined using the Milliplex® XMAP kit (EMD Millipore, Massachusetts, USA) according to manufacturer’s protocol. The kit was stored at 4°C and brought to room temperature (RT) before use. A low protein binding Durapore® membrane 96-well Multiscreen® filter plate (Merck Millipore, Ireland) was prepared with wash buffer supplied in the kit, and sealed and incubated on a plate.
shaker at 500 rpm for 10 mins at RT. The plate was kept on a plate holder and wrapped in foil at all times. Two vials of Quality Controls and the Human Cytokine Standard (10,000 pg/mL) provided by the kit were reconstituted with 250 μL of deionised water and left on a roller for 10 mins. Assay Buffer was used make up the standards by performing five-fold serial dilutions (2,000 pg/mL, 400 pg/mL, 80 pg/mL, 16 pg/mL, and 3.2 pg/mL). Standards, quality controls and assay buffer (background) were added to the respective wells on the plate. R10 medium (25μL/well) was added to all standard and control wells and Assay Buffer (25μL) was added to all sample wells. Twenty-five microliters of PBMC sample supernatants were added to the respective wells on the plate for each of the five PBMC conditions in singlicate. Each antibody-bead vial was sonicated for 30 sec then vortexed for 1 min. Sixty microliters from each antibody-bead vial and 2.7 mL of bead diluent were added to the mixing bottle supplied in the kit and vortexed for 1 min. Twenty-five microliters of the mixed beads were then added to all wells and the plate was sealed and incubated on a plate shaker for 2 h at RT. The plate was then washed twice with wash buffer (200 μL/well) via vacuum filtration, followed by the addition of 25μL/well of detection antibodies and a 1 h incubation on a plate shaker at RT. Twenty-five microliters of Streptavidin-Phycoerythrin was added to all wells and incubated on a plate shaker at RT for 30 min. The plate was then washed twice followed by the addition of 150μL/well of sheath fluid and incubated on shaker for 5 min. The plate was then read on a Luminex 200 instrument and analysed using Luminex xPONENT software version 3.1 (Luminex Corporation, Texas, USA).

5.3.2.3. Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 software. Comparison of each cytokine pre- and post-2vHPV, between three-dose group and two, one or zero-dose group, and between iTaukei and FID girls within each dosage group were compared using the Mann-Whitney U test. Comparison of each cytokine within each dosage groups pre- and post-2vHPV were performed using the Wilcoxon matched-pairs signed rank test. Characterisation of the cytokine profiles of girls vaccinated with different doses of 4vHPV were performed by calculating the ratio of the principal effector cytokines for Th1 (IFNγ) and Th2 (IL-5) populations, and comparison between the three-dose group and two, one or zero-dose group using Mann-Whitney U test. Correlation test between each cytokine and HPV-specific IFNγ producing cells (from ELISPOT), and each cytokine with the HPV genotype-specific NAb titres were done using the Spearman’s rank test. For all statistical analyses, a p value <0.05 was considered significant.
5.4. Results

5.4.1. Cytokine responses following reduced-dose 4vHPV schedules and subsequent responses to a dose of 2vHPV

Six years after receiving the last dose of 4vHPV, the two-dose group had significantly lower IFN$\gamma$ for HPV18 ($p=0.002$) when compared with the three-dose group (Figure 5.1). Both two- and one-dose groups had significantly lower IL-2 for HPV16 (two-dose: $p=0.022$; one-dose: $p=0.040$) and 18 (two-dose: $p=0.013$; one-dose: $p=0.031$) when compared with the three-dose group. In addition, significantly lower TNF$\alpha$ ($p=0.016$) and IL-10 ($p=0.018$) were observed for HPV18, but not HPV16 in the two-dose group compared with the three-dose group; significantly lower IL-10 was also observed in the one-dose group for HPV18 ($p=0.014$) but not HPV16. The zero-dose group had significantly lower levels across all cytokines for both HPV16 and 18 except IL-5 and IL-10 (HPV16 only).

Following the administration of a single dose of 2vHPV, no significant differences were observed across all cytokines measured for both HPV types between the two- and three-dose groups. Overall, the cytokine responses post-2vHPV were higher than pre-2vHPV, but were not significant in all cases, while the HPV16 response was generally higher than HPV18. HPV16 and 18-specific IFN$\gamma$ levels were significantly lower in the one-dose group compared with the three-dose group (HPV16: $p=0.029$; HPV18: $p=0.043$). This was also observed for IL-10 for HPV16 ($p=0.032$) but not HPV18 when the same groups were compared (Figure 5.1). Similar to pre-2vHPV, the zero-dose group had significantly lower levels for all cytokines for both HPV16 and 18, except for IL-5 (both HPV16/18) and TNF$\alpha$ (HPV18), which were lower but not significant.

When the background level of cytokines was taken into consideration, significantly lower cytokine fold-change responses (HPV16 IL-2, HPV16/18 TNF$\alpha$ and IL-10) were observed in the one-dose but not the two-dose group (except HPV18 IL-10 and IL-5 which was also significantly lower), when compared with the three-dose group six years after the last dose of 4vHPV (Appendix 10). This was consistent for HPV16/18 TNF$\alpha$, HPV16 IL-10 following a dose of 2vHPV (Appendix 10).
4vHPV dosage groups pre/post 2vHPV
Figure 5.1: HPV cytokine response of girls six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and cytokines measured in supernatants by Milliplex bead array. Red bars represent three-dose group, n=15; blue bars represent two-dose group, n=14; purple bars represent one-dose group, n=15 and green bars represent zero-dose group, n=15. Bars represent mean ± standard error of mean (SEM).

5.4.2. Cytokine response differences between ethnic groups following reduced-dose 4vHPV schedules

To further examine the basis for the differential NAb responses observed between the two ethnic groups, the girls were stratified based on their ethnicity and their cytokine responses compared. There were no clear differences for any cytokines measured between the iTaukei girls and FID girls for all dosage groups (Figure 5.2). The FID girls had higher but not significantly different levels of IFNγ and IL-2 for both HPV16 and 18 than iTaukei girls in all dosage groups except the one-dose group. Although higher levels of TNFα were observed in FID girls than iTaukei girls post-2vHPV (three-dose group for HPV16 and HPV18, one-dose and zero-dose group for HPV18), no significant difference was observed across all dosage groups. Significantly higher IL-10 only for HPV16 was found in iTaukei girls compared to FID girls in the three-dose group six years following the last dose of 4vHPV. In contrast, FID girls had significant higher IL-10 than iTaukei girls for both HPV16 and 18 in the zero-dose group after a dose of 2vHPV. Although not statistically significant, IL-5 was higher in iTaukei girls than FID girls for HPV16 but not HPV18 for all dosage groups.
**IFN-γ**

![IFN-γ Graph](image)

**IL-2**

![IL-2 Graph](image)

**TNF-α**

![TNF-α Graph](image)

**IL-10**

![IL-10 Graph](image)

**IL-5**

![IL-5 Graph](image)

*4vHPV dosage groups pre/post 2vHPV*
Figure 5.2: HPV cytokine response of iTaukei and FID girls six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. PBMCs were stimulated with HPV16 or HPV18 peptides for two days and cytokines measured in supernatants by Milliplex bead array. Red bars represent iTaukei (indigenous Fijians) girls, n=7 for all dosage groups; blue bars represent FID (Fijians of Indian Descent) girls, n=8 for all dosage groups except two-dose group, n=7. Bars represent mean ± SEM.

5.4.3. Characterisation of cytokine responses following reduced-dose 4vHPV vaccination

Higher Th1 cytokine responses against HPV16 and 18 were observed across all HPV vaccine dosage groups six years after the last dose of 4vHPV and following a dose of 2vHPV (Figure 5.3). No significant differences in Th1/Th2 ratio were observed between the one- or two-dose group and the three-dose group pre- and post-2vHPV for HPV16 and 18. Only the zero dose group had significantly lower Th1/Th2 ratio differences when compared with the three dose groups pre- and post-2vHPV for both HPV16 and 18.

Figure 5.3: Comparison of IFNγ/IL-5 ratio (representative Th1/Th2 cytokines) to HPV16 (A) and HPV18 (B) six years after zero, one, two or three doses of 4vHPV and one month after a single booster dose of 2vHPV. Red bars represent three-dose group, n=15; blue bars represent two-dose group, n=14; purple bars represent one-dose group, n=15 and green bars represent zero-dose group, n=15. Bars represent mean ± standard error of mean (SEM).
5.4.4. **Correlation of cytokine responses with HPV type-specific T cell responses**

For all five cytokines measured, there was a moderate to strong correlation with HPV-specific IFNγ secreting cells when all data (regardless of dosage groups) were pooled (p<0.0001) (Figure 5.4). This was also observed for all cytokines when stratified by dosage groups except for the following analyses: HPV18 TNFα in the zero-dose group, HPV16/18 IL-10 in the three-dose group, and HPV16 IL-5 in the zero-dose group (Table 5.2).

5.4.5. **Correlation of cytokine responses with HPV type-specific NAb responses**

All five HPV-type specific cytokines had weak to moderate correlation with HPV type-specific NAb titres for all individuals regardless of dosage groups, and were statistically significant (p<0.001 for all comparisons except for HPV18 TNFα; p=0.042), except for IL-5 (HPV18) (Figure 5.5). When the analyses were stratified by 4vHPV dosage group, moderate to strong correlations that were statistically significant (p<0.05) were observed for all five cytokines against HPV16 for the three- and two-dose groups, except for IFNγ in the two-dose group, and TNFα in the three-dose group (Table 5.3). The zero-dose group had moderate correlations that were statistically significant between the HPV16 cytokines IFNγ, IL-2 and IL-10 and HPV16 NAb levels (Table 5.3). No correlation of any cytokines and NAb were observed against both HPV16 and 18 in the one-dose group. There were fewer correlations that were statistically significant between the HPV18 cytokine responses and NAb: for IL-2 (two and zero dose group) and IL-10 (two-dose group) (Table 5.3).
IFN-γ producing cells/10^6 PBMCS

cytokines: pg/ml

r = 0.87
p < 0.0001

r = 0.77
p < 0.0001

r = 0.63
p < 0.0001

r = 0.57
p < 0.0001

r = 0.45
p < 0.0001

r = 0.57
p < 0.0001

r = 0.74
p < 0.0001

r = 0.45
p < 0.0001

IFN-γ

IL-2

TNF-α

IL-10

IL-5

HPV16

HPV18
**Figure 5.4:** Correlation analyses between PBMC supernatant cytokines (IFNγ, IL-2, TNFα, IL-10 and IL-5) and IFNγ producing cells for HPV16 and HPV18 for all individuals regardless of dosage groups pre- and post-2vHPV (n=59).

**Table 5.2:** Correlation analyses between HPV-specific cytokines and T cells to HPV16 and 18 when stratified by dosage groups (data included from both pre- and post-2vHPV)

<table>
<thead>
<tr>
<th>Dosage groups/ Cytokines</th>
<th>3 dose (n=15)</th>
<th>2 dose (n=14)</th>
<th>1 dose (n=15)</th>
<th>0 dose (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HPV16</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.75</td>
<td>&lt;0.0001</td>
<td>0.62</td>
<td>0.001</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.45</td>
<td>0.013</td>
<td>0.77</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.58</td>
<td>0.001</td>
<td>0.56</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.28</td>
<td>0.128</td>
<td>0.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.45</td>
<td>0.013</td>
<td>0.46</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>HPV18</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.75</td>
<td>&lt;0.0001</td>
<td>0.67</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.82</td>
<td>&lt;0.0001</td>
<td>0.71</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.36</td>
<td>0.049</td>
<td>0.51</td>
<td>0.006</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.21</td>
<td>0.258</td>
<td>0.47</td>
<td>0.012</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.56</td>
<td>0.001</td>
<td>0.51</td>
<td>0.005</td>
</tr>
</tbody>
</table>

R: correlation coefficient
**Figure 5.5:** Correlation between cytokines (IFNγ, IL-2, TNFα, IL-10 and IL-5) and neutralising antibodies for HPV16 and HPV18 for all individuals regardless of dosage groups pre- and post-2vHPV (n=59).

**Table 5.3:** Correlation analyses between HPV-specific cytokines and NAb to HPV16 and 18 when stratified by dosage groups (data included from both pre- and post-2vHPV)

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Dosage groups/</th>
<th>3 dose (n=15)</th>
<th>2 dose (n=14)</th>
<th>1 dose (n=15)</th>
<th>0 dose (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R</td>
<td>p value</td>
<td>R</td>
<td>p value</td>
<td>R</td>
</tr>
<tr>
<td>HPV16</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.52</td>
<td>0.003</td>
<td>0.37</td>
<td>0.051</td>
<td>0.23</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.52</td>
<td>0.003</td>
<td>0.61</td>
<td>&lt;0.001</td>
<td>0.32</td>
</tr>
<tr>
<td>TNFα</td>
<td>0.34</td>
<td>0.062</td>
<td>0.43</td>
<td>0.023</td>
<td>0.12</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.58</td>
<td>&lt;0.001</td>
<td>0.59</td>
<td>0.011</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.43</td>
<td>0.017</td>
<td>0.46</td>
<td>0.014</td>
<td>0.29</td>
</tr>
<tr>
<td>HPV18</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.17</td>
<td>0.365</td>
<td>0.27</td>
<td>0.172</td>
<td>-0.001</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.30</td>
<td>0.100</td>
<td>0.66</td>
<td>&lt;0.001</td>
<td>0.09</td>
</tr>
<tr>
<td>TNFα</td>
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<td>0.518</td>
<td>0.28</td>
<td>0.147</td>
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</tr>
<tr>
<td>IL-10</td>
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<td>0.088</td>
<td>0.58</td>
<td>0.001</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-5</td>
<td>0.25</td>
<td>0.188</td>
<td>0.34</td>
<td>0.081</td>
<td>0.16</td>
</tr>
</tbody>
</table>
5.5. Discussion

This chapter demonstrated that girls who were vaccinated with one or two doses of 4vHPV six years previously had comparable cytokine profiles against HPV16 with girls vaccinated with three doses, although a larger sample size is needed to confirm this finding. For HPV18, four (IFNγ, IL-2, TNFα and IL-10) out of the five cytokines measured were significantly lower in the two-dose group compared with the three-dose group, suggesting that this response may be inferior following two doses of 4vHPV. In addition, cytokine responses to HPV18 were less immunogenic than HPV16 in general, which was consistent with the IFNγ-secreting cell response (Chapter 4) and NAb (Chapter 3). Interestingly, only two out of the five cytokines (IL-2 and IL-10) in response to HPV18 were significantly lower in the one-dose group compared with the three-dose group. This is likely due to the variability between individual responses in each dosage group, as evident by the large error bars (IFNγ against HPV18 in the one-dose group and TNFα against HPV18 in the two-dose group). Whether these inferior cytokine responses, particularly for HPV18 following fewer 4vHPV doses are associated with an increased risk of HPV infection in the long-term are unknown.

The cytokine responses across all five cytokines following a dose of 2vHPV were not significantly different between the two- and three-dose groups. However, this was not observed in the one-dose group, suggesting that there might be some inferior cellular memory responses. In addition, despite moderate to strong correlations between each of the five cytokines and the number of IFNγ producing cells for all dosage groups, no correlation was found between cytokine levels and HPV16 and 18 specific NAs in the one-dose group, possibly due to weaker priming. These observations suggest that the inferior cellular responses following one dose of 4vHPV may be due to immune cells other than IFNγ producing cells, which might have an impact on long-term protection. It is important to note that some of these cytokines may also be produced by other immune cells (i.e. monocytes and tissue macrophages secrete TNFα and to a lesser extent IL-10; NK cells produce IFNγ and regulatory T cells produce IL-10) (Striz, Brabcova et al. 2014). Therefore, detailed studies that investigate the role of different immune cell populations following HPV vaccination may elucidate important immune mechanisms associated with protection against HPV infection. The cytokines measured in this study correlate better with the IFNγ producing cell responses than NAb. The lack of correlation between cytokines and NAb in this study is somewhat consistent with previous studies that found inconclusive outcomes on correlation analyses between cytokines (i.e. IFNγ, IL-5 and
IL-10) and antibody responses following HPV vaccination (Pinto, Edwards et al. 2003, Pinto, Viscidi et al. 2006, Garcia-Pineres, Hildesheim et al. 2007).

This is the first study to examine *ex vivo* cytokine responses following reduced-dose schedules, and also the longest duration of follow-up of reduced-dose schedules since the last dose of 4vHPV. Previous studies that have evaluated cytokine responses following HPV vaccination in women have found significant increases of Th1 and Th2 cytokines one month following one or three doses of the first generation HPV16 VLP vaccine, which did not contain any adjuvant (Pinto, Castle et al. 2005, Garcia-Pineres, Hildesheim et al. 2007). Another study also found significant up-regulation of mRNA for several cytokines (IFNβ, IL-12, IFNγ, IL-6, IL-17, IL-10, TNFα) in PBMCs from individuals who received three doses of 2vHPV but not from unvaccinated individuals (Goncalves, Giraldo et al. 2015). These findings (an increase in Th1 and Th2 cytokines) were similar to the findings of this chapter. However, it is difficult to compare these results due to differences in the HPV vaccine used (i.e. first generation HPV16 VLP vaccine, 4vHPV and 2vHPV), culture conditions (i.e. different timing and antigen stimulation) and cytokine detection assays (i.e. cytokine mRNA, multiplex bead array and ELISA).

Although both Th1 and Th2 cytokines were induced pre- (six years after the last dose of 4vHPV) and post-2vHPV, higher Th1 than Th2 cytokines were produced based on the IFNγ/IL-5 ratios. It is widely recognised that IFNγ is a typical Th1 cytokine and IL-5 is a typical Th2 cytokine, therefore these cytokines were selected for this analysis; IL-4 is also a typical Th2 cytokine, but was not chosen for this analysis as it is known to be produced at low levels based on the experience of the laboratory and other investigators (Bradley, Dalton et al. 1996, Kouro and Takatsu 2009, Licciardi, Ismail et al. 2013). The higher Th1 response observed is consistent with a study that demonstrated predominant Th1 cytokine profiles following immunisation with the measles virus vaccine (Ovsyannikova, Reid et al. 2003), although this is a different type of vaccine (live-attenuated) compared to the HPV vaccine (subunit). The role of cytokines induced by HPV vaccination is unclear and remains to be determined. It is likely that both the Th1 and Th2 cytokines produced help support B cell activation and differentiation following vaccination (Siegrist 2008), since the mechanism of protection by HPV vaccine is thought to be via NAb. In addition, Th1 and Th2 cytokines are counter-regulated, which may explain why both Th1 and Th2 cytokines were elevated following vaccination (Van Eden, Van Der Zee et al. 2002).
Another possible role for Th1 cytokines induced by HPV vaccination is the involvement of cellular immunity that includes the elimination of HPV via their support to CD8+ T cell differentiation or through creating an inflammatory environment to facilitate clearance (Siegrist 2008). However, one previous study showed no accelerated clearance of pre-existing HPV16/18 infections or any therapeutic effect six or twelve months following three doses of 2vHPV (Hildesheim, Herrero et al. 2007). The different adjuvants in the 4vHPV/9vHPV and 2vHPV may also contribute to the different cytokines being produced and this has not been examined previously. Whether the higher antibody and T cell responses induced by 2vHPV than 4vHPV are due to difference in cytokine responses as a result of the AS04 adjuvant is unknown.

In terms of HPV clearance, there seems to be a better understanding of the role of cytokines. In other studies, it was observed that a Th1 pattern of cytokine expression (represented by expression of IFNγ) was needed for clearance of HPV infection, and that HPV persistence was often associated with a low Th1 response (Scott, Stites et al. 1999). This was somewhat supported by other studies that examined ex vivo cultures of PBMCs stimulated with various antigens, with lower production of Th1 cytokines (IL-2 and IFNγ), and higher Th2 cytokines (IL-4 and IL-10) in patients with cervical intraepithelial neoplasia (CIN) when compared with healthy controls (Tsukui, Hildesheim et al. 1996, Clerici, Merola et al. 1997). The authors also found that these differences were more pronounced in CIN patients with HPV infection extending beyond the cervix, suggesting that a more prolonged/extensive HPV infection may be associated with defective Th1 and augmented Th2 cytokine production. More recent cohort studies have also demonstrated a shift in cytokine profiles in women between acquisition and clearance of incident HPV infection, and between high-risk and low-risk HPV samples; an increase in pro-inflammatory (MIP-1α and TNF cytokine family), Th1 (IL-12), and regulatory (IL-10) cytokines from the cervicovaginal lavage samples were associated with a reduced likelihood of HPV clearance (Scott, Shvetsov et al. 2013). Higher concentrations of IL-5, IL-9, IL-13, IL-17, eotaxin, GM-CSF, and MIP-1α were also observed in women infected with high-risk HPV types when compared with women infected with low-risk HPV types (Marks, Viscidi et al. 2011). These studies indicate a role for Th1 cytokines in HPV clearance. So, the question of whether the cytokines (both Th1 and Th2) induced by HPV vaccination have a role in HPV clearance or creates a cellular environment that clears or prevents HPV infection is an important question that remains to be elucidated.
There were several limitations to this study. Firstly, this was a secondary analysis and therefore the sample size may be too small to make any firm conclusions. Secondly, there is no clinical data (e.g. HPV DNA) from these girls to correlate with these immune parameters, making it difficult to draw any clinical significance. Thirdly, cytokines can be influenced by a range of different factors (i.e. ongoing/past infections by pathogens, inflammation and allergies), including the timing of sample collection and between individuals, which means that the cytokine results may not be a true representation of their responses to HPV vaccination, although all girls were otherwise healthy when they were enrolled into the study. Fourthly, potentially important contributions of other cytokines may have been missed since only five cytokines were measured. Other cytokines and chemokines of interest include Th1: IL-12 (drives Th1 differentiation) (Trinchieri 2003, Hamza, Barnett et al. 2010) Th2: IL-4 (drives Th2 differentiation), IL-13 (antibody production) (Siegrist 2008); Treg: TGFβ (regulation of T cells) (Li and Flavell 2008); IP-10 (recruitment of Th1 and other immune cells) (Liu, Guo et al. 2011) and MCP-1 (regulates the migration and infiltration of monocytes, memory T lymphocytes, and natural killer (NK) cells) (Deshmane, Kremlev et al. 2009). However, the cytokines selected for this analysis represent the most likely Th1/Th2 cytokines involved in this response.

In summary, girls who were previously vaccinated with at least one dose of 4vHPV had higher Th1 and Th2 cytokines than unvaccinated girls, six years following the last dose of 4vHPV and one month after a dose of 2vHPV, suggesting the persistence and induction of memory responses after at least one dose of 4vHPV. Lower cytokine responses to HPV18 were observed in the two- and one-dose groups when compared with three-dose group after six years. Whether these lower cytokine responses affect long-term protection against HPV, particularly HPV18, is unknown, and longer follow-up studies are needed to examine the immunological basis of long-term protection following HPV vaccination.
6. General Discussion

The overall objective of this thesis was to comprehensively investigate the immune response following HPV vaccination in a group of Fijian girls who were previously vaccinated with zero, one, two or three doses of 4vHPV six years ago, and their responses to a ‘booster’ dose of 2vHPV. Previous studies on reduced-dose HPV schedules focussed largely on antibody responses in two-dose HPV schedules. Research into cellular immunity following prophylactic HPV vaccination is a relatively new field with only a limited number of studies that have examined this aspect. This is not surprising since neutralising antibodies are thought to be the primary mechanism of protection. Despite that, cellular immune responses have important roles in the production of robust antibodies and memory responses. The long-term immunogenicity (six years) involving both neutralising antibody and cellular responses of one- and two- and three-dose schedules were examined in this study.

6.1. Key findings

This prospective cohort study represents the longest follow-up study to date on reduced-dose HPV vaccine schedules (six years), focussing on both antibody and cellular immune responses. It is also the first study to characterise the memory response following only one dose of 4vHPV. Using the gold-standard HPV PsV neutralisation assay to assess NAb responses, and PBMC ex vivo assays to examine cellular immunity, a number of key findings were observed.

For antibody responses, one dose of 4vHPV induced vaccine-type antibodies in Fijian girls that persists for at least six years, with levels five- to nine-fold higher than unvaccinated girls. In addition, one dose of 4vHPV elicits sufficient memory responses against 4vHPV types as demonstrated by a similar anamnestic antibody response between girls who previously received one, two or three doses of 4vHPV following a dose of 2vHPV. Two doses of 4vHPV induced antibody responses that were not significantly different compared with three doses after six years and one month after a ‘booster’ dose of 2vHPV. No cross reactive NAb to HPV6 and 11 were induced by 2vHPV, with FID girls having significantly higher antibody responses to 4vHPV types than iTaukei girls six years after the last dose of 4vHPV and one month after a dose of 2vHPV in the two- and three-dose group.

For cellular immune responses, the number of HPV16/18-specific T cells were not significantly different between one- or two-dose recipients of 4vHPV and three-dose recipients after six
years, with the exception of two-dose recipient’s response to HPV18. Following a single dose of 2vHPV, HPV16/18-specific T cell responses were not significantly different in girls who received one, two or three doses of 4vHPV six years previously. In terms of cytokine responses, six years since the last dose of 4vHPV, HPV16-specific IL-2, and HPV18-specific IL-2 and IL-10 were significantly higher in three-dose recipients compared with one- or two-dose recipients. In addition, IFNγ and TNFα against HPV18 were also significantly lower in the two-dose group compared with the three-dose group. Following a dose of 2vHPV, IFNγ cytokine response to HPV16 and 18, and IL-10 to HPV16 were significantly lower in the one-dose group when compared with the three-dose group. In general, no significant differences between ethnicities were observed for the HPV-specific T cell and cytokine responses. While the cytokine responses had moderate to strong correlation with HPV-specific T cell responses, poor to moderate correlation with the antibody responses were observed. It is important to note that the cellular immune response analysis was a secondary analysis of the study, and therefore the sample size may be too small to make any firm conclusion. A larger sample size is needed to confirm some of these findings.

A major limitation of this study was that no virological endpoint was measured and no information on sexual behaviour was collected (due to ethical issues). These were potentially major confounders of the study as a proportion of the girls could have been exposed to HPV prior to vaccination, and a substantial proportion could have been immunologically boosted from sexual exposures during the study. Therefore, careful interpretation of these results is needed. This information may have been useful for understanding the cellular immune responses described (Chapter 4 and 5), in which the one-dose group was observed to have higher responses than the two-dose group, which could be due to previous or current HPV infection. However, the preliminary results from an ongoing study on HPV infection in pregnant women who previously received one, two or three doses of 4vHPV in Fiji did not find any significant differences on crude HPV16/18 infection rates in women who received one (n=4/70, 5.7%) or two (n=0/77, 0%) doses of 4vHPV when compared with women who received three doses (n=3/187, 1.6%) six years after the last dose of 4vHPV (1 vs 3 dose, p=0.072; 2 vs 3 dose, p=0.853; ) (The crude HPV16/18 infection rates were significantly higher in the 0 dose group: n=53/370, when compared with the 3 dose, p <0.001). This might suggest that the higher responses observed in the one-dose group is unlikely to be caused by HPV16/18 infection, assuming the HPV16/18 infection rates are consistent between study groups. However, the influence of previous HPV16/18 infection that have subsequently cleared cannot be excluded, or infections due to other types related to HPV16/18 (i.e. HPV31 and HPV45).
6.2. Implications of these findings:

Majority of cervical cancers occur in LMICs where HPV vaccination is not available due to the high costs and limited logistics to deliver multiple doses of the vaccine. Reduced-dose HPV schedules is one way to alleviate these issues. Immuno-bridging studies have demonstrated similar immunogenicity between girls (<15 years old) who received two doses compared with women (16-26 years old) who received three doses (Toh, Licciardi et al. 2015). This led the WHO to revise its recommendation from administering three to two doses of HPV vaccine to girls <15 years old provided the second dose is given six months apart (World Health Organization 2014). Since then, many high-income and some LMICs have adopted this recommendation. The NAb data in this thesis supports the two-dose schedule, and provided evidence that one dose of HPV vaccine may be sufficient to protect against HPV infection and cervical cancers over the long-term (Toh, Russell et al. 2017). The correlate of protection against HPV infection has not been identified but is thought to be NAb.

The antibody response following three doses of HPV vaccine were found to persist for at least 9.4 years, and approximately 10-fold higher than natural infection (Naud, Roteli-Martins et al. 2014, Nygard, Saah et al. 2015). On the basis of these data, it suggests that a booster dose is not needed for at least 10 years in women who were vaccinated with three doses based on the high level of persisting antibody and no breakthrough infections. In the case of two-dose HPV schedules, the longest duration of follow-up of girls with persisting antibodies to vaccine types prior to this study was three and four years for 4vHPV and 2vHPV, respectively (Dobson, McNeil et al. 2013, Romanowski, Schwarz et al. 2014). It is not known if a two-dose schedule will induce robust antibodies that persist as long as three doses and if a booster dose is needed in future. However, based on the NAb data in this thesis and previous studies that documented similar waning NAb levels between two- and three-dose schedules (Toh, Licciardi et al. 2015, Toh, Russell et al. 2017), it is likely that a two-dose schedule (given at zero and six/twelve months to girls <15 years old) induces similar long-term immunogenicity and protection as three-dose schedules. Studies are ongoing to evaluate reduced-dose 9vHPV in the long-term. To date, similar/higher antibody responses were demonstrated one month after the last dose of 9vHPV in girls who received two doses compared with women who received three doses (Iversen, Miranda et al. 2016). These similar/higher antibody responses to all nine HPV types were also demonstrated in girls of the same age group who received either two or three doses.
of 9vHPV one month after the last dose (Luxembourg 2017). One other important question that remains to be determined is whether the two-dose schedule can be extended to women >15 years old. This will avoid having two different vaccination schedules for different age groups, which would make vaccine introduction more straightforward, particularly in LMICs. It is still unclear why higher antibody responses were observed in younger girls than in older women who are both HPV-DNA negative following HPV vaccination. Differences in the number of B cells could be one explanation, where one study in healthy adolescents (12-18 years old) found a higher percentage of B cells in the whole blood of younger adolescents than older adolescents (Bartlett, Schleifer et al. 1998). Another study on the effect of age on host immunity (measured monocytes, neutrophils B and T cells) in rhesus macaques also found a higher number of lymphocytes including B and T cells in adolescent macaques compared with adult macaques (Oxford, Dela Pena-Ponce et al. 2015).

The timing of the second dose in a two-dose schedule is important in generating similar antibody responses as three-dose schedules. It is thought that the generation of memory B cells requires at least four to six months to mature and differentiate to high-affinity B cells (World Health Organization 2014). Higher immunogenicity was observed in clinical studies when the timing of the second dose was greater than six months (Romanowski, Schwarz et al. 2011, Kreimer, Struyf et al. 2015, Puthanakit, Huang et al. 2016, Sankaranarayanan, Prabhu et al. 2016) compared to less than six months, although this was not observed in this study. The vaccine efficacy against HPV infection in relation to timing of second dose is unclear. Sankaranarayanan et al. found no significant difference in HPV16/18 infections between girls whose second dose was given less than six months or six or more months apart at a median follow-up period of 4.7 years (Sankaranarayanan, Prabhu et al. 2016). However, in a post-hoc analysis of the PATRICIA and Costa Rica vaccine trial, vaccine efficacy against cross-NAb to HPV31, 33 and 45 were only observed in women who received the second dose at six months, but not in those who received the second dose at one month apart (Kreimer, Struyf et al. 2015). This study suggest that cross-NAb may be lower/non-functional if the intervals between doses were less than six months, and requires optimal timing between doses to generate effective NAb/memory responses. In addition, a cohort study in Denmark also found a decreased risk of genital warts with increasing timing between doses in girls who received two doses of 4vHPV. The WHO recommends that girls (<15 years old) who receive their second dose less than six months apart are required to receive a third dose.
Despite the similar NAb data between the two and three-dose groups, analyses of cellular immunity (HPV-specific T cell and cytokine responses) were less conclusive. Lower HPV18 cellular responses were found in girls who received two doses compared with girls who received three doses. The clinical significance of this (lower HPV18 cellular responses) is unknown and whether it has any effect on long-term immunity against HPV infection, particularly adenocarcinomas (more frequently associated HPV18 and 45 remains to be determined (Cubie 2013). Although the sample size for the cellular immunity studies was small and unable to provide firm conclusion, a previous study that examined cellular responses following reduced-dose schedules had also demonstrated lower HPV18 T cell responses (Smolen, Gelines et al. 2012). This suggests that memory responses may be inferior following a two-dose schedule. The T cell and cytokine responses were found to have poor to moderate correlation with the NAb, suggesting that the immune response generated following HPV vaccination is likely to be complex and involve other immune cells apart from the T cells, cytokines and NAb examined in this study. Another possible reason for the poor-moderate correlation between T cell/cytokines and NAb is that the stimulation assay used to examine T cell responses may have selected cells (i.e. NK cells, macrophages and Th17) that are not directly involved in the production of NAb, and were not measured in this study. In addition, important T cell responses may have been missed due to the time of sample collection (day 28 instead of day 7). More studies are needed to examine cellular immune responses to fully understand the immune mechanism of HPV vaccines, and to make sure that the inferior cellular immunity following reduced-dose schedules do not impact on long-term protection against HPV infection.

In terms of type-specific immune responses generated following 4vHPV vaccination, lower HPV18 immune responses (both antibody and cellular responses) were generally observed compared to HPV16. This is consistent with previous studies that have documented lower HPV18 antibody responses than the other three HPV vaccine types one month following last dose of 4vHPV vaccination (Block, Nolan et al. 2006, Castellsague, Munoz et al. 2011, Dobson, McNeil et al. 2013, Gilca, Sauvageau et al. 2014). Lower HPV18 than HPV16 antibody responses were also observed for 2vHPV (Einstein, Baron et al. 2011, Leung, Liu et al. 2015). In addition, inferior HPV18 antibody responses were also observed in girls who received two 4vHPV doses when compared with women who received three 4vHPV doses after three years (Dobson, McNeil et al. 2013). Despite the lower HPV18 antibody responses observed following HPV vaccination, this has not been associated with poorer clinical
outcomes so far (ten years for three-dose schedule and five years for reduced-dose schedules involving one and two dose) (Kreimer, Struyf et al. 2015, Garland, Kjaer et al. 2016, Sankaranarayanan, Prabhu et al. 2016). It is still unknown whether this will affect protection against HPV18 in the long term (greater than ten years), thus longer term studies are still needed.

It is also important to note that individuals from different ethnic backgrounds may respond to HPV vaccine differently, and reduced-dose schedules may not induce sufficient immunity to protect against HPV infection in the long-term for certain ethnic groups. This was observed in the NAb data across all four HPV types in this study. The exact reasons for lower NAb levels in the iTaukei girls than in FIDs girls are unknown and may be due to sexual behaviours (although these information was not collected), or due to genetics or differences in body weight (iTaukei girls have higher BMI, and higher BMI has been associated with reduced vaccine responses against Hepatitis B; discussed in Chapter 3). There are no data on sexual activity between the two ethnic groups, but generally higher HIV rates (81% iTaukei compared to 13% FID(Ministry of Health 2012)) and number of sexual workers are reported in iTaukei (75% iTaukei compared to 18% FIDs (Tokalau 2014)) than in FIDs. However, these reports does not explain the lower NAb levels observed in iTaukei when compared with FIDs. These ethnic differences in NAb may not only apply to Fiji but also other ethnic groups in different countries around the world. Taken together, these ethnic differences argue for continued surveillance of vaccine efficacy against HPV infection following reduced-dose HPV schedules in these groups. This is essential to make sure that individuals who receive reduced-dose HPV schedules are not susceptible to vaccine-type HPV infection and HPV-associated diseases including cervical cancers in the future, and may also provide the rationale for the use of later booster dose of HPV vaccine. There is an ongoing surveillance study on HPV infection in Fiji in pregnant women who were previously vaccinated with reduced-dose 4vHPV schedules six years previously. This study will provide long-term efficacy data following reduced-dose 4vHPV schedule.

Nevertheless, the recommendation of two-dose HPV schedules to girls <15 years of age is likely to be adopted in most countries, including some LMICs due to the alleviation of vaccine cost and logistics in delivering multiple doses. The fact that similar antibody responses were observed in previous studies when the second dose was given at six or twelve months after the first dose (Lamontagne, Thiem et al. 2013, Iversen, Miranda et al. 2016, Puthanakit, Huang et
al. 2016) also provides flexibility and logistical relief in the case of school vaccination programs, where the school health team visits every year to vaccinate the girls. School attendance may be lower in rural LMICs, hence community outreach programs will be important in this case. Cost is a major issue for HPV vaccine introduction, particularly in LMICs. It was estimated that if a two-dose schedule can provide at least 20 years of protection, this would be the most cost-effective schedule and the additional benefit of three-dose schedule would be minimal (Jit, Brisson et al. 2015, Laprise, Markowitz et al. 2016). Most LMICs in Asia and Africa have not introduced this vaccine into their national immunisation schedule (Bruni, Diaz et al. 2016). With the adoption of reduced-dose schedules, girls in LMICs will have a higher chance of accessing this life-saving vaccine.

A single-dose HPV schedule has generated significant interest in recent years and data from previous publications are encouraging in terms of sustained antibody response (five-seven fold higher than unvaccinated), and similar vaccine efficacies against HPV infection between fully vaccinated (three doses) individuals and individuals vaccinated with one dose (Kreimer, Rodriguez et al. 2011, Safaeian, Porras et al. 2013, Kreimer, Struyf et al. 2015, Sankaranarayanan, Prabhu et al. 2016). This could potentially be a ‘game changer’ in terms of HPV vaccine introduction or providing access to this vaccine for women in LMICs. A pooled analysis of the Costa Rica and PATRICIA studies of 2vHPV (two largest clinical studies that evaluated 2vHPV) found similar vaccine efficacies against incident HPV16/18 infection in women who received one, two or three doses of 2vHPV after four years (Kreimer, Struyf et al. 2015). In addition, one dose of 2vHPV induced antibodies that were five to nine-fold higher than levels of natural infection after four years (Safaeian, Porras et al. 2013). These levels were maintained for at least seven years (Kreimer 2017). Similar vaccine efficacies against persistent HPV16/18 infection were also found in girls vaccinated with one, two or three doses of 4vHPV after 4.7 years despite lower NAb responses in the one-dose group in a prospective cohort study in India (Sankaranarayanan, Prabhu et al. 2016). These studies suggest one dose of HPV vaccine may be sufficient to protect against HPV infection. Taken together, these studies and the NAb data in this thesis support the continued assessment of one-dose HPV schedules. Several studies have planned to formally assess the vaccine efficacy of one dose HPV vaccine against HPV infection (Kreimer 2017): a randomised controlled trial is planned to evaluate single-dose efficacy by comparing non-inferiority responses of one and two doses of HPV vaccine (2vHPV and 9vHPV) in the prevention of six-month persistent HPV16/18 infections for four years (ESCUDDO study by the National Cancer Institute, USA). Another study
planned is the African-based HPV immunogenicity study (study name: Doris Tannzania) in which girls are randomised to receive one, two or three doses of either 2vHPV or 9vHPV, and will be followed up for three years. The results of these studies are greatly anticipated and will have significant implications for LMICs if one-dose HPV schedule proves to be effective against HPV infection/cervical pre-cancers.

A unique aspect of this study was that 4vHPV was given six years ago and a 2vHPV was given as a ‘booster’ dose; this is known as a mixed vaccine schedule. This study has demonstrated that such mixed HPV vaccine schedules are safe and can be given to complete a primary series or as a ‘booster’ dose. Only one other study has examined a mixed HPV schedule and was demonstrated to be safe with only local adverse events reported (i.e. pain at injection site, and redness and swelling) that required no medical intervention (Gilca, Sauvageau et al. 2015). With the introduction of 9vHPV, it was postulated that 9vHPV could be used to complete a primary series of HPV vaccination in countries transitioning from either 2vHPV or 4vHPV to 9vHPV (Van Damme, Bonanni et al. 2016). The safety data (shown in Chapter 3 of this thesis) from this prospective study in Fiji contributes to safety and immunogenicity data of such mixed HPV schedules.

6.3. Future directions

There is ongoing surveillance currently in Fiji to determine the HPV prevalence in a group of pregnant women who were previously vaccinated with zero, one, two or three doses of 4vHPV during the vaccination campaign of 2008/09. This data is important to determine the vaccine efficacy of reduced-dose 4vHPV schedule in the long-term, and may allow correlation with the immunogenicity data obtained in this study.

There are still several gaps in knowledge for reduced-dose schedules. One of these questions is the durability of antibody response that is affected by the circulating HPV in the population. It is likely that exposure to subclinical HPV may boost the immune response within a population that have received HPV vaccination, however whether this population immunity to circulating HPV will be maintained when the circulating strains are diminished as the population gets increasingly vaccinated is unknown. Therefore, continued surveillance of circulating HPV types and type-specific immune responses in a population with high HPV vaccine coverage is important. Another question is whether reduced-dose HPV schedules
generates similar cross-reactive immune responses to the standard three-dose schedules. Three
doses of either 2vHPV or 4vHPV were found to induce antibodies to HPV31, 33 or 45 one to
six months after their last vaccine dose (Einstein, Baron et al. 2011, Barzon, Squarzon et al.
2014, Bissett, Draper et al. 2014, Toft, Tolstrup et al. 2014), and were also found to decrease
the risk of HPV infection due to these HPV types (HPV31, 33, 45, 52, 58 and 59) by 20-40%
(Brown, Kjaer et al. 2009, Wheeler, Kjaer et al. 2009). However, this may not occur following
two- or one-dose schedules, as it was previously demonstrated that cross-protection was only
observed against HPV31, HPV33, and HPV45 infection for those vaccinated with three doses
but not two doses (Kreimer, Rodriguez et al. 2011, Kavanagh, Pollock et al. 2014). Cross-
protection against HPV31, 33, 45, 52, 58 and 59 are important as these types along with HPV16
and 18 accounts for 90% of all cervical cancers globally (de Sanjose, Quint et al. 2010, Serrano,
de Sanjose et al. 2015). Further studies of cross-NAb in this cohort are planned. This will add
critical information to add support to the use of reduced-dose schedules, particularly in those
countries that are unable to introduce the newly licensed 9vHPV.

The immune mechanism of HPV vaccines is not fully understood, and the correlate of
protection has not been identified to date. With techniques such as microarray, real-time PCR
and RNA/whole genome sequencing available to examine gene expression analyses, it is
possible to identify early immune gene ‘signatures’ that correlate later with T/B cell, cytokine
or NAb responses to predict effective HPV immunity. This has been demonstrated for the
yellow fever vaccine (Querec, Akondy et al. 2009). Using a systems biology approach, the
identification of genes and other markers of protection associated with HPV vaccination is
possible, and may elucidate important, as yet unidentified immune mechanisms as well as
different immune cell populations involved in HPV vaccination. In addition, using this
approach may also help to determine why higher persisting NAb responses were observed in
FID girls than iTaukei girls. Memory B cells are surrogates for predicting long-term immunity
and would be important in the case of reduced-dose schedules, but this was not measured in
this study due to issues with obtaining the VLPs required for these assays. This is something
that can be done in the future once there is a source of these VLPs, or another assay can be
developed that allows the measurement of this parameter using our biobank of cryopreserved
PBMCs from this study.

The NAb induced by HPV vaccination are postulated to transudate into mucosal surfaces of
the cervix or oral cavity and prevent HPV from binding and infecting epithelial cells. This is
supported by various *in vitro* and *in vivo* mouse model studies that found inhibition of virus binding to target cells directly or indirectly (Day, Thompson et al. 2007, Day, Gambhira et al. 2008), as well as the detection of HPV antibodies following HPV vaccination in oral fluids and cervical secretions in various clinical studies (Rowhani-Rahbar, Carter et al. 2009, Scherpenisse, Mollers et al. 2013, Handisurya, Schellenbacher et al. 2016, Pinto, Kemp et al. 2016). Although these samples (i.e. saliva and cervical secretions) were not collected in this study, future studies evaluating reduced-dose schedules may incorporate measurements of HPV antibodies in these mucosal samples and compare their antibody levels with those vaccinated with three-dose schedules. This will provide important information on whether there is any effect on mucosal immunity following reduced-dose HPV vaccination.

The use of reduced-dose ‘mixed’ schedules consisting of priming with 9vHPV and then boosting with 2vHPV is a unique vaccination schedule for LMICs that are considering a wider range of protection but cannot afford a full 9vHPV schedule. Theoretically, the 9vHPV covers a wider range of vaccine types, while 2vHPV is thought to induce stronger vaccine- and cross-NAb (HPV31, 33 and 45) responses, possibly due to the AS04 adjuvant activity (Garcon, Wettendorff et al. 2011, Barzon, Squarzon et al. 2014, Leung, Liu et al. 2015). It would be interesting to see if boosting with 2vHPV will induce similar boosting responses to cross-neutralising types (HPV31, 33 and 45) present in 9vHPV as compared with boosting with 9vHPV. Reduced-dose mixed HPV vaccine schedules may therefore offer the advantage of increased protection against more HPV types (i.e. HPV31, 33 and 45) than standard HPV vaccine schedules, and may possibly be a cheaper schedule for countries that cannot afford a full 9vHPV schedule. However, this schedule has not been studied in detail and it is important to note that the logistics involved in administration of two different vaccines is challenging depending on the strength of the health system, and a comprehensive assessment of immunogenicity, vaccine efficacy, safety and feasibility in large clinical trials are needed. This is potentially worth investigating in LMICs that are considering broadening the range of protection against HPV infection while reducing cost.

### 6.4. Conclusions

Reduced-dose HPV vaccine schedules has many advantages in terms of lower overall vaccine cost and logistic challenges. However, long-term protection data relating to reduced-dose HPV schedules are currently lacking. This is important as reduced-dose schedules should not
compromise long-term protection against HPV infection. The findings presented in this thesis represents the longest follow-up immunogenicity study to date for 4vHPV and have provided important information on the long-term humoral and cellular immune responses following reduced-dose schedules. This is also the first study to document the induction of memory responses following one dose of 4vHPV and the first to examine cellular immunity in girls who received one- and two-dose schedules. Measurement of cellular immunity is important to fully understand the mechanism of long-term protection provided by HPV vaccines. In addition, long-term immunogenicity and vaccine efficacy studies are still needed to make sure that reduced-dose HPV schedules are protective against HPV infections in the long-term. The results of this thesis are likely to make a significant contribution to the evidence-base for countries that are considering the implementation of reduced-dose HPV vaccine schedules, and has supported the recent decision by the Fiji Ministry of Health to revise their national HPV vaccine schedule in 2016 from three to two doses over six months.
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Appendices


3. Participant’s information sheet and consent form

4. Parent/guardian information sheet and consent form

5. Questionnaires for Day 0, Day 2-3, Day 28

6. Optimisation of dose and time for HPV-specific T cell assay

7. Flow cytometry gating strategy

8. Number of HPV-specific IFNγ producing cells to PHA and TT for each dosage groups before and after a dose of 2vHPV

9. Fold-change of IFNγ+ producing cells stratified into CD4+ and CD8+ cells against HPV16 and 18

10. Fold-change in PBMCs cytokine responses against HPV16 and 18
Human papillomavirus (HPV) vaccines have been introduced in more than 100 countries worldwide to prevent cervical cancer. Clinical trials and observational studies have shown that HPV vaccines have been 100% effective in preventing vaccine-type pre-cancerous cervical dysplasia in mostly affluent, Western countries (FUTURE II Study Group, 2007; Kjaer et al., 2009; Paavonen et al., 2009). Moreover in countries such as Australia where vaccine coverage is high, reductions in HPV vaccine-type related infections and histologically-proven cervical intraepithelial neoplasia grade III (CIN3) have already been observed in the order of 77% (Gertig et al., 2013; Tabrizi et al., 2012). However, in resource-limited countries such as Fiji, the high cost of HPV vaccines and the challenges of implementing a three-dose schedule are significant. Reduced-dose HPV vaccines may be one way to overcome these issues, although current evidence to justify a change in the HPV vaccine schedule is limited. In this perspective piece, we propose that using schedules involving fewer than the recommended three doses of the HPV vaccine is worth investigating to add to the evidence from other countries which show the benefit of a reduced dose schedule, and that this may be beneficial in countries such as Fiji where there is a high burden of cervical cancer.

Burden of cervical cancer

Based on the latest statistics from the World Health Organization (WHO), cervical cancer is the fourth most common cancer in women worldwide. There are approximately 520,000 new cases every year, with 85% of these cases occurring in developing countries (GLOBOCAN 2012). In Fiji, cervical cancer rates (3.3 per 100,000) and mortality (20.6 per 100,000) are one of the highest in the world, ranking as number one for cancer mortality in women, with a higher burden in 1-Taukei women compared with Fijians of Indian descent (Kiehn et al., 2012; Law et al., 2013), although these figures are likely to be a substantial underestimate. Cervical cancer rates in Fiji are approximately 10-fold higher than those of Australia or New Zealand. This is mainly due to effective comprehensive cervical cancer screening strategies which detect and treat early pre-cancerous lesions in both these countries. Prevention of cervical cancer requires a comprehensive program of providing HPV vaccine for girls prior to sexual debut to prevent infection with HPV, and cervical cancer screening which aims to pick up pre-cancerous lesions so that curative treatment can be provided early before cancer develops. The two most common genotypes associated with cervical cancer in Fiji are HPV 16 and 18 (Tabrizi et al., 2011), which are similar to the ones described worldwide. In this article we will focus on primary prevention with HPV vaccine, which provides protection against these two common cervical cancer causing genotypes.

HPV vaccines

There are currently two licensed HPV vaccines. Gardasil® (Merck & Co., USA) is a quadrivalent vaccine that protects against four HPV genotypes (6, 11, 16 and 18). HPV 6 and 11 causes 90% of genital warts cases worldwide (Garland et al., 2007; Garland et al., 2009; FUTURE II Study Group, 2007). The other vaccine is Cervarix® (GliaSmithKline, UK), a bivalent vaccine with the adjuvant AS04 (made up of an aluminium salt and monophosphoryl lipid A) that protects against infection with HPV 16 and 18. The introduction of one of these two HPV vaccines could potentially reduce the burden of cervical cancer (caused by HPV 16 and 18) by almost 80% within Fiji (Tabrizi et al., 2011). Both vaccines are given as a three-dose schedule intramuscularly; Gardasil® vaccine is administered at 0, 2 and 6 months, while Cervarix® vaccine is administered at 0, 1 and 6 month schedule. Both vaccines are highly efficacious against the genotypes included in the vaccines (Dillner et al., 2010; Herrero et al., 2011; Paavonen et al., 2009), and both stimulate long-lasting neutralising antibodies that persist for at least 5 and 8.4 years post vaccination with Gardasil® and Cervarix® vaccine, respectively (Romanowski, 2011). The vaccine efficacy against pre-cancer lesions is reported to be greater than 99%, and seroconversion occurs in 99 to 100% of those vaccinated (Bonanni et al., 2009; Harper et al., 2006; Villa et al., 2005). The magnitude of antibody responses seems to be age-dependent as significantly higher antibody titres were observed in young adolescents (9 to 13 years old), as compared to young women (16 to 26 years old) (Dobson et al., 2013). As HPV infection is very common and initially asymptomatic, it is important to vaccinate prior to HPV exposure i.e. immunise young adolescents (9-12 years old) before their sexual debut, often via a school-based program. Although the vaccine is also recommended for boys, most countries elect to only vaccinate girls due to the cost of the vaccine.

Issues with implementing a three-dose HPV schedule

In 2013, the Fiji MoH with Australian aid support introduced the Cervarix® vaccine as a three-dose schedule (0, 1 and 6 months) to be given to all girls in the last year of primary school as part of the national immunisation program. However, the high cost of the HPV vaccine, representing a substantial cost to the Fiji MoH budget, and the issues surrounding the implementation and maintaining high coverage of a three-dose schedule is challenging. In response to these issues, recent interest has been focused on whether reduced dose HPV vaccines may be equally efficacious to the standard three-dose schedule. In fact, the European Commission has already granted the marketing authorisation for Cervarix® vaccine to be implemented as a two-dose schedule for girls aged 9-14 years old (Landes Bioscience, 2010) based on non-inferior antibody responses (Puthakat et al., 2013; Romanowski et al., 2014). Apart from Europe, other countries such as Panama, Chile, Canada, Pakistan and Bangladesh have already implemented a two-dose schedule for the Cervarix® vaccine at 0 and 6 months. We await the outcome of disease protection over time.
Studies of reduced dose HPV schedules

Studies of reduced dose HPV schedules demonstrating non-inferiority to the standard three-dose schedules are emerging. A trial in Costa Rica found no difference in vaccine efficacy against new HPV infections for women aged 18-25 years old who received one, two or three doses of Cervarix® vaccine four years after receiving the vaccine (Kremer et al., 2011). Similarly, a study in Germany found non-inferior antibody responses in healthy girls (in the age group of 9-14 and 15-19 years old) who received the two-dose schedule (0 and 6 months), one month after the last vaccine dose (Romanowski et al., 2011). The antibody responses to all genotypes in the vaccine (in girls aged 9-14 years old) were found to last for up to four years since the first vaccination (Romanowski et al., 2014).

The most recent study on reduced dose HPV schedules using the Gardasil® vaccine found non-inferior antibody responses to HPV types 6, 11 and 16 and 18 one month after the last dose in girls (aged 9-13 years old) receiving the two-dose schedule, when compared with either girls (aged 9-13 years old) or women (aged 16-26 years old) receiving the three-dose schedule. These antibody responses remained similar for all genotypes up to 36 months post-vaccination (Dobson et al., 2013).

These studies have demonstrated promising results on the potential utility of reduced dose HPV schedules in Fiji and other similar resource-limited settings. The challenges of cost, coverage rates and vaccine delivery would be alleviated by schedules requiring the administration of fewer doses, and facilitate the implementation of national HPV vaccination programs in resource-limited settings. It may also allow the additional advantage of vaccinating boys with these cost savings. However, despite inferiority of the antibody responses for some HPV types reported in some studies, it is still unclear whether reduced dose HPV schedules can generate long-lasting immune responses similar to the standard three-dose schedule. Reduced dose schedules need to be evaluated in order to provide protection over the very long exposure period. More longitudinal studies of reduced dose HPV schedules are therefore required.

Reduced dose HPV schedules: the Fijian context

In 2008/9, the MoH in Fiji accepted a one-off donation of 110,000 doses of quadrivalent HPV vaccine (Gardasil® Merck & Co.) based on the high cervical cancer disease burden. There was enough vaccine to vaccinate four birth cohorts of girls (30,338 girls aged 9-12 years old) with a three-dose schedule via a school-based program. However, not all the girls received the three-dose schedule, mainly due to absence from school on the day the school health team were visiting. The Gardasil® vaccine coverage following the initial and the subsequent mop-up campaign was: 62%, 56%, and 35% for doses one, two, and three respectively.

With Australian Aid support, follow-up of these girls 5 years since the last HPV vaccination provides a unique opportunity to rigorously investigate the question of whether reduced dose schedules are non-inferior in terms of long-term immunity compared to the standard three-dose schedule. We have funding from the Fiji Health Sector Support Project to compare antibody responses and memory cells (specialised long-lived immune cells that produces specific antibodies upon re-exposure to the same antigen) to the four genotypes in the Gardasil® vaccine. In addition, we plan to compare the gene expression profile in immune cells from different individuals and/or dosage groups to examine genes and their pathways that may be switched on or off following a reduced dose schedule to help elucidate those critical genes involved in a protective immune response.

This study also has an additional benefit to determine the long-term immunity of the HPV genotypes that cause genital warts, as this is a neglected area of research. Genital warts are a common sexually transmitted disease caused by HPV, and although not life-threatening, it causes many social and economic issues. This is particularly so in immunocompromised individuals such as those infected with human immunodeficiency virus (HIV). In Fiji, genital warts are believed to be a very common but under reported disease.

This follow-up cohort study will contribute significantly to the growing international literature on the minimum number of HPV doses required for optimal protective immunity in high disease burden settings. The goal for exploring reduced dose HPV schedules is to reduce vaccine cost and to increase HPV vaccine coverage with the ultimate goal to reduce the burden of cervical cancer. We plan on starting the study in July 2014, with results available in the near future. Given the high burden of cervical cancer in Fiji, and the significant cost of HPV vaccines, we believe that examination of reduced dose HPV schedules is a critical area of research that has the potential to provide substantial health and economic benefit to the community.

Reference


Review dose human papillomavirus vaccination: An update of the current state-of-the-art

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Long term protection

ABSTRACT
Human papillomavirus (HPV) infection is the primary cause of genital warts, some oropharyngeal cancers and anogenital cancers, including cervical, vagina, vulva, anal and penis cancers. Primary prevention of cervical cancer requires the prevention of high-risk HPV infections, particularly HPV genotypes 16 and 18. Both Gardasil® and Cervarix® vaccines when administered by a three-dose schedule have been demonstrated to be effective against cervical, vulva, and vaginal cancer precursors from vaccine genotypes in phase III clinical trials, and post-marketing studies. Gardasil® vaccine also offers additional protection against anal cancer precursors. However, high costs of HPV vaccines and the logistics of delivering a three-dose schedule over 6 months are challenging in countries with limited resources. Several studies have demonstrated non-inferiority in antibody response between adolescents (9–15 years old) who received two doses (6 months apart) and women (>15 years old) who received the standard three-dose schedule. These studies provided evidence for the World Health Organization and European Medical Association to revise its recommendation to give two instead of three doses of HPV vaccine to adolescents below 15 years of age, provided the 2nd dose is given 6 months apart. Although reduced dose schedules can alleviate costs and logistics associated with HPV vaccination, especially in resource-poor countries, there are still gaps in this area of research, particularly regarding long-term protection. This review discusses the findings on antibody response and clinical outcomes in studies evaluating reduced dose HPV schedules, and highlights the important considerations of its implementation. In addition, other important immunological biomarkers that may be associated with long-term protection are highlighted and discussed.

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1. Introduction
Human papillomavirus (HPV) is the main causal agent of cervical cancers. This association was first reported by Harald zur Hausen and his team in the early 1980s [1]. HPV was then verified as the cause of cervical cancer, following a number of molecular epidemiological studies [2,3]. Since then, research has focused on the prevention of cervical cancer, including the prevention of HPV infection through vaccination. The major breakthrough in vaccine development was the discovery of the self-assembly L1 capsid viral proteins into virus-like particles (VLPs), which induced the production of high-level neutralizing antibodies, forming the basis for the current HPV vaccines [4].

There are currently more than 170 HPV types identified, with at least 13 types classified as carcinogenic. With the exception of the newly licensed nonavalent HPV vaccine (Gardasil® 9), which requires more long-term studies to evaluate its efficacy and impact
on cervical cancers globally (particularly to the 5 new cancer causing types included in the vaccine), the two HPV vaccines (Cervarix® and Gardasil®) currently used in most developed countries only protects against two oncogenic HPV types (HPV 16 and 18; accounting for 70% of cervical cancers) [5]. Also, as HPV vaccines are only available to a minority of the world's women, and many of these countries do not have a comprehensive effective cervical screening program with treatment of precursor lesions, cervical cancer is the fourth most common cancer in women worldwide, according to recent data from the World Health Organization (WHO) [6]. Approximately 530,000 new cases of cervical cancer occur every year, with 85% of these cases occurring in developing countries [6]. Highly successful cervical cancer secondary prevention activities are often not available or accessible to the world's poorest women. Furthermore, the high cost of HPV vaccines is a major obstacle in low- and middle-income countries. The GAVI Alliance (formerly known as Global Alliance of Vaccines and Immunization) has subsidized and supported the introduction of HPV vaccine in low-income countries, but to date, Bhutan and Laos PDR are the only GAVI-eligible Asian countries to take up this offer. Although low-income countries are eligible for GAVI support, middle-income countries are ineligible and struggle to afford the vaccines. There are also logistical issues in administering the vaccine in a three-dose schedule over 6 months. These issues have led to the exploration of alternative schedules, which could potentially alleviate the issues of vaccine cost and delivery. Based on the increasing evidence of non-inferior antibody responses between girls receiving two doses of HPV vaccine and women receiving three doses, the WHO and European Medical Association (EMA) have revised their recommendation from giving three doses to two doses to girls below the age of 15, provided the second dose is administered 6 months apart. Several countries (i.e. Canada and United Kingdom) have already implemented a two-dose HPV schedule. However, long-term protection is yet to be determined and the immunology of reduced dose HPV vaccine schedules is still unclear. This review will focus on the immunology of evaluating reduced dose HPV vaccine schedules as compared with the standard three-dose schedule.

2. Immune response to HPV during natural infection

The immune response to natural infection with HPV is weak and very slow (up to 18 months), as important signals for the induction of immune responses are absent, due to the ability of the virus to evade the immune response. HPV's replication is exclusively intraepithelial, and there is minimal or no exposure of viral proteins in infected cells, preventing the activation of the immune system [7]. Furthermore, HPV infection is not blood borne, and does not induce cell death, resulting in very weak inflammatory responses [7].

The majority of people who become infected with HPV do not exhibit any clinical symptoms, and most HPV infections are cleared by the host response within a few months, with 50% of infected cases cleared within 2 years [8]. Those women who develop benign cervical lesions usually mount a late, but successful cell mediated immune response, which causes the lesion to regress [9]. The role of cell-mediated immunity in the clearance of HPV infection is evident in immunocompromised individuals (i.e. HIV-infected individuals), who have multiple recurrences of HPV infection, higher incidence of genital warts, and an increased risk of progression from sub-clinical to clinical disease [10–12]. Serum neutralizing antibodies against the L1 capsid protein are generated in approximately 50–70% of infected individuals [13]. However, the level of antibodies generated even at peak titers is low, reflecting the ability of the virus to cause an exclusive intraepithelial infection [13,14]. The long term significance of immunity induced by natural infection is still uncertain, with some clinical studies suggesting that antibodies elicited by natural infection from the virus may not provide complete protection in the long term [15]. Hence, it is recognized that antibody levels detected following natural infection may be less reliable in predicting protection from infection by the HPV types.

3. Immune responses induced by HPV vaccination

There are currently three licensed HPV vaccines (Table 1): Gardasil® (Merck & Co., Inc.; 4vHPV) a quadrivalent vaccine with Alum adjuvant that protects against four genotypes (HPV 6, 11, 16 and 18) and Cervarix® (GlaxoSmithKline; 2vHPV), a bivalent vaccine with the novel adjuvant AS04 (made up of an aluminum salt and monophosphoryl lipid A) that activates innate immunity [16], and protects against infection with HPV 16 and 18. A new next-generation nonavalent HPV vaccine, Gardasil® 9 (Merck & Co., Inc.; 9vHPV), which contains an additional five cancer-causing HPV types (HPV 31, 33, 45, 52, and 58) in addition to the four types in Gardasil®, was recently approved by the U.S. Food and Drug Administration (FDA) (December 2014) and Canada Health (February 2015). This vaccine may potentially prevent 90% of cervical cancers. In a randomized, international, double-blind study, greater than 95% vaccine efficacy against cervical, vulva and vaginal pre-cancer and cancer caused by HPV 31, 33, 45, 52, and 58 was reported for 9vHPV, when compared with 4vHPV in a phase III trial. Efficacy population of 16–26 years old women [17,18]. In addition, similar antibody responses against the common vaccine types were also reported in women vaccinated with 5vHPV or 4vHPV, 1 month post-dose 3 [16]. Due to the recent licensure and limited reduced dosage studies on 9vHPV, this review will focus on data from 2vHPV and 4vHPV.

Immunization against HPV is achieved by a dose of three doses of vaccine, delivered intramuscularly at 0, 2, and 6 months (4vHPV) or 0, 1, and 6 months (2vHPV). The administration of the vaccine intramuscularly helps to circumnavigate the virus's strategies of intraepithelial evasion of the immune system. The prophylactic vaccines are virus-like particle (VLP) based vaccines, which does not contain the viral genome, so the particles contain no DNA and are not infectious. Studies have shown that administration of 4vHPV to 15–26 years old women had 96–100% effectiveness in the prevention of HPV 16- and HPV 18-related grade 1–3 cervical intraepithelial neoplasia, adenocarcinoma in situ, invasive cervical carcinoma, vulvar intraepithelial neoplasia, vulvar cancer, and vaginal cancer [19–21]. It demonstrated a higher prevention rate (98–100%) against HPV 6- and HPV 11-related genital warts and cervical, vulva, vaginal, and anal intraepithelial neoplasia as surrogates to respiratory cancers [19,20]. Similar vaccine effectiveness against cervical intraepithelial neoplasia grade 3 or greater (CIN3+) was also shown with the administration of 2vHPV to 15–26 years old women [22–24].

Antibody responses peak at month 7 (1 month after dose three) at titers between 10 and 100 fold higher than following natural infection, depending on the HPV type and vaccine [24–26]. Following an initial decline, they appear to plateau at 18–24 months, remaining stable for at least 5 years at levels above or at least equivalent to those seen with natural infection [25–28]. The longest duration of antibody response induced by HPV vaccination for 2vHPV and 4vHPV were 9.3 and 8 years, respectively [29,30]. Overall, seroconversion occurs in 99–100% of those vaccinated [27,31].

4. HPV vaccination immunobridging studies

Due to ethical issues of evaluating clinical outcomes in adolescents below the age of 15 years, safety and immunogenicity bridging studies of HPV vaccination are conducted instead, so that
Table 1
Charateristics of HPV VLP vaccines.

<table>
<thead>
<tr>
<th>Manufacturer</th>
<th>Merck™ (Gardasil™)</th>
<th>GlaxoSmithKline™ (Cervarix™)</th>
<th>Merck™ (Gardasil® 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LI VLP types</td>
<td>6, 11, 16 and 18</td>
<td>16 and 18</td>
<td>6, 11, 16, 18, 31, 33, 45, 52, and 58</td>
</tr>
<tr>
<td>Dose</td>
<td>20 µg</td>
<td>20 µg</td>
<td>30 µg</td>
</tr>
<tr>
<td>Producer cell</td>
<td>Saccharomyces cerevisiae (baker's yeast) expressing LI</td>
<td>Trichoplusia ni (silkworm) insect cell line infected with LI</td>
<td>Saccharomyces cerevisiae (baker's yeast) expressing LI</td>
</tr>
<tr>
<td>Adjuvant</td>
<td>325 µg aluminum hydroxyphosphate sulfate</td>
<td>50 µg aluminum hydroxide, 50 µg aluminum monophosphoryl lipid A</td>
<td>500 µg aluminum hydroxyphosphate sulfate</td>
</tr>
<tr>
<td>Vaccination schedule</td>
<td>0, 2 and 6 months</td>
<td>0, 1 and 6 months</td>
<td>0, 2 and 6 months</td>
</tr>
</tbody>
</table>

Source of data [60].

HPV, human papillomavirus; VLP, virus-like particles.

the licensing of the vaccines can be extended for use in early adolescent girls or boys before the onset of sexual activity.

In immunobringing studies for HPV vaccines, both 4vHPV and 2vHPV have demonstrated at least 1–2 fold higher levels of HPV vaccine genotype-specific antibodies in adolescent girls and boys when compared with women, following a three-dose schedule. Moreover, higher antibody levels were observed in the adolescent group at all time points (1 month post-dose 1, 2 or 3) [32–35]. The reason for these different responses could be due to a combination of greater immune responses in young adolescents than adults, and also prior exposure to HPV infection in women, resulting in lower HPV antibody responses. As HPV vaccines are most efficacious in girls/women naive to vaccine-type HPV infection [15], this highlights the need for vaccination prior to HPV infection exposure, and therefore preferably before sexual debut. Since the immunization of adolescent girls yields a much higher antibody response than women, this raises the question of whether a two-dose HPV schedule in adolescents may have similar or non-inferior level of antibodies as a three-dose schedule in women. If so, the longevity of the immune response needs to be determined.

5. Evidence of non-inferior antibody responses in reduced dose HPV schedules

The use of reduced dose schedules in vaccinology is not unusual as other vaccines such as Pneumococcal, hepatitis A and Meningococcal C dosing schedules have been reduced from three to two doses, following clinical studies that have demonstrated non-inferiority in immunogenicity and safety [36–38]. High costs of HPV vaccines and logistical issues with delivery, together with higher antibody responses observed in young adolescents, has prompted the exploration of reduced dose HPV schedules. However, since there have been no breakthrough infections from vaccinated individuals in clinical trials, and no identified correlates of protection, it is hard to establish any protective threshold level.

There are a number of clinical studies that have evaluated antibody responses following either 2vHPV or 4vHPV in the context of reduced dose HPV vaccination. These studies have demonstrated non-inferior antibody responses in healthy girls (aged 9–14 years) who received two doses (month 0 and 9), compared with women (range from 15 to 25 years old) who received three doses (2vHPV 0, 1 and 6 months; 4vHPV 0, 2 and 6 months) [39–43] (summarized in Table 2). These non-inferior antibody responses against the HPV vaccine genotypes were found to last for at least 4 and 3 years for 2vHPV and 4vHPV, respectively, after the last vaccine dose [39,42]. However, HPV 18 and 6 antibody levels were reported to be lower in girls aged 9–13 years old who received two doses of 4vHPV when compared with girls of the same age who received three doses, after 24 and 36 months of the last vaccine dose, respectively, which might suggest waning protection [39]. In an observational study, girls (10 years old) who received either one or two doses of 2vHPV and were compared with girls who received three doses also observed lower HPV 16- and 18-antibody levels, 24 months following the last dose of the vaccine [44]. Whether this inferior response will affect long-term protection in girls receiving reduced dose HPV schedules remains to be determined. The most recent study on reduced dose schedules using 2vHPV reported similar antibody levels against the vaccine types, as well as HPV 31 and 45, and cell-mediated responses (specific T- and B-cell responses) in girls (9–14 years) receiving two doses (month 0, 6) when compared with women (15–25 years) who received three doses (month 0, 1, 6) [45]. A randomized trial of two vs three doses of 4vHPV in India is currently ongoing.

6. Clinical outcomes following reduced dose HPV schedules

A number of studies have investigated the impact of reduced dose HPV schedules on clinical outcomes (Table 3). The study in Costa Rica reported no significant difference against newly acquired HPV 16/18 infection in women aged 18–25 years old, who received either one, two or three doses of 2vHPV when compared with the control group (received modified Hepatitis A vaccine) post-vaccination. However, the analysis for the group who received one dose should be interpreted with caution due to the small sample size and limited follow-up period [40]. Apart from the Costa Rica trial, most of the other studies are based in developed nations (i.e., Australia, Sweden and Scotland) and are observational case-control studies following national HPV vaccine introduction. In Scotland, there was a significant reduction in HPV 16 and 18 infection for those vaccinated with two (OR = 0.68; 0.42-1.12) or three doses (OR = 0.43; 0.34-0.55) of 2vHPV when compared with unvaccinated women [46]. Although there were reduction in HPV 31, 33 and 45 infections for those vaccinated with either two or three doses, only the latter were statistically significant. A study in Australia found that women immunized with either one, two or three doses of 4vHPV had a reduced risk (hazard ratio of 0.76; 95% CI 0.72-0.80) of developing cytological-confirmed low-grade cervical disease (low-grade squamous intraepithelial lesions (LSIL) and/or atypical endocervical cells of uncertain significance) when compared with unvaccinated women [47]. However, only those women who received three doses of 4vHPV had a significant reduction in histological- and cytological-confirmed high-grade cervical diseases (CIN2, CIN3, and/or AIS: high-grade squamous intraepithelial lesion (HSIL) and/or adenocarcinoma), suggesting that a reduced dose schedule may have limited protection against high-grade cervical diseases. Although the other dosage group did not have any significant reduction, the sample size was insufficient to make firm conclusions. A similar observation was also seen in a
Table 2: Clinical studies of reduced dose HPV schedules in terms of antibody responses.

<table>
<thead>
<tr>
<th>Study</th>
<th>Vaccine</th>
<th>Primary endpoints</th>
<th>No. of participants and vaccine dose</th>
<th>Non-inferiority criteria</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00128631</td>
<td>2vHPV</td>
<td>Vaccine efficacy between reduced dose 2vHPV and control (HPV A vaccine)</td>
<td>2vHPV vs control vaccine (18–25 yo women); 3 doses (n=1065 vs 3021); 2 doses (n=488 vs 441); 3 doses (n=277 vs 274); 2 doses (1 m apart); 2 doses (6 m apart): (n=140), 2 doses (6 m apart): (n=52); 1 dose (n=938); +Natural infection (n=113)</td>
<td>Lower limit of the 95% CI of the GMT ratio &lt;0.5</td>
<td>No difference in vaccine efficacy between different dosage groups when compared to control group. Cross protection against 1 year persistent infection with HPV 31, 33 and 45 for three dose but not two dose group; numbers in one dose group were too small for analysis.</td>
</tr>
<tr>
<td>NCT00541070</td>
<td>2vHPV</td>
<td>Noninferior ab response between 2-dose schedules (with or without adjuvant formulations) and 3-dose schedule</td>
<td>Healthly girls/women (9–14, 15–19, 20–25 yd); 2 doses (20/20 μg): (0.6 μg) = 240; (0.6/0.6 μg) = 241; (0.6/0.6 μg) = 240; compared with 3 doses (20/20 μg): (0.1, 0.6 μg)</td>
<td>Lower limit of the 95% CI of the GMT ratio &lt;0.5, upper limit of the 95% CI of the GMT ratio &lt;2</td>
<td>Non-inferior HPV 16 and 18 ab titers between 2 doses and 3 dose except for HPV 16 in 2 dose group (90% CI: 0.82, 0.92). Compared to women (15–25 yo) 1 m after the last dose, non-inferior HPV 16 and 18 ab titers were reported in: - 9–14 yo girls (for all 2 dose groups): - 15–19 yo girls in all 3 dose groups except HPV 16 ab titers in the 2 dose (40/40 μg) (0.2 m) group: - 20–25 yo women in 2 dose group (40/40 μg) (0.2 m) Non-inferiority maintained up to 24 m for 9–14 yo girls except for 2 dose group (40/40 μg) (0.2 m) when compared with women (15–25 yo) (3 dose): non-inferiority maintained up to 48 m for a subset of girls and women.</td>
</tr>
<tr>
<td>NCT01711718</td>
<td>2vHPV</td>
<td>Noninferior ab response between standard schedule (0, 1, 6 m) and extended schedule (0, 6, 60 m)</td>
<td>Standard schedule: Girls (9–10 yo) = 474; Women (18–24 yo) = 500; Extended schedule: Girls (10–16 yo) = 1025</td>
<td>Upper limit of the 95% CI of the GMT ratio &lt;2</td>
<td>Non-inferior HPV 16 and 18 ab titers between 2 and 3 dose groups at 7 and 21 m. Higher but noninferior ab titers in girls who received 3 doses than girls who received 2 doses. Higher but noninferior ab titers in girls who received 2 doses than women who received 3 doses.</td>
</tr>
<tr>
<td>NCT00501137</td>
<td>4vHPV</td>
<td>Noninferior ab response between 2-dose and three dose schedules in girls (9–13 yo) and women (16–28 yo)</td>
<td>Girls: 3 doses: n = 261; 2 doses: n = 259; Women: 3 doses: n = 310</td>
<td>Lower limit of the 95% CI of the GMT ratio &gt;0.5</td>
<td>Non-inferior HPV 6, 11, 16, and 18 ab titers between girls who received 2 doses and women who received 3 dose at 7 m, and this non-inferiority remained up to 36 m. Inferior ab titers for HPV 18 and HPV 6 by 24 m, and 36 m, respectively between girls who received 2 doses and girls who received 3 doses. Inferior HPV 16 and 18 ab titers in girls who received 1 or 2 doses when compared with 3 doses. Higher HPV 16 and 18 ab titers in vaccinated individuals (at least 1 dose) than those that had natural infection. Higher HPV 16 and 18 ab titers in girls than women (from CVT study) who received the same number of vaccine doses.</td>
</tr>
<tr>
<td>Uganda</td>
<td>2vHPV</td>
<td>Ab response between girls (10 yo) who received 1, 2, or 3 doses of vaccine, more than 24 months after last dose</td>
<td>Girls: 3 doses: n = 195; 2 doses: n = 145; 1 dose: n = 36</td>
<td>Lower limit of 95% CI (adjusted of the GMT ratio &lt;0.5</td>
<td>Non-inferior HPV 16 and 18 ab titers in girls who received 2 doses when compared with 3 doses. Higher HPV 16 and 18 ab titers in vaccinated individuals (at least 1 dose) than those that had natural infection. Higher HPV 16 and 18 ab titers in girls than women (from CVT study) who received the same number of vaccine doses.</td>
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</table>

* type years old; Ab: antibody; CI: confidence interval; GMT: geometric mean titer; m: month.

* Costa Rica Vaccine Trial (CVT).

* A separate paper of the same study that compared the antibody responses of different dosage groups.

* HPV16 or HPV18 L1 seropositive women vaccination arm; simulate the "natural infection" group.

Case-control study: compared to unvaccinated individuals, individuals vaccinated with three doses of 4vHPV had a lower risk of acquiring HPV infection, and the risk increased as the number of doses decreased [48]. However, the women receiving less than three doses in this study were older than those who were fully vaccinated, and are more likely to be infected with HPV, which might have contributed to the reduced vaccine efficacy (in those partially vaccinated).

The effectiveness of a reduced dose HPV vaccine schedule on genital warts is unclear. A study in Denmark assessed the population effects of HPV vaccination (4vHPV) on the subsequent risk of genital warts. It was reported that there was a significant reduction.
## Table 3
Clinical studies of reduced dose HPV schedules in terms of clinical outcomes.

<table>
<thead>
<tr>
<th>Country of study</th>
<th>Vaccine</th>
<th>Primary endpoints</th>
<th>No. of participants and vaccine dose</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Costa Rica</td>
<td>2xHPV vs control vaccine (Hepatitis A)</td>
<td>Newly detected HPV16 or HPV18 infection that persisted for at least 10 months</td>
<td>3 doses (2xHPV: 2957; control: 3010); 2 dose (2xHPV: 423; control: 380); 1 dose (2xHPV: 196; control: 185)</td>
<td>No significant difference between dosage group</td>
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<td>Vf for:</td>
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<td>-3 doses: 80.8% (95% CI: 71.1-87.7%)</td>
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<td>-2 doses: 81.1% (95% CI: 70.2-85.1%)</td>
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<td>-1 dose: 100% (95% CI: 66.5-100%)</td>
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<td></td>
<td>Cross-protection against HPV31, HPV33, and HPV45 for 3 dose group (43.3%); 95% CI: 18.9-67.0%), but not for 2 dose group (17.0%); 95% CI: 31.4-44.1% in number of events too small for analysis for 1 dose group</td>
</tr>
<tr>
<td>Scotland</td>
<td>2xHPV</td>
<td>HPV prevalence between vaccinated and unvaccinated women in Scotland population</td>
<td>3 doses - 1100; 2 doses - 105; 1 dose - 35; 0 dose - 3418</td>
<td>0% for HPV 16 and 18 exposure</td>
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<td>-3 doses: 0.4% (95% CI: 0.04-0.65%)</td>
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<td>-2 doses: 0.08% (95% CI: 0.04-0.22%)</td>
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<td>-1 dose: 0.05% (95% CI: 0.01-0.17%)</td>
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<td>-0 dose: 1.00%</td>
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<td></td>
<td>Only women vaccinated with 2 doses have significant reduction against cross-protective types (HPV 31, 33 and 45)</td>
</tr>
<tr>
<td>Australia</td>
<td>4xHPV</td>
<td>Effectiveness against cervical abnormalities in a screening population of women eligible for vaccination in the school-based cohorts (aged 17 or younger in 2007)</td>
<td>3 dose - 21,199; 2 dose - 3412; 1 dose - 2568; 0 dose - 15,192</td>
<td>Hazard ratio</td>
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<td>Any high grade histological abnormalities (CIN2+)</td>
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<td>-3 doses: 0.7% (95% CI: 0.56-0.89%)</td>
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<td>-2 doses: 1.0% (95% CI: 0.88-1.15%)</td>
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<td>-1 dose: 1.47% (95% CI: 0.97-2.23%)</td>
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<td>No significant difference in risk ratio for women who received less than 3 doses</td>
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<td>High grade cervical abnormalities</td>
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<td>-3 doses: 0.71% (95% CI: 0.61-0.83%)</td>
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<td>-2 doses: 0.06% (95% CI: 0.03-0.13%)</td>
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<td>-1 doses: 0.85% (95% CI: 0.62-1.17%)</td>
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<td>Low grade cervical abnormalities</td>
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<td>-3 doses: 0.79% (95% CI: 0.72-0.84%)</td>
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<td>-2 doses: 0.64% (95% CI: 0.57-0.72%)</td>
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<td>-1 dose: 0.67% (95% CI: 0.56-0.76%)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Reduced risk of low-grade cervical abnormalities in women who received one or two doses of vaccine compared with unvaccinated women</td>
</tr>
<tr>
<td>Australia</td>
<td>4xHPV</td>
<td>Effectiveness of 4xHPV against cervical abnormalities four years following vaccine introduction</td>
<td>3 doses - 25,119; 2 doses - 12,073; 1 dose - 10,879; 0 dose - 68,282</td>
<td>Odds ratio for exposure to CIN2+ or AIS</td>
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<tr>
<td></td>
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<td>-3 doses: 0.34% (95% CI: 0.46-0.63%)</td>
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<td>-2 doses: 0.79% (95% CI: 0.64-0.95%)</td>
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<td>-1 dose: 0.05% (95% CI: 0.07-1.16%)</td>
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<td>Odds ratio for exposure to low grade abnormalities</td>
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<td>-3 doses: 0.08% (95% CI: 0.02-0.30%)</td>
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<td></td>
<td>-2 doses: 0.79% (95% CI: 0.74-0.85%)</td>
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<td></td>
<td>-1 dose: 0.05% (95% CI: 0.03-0.10%)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Decreased exposure odds ratio in older women (0.05; 95% CI: 0.63-1.45)</td>
</tr>
<tr>
<td>Denmark</td>
<td>4xHPV</td>
<td>HPV vaccination status and the effect on risk of CIN2 in Denmark population</td>
<td>Vaccinated - 248,403; Unvaccinated - 151,087</td>
<td>Reduced risk of CIN2 in vaccinated girls of least one dose: 0.12-0.62 (depending on age; lower risks in younger birth cohorts)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>No dose stratification results</td>
</tr>
<tr>
<td>Sweden</td>
<td>4xHPV</td>
<td>Estimation of incidence rates per 100,000 person-years of GWI in 5 different age groups</td>
<td>3 doses - 89,836; 2 doses - 107,338; 1 dose - 115,197; 0 dose - 1,045,192</td>
<td>IRR for exposure to GWI</td>
</tr>
<tr>
<td></td>
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<td>-3 doses: 0.20% (95% CI: 0.17-0.23)</td>
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<td>-2 doses: 0.32% (95% CI: 0.26-0.40)</td>
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<td>-1 dose: 0.04% (95% CI: 0.01-0.16)</td>
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<td></td>
<td></td>
<td></td>
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<td>-2 doses associated with risk reduction</td>
</tr>
</tbody>
</table>

CIN1+CIN2+AIS, cervical intraepithelial neoplasia grade 1 or worse; CIN3+CIN2+CIN1, cervical intraepithelial neoplasia grade 2 or worse; CI, confidence interval.
59 genital warts cases per 100,000 person-years will be prevented with three doses of 4vHPV as compared with two doses. However, whether this observation relates to more serious diseases, such as cervical cancers, is not known. Nevertheless, some of these studies suggest that there may be some protection against HPV vaccine types after having one dose of HPV vaccine, particularly in younger people, and pre-HPV exposure. Studies examining the immunogenicity of a single dose schedule are at an early-stage, with no information available to date on their long-term protective efficacy. The results of these studies will be eagerly anticipated over the next few years.

These studies, including those that reported non-inferior antibody responses have provided evidence for the WHO to change its recommendations to a two-dose HPV vaccine schedule for girls below the age of 15 years old, provided the interval between each dose is at least 6 months [51]. However, it is important to note that for girls above the age of 15 years and immunocompromised individuals (i.e. HIV-infected), the recommendation of a three dose HPV schedule remain unchanged [51]. In addition, individuals whose first and second doses of the HPV vaccine are within 6 months are also recommended to receive a third dose. Although unclear, it is postulated that a third dose would improve immune maturation including antibody class-switching and the induction of long-lived memory responses.

7. Gaps in the knowledge of reduced dose HPV schedules

Despite the recommendation of two-dose HPV schedules, further research is required to assess the clinical effectiveness of this schedule, particularly in low- and middle-income countries, where disease burden is greatest [52]. The evidence for non-inferior immunogenicity of two- vs three-dose HPV schedules in adolescents was inconclusive according to a recent systematic review and meta-analysis of the current literature [53]. It is also important to note that the vaccines are not licensed as a two-dose schedule in all countries.

Whether the duration of antibody response generated by reduced dose schedules is similar to three doses is still not known. A study by Dohson et al. [39] reported lower HPV 18 and HPV 6 antibody levels in girls aged 9–13 years old who received two doses of 4vHPV when compared with girls who received three doses, 24 and 36 months respectively, after receiving the last vaccine dose. Despite that, clinical efficacy against HPV infection remains high in this group of girls. Several clinical studies have also reported the generation of cross-protective antibodies/cross-protection by the 4vHPV or 2vHPV (i.e. antibodies to HPV 31 and 45) when administered with a three-dose schedule [54,55]. A cross-sectional study in Australia demonstrated some cross-protection only on fully vaccinated women 6 years following the introduction of the HPV vaccination programme [56]. So, the question of whether reduced dose HPV schedules generate a similar level and function of cross-protective antibodies as the three-dose HPV schedule is still unknown and needs to be determined. This is important because both 4vHPV and 2vHPV does not protect against all oncogenic HPV types. A study by Kreimer et al. [40] found cross-protection against one year persistent infection with HPV 31, 33 and 45 in women who received three doses of 2vHPV, but not in those who received two doses. This has important implications as these are high-risk HPV genotypes, and a reduced dose schedule may not protect against these HPV types. It is possible that a two-dose HPV schedule may
be less potent against a limited number of HPV types. The new 9vHPV may potentially address the issue of cross-protection, but require more studies to fully understand the long-term protection afforded by this vaccine following reduced doses. Another important consideration is the potential for HPV genotype replacement following HPV vaccination, although to date there is limited data to support this [57]. Moreover, as the virus is relatively stable, it is less likely that type replacement will be seen, but surveillance on the prevalence of non-vaccine types causing diseases is important.

Other issues regarding reduced dose HPV schedules include whether the two-dose vaccination schedule will be extended to people older than 15 years, and whether the implementation of two different dose schedules for different age groups is feasible due to complicated logistics, which would be difficult particularly in developing countries [58].

In most of the non-inferiority and clinical studies on HPV vaccination, the only outcome measure is antibody response. This is not surprising since antibody response is thought to be the primary mechanism of protection against HPV infection. Since there has been no breakdown infection following HPV vaccination from clinical trials, a protective antibody threshold is hard to estimate, although waning antibody levels following HPV vaccination is reported to be much higher than antibody levels generated from natural infection. The antibody response is mediated by a coordinated immune system, and the role of other immune cells in the modulation of the immune response is not known. HPV vaccination is a relatively new area of research and the immune mechanisms of protection are still not fully understood.

8. Novel immunological markers of vaccine-induced protection

The major cell subsets involved in the immune response generated by HPV vaccination are depicted in Fig. 1. While antibody is the primary mechanism of protection against HPV infection, the role of T and B cell populations are also important, particularly for long-term protection. Identification of these markers of protection induced by HPV vaccines will be important in monitoring vaccine effectiveness. The current understanding for generation of robust antibodies from B cells requires help from T helper cells in the form of cytokines, in particular Th2 cytokines (I.e. IL-4, IL-5, IL-6, IL-9, IL-10, IL-13) to assist in maturation of naive B cells into effector cells (plasma cells) and memory B cells [59]. This is likely the case for HPV vaccination for the generation of long-lived HPV-specific B cell memory responses for long-term protection. The evidence for these immune correlates (i.e. cytokines, memory B and T cells) in HPV vaccination is summarized in Table 4.

Importantly, these immune correlates have not been explored following reduced dose HPV vaccination. In an in vivo mouse model study, a single dose of HPV 16 vaccine was able to induce some IFN-γ levels in mice, but three doses of the vaccine significantly boosted the levels 6-fold higher. In comparison, the levels of IL-4 (at much lower levels) were only detectable after three doses but not with one dose [60]. These observations highlight the potential weakened immune response due to reduced doses. Despite this, it is important to note that these studies have evaluated HPV 16 vaccine without adjuvant (which is different from the commercially available HPV vaccines), and that some of these studies are carried out in mouse models, thus the immune response might be different with current vaccines and in humans. Nevertheless, the cytokines induced by HPV vaccination clearly play an important role in the immune response against HPV infection, and whether this parameter is associated with long-term protection remains to be elucidated.

The rationale of implementing a two-dose schedule separated by 6 months is based on effective boosting responses, whereby it is believed that memory B cells elicited by the first dose of vaccine requires at least 4–6 months to mature and differentiate into high affinity memory B cells [61]. This suggests that the second dose should be given at least 4 months after the primary dose to reactivate these memory B cells and induce differentiation into antibody-secreting plasma cells. To date, there is only one published study that has assessed these immune markers in the context of reduced dose HPV schedules. As part of the larger study assessing non-inferior antibody levels between girls receiving two doses and girls/women receiving three doses, Smolen et al.
observed significantly lower HPV 16-specific memory B cells in women compared with girls receiving three doses, suggesting that the age of vaccination may influence memory B cell formation. In contrast, significantly lower numbers of IFN-γ-producing T cells were observed in girls receiving two doses when compared with girls or women receiving three doses. This indicates that the formation of IFN-γ producing T cells may be influenced by the number of vaccine doses [62]. Whether these observations are seen in other studies/populations remains to be determined.

As technology advances, gene analysis is becoming more feasible and affordable as a diagnostic tool. This technology has the potential to elucidate genes and gene pathways that have not yet been characterized in the protective immune response following HPV vaccination. One study has found genes associated with inflammatory/defense response, cytokine, IFN, and cell cycle pathways in individuals administered with the HPV 16 L1 VLP vaccine (highlighted in Table-4) [63]. These genes may help to identify early markers of long-lasting vaccine responses, especially in the context of reduced dose schedules. We will explore some of these immune correlates, including antibody responses in a cohort of Fijian girls (now aged 15–17 years old) who were previously vaccinated with one, two or three doses of 4HPV 5–6 years ago [64]. Results from this study are anticipated to be available in 2016. Currently, this study may be the longest follow-up study for reduced dose HPV vaccination (2x4HPV and 4xHPV were 4 and 3 years, respectively, following the primary vaccination series) [39,42].

9. Conclusion

Preliminary data from reduced dose HPV schedules are encouraging but significant further developments to determining country's specific logistics and cost of the vaccine are major issues. Despite the potential benefits of reduced dose HPV schedules, there are still important research questions to evaluate long-term protection, and the immunological correlates associated with clinical effectiveness. Future studies aimed at addressing these important concepts will be of greatest benefit.

Acknowledgements

The work was part of the New Vaccine Evaluation Project (NVEP), funded by the Fiji Health Sector Support Project and Australian Aid–FTA, the Department of Foreign Affairs and Trade, Australia. QTI is supported by a Melbourne Research Scholarship from The University of Melbourne and a Murdoch Childrens Research Institute top-up scholarship. FMR is a recipient of an NHMRC Early Career Fellowship. This work was also supported in part by the Victorian Government’s Operational Infrastructure Support Program.

Conflict of interest: SMG has received grant support through her institution from CSL Bio, Merck and GlaxoSmithKline and has received funding through her institution to conduct HPV vaccine studies for MSD, GSK (PATRICIA, VIVIANNE) trials. She is also a member of the Merck Global Advisory Board as well as the Merck Scientific Advisory Committee for HPV (unpaid position).

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vaccine programme in Australia: a cross-sectional survey. Lancet Infect Dis

Conclusive evidence for non-inferior immunogenicity of two compared with three
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vaccine programme in Australia: a cross-sectional survey. Lancet Infect Dis

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vaccine programme in Australia: a cross-sectional survey. Lancet Infect Dis
Appendix 3: Participant’s information sheet and consent form

**HREC Project Number:** 2014.5.FNRERC.5.SU  
**Research Project Title:** Evaluation of long term immunity following HPV vaccination  
**Principal Researcher:** Professor Edward Kim Mulholland and Associate Professor Fiona Russell  
**Version Number:** 4  
**Version Date:** 25/11/2014

Thank you for taking the time to read this Participant Information Statement and Consent Form. We would like to invite you to participate in a research project that is explained below. This document is 6 pages long. Please make sure you have all the pages.

**What is an Information Statement?**
These pages tell you about the research project. It explains to you clearly and openly all the steps and procedures of the project. The information is to help you to decide whether or not you would like to take part in the research. Please read this Information Statement carefully.

Before you decide to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

- understand what you have read
- had a chance to ask questions and received satisfactory answers
- consent to taking part in the project.

We will give you a copy of this information and consent form to keep.
1. What is the research project about?
In Fiji, cervical cancer (cancer affecting part of the baby bag) is the second commonest cancer in women. Most cases of cervical cancer are caused by a germ called the human papillomavirus (HPV). There are many types of HPV but types 16 and 18 are the commonest causes of cervical cancer in Fiji and other countries. Two vaccines are currently available to prevent infection against HPV 16 and 18. These vaccines are called Gardasil® and Cervarix®. HPV vaccines are routinely used in many other countries, including Fiji, to prevent cervical cancer. Both vaccines provide excellent protection against HPV types 16 and 18 while Gardasil® also provides protection against HPV types 6 and 11 (the commonest types that cause genital warts).

You may have been offered vaccination with Gardasil® vaccine at school as part of the Ministry of Health’s vaccination campaign in 2008-2009. Three doses are given for protection, but not all girls in this campaign received all three doses. This means that the girls that did not receive three doses of Gardasil® vaccine may not be fully protected against the cancer causing HPV types 16 and 18. In this study we are interested in finding out how well you have responded to the Gardasil® vaccine in 2008/9, and whether you are still likely to be protected.

In 2013 the Fiji Ministry of Health introduced the Cervarix® vaccine to be given to all girls at the end of primary school. If you have not been vaccinated with any HPV vaccine before, you are strongly recommended to receive three doses of Cervarix® vaccine to protect against HPV16 and 18. Although this vaccine protects against most cervical cancer, it does not prevent all cervical cancer so you would still need to get any health checks recommended by the Ministry of Health.

For this study, we will ask you to provide two blood samples and receive Cervarix® vaccine. This vaccine is given to extend your protection, and also allow us work out how well you are protected. If you have not received the recommended three doses of HPV vaccine, we will arrange for you to receive the remaining doses by the end of the study, so that you will be fully vaccinated with three doses of HPV vaccine.

This is a phase II/III clinical trial study that will involve about 200 girls in Suva, including girls who were previously vaccinated with the Gardasil® vaccine.

2. Who is funding this research project?
The Murdoch Childrens Research Institute (MCRI). This study is part of the New Vaccine Evaluation Project (NVEP), a collaborative project between the Fiji Ministry of Health and the MCRI. The NVEP is funded by the Fiji Health Sector Support Project, which is funded by Australian Aid, the Department of Foreign Affairs and Trade, Australia.

3. Why am I being asked to be in this research project?
As part of the Ministry of Health’s vaccination campaign in 2008-2009, you may have been offered the HPV vaccine at school, and may have been vaccinated with three or less doses of the Gardasil® vaccine. In this study we are interested in finding out how well you have responded to this vaccine and whether you are likely to be protected. You will also receive a dose of the Cervarix® vaccine to extend your protection, and allow us to work out how well you are protected.
4. What does participation in this research involve?
If you agree to take part in this study you will need:

- to come for two visits to either Nausori or Valelevu health centre, or the Colonial War Memorial Hospital (CWMH)
  - the first visit will take about one hour
  - the second visit will take place 28 days later and will take about 30 minutes
- to bring your health record number/health care card to the clinic if you have it available (it is still OK to be in the study if you don’t have it)

The study staff will contact you to arrange a suitable time for you to come into the clinic.

On the first visit, we will ask you and your parent/guardian’s permission to be included in the study. We will then ask some questions to see if you are suitable to be included in the study. If you are suitable, we will ask you and your parent/guardian some questions about your family, what your job is, and your education. We will ask you to have a blood test (25 ml, about 4½ teaspoons), and then give you one dose of the Cervarix® vaccine. We will phone you 2-3 days after receiving the vaccine to make sure you are feeling well. In case you are not well after the vaccine, you will be immediately referred to the health centre’s doctor or CWMH for assessment and treatment.

One month later, we will take a second blood test (25 ml, about 4½ teaspoons). The blood tests and vaccine dose will allow us to measure how well you respond to the vaccine and how well you are protected.

To make sure you are fully vaccinated by the end of the study, we will arrange for you to have any additional doses you may need so you don’t miss out on being fully vaccinated. If you have not received any HPV vaccine previous to this study, you will receive two more doses of Cervarix® vaccine according to the recommended schedule (1 and 6 month); if you received one dose of Gardasil® vaccine in 2008/9, you will receive one more dose of Cervarix® vaccine 5 months following the first study visit; if you have received two or three doses of Gardasil® vaccine in 2008/9, you will not receive any more Cervarix® vaccine after the first study visit. All your samples will be kept for 15 years at MCRI, Melbourne, Australia, according to ethics requirements for a clinical trial.

5. Optional consent
You can choose to consent for your blood sample to be kept and used for future ethically approved research, only related to this study. On the consent form:

If you tick the box ‘I do consent’,
Your blood sample and the data collection sheet will be stored at the MCRI, Melbourne Australia indefinitely. The samples will only be identified by their unique study number. It will be only be used in future ethically-approved HPV-related research. You will not be informed if the sample is used in any future studies.

If you tick the box ‘I do not consent’,
Your blood sample will be used for this study only and will be kept for 15 years according to ethics requirements for a clinical trial.
6. What are my alternatives to taking part?
Participation in a research project is voluntary. It is your choice to take part in this research. You do not have to agree if you do not want to.

You can withdraw from the project at any time. You do not need to tell us the reason why you want to stop being in the project. If you leave the project we will use any information already collected unless you tell us not to.

Whatever your decision, you will still get your normal access to medical care.

7. What are the possible benefits for me and other people in the future?
You will be fully vaccinated with the HPV vaccine by the end of the study, which will give you the best possible protection against infection caused by HPV 16 and 18. You will be reimbursed for your time and travel expenses. This study will provide evidence to the key question as to whether less than three-dose HPV vaccination will protect against the major HPV types, and will eventually influence policy making of the HPV vaccination globally.

8. What are the possible risks, side-effects, discomforts and/or inconveniences?
The HPV vaccine is not recommended for pregnant women because it is not known if it will cause harm to the baby. If you are pregnant you should not receive the HPV vaccine. You are advised to see a doctor at your nearest health centre.

There are no major risks related with a blood test. It is possible you may feel some discomfort, such as a sting when the needle is put in your arm to take the blood. If required, we will use a cream to numb the skin before the blood is taken. It is possible there may be some bruising, swelling or bleeding where the needle enters the skin for the blood test. Some people can feel a little light-headed when blood is taken so we will ask you to seat or lie down. We will observe you for 30 minutes after the vaccination. The nurses are trained to recognise and manage these cases, and doctors will be referred to if needed.

The HPV vaccine is generally very safe. Serious problems caused by the vaccine are rare. At least 1 in 10 people who have the vaccine have mild side effects for a day or so. These include some pain, swelling and redness around the site of the injection, headache, aching muscles and tiredness. Occasionally, there is some bleeding or itchiness around the area of the injection. Less common side effects include slightly raised temperature, sickness (vomiting), dizziness, diarrhoea and muscle aches. More rarely, people can develop hives (urticaria). Extremely rarely, a severe allergic reaction may occur. If this severe reaction were to occur, it would develop within the 30 minutes that we will monitor you in the clinic. In the extremely unlikely event that this occurs, medication and treatment will be immediately available in the clinic.

9. What will be done to make sure my information is confidential?
Each participant will be given a study number. The health information we collect about you will be kept private, unless for legal reasons we need to disclose it. Information can only be accessed by the Fiji National Research Ethics Review Committee and the study team. All study forms will be kept in a locked study office. Information will also be kept on a password-protected computer with access only by study staff. Your name will not be entered into the database. Information will be stored for 15 years.
The results of the study will be published in scientific journals, and presented at conferences. The results will not identify you.

Information collected for this study will be sent to the research team at the MCRI, Melbourne. This information will not contain your name.

10. Will I be informed of the results when the research project is finished?  
   We will send you a letter about the study’s results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: Tupou Ratu  
Contact telephone: 679 3317670

If you have any concerns and/or complaints about the project, the way it is being conducted or your rights as a research participant, and would like to speak to someone independent of the project, please contact:

Mere Y Delai  
FNRERC Secretariat  
Health Research Unit  
Ministry of Health  
Suva, Fiji  
Ph: (679) 32157-70  
Email: mere.delai@govnet.gov.fj
CONSENT FORM

HREC Project Number: 2014.5.FNRERC.5.SU
Research Project Title: Evaluation of Immune Memory following HPV vaccination
Version Number: 4 Version Date: 25/11/2014

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my involvement in this project.
- I voluntarily consent to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that my samples will be kept for 15 years at MCRI, Melbourne, Australia.
- I understand that this project has been approved by the Fiji National Health Research Committee (NHRC) and Fiji National Research Ethics Review Committee (FNRERC)
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT

☐ I do ☐ I do not consent to the storage of my blood sample to be kept indefinitely for use in future ethically approved research related to this study only

Participant Name ___________________________ Participant Signature ___________________________ Date ______

Name of Witness to Participant’s Signature ___________________________ Witness Signature ___________________________ Date ______

Declaration by researcher: I have explained the project to the participant who has signed above, and believe that they understand the purpose, extent and possible risks of their involvement in this project.

Research Team Member Name ___________________________ Research Team Member Signature ___________________________ Date ______

Note: All parties signing the Consent Form must date their own signature.
Appendix 4: Parent/guardian information sheet and consent form

HREC Project Number: 2014.5.FNRERC.5.SU
Research Project Title: Evaluation of long term immunity following HPV vaccination
Principal Researcher: Professor Edward Kim Mulholland and Associate Professor Fiona Russell
Version Number: 7

Thank you for taking the time to read this Parent/Guardian Information Statement and Consent Form. We would like to invite your child to participate in a research project that is explained below. This document is 6 pages long. Please make sure you have all the pages.

What is an Information Statement?
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Before you decide if you want your child to take part or not, you can ask us any questions you have about the project. You may want to talk about the project with your family, friends or health care worker.

If you would like your child to take part in the research project, please sign the consent form at the end of this information statement. By signing the consent form you are telling us that you:

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- had a chance to ask questions and received satisfactory answers
- consent to your child taking part in the project.

We will give you a copy of this information and consent form to keep.
1. What is the research project about?
In Fiji, cervical cancer (cancer affecting part of the baby bag) is the second commonest cancer in women. Most cases of cervical cancer are caused by a germ called the human papillomavirus (HPV). There are many types of HPV but types 16 and 18 are the commonest causes of cervical cancer in Fiji and other countries. Two vaccines are currently available to prevent infection against HPV 16 and 18. These vaccines are called Gardasil® and Cervarix®. HPV vaccines are routinely used in many other countries, including Fiji, to prevent cervical cancer. Both vaccines provide excellent protection against HPV types 16 and 18 while Gardasil® also provides protection against HPV types 6 and 11 (the commonest types that cause genital warts).

Your child may have been offered vaccination with Gardasil® vaccine at school as part of the Ministry of Health’s vaccination campaign in 2008-2009. Three doses are given for protection, but not all girls in this campaign received all three doses. This means that the girls that did not receive three doses of Gardasil® vaccine may not be fully protected against the cancer causing HPV types 16 and 18. In this study we are interested in finding out how well your child has responded to the Gardasil® vaccine, and whether they are still likely to be protected.

In 2013 the Fiji Ministry of Health introduced the Cervarix® vaccine to be given to all girls at the end of primary school. If your child has not been vaccinated with any HPV vaccine before, she is strongly recommended to receive three doses of Cervarix® vaccine to protect against HPV16 and 18. Although this vaccine protects against most cervical cancer, it does not prevent all cervical cancer so your child would still need to get any health checks recommended by the Ministry of Health.

For this study, we will ask your child to provide two blood samples and receive Cervarix® vaccine. This vaccine is given to extend their protection, and also allow us work out how well your child is protected. If your child has not received the recommended three doses of HPV vaccine, we will arrange for her to receive the remaining doses by the end of the study, so that she will be fully vaccinated with three doses of HPV vaccine.

This is a phase II/III clinical trial study that will involve about 200 girls in Suva, including girls who were previously vaccinated with the Gardasil® vaccine.

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3. Why is my child being asked to be in this research project?
As part of the Ministry of Health’s vaccination campaign in 2008-2009, your child may have been offered the HPV vaccine at school, and may have been vaccinated with three or less doses of the Gardasil® vaccine. In this study we are interested in finding out how well your child has responded to this vaccine and whether she is likely to be protected. Your child will also receive a dose of the Cervarix® vaccine to extend her protection, and allow us to work out how well she is protected.
4. What does participation in this research involve?
If you agree for your child to take part in this study you will need:

- to bring your child to two visits to either Nausori or Valelevu health centre, or the Colonial War Memorial Hospital (CWMH)
  - the first visit will take about one hour
  - the second visit will take place 28 days later and will take about 30 minutes
- to bring your child’s health record number/health care card to the clinic if you have it available (it is still OK to be in the study if you don’t have it)

The study staff will contact you to arrange a suitable time for your child to come into the clinic.

On the first visit, we will ask you and your child’s permission to be included in the study. We will then ask some questions to see if your child is suitable to be included in the study. If she is suitable, we will ask both of you some questions about your family, what your job is, and your education. We will ask your child to have a blood test (25 ml, about 4½ teaspoons), and then give her one dose of the Cervarix® vaccine. We will phone you 2-3 days after receiving the vaccine to make sure your child is well. In case your child is not well after the vaccine, she will be immediately referred to the health centre’s doctor or CWMH for assessment and treatment.

One month later, we will take a second blood test (25 ml, about 4½ teaspoons). The blood tests and vaccine dose will allow us to measure how well your child responds to the vaccine and how well your child is protected.

To make sure your child is fully vaccinated by the end of the study, we will arrange for your child to have any additional doses she may need so she doesn’t miss out on being fully vaccinated. If your child did not receive any HPV vaccine previous to this study, she will receive two more doses of Cervarix® vaccine according to the recommended schedule (1 and 6 month); if your child received one dose of Gardasil® vaccine in 2008/9, she will receive one more dose of Cervarix® vaccine 5 months following the first study visit; if your child have received two or three doses of Gardasil® vaccine in 2008/9, she will not receive any more Cervarix® vaccine after the first study visit. All your child samples will be kept for 15 years at MCRI, Melbourne, Australia, according to ethics requirements for a clinical trial.

5. Optional consent
You can choose to consent for your child’s blood sample to be kept and used for future ethically approved research, only related to this study. On the consent form:

If you tick the box ‘I do consent’,
Your child’s blood sample and the data collection sheet will be stored at the MCRI, Melbourne Australia indefinitely. The samples will only be identified by their unique study number. It will be only be used in future ethically-approved HPV-related research. You will not be informed if the sample is used in any future studies.

If you tick the box ‘I do not consent’,
Your child’s blood sample will be used for this study only and will be kept for 15 years according to ethics requirements for a clinical trial.
6. **What are my child’s alternatives to taking part?**
Participation in a research project is voluntary. It is your choice to let your child take part in this research. You do not have to agree if you do not want to. You or your child can withdraw from the project at any time. You do not need to tell us the reason why you or your child wants to stop being in the project. If your child leaves the project we will use any information already collected unless you tell us not to.

Whatever your decision, your child will still get normal access to medical care.

7. **What are the possible benefits for my child and other people in the future?**
Your child will be fully vaccinated with the HPV vaccine by the end of the study, which will give your child the best possible protection against infection caused by HPV 16 and 18. You will be reimbursed for your time and travel expenses. This study will provide evidence to the key question as to whether less than three-dose HPV vaccination will protect against the major HPV types, and will eventually influence policy making of the HPV vaccination globally.

8. **What are the possible risks, side-effects, discomforts and/or inconveniences?**
The HPV vaccine is not recommended for pregnant women because it is not known if it will cause harm to the baby. If your child is pregnant, she should not receive the HPV vaccine. Your child is advised to see a doctor at your nearest health centre.

There are no major risks related with a blood test. It is possible your child may feel some discomfort, such as a sting when the needle is put in their arm to take the blood. If required, we will use a cream to numb the skin before the blood is taken. It is possible there may be some bruising, swelling or bleeding where the needle enters the skin for the blood test. Some people can feel a little light-headed when blood is taken so we will ask your child to seat or lie down. We will observe your child for 30 minutes after the vaccination, just in case your child faints or has a severe allergic reaction. The nurses are trained to recognise and manage your child in these cases, and doctors will be referred to if needed.

The HPV vaccine is generally very safe. Serious problems caused by the vaccine are rare. At least 1 in 10 people who have the vaccine have mild side effects for a day or so. These include some pain, swelling and redness around the site of the injection, headache, aching muscles and tiredness. Occasionally, there is some bleeding or itchiness around the area of the injection. Less common side effects include slightly raised temperature, sickness (vomiting), dizziness, diarrhoea and muscle aches. More rarely, people can develop hives (urticaria). Extremely rarely, a severe allergic reaction may occur. If this severe reaction were to occur, it would develop within the 30 minutes that we will monitor your child in the clinic. In the extremely unlikely event that this occurs, medication and treatment will be immediately available in the clinic.

9. **What will be done to make sure my child’s information is confidential?**
Each participant will be given a study number. The health information we collect about your child will be kept private, unless for legal reasons we need to disclose it. Information can only be accessed by the Fiji National Research Ethics Review Committee and the study team. All study forms will be kept in a locked study office. Information will also be kept on a password-protected computer with access only by study staff. Your child’s name will not be entered into the database. Information will be stored for 15 years.
The results of the study will be published in scientific journals, and presented at conferences. The results will not identify your child.

Information collected for this study will be sent to the research team at the MCRI, Melbourne. This information will not contain your child’s name.

10. Will we be informed of the results when the research project is finished?
We will send you a letter about the study’s results.

If you would like more information about the project or if you need to speak to a member of the research team in an emergency please contact:

Name: Tupou Ratu
Contact telephone: 679 3317670

If you have any concerns and/or complaints about the project, the way it is being conducted or your child’s rights as a research participant, and would like to speak to someone independent of the project, please contact:

Mere Y Delai
FNRERC Secretariat
Health Research Unit
Ministry of Health
Suva, Fiji
Ph: (679) 32157-70
Email: mere.delai@govnet.gov.fj
CONSENT FORM

HREC Project Number: 2014.5.FNRERC.5.SU
Research Project Title: Evaluation of Immune Memory following HPV vaccination
Version Number: 7  Version Date: 25/11/2014

- I have read, or had read to me in my first language, the information statement version listed above and I understand its contents.
- I believe I understand the purpose, extent and possible risks of my child’s involvement in this project.
- I voluntarily consent for my child to take part in this research project.
- I have had an opportunity to ask questions and I am satisfied with the answers I have received.
- I understand that my child’s samples will be kept for 15 years at MCRI, Melbourne, Australia.
- I understand that this project has been approved by the Fiji National Health Research Committee (NHRC) and Fiji National Research Ethics Review Committee (FNRERC).
- I understand I will receive a copy of this Information Statement and Consent Form.

OPTIONAL CONSENT

<table>
<thead>
<tr>
<th>I do</th>
<th>I do not</th>
<th>consent to the storage of my child’s blood sample to be kept indefinitely for use in future ethically approved research related to this study only</th>
</tr>
</thead>
</table>

Child’s Name

Parent/Guardian Name  Parent/Guardian Signature  Date

Name of Witness to Parent/Guardian’s Signature  Witness Signature  Date

Declaration by researcher: I have explained the project to the parent/guardian who has signed above, and believe that they understand the purpose, extent and possible risks of their child’s involvement in this project.

Research Team Member Name  Research Team Member Signature  Date

Note: All parties signing the Consent Form must date their own signature.
Appendix 5: Questionnaires for Day 0, Day 2-3 and Day 28

STUDY VISIT DAY 0

<table>
<thead>
<tr>
<th>ID Number</th>
<th>FHPV- [<em><strong>][</strong></em>][___]</th>
<th>DATE □□/□□/□□□□ (Day/Month/Year)</th>
<th>Weight __ __ __ . __kg</th>
<th>Height __ __ __ . __ cm</th>
<th>DOB □□/□□/□□□□ (Day/Month/Year)</th>
<th>Ethnicity? i-Taukei □ Indo Fijian□ Other□</th>
</tr>
</thead>
</table>

Questions to ask the parents

**MOTHER**

*Does the girl have a:*  
Mother □ Legal guardian □ None □

*What is their highest level of education?*  
□None □Primary school not complete □Primary school □Secondary school □Secondary school not complete □Diploma □Degree □Other, specify:________

*Are they employed?* □Yes □No

If yes, which of the following best describes your employment: (read out categories)
□Self-employed (own business) □Private sector employed □Government □Factory worker □Commercial farmer □Other, specify:________________

**FATHER**

*Does the girl have a:*  
Father □ Legal guardian □ None □

*What is their highest level of education?*  
□None □Primary school not complete □Primary school □Secondary school □Secondary school not complete □Diploma □Degree □Other, specify:________

*Are they employed?* □Yes □No

If yes, which of the following best describes your employment: (read out categories)
□Self-employed (own business) □Private sector employed □Government □Factory worker □Commercial farmer □Other, specify:________________
<table>
<thead>
<tr>
<th>Question</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>What are the outer walls of your house made of?</td>
<td>□ Iron  □ Wood  □ Concrete  □ Bure</td>
</tr>
<tr>
<td>What type of cooking fuel does your household use?</td>
<td>□ Kerosene  □ Wood  □ LPG (gas)  □ Electricity</td>
</tr>
<tr>
<td>Does your household have the following items?</td>
<td>Car or truck □ Yes □ No  Electricity □ Yes □ No □ Shared</td>
</tr>
<tr>
<td>Fridge □ Yes □ No  Washing machine □ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>Computer □ Yes □ No  Television or video player □ Yes □ No</td>
<td></td>
</tr>
<tr>
<td>How many people live in your house? ________</td>
<td></td>
</tr>
<tr>
<td>Confidential questions to ask the girl in private</td>
<td></td>
</tr>
<tr>
<td>How many siblings do you have? ________</td>
<td>What is your number in the birth order of your family? ________</td>
</tr>
<tr>
<td>Do you have a boyfriend or are you married? □ Yes □ No</td>
<td>Do you have any children? □ Yes □ No</td>
</tr>
<tr>
<td>Does anyone in your household smoke? □ Yes □ No</td>
<td>Have you smoked 100 cigarettes or more in your entire life? □ Yes □ No</td>
</tr>
<tr>
<td>Does anyone in your household drink cava? □ Yes □ No</td>
<td>How often do you drink cava? □ Never □ Less than once per week □ Once per week or more</td>
</tr>
<tr>
<td>Does anyone in your household drink alcohol? □ Yes □ No</td>
<td>How often do you have a drink containing alcohol? □ Never □ Less than once per week □ Once per week or more</td>
</tr>
<tr>
<td>Could you be pregnant? □ Yes □ No □ Possibly □</td>
<td>(If Yes or Possibly, stop the visit as the girl is not eligible)</td>
</tr>
<tr>
<td>Axillary Temperature □ □ °C</td>
<td>Is the study participant well today? □ Yes □ No</td>
</tr>
<tr>
<td>If No, record which symptoms the participant is experiencing below.</td>
<td></td>
</tr>
<tr>
<td>Faint / lightheaded □□   □□   □□ (If yes, rate reaction) 1.□   2.□   3.□</td>
<td>Yes □ No □ Unknown □</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>Headache</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Fatigue</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Nausea</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Vomiting</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Muscle or joint pain</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Lack of appetite</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>(If yes, rate reaction) 1.□ 2.□ 3.□</td>
</tr>
<tr>
<td>Other (please specify)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Age of first menses?</td>
<td>□□ years □ Not applicable</td>
</tr>
<tr>
<td>The total number of HPV vaccine doses received in 2008/9?</td>
<td>By parent/child recall 1□ 2□ 3□ or 0□</td>
</tr>
<tr>
<td></td>
<td>By school vaccination list 1□ 2□ 3□ or 0□</td>
</tr>
<tr>
<td>If known, record the dates the vaccines were given.</td>
<td>HPV 1 □□/□□/□□□□ (Dy/Mon/Year) by parent/child recall □ OR school list □</td>
</tr>
<tr>
<td></td>
<td>HPV 2 □□/□□/□□□□ (Dy/Mon/Year) by parent/child recall □ OR school list □</td>
</tr>
<tr>
<td></td>
<td>HPV 3 □□/□□/□□□□ (Dy/Mon/Year) by parent/child recall □ OR school list □</td>
</tr>
<tr>
<td>Has the participant had a reaction following any vaccine?</td>
<td>Yes □ No □ Unknown □</td>
</tr>
</tbody>
</table>

*If Yes, record the reaction below. Stop the visit and discuss with the study PI before proceeding.*
<table>
<thead>
<tr>
<th>Question</th>
<th>Yes □</th>
<th>No □</th>
</tr>
</thead>
<tbody>
<tr>
<td>Was a 25 mL blood sample obtained?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>If, no record the volume obtained</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[<em><strong>].[</strong></em>] mls</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Was the HPV vaccine given?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Were there any reactions during 30 minutes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>post vaccination?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[<em><strong>].[</strong></em>] mls</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*If Yes, contact the study coordinator immediately and document details:*

<table>
<thead>
<tr>
<th>Nurse’s name:</th>
<th>Monitor’s name:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature:</td>
<td>Signature:</td>
</tr>
<tr>
<td>Date:</td>
<td>Date:</td>
</tr>
</tbody>
</table>
### STUDY VISIT DAY 2-3 post vaccination

<table>
<thead>
<tr>
<th>ID Number</th>
<th>FHPV- [<strong>] [</strong>] [__]</th>
<th>DATE [<strong>]/[</strong>]/[__] (Day/Month/Year)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Did the participant have any symptoms post vaccination?</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>If yes, record and rate the reaction.</em></td>
<td><em>1 mild. 2 moderate. 3 severe.</em></td>
<td></td>
</tr>
<tr>
<td><strong>Felt hot</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If temperature taken record here) ☐ ☐ °C</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Faint / lightheaded</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Headache</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fatigue</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Nausea</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vomiting</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diarrhoea</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Muscle or joint pain</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lack of appetite</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Upper respiratory tract infection</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Redness, swelling or pain at injection site</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
<tr>
<td><em>(If yes, rate reaction) 1. ☐ 2. ☐ 3. ☐</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Convulsion</strong></td>
<td><strong>Yes</strong> ☐ <strong>No</strong> ☐ <strong>Unknown</strong> ☐</td>
<td></td>
</tr>
</tbody>
</table>
### Rash
(If yes, rate reaction) 1. □  2. □  3. □

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
<th>Unknown □</th>
</tr>
</thead>
</table>

Other (please specify) ________________

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
</table>

Was the participant hospitalisation or sort medical attention post vaccination?.

(If Yes, please specify below).

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
<th>Unknown □</th>
</tr>
</thead>
</table>

Was any medicine taken for any of the above symptoms?

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
<th>Unknown □</th>
</tr>
</thead>
</table>

If yes, record the name of medicine taken and the start end dates.

Medication_________________________

Start date: ☐☐/☐☐/☐☐☐☐   End date: ☐☐/☐☐/☐☐☐☐

(Day/Month/Year)   (Day/Month/Year)

Is the condition resolved?

<table>
<thead>
<tr>
<th>Yes □</th>
<th>No □</th>
<th>Unknown □</th>
</tr>
</thead>
</table>

If no, call the participant the following day and complete another post vaccination form.

---

**Nurse’s name:**

**Monitor’s name:**

**Signature:**

**Signature:**

**Date:**

**Date:**
**STUDY VISIT DAY 28**

<table>
<thead>
<tr>
<th>Was a 25 mL blood sample obtained?</th>
<th>Yes□</th>
<th>No□</th>
</tr>
</thead>
</table>

If no, record the volume obtained  
[___].[___]mls

<table>
<thead>
<tr>
<th>Did the participant have any further reactions post vaccination?</th>
<th>Yes□</th>
<th>No□</th>
</tr>
</thead>
</table>

*If yes, record reaction, end date or unresolved*

| Reaction:___________________ | ☐☐/☐☐/☐☐☐☐ | □ |
| End date (Day/Month/Year) |      |    |

Unresolved

| Reaction:___________________ | ☐☐/☐☐/☐☐☐☐ | □ |
| End date (Day/Month/Year) |      |    |

Unresolved

<table>
<thead>
<tr>
<th>Is the participant due for a dose of HPV vaccine?</th>
<th>Yes□</th>
<th>No□</th>
</tr>
</thead>
</table>

*(Only applicable for groups 0 and 1)*

If yes, complete the table below.
If no, sign and date the end of the form.

<table>
<thead>
<tr>
<th>Is the study participant well today?</th>
<th>Yes□</th>
<th>No□</th>
</tr>
</thead>
</table>

If No, record which symptoms the participant is experiencing below.

<table>
<thead>
<tr>
<th>Faint / lightheaded</th>
<th>Yes□</th>
<th>No□</th>
<th>Unknown□</th>
</tr>
</thead>
</table>

*(If yes, rate reaction)* 1.□ 2.□ 3.□

<table>
<thead>
<tr>
<th>Headache</th>
<th>Yes□</th>
<th>No□</th>
<th>Unknown□</th>
</tr>
</thead>
</table>

*(If yes, rate reaction)* 1.□ 2.□ 3.□

<table>
<thead>
<tr>
<th>Fatigue</th>
<th>Yes□</th>
<th>No□</th>
<th>Unknown□</th>
</tr>
</thead>
</table>

*(If yes, rate reaction)* 1.□ 2.□ 3.□

<table>
<thead>
<tr>
<th>Nausea</th>
<th>Yes□</th>
<th>No□</th>
<th>Unknown□</th>
</tr>
</thead>
</table>

*(If yes, rate reaction)* 1.□ 2.□ 3.□
### Vomiting

*(If yes, rate reaction)*

<table>
<thead>
<tr>
<th>1 □</th>
<th>2 □</th>
<th>3 □</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Unknown□</strong></th>
</tr>
</thead>
</table>

### Diarrhoea

*(If yes, rate reaction)*

<table>
<thead>
<tr>
<th>1 □</th>
<th>2 □</th>
<th>3 □</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Unknown□</strong></th>
</tr>
</thead>
</table>

### Muscle or joint pain

*(If yes, rate reaction)*

<table>
<thead>
<tr>
<th>1 □</th>
<th>2 □</th>
<th>3 □</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Unknown□</strong></th>
</tr>
</thead>
</table>

### Lack of appetite

*(If yes, rate reaction)*

<table>
<thead>
<tr>
<th>1 □</th>
<th>2 □</th>
<th>3 □</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Unknown□</strong></th>
</tr>
</thead>
</table>

### Upper respiratory tract infection

*(If yes, rate reaction)*

<table>
<thead>
<tr>
<th>1 □</th>
<th>2 □</th>
<th>3 □</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Unknown□</strong></th>
</tr>
</thead>
</table>

### Other (please specify)

______________________________

---

<table>
<thead>
<tr>
<th>Has the participant had a reaction following any vaccine?</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
</tr>
</thead>
</table>

*If Yes, record the reaction below. Stop the visit and discuss with the study PI before proceeding.*

<table>
<thead>
<tr>
<th>Could the participant be pregnant?</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
<th><strong>Possibly□</strong></th>
</tr>
</thead>
</table>

*(If Yes or Possibly, stop the visit as the girl is not eligible)*

<table>
<thead>
<tr>
<th>Was the HPV vaccine given?</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
</tr>
</thead>
</table>

*(Only applicable for group 0 and 1)*

<table>
<thead>
<tr>
<th>Were there any reactions during 30 minutes post vaccination?</th>
<th><strong>Yes□</strong></th>
<th><strong>No□</strong></th>
</tr>
</thead>
</table>

*If Yes, contact the study coordinator immediately and document details:*

<table>
<thead>
<tr>
<th>If the participant is in group 0 make another appointment for 5 months’ time, to receive the third dose of HPV.</th>
<th><strong>HPV due DATE</strong> □□/□□/□□□□  <em>(Day/Month/Year)</em></th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Nurse’s name:</th>
<th>Monitor’s name:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature:</td>
<td>Signature:</td>
</tr>
<tr>
<td>Date:</td>
<td>Date:</td>
</tr>
</tbody>
</table>
Appendix 6: Optimisation of dose and time for HPV-specific T cell assay

Optimisation of dose and time of HPV16 peptide stimulation: Three doses (1 µg/mL, 2.5 µg/mL and 5 µg/mL) of pooled HPV16 peptides were stimulated at two time points (2 days; 2D and 6 days; 6D). Low cell viability and no IFNγ producing cells were observed for 5 µg/mL for both time points.
Appendix 7: Flow cytometry gating strategy

1. Gating on live cells

2. Gating on lymphocytes

3. Exclusion of aggregates

4. Gating on Total IFNγ+ cells

5. Gating on CD4+ and CD8+ cells
6. Gating of CD4+45RO+IFNγ+ and CD4+45RO+IFNγ− cells

7. Gating of CD8+45RO+IFNγ+ and CD8+45RO+IFNγ− cells
Appendix 8: Number of HPV-specific IFNγ producing cells to PHA and TT for each dosage groups before and after a dose of 2vHPV

Total mean number of HPV-specific IFNγ producing cells per 10⁶ PBMCs to phytohaemagglutinin (PHA; 5 µg/mL) and tetanus toxoid (TT; 10 µg/mL) pre- (solid bar) and one month post-2vHPV (stripes bar) for all dosage groups. PBMCs were stimulated with PHA or TT for two days and IFNγ producing cells were measured by ELISPOT. Each bar represent n=15, except two-dose group, n=14. Red bars represent three-dose group (3D); blue bars represent two-dose group (2D); purple bars represent one-dose group (1D) and green bars represent zero-dose group (0D). Bars represent mean ± standard error of mean (SEM).
Appendix 9: Fold-change of IFNγ+ producing cells stratified into CD4+ and CD8+ cells against HPV16 and 18

Fold-change of IFNγ+ producing cells (consisting of both CD45RO+ and CD45RO- cells) stratified into CD4+ cells (CD4+IFNγ+) cells against HPV16 (A) and HPV18 (B) and CD8+ (CD8+IFNγ+) against HPV16 (C) and HPV18 (D), pre- (six years after last vaccine dose) and post-2vHPV. Red bars represent three-dose group, n= 7 (pre), n= 8 (post); blue bars represent two-dose group, n= 9 (both pre and post); purple bars represent one-dose group, n= 8 (pre), n= 7 (post) and green bars represent zero-dose group, n=11 (pre), n= 10 (post). Bars represent mean ± SEM.
Appendix 10: Fold-change in PBMCs cytokine responses against HPV16 and 18

**HPV16**

- IFNγ: p = 0.0027
- IL-2: p = 0.0001
- TNFα: p = 0.0001
- IL-10: p = 0.0001
- IL-5: p = 0.0001

**HPV18**

- IFNγ: p = 0.0021
- IL-2: p = 0.0027
- TNFα: p = 0.0054
- IL-10: p = 0.0054
- IL-5: p = 0.0054

4vHPV dosage groups pre/post 2vHPV
Fold-change in PBMCs cytokine responses against HPV16 and HPV18 pre- (six years after last vaccine dose) and post-2vHPV. Red bars represent three-dose group, n=15; blue bars represent two-dose group, n=14; purple bars represent one-dose group, n=15 and green bars represent zero-dose group, n=15. Bars represent mean ± standard error of mean (SEM).