HPV vaccine-induced cross-neutralising antibodies target complex epitopes on the major capsid protein

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A thesis presented for the degree of

Doctor of Philosophy

Declaration of Originality

I, Sara Louise Bissett, declare that the work presented in this thesis is my own and any additional sources of information have been duly acknowledged and referenced.

Copyright Declaration

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Abstract

The current human papillomavirus (HPV) vaccines consist of major capsid protein (L1) virus-like particles (VLP) and target the two most prevalent oncogenic genotypes, HPV16 and HPV18. Prophylactic HPV vaccination is highly effective at preventing HPV16 and HPV18 infection and associated cervical disease, with type-specific neutralising antibodies thought to be the immune mediators of vaccine type protection. A degree of vaccine-induced cross-protection has also been demonstrated against genetically-related genotypes in the Alpha-7 (HPV18-like) and Alpha-9 (HPV16-like) species groups and although the underlying immune mechanism is uncertain, cross-protection is coincident with the detection of cross-neutralising antibodies. The aim of this thesis was to delineate the HPV L1 domains that are recognised by inter-genotype cross-neutralising antibodies. The formal analysis of the vaccine-induced A9 L1 antibody response demonstrated that cross-neutralising antibodies were a minor component of the total HPV16 antibody response and comprised antibody specificities which recognised single and multiple non-vaccine genotypes. The bioinformatic examination of A9 capsid amino acid sequences demonstrated that the L1L2 pseudovirions (PsV) used to measure cross-neutralising responses were generally representative of available contemporary sequences. The potential impact of amino acid variation within the L1 capsid protein was investigated for HPV31 and found differences in cross-neutralising antibody recognition of the L1 variants; however, this was of a low magnitude. L1 crystallographic homology models predicted structural changes in the loops between HPV16 and the non-vaccine A9 genotypes, informing the design and generation of chimeric PsV with inter-genotype loop swaps. These chimeric PsV demonstrated that cross-neutralising antibodies recognise DE and FG loop amino acid residues within close proximity to each other on the capsid surface. These data contribute to our understanding of the antigenicity of the L1 major capsid protein of HPV by identifying the L1 regions recognised by vaccine-induced cross-neutralising antibodies. Such specificities may play a critical role in vaccine-induced cross-protection.

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Abbreviations

Å	Angstrom
AAHS	Amorphous aluminium hydroyxphosphate sulphate
AIN	Anal intraepithelial neoplasia
AIS	Adenocarcinoma *in situ*
AS04	Adjuvant system 04
ATP	According-to-protocol
ATP-E	According-to-protocol for efficacy
BCR	B cell receptor
BPV1	Bovine papillomavirus type 1
BSA	Bovine serum albumin
cDMEM	Complete Dulbecco's modified Eagle's medium
CIN	Cervical intraepithelial neoplasia
cLIA	Competitive Luminex immunoassay
CI	Confidence interval
COPV	Canine oral papillomavirus
CRPV	Cottontail rabbit papillomavirus
cSf-900	Complete Sf-900
CVC	Cervicovaginal challenge
dH_20	Distilled water
DMEM	Dulbecco's modified Eagle's medium
DTT	Dithiothreitol
E	Early
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FAP	Final aqueous preparation
FBS	Foetal bovine serum
FDA	Food and drug administration
FUTURE	Females united to unilaterally reduce endo/ectocervical disease
GFP	Green fluorescent protein
GMQE	Global model quality estimation
GMT	Geometric mean titre
HA	Haemagglutinin
HAI	Haemagglutination inhibition assay
HBV	Hepatitis B virus
HBsAg	Hepatitis B surface antigen
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
HSPG	Heparin sulphate proteoglycans
HTLV	Human T cell lymphotropic virus
IARC	International Agency for Research on Cancer
ICTV	International Committee on Taxonomy of Viruses
IQR	Interquartile range
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Late
LBC	Liquid-based cytology
LCR	Long control region
LLETZ	Large loop excision of the transformation zone
LLPC	Long-lived plasma cells
LPS	Lipopolysaccharide

MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MPL	Monophosphoryl lipid A
mRNA	Messenger RNA
NCBI	National centre for biotechnology information
NCI	National cancer institute
nDMEM	Neutralisation Dulbecco's modified Eagle's medium
NIBSC	National institute of biological standards and control
ORF	Open reading frame
PATRICIA	Papilloma trial against cancer in young adults
PBS	Phosphate-buffered saline
PDB	Protein data bank
PI	Particle-to-infectivity
PsV	Pseudovirus
PV	Papillomavirus
REC	Research ethics committee
RMS	Root mean squared
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris-buffered saline
$TCID_{50}$	Tissue culture infectious dose 50%
TLR	Toll-like receptor
VIN	Vulva intraepithelial neoplasia
VIVIANE	Human papillomavirus: vaccine immunogenicity and efficacy
VLP	Virus-like particle
WGS	Whole genome sequence

1. Introduction

1.1 Oncoviruses

Viruses have an aetiological role in the development of approximately 12% of human cancers (Parkin, 2006). The highest percentage of virus-associated cancer cases (5.2%) are attributable to infection with human papillomavirus (HPV), followed by hepatitis B virus (HBV) and hepatitis C virus (HCV) (4.9% accumulatively), Epstein-Barr virus (EBV) (1-2%), Kaposi's sarcoma-associated herpesvirus (KSHV) (1%), and human T cell lymphotropic virus (HTLV) (0.5%) (Parkin, 2006). The recognition that infectious agents are the principal cause of certain human cancers, such as cancer of the cervix (HPV) and liver (HBV), offers a unique opportunity whereby preventing the initial infection would also protect against the development of the associated cancer. A prophylactic HBV vaccine for the prevention of hepatocellular carcinoma (HCC) was first licensed in 1981-82 and subsequent generations of the vaccine have proven highly effective at reducing the burden of acute HBV and subsequent chronic carriage, the main predictor for the development of HBV-associated HCC (Schiller and Lowy, 2010). Since 2006, two prophylactic HPV vaccines have been licensed in over 100 countries worldwide with the primary aim of decreasing the burden of cervical cancer associated with infection by the two most prevalent HPV genotypes (Markowitz et al., 2012).

1.2 Papillomavirus phylogeny and classification

Papillomaviruses (PV) were originally classified in the *Papovaviridae* virus family alongside polyomaviruses (e.g. SV40 and JC virus) due to both viruses having a non-enveloped icosahedral capsid containing a double-stranded DNA genome as observed by electron microscopic analysis (Klug and Finch, 1965). The advancement of molecular technology demonstrated that PV and polyomaviruses have different genome sizes and organisations with the only sequence homology between the two viruses limited to a single protein in each virus, the E1 of PV (a region of *ca.* 230 amino acids) and the T-antigen of polyomavirus (Clertant and Seif, 1984).

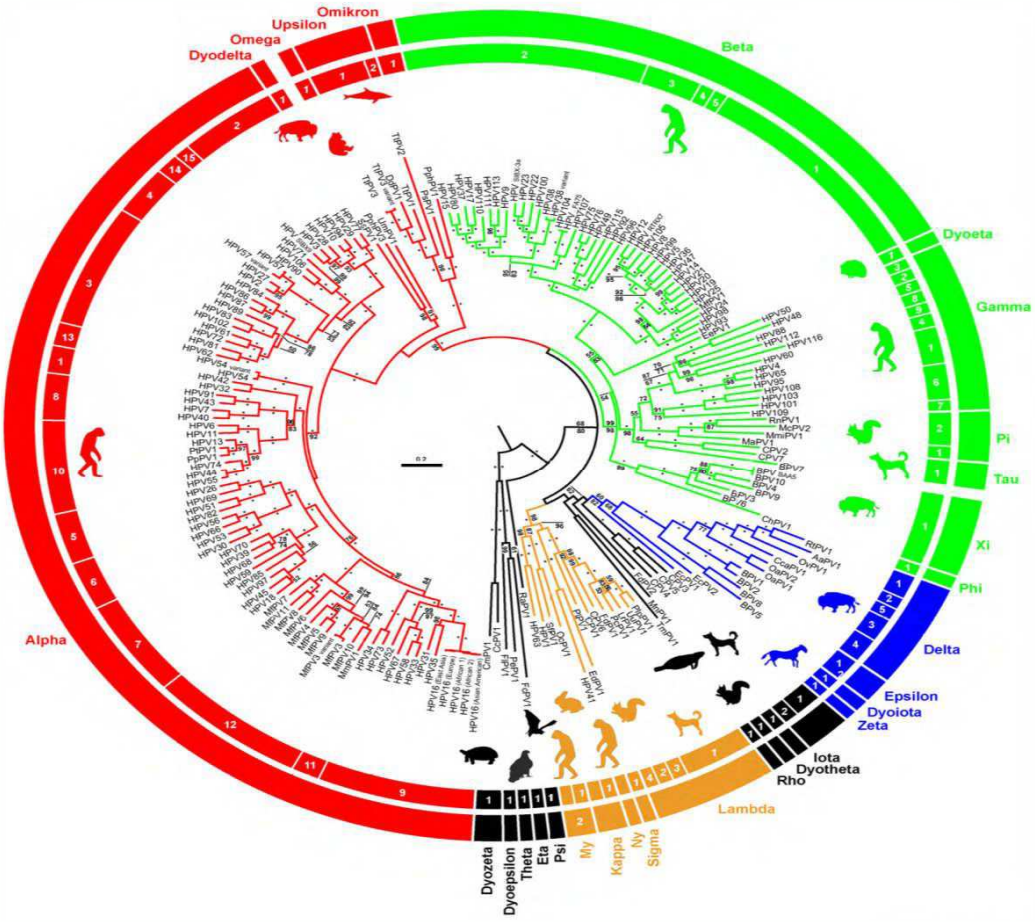
In 2002 the International Committee on Taxonomy of Viruses (ICTV) reclassified PV into a distinct family, the *Papillomaviridae* and a classification system was proposed whereby the family was first divided into genera designated by Greek letters and then sub-divided into numbered species groups containing the individual PV genotypes (de Villiers et al., 2004) (**Figure 1A**). The classification of PV is based upon the nucleotide sequence of the most conserved region of the viral genome, the L1 open reading frame (ORF), which encodes the major capsid structural protein (Bernard et al., 1994). Different PV genera (e.g. Alpha and Beta) share a L1 nucleotide sequences identity of <60% and species groups (e.g. Alpha-7 and Alpha-9) within a genera share a 60-70% sequence identity (Bernard, 2013). Individual PV genotypes (e.g. HPV16 and HPV31) within a species group are classified by a difference in L1 sequence identity of >10% (**Figure 1B**). PV have been isolated from a diverse range of mammals, as well as birds and reptiles (Bernard et al., 2010) but the vast majority of PV have been isolated from humans (170 genotypes isolated to date) and fall into one of five genera: Alpha, Beta, Gamma, Mu, and Nu (de Villiers, 2013). PV also demonstrate tissue tropism and are generally divided into PV which predominantly infect mucosa (e.g. Alpha-PV) or cutaneous epithelium (e.g. Beta-PV) (de Villiers et al., 2004; Mistry et al., 2008)

1.3 PV evolution and viral variants

It has been estimated that the *ca.* 8 kb double-stranded DNA genome of PV is replicated via host cell polymerases with an error rate of *ca.* $2x10^{-7}$ base substitutions, per site, per year (Bernard, 2013). This rate is substantially lower than that found in the majority of single-stranded RNA viruses (*ca.* $1x10^{-3}$ base substitutions/site/year) (Duffy et al., 2008) and is more closely related to the slower rate of the host species, suggesting an evolutionary link. Both HPV and ape PV are found within the Alpha-PV genera whilst other mammal groups (e.g. hooved) fall into separate distinct genera (e.g. Delta-PV) (**Figure 1A**). This infers that humans and apes shared a common PV ancestor and that the evolution into HPV and ape PV occurred along the same time lines as the speciation of the host (Bernard, 2013).

Figure 1

A



B

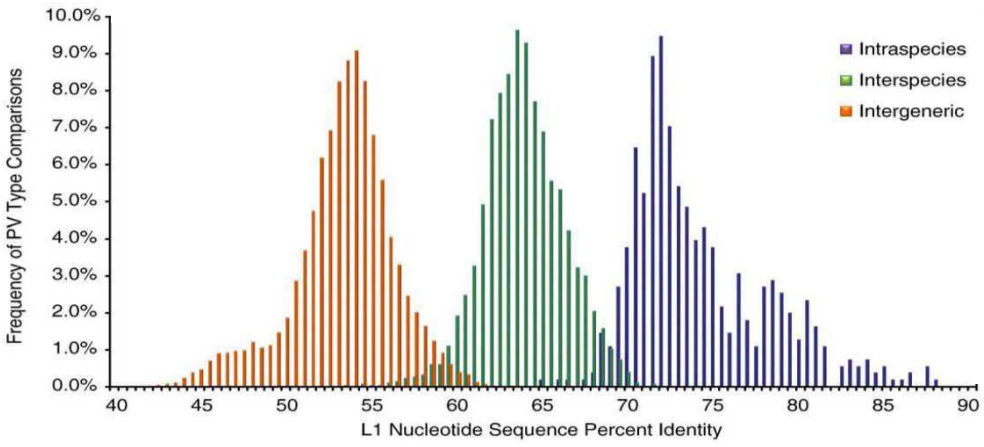


Figure 1 PV phylogeny. (A) Maximum likelihood phylogenetic tree for PVs. The four PV supertaxa are colour coded, with PVs not yet assigned to a supertaxa labelled in black. Silhouettes represent the hosts infected by the corresponding viruses. (B) Intergeneric, interspecies and intraspecies L1 nucleotide sequence percentage identity. The 189 L1 nucleotide sequences were used to evaluate the distribution of intraspecies: comparisons of PV genotypes within the same species; interspecies: comparisons of PV genotypes within the same genus; intergeneric: comparisons of all PV genotypes within different genera. Bravo et al., Trends in Microbiology, 18:432 (2010); Bernard *et al.,* Virology, 401:70 (2010).

Despite the low evolutionary rate of the HPV genome, sequence variants have arisen over time leading to the generation of distinct intra-genotype variant lineages and sublineages (Burk et al., 2013). Initial studies of HPV16 and HPV18 identified that variant lineages evolved as the human population spread out of Africa and that the lineages became associated with distinct ethnic groups: for example, HPV16 variants were categorised as African (Af1 and Af2), Asian-American (AA), European (E) and Asian (As) (Bernard, 1994) (**Figure 1A**). However, such geographical niches are not overtly obvious for variants of the other A9 HPV genotypes (Chen et al., 2011) and subsequently these categories have been replaced by alphabetical designations (A to D) to bring HPV16 variant classification in line with other genotypes (Burk et al., 2013).

1.4 History of HPV

References in the medical literature to warts, a clinical manifestation of HPV infection, can be found as far back as the ancient Greeks and Romans with both civilisations recognising that such warts could be sexually transmitted (Onon, 2011). A treatment for genital warts was documented by the Greek Hippocrates, who lived 400 years BC, describing how plant extracts could be used for the removal of penile warts. An initial understanding of the HPV epidemiology began to emerge in the 19th century, when Italian physician Domenico Rigoni-Stern studied the cause of cancer death in married, widower and single women including nuns (Scotto and Bailar, 1969). He observed that in the latter group death due to cancer of uterine was substantially lower whilst similar rates of death due to breast cancer were seen across the three groups. By the late 19th century it was recognised that the risk factors for contracting a sexually transmitted disease were the same risk factors associated with the development of cervical cancer, these included the early onset of sexual activity and having multiple sexual partners.

The link between an infectious agent and cancer was established by work carried out in chickens by Peyton Rous which demonstrated that inoculation with cell-free extract,

prepared from a spontaneous malignant sarcoma, resulted in the development of malignant tumours in inoculated chickens (Rous, 1911). The infectious agent responsible for tumour development in healthy chickens was an RNA virus which was subsequently called Rous sarcoma virus. Work carried out by Richard Shope, a colleague of Rous's at the Rockefeller Institute, demonstrated that PV could also induce malignant tumours. Shope prepared cell-free extracts of a malignant tumour taken from a rabbit infected with cottontail rabbit papillomavirus (CRPV) and inoculated healthy rabbits with the cell-free extracts, resulting in the formation of benign papillomas which had the potential to progress to malignant tumours (Shope, 1932a). The development of papillomas was found to coincide with resistance to homologous viral challenge at other cutaneous sites and the detection of neutralising antibodies in the serum of the inoculated rabbits (Shope and Hurst, 1933). Further work carried out using CRPV demonstrated both the strict species and tissue tropism of PV (Shope, 1932b) (Shope and Hurst, 1933). These early discoveries were subsequently followed by a reduced interest in PV research due in part to the inability to propagate the virus *in vitro* and the belief that HPV was of limited medical importance.

1.5 HPV cervical infection, disease and cancer

A role for HPV in the development of human cancers was postulated by Harald zur Hausen in the 1970s (zur Hausen, 1977) and his seminal work in the field culminated in the award of the Nobel Prize for Medicine in 2008. Today the WHO International Agency for Research on Cancer (IARC) recognises 12 HPV genotypes as human carcinogens: HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59 because of their fundamental role in the development of cervical cancer (**Figure 2A**) (Bouvard et al., 2009), the third commonest malignancy in women worldwide (Forman et al., 2012).

Sexually active women have a >80% risk of acquiring an HPV infection at least once during their lifetime (Brown et al., 2005; Koutsky, 1997) with 75% of these infections attributable to oncogenic HPV genotypes (Peto et al., 2004). Cervical HPV infection is usually acquired

Figure 2

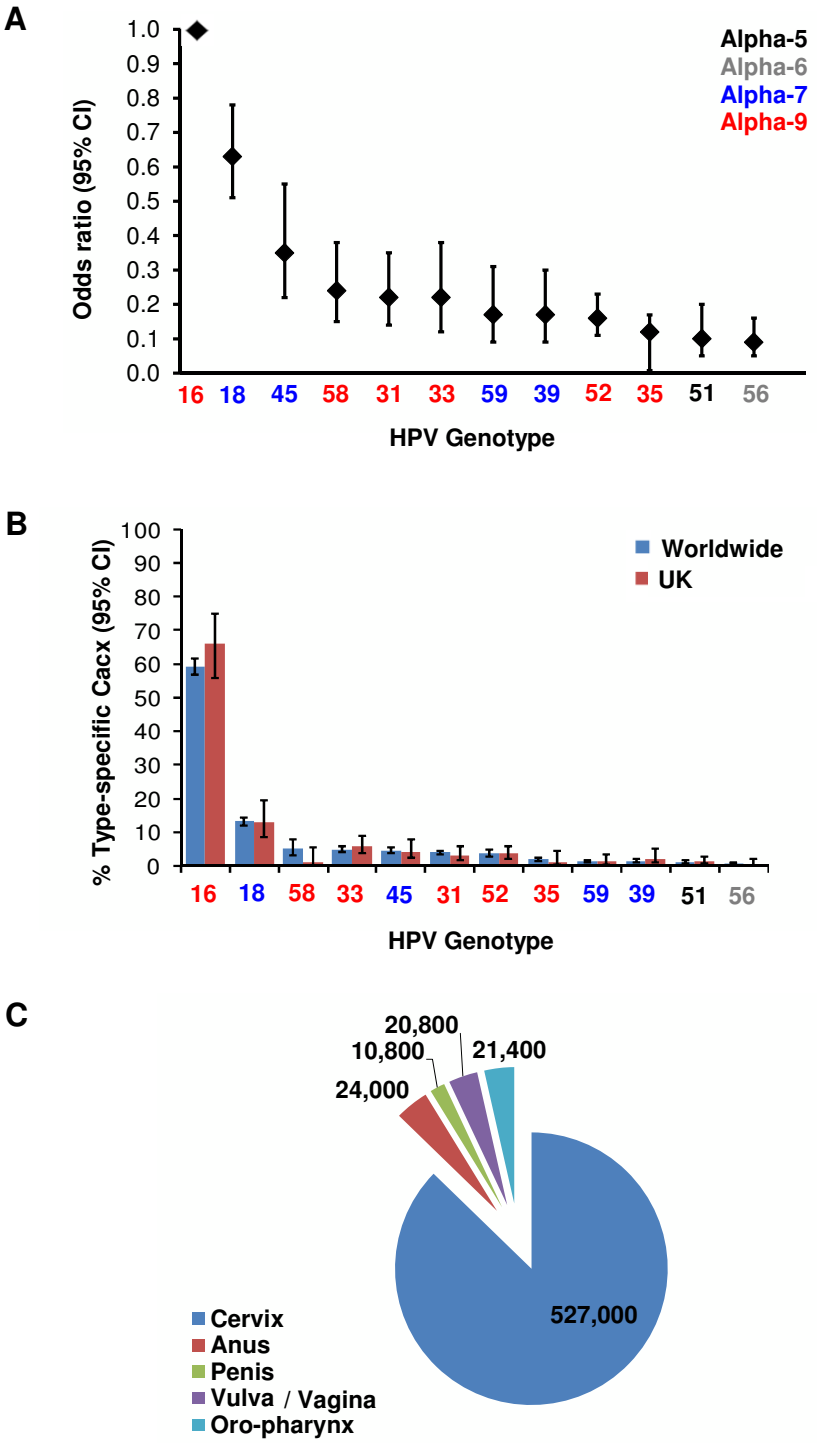


Figure 2 Carcinogenic HPV genotypes. (A) Genotype-specific prevalence in cervical cancer reported as odds ratio relative to HPV16. (B) Genotype-specific prevalence in cervical cancer cases worldwide and in the UK. (C) Estimated HPV attributable cancer cases per annum. Bernard *et al.,* BMC Infectious Diseases, 13:373 (2013); Li *et al*., Int J Cancer, 128:927 (2011); Howell-Jones *et al*., Br J Cancer, 103:209 (2010); Forman *et al .,* Vaccine, 30S F12 (2012).

soon after initiating sexual activity with *ca.* 90% of infections cleared within 2 years (Winer et al., 2011). The prevalence of HPV infection reaches a peak in women below the age of 25 followed by a slow decline (Cuschieri et al., 2004; Dunne et al., 2007) with a second, lower peak in HPV infection observed in women aged 45 or older (de Sanjose et al., 2007). This secondary peak in older women has mainly been attributed to the reactivation of latent HPV infections rather than incident infections due to new sexual exposures (Rositch et al., 2012).

Infection with HPV can cause dysplasia of the cervical epithelium, defined as cervical intraepithelial neoplasia (CIN) and graded as mild (CIN1), moderate (CIN2) and severe (CIN3) changes in the cells of the cervix. Persistent infection with an oncogenic HPV genotype increases the risk of progression to CIN3, the precursor to cervical cancer (Dahlstrom et al., 2010; Moscicki et al., 2012). The detection of CIN3+ (CIN3 or worse) lesions peaks in women aged 25-29 years indicating that the time from first acquisition to the development of lesions is considerably shorter than the decades that generally precede the development of cancer (Moscicki et al., 2012; Winer et al., 2005). The persistence of CIN3+ lesions does not necessitate the development of cancer since lesions can regress or persist without clonal expansion, which is the final step towards the development of invasive cervical cancer (Moscicki et al., 2012).

Infection with either HPV16 or HPV18 accounts for *ca.* 70% of cervical cancer cases worldwide and nearly all cases are attributable to infection with either an HPV18-related Alpha-7 (A7) or HPV16-related Alpha-9 (A9) genotype (**Figure 2B**) (Li et al., 2010). Infection with oncogenic HPV genotypes is also an identified risk factor associated with the development of other anogenital cancers and head and neck cancers (**Figure 2C**) (Forman et al., 2012). All cervical cancer cases are attributable to infection with HPV; however, the number of cases attributed to HPV varies between the other anatomical sites: anus (88%), vagina (70%), penis (50%), vulva (43%) and oropharynx (26%) (Forman et al., 2012).

1.6 The HPV genome and gene expression

HPV have a closed circular double-stranded DNA genome of approximately 8kb which can be divided into three regions: early (E), late (L) and long control region (LCR) (**Figure 3A**).

The early regions encode non-structural viral proteins (E1, E2, E4, E5, E6 & E7) whilst the late regions encode genes which express the viral structural proteins (L1 & L2) (Johansson and Schwartz, 2013). The LCR is a non-coding region located between the L1 and E6 ORFs which contains the origin of DNA replication (*ori*) and the early promoter p97 (HPV16 designation). Activation of the p97 promoter during the early stages of viral replication generates messenger RNA (mRNA) transcripts representing all the early genes (Schwartz, 2013; Smotkin and Wettstein, 1986). The late promoter, p670, is located within the E7 gene and its differentiation-dependent activation results in increased production of E1, E2 and E4 mRNA transcripts followed by the subsequent transcription of the L1 and L2 ORFs (Grassmann et al., 1996). HPV increases the coding capacity of its viral genome by generating multiple polycistronic mRNA species and full transcription maps have been generated for HPV16, HPV18 and HPV31 with up to 23 different mRNAs being detected in HPV31-infected cell lines (**Figure 3B**) (Ozbun and Meyers, 1997; Wang et al., 2011; Zheng and Baker, 2006). This diversity results from the combined effects of alternative promoter and termination site usage and differential use of splice donor and splice acceptor sites within the mRNA transcripts.

Expression of the E1 ORF produces the only viral encoded enzyme, an ATP-dependent DNA helicase which is essential for viral DNA synthesis and subsequent elongation (Liu et al., 1995). The E1 protein binds the *ori* located in the LCR, an interaction facilitated by the E2 protein which loads the E1 onto the *ori* (Mohr et al., 1990). Aside from its role in the initiation of DNA replication, the primary function of the E2 protein is the regulation of viral transcription. The E2 can activate or repress transcription dependent upon its binding motif, associated cellular factors and the specific isoform of E2 interacting with the viral genome

Figure 3

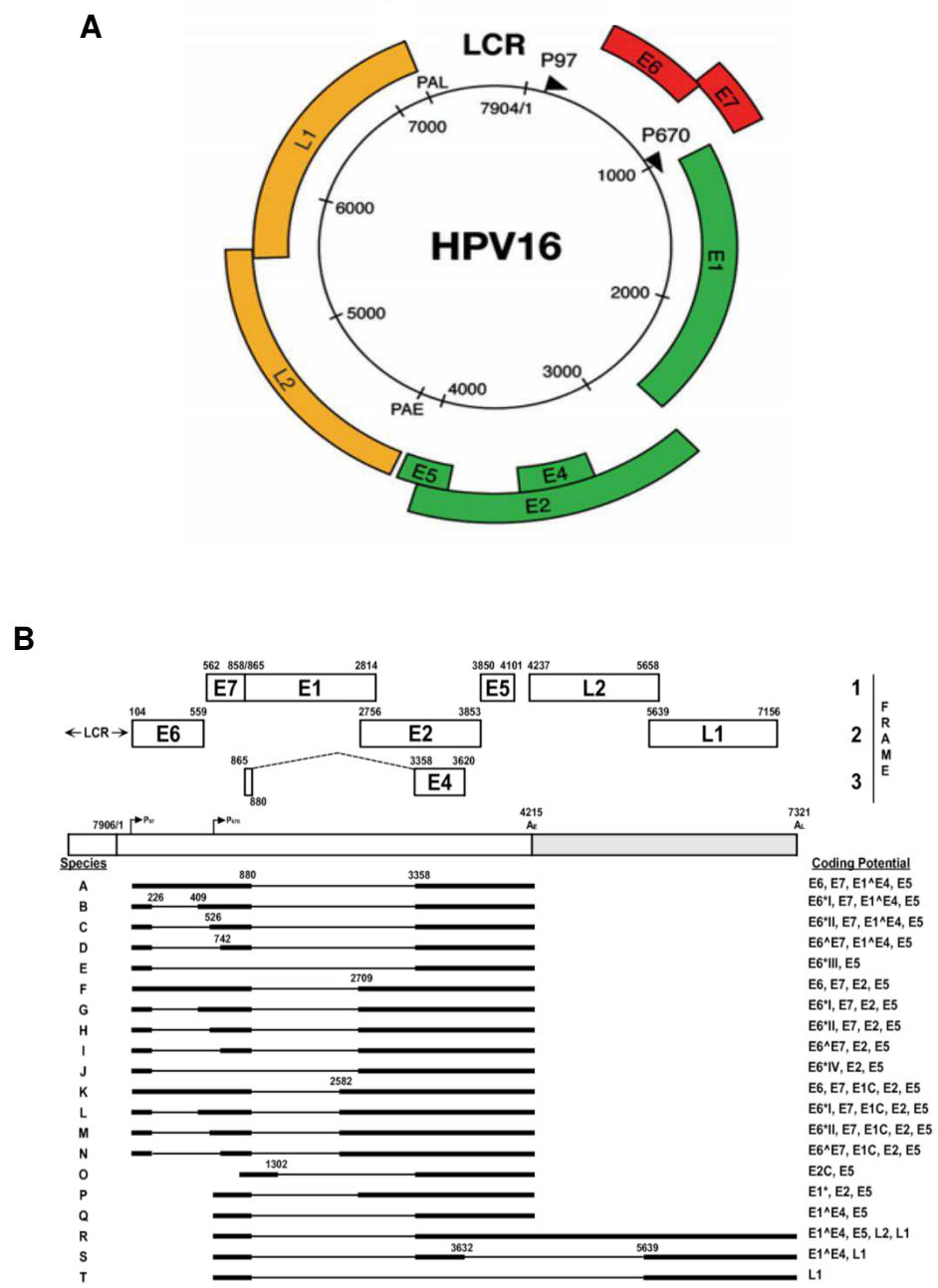


Figure 3 Graphic representations of HPV16 genome and transcriptional map. (A) L1 and L2 structural genes labelled in yellow. E1. E2, E4 and E5 regulatory genes labelled in green. E6 and E7 oncogenes labelled in red. Genes are expressed from a double-stranded DNA genome of ~8kb. (B) Linear form of virus genome positioned above reported RNA species derived from alternative promoter usage and alternative splicing. Doorbar *et al.,* Clinical Science, 110:525 (2006); Zheng *et al.,* Frontiers in Biosciences, 11:2286 (2006).

(McBride, 2013). For example, following the upregulation of p670 the E2 protein represses the transcription of the E6 and E7 genes by direct binding to p97 (Thierry, 2009). The E2 protein exerts control in a dose-dependent manner whereby low concentrations activate viral transcription whilst high concentrations repress (Steger and Corbach, 1997).

The E6 and E7 proteins disrupt checkpoints that regulate cell cycle progression thereby promoting cellular proliferation which facilitates the process of viral DNA synthesis in differentiating cells which would normally exit the cell cycle (Howie et al., 2009). The E6 and E7 proteins of oncogenic HPV genotypes are commonly referred to as 'oncoproteins' because they have the potential to transform the host cell and they are the only viral proteins consistently expressed in HPV-associated cancers (Munger et al., 2004).

The E6 protein contains two zinc-like finger motifs (Barbosa et al., 1989) and the majority of E6 activity is mediated via protein-protein interactions. The E6 protein can alter multiple cellular pathways by binding host cell proteins affecting functions such as G protein signalling, chromosome stability, polarity adhesions and modulation of immune signalling which facilitates immune avoidance (Howie et al., 2009). The best characterised E6 protein interaction is that with p53 (Thomas et al., 1999), a tumour suppressor protein, which is activated in response to cellular stress resulting in the initiation of DNA repair, cell cycle arrest and/or apoptosis (Zilfou and Lowe, 2009). HPV-mediated stimulation of DNA synthesis outside the S phase of the cell cycle also triggers p53 activation and the E6 protein acts to block p53 function by binding the cellular protein E3 ubiquitin ligase (E6AP), an interaction which permits E6AP to bind p53 which is subsequently degraded (Scheffner et al., 1993).

The E7 protein contains a single zinc-like finger motif (Barbosa et al., 1989) which shares sequence homology with control protein EA1 of Adenovirus and the large T antigen of SV40 (Phelps et al., 1988; Vousden and Jat, 1989). The E7 protein, like E6, interacts with host cell

proteins resulting in the alteration or disruption of cellular processes including cell death, cytostatic cytokine signalling, cellular metabolism and epigenetic programming (McLaughlin-Drubin and Munger, 2009). The major action of E7 is the association with, and subsequent degradation of, the retinoblastoma tumour protein, pRB (Roman and Munger, 2013). The pRB binds transcriptional factors of the E2F family forming a repressor complex which inhibits cell cycle progression from G1 to S phase and subsequent cellular DNA replication (Frolov and Dyson, 2004). The E7 protein associates with, and disrupts, the pRB/E2F repressor complex (Dyson et al., 1992) resulting in the release of E2F and as a consequence the cell cycle exits G1 and enters the S phase. The pRB is subsequently inactivated via proteasomal degradation in a process mediated by the E7 protein (Boyer et al., 1996).

The E5 is a small hydrophobic protein of *ca.* 83 amino acids (HPV16) which is primarily expressed during the late stage of the viral replication cycle (Longworth and Laimins, 2004). The E5 protein acts via interaction with cellular proteins and is required for the successful completion of viral genome amplification and the subsequent activation of the late genes (DiMaio and Petti, 2013). The E5 protein directly interacts with the epidermal growth factor receptor (EGFR) (Tomakidi et al., 2000), activating the receptor and initiating a cascade of mitogenic signalling which results in the proliferation of the HPV infected cell.

The E4 gene is located within the E2 ORF and the most abundant E4 gene product is E1^E4 which is expressed from a spliced mRNA containing a 5' E1 region with transcription initiated from the E1 start codon (Doorbar, 2013). An increase in E1^E4 protein expression precedes the expression of the late proteins and coincides with the initiation of viral genome amplification (Doorbar et al., 1997). The role(s) of the E1^E4 protein during the earlier stages of viral replication, where limited amounts of the protein are expressed, are undefined (Doorbar, 2013). In the later stages of viral replication the E1^E4 protein facilitates viral genome amplification and protein synthesis by stimulating cell cycle arrest in G2 (Davy et al.,

2002). The E1^E4 has also been shown to disrupt cellular cytokine networks indicating that it may have a role in facilitating virion release from the host cell (Doorbar et al., 1991).

The late viral genes express two structural proteins, the major capsid protein L1 and the minor capsid protein L2. The L1 and L2 form a non-enveloped, icosahedral structure which encapsidates the viral genome and associated cellular histones (Buck et al., 2013; Wang and Roden, 2013). The L1 protein is *ca.* 500 amino acids in length and consists of a core of β-strand and α-helix structures which support the surface exposed loop regions designated BC, DE, EF, FG and HI (Chen et al., 2000). Five L1 proteins, or monomers, form an intermediate capsomer structure, then these pentameric subunits associate to form an icosahedral structure of 72 capsomers (Modis et al., 2002). The C-terminus of the L1 extends outwards from the core and interacts with L1 monomers in adjacent capsomers resulting in 12 pentavalent and 60 hexavelent positioned capsomers (Modis et al., 2002). The L2 protein is *ca.* 450 amino acids in length and the HPV capsid is capable of incorporating up to 72 L2 monomers, thought to be positioned in the axial lumen of each L1 capsomer (Buck et al., 2008), although as few as 12 monomers per capsid has also been reported (Roden et al., 1996a; Volpers et al., 1994).

1.7 HPV replication cycle

The replication cycle of HPV is strictly linked to the differentiation program of epithelial cells (Stubenrauch and Laimins, 1999) and to establish a productive infection HPV must infect cells capable of undergoing cell division such as the basal cells of the epithelium which are accessed via microtraumas, one or two cell-length tears in the stratified epithelium (Schiller et al., 2010). Additionally, the outermost cell layer of the cervical squamo-columnar junction is also capable of undergoing differentiation in a novel 'top down' process (Herfs et al., 2013) and it has been demonstrated that HPV are capable of infecting this superficial layer of cells (Mirkovic et al., 2015).

The L1 protein mediates initial viral attachment via heparin sulphate proteoglycans (HSPG) on the basement membrane (Giroglou et al., 2001a; Johnson et al., 2009; Joyce et al., 1999). At least three L1 sites are involved in virus interaction with the host heparin sulphate moieties, with lysine residues in site 1 (Lys^{278} & Lys^{361}: HPV16 designation) mediating primary attachment following which sites 2 (Lys^{54} & Lys^{356}) and 3 (Asn^{57}, Lys^{59}, Lys^{442} & Lys^{443}) are engaged (Knappe et al., 2007; Richards et al., 2013). L1-mediated attachment triggers a conformational change in the viral capsid, exposing the N-terminus of the L2 protein which is subsequently cleaved by cellular furin proteases (Richards et al., 2006). The cleavage of the L2 protein causes an additional conformational change in the capsid which reduces L1 affinity for the primary HSPG receptor resulting in the subsequent transfer to an as yet unidentified secondary receptor on the host cell surface (Buck et al., 2013). These series of events necessitate that the time from initial HPV attachment to particle endocytosis is protracted (Giroglou et al., 2001a; Schelhaas et al., 2012) (**Figure 4**).

There are conflicting data on the pathways used by individual HPV genotypes with HPV16 and HPV31 the most intensely studied. The entry process of HPV16 has been classified as both clathrin-dependent (Bousarghin et al., 2003; Hindmarsh and Laimins, 2007; Smith et al., 2008b) and clathrin- and caveolar-independent (Schelhaas et al., 2012; Spoden et al., 2008) whereas the entry of HPV31 has been identified as caveolar-dependent in some studies (Bousarghin et al., 2003; Smith et al., 2008b) and clathrin-dependent in others (Hindmarsh and Laimins, 2007). Following endocytosis, HPV is trafficked through the endosomal system as particle uncoating necessitates an acidified environment (Day et al., 2003; Smith et al., 2008b). The L1 protein is degraded by lysosomes in the late endosomal compartments whilst the L2 protein facilitates viral genome egress from the endosome, forming a complex with the genome whilst it is translocated to the host cell nucleus (Bergant Marusic et al., 2012; Day et al., 2004).

Figure 4

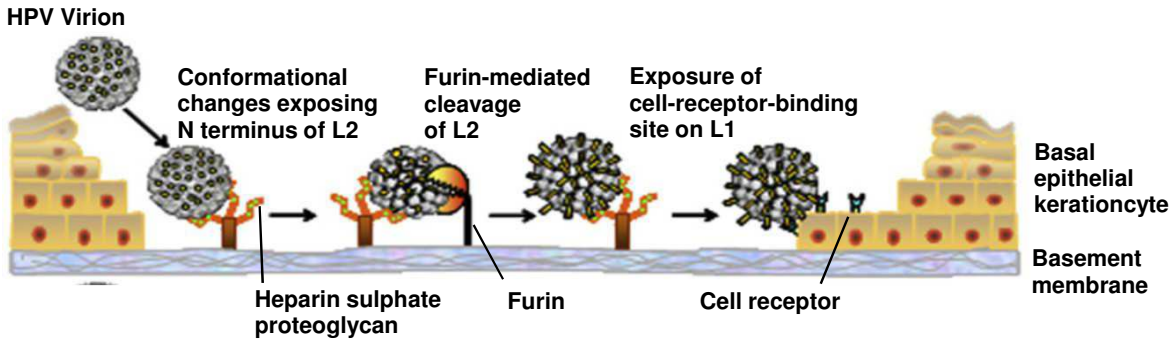


Figure 4 Steps in HPV binding to host epithelial cells. Initially attachment to basement membrane heparin sulphate proteoglycans mediates a conformational change in the viral capsid exposing L2, which is cleaved, subsequently an L1 cell-receptor binding site is exposed and the virus attaches to basal epithelial keratinocytes. Day *et al.,* Cell Host and Microbe, 8:260 (2010); Schiller & Lowy Nat Rev Mirco, 10:681 (2012).

An initial phase of viral genome amplification occurs within the infected basal cell facilitated by viral replication proteins, E1 and E2 (Lambert, 1991). The viral genome is subsequently maintained within the nucleus as a low copy number episome (Stubenrauch and Laimins, 1999). The infected basal cell divides and the daughter cell migrates into the upper layers of epithelium and begins the process of differentiation which coincides with exit from the cell cycle and the down regulation of cellular replication factors. However, HPV-infected cells do not exit the cell cycle but instead re-enter the S-phase (Stubenrauch and Laimins, 1999) principally due to the action of the E7 protein which binds and degrades pRB, releasing the E2F transcription factor which induces host cell replication machinery overcoming the block on DNA synthesis (Flores et al., 1999). The E6 and E5 proteins also contribute to the maintenance of this favourable cellular environment for HPV genome amplification. The activity of p53, which increases in response to the activity of the E7 protein, is counteracted by the E6 protein which targets p53 for degradation via the ubiquitin-proteasome pathway (Scheffner et al., 1993). The E5 protein binds the EGFR resulting in receptor dimerisation and the initiation of a signalling cascade which stimulates cell growth (Venuti et al., 2011).

The concentration of the E1 and E2 proteins increases with the differentiation-dependent activation of the late promoter, p670 (Grassmann et al., 1996). The E2 protein subsequently downregulates the early promoter (p97) resulting in the reduced expression of E6 and E7 proteins and subsequent progression in cell differentiation (Thierry, 2009). Upregulation of the p670 increases the expression of the E1^E4 protein which stimulates cell cycle arrest in G2 (Davy et al., 2002) facilitating the synthesis of the viral late proteins, L2 followed by L1, in the uppermost layers of the epithelium. The E2 protein recruits the L2 to the newly replicated viral genome which is subsequently encapsidated, alongside cellular histones, within an icosahedral capsid composed of the L1 and L2 proteins (Buck et al., 2013; Wang and Roden, 2013). Progeny virions are shed from the top layer of epithelium, released from cells undergoing a programmed cell death, a process which is facilitated by disruption of the cellular cytokine network by the E1^E4 protein (Doorbar et al., 1991).

1.8 HPV oncogenesis

Oncogenesis is the process by which normal cells are transformed into cancer cells following a breakdown in the regulatory mechanisms which govern cell division. Multiple steps are involved in the development of cancer beginning with initiation whereby acquisition of DNA damage and/or mutations causes the cell to become abnormal; this may happen as a result of exposure to carcinogens such as certain chemicals or radiation but can also occur spontaneously (Bertram, 2000). Cellular mechanisms are in place which identify and repair abnormal DNA and if these mechanisms fail the cell undergoes a programmed cell death; however, the accumulation of DNA damage and/or mutations over time, which can be promoted by certain hormones and drugs (Yager and Davidson, 2006), results in the loss of cellular growth control checks and transformation of the cell into a cancer cell. The uncontrolled proliferation of cancer cells leads to the formation of malignant tumours which interfere with the normal functions of their resident tissue or organ. Ultimately, cancer cells can spread throughout the body and form secondary tumours or metastases (Leber and Efferth, 2009).

Cancers attributable to oncoviruses share common traits such as virus infection being necessary but not sufficient for cancer development and viral cancers occurring in the context of persistent infection (Mesri et al., 2014); however the specific mechanism of oncogenesis differs between oncoviruses with multiple factors contributing towards cancer development. The development of HCC as a result of a chronic infection with HBV is multifactorial with oncogenesis promoted by HBV DNA integration in the host genome, the expression of the HBV X protein and HBV-specific T cell-meditated hepatic inflammation (Sung et al., 2012). These three events contribute to the induction of chromosomal instability and altered gene functions, modulation of cell viability and proliferation and the accumulation of genetic damage, respectively.

A persistent infection with an oncogenic HPV genotype can lead to integration of viral DNA into the host genome (Klaes et al., 1999), a key event in HPV oncogenesis. Integration results in the maintenance of E6 and E7 oncogene expression whilst other regions of the viral genome are lost or their expression impaired. Significantly the expression of the E2 transcriptional repressor protein is lost resulting in deregulation of E6 and E7 oncoprotein expression (Baker et al., 1987). The integration of HPV DNA alters host cell gene expression (Alazawi et al., 2002) whilst the expression of E6 and E7 confers a selective growth advantage over cells which contain HPV DNA in episomal form (Jeon et al., 1995). The major action of the E6 and E7 proteins during the HPV replication cycle is the facilitation of viral DNA synthesis by blocking the functions of p53 and pRB, respectively (Roman and Munger, 2013; Thomas et al., 1999). This in turn promotes cellular proliferation since the cell cycle remains in the S phase without triggering DNA repair pathways and ultimately apoptosis (Howie et al., 2009). The absence of E2-mediated regulation of E6 and E7 protein expression following HPV DNA integration allows the accumulation of secondary mutations within the host DNA contributing to the malignant progression of the cell (McBride, 2013).

1.9 Host response to HPV infection

The high rates of exposure in contrast to the low numbers of women who develop cervical cancer indicate that an effective immune response is generated against the majority of HPV infections. The humoral immune response to HPV predominantly targets the L1 major capsid protein; however, antibodies targeting the early proteins E2, E6 and E7 can be detected in patients with high grade cervical disease (Lehtinen et al., 1992; Muller et al., 1992) suggesting that the detection of such antibody specificities may have utility for monitoring disease progression. Low levels of L1 serum antibodies are detectable in *ca.* 60% of HPV infected individuals (Gravitt, 2011) with one study finding that seroconversion rates at 18 months following incident infection with either HPV16, HPV18 or HPV6 were 60%, 54% and 69% respectively (Carter et al., 2000). A recent systematic review demonstrate that HPV16 natural infection antibodies offered significant protection against reinfection with HPV16 in

women, although this effect was not apparent in male subjects (Beachler et al., 2015). Seroconversion rates following natural HPV infection are lower in males which may contribute towards this lack of protection (Dunne et al., 2006). Protection also seems to be dependent upon the magnitude of the antibody response since higher levels of HPV16 and HPV18 natural infection antibodies have been shown to be associated with a reduced risk of subsequent reinfection in women (Safaeian et al., 2010).

The regression of HPV-induced lesions is thought to occur as a result of a successful cell-mediated response targeting the viral early proteins, particular E2 and E6 (Woo et al., 2010). This was first demonstrated by an immunohistologic study which found significantly higher levels of T lymphocytes (CD4+ and CD8+) in regressing genital warts compared to non-regressing (Coleman et al., 1994), an observation which was subsequently confirmed using serial wart biopsies taken from the canine oral papillomavirus (COPV) infection model. Immunohistochemical staining of these biopsies showed the presence of T-lymphocytes just prior to regression with levels reaching peak concentrations during the resolution of the wart (Nicholls et al., 2001).

HPV has evolved strategies to avoid detection by the innate immune system. thereby delaying the activation of the adaptive immune system resulting in the maintenance of a persistent infection (Schiffman and Kjaer, 2003). The replication of HPV is linked to epithelial differentiation and the establishment of a productive infection depends upon the infection of basal epithelium cells. Basal cells are the only cell type capable of undergoing cell division with one daughter cell, carrying the viral episome, migrating away to begin the program of terminal differentiation whilst the other daughter cell becomes a new basal cell maintaining the viral episome (Lowy and Schiller, 2006). The expression of high levels of viral proteins, which would be detectable by immune surveillance, only occurs in highly differentiated cells which have migrated away from the basal cell layer leaving the viral episome-containing cell undetected (Lowy and Schiller, 2006). Critically there is no inflammation and therefore no

danger signals are raised when the infectious virus particles are shed from the top layer of epithelium since progeny virions are released from cells undergoing apoptosis. There is also no viraemic phase during the normal course of HPV infection, with the virus shed into the local environment of the genital mucosa where transport to lymph nodes via vascular and lymphatic channels is limited (Mariani and Venuti, 2010). HPV DNA is detectable in the peripheral blood of women with advanced cervical cancer, however this DNA is thought to originate from circulating tumour cells rather than the release of progeny virions (Kay et al., 2005).

HPV also targets specific components of both the innate and adaptive immune system. Virus infected cells should stimulate the production of type 1 interferons, IFN-α and IFN-β, which have broad-spectrum antiviral activity and act as a bridge between the innate and adaptive arms of the immune system (Le Bon and Tough, 2002). HPV lesions exhibit a degree of clinical resistance to IFN-α treatment and it has been demonstrated that the E7 protein can inhibit the antiviral activities of INF-α by direct interaction with protein components of the interferon signalling pathway (Barnard and McMillan, 1999; Barnard et al., 2000). Langerhans cells are a subset of antigen-presenting dendritic cells which reside in the epithelium, where they act as the first line of defence against invading pathogens alongside other elements of the innate system (e.g. cytokines, neutrophils and macrophages). Langerhans cells should be activated by the uptake of HPV capsids triggering an anti-HPV immune response; however, it has been shown that Langerhans cells are not activated by HPV16 L1 VLPs (Fausch et al., 2002) demonstrating the ability of HPV to silence another component of the innate immune response. Toll-like receptors (TLR) which play a fundamental role in pathogen recognition and subsequent activation of the innate immune system are also a target for the E6 and E7 proteins which downregulate TLR9 mRNA inhibiting the activation of the TLR9 pathway (Hasan et al., 2007). HPV can also subvert the adaptive immune system, impairing cytotoxic T-cell targeting of HPV infected cells by the

action of the E7 and E5 viral proteins which reduce the expression of the major histocompatibility complex (MHC) I (O'Brien and Saveria Campo, 2002).

1.10 Medical and therapeutic HPV interventions

The link between persistent infection with an oncogenic HPV genotype and subsequent development of cervical cancer has resulted in many countries establishing national screening programmes to identify and treat pre-cancerous cervical disease resulting in a subsequent decrease in the numbers of cervical cancer cases (Hakama et al., 1985). However, resources for such programmes are not available, or limited, in many low- and middle-income countries contributing to the heavier burden of cervical cancer incidence (86% of worldwide incidence) and mortality (88%) in these regions (Arbyn et al., 2011).

The UK national cervical screening programme was launched in 1988 supported by a computerised call/recall system and is estimated to prevent up to 5,000 deaths per year in the UK (Peto et al., 2004). The programme invites 25 to 64 year old women to attend for screening every 3 years between the ages 25 to 49 and every 5 years between the ages of 50 to 64. Cell samples are collected from the surface of the cervix and screened for abnormalities, which if found trigger follow-up investigations. HPV DNA testing is carried out on samples with cellular abnormalities classified as borderline or mild cervical dyskaryosis and women with HPV DNA positive samples are referred for colposcopy examination. Women with cellular abnormalities classified as moderate or severe cervical dyskaryosis are referred straight to colposcopy for biopsy and treatment of abnormal cells, which are usually removed by large loop excision of the transformation zone (LLETZ). Cervical treatment is successful in *ca.* 90% of women, with no cellular abnormalities detected at follow up screenings (Kitchener et al., 2006).

The development of therapeutic vaccines for the treatment of precancerous HPV lesions without the need for surgical excision has focussed on the E6 and E7 oncoproteins (van der

Sluis et al., 2015). It is the expression of these two proteins that is required for the initiation and maintenance of precancerous, high grade lesions and their subsequent immortalisation (Hudson et al., 1990). Clinical trials have been carried out with various forms of the E6 and/or E7 as immunogens (Stern et al., 2012) with vaccination strategies generally aimed at stimulating T lymphocytes since this is primarily the immune response implicated in the regression of HPV lesions (Coleman et al., 1994; Nicholls et al., 2001). It has been demonstrated that therapeutic vaccines are safe and are well tolerated in patient cohorts ranging from healthy individuals to those with end-stage cervical cancer; however, their efficacy to date has been limited (Stern et al., 2012). Cervical cancer tumours commonly have mutations within the genes involved in antigen processing and presentation which may reduce the effectiveness of E6 and/or E7 antigen-specific vaccines in late stage disease (Brady et al., 2000; Evans et al., 2001). However, clinical efficacy has been demonstrated for two E6/E7 vaccines against HPV16-associated vulva intraepithelial neoplasia (VIN) in separate phase II clinical trials with lesion regression correlating with the detection of vaccine-induced HPV-specific T cell responses (Daayana et al., 2010; Welters et al., 2010). More recently the therapeutic vaccine candidate VGX-3100, which consists of synthetic plasmids encoding the E6 and E7 genes, demonstrated therapeutic efficacy against CIN2/3 associated with HPV16 and HPV18 (Trimble et al., 2015).

1.11 L1 virus-like particle prophylactic vaccines

1.11.1 L1 virus-like particles

The L1 major capsid protein of HPV can self-assemble to form icosahedral virus-like particles (VLP) when over-expressed in various *in vitro* systems including bacterial and yeast cells which are transformed by L1 expression plasmids and by L1 recombinant vaccinia or baculovirus infection of eukaryotic cell lines (Kirnbauer et al., 1992; Nardelli-Haefliger et al., 1997; Sasagawa et al., 1995; Zhou et al., 1993). L1 VLP form in a step-wise process whereby five L1 monomers associate to form a capsomer, then 72 capsomers associate to form the icosahedral capsid structure (Modis et al., 2002) (**Figure 5A**). The five L1 loop

Figure 5

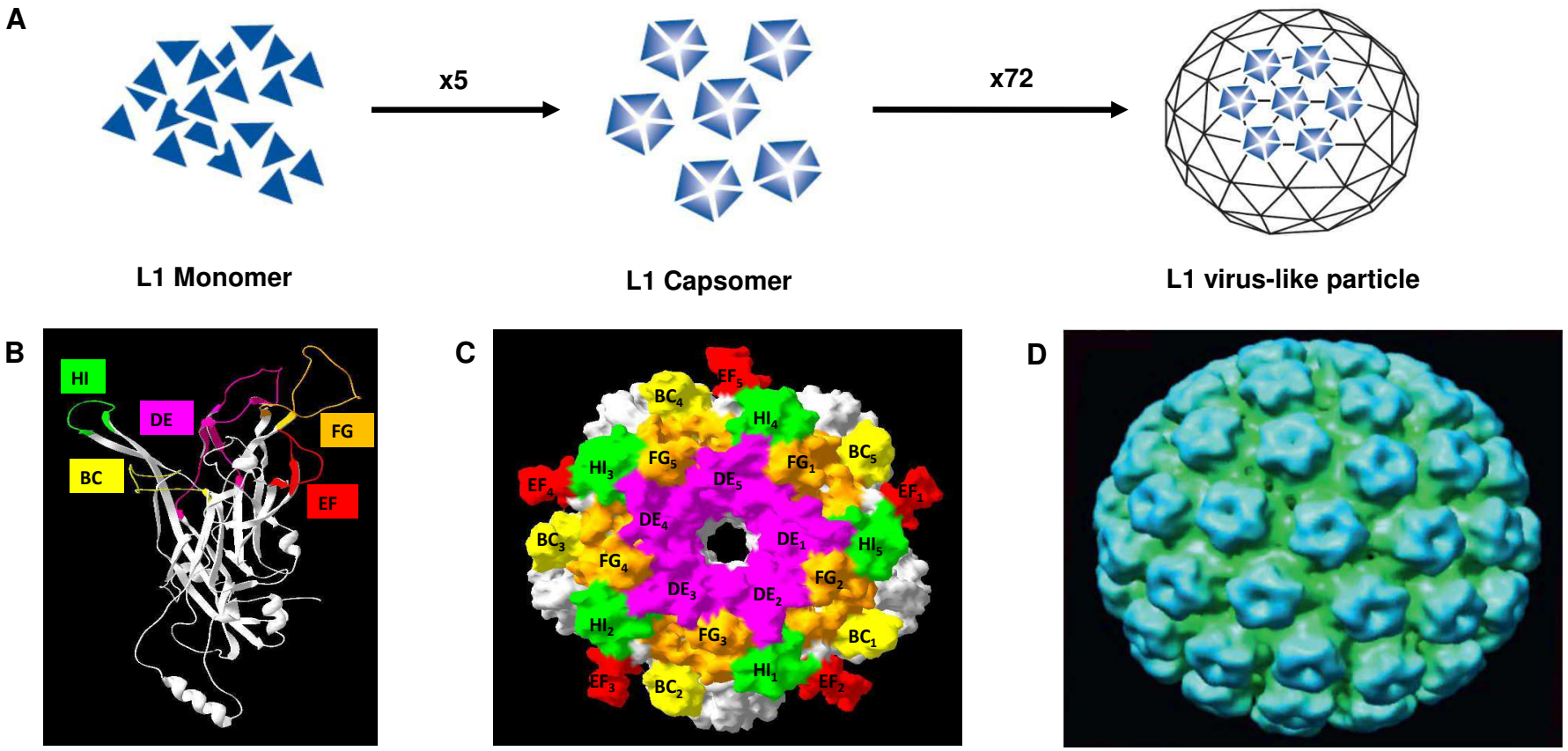


Figure 5 HPV L1 virus-like particles. (A) L1 VLP form in a step-wise manner whereby five L1 monomers associate to form a capsomer, then 72 capsomers associate to form the icosahedron capsid structure. (B) Crystal structure of HPV16 L1 monomer (PDB code: 2R5H) and (C) capsomer (PDB code: 1DZL) with L1 loops BC, DE, EF FG and HI color-coded and labelled. (D) Cryo-electron microscopy structure of HPV16. Schiller & Muller, Lancet Oncology, 16:e217 (2015); Zhao et al., Trends in Biotech, 31:654 (2013).

regions, BC, DE, EF, FG and HI (**Figure 5B**), are surface exposed on the pentameric capsomer structure. The DE loop, centrally positioned around the capsomer lumen, is encircled by the FG and HI loops with the BC and EF loops located on the outer rim of the capsomer (Chen et al., 2000) (**Figure 5C**). The L1 loops from different monomers are intertwined within the capsomer structure. For example, the BC loop of monomer 1 is in close proximity to the EF loop of monomer 2 whilst the HI loop of monomer 1 inserts between the FG and EF loops of monomer 2 and extends as far as the FG loop of monomer 3 (Bishop et al., 2007). This repetitive external structure makes L1 VLP (**Figure 5D**) immunogenic even in the absence of adjuvant (Suzich et al., 1995). Most of the monoclonal antibodies (MAbs) known to neutralise HPV infection *in vitro* recognise conformational epitopes on one or more of these L1 loops (Carter et al., 2003; Rizk et al., 2008; Zhang et al., 2015a; Zhang et al., 2015b).

1.11.2 L1 VLP mediated protection in PV preclinical disease models

Two seminal studies carried out on the CRPV and COPV disease models demonstrated that active immunisation with L1 VLP protected animals from homologous viral challenge at both cutaneous and mucosal epithelium sites of infection (Breitburd et al., 1995; Suzich et al., 1995). L1 VLP-induced protection was concomitant with the detection of high titre, type-specific anti-L1 serum antibodies which recognised the L1 VLP used as an immunogen. Passive transfer of sera from immunised animals, or the purified IgG component, was found to protect naïve animals from subsequent viral challenge. Protection was type-specific since immunisation did not protect animals against heterologous challenge; however, in both instances the heterologous PV used for challenge were from a diverse PV genus (Breitburd et al., 1995; Suzich et al., 1995). No protection from viral challenge was observed when animals were immunised with denatured L1 VLP, implying that the anti-L1 antibodies which conferred protection target conformational epitopes of the L1 capsid protein. These studies highlighted the utility of the L1 VLP as a candidate prophylactic HPV vaccine.

1.11.3 L1 VLP based vaccines

The HPV prophylactic vaccines Cervarix® and Gardasil® both contain L1 VLP representing the oncogenic genotypes HPV16 and HPV18; additionally Gardasil® also contains VLP representing genotypes HPV6 and HPV11 which are associated with *ca.* 90% of external genital warts cases (Lacey et al., 2006) (**Table 1**). The two vaccine preparations use different adjuvants: Gardasil® is adjuvanted with amorphous aluminium hydroyxphosphate sulphate (AAHS) whilst Cervarix® is formulated with the proprietary Adjuvant System 04 (AS04). AAHS was demonstrated to have a greater binding capacity for L1 VLP and induced higher anti-L1 VLP antibody titres when compared to aluminium phosphate ($AlPO_4$) and aluminium hydroxide (AlOH) in small animal immunisation studies (Caulfield et al., 2007). AS04 contains AlOH in combination with 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL). MPL is a detoxified derivative of the lipopolysaccharide (LPS) cell wall of *Salmonella minnesota* strain R595 (Garcon et al., 2007) and activates TLR4 resulting in activation of innate system immune cells (Hoshino et al., 1999). This non-specific immunodulatory effect of MPL enhances antigen presentation and in doing so links the innate and adaptive immune system responses (Garcon et al., 2007). HPV16 and HPV18 L1 VLP adjuvanted with AS04 were shown to induce higher anti-L1 VLP antibody titres in humans than the corresponding VLP adjuvanted with AlOH alone (Giannini et al., 2006). The quality of the immune response, as measured by the frequency of HPV16 and HPV18 specific memory B cells, was also higher in the AS04 adjuvanted group (Giannini et al., 2006).

1.11.4 L1 VLP vaccine efficacy and immunobridging trials

The HPV L1 VLP-based vaccines were primarily developed to prevent cervical cancer and vaccine efficacy trials were carried out in young women (15–26 years) with surrogate clinical endpoints of infection and disease (Schiller et al., 2008). The Cervarix® phase II trial (GSK001/007) investigated safety, immunogenicity and efficacy against incident and persistent HPV16/18 infection (Harper et al., 2006). In the according-to-protocol (ATP) analysis comprising women who were negative for HPV16 and HPV18 antibodies and

Table 1. Characteristics of HPV VLP vaccines

	Cervarix®	Gardasil®
Manufacturer	GlaxoSmithKline	Merck
VLP genotypes	16/18	6/11/16/18
Dose of L1 protein	20/20µg	20/40/40/20µg
Producer cells	*Trichoplusia ni* Insect cells	*Saccharomyces cerevisiae* Yeast cells
Adjuvant	Adjuvant system 04 (AS04)	Amorphous aluminium hydroyxphosphate sulphate (AAHS)
	500µg aluminium hydroxide 50µg 3-O-deacylated-4'-monophosphoryl lipid A (MPL)	225µg aluminium hydroyxphosphate sulphate
Injection schedule	0, 1, 6 months	0, 2, 6 months

Source: Schiller *et al.,* Vaccine, 30S F123 (2012)

oncogenic HPV DNA at entry and who had received all three doses of vaccine, efficacy was demonstrated to be 96.0% (95% confidence interval [CI], 75.2 to 99.9) against 6 month persistent infection (Harper et al., 2006) (**Table 2**). The Merck 007 phase II trial measured Gardasil® efficacy against HPV6/11/16/18 persistent infection and cervical, external anogenital or vaginal disease (Villa et al., 2006b). In the preprotocol population analysis of women negative for HPV6/11/16/18 antibodies and DNA at entry who received all three doses, efficacy was 95.6% (95% CI, 83.3 to 99.5) against 4 month persistent infection (Villa et al., 2006b) (**Table 2**).

Larger phase III vaccine efficacy trials followed, recruiting tens of thousands of women from multiple sites in Europe, North America, South America, Asia and Australia (Schiller et al., 2008). The papilloma trial against cancer in young adults (PATRICIA) study enrolled 18,644 women aged 15-25 years randomly assigned to receive either Cervarix® or the hepatitis A vaccine. In the 4-year end-of-study analysis, efficacy against HPV16/18 CIN3+ of 91.7% (95% CI, 66.6 to 99.1) was reported in the ATP cohort for efficacy (ATP-E) (Lehtinen et al., 2011) (**Table 2**). The FUTURE (females united to unilaterally reduce endo/ectocervical disease) I and II trials investigated the efficacy of Gardasil® against anogenital disease associated with HPV6/11/16/18 in a study cohort of 17,222 women aged 15-26 years. Vaccine efficacy against HPV6/11/16/18 CIN3+ was 100% (95% CI, 90.5 to 100), in the study cohort DNA negative for 14 oncogenic HPV genotypes at enrolment (Munoz et al., 2010) (**Table 2**).

Efficacy trials have also been carried out in other populations where the HPV vaccines offer potential health benefits. A Gardasil® study (Protocol 019) carried out in older women (24-45 years) reported an end-of-study efficacy against 6 month persistent infection of 89.6% (95% CI, 79.3 to 95.4), in women who were seronegative for HPV6/11/16/18 antibodies and DNA at enrolment and who remained DNA negative up to 7 months (Castellsague et al., 2011) (**Table 2**). The ongoing human papillomavirus: vaccine immunogenicity and efficacy

Table 2. HPV vaccine efficacy against infection and lesions related to vaccine targeted genotypes

Vaccine	Study	Participants (years)	Endpoints[a]	Efficacy (95% CI)[b]
Cervarix®	GSK 001/007	Women (15 to 25)	Persistence infection (6M) Persistence infection (12M)	96.0 (75.2 to 99.9) 100 (52.5 to 100)
	PATRICIA	Women (15 to 25)	CIN2+ CIN3+	94.9 (87.7 to 98.4) 91.7 (66.6 to 99.1)
	VIVIANE	Women (26 to 45)	Persistence infection (6M) CIN1+	82.9 (53.8 to 95.1) 86.1 (-35.4 to 99.9)
Gardasil®	Merck 007	Women (16 to 23)	Persistence infection (4M) CIN1+	95.6 (83.3 to 99.5) 100 (<0.0 to 100)
	FUTURE I and II	Women (15 to 26)	CIN2+ CIN3+	100 (91.9 to 100) 100 (90.5 to 100)
	Protocol 019	Women (24 to 45)	Persistence infection (6M) CIN, any grade	89.6 (79.3 to 95.4) 94.1 (62.5 to 99.9)
	V501-020	Men (16 to 26)	Persistence infection (6M) AIN, any grade	94.9 (80.4 to 99.4) 77.5 (39.6 to 93.3)

CI: confidence interval, CIN: Cervical intraepithelial neoplasia; AIN: Anal intraepithelial neoplasia.

[a] Protection against persistent infection over 4 (4M), 6 (6M) and 12 (12M) months, cervical infection in women and anal infection in men.

[b] The Cervarix® efficacy data reported from the according to protocol (ATP) group. The Gardasil® efficacy data reported from the pre-protocol population for the Merck 007, Protocol 019 and V501-020 trials. Efficacy data from the FUTURE I and II trials reported from women DNA negative for 14 oncogenic HPV genotypes are enrolment.

Source: Schiller *et al.,* Vaccine, 26S K53 (2008); Schiller *et al.,* Vaccine, 30S F123 (2012); Skinner *et al.,* Lancet, 384:2213 (2014).

(VIVIANE) study assessing the efficacy, safety, and immunogenicity of Cervarix® in 26-45 year old women reported interim efficacy data of 82.9% (97.7% CI, 53.8 to 95.1) against 6 month persistent infection with HPV16/18 in the ATP-E cohort (Skinner et al., 2014) (**Table 2**). A Gardasil® efficacy study (V501-020) carried out in males (16-26 years) demonstrated efficacy of 77.5% (95% CI, 39.6 to 93.3) against anal intraepithelial neoplasia (AIN) attributable to HPV6/11/16/18, in the study cohort DNA negative for 14 oncogenic HPV genotypes and HPV6/11/16/18 antibody negative at enrolment (Giuliano et al., 2011; Palefsky et al., 2011) (**Table 2**).

The HPV vaccines induce high titre, neutralising serum antibody responses which target the L1 of HPV16 and HPV18. Antibody titres increased in a step-wise manner following each vaccine dose, peaking at levels 10 to 100-fold higher than those induced by natural infection at 1 month post final dose (Einstein et al., 2011b; Villa et al., 2006a). Antibodies specific for HPV16 and HPV18 L1 VLP can also be detected in the cervicovaginal secretions of vaccine recipients but at titres several fold lower (Einstein et al., 2009; Kemp et al., 2008) and are thought to be serum antibodies which have accessed the site of HPV infection via the mechanisms of transudation or exudation (Schiller and Lowy, 2012). Serum antibody titres subsequently wane over the following 18 months plateauing at a level *ca.* 10-fold higher than the level of natural infection antibody (Einstein et al., 2011b). This level of antibody is maintained over several years with Gardasil® immunogenicity demonstrated through 9 years of follow up (Nygard et al., 2015) and Cervarix® through 9.4 years (Naud et al., 2014).

Immunobridging studies were carried out in young adolescents (9-15 years), the target age group for vaccination but a population for which efficacy data could not be obtained (Block et al., 2006; Pedersen et al., 2007; Petaja et al., 2009). The adolescent age groups demonstrated non-inferiority of vaccine-induced immunogenicity, measured in the form of L1 serum antibody responses, compared to older women from the age group (15-25 years) for which vaccine efficacy had been proven. It is inferred that the non-inferior immunogenicity of

the younger age group will translate into non-inferior vaccine efficacy (Lowy et al., 2015). These studies alongside the vaccine efficacy trials have supported the successful licensure of both Cervarix® and Gardasil® in over 100 countries worldwide since 2006 (Markowitz et al., 2012). Cervarix® is indicated for use in 9-25 year old females for the prevention of CIN, cervical cancer and adenocarcinoma *in situ* (AIS). Gardasil® is indicated for 9-26 year olds for the prevention of cervical, vulvar and vaginal cancers in females, and anal cancer, external genital warts, precancerous and dysplastic lesions in both females and males.

1.11.5 L1 VLP vaccine-induced cross-protection

L1 VLP vaccine-induced cross-protection was considered unlikely due to L1 VLP type-specific protection seen in animal models (Breitburd et al., 1995; Suzich et al., 1995); however, data from the vaccine efficacy trials demonstrated a degree of cross-protection against some non-vaccine genotypes closely related to either HPV16 or HPV18 within the A9 or A7 species groups.

Efficacy against non-vaccine genotypes was first reported for Cervarix® in 2006 as part of the phase II trial, GSK 001/007, where a reduction in incident infection caused by HPV31 (54.5% [95% CI, 11.5 to 77.7]) and HPV45 (94.2% [95% CI, 63.6 to 99.9]) was observed (Harper et al., 2006). The larger phase III PATRICIA trial of Cervarix® extended the analysis of vaccine efficacy to include 12 oncogenic HPV genotypes (Wheeler et al., 2012). In the ATP-E cohort efficacy against 6-months persistent infection was demonstrated for HPV31, HPV33 and HPV45 (**Table 3**). Furthermore, efficacy against CIN2+ was demonstrated for HPV31 (84.3% [95% CI, 59.5 to 95.2]) and HPV33 (59.4% [95% CI, 20.5 to 80.4]) (Wheeler et al., 2012). The cross-protective efficacy of Cervarix® has also been demonstrated for older women (26-45 years) in the VIVANE study which reported efficacy in its ATP-E cohort against 6 month persistent infection attributable to HPV31 (79.1% [97.7% CI, 27.6 to 95.6]) and HPV45 (76.9% [97.7% CI, 18.5 to 95.6]) (Skinner et al., 2014). Cross-protection against infection with non-vaccine genotypes was also evaluated in the phase III trials of Gardasil®.

Table 3. HPV vaccine efficacy against 6-month persistent infection related to non-vaccine genotypes

	Efficacy (95% CI)[a]	
Genotype	Cervarix® - PATRICIA	Gardasil® - FUTURE I and II
HPV31	76.8 (69.0 to 82.9)	46.2 (15.3 to 66.4)
HPV33	44.8 (24.6 to 59.9)	28.7 (-45.1 to 65.8)
HPV35	-19.8 (-74.1 to 17.2)	17.8 (-77.1 to 62.5)
HPV52	8.3 (-6.5 to 21.0)	18.4 (-20.6 to 45.0)
HPV58	-18.3 (-51.8 to 7.7)	5.5 (-54.3 to 42.2)
All non-vaccine A9	22.0 (13.2 to 30.0)	21.9 (0.6 to 38.8)
HPV39	4.8 (-17.7 to 23.1)	NR
HPV45	73.6 (58.1 to 83.9)	7.8 (-67.0 to 49.3)
HPV59	-7.5 (-51.8 to 23.8)	18.7 (-22.8 to 46.4)
HPV68	2.6 (-21.5 to 21.9)	NR
Non-vaccine A7	11.6 (-1.0 to 22.7)	14.8 (-19.9 to 39.6)[b]

CI: confidence interval; NR: Not reported

[a] The Cervarix® efficacy data reported from the according to protocol (ATP) group from the PATRICIA trial. The Gardasil® efficacy data from the FUTURE I and II trials reported from women DNA negative for 14 oncogenic HPV genotypes are enrolment.

[b] Date for HPV45 and HPV59 only

Source: Brown *et al.,* Journal of Infectious Diseases, 199:926 (2009); Wheeler *et al.,* Lancet Oncology, 13:100 (2012); Schiller *et al.,* Vaccine, 30S F123 (2012).

In the cohort of women HPV-naïve at enrolment, efficacy against 6-months persistent infection was most notable for HPV31 (**Table 3**), with efficacy against CIN2-3 or AIS attributable to HPV31 infection (70.0% [95% CI, 32.1 to 88.2) also reported (Brown et al., 2009).

1.12 L1 antibody serology

The antibody responses following L1 VLP immunisation have been measured using a variety of serological assays and although the antibody specificities measured by each assay do overlap the direct comparison of each system output is problematic (Schiller and Lowy, 2009). The enzyme-linked immunosorbent assay (ELISA) has probably been the most widely used with the assay generally taking the form of an indirect ELISA (Giannini et al., 2006; Nardelli-Haefliger et al., 2003). The target L1 VLP is immobilised on the solid phase and subsequently bound by anti-L1 antibodies, a reaction which is resolved by the addition of a secondary enzyme-conjugated antibody which allows Ig class and subclass differentiation. However, neutralising and non-neutralising antibodies cannot be discriminated using the ELISA as all antibodies which bind to the L1 capsid, irrespective of functionality, will be detected.

The competitive Luminex immunoassay (cLIA) was developed by Merck to monitor vaccine immunogenicity (Opalka et al., 2003). L1 VLP are immobilised on microspheres, then anti-L1 antibodies compete with a fluorescent tagged type-specific, neutralising murine L1 MAb for binding to the VLP. The fluorescent output is inversely proportional to the amount of anti-L1 antibody able to block MAb binding. This assay is highly specific as it only detects antibodies which can abrogate the binding of single antibody specificity. Antibodies which can successful compete off the MAb are assumed to be type-specific and neutralising in nature.

The L1 protein's innate ability to self-assemble was exploited to generate high titre, infectious pseudovirions (PsV) with capsids comprising the L1 and the L2 minor capsid

protein (Buck et al., 2004) which is essential for HPV infectivity (Yang et al., 2003) (**Figure 6A**). PsV can encapsidate exogenous DNA such as luciferase or green fluorescent protein (GFP) expression plasmids (**Figure 6B**) and act as viral vectors since the encapsidated plasmid is chaperoned to the host cell nucleus by the L2 minor capsid protein (Day et al., 2004). The reporter protein is then expressed and the level of expression is used as a surrogate marker for PsV infectivity.

L1L2 PsV are utilised in the HPV neutralisation assay (Pastrana et al., 2004). The PsV are pre-incubated with anti-L1 antibodies before addition to immortalised cell lines that stably express the SV40 large T antigen which in turn drives the expression of the reporter protein. If functional anti-L1 antibodies are present the PsV are neutralised; however, if such antibody specificities are not present the PsV will be taken up by the cell and the reporter plasmid delivered resulting in the expression of the reporter protein. This assay format only detects functional, neutralising antibodies, the antibody specificity thought to most likely play a role in protection against HPV infection.

The ability of HPV L1L2 PsV to bind and transduce cells of the murine genital epithelium *in vivo* to express reporter protein led to the establishment of the murine cervicovaginal challenge (CVC) model (Roberts et al., 2007). Murine genital epithelium becomes susceptible to transduction with L1L2 PsV following gentle abrasion, a situation which is thought to parallel the epithelium micro-traumas necessary for the establishment of authentic HPV infection. The murine CVC model is a more sensitive measurement of neutralising antibodies than the standard *in vitro* L1L2 PsV neutralisation assay (Longet et al., 2011).

1.13 Type-specific L1 antigenicity

1.13.1 L1 MAbs

MAbs raised against HPV L1 VLP have utility in the assessment of the antibody responses induced by vaccination and natural infection (Smith et al., 2008a; Wang et al., 1997a), the

Figure 6

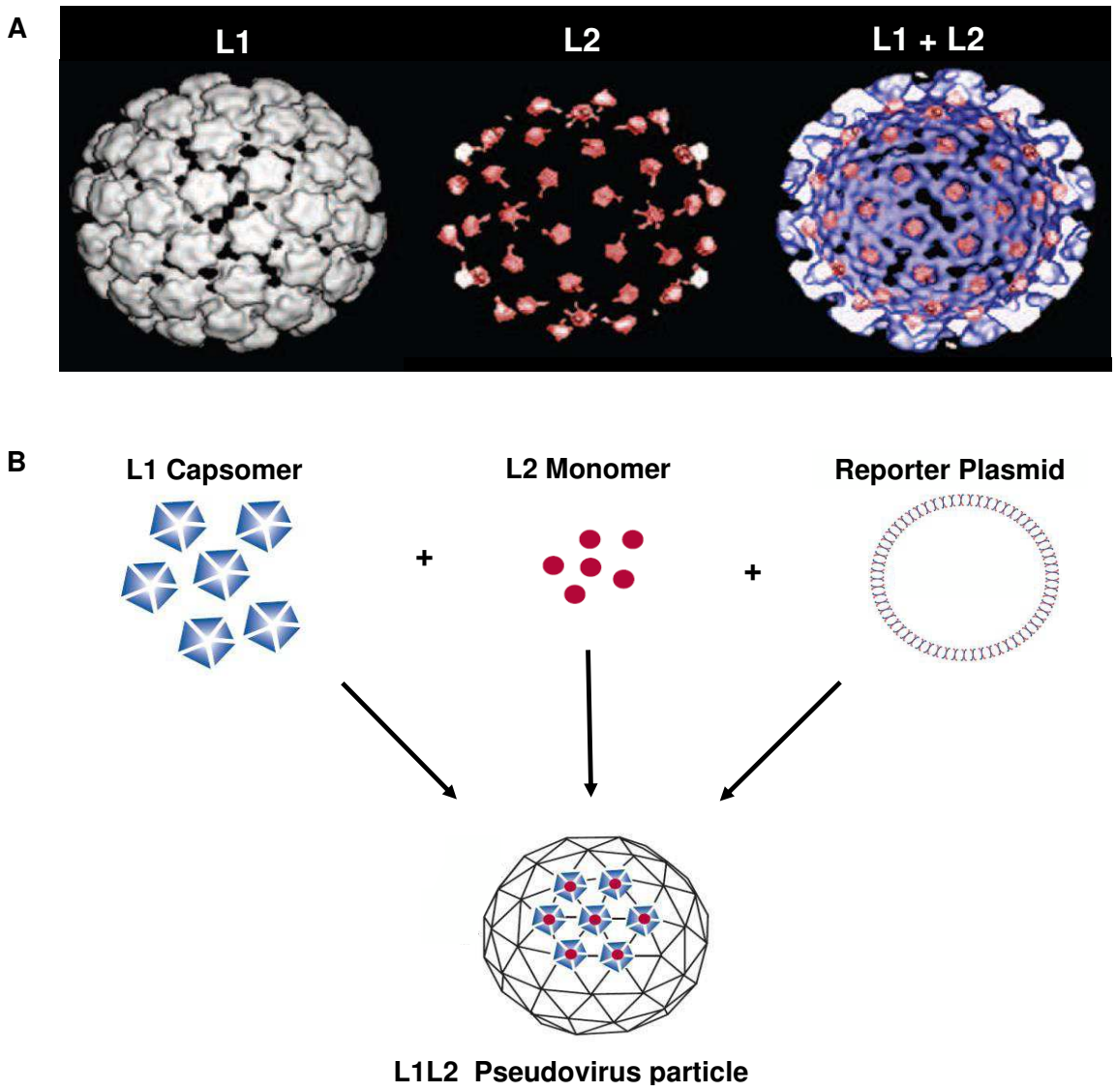


Figure 6 HPV L1L2 pseudovirus particles. (A) Computerised reconstruction of exterior view of L1-only capsid, L2-specific density alone and superimposed in interior of L1-only capsid. (B) The PsV particle is comprised of the L1 and L2 proteins and is able to encapsidate a reporter plasimd. Schiller & Muller, Lancet Oncology, 16:e217 (2015); Buck *et al.,* Journal of Virology, 82:5190 (2008).

identification and characterisation of L1 epitopes (Christensen et al., 1996a; Christensen et al., 1996b; Fleury et al., 2009) and the monitoring of L1 VLP quality and vaccine potency (Deschuyteneer et al., 2010; Shank-Retzlaff et al., 2005; Shank-Retzlaff et al., 2006; Zhao et al., 2012). The murine MAbs which have been generated against L1 VLP immunogens are almost exclusively of a type-specific nature and recognise conformational, neutralising epitopes on the surface-exposed loops of the L1 protein (Carter et al., 2003; Fleury et al., 2006; Rizk et al., 2008). A number of cross-reactive L1 MAbs have also been generated supporting the concept of common L1 epitopes but only a minority are neutralising (Rizk et al., 2008). HPV16 has historically been the most intensively studied HPV due to its high prevalence in cervical cancer and subsequently the majority of well characterised murine MAbs that are available have been generated against HPV16 L1 VLP (Christensen et al., 2001; Christensen et al., 1996a). Panels of MAbs targeting HPV6, HPV11 (Christensen et al., 1996b) and HPV18 (Christensen et al., 1996a) were also developed in parallel with the HPV16 MAbs and more recently L1 MAbs raised against HPV31, HPV33, HPV45, HPV52 and HPV58 have been generated and characterised (Brendle et al., 2010; Brown et al., 2014; Fleury et al., 2006).

The neutralising, type-specific MAb H16.V5 binds a conformational epitope comprised of amino acid residues from the DE, EF, FG and HI loops of the HPV16 L1 protein (Guan et al., 2015; Lee et al., 2015) and is able to block *ca.* 75% of the binding antibodies generated in response to HPV16 natural infection (Wang et al., 1997a). This demonstrated that some components of the murine and human L1 antibody repertoires overlapped and that murine MAbs had utility for monitoring of the HPV antibody response in humans. H16.V5 was incorporated into the cLIA, developed to monitor the type-specific antibody response following HPV vaccination (Smith et al., 2008a). This assay also incorporated type-specific MAbs which bound L1 conformation-dependent epitopes of HPV6 (H6.M48), HPV11 (H11.B2) and HPV18 (H18.J4) (Smith et al., 2008a). Like H16.V5, these MAbs were able to block type-specific anti-L1 reactivity in human vaccinee sera exploiting the overlap in human

and murine antibody repertoires, albeit to only a single antibody specificity for each genotype.

Cross-reactive L1 MAbs recognise epitopes common between HPV genotypes but rarely neutralise their prototype genotype or cross-neutralise other genotypes (Rizk et al., 2008). A limited number of L1 MAbs, which target the A10 genotype HPV6, and the A7 genotypes HPV18 and HPV45 (Brown et al., 2014; Christensen et al., 1996b; Smith et al., 2007), have demonstrated cross-neutralisation of closely related genotypes from within the same species group. For example, MAb H18.R5 was able to cross-neutralise L1L2 PsV representing HPV45 whilst MAbs H45.6G6 and H45.3C3 were able to cross-neutralise L1L2 PsV representing HPV18; however, higher IgG concentrations (*ca.* 5 to 30-fold) were required for cross-neutralisation compared to the concentration required to neutralise the prototype PsV (Smith et al., 2007). This observation indicates that even though these monoclonal specificities recognise common L1 epitopes between HPV18 and HPV45, they have reduced recognition of these epitopes when presented on the heterologous L1 protein. No L1 MAbs which cross-neutralise genotypes within the HPV16 containing A9 group have been identified to date.

1.13.2 L1 MAb epitope identification

The majority of studies aimed at identifying and characterising the epitopes of the L1 protein have utilised MAbs in conjunction with L1 VLP harbouring either individual amino acid residue substitutions or the replacement of entire sections of the L1 protein. Initial studies mapped conformationally dependent epitopes of HPV11 by transferring MAb reactivity from HPV11 to either HPV6 or HPV16 L1 VLPs via the introduction of amino acid residues specific to HPV11 into the heterologous backbones (Ludmerer et al., 1996; Ludmerer et al., 1997). These substitutions demonstrated that residues within a *ca.* 20 amino acid stretch of the DE loop were important for the binding of a subset HPV11 neutralising MAbs. Utilising chimeric L1 VLP with inter-genotype loop swaps, a second panel of HPV11 MAbs

demonstrated more complex epitope footprints which encompassed both the FG and HI loops (Ludmerer et al., 2000).

Similar work carried out with HPV6, HPV16, and HPV33 L1 MAbs provided further evidence that the L1 capsid of HPV harbours epitopes which can be restricted to a single loop and those that have more complex, multiple loop epitopes. The BC loop alone, and in conjunction with EF loop, was identified as contributing towards the epitope footprints of HPV6 MAbs (McClements et al., 2001) whilst HPV16 L1 MAbs appeared to target BC and HI single loop epitopes and FG/HI multi-loop epitopes (Christensen et al., 2001). Two HPV33 L1 MAbs targeted residues in the DE and FG loops with the BC loop alone containing the epitope of a third MAb (Roth et al., 2006). The epitope of the H16.V5 MAb has been extensively studied with several studies confirming the requirement for the FG loop (Carpentier et al., 2005; Slupetzky et al., 2001) in conjunction with the HI (Carter et al., 2003; Ryding et al., 2007) for H16.V5 binding whilst fine mapping has identified specific FG loop amino acid residues (Asn^{270}, Asn^{285} and Ser^{288}) which appeared to contribute towards the epitope footprint (Carpentier et al., 2005; Carter et al., 2003). Recently, the epitope footprint of HPV16.V5 was resolved further with cryo-electron microscope data corroborating the requirement of residues in the FG and HI loops, and identifying additional residues in the DE and EF loops (Guan et al., 2015; Lee et al., 2015).

Limited data are available on whether L1 epitope presentation is altered by the inclusion of L2 in the capsid. A number of HPV16 L1 MAbs demonstrate differential recognition of their epitopes displayed on L1 VLP compared to L1L2 PsV (Culp et al., 2007). There are structural differences between L1 VLP and L1L2 PsV, with the latter containing a higher degree of disulphide cross-linking between L1 monomers within the capsid (Fligge et al., 2001). However, the epitopes recognised by the majority of type-specific neutralising L1 MAbs appear not to be affected by the structural, and possible antigenic, changes in the capsid resulting from the inclusion of the L2 protein (Culp et al., 2007).

1.13.3 L1 domains recognised by natural infection antibodies

The L1 epitopes recognised by the antibodies generated in response to natural infection with HPV16 and HPV6 have been mapped using chimeric L1 VLP or L1 capsomers. HPV6 L1 capsomers with HPV11 loop swaps, either in single or multiple combinations, demonstrated that the antibody response to HPV6 infection targets the BC, DE and FG loops, singly or in combination with reactivity against the C-terminal portion (HPV6 aa 361-500) also observed (Orozco et al., 2005). A study mapping the antibody response to HPV16 natural infection found that the majority of the seroreactivity targeted the C-terminal portion of the L1 (HPV16 aa 172-505) with reactivity against the N-terminal portion (HPV16 aa 1-173) only observed in a limited number of sera (Wang et al., 2003). A chimeric HPV11 L1 VLP with HPV16 FG and HI loops, which retained reactivity against the H16.V5 MAb, was recognised by 84% of the sera which demonstrated C-terminal portion reactivity implying the FG and HI loops of HPV16 are immunodominant regions (Wang et al., 1997b). An attempt to fine map the L1 regions targeted by HPV16 neutralising antibodies induced in response to natural infection demonstrated that most sera targeted epitopes spanning two or more loops with the DE, FG and HI most frequently recognised (Carter et al., 2006). These data illustrate that the antibody response to HPV natural infection is polyclonal in nature and although reactivity patterns can be grouped, antibody specificities differ from one individual to another and target complex sets of L1 epitopes.

1.14 L1 cross-neutralising antibody responses in vaccine recipients

The potential of L1 VLP vaccines to induce a cross-neutralising antibody response in vaccinees was first reported in two small scale studies. HPV31 (closely related to HPV16 within the A9 species group) cross-neutralising antibodies were detected following immunisation with a monovalent L1 HPV16 VLP vaccine candidate (Pinto et al., 2006) and HPV45 (closely related to HPV18 within the A7 species group) cross-neutralising antibodies were detected following immunisation with Gardasil® or a monovalent L1 HPV18 VLP vaccine candidate (Smith et al., 2007). The potential to generate cross-neutralising

antibodies which recognise HPV31 and HPV45 following vaccination was confirmed by two larger scale studies: the first compared Cervarix® and Gardasil® immunogenicity against HPV31 and HPV45 in women aged 18-45 years (Einstein et al., 2011a) and the second demonstrated that Cervarix® sera had the potential to cross-neutralise the L1L2 PsV representing the non-vaccine A9 genotypes HPV52 and HPV58, in addition to HPV31 and HPV45 (Kemp et al., 2011).

Cervarix® sera from young adolescent girls (13-14 years) tested against a complete panel of L1L2 PsV representing the oncogenic A7 (HPV18, HPV39, HPV45, HPV59 and HPV68) and A9 (HPV16, HPV31, HPV33, HPV35, HPV52 and HPV58) genotypes demonstrated cross-neutralisation of all the A9 genotypes but recognition in the A7 species group appeared to be limited to HPV45 (Draper et al., 2011) (**Figure 7**). The difference in the breadth of response between the A9 and the A7 groups may be due to differences in the L1 amino acid sequence homology of the non-vaccine genotypes compared to the vaccine types HPV16 and HPV18. For example, HPV45 has a 88% L1 amino acid sequence homology with HPV18; however the remaining A7 non-vaccine genotypes (HPV39, HPV59 and HPV68) have L1 sequence homology to HPV18 of <80% whilst the non-vaccine A9 genotypes all share an L1 amino acid sequence homology of *ca.* 80% with HPV16 (Brown et al., 2009).

Data from these immunogenicity studies also demonstrate that whilst Cervarix® and Gardasil® vaccines have the potential to generate cross-neutralising antibodies, seropositivity rates against non-vaccine genotypes are lower than those observed against HPV16 and HPV18 and cross neutralising antibody titres are considerably lower, generally representing <1% of the vaccine type neutralising antibody titre (Draper et al., 2011; Kemp et al., 2011). It is not known whether the vaccine-induced cross-neutralising antibody response is a consequence of a low affinity interaction of an otherwise predominantly type-specific antibody. For example, the cross-neutralisation of A9 genotypes may be attributable to the human antibody equivalent of H16.V5 which exhibits some degree of cross-

Figure 7

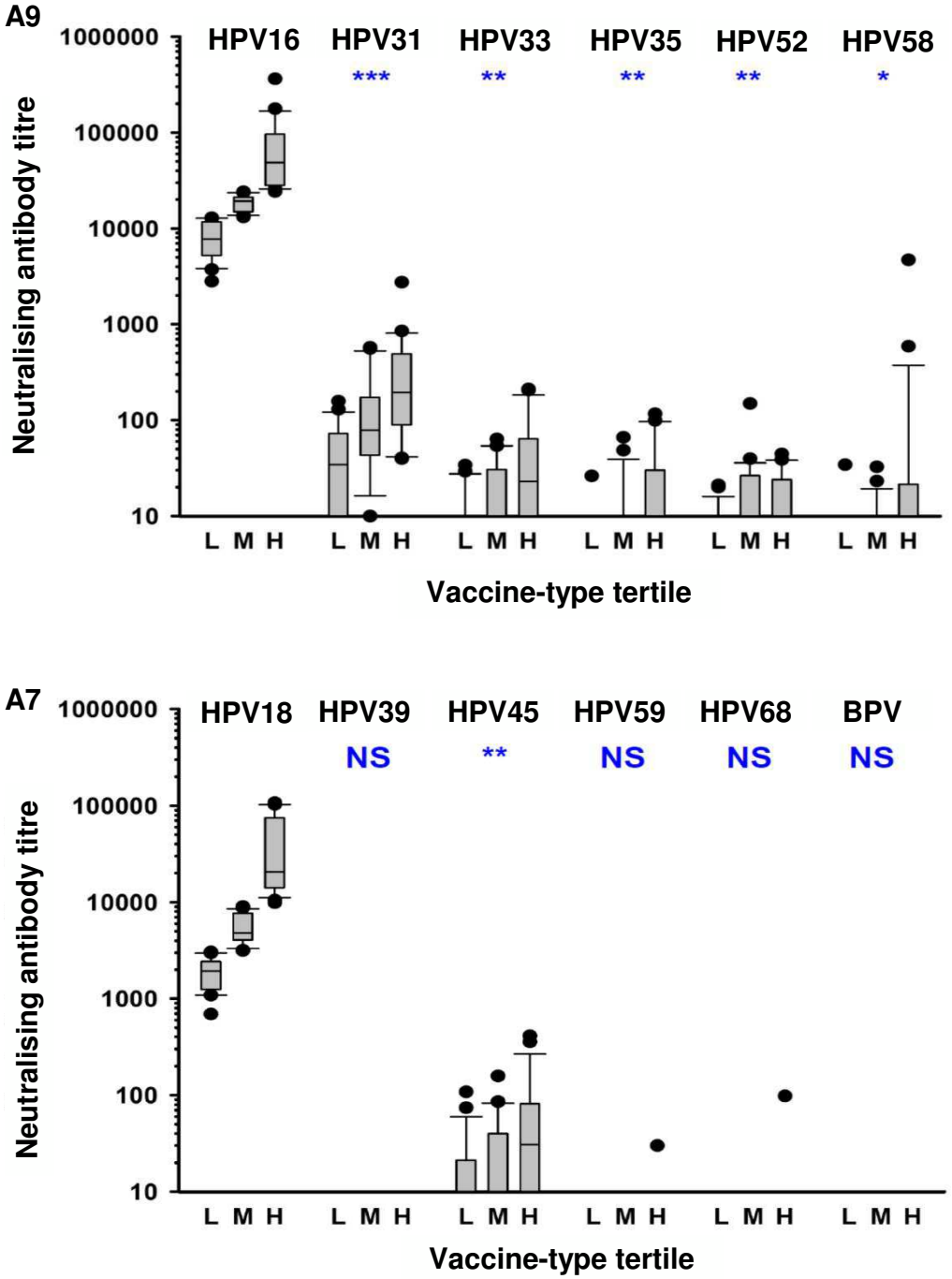


Figure 7 Cross-neutralising antibody titres related to vaccine-type neutralising antibody titres for A9 and A7 HPV genotypes. Neutralising antibody data from non-vaccine genotypes segregated according to Low (L), Middle (M) and High (H) vaccine-type tertiles. Plot shows box (median, IQR), whisker (±1.5 IQR) and outliers (>1.5 IQR). *p* values represent associated by Pearson's correlation across tertiles: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS $p > 0.05$. Draper *et al.,* Vaccine, 29:8585 (2011).

recognition not present in the murine version. Alternatively, the cross-neutralising antibody response may represent a minor antibody specificity (or specificities).

1.15 Cross-reactive L1 antigenicity

Several studies have generated L1 VLP antisera in animals in order to investigate whether common L1 epitopes existed between HPV genotypes. Initial studies used diverse means of testing the neutralising potential of the antisera including a L1 VLP haemagglutination inhibition assay and HPV16 virion infection inhibition as measured by the detection of HPV16 spliced mRNA (Roden et al., 1996b; White et al., 1998). Subsequent studies have tested antisera against PsV representing HPV genotypes *in vitro* neutralisation assays (Bousarghin et al., 2002; Combita et al., 2002; Giroglou et al., 2001b; Ochi et al., 2008). However, due to the limited number of genotypes tested, the general lack of reciprocal data and the inconsistency in choice of antigen (L1 PsV vs L1L2 PsV), assay and animal used, it is difficult to directly compare these studies.

Data from these preclinical studies did however demonstrate that even though the majority of the antibody response to L1 VLP is type-specific, cross-neutralisation was observed sporadically within the A7 and A9 species groups at antibody titres substantially lower than the type-specific titres (**Figure 8**). For example, antisera generated against L1 VLP representing A9 genotypes HPV16 and HPV31 demonstrated reciprocal cross-neutralisation which was reproducible across studies (Bousarghin et al., 2002; Combita et al., 2002; Ochi et al., 2008). In one study, rabbit antisera raised against HPV16 L1 VLP neutralised HPV16 and HPV31 at antibody titres of 204,800 and 200 respectively whilst antisera raised against HPV31 neutralised HPV16 and HPV31 at antibody titres of 800 and 1,638,400 respectively, demonstrating the difference in magnitude between the type-specific and cross-neutralising antibody responses (Ochi et al., 2008). These data imply that common L1 epitopes which are targeted by neutralising antibodies exist between closely related genotypes.

Figure 8

	Pseudovirus										
	Alpha-9						Alpha-7				
	16	31	33	35	52	58	18	39	45	59	68
VLP16	4.7	2.5	1.3		1.0	1.0	1.0	1.0	1.0	1.0	
VLP31	2.1	5.7	3.0		1.0	1.0	1.0	1.0	1.0	1.0	
VLP33	1.7	1.0	3.7			1.3	1.0	1.0	1.0	1.0	
VLP35	1.0		1.0			1.0					
VLP52	1.0	1.0			5.6	2.6	1.0				
VLP58	1.5	1.0	1.5		4.4	5.3	1.0				
VLP18	1.0		1.0		1.0	1.0	5.9				
VLP39	1.0		1.0				1.0				
VLP45	1.0	1.0	1.0			1.0	3.0	1.0	2.3	1.0	
VLP59	1.0	1.0	1.0			1.0		1.6	1.0	3.0	
VLP68											

Log_{10}
6.0
5.0
4.0
3.0
2.0
1.0

Figure 8 Heatmap summarising A7 and A9 type-specific and cross-neutralising antibody responses from preclinical L1 antigenicity studies. Data from preclinical L1 VLP immunisation studies (n=4) carried out in small mammals were pooled and presented as a heatmap representing the log_{10} transformed average neutralising titre against indicated PsV. Key indicates log_{10} heatmap gradient. Combinations which were not tested are greyed out. Combita *et al.,* J Virol, 76:6480 (2002); Giroglou *et al.,* Vaccine, 19:1783 (2001); Bousarghin *et al.,* J Clin Microbiol, 40:926 (2002); Ochi *et al.,* Clin Vaccine Immunol, 15:1536 (2008).

1.16 L1 VLP vaccine-induced protection

Neutralising antibodies in the serum or on the mucosa are the correlates or surrogates of protection for almost all prophylactic viral vaccines (Plotkin, 2008); however, no immune correlate or surrogate of protection has been defined for the HPV prophylactic vaccines since both vaccines are high efficacious and no vaccine type breakthrough infections have been reported (Stanley et al., 2012). L1 type-specific neutralising antibodies are assumed to be the immune effectors of HPV vaccine-induced type-specific protection based upon data from preclinical studies carried out in CRPV and COPV which demonstrated that passive transfer of neutralising antibodies protected animals against PV challenge (Breitburd et al., 1995; Suzich et al., 1995). More recently, work carried out using the murine CVC model demonstrated that the passive transfer of serum from Gardasil®-immunised mice conferred protection against *in vivo* genital challenge with L1L2 PsV representing the vaccine types, HPV16 and HPV18 (Longet et al., 2012).

Cross-neutralising antibodies are assumed to be the mediators of cross-protection based upon the observation that the *in vitro* detection of cross-neutralising antibodies is coincident with the cross-protection data from vaccine efficacy studies (Schiller and Lowy, 2012). If cross-neutralising antibodies generated against L1 VLP are cross-protective, then the examination of the functional characteristics and antigenic targets of these antibody specificities may help to elucidate the immunological mechanism(s) supporting the partial cross-protection induced by the HPV vaccines and contribute to our understanding of vaccine-induced host-virus interactions.

1.17 Aims and Objectives

1.17.1 Hypothesis: Common antigenic L1 domains exist between related HPV genotypes

1.17.2 Aim of thesis: To delineate the L1 domains which are recognised by inter-genotype cross-neutralising antibodies

1.17.3 Objectives:

1. **Perform a formal analysis of the vaccine-induced A9 cross-reactive L1 antibody response**

   Vaccine sera will be tested against antigens representing the A9 genotypes in both antibody binding and neutralisation assays, with the resulting serological data subjected to hierarchical clustering in order to get an overview of HPV16 vaccine-induced cross-reactive antibody response and the specificities therein.

2. **Examine the capsid amino acid sequences of the A9 genotypes using appropriate bioinformatic tools**

   The L1 and L2 protein sequences of the A9 antigens will be compared alongside available database sequences to determine how representative these antigens are of their respective genotype.

3. **Design and generate novel antigens to test cross-neutralising antibody recognition of specific L1 domains**

   L1 proteins harbouring point mutations, inter-genotype loop swaps or foreign epitope insertions, alone or in combination, will be utilised to generate VLP and/or PsV antigens.

2. Materials and Methods

2.1 Study Samples

Study-01 wherein 69 serum samples were collected from girls aged 13-14 years, who had received three doses of the Cervarix® vaccine as part of the UK's school-based National HPV Immunisation Programme (Research Ethics Committee (REC) number 09/H1013/33). Serum samples were collected in October and November 2009, a median of 5.9 months (Interquartile range [IQR] 5.7 to 6.0) after the girls received their final dose (Draper et al., 2011). Study-02 wherein 198 girls aged 12-15 years were randomised to receive either three doses of the Cervarix® vaccine or the Gardasil® vaccine as part of a Phase IV clinical trial comparing HPV vaccine immunogenicity (www.clinicaltrials.gov: NCT00956553; REC number 09/H0720/25). Study enrolment commenced October 2009 and the last sample was collected in December 2011. Month 7 serum samples from 46 study participants were selected based upon HPV31 cross-neutralising antibody titres of >450 (n=22 Cervarix® and n=24 Gardasil®) (Draper et al., 2013). Anonymised HPV DNA samples were available from liquid-based cytology (LBC) samples collected from women aged 25 years or older, attending cervical screening at eight centres in England (REC number 06/MRE01/48) (Howell-Jones et al., 2010).

2.2 Control Material

2.2.1 Antibody-control reagent

The high HPV16/18 and HPV negative plasma pools were used as positive and negative serological control reagents (Bissett et al., 2011). These reagents were generated from plasma samples taken from females of 18 years of age in September 2009, who would have been targeted for vaccination with the Cervarix® vaccine as part of the National HPV Immunisation "Catch-up" Campaign. The HPV antibody specificities of the plasma samples were evaluated by three independent laboratories and pooled to create a reagent which was not reactive against any of the HPV genotypes tested (HPV negative) and a reagent with high levels of antibody to HPV16, HPV18, HPV31, and HPV45 (High HPV16/18).

2.2.2 Heparin

Heparin (H-4784; Sigma) was included in the L1L2 PsV neutralisation assay as a positive inhibition control and as an indicator of inter-assay reproducibility.

2.3 Cell lines

The human embryonic kidney cell line, 293TT (National Cancer Institute [NCI], Bethesda, MD, USA) was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids (all Thermo Fisher Scientific) and 400µg/mL of hygromycin B (Roche), herein referred to as complete DMEM (cDMEM). Cell culture assays were performed using cDMEM without phenol red and hygromycin B, herein referred to as neutralisation DMEM (nDMEM). The *Spodoptera frugiperda* insect cell line Sf21 (Thermo Fisher Scientific) was maintained in Sf-900 II serum-free media supplemented with 5% FBS and 0.5% penicillin-streptomycin (all Thermo Fisher Scientific) herein referred to as complete Sf-900 (cSf-900).

2.4 HPV L1 and L2 gene amplification and sequencing

The L1 and L2 genes from HPV DNA positive LBC samples were amplified on a PTC-200 thermal cycler machine (Bio-Rad) using an initial denaturation step of 95^{o}C 1 min, followed by 30 to 40 cycles of 95^{o}C for 1 min, primer-specific melting temperature for 1 min and target-specific extension time at 72^{o}C, with a final extension step of 72^{o}C for 5 mins. Each amplification was carried out as standard, in a 50µL reaction volume containing: 1X High Fidelity PCR buffer, 3mM $MgSO_4$, 0.2mM dNTPs, 1.25 U Platinum® Taq DNA polymerase High Fidelity (all Thermo Fisher Scientific), 20 pmol each of target-specific forward and reverse primers and 10 µL of sample DNA. Amplification products were visualised by agarose gel electrophoresis stained with RedSafe™ nucleic acid staining solution (iNtRON Biotechnology). Amplicons were purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced using an ABI 3730 genetic analyser using target-specific sequencing primers. For primer sequences see **Supplementary Table 1**.

2.5 L1L2 PsV

2.5.1 L1L2 PsV expression plasmids

Bicistronic pXsheLL vectors, where X is the HPV genotype, containing L1 and L2 codon optimised genes for mammalian expression were available for HPV16, HPV31, HPV52 and HPV58 (JT Schiller and C Buck, NCI) and HPV33 (H Faust and J Dillner, Malmö University Hospital, Malmö, Sweden). A novel L1L2 PsV construct representing HPV35 was generated in order to expand the panel to cover all oncogenic A9 genotypes. HPV35 L1 and L2 amino acid sequences were collected from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/) database (GenBank accession numbers: M74117 (Marich et al., 1992) and X74477) and additional contemporary sequences were derived from LBC samples (JN104062-67 (Draper et al., 2011)). The consensus L1 and L2 sequences were then codon optimised for mammalian expression and the resulting genes were synthesised (Blue Heron) and inserted into the p5sheLL backbone (JT Schiller and C Buck) following a protocol provided by Chris Buck (personal communication). Briefly, the L1 and L2 genes were excised from separate Blue Heron plasmids following restriction enzyme digestion (L1: BspEI and PspXI; L2: NotI and NheI) and subcloned into the p5sheLL plasmid in conjunction with the Rapid DNA Dephos & Ligation Kit (Roche). The HPV35 L1L2 PsV construct is available from Addgene (www.addgene.org) and the sequence has been deposited on the NCI website.

Novel L1L2 PsV expression plasmids were generated throughout the study. These included L1L2 PsV representing variant lineage and chimeric L1L2 PsV with inter-genotype L1 loop swaps. Codon-optimised L1 and L2 genes were either synthesised (GeneArt®, Thermo Fisher Scientific) or generated by QuikChange® Site-Direct Mutagenesis (Stratagene), prior to subcloning into the appropriate psheLL backbone. The accuracy of the L1 and L2 genes within the PsV expression plasmids were confirmed by sequencing. For sequencing and mutagenesis primer sequences see **Supplementary Table 1**.

2.5.2 L1L2 PsV expression and purification

L1L2 PsV stocks were expressed and purified as previously described (Buck and Thompson, 2007) using an alternative protocol for capsid maturation (http://home.ccr.cancer.gov/lco/ripcord.htm) and a luciferase plasmid (pGL4.51 [luc2/CMV/Neo]; Promega) as the encapsidated reporter DNA. The alternative protocol removes L1L2 PsV 'cold capsids' that contain encapsidated cellular DNA instead of reporter DNA with the intent of improving the particle-to-infectivity (PI) ratio of the resulting L1L2 PsV stocks. Since the alternative protocol removes 'cold capsids' the L1 protein concentration in the L1L2 PsV stock is decreased, restricting the use of these antigens in assays which require a higher protein input. In order to carry out an ELISA using L1L2 PsV as the target antigen, PsV stocks with higher L1 protein concentrations were generated following the original protocol for capsid maturation (Buck and Thompson, 2007).

2.5.3 Transfection of 293TT cells

A 75cm^2 flask was seeded with 7.5 x 10^6 293TT cells in cDMEM without hygromycin B and incubated at 37°C with 5% CO_2 overnight. The cells were transfected with 19µg of pXsheLL plasmid DNA and 19µg of luciferase plasmid DNA using Lipofectamine 2000 (Thermo Fisher Scientific) before incubation at 37°C with 5% CO_2 for 6 hrs. The transfection mixture was then removed and replaced with cDMEM and protein expression occurred during a 48 hr incubation at 37°C with 5% CO_2.

2.5.4 Cell lysis and capsid maturation

The cDMEM was removed and the transfected cells were pelleted (500 x *g* for 5 mins) following trypsin (Thermo Fisher Scientific) treatment. The cell pellet was resuspended in phosphate-buffered saline (PBS) (Thermo Fisher Scientific) supplemented with 9.5mM $MgCl_2$ (Sigma) (PBS-Mg) and transferred to a 2.0mL low protein binding tube (Eppendorf) before re-pelleting (500 x *g* for 5 mins) and disposal of the supernatant. In the alternative protocol, the cell pellet was resuspended in a 1X cell pellet volume of lysis buffer containing

a final concentration of 0.4% Brij-58 (Sigma) and 0.1% RNase Cocktail (Ambion) in PBS-Mg. In the original protocol, the 1X cell pellet volume of lysis buffer contained a final concentration of 0.5% Brij-58, 0.25% Benzonase (Sigma) and 0.25% Plasmid Safe (Epicentre) in PBS-Mg. The cell lysate was then 'matured' at 37^{o}C for 24 hrs to allow disulphide bond formation between neighbouring L1 monomers which is required for the stabilisation of the capsid structure (Buck et al., 2005).

2.5.5 Purification

Iodixanol (Optiprep; Sigma) gradients were prepared by layering three concentrations (39%, 33% and 27%) on top of each other in 13 x 51mm polyallomer centrifuge tubes (Beckman Coulter), before incubation at room temperature for 2 hrs to allow a continuous gradient to form. The cell lysate produced by the alternative protocol was clarified twice by centrifugation (14,000 x *g* for 10 mins) before the double clarified supernatant was applied to an iodixanol gradient. In the original protocol, NaCl was added to the cell lysate to a final concentration of 0.85M before incubation on ice and addition of the cell lysate to an iodixanol gradient. The L1L2 PsV were subjected to fractionation at 234,000 x *g* for 3.5 hrs using a SW55Ti rotor and the Optima L-100 XP Ultracentrifuge (Beckman Coulter). Gradient fractions were collected by puncturing the bottom of the centrifugation tube. Individual fractions spanning the expected peak of infectivity were pooled and 50µL aliquots of infectious L1L2 PsV stocks were prepared and stored at -80^{o}C.

2.5.6 Protein quantification

The L1 protein concentration of the original protocol L1L2 PsV stocks, generated for use as target antigens for ELISA, were determined by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using reagents from Thermo Fisher Scientific unless otherwise stated. A sample volume of 8µL was added to 1X Novex® Tris-Glycine SDS Sample Buffer and 1X NuPAGE® Reducing Agent and denatured at 85^{o}C for 2 mins. After denaturing the sample was run on a Novex® Tris-Glycine 4-20% Gel alongside a

protein molecular weight marker (SeeBlue® Plus2 Pre-Stained Standard), bovine serum albumin (BSA) standard (1600, 800, 400 and 200 ng/well) (Pierce) and HPV16 L1 VLP final aqueous preparation (FAP) (National Institute of Biological Standards and Control [NIBSC]) at a concentration of 800ng/well. Gels were washed twice in distilled water (dH_2O) before staining with SimplyBlue™ SafeStain for 60 mins shaking at room temperature. Gels were subsequently destained and dried using a 65% methanol (Prolabo Chemicals, VWR International) and 5% glycine (Sigma) solution in conjunction with the DryEase® Mini-Gel Drying System (Thermo Fisher Scientific). The amount of L1 protein was quantified against the BSA standard using ImageJ software (U. S. National Institutes of Health, http://imagej.nih.gov/ij).

2.5.7 Electron microscopic analysis

Particle formation and size were confirmed using a JEM-1400 electron microscope (JEOL). Particles were negatively stained with phosphotungstic acid (Sigma) and adsorbed onto copper grids coated with formvar (Sigma) and carbon (**Figure 9A**).

2.5.8 Infectivity assay

The relative infectivity of the L1L2 PsV stocks generated by the alternative protocol for use in the neutralisation assay (**Figure 9B**) were determined. The inner 60 wells of a clear 96-well plate were seeded with 293TT cells at 1 x 10^4 cells per well in nDMEM and incubated at 37°C with 5% CO_2 overnight. Purified L1L2 PsV stocks were subjected to five-fold serial dilutions with each dilution tested in quadruplicate. A 100µL volume, made up of 50µL of diluted L1L2 PsV stock and 50µL of nDMEM, was added to the cells and the assay was incubated for 72 hrs at 37°C with 5% CO2. Luciferase reporter gene transduction was detected using the Steady-Glo Luciferase Assay Reagent (Promega) and the luminescent signal output read using the GloMax Multi Detection System (Promega) in accordance with manufacturer's instructions. The equivalent of a Tissue Culture Infectious Dose 50% ($TCID_{50}$) was estimated for the L1L2 PsV stock using the Spearman-Karber equation.

Figure 9

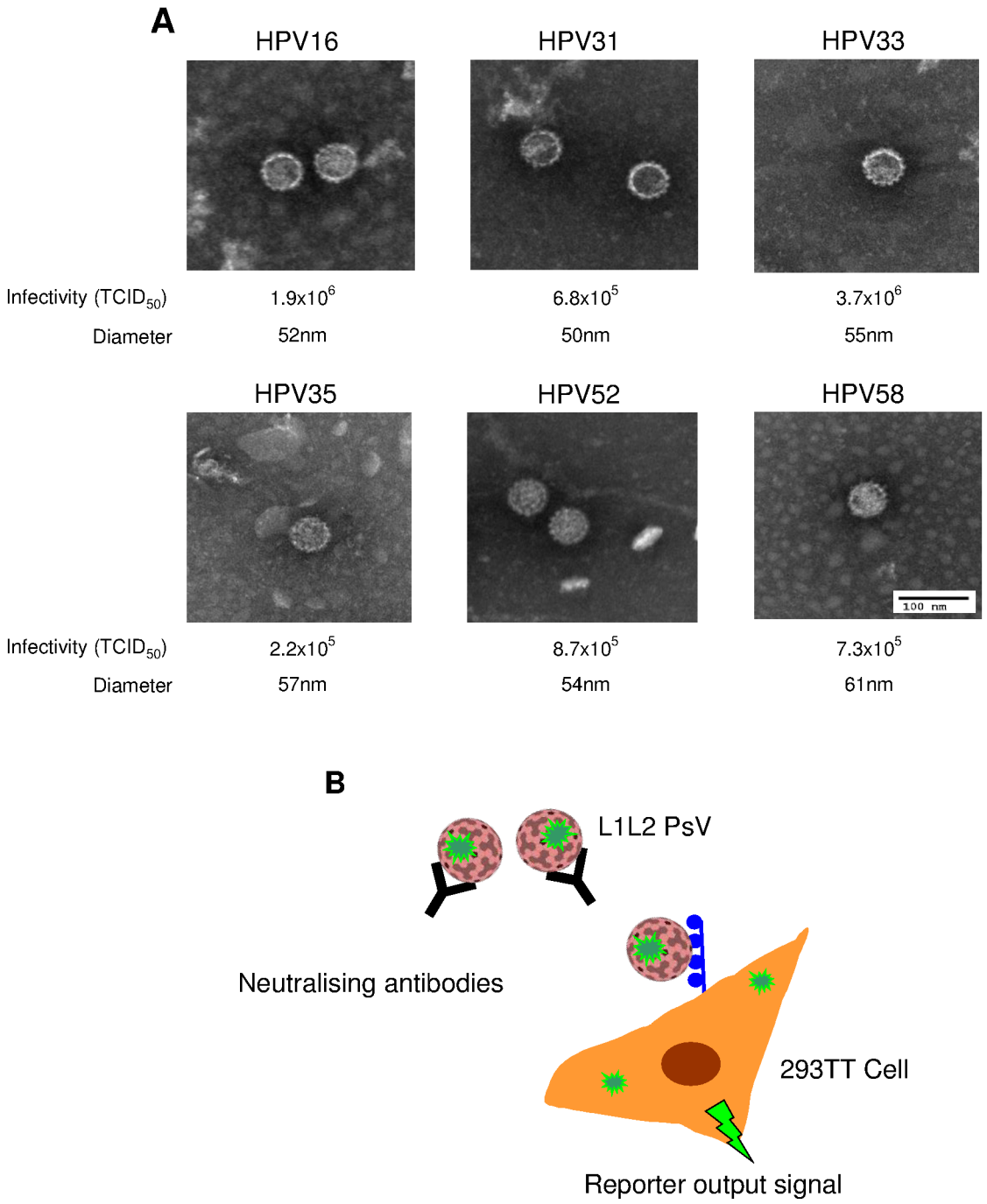


Figure 9. L1L2 PsV (A) Negatively stained EM images of A9 L1L2 PsV preparations. Infectivity represented by the $TCID_{50}$ and particle diameters are indicated for each genotype. (B) Graphical representation of L1L2 PsV utilisation in an *in vitro* neutralisation assay. Neutralising antibodies are able to recognise and bind L1L2 PsV. L1L2 PsV which are not neutralised attach and enter the host cell which is transduced to express the reporter gene producing an output signal.

2.6 L1 VLP

2.6.1 L1 VLP expression plasmids

Recombinant bacmid vectors were generated by the transposition of L1 genes representing the oncogenic A9 HPV genotypes. The L1 genes were derived from HPV DNA positive LBC samples (section 2.1) as previously described (section 2.4) and had a 100% amino acid sequence identity to the L1 gene of the homologous L1L2 PsV construct (**Figure 10**). The purified L1 PCR products were ligated into TOPO® vectors in accordance with the manufacturer's instructions and subsequently used to transform One Shot® TOP10 Chemically Competent E.coli (Thermo Fisher Scientific). L1 genes were excised from the TOPO® Vector following restriction enzyme digestion and subcloned into the pFastBac™1 (Thermo Fisher Scientific), in conjunction with Rapid DNA Dephos & Ligation Kit. Recombinant bacmid vectors were generated by the transformation of MAX Efficiency® DH10Bac™ Competent Cells (Thermo Fisher Scientific) by the recombinant L1 pFastBac™1, in accordance with the manufacturer's instructions. The accuracy of the L1 genes within the TOPO®, pFastBac™1 and recombinant bacmid vectors were confirmed by sequencing. For sequencing primer sequences see **Supplementary Table 1**.

2.6.2 Transfection of Sf21 cells

A 6-well plate was seeded with Sf21 cells at 8 x 10^5 cells per well in cSf-900 and incubated for 1 hr at 27^{o}C to allow the cells to adhere. The media was removed prior to transfection and replaced with 2.5mL of plating medium consisting of Grace's Insect Medium Unsupplemented containing 15% Supplemented Grace's Insect Medium and 10% FBS (Thermo Fisher Scientific). Each well was transfected with 1µg of recombinant bacmid DNA using Cellfectin® II (Thermo Fisher Scientific) and incubated at 27^{o}C for 5 hrs. The transfection mixture was subsequently removed and replaced with cSf-900 and the cells were incubated for a further 72 hrs at 27^{o}C

Figure 10

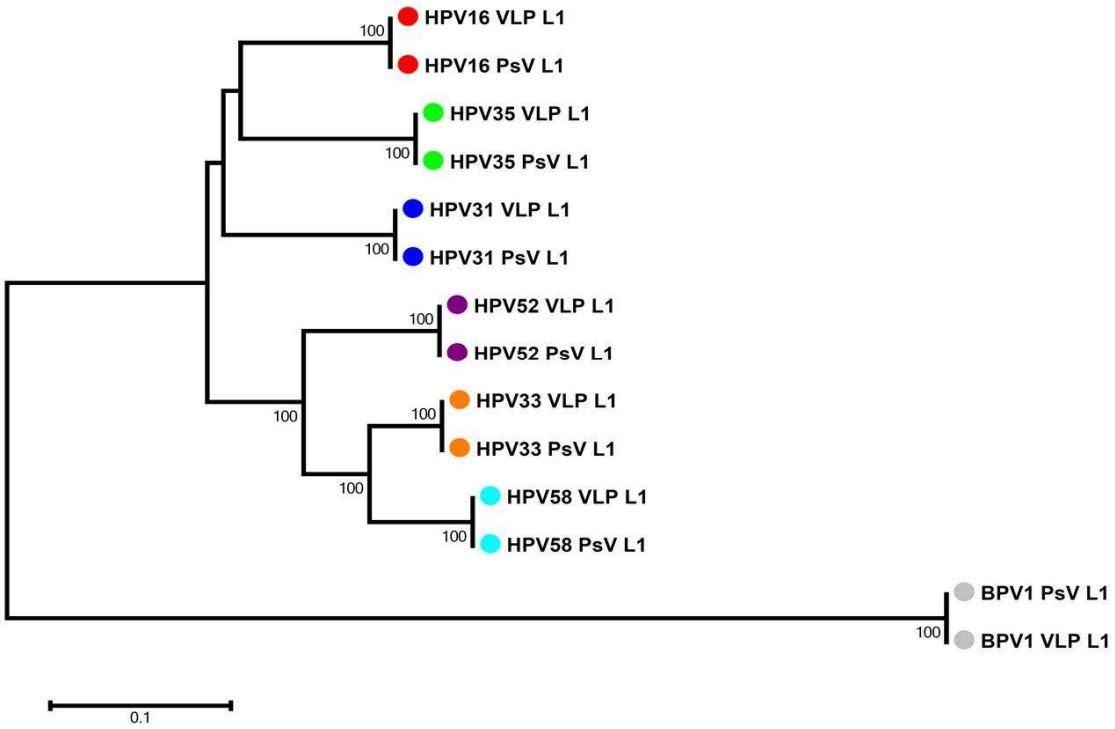


Figure 10. A9 L1 phylogenetic tree. Neighbouring joining tree generated from the L1 amino acid sequence from the VLP and PsV representing the A9 genotypes. BPV1 is used as an outlier.

2.6.3 Isolation of infectious recombinant baculovirus

The supernatant containing the infectious recombinant baculovirus was removed from the transfected wells and cellular debris pelleted by centrifugation (500 x *g* for 5 mins). The supernatant was transferred to a clean tube and stored at 4°C, protected from light.

2.6.4 Infection of Sf21 cells with recombinant baculovirus

A 175cm^2 flask was seeded with 2 x 10^7 Sf21cells in cSf-900 II SFM and incubated at 27°C for 24 hrs prior to infection. The media was then removed and replaced with 5mL of infectious recombinant baculovirus stock and the cells were incubated at room temperature for 1 hr whilst gently rocking. Subsequently, an additional 10mL of cSf-900 II SFM was added to the flask before the infected cells were incubated for a further 72 hrs at 27°C.

2.6.5 L1 VLP maturation

The infected cells were scraped off the flask and pelleted by centrifugation (500 x *g* for 5 mins). The cell pellet was resuspended in 1mL of PBS, transferred to a low protein binding tube and re-pelleted (500 x *g* for 5 mins). The supernatant was removed and the cell pellet resuspended in 1X cell pellet volume of lysis buffer containing a final concentration of 0.5% IGEPAL® CA-630 (Sigma), 1X Complete Mini Protease Inhibitor (Roche) and 10µM E-64 (Thermo) in PBS-Mg. The cell lysate was then incubated at 27°C for 24 hrs.

2.6.6 L1 VLP purification

The cell lysate was clarified twice by centrifugation (10,000 x *g* for 10 mins) before the double clarified supernatant was applied to an iodixanol gradient. The L1 VLP were subjected to ultracentrifugation and gradient fractions collected following the same method used for the L1L2 PsV (see section 2.5.5).

2.6.7 Characterisation of L1 VLP stocks

L1 VLP formation and particle size were also confirmed using a JEM-1400 electron microscope (see section 2.5.7) (**Figure 11A**). The L1 protein concentration and purity of the VLP stocks were determined using the ImageJ software following SDS-PAGE and total protein staining with SimplyBlue™ SafeStain (see section 2.5.6).

2.7 Serological Assays

2.7.1 L1 VLP & L1L2 PsV ELISA

Nunc-Immuno™ Polysorp 96-well plates were coated overnight at 4°C with either L1 VLP (**Figure 11B**) or L1L2 PsV representing a L1 protein concentration of 25ng per well diluted in tris-buffered saline (TBS) (Sigma). Wells were washed 3 times with 300µL of wash buffer (TBS and 0.05% Tween 20; Sigma) and blocked at room temperature for 3 hrs with 300µL of blocking buffer (TBS, 5% non-fat milk and 5% normal sheep serum; Abcam) followed by 3 washes. Samples were subjected to 4 to 5 serial dilutions carried out in sample buffer (TBS, 2% non-fat milk, 20% normal sheep serum and 0.05% Tween 20) before 50µL was added to wells and incubated for 1 hr at 37°C. The wells were washed 4 times before a further incubation at 37°C for 1 hr with 50µL of goat anti-human IgG alkaline phosphatase conjugated secondary antibody (Thermo Fisher Scientific) diluted in sample buffer. A final 4 washes preceded detection using the ELISA Amplification System (Thermo Fisher Scientific) according to the manufacturer's instructions with absorbance read at 490nm using the GloMax Multi Detection System (Promega). The antibody titre derived using the 50% maximal binding optical density was estimated by interpolation. The positive (High HPV16/18) and negative (HPV negative) antibody-control reagents were included in every assay (**Table 4**) and additionally a panel of six human vaccinee sera were retested against L1 VLP and L1L2 PsV representing the A9 genotypes and demonstrated good inter-assay reproducibility: L1 VLP ELISA (n=36; Pearson's r = 0.947 p <0.001) and L1L2 PsV ELISA (n=36; Pearson's r = 0.819; p <0.001). The denatured L1 VLP ELISA were carried out following the above method except that the L1 VLP were denatured prior to coating by

Figure 11

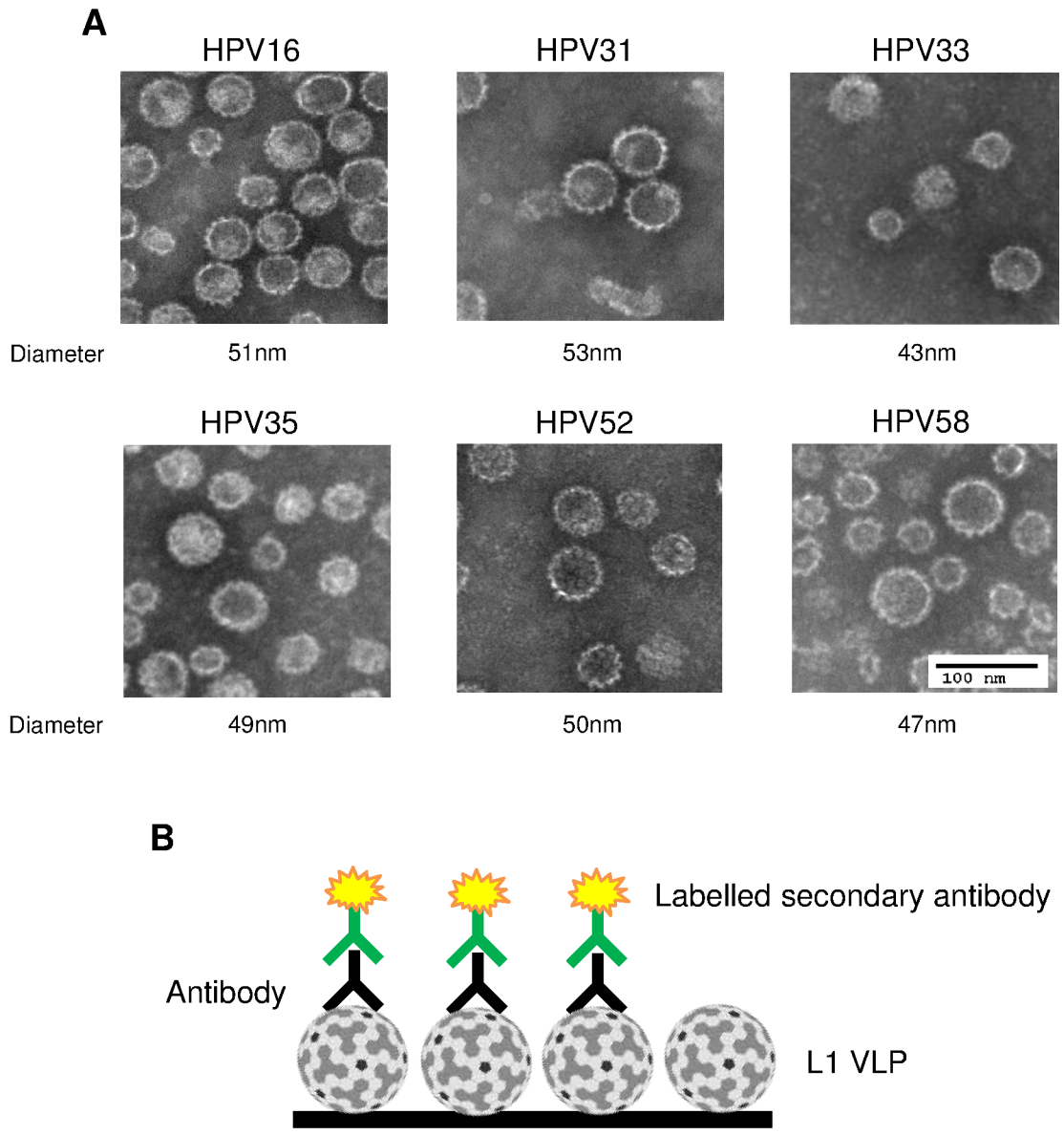


Figure 11. L1 VLP (A) Negatively stained EM images of A9 L1 VLP preparations. Particle diameters are indicated for each genotype. (B) Graphical representation of L1 VLP utilisation as the target antigen in an indirect ELISA.

incubation at 68°C for 30 mins in a solution of 0.1M $NaHCO_3$ (Sigma) containing 15mM dithiothreitol (DTT) (Thermo Fisher Scientific). The denatured L1 VLP were subsequently diluted to a concentration of 500ng/mL of L1 protein in TBS and Polysorp 96-well plates were coated at 25ng per well overnight at 4°C.

2.7.2 L1L2 PsV neutralisation assay

The assay was performed as originally described (Pastrana et al., 2004) with some modifications. The inner 60 wells of a clear 96-well plate were seeded with 293TT cells at 1 x 10^4 cells per well in nDMEM and incubated at 37°C with 5% CO_2 overnight. Serum samples were heat inactivated (56℃ for 30 mins) prior to testing. Samples were subjected to 4 to 5 serial dilutions before a volume of 55µL was incubated at room temperature for 1 hr with 55µL of L1L2 PsV at a standard input of 300 $TCID_{50}$. Subsequently 100µL was transferred to the cells and the assay was incubated for 72 hrs at 37°C with 5% CO_2. Luciferase reporter gene transduction was detected as previously described (see section 2.5.8). The antibody titre resulting in an 80% reduction of luciferase signal produced by the control wells containing L1L2 PsV only was estimated by interpolation. Heparin, either titrated or at a single input concentration (1mg/mL), was tested alongside positive (High HPV16/18) and negative (HPV negative) antibody-control reagents in every assay (**Table 4**). Good inter-assay reproducibility was demonstrated by testing a panel of six sera against the A9 L1L2 PsV (n=36; Pearson's $r = 0.976$; $p < 0.001$).

2.7.3 L1 VLP competition of neutralising antibodies

The L1L2 PsV neutralisation assay was carried out following the above method except the serial diluted samples were pre-incubated for 1 hr at room temperature with 1µg of L1 VLP representing HPV16, HPV31 or HPV33 prior to incubation with the L1L2 PsV. A 100µL volume of the antibody/L1 VLP/L1L2 PsV mixture was transferred to 293TT cells, incubated for 72hrs at 37°C before the 80% reciprocal neutralisation titres were estimated by interpolation.

Table 4. HPV control reagent reproducibility data against A9 L1 and L1L2 targets

HPV	Assay	High HPV16/18[a]		HPV Negative[b]		Heparin[c]	
		N	Median (IQR)	N	Median (IQR)	N	Median (IQR)
16	L1L2 PsV Neutralisation	19	64,162 (40,034 - 74,499)	34	10 (10 - 10)	8	12.1 (8.1 - 15.9)
	L1L2 PsV Binding	10	63,605 (55,806 - 80,794)	32	10 (10 - 10)	-	-
	L1 VLP Binding	15	64,192 (45,627 - 70,535)	51	10 (10 - 10)	-	-
31	L1L2 PsV Neutralisation	18	489 (402 - 593)	32	10 (10 - 10)	7	3.1 (2.7 - 5.1)
	L1L2 PsV Binding	6	851 (758 - 955)	11	10 (10 - 10)	-	-
	L1 VLP Binding	10	1,784 (1,240 - 1,907)	19	10 (10 - 10)	-	-
33	L1L2 PsV Neutralisation	10	71 (50 - 78)	16	10 (10 -10)	5	6.7 (6.6 - 18.1)
	L1L2 PsV Binding	6	322 (305 - 406)	11	10 (10 -10)	-	-
	L1 VLP Binding	8	916 (853 - 2,101)	21	10 (10 -10)	-	-
35	L1L2 PsV Neutralisation	3	37 (36 - 38)	3	10 (10 - 10)	5	2.3 (2.2 - 2.8)
	L1L2 PsV Binding	3	381 (338 - 460)	5	10 (10 - 10)	-	-
	L1 VLP Binding	5	3,195 (2,988 - 4,812)	3	10 (10 - 10)	-	-
52	L1L2 PsV Neutralisation	3	10 (10 - 10)	3	10 (10 - 10)	5	22.8 (7.7 - 24.5)
	L1L2 PsV Binding	4	128 (91 -139)	6	10 (10 - 10)	-	-
	L1 VLP Binding	5	846 (829 - 5,361)	15	10 (10 - 10)	-	-
58	L1L2 PsV Neutralisation	9	20 (10 - 30)	12	10 (10 - 10)	5	8.3 (3.5 - 12.5)
	L1L2 PsV Binding	4	10 (10 - 73)	6	10 (10 - 10)	-	-
	L1 VLP Binding	5	960 (923 - 4,349)	15	10 (10 - 10)	-	-

N, Number of data sets used to calculate median and inter-quartile range (IQR).

[a] Median (IQR) of 80% inhibition titre (L1L2 PsV Neutralisation) or 50% binding titre (L1L2 PsV and L1 VLP Binding). For calculation purposes High HPV16/18 neutralisation titres of <20 and ELISA titres of <100 were assigned a value of 10.

[b] Median (IQR) of 80% inhibition titre (L1L2 PsV Neutralisation) or 50% binding titre (L1L2 PsV and L1 VLP Binding). For calculation purposes HPV Negative neutralisation titres of <40 and ELISA titres of <100 were assigned a value of 10.

[C] Median (IQR) of 50% inhibition concentration (µg/mL) L1L2 PsV Neutralisation only.

2.8 Antibody enrichment on L1 VLP

2.8.1 L1 VLP coupling to magnetic sepharose beads

The coupling reaction was carried out using NHS Mag Sepharose beads (GE Healthcare) in conjunction with the NHS HP SpinTrap Buffer Kit (GE Healthcare). A single reaction volume of NHS Mag Sepharose beads (25µL slurry volume containing a 5µL bead volume) was transferred to a 1.5mL tube and placed on a magnetic rack. The storage solution was removed from the beads and replaced with 500µL of ice cold equilibration buffer (0.1M HCl). L1 VLP representing HPV16 and non-vaccine A9 genotypes were prepared at a concentration of 10µg and 5µg, respectively, in coupling buffer (0.15M triethanolamine, 0.15M NaCl, pH 8.3). The equilibration buffer was removed from the beads and replaced with 500µL of L1 VLP solution and the coupling reaction was subjected to end-over-end mixing overnight at 4^{o}C. The coupling solution was removed and the beads were blocked using three alternative 500µL incubations with Blocking Buffer A (0.5M ethanolamine, 0.5M NaCl, pH 8.3) and Blocking Buffer B (0.1M NaAc, 0.5M NaCl, pH 4.0), followed by three additional 500µL incubations with DMEM supplemented with 10% FBS. The L1 VLP coupled beads were stored in a 500µL volume of DMEM supplemented with 10% FBS at 4^{o}C.

2.8.2 Antibody depletion on L1 VLP

The serum samples for adsorption were diluted 5-fold in DMEM supplemented with 10% FBS and a 350µL volume incubated with the L1 VLP coupled beads by end-over-end mixing for 1 hr at room temperature. The post absorption serum fraction was separated from the beads using a magnetic rack and transferred to a fresh L1 VLP coupled bead set for a second round of adsorption. The serum fraction was subsequently separated from the second bead set and clarified twice using the magnetic rack to ensure that no beads were carried over, before storage at -20^{o}C.

2.8.3 Antibody elution from L1 VLP

Both L1 VLP coupled bead sets were washed 3 times in 500µL of DMEM supplemented with 10% FBS. The residual antibody activity in the final washes was below the detection threshold of the L1L2 PsV neutralisation assay. Antibody elution was performed as described elsewhere with minor modifications (Li et al., 2009). Antibodies were eluted from the beads using 0.1M glycine-HCl (Sigma) with a step-wise reduction in pH: 3 x 50µL at pH 2.9, 3 x 50µL at pH 2.4 and 3 x 50µL at pH 1.9. The beads were vortexed for 30 seconds after each 50µL addition and the eluted antibody fraction was separated from the beads using a magnetic rack and neutralised with 1M Tris-HCl pH 9 (Sigma). The eluted antibody fractions from both bead sets were then combined and concentrated using a Vivaspin 500 column (GE Healthcare) and stored at -20°C. To control for non-specific adsorption each serum was also subjected to two rounds of adsorption on, and elution from, beads coupled with 10 µg BSA. The BSA eluted fractions were found to have levels of neutralising antibody below the detection threshold of the neutralisation assay when tested against HPV16 L1L2 PsV.

2.9 Bioinformatics and statistical analyses

2.9.1 Hierarchical clustering of serological data

Pairwise Euclidean distances were calculated for the Log_{10}-transformed serological data generated from the L1L2 PsV neutralisation assay, L1 VLP and L1L2 PsV ELISA which generated distance matrices which were then clustered using a neighbour joining algorithm (http://evolution.genetics.washington.edu/phylip.html) producing the serological and viral dendrograms. The resulting viral dendrograms were bootstrapped by resampling the sera data to generate 500 pseudoreplicates. Due to the limited number of viral targets sampled relative to the number of sera, it was not possible to bootstrap the serological dendrograms. Dendrograms were viewed using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The serological data were then represented by a heat map ordered according to the resulting serological and viral dendrograms.

2.9.2 L1 and L2 amino acid sequence analysis

Available L1 and L2 gene sequences representing the A9 genotypes HPV16, HPV31, HPV33, HPV35, HPV52 and HPV58 were downloaded from the NCBI database. Only whole genome sequences (WGS) (n=265) or partial sequences encompassing both the L1 and L2 genes (n=34) were considered for this analysis. The full length L1 and L2 amino acid sequences were extracted separately and then adjoined so that both sequences were in the same reading frame, with the L1 sequence positioned in front of the L2 sequence. The L1 and L2 amino acid sequences representing the A9 PsV were downloaded from the NCI database (http://home.ccr.cancer.gov/LCO/packaging.htm). The A9 genotype reference sequences were the same as those given in the Papillomavirus Episteme database (http://pave.niaid.-nih.gov) and the representative sequences for each A9 variant lineage and sublineage were taken from the paper by Burk and colleagues (Burk et al., 2013). For sequence accession numbers and source references see **Supplementary Table 2**. Using MEGA v6 (Tamura et al., 2013) the adjoined L1 and L2 amino acid sequences were aligned and analyzed using a Neighbour-Joining tree algorithm with the resulting phylogenetic tree supported by bootstrap values of ≥80% (n=500 iterations). L1 and L2 amino acid sequence diversity of the A9 variant lineages from their representative L1L2 PsV were also calculated using MEGA v6.

2.9.3 L1 amino acid diversity analysis

The L1 amino acid sequence of the A9 L1L2 PsV were aligned and exported as a FASTA formatted file using MEGA v6, this FASTA file was subsequently used as the input file for further analysis. Amino acid charge was calculated using EMBOSS Pepinfo (www.ebi.ac.uk/Tools/seqstats/emboss_pepinfo/) whilst hydrophobicity and molecular weight scores were determined using ProtScale software (web.expasy.org/protscale/) with a scale normalised from 0 to 1. The hydrophobicity scale used was determined by Eisenberg (Eisenberg et al., 1984). All analyses were carried out using a window size of 9.

2.9.4 L1 modelling

L1 homology models were created from the L1 amino acid sequence of each A9 PsV using SWISS MODEL (http://swissmodel.expasy.org/) (Bordoli et al., 2009; Schwede et al., 2003). The crystal structure of the HPV16 L1 capsomer (Protein Data Bank [PDB] code: 2R5H) (Bishop et al., 2007) was used as the template to which the target amino acid sequences were modelled. The quality of the predictive models was measured by the Global Model Quality Estimation (GMQE) score which combines properties from the target-template alignment and represents the expected accuracy of the resulting model. The GMQE score ranges from 0 to 1, with a score of 1 indicating the highest level of quality estimation reliability (Biasini et al., 2014). DeepView Swiss-Pdb viewer v4.0 (Guex and Peitsch, 1997) was used to model the positions of amino acid residues of interest on to the crystal structure of the HPV16 L1 capsomer and the L1 homology models. Additionally, pairwise L1 model comparisons were performed by superimposition and predicted structural differences between models were measured in angstroms (Å). The superimposition of L1 homology models was supported by a Root Mean Squared (RMS) value. The RMS value is a measure of the degree of relatedness between the two models and represents the average distance in Å between corresponding atoms in the two models. A model compared to itself would have an RMS value of 0 and the lower the RMS value the closer two models are related (Guex and Peitsch, 1997).

2.9.5 Statistical methods

Tests were 2-tailed where appropriate and performed using the statistical package Stata 12.1 (StataCorp LP). The Wilcoxon paired signed-rank test was used for the comparison of antibody titres between different assay systems and antigen targets. The Fisher's Exact test was used to determine whether there was a difference in seropositivity rates between the L1L2 PsV neutralisation assay and the L1 VLP ELISA or L1L2 PsV ELISA for non-vaccine A9 genotypes. Pearson's correlation was used to evaluate the relationship between HPV16 antibody titres and the inter-assay reproducibility of antibody titres. Inter-rater agreement

between the L1L2 PsV neutralisation assay and the L1 VLP ELISA or L1L2 PsV ELISA were generated using Kappa (k) statistics wherein a k of ≤0.20 is generally considered *Poor*, a k between 0.21 - 0.40 is considered *Fair*; a k between 0.41 - 0.60 *Moderate*, a k between 0.61 - 0.80 *Substantial* and k of between 0.81 - 0.99 an *Almost Perfect* agreement. Sensitivity and specificity determinations (including 95% CI) were also generated. The McNemar test was used to assess discordance between the L1 VLP and L1L2 PsV binding results and the L1L2 PsV neutralisation assay results. The Mann-Whitney test was used for the comparison of L1 amino acid hydrophobicity and molecular weight scores between HPV16 and the non-vaccine A9 genotype.

3. Results

3.1 Cross-neutralising antibodies display a range of A9 inter-genotype specificities

3.1.1 Background

The current HPV L1 VLP vaccines, Cervarix® and Gardasil®, demonstrated a degree of cross-protection in clinical trials against genotypes closely related to the vaccine types, particularly HPV31 and HPV33 which are related to HPV16 in the A9 species group and HPV45 which is related to HPV18 in the A7 species group (Brown et al., 2009; Wheeler et al., 2012). Cross-protection is coincident with the detection of L1 cross-neutralising antibodies in the serum of vaccine recipients (Draper et al., 2011; Einstein et al., 2011a; Kemp et al., 2011) raising the possibility that cross-neutralising antibodies may be a surrogate, if not the immune effector, of vaccine-induced cross-protection. HPV vaccine-type immunogenicity studies use the detection of L1 binding antibodies as a surrogate for the presence of neutralising antibodies since a good correlation exists between the results obtained for the L1L2 PsV neutralisation assay and the less laborious L1 VLP ELISA (Dessy et al., 2008; Kemp et al., 2008) even though the different antigenic targets measure different antibody specificities (Schiller and Lowy, 2009). It is not known whether cross-reactive L1 binding antibodies would act as a good surrogate for cross-neutralisation antibodies should the monitoring of such antibody specificities be a desirable adjunct to future vaccine immunogenicity studies or post-vaccine surveillance.

The L1 VLP induced cross-reactive antibody response is poorly understood. The limited data from pre-clinical studies demonstrates that the generation of cross-neutralising antibodies is less frequent than type-specific neutralising antibodies and that titres are substantially lower (Bousarghin et al., 2002; Combita et al., 2002; Giroglou et al., 2001b; Ochi et al., 2008). These observations are in agreement with the cross-neutralisation data generated from testing human vaccinee sera (Draper et al., 2011; Kemp et al., 2011). It is not known

whether the cross-neutralising antibody response is a consequence of a low affinity interaction of an otherwise predominantly type-specific antibody or whether it represents a minor antibody specificity (or specificities), the generation and maintenance of which may be precarious over time. The HPV16 L1 VLP cross-neutralising antibody response also has a greater breadth, demonstrated by the potential to recognise all the non-vaccine A9 genotypes whilst the HPV18 L1 VLP cross-neutralising response was essentially limited to recognition of HPV45 (Draper et al., 2011).

3.1.2 Aim of chapter: To delineate the L1 VLP induced A9 cross-reactive antibody response

3.1.3 Specific objectives

1. Carry out a formal analysis of the vaccine-induced A9 cross-reactive L1 antibody response
2. Use these data to describe the antigenic relationship between A9 L1 proteins

3.1.4 Results

The serum samples utilised in this project originated from two separate studies: Study-01 and Study-02. In Study-01, serum samples were retrospectively collected from girls aged 13-14, *ca.* 6 months after receiving the third dose of the Cervarix® vaccine as part of the UK's school-based National HPV Immunisation Programme which commenced in September 2008 (Draper et al., 2011). In Study-02, a Phase IV clinical trial comparing the immunogenicity of Cervarix® versus Gardasil®, serum samples were available from girls aged 12-15, collected 1 month after receiving the third vaccine dose (Draper et al., 2013).

3.1.4.1 Seroreactivity of A9 Cervarix® vaccine antibodies to L1 and L1L2 antigens

Study-01 serum samples (n=69) had previously been tested in the L1L2 PsV neutralisation assay for the presence of neutralising antibodies targeting the A9 HPV genotypes (16, 31,

33, 35, 52 & 58) (Draper et al., 2011) and were subsequently tested here for binding antibodies against L1L2 PsV and L1 VLP representing the A9 genotypes. The antigens used in the binding and neutralisation assays that represent a particular A9 genotype shared 100% L1 amino acid sequence homology.

All samples (n=69, 100%) were positive for antibodies targeting L1 and L1L2 antigens representing the vaccine type HPV16 in all three assay systems; however, differences in assay-specific seropositivity rates were apparent for the non-vaccine A9 genotypes (**Table 5**). The L1 VLP binding assay had higher rates of seropositivity for HPV33, HPV35, HPV52 and HPV58 in comparison to the L1L2 PsV neutralisation assay. The L1L2 PsV binding assay demonstrated similar rates of seropositivity compared to the L1L2 PsV neutralisation assay except for HPV58 where a significantly higher number of samples were positive for binding antibodies (**Table 5**). In addition all samples were tested for binding antibodies which target denatured L1 VLP representing HPV16 and HPV31. No samples were positive for antibodies which recognised denatured HPV16 L1 VLP and only one sample was positive against denatured HPV31 L1 VLP (n=1, 1.4%).

There were good correlations observed between HPV16 antibody titres in the L1L2 PsV binding (Pearson's r = 0.912; p <0.001) and L1 VLP binding (r = 0.833; p <0.001) assays compared to the L1L2 PsV neutralisation assay. However, there were minor differences in the titres generated by each assay system with a median 1.3-fold (IQR, 0.9 to 1.7; Wilcoxon paired signed-rank test, p = 0.005) increase in the HPV16 antibody titre observed in the L1L2 PsV binding assay compared to the L1L2 PsV neutralisation assay. In contrast a median 1.3-fold (IQR, 0.8 to 2.5; p = 0.051) decrease in antibody titre was observed in the L1 VLP binding assay (**Table 5**) compared to the L1L2 PsV neutralisation assay.

Differences in the magnitude of the antibody titres between the three assay systems were more apparent for the non-vaccine genotypes (**Table 5**). Whilst HPV31 seropositivity for

Table 5. Seroreactivity of L1 antibodies against A9 L1 and L1L2 targets in binding and neutralisation assays Study-01 serum samples: Cervarix® vaccine recipients n=69

HPV	Assay	Seropositivity		Antibody titre		% of 16 titre[c]
		N (%)	*p* value[a]	Median (IQR)	*p* value[b]	Median (IQR)
16	L1L2 PsV Neutralisation	69 (100)	-	19,258 (11,730 - 28,132)	-	-
	L1L2 PsV Binding	69 (100)	-	23,031 (11,129 - 43,392)	**0.005**	-
	L1 VLP Binding	69 (100)	-	9,279 (7,290 - 44,719)	0.051	-
31	L1L2 PsV Neutralisation	60 (87.0)	-	78 (40 - 173)	-	0.38 (0.23 - 0.94)
	L1L2 PsV Binding	55 (79.7)	0.361	229 (122 - 526)	**<0.001**	0.82 (0.49 - 1.40)
	L1 VLP Binding	58 (84.1)	0.810	623 (503 - 713)	**<0.001**	5.45 (1.13 - 8.12)
33	L1L2 PsV Neutralisation	29 (42.0)	-	10 (10 - 27)	-	0.09 (0.05 - 0.19)
	L1L2 PsV Binding	24 (34.8)	0.484	10 (10 - 197)	**0.009**	0.11 (0.04 - 0.38)
	L1 VLP Binding	47 (68.1)	**0.003**	378 (10 - 640)	**<0.001**	1.12 (0.20 - 3.85)
35	L1L2 PsV Neutralisation	15 (21.7)	-	10 (10 - 10)	-	0.07 (0.04 - 0.12)
	L1L2 PsV Binding	20 (29.0)	0.434	10 (10 - 113)	**<0.001**	0.10 (0.04 - 0.22)
	L1 VLP Binding	42 (60.9)	**<0.001**	329 (10 - 571)	**<0.001**	0.75 (0.13 - 3.60)
52	L1L2 PsV Neutralisation	22 (31.9)	-	10 (10 - 21)	-	0.08 (0.04 - 0.13)
	L1L2 PsV Binding	12 (17.4)	0.075	10 (10 - 10)	0.813	0.05 (0.03 - 0.12)
	L1 VLP Binding	41 (59.4)	**0.002**	230 (10 - 485)	**<0.001**	0.70 (0.13 - 3.43)
58	L1L2 PsV Neutralisation	10 (14.5)	-	10 (10 - 10)	-	0.06 (0.04 - 0.12)
	L1L2 PsV Binding	26 (37.7)	**0.003**	10 (10 - 173)	**<0.001**	0.10 (0.04 - 0.34)
	L1 VLP Binding	45 (65.2)	**<0.001**	282 (10 - 612)	**<0.001**	0.97 (0.16 - 3.82)

For calculation purposes neutralisation titres of <20 and ELISA titres of <100 were assigned a value of 10
IQR, inter-quartile range
[a] *p* values generated by Fisher's Exact with significant difference in seropositivity rates from the L1L2 PsV neutralisation assay highlighted in bold type
[b] *p* values obtained using the Wilcoxon paired signed-rank test represent differences in L1L2 PsV binding and L1 VLP binding antibody titres from L1L2 PsV neutralisation titres with significant differences highlighted in bold.
[c] Non-vaccine antibody titres represented as a % of the corresponding HPV16 titre

L1L2 PsV binding (n = 55; 80%; Fisher's exact test $p = 0.361$) and L1 VLP binding (n = 58; 84%; $p = 0.810$) were similar to the L1L2 PsV neutralisation assay (n = 60; 87%), antibody titres increased by a median 2.3-fold (IQR, 1.0 to 3.7; Wilcoxon paired signed-rank test, $p < 0.001$) in the L1L2 PsV binding assay and by median 5.9-fold (IQR, 2.4 to 9.6; $p < 0.001$) in the L1 VLP binding assay compared to the L1L2 PsV neutralisation assay. This tendency towards a stepwise increase in antibody titres between the L1L2 PsV neutralisation assay, the L1L2 PsV binding assay and the L1 VLP binding assay was also apparent with antigens representing HPV31, HPV35, HPV52 and HPV58 (**Table 5**).

The antibody titres against non-vaccine A9 genotypes were very low in comparison with the titres against HPV16, with cross-reactive antibodies generally representing <1% of the HPV16 antibody titre in all three assay systems (**Table 5**). There was a trend towards an increase in the proportion of cross-reactive antibodies relative to the HPV16 response from the L1L2 PsV neutralisation, through the L1L2 PsV binding assay to the L1 VLP binding assay. For example, for HPV31 the median percentage of HPV16 titre was 0.38% (IQR, 0.23 to 0.94%) in the L1L2 PsV neutralisation assay, increasing to 0.82% (IQR, 0.49 to 1.40%) in the L1L2 PsV binding assay and 5.45% (IQR, 1.13 to 8.12%) in the L1 VLP binding assay (**Table 5**).

The utility of vaccine-induced cross-reactive binding antibody detection as a surrogate marker for the presence of cross-neutralising antibodies was assessed for L1 VLP and L1L2 PsV antigens (**Table 6**). The L1 VLP and the L1L2 PsV binding assays demonstrated reduced sensitivity and specificity compared to the L1L2 PsV neutralisation assay. For example, both the HPV31 L1L2 PsV and L1 VLP binding assays had a sensitivity of 90% (95% CI, 80 to 96) and a specificity of 89% (95% CI, 52 to 100) and 56% (95% CI, 21 to 86) respectively, compared to the L1L2 PsV neutralisation assay. The lower specificity of the two binding antigens resulted partly from the detection of cross-reactive binding antibodies in the absence of cross-neutralising antibodies. For example, 4 serum samples tested positive for

Table 6. Sensitivity and specificity of binding antibodies as a surrogate for A9 cross-neutralising antibodies Study-01 serum samples: Cervarix® vaccine recipients n=69

HPV	Binding antigen	Both positive	Neutralisation + Binding -	Neutralisation - Binding +	Both negative	Sensitivity (95% CI)	Specificity (95% CI)	κ (95% CI)	*p* Value
31	L1L2 PsV	54	6	1	8	90% (80-96)	89% (52-100)	0.638 (0.397 - 0.879)	0.125
	L1 VLP	54	6	4	5	90% (80-96)	56% (21-86)	0.416 (0.118 - 0.714)	0.754
33	L1L2 PsV	17	12	7	33	59% (39-77)	83% (67-93)	0.421 (0.205 - 0.638)	0.360
	L1 VLP	24	5	23	17	83% (64-94)	43% (27-60)	0.233 (0.038 - 0.427)	**<0.001**
35	L1L2 PsV	11	4	9	45	73% (45-92)	83% (71-92)	0.506 (0.276 - 0.736)	0.267
	L1 VLP	13	2	29	25	87% (60-98)	46% (33-60)	0.200 (0.045 - 0.355)	**<0.001**
52	L1L2 PsV	5	17	7	40	23% (8-45)	85% (72-94)	0.089 (-0.104 - 0.318)	0.064
	L1 VLP	15	7	26	21	68% (45-86)	45% (30-60)	0.105 (-0.093 - 0.303)	**0.001**
58	L1L2 PsV	6	4	20	39	60% (26-88)	66% (53-78)	0.157 (-0.050 - 0.364)	**0.001**
	L1 VLP	10	0	35	24	100% (69-100)	41% (28-54)	0.166 (0.059 - 0.273)	**<0.001**

p values obtained using the M^{c}Nemar test represent discordance between the L1 VLP and L1L2 PsV binding results and the L1L2 PsV neutralisation assay results with significant discordance highlighted in bold.

HPV31 L1 VLP binding antibodies but negative for HPV31 cross-neutralising antibodies. This trend was apparent for the other non-vaccine genotypes and contributed to a moderate to poor inter-rater agreement between the L1 VLP binding assay and the L1L2 PsV neutralisation assay for the non-vaccine genotypes (**Table 6**). A lower number of samples were neutralisation negative but L1L2 PsV binding positive. For example, only 1 serum sample tested positive for HPV31 L1L2 PsV binding antibodies in the absence of HPV31 cross-neutralising antibodies; however, the number of serum samples discordant in the same manner increased for HPV33 (n=7), HPV35 (n=9), HPV52 (n=7) and HPV58 (n=20).

These data suggest that there are quantitative differences in the cross-reactive antibody responses measured by each system and/or target antigen.

3.1.4.2 Hierarchical clustering of L1 and L1L2 antigen-derived serological data

The serological data generated from the testing of the 69 Cervarix® vaccine sera from Study-01 against L1 and L1L2 antigens representing the A9 genotypes were used to evaluate whether qualitative differences between the assay systems and/or antigens underpinned the observed quantitative differences. The approach used for this analysis involved the calculation of the Pairwise Euclidean distances from the L1L2 PsV neutralisation assay, L1 VLP and L1L2 PsV ELISA serological data, generating distance matrices that were then clustered using a neighbour joining algorithm resulting in the creation of a serological and target antigen dendrogram for each assay system. The $\log_{10}$-transformed antibody titre data from each assay system were then represented by a heat map ordered according to the resulting serological and target antigen dendrograms (**Figure 12**).

The increase in heat map colour intensity, from the L1L2 PsV neutralisation assay through the L1L2 PsV binding assay to the L1 VLP binding assay provides a visual representation of both the higher magnitude and breadth of the binding antibody response targeting the non-vaccine A9 genotypes compared to the cross-neutralising antibody response. It also made

Figure 12

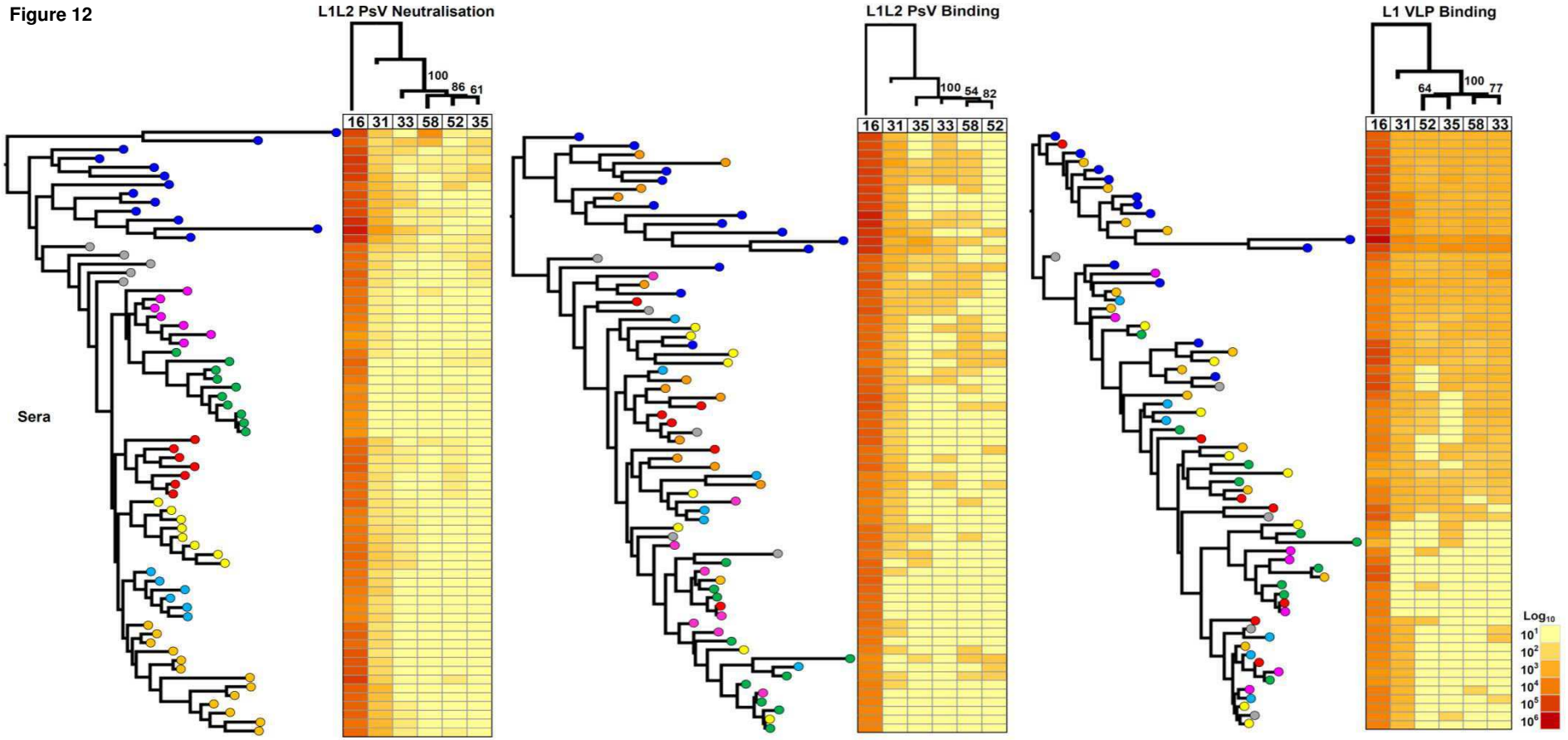


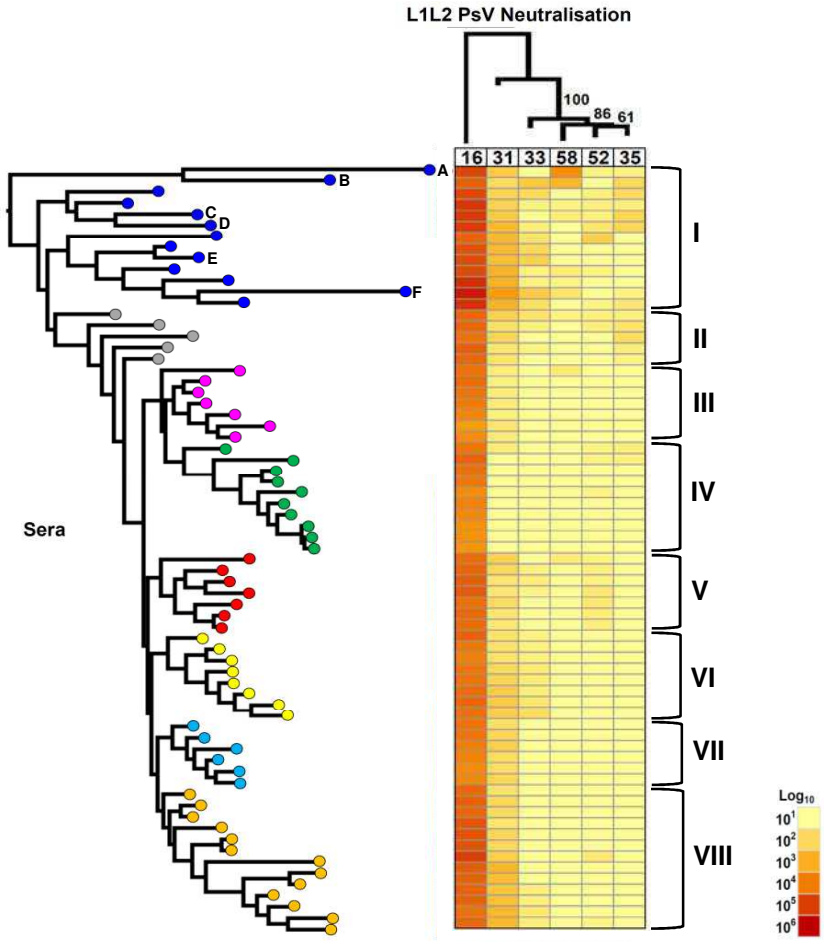
Figure 12 Hierarchical clustering of L1 and L1L2 antigen derived serological data. Log_{10}-transformed serological data (centre, heat map) were subjected to two-dimensional hierarchical clustering and re-ordered according to serological (left) and target antigen (top) dendrograms constructed from the resulting distance matrix. The dendrograms were generated using a neighbour joining algorithm and the antigen dendrograms are supported by bootstrapping of 500 pseudoreplicates. Distinctive clusters within the L1L2 PsV neutralisation serological dendrogram are colour coded, with the corresponding sample in the L1 VLP and L1L2 PsV binding serological dendrogram retaining this colour designation. Key indicates log_{10} heat map gradient. Bissett *et al.,* Vaccine, 32:1139 (2014).

apparent that the serological responses against an antigen representing a particular genotype were not uniform across the three assay systems. Eight clusters of sera with similar magnitude and breadth profiles were identified in the serological dendrogram generated from the L1L2 PsV neutralisation data. These clusters were labelled by colour in order to track how the sera clustered according L1L2 and L1 VLP binding profiles. The serological dendrograms based upon L1L2 PsV and L1 VLP binding titres permitted the formation of clusters but the ordering of individual sera bore little relation to each other or to the order within the serological dendrograms based upon L1L2 PsV neutralisation data (**Figure 12**). These observations indicated that the differences between each system and/or antigen were not purely quantitative and that qualitative differences also existed since the rank order of serum and target antigen were not duplicated across the three assays.

Eight clusters (I – VIII) made up the serological dendrogram produced from the L1L2 PsV neutralisation data (**Figure 13A**), with samples in cluster I displaying the highest HPV16 neutralisation titres and the broadest response, recognising all A9 non-vaccine genotypes (**Figure 13B**). In comparison, the samples in cluster IV had the lowest HPV16 titres and had a primarily type-specific response. These data support a generally quantitative relationship between the magnitude of the antibody response against HPV16 and the ability to recognise non-vaccine genotypes. However, a number or different antibody specificities are displayed, for example the serum samples within clusters II, V and VIII have similar intermediate titres against HPV16 but differ in breadth of response (**Figure 13B**). Cluster VIII samples predominantly recognising HPV31 only, whilst cluster V samples also recognise HPV52 in addition to HPV31 and cluster II samples recognise HPV31, HPV33 and HPV35. These data suggest that multiple cross-reactive antibody profiles are generated in response to vaccination with Cervarix®.

Figure 13

A



B

			Median (IQR) serum neutralisation titers against indicated HPV pseudovirus					
Cluster	N	Breadth	HPV16	HPV31	HPV33	HPV35	HPV52	HPV58
I	13	High	74,295 (55,880 – 122,896)	482 (195 – 665)	54 (24 – 87)	22 (10 – 68)	21 (10 – 25)	20 (10 – 32)
II	5	High	20,556 (20,032 – 20,559)	58 (51 – 98)	23 (10 – 27)	27 (25 – 49)	10 (10 – 25)	10 (10 – 10)
III	7	Low	9,721 (5,959 – 12,954)	31 (27 – 33)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)
IV	10	Low	6,953 (4,366 – 11,584)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)	10 (10 – 18)	10 (10 – 10)
V	7	Medium	18,351 (17,026 – 25,055)	45 (42 – 84)	10 (10 – 21)	10 (10 – 10)	28 (25 – 35)	10 (10 – 10)
VI	8	Medium	13,302 (11,612 – 17,578)	108 (57 – 166)	30 (25 – 37)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)
VII	6	Low	8,275 (6,386 – 11,407)	87 (70 – 107)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)
VIII	13	Medium	25,962 (21,195 – 40,113)	152 (90 – 399)	10 (10 – 26)	10 (10 – 10)	10 (10 – 10)	10 (10 – 10)

Figure 13 Clustered analysis of L1L2 PsV neutralisation data. (A) Log_{10}-transformed serological data (centre, heat map) were subjected to two-dimensional hierarchical clustering and re-ordered according to serological (left) and target antigen (top) dendrograms constructed from the resulting distance matrix. The antigen dendrogram was generated using a neighbour joining algorithm and is supported by bootstrapping of 500 pseudoreplicates. The serological dendrogram is labelled I-VIII based upon intuitive clustering of the serological data. Samples labelled A-F within cluster I subsequently used for enrichment. (B) Median (IQR, Interquartile range) neutralising antibody titres of sera within indicated intuitive clusters against indicated A9 L1L2 PsV. Key indicates log_{10} heatmap gradient. Bissett *et al.,* Vaccine, 32:1139 (2014).

3.1.4.3 The antigenic relationship between A9 genotypes based upon Cervarix® vaccine antibodies

The hierarchical clustering of serological data permitted ranking of the L1 VLP and L1L2 PsV target antigens (**Figure 12**). This ranking was not influenced by differences in L1 amino acid sequence between the VLP and PsV representing a particular A9 genotype since both antigens shared a 100% sequence identity. HPV31 was the nearest antigenic relative to HPV16 independent of the representative HPV31 antigen or assay system; however, the order of the remaining non-vaccine genotypes did differ between systems. For example, in the L1L2 PsV neutralisation viral dendrogram, after HPV31, HPV33 was the next nearest antigenic relative to HPV16 but in the L1 VLP binding viral dendrogram HPV33 was the furthest relative from HPV16. HPV35 and HPV52 clustered together in the viral dendrograms produced from the L1L2 PsV neutralisation and L1 VLP binding data, suggesting a close antigenic relationship between these two genotypes; however, this relationship was not duplicated in the L1L2 PsV binding viral dendrogram. Bootstrap values supported these inter-genotype antigenic relationships which all differed somewhat from the inter-genotype genetic distance based upon L1 amino acid sequence (see Chapter 2, Figure 10).

3.1.4.4 Enrichment of Cervarix® vaccine-induced A9 genotype antibody specificities

The serological dendrogram analysis demonstrated multiple cross-reactive antibody profiles. To directly address whether the cross-reactive response consisted of multiple antibody specificities, selected sera were adsorbed on, and eluted from, L1 VLP representing the individual non-vaccine A9 genotypes. If cross-reactive antibodies are a minority population consisting of multiple specificities then such an approach should enrich for these specificities in preference to HPV16 type-specific antibodies (**Figure 14A**). For example, enrichment on HPV31 L1 VLP should result in the generation of an enriched fraction of antibodies with equivalent recognition for HPV31 and HPV16, and which may or may not also recognise other non-vaccine genotypes. If the cross-reactive antibody response is the consequence of a low affinity interaction of an otherwise predominantly HPV16 type-specific antibody, then

Figure 14

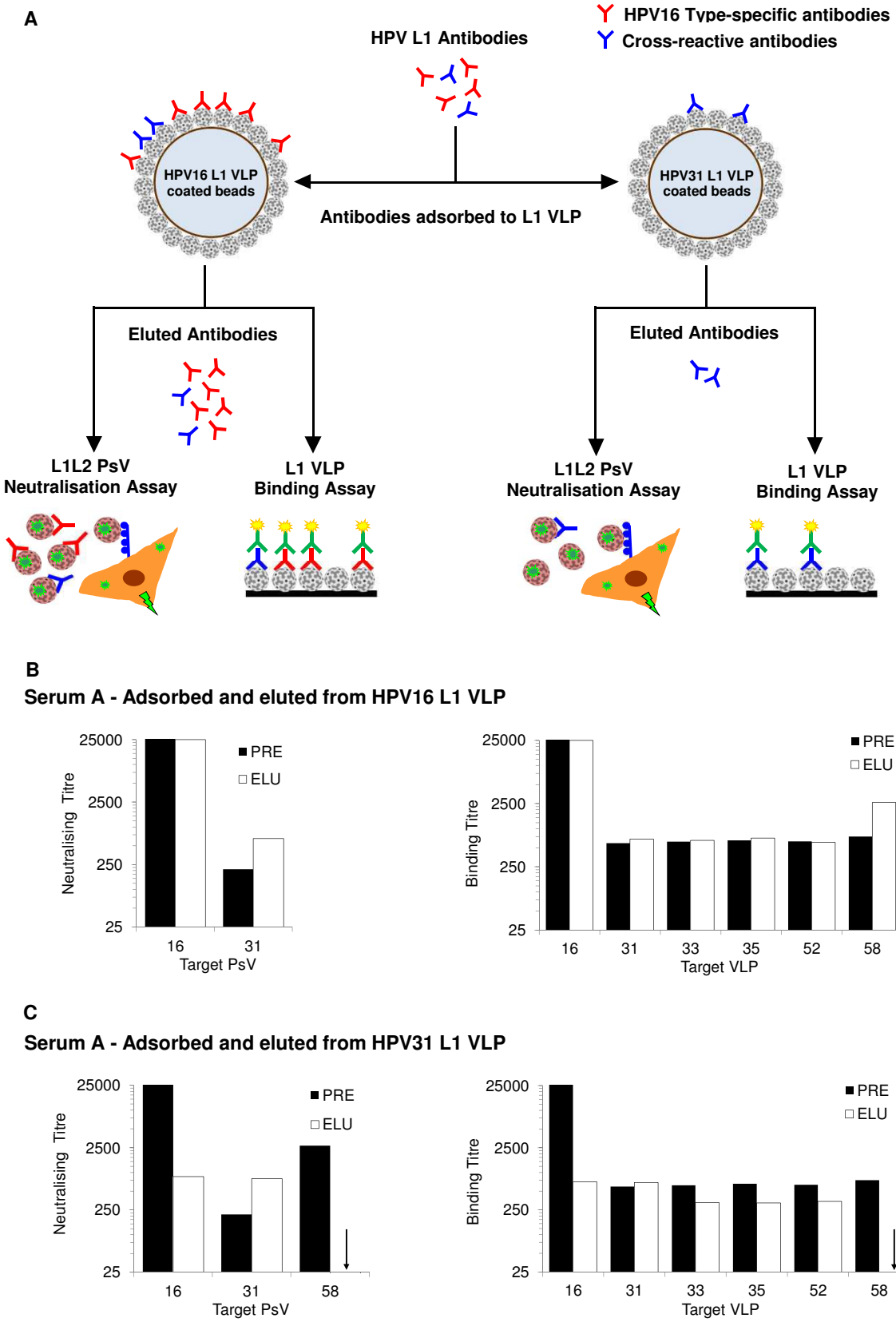


Figure 14 L1 VLP enrichment. (A) Schematic of antibody adsorption and elution from L1 VLP coated beads and subsequent application. (B) Serum A neutralisation and binding antibody profile following enrichment on HPV16 L1 VLP. (C) Serum A neutralisation and binding antibody profile following enrichment on HPV31 L1 VLP.

enrichment on HPV31 L1 VLP would yield antibodies which recognised HPV16 and HPV31 at titres separated by a fold-difference similar in magnitude to the difference separating the pre-enrichment HPV16 and HPV31 titres.

Six serum samples (A-F) were selected from cluster I of the L1L2 PsV neutralisation serological dendrogram for enrichment (**Figure 13**) since samples within cluster I demonstrated the greatest breadth of cross-neutralisation. The enriched fractions were subsequently tested in the L1L2 PsV neutralisation assay against HPV16, HPV31 and another relevant genotype which was determined from the pre-enriched neutralisation profile of each serum sample. HPV31 was chosen as the representative non-vaccine genotype since all 6 serum samples demonstrated cross-neutralisation of HPV31 pre-enrichment therefore HPV31 could be used to probe the separation of vaccine and non-vaccine antibody responses. The higher input volume required for the L1L2 PsV neutralisation assay restricted testing to three A9 genotypes; however, L1 VLP binding titres could be determined against all A9 genotypes prior to and post enrichment.

The six sera were also enriched on HPV16 L1 VLP. As expected, enrichment yielded antibodies capable of neutralising (HPV16 & HPV31) and binding (all A9 genotypes) at equivalent titres compared to pre-enrichment titres. For example, serum A neutralised HPV31 at a titre of 211 prior to enrichment and at a titre of 621 post-enrichment on HPV16 L1 VLP. Similar binding titres against HPV31 (Pre: 591; Post: 685) were also observed following enrichment on HPV16 (**Figure 14B**), confirming that cross-reactive A9 specificities are induced in response HPV16. The HPV16 neutralisation titre was reduced by a median 1.6 $\log_{10}$-fold (IQR, 1.5 to 2.8; n=30) following enrichment on L1 VLP representing non-vaccine A9 genotypes, confirming that cross-neutralising antibodies represent a minority population. Non-vaccine VLP enriched neutralising antibody titres against HPV16 were similar to the titres observed against the non-vaccine genotype used for enrichment. For example, antibodies in serum A when enriched on HPV31 VLP neutralised HPV16 and

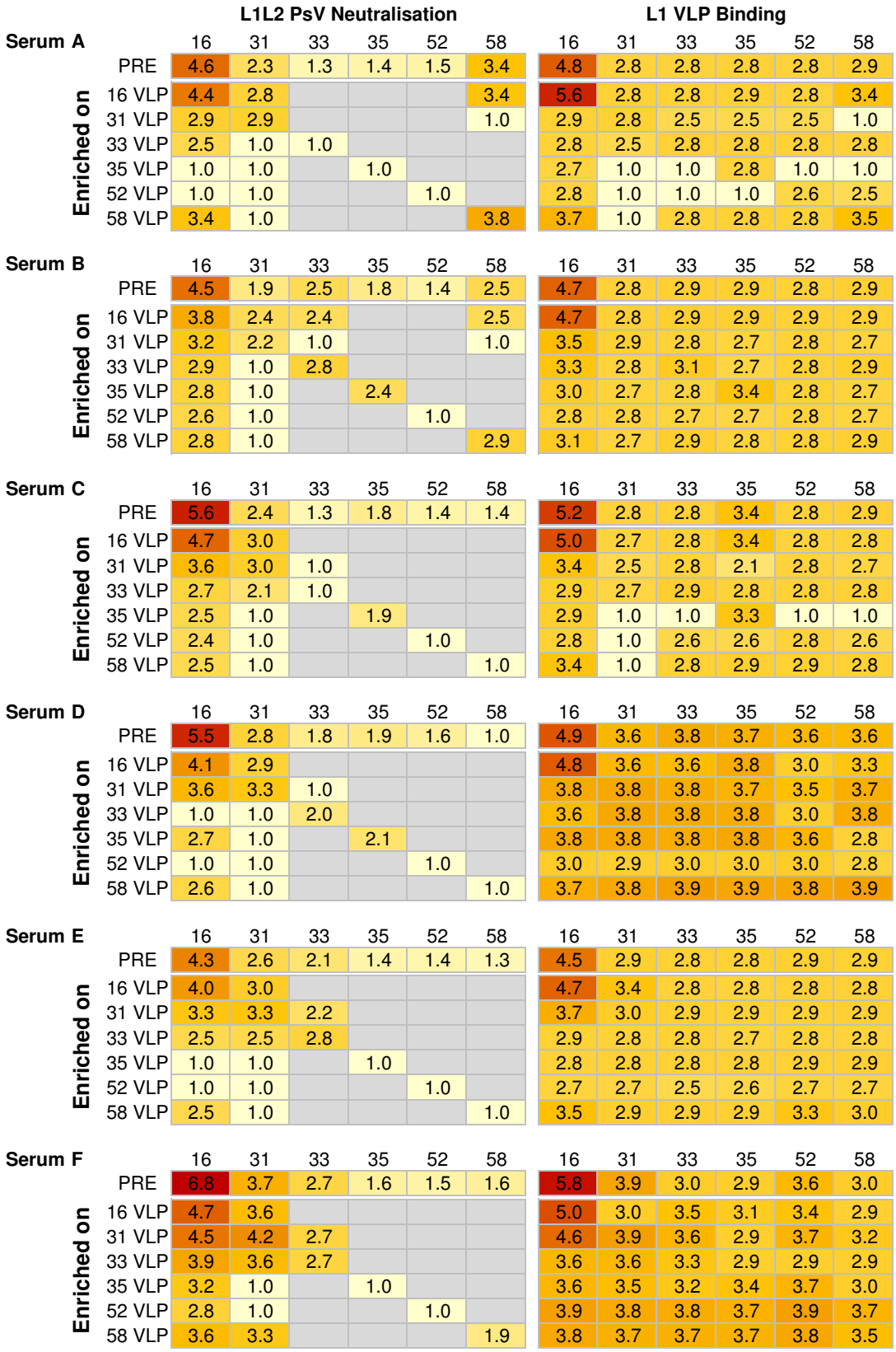
HPV31 at titres of 861 and 795 respectively. Similar binding titres against HPV16 (709) and HPV31 (692) were also observed (**Figure 14C**) indicating that cross-neutralising antibodies have equivalent recognition of HPV16 and non-vaccine genotypes**.**

The log_{10} transformed neutralisation and binding titres of the six sera (A-F) prior to enrichment and post L1 VLP enrichment were represented in heat maps, with the target A9 antigens across the top and the L1 VLP used for enrichment down the left hand side (**Figure 15**).

The enrichment of sera A and B on L1 VLP representing non-vaccine A9 genotypes did not enrich for cross-neutralising antibodies which recognised another non-vaccine genotype. Enrichment of serum A on HPV31 or HPV58 yielded neutralising antibodies capable of recognising HPV16 and only the genotype used for enrichment. The pre-enrichment HPV31 neutralisation titre of serum A was 211, increasing to 795 post-enrichment on HPV31 L1 VLP; however, no HPV58 neutralising reactivity was detectable. Similarly for HPV58, the post-enrichment neutralisation titre of serum A increased to 6,188 (2,696 pre-enrichment) but HPV31 neutralising reactivity was not detectable. Enrichment of serum B on HPV31, HPV33, HPV35 and HPV58 yielded neutralising antibodies which appear to represent multiple antibody specificities that neutralised HPV16 and only the indicated non-vaccine genotype.

Sera C and D antibodies enriched on HPV31 L1 VLP only neutralised HPV16 and HPV31 whilst enrichment on HPV35 L1 VLP yielded neutralising antibodies capable of recognising HPV16 and HPV35 but not HPV31 (**Figure 15**). Serum C neutralising antibodies enriched on HPV33 L1 VLP demonstrated recognition of HPV31; however, this was in the absence of detectable HPV33 neutralisation.

Figure 15



Serum A	16	31	33	35	52	58	16	31	33	35	52	58
PRE	4.6	2.3	1.3	1.4	1.5	3.4	4.8	2.8	2.8	2.8	2.8	2.9
Enriched on 16 VLP	4.4	2.8				3.4	5.6	2.8	2.8	2.9	2.8	3.4
Enriched on 31 VLP	2.9	2.9				1.0	2.9	2.8	2.5	2.5	2.5	1.0
Enriched on 33 VLP	2.5	1.0	1.0				2.8	2.5	2.8	2.8	2.8	2.8
Enriched on 35 VLP	1.0	1.0		1.0			2.7	1.0	1.0	2.8	1.0	1.0
Enriched on 52 VLP	1.0	1.0			1.0		2.8	1.0	1.0	1.0	2.6	2.5
Enriched on 58 VLP	3.4	1.0				3.8	3.7	1.0	2.8	2.8	2.8	3.5

Serum B	16	31	33	35	52	58	16	31	33	35	52	58
PRE	4.5	1.9	2.5	1.8	1.4	2.5	4.7	2.8	2.9	2.9	2.8	2.9
Enriched on 16 VLP	3.8	2.4	2.4			2.5	4.7	2.8	2.9	2.9	2.9	2.9
Enriched on 31 VLP	3.2	2.2	1.0			1.0	3.5	2.9	2.8	2.7	2.8	2.7
Enriched on 33 VLP	2.9	1.0	2.8				3.3	2.8	3.1	2.7	2.8	2.9
Enriched on 35 VLP	2.8	1.0		2.4			3.0	2.7	2.8	3.4	2.8	2.7
Enriched on 52 VLP	2.6	1.0			1.0		2.8	2.8	2.7	2.7	2.8	2.7
Enriched on 58 VLP	2.8	1.0				2.9	3.1	2.7	2.9	2.8	2.8	2.9

Serum C	16	31	33	35	52	58	16	31	33	35	52	58
PRE	5.6	2.4	1.3	1.8	1.4	1.4	5.2	2.8	2.8	3.4	2.8	2.9
Enriched on 16 VLP	4.7	3.0					5.0	2.7	2.8	3.4	2.8	2.8
Enriched on 31 VLP	3.6	3.0	1.0				3.4	2.5	2.8	2.1	2.8	2.7
Enriched on 33 VLP	2.7	2.1	1.0				2.9	2.7	2.9	2.8	2.8	2.8
Enriched on 35 VLP	2.5	1.0		1.9			2.9	1.0	1.0	3.3	1.0	1.0
Enriched on 52 VLP	2.4	1.0			1.0		2.8	1.0	2.6	2.6	2.8	2.6
Enriched on 58 VLP	2.5	1.0				1.0	3.4	1.0	2.8	2.9	2.9	2.8

Serum D	16	31	33	35	52	58	16	31	33	35	52	58
PRE	5.5	2.8	1.8	1.9	1.6	1.0	4.9	3.6	3.8	3.7	3.6	3.6
Enriched on 16 VLP	4.1	2.9					4.8	3.6	3.6	3.8	3.0	3.3
Enriched on 31 VLP	3.6	3.3	1.0				3.8	3.8	3.8	3.7	3.5	3.7
Enriched on 33 VLP	1.0	1.0	2.0				3.6	3.8	3.8	3.8	3.0	3.8
Enriched on 35 VLP	2.7	1.0		2.1			3.8	3.8	3.8	3.8	3.6	2.8
Enriched on 52 VLP	1.0	1.0			1.0		3.0	2.9	3.0	3.0	3.0	2.8
Enriched on 58 VLP	2.6	1.0				1.0	3.7	3.8	3.9	3.9	3.8	3.9

Serum E	16	31	33	35	52	58	16	31	33	35	52	58
PRE	4.3	2.6	2.1	1.4	1.4	1.3	4.5	2.9	2.8	2.8	2.9	2.9
Enriched on 16 VLP	4.0	3.0					4.7	3.4	2.8	2.8	2.8	2.8
Enriched on 31 VLP	3.3	3.3	2.2				3.7	3.0	2.9	2.9	2.9	2.9
Enriched on 33 VLP	2.5	2.5	2.8				2.9	2.8	2.8	2.7	2.8	2.8
Enriched on 35 VLP	1.0	1.0		1.0			2.8	2.8	2.8	2.8	2.9	2.9
Enriched on 52 VLP	1.0	1.0			1.0		2.7	2.7	2.5	2.6	2.7	2.7
Enriched on 58 VLP	2.5	1.0				1.0	3.5	2.9	2.9	2.9	3.3	3.0

Serum F	16	31	33	35	52	58	16	31	33	35	52	58
PRE	6.8	3.7	2.7	1.6	1.5	1.6	5.8	3.9	3.0	2.9	3.6	3.0
Enriched on 16 VLP	4.7	3.6					5.0	3.0	3.5	3.1	3.4	2.9
Enriched on 31 VLP	4.5	4.2	2.7				4.6	3.9	3.6	2.9	3.7	3.2
Enriched on 33 VLP	3.9	3.6	2.7				3.6	3.6	3.3	2.9	2.9	2.9
Enriched on 35 VLP	3.2	1.0		1.0			3.6	3.5	3.2	3.4	3.7	3.0
Enriched on 52 VLP	2.8	1.0			1.0		3.9	3.8	3.8	3.7	3.9	3.7
Enriched on 58 VLP	3.6	3.3				1.9	3.8	3.7	3.7	3.7	3.8	3.5

Figure 15 Heatmaps of neutralising and binding antibody responses against A9 target antigens by L1 VLP enriched antibody fractions. The $\log_{10}$ transformed neutralisation and binding titres of the six sera (A-F) prior to enrichment and post L1 VLP enrichment were represented in heat maps, with the target A9 antigens across the top and the L1 VLP used for enrichment down the left hand side.

Neutralising antibodies enriched from serum E and F exhibited cross-recognition of more than one non-vaccine A9 genotype (**Figure 15**). Enrichment of both serum E and F on HPV31 and HPV33 L1 VLP yielded antibodies capable of neutralising HPV16, HPV31 and HPV33. Enrichment of serum F on HPV58 L1 VLP also yielded antibodies capable of neutralising HPV31 in addition to HPV16 and HPV58. The HPV31 enriched antibodies from serum E and F neutralised HPV16 and HPV31 at similar titres but neutralised HPV33 at a lower titre, whilst antibodies enriched on HPV33 neutralised HPV16, HPV31 and HPV33 at equivalent titres. These differences in cross-recognition appear to demonstrate yet another level of antibody complexity induced in response to the Cervarix® vaccine.

L1 VLP enrichment of serum B, D, E and F yielded binding antibodies which recognised all A9 genotypes independent of the L1 VLP used for the enrichment process (**Figure 15**), highlighting again the discrepancy between binding and neutralisation antibody specificity. The L1 VLP enrichment of serum A and C did not exclusively yield pan-reactive A9 binding antibodies since enrichment of both sera on HPV35 yielded binding antibodies which only recognised HPV16 and HPV35, a pattern which was duplicated in the L1L2 PsV neutralisation data derived from HPV35 enrichment of serum C. Both sera also yielded binding antibodies which recognised all A9 genotypes except HPV31 following enrichment on HPV58, a pattern which was also duplicated in the L1L2 PsV neutralisation data derived from HPV58 L1 VLP enrichment of serum A.

Overall, these data suggest that cross-reactive antibodies represent a minor population of multiple specificities which exist within the total HPV16 antibody response generated against the Cervarix® vaccine.

3.1.4.5 A9 L1 and L1L2 antigen serological bridging studies

During the course of these studies a clinical trial was carried out, referred to as Study-02, from which both Cervarix® and Gardasil® vaccinee sera could be accessed. Study-02 serum

samples had previously been tested in the L1L2 PsV neutralisation assay for the presence of neutralising antibodies targeting all the A9 HPV genotypes (Draper et al., 2013). A group of forty-six sera (Cervarix® n=22; Gardasil® n=24), with high HPV31 cross-neutralising antibody titres, were selected for the remainder of the project and serological bridging studies were subsequently carried out using L1 and L1L2 antigens representing HPV16, HPV31 and HPV33.

All samples, independent of HPV vaccine, were seropositive for antibodies targeting L1 and L1L2 antigens representing HPV16, HPV31 and HPV33 (**Table 7**). The exceptions to this were the detection of HPV33 cross-neutralising antibodies in the L1L2 PsV neutralisation assay (n=41; 89%) and the detection of antibodies which recognised denatured HPV16 or HPV31 L1 VLP. Sixteen samples, 5 Cervarix® and 11 Gardasil®, were found to be positive for antibodies which recognised denatured HPV16 L1 VLP with median 50% binding titres of 234 (IQR, 161 to 276) and 157 (IQR, 137 to 250) respectively (calculated from the positive samples only), whilst a single Gardasil® sample (binding titre: 138) was positive against denatured HPV31 L1 VLP.

A correlation was observed between the HPV16 antibody responses in the L1L2 PsV binding (Pearson's $r = 0.650$; $p < 0.001$) and L1 VLP binding ($r = 0.727$; $p < 0.001$) assays compared to the L1L2 PsV neutralisation assay, similar to the HPV16 data derived from the Study-01 samples. Differences in the magnitude of the HPV16 antibody response between the assay systems was also apparent again with the antibody titre decreasing by a median 1.5-fold (IQR, 1.2 to 2.6; Wilcoxon paired signed-rank test, $p < 0.001$) in the L1L2 PsV binding assay and a median 3.0-fold (IQR, 2.2 to 5.0; $p < 0.001$) in the L1 VLP binding assay compared to the L1L2 neutralisation assay.

For HPV31 and HPV33, the L1 VLP binding assay generated the highest antibody titres, followed by a stepwise decrease in the L1L2 PsV binding assay then the L1L2 neutralisation

Table 7. Seroreactivity against HPV16, HPV31 and HPV33 L1 and L1L2 targets in binding and neutralisation assays Study-02 serum samples: Cervarix® n=22 and Gardasil® n=24 vaccine recipients

HPV	Assay	Vaccine	Seropositivity	Antibody titre[a]		% of 16 titre[c]
			N (%)	Median (IQR)	*p* value[b]	Median (IQR)
16	L1L2 PsV Neutralisation	Cervarix	22 (100)	244,460 (159,575 - 360,654)	-	-
		Gardasil	24 (100)	104,440 (79,636 - 220,963)	-	-
		All	46 (100)	168,073 (87,716 - 333,266)	-	-
	L1L2 PsV Binding	Cervarix	22 (100)	174,668 (114,741 - 230,299)	**<0.001**	-
		Gardasil	24 (100)	71,073 (42,358 - 91,178)	**<0.001**	-
		All	46 (100)	92,880 (56,843 - 213,869)	**<0.001**	-
	L1 VLP Binding	Cervarix	22 (100)	51,849 (39,675 - 117,928)	**<0.001**	-
		Gardasil	24 (100)	38,352 (32,906 - 46,728)	**<0.001**	-
		All	46 (100)	44,857 (34,392 - 100,440)	**<0.001**	-
31	L1L2 PsV Neutralisation	Cervarix	22 (100)	1,072 (693 - 2,273)	-	0.59 (0.33 - 0.95)
		Gardasil	24 (100)	767 (543 - 1,876)	-	0.77 (0.56 - 1.21)
		All	46 (100)	938 (618 - 2,070)	-	0.70 (0.46 - 1.01)
	L1L2 PsV Binding	Cervarix	22 (100)	2,051 (1,680 - 3,155)	**0.031**	1.70 (1.06 - 2.74)
		Gardasil	24 (100)	1,421 (971 - 2,137)	**0.046**	2.40 (1.62 - 3.40)
		All	46 (100)	1,912 (1,265 - 2,299)	**0.003**	2.17 (1.12 - 2.84)
	L1 VLP Binding	Cervarix	22 (100)	2,326 (1,673 - 5,368)	**<0.001**	8.88 (6.39 - 20.44)
		Gardasil	24 (100)	2,301 (1,939 - 4,785)	**<0.001**	8.78 (7.40 - 18.26)
		All	46 (100)	2,312 (1,814 - 5,158)	**<0.001**	8.82 (6.92 - 19.69)
33	L1L2 PsV Neutralisation	Cervarix	20 (90.9)	97 (35 - 151)	-	0.04 (0.01 - 0.06)
		Gardasil	21 (87.5)	85 (36 - 174)	-	0.07 (0.02 - 0.13)
		All	41 (89.1)	86 (35 - 165)	-	0.05 (0.02 - 0.10)
	L1L2 PsV Binding	Cervarix	22 (100)	372 (305 - 487)	**<0.001**	0.25 (0.16 - 0.48)
		Gardasil	24 (100)	372 (288 - 533)	**<0.001**	0.64 (0.49 - 1.02)
		All	46 (100)	372 (297 - 502)	**<0.001**	0.50 (0.21 - 0.82)
	L1 VLP Binding	Cervarix	22 (100)	458 (376 - 1,304)	**<0.001**	1.75 (1.43 - 4.98)
		Gardasil	24 (100)	957 (489 - 1,345)	**<0.001**	3.65 (1.87 - 5.13)
		All	46 (100)	749 (442 - 1,362)	**<0.001**	2.86 (1.69 - 5.20)

IQR, inter-quartile range. For calculation purposes neutralisation titres of <20 were assigned a value of 10

[a] Antibody titres are presented as the median (IQR) 80% neutralisation titre or 50% binding titres

[b] *p* values, obtained using the Wilcoxon paired signed-rank test, represent differences in L1L2 PsV binding and L1 VLP binding antibody titres from L1L2 PsV neutralisation titres. Significant differences are highlighted in bold.

[c] HPV31 and HPV33 antibody titres represented as a % of the corresponding HPV16 titre

assay. This corresponded with a step-wise increase in the percentage of HPV31 and HPV33 antibodies relative to the HPV16 response (**Table 7**). For example, for HPV31 the median percentage of the HPV16 titre was 0.70% (IQR, 0.46 to 1.01%) in the L1L2 PsV neutralisation assay, increasing to 2.17% (IQR, 1.12 to 2.84%) in the L1L2 PsV binding assay and 8.82% (IQR, 6.92 to 19.69%) in the L1 VLP binding assay. These data are in general agreement with the Study-01 serological data derived against antigens representing HPV16 and non-vaccine A9 genotypes.

3.1.4.6 Neutralising specificities of HPV vaccine-induced antibodies

L1 VLP enrichment of Study-01 serum samples demonstrated that cross-reactive specificities are a minority antibody population which consists of multiple specificities. A simpler method whereby L1 VLP representing HPV16, HPV31 and HPV33 were used as competing antigens in L1L2 PsV neutralisation assays was employed to corroborate these observations using Study-02 serum samples (n=12). This approach should reduce the antibody pool which targets the competing L1 VLP therefore reducing neutralisation potential of these antibody specificities in the downstream assay.

Pre-incubation with HPV16 L1 VLP reduced neutralising antibody titres against HPV16, HPV31 and HPV33; however, pre-incubation with HPV31 or HPV33 L1 VLP did not reduce the HPV16 neutralisation titre (**Table 8**). Pre-incubation with HPV31 L1 reduced the neutralising antibody titres against HPV31 and HPV33 with 7 of the 12 serum samples demonstrating a ≥3-fold reduction in HPV33 neutralisation titres following pre-incubation with HPV31 L1 VLP. Pre-incubation with HPV33 L1 VLP reduced the neutralising antibody titres against HPV33 but had little impact upon HPV16 or HPV31 neutralisation titres. Antibody competition against L1 VLP demonstrated reduced sensitivity compared to L1 VLP antibody enrichment, in that the neutralising antibodies against the competing L1 VLP were not completely removed. Nevertheless these data are consistent with Study-01 antibody

Table 8. Specificity of neutralising antibodies induced by HPV vaccines
Study-02 serum samples: Cervarix® n=6 and Gardasil® n=6 vaccine recipients

Competing antigen	Median (IQR) in neutralising antibody titre to indicated PsV Pre and Post addition of competing VLP								
	PsV 16			PsV 31			PsV 33		
	Pre	Post	Fold[a]	Pre	Post	Fold[a]	Pre	Post	Fold[a]
VLP 16	138,737 (121,551 - 374,486)	891 (683 - 1,873)	**166** (108 - 191)	2,100 (1,065 - 4,467)	101 (89 - 130)	**20** (12 - 42)	498 (384 - 843)	89 (29 - 173)	**6** (4 - 8)
VLP 31	138,737 (121,551 - 374,486)	179,942 (125,149 - 439,678)	≤ 1	2,100 (1,065 - 4,467)	191 (169 - 415)	**8** (5 - 11)	498 (379 - 722)	82 (27 - 538)	**5** (2 - 9)
VLP33	138,737 (121,551 - 374,486)	383,132 (139,921 - 781,250)	≤ 1	2,100 (1,065 - 4,467)	1,182 (723 - 3,825)	1.2 (0.9 -1.9)	498 (379 - 722)	24 (20 - 32)	**17** (10 - 27)

Interquartile range (IQR)
[a] Fold reduction (Median and IQR) in neutralising antibody titre to indicated PsV by addition of VLP compared to no VLP control; Median and IQR are not presented when reduction in neutralisation titre ≤1-fold; Reductions of ≥3-fold are indicated in bold type.

enrichment data which demonstrated that multiple cross-neutralising antibody specificities appear to be present as a minor population within the total HPV16 antibody repertoire.

3.1.5 Discussion

The cross-neutralising antibody responses generated against HPV16 L1 VLP in the Cervarix® and Gardasil® vaccines were compared with the responses measured against L1 VLP and L1L2 PsV antigens in an indirect ELISA. The L1L2 PsV neutralisation assay only detects functional, neutralising antibodies whilst an indirect ELISA will detect all antibodies capable of binding to the target antigen irrespective of functionality. A good correlation was observed between the HPV16 antibody responses measured by ELISA (L1 VLP and L1L2 PsV) and neutralisation assay, in agreement with the findings of previous studies (Dessy et al., 2008; Kemp et al., 2008; Safaeian et al., 2013b).

Agreement between non-vaccine antibody responses measured by L1L2 PsV neutralisation assay and the L1L2 PsV or L1 VLP binding assays were weaker, with a stepwise increase in antibody titres observed from the L1L2 PsV neutralisation assay, through the L1L2 PsV binding assay to the L1 VLP binding assay. The higher antibody titres in the L1 VLP binding assay were accompanied by increased seropositivity of HPV33, HPV35, HPV52 and HPV58 compared to the L1L2 PsV neutralisation assay. Data regarding the quantitative differences between ELISA and neutralisation assay formats for the measurement of cross-reactive antibody titres is limited. In one study, the immunisation of New Zealand white rabbits with L1L2 VLP representing HPV31 generated polyclonal anti-serum which cross-neutralised HPV16 L1 VLP in a haemagglutination inhibition assay (HAI) at an antibody level *ca.* 50-fold lower than the level which cross-reacted with HPV16 L1 VLP by ELISA (Xu et al., 2007).

Increased seroreactivity measured by the L1 VLP ELISA has been attributed to the binding of antibodies to linear L1 epitopes exposed on denatured protein within the VLP preparations (Du et al., 2015; Schiller and Lowy, 2009). However, very limited reactivity was

observed when the vaccine sera were tested against denatured L1 VLP representing HPV31. These data indicate that whilst non-vaccine genotype binding antibodies primarily target conformational epitopes, their detection may not be an appropriate surrogate measurement for the magnitude and specificity of the cross-neutralising antibody response of vaccine sera.

Study-01 serological data sets were subjected to hierarchical clustering in order to further examine the discrepancies between the cross-reactive antibody profiles. Such an approach has been used to evaluate the antibody specificities targeting HIV (Binley et al., 2004; Gray et al., 2009; Seaman et al., 2010; Shang et al., 2011), foot-and-mouth disease virus (Reeve et al., 2010) and avian influenza virus H5N1 (Lai et al., 2012). The serological profiles differed starkly between the L1L2 PsV neutralisation assay, the L1L2 PsV binding assay and L1 VLP binding assay. Samples which clustered together based upon similar A9 neutralisation profiles subsequently cluster differently in the serological dendrograms produced by both sets of binding data. The hierarchical clustering also permitted the antigenic inter-genotype ranking of the A9 targets and found that HPV31 was always the nearest antigenic relative to HPV16 but the order of the remaining genotypes differed between the three assay systems. These data indicate that the quantitative differences between the assays were underpinned by qualitative differences in the antibody specificities measured.

A number of L1 MAbs demonstrate differential recognition of their epitopes displayed on L1 VLP compared to L1L2 PsV (Christensen et al., 1996a; Christensen et al., 1996b; Culp et al., 2007; Rizk et al., 2008). For example, H16.J4 cross-reacts with L1 VLP representing A9 genotypes HPV31, HPV33 and HPV35 (Christensen et al., 1996a) and cross-neutralises HPV31, HPV33 and HPV58 in an L1-based reporter transduction assay (Combita et al., 2002) but poorly recognises its epitope on HPV16 L1L2 PsV antigens used in either an ELISA or neutralisation assay (Culp et al., 2007; Rizk et al., 2008). Structural differences are

apparent between L1 VLP and L1L2 PsV, with the latter containing a higher degree of disulphide cross-linking between L1 monomers within the capsid (Fligge et al., 2001). However, the epitopes recognised by the majority of type-specific neutralising L1 MAbs, such as H16.V5, appear not to be affected by the structural, and possible antigenic, changes to the capsid due to L2 inclusion (Culp et al., 2007). Consistent with this observation for type-specific MAbs, the polyclonal vaccine sera were all able to recognise both L1 VLP and L1L2 PsV antigens representing the vaccine type HPV16. It could reasonably be assumed that the majority of non-neutralising, cross-reactive antibodies bind conformational regions of L1 proteins which are not involved in (pseudo)virus entry as this would account for the increased seroreactivity in both binding assays compared to the neutralisation assay. In addition, the increased seroreactivity in the L1 VLP binding assay compared to the L1L2 PsV binding assay could be accounted for if a proportion of these antibodies targeted domains that are altered or occluded by the incorporation of L2 into the capsid, similar to the H16.J4 MAb (Culp et al., 2007).

Cross-reactive antibody titres were very low in comparison with the HPV16 antibody titres in all three assay systems. It was not clear from the antibody titre data alone whether cross-reactivity was the consequence of antibodies that made up a minor percentage of the total HPV16 antibody repertoire or whether it was the consequence of reduced recognition by an otherwise HPV16 type-specific antibody specificity. This latter phenomenon was observed in the sole instance where L1 cross-neutralising MAbs have been identified, with effective cross-neutralisation of the A7 genotypes HPV18 and HPV45 requiring a higher IgG concentration compared to type-specific neutralisation (Smith et al., 2007).

This uncertainty was addressed by utilising immobilised L1 VLP for the enrichment of a small panel of broadly cross-neutralising Study-01 sera. It was reasoned that since L1 VLP are the immunogens of the HPV vaccines this approach would allow the capture of the majority of L1-specific antibodies generated. This approach has previously been used to enrich for

broadly-neutralising antibodies induced in response to HIV infection (Gray et al., 2009; Li et al., 2007; Sather et al., 2009). HPV16 neutralising antibodies and cross-neutralising antibodies could be detected in the enriched fractions indicating that the immobilised L1 VLP appeared to maintain a reasonable degree of conformational integrity. Serum enriched on L1 VLP representing non-vaccine genotypes demonstrated equivalent recognition of HPV16 and the non-vaccine genotype used for the enrichment. This suggests that cross-neutralising antibodies form a distinct, minor component within the total vaccine-type HPV16 antibody repertoire and that cross-neutralisation is not the result of HPV16 type-specific antibody which exhibits low affinity interactions with non-vaccine genotypes.

If cross-neutralising antibodies are a minority population and only a small pool of memory B cells express these specificities (Godi et al., 2015a), it is possible that their generation and maintenance over time is more precarious than those immune components that recognise vaccine type antigens. Consequentially, any potential contribution which these antibody specificities have to vaccine-induced cross-protection may also diminish over time.

Cross-neutralising antibodies targeting HPV31 have been detected at 24 months in women who received three doses of the Cervarix® vaccine suggesting that these specificities do have a degree of longevity (Einstein et al., 2011a). A reduced-dose schedule did appear to effect the generation and/or maintenance of HPV31 cross-neutralising antibodies since seroconversion rates decreased from 63% following a three-dose schedule of the Cervarix® vaccine, to 50% following two doses with a further reduction to 24% following one dose (Safaeian et al., 2013b). However, both these studies were carried out in women, aged 18-45 years, and it has been demonstrated that antibody responses to HPV vaccine decreases with age (Einstein et al., 2014). A two-dose schedule of Gardasil® in girls aged 9-13 years, who are the target age group for vaccination, demonstrated non-inferior vaccine type antibody levels compared to a three-dose schedule in women (aged 16-26) (Dobson et al.,

2013). This increased immunogenicity observed in the younger age group may better support the maintenance of the cross-neutralisation antibody response.

The hierarchical clustering of the serological data and enrichment data suggest that vaccination has the potential to elicit multiple cross-reactive antibody specificities but that these specificities vary between vaccine recipients. Variation in individual immune responses to vaccination has been observed for other vaccine-preventable viral infections. Vaccination with the recombinant HBV vaccine fails to elicit protective antibody levels in 5-10% of healthy adult recipients, with the differences in individual responses associated with diversity in human leukocyte antigen (HLA) genes (Wang et al., 2004). Polymorphisms in HLA genes are also associated with variation in the immune response to the live attenuated measles vaccine, where a proportion of individuals fail to either mount or maintain a protective response (Haralambieva et al., 2013). Such genetic components could impact upon an individual's ability to process and present certain L1 epitopes following HPV vaccination.

These data also suggest that the HPV16 L1 protein harbours multiple surface-exposed, immunogenic domains that share sequence and/or structural homology with other A9 genotypes. Such domains appear to be common between HPV16, HPV31 and HPV33. The surface-exposed loops of the HPV16 L1 protein are antigenic targets for both neutralising natural infection antibodies and L1 MAbs (Carter et al., 2006; Fleury et al., 2009; Rizk et al., 2008). Neutralising antibodies generated in response to HPV16 natural infection appear to preferentially target the DE, FG and HI loops over the EF loop, with antibody recognition of the BC loop infrequent (Carter et al., 2006). Cryo-electron microscopy has been used to identify the precise epitope footprints of several HPV16 L1 murine MAbs, which encompass amino acid residues in all five L1 loops (Guan et al., 2015; Lee et al., 2015) and recently a human HPV16 L1 MAb has been produced from a HPV vaccine recipient which recognises amino acid residues in DE and FG loops (Xia et al., 2016).

Overall these data support the notion that HPV vaccine-induced L1 cross-neutralising antibodies are a minor component of the total HPV16 antibody response, consisting of multiple antibodies with both distinct and overlapping specificities which exhibit equivalent recognition for HPV16. These findings indicate that HPV16 harbours immunogenic L1 domains which share sequence and/or structural homology with the L1 proteins of the other A9 genotypes. Identification of such common domains will improve our understanding of L1 capsid protein antigenicity and may offer the opportunity to improve the immunogenicity of such domains in future vaccines.

3.2 A9 intra-genotype L1 amino acid diversity is located in the surface-exposed loops

3.2.1 Background

The capsid of HPV PsV contains both viral structural proteins, the L1 which mediates attachment to the host cells (Buck et al., 2013) and the L2 which is essential for viral infectivity (Wang and Roden, 2013). HPV PsV resemble authentic HPV virions (Buck et al., 2005) and are employed as surrogates in a range of *in vitro* and *in vivo* systems for the study of HPV antibody-mediated neutralisation and entry kinetics (Pastrana et al., 2004; Roberts et al., 2007). Each Alpha-7 and Alpha-9 species group genotype is represented by a single PsV. The majority of PsV also represent the genotype reference sequence, for example the L1 sequences of the PsV representing HPV18, HPV31, HPV33, HPV45, HPV52 and HPV58 have a 100% amino acid sequence identity to the reference sequence L1 of their respective genotype (Ahmed et al., 2013).

The utilisation of WGS technologies has increased the available data for HPV and permitted the classification of variant lineages and sublineage within a genotype based upon single nucleotide polymorphisms identified across the whole HPV genome (Chen et al., 2011, 2013). The potential impact of this genetic variation on HPV capsid antigenicity has only been investigated for HPV16, where L1L2 PsV representing lineage-specific L1 variants demonstrated similar susceptibility to neutralisation by antibodies elicited against a L1 VLP representing a single L1 variant lineage (Pastrana et al., 2001). However, this study did not evaluate the impact of lineage-specific variation within the L2 protein. Given the increasing number of identified variant lineages and sublineages within a genotype it is unclear the potential impact that such variation may have upon capsid recognition by antibodies and how representative the PsV L1 and L2 amino acid sequences are of their respective genotypes.

3.2.2 Aim of chapter: To evaluate how representative the A9 PsV L1 and L2 protein sequences are of their designated genotype

3.2.3 Specific objective

- To carry out an analysis of intra-genotype A9 L1 and L2 amino acid variation

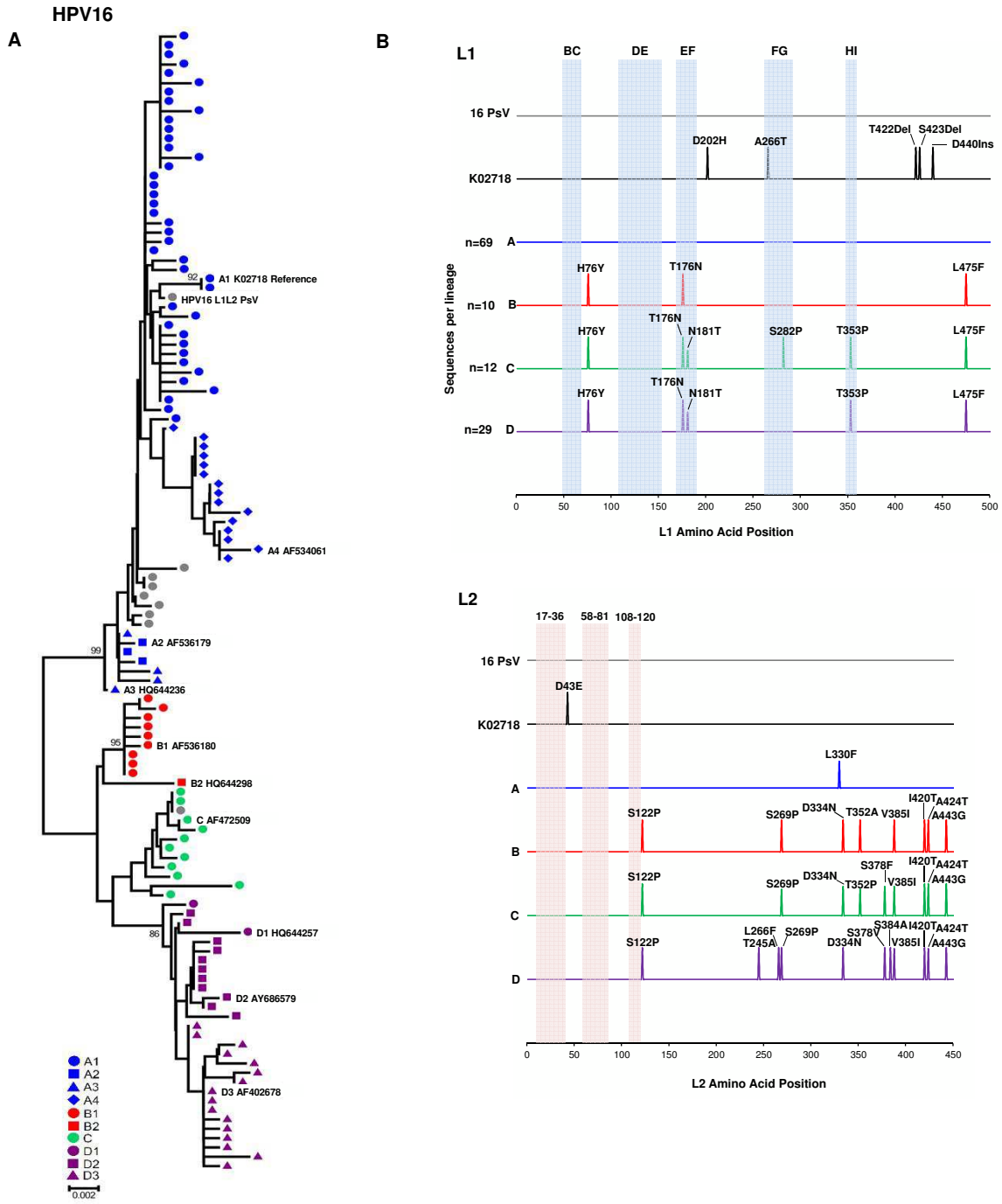
3.2.4 Results

3.2.4.1 Phylogenetic analysis of A9 L1 and L2 amino acid sequences

HPV WGS derived (n=265) or partial sequences encompassing both the L1 and L2 genes (n=34) were identified and downloaded from the NCBI database for the A9 genotypes (HPV16/31/33/35/52/58). The L1 and L2 amino acid sequences were extracted and adjoined so both sequences were in the same reading frame. For example, the L1 (nucleotides from positions 5637 to 7154) and L2 (4235 to 5656) of the HPV16 reference sequence K02718 were extracted and adjoined so that the stop codon of the L1 was directly followed by the start codon of the L2. The adjoined L1 and L2 amino acid sequences were analysed alongside their representative L1L2 PsV with the resulting phylogenetic trees, supported by bootstrap values of ≥80%, generated using a neighbour-joining algorithm.

The segregation of A9 genotypes into variant lineages was generally supported by the L1 and L2 amino acid sequences (**Figures 16A** to **21A**) and consensus sequences were derived for each variant lineage for comparison against the L1 and L2 sequences of their respective PsV (**Figures 16B** to **21B**). There were, however, instances where the distinction between lineages were lost, for example the HPV52 sequences designated as variant lineage A, B or C by WGS analysis became intermingled and no longer segregated into three separate lineages based upon L1 and L2 amino acid sequence (**Figure 20A**). The further segregation of the A9 genotypes into variant sublineages was not supported by L1 and L2 amino acid sequence (**Figures 16A** to **21A**). For example, whilst the sequences of HPV16 variant lineage A generally clustered with sequences of the same sublineage (A1,

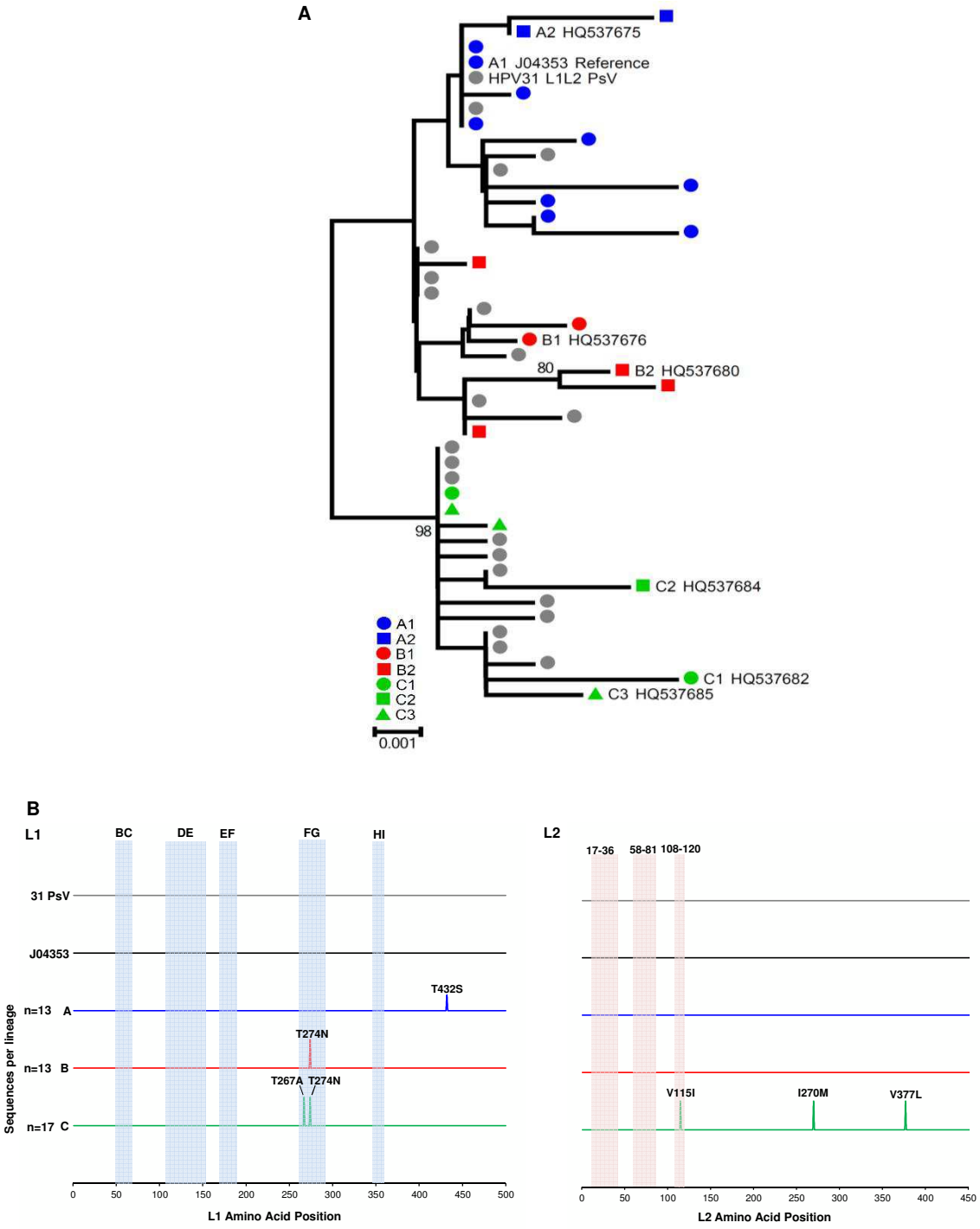
Figure 16



Figures 16 HPV16 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV16 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A, 42/69 = 61%; B, 7/10 = 70%; C, 6/12 = 50%; D, 3/29 = 10%. L2 sequences represented by lineage consensus: A, 13/69 = 19%; B, 5/10 = 50%; C, 6/12 = 50%; D, 10/29 = 34%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

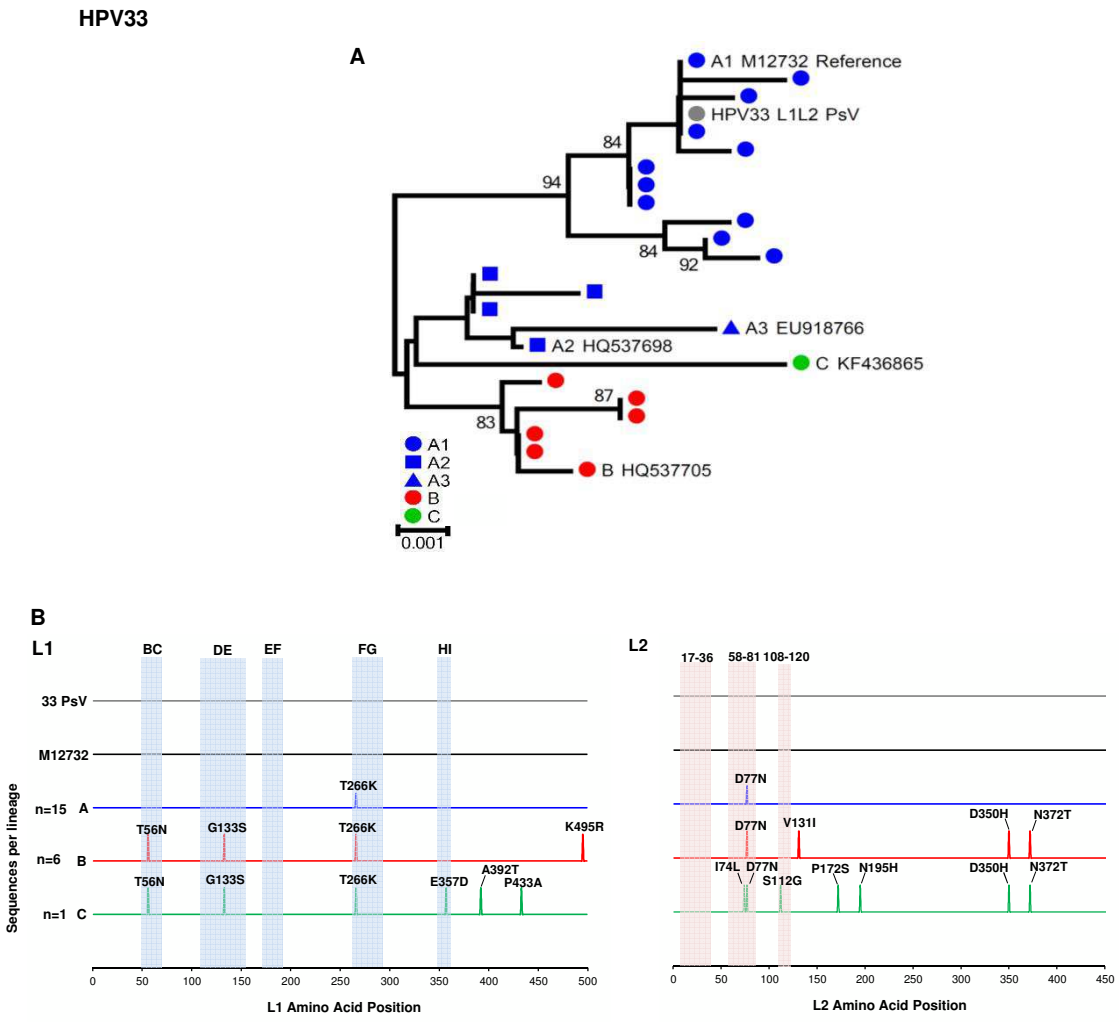
Figure 17

HPV31



Figures 17 HPV31 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV31 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A, 4/13 = 31%; B, 8/13 = 62%; C, 10/17 = 59%. L2 sequences represented by lineage consensus: A, 4/13 = 31%; B, 5/13 = 38%; C, 8/17 = 47%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

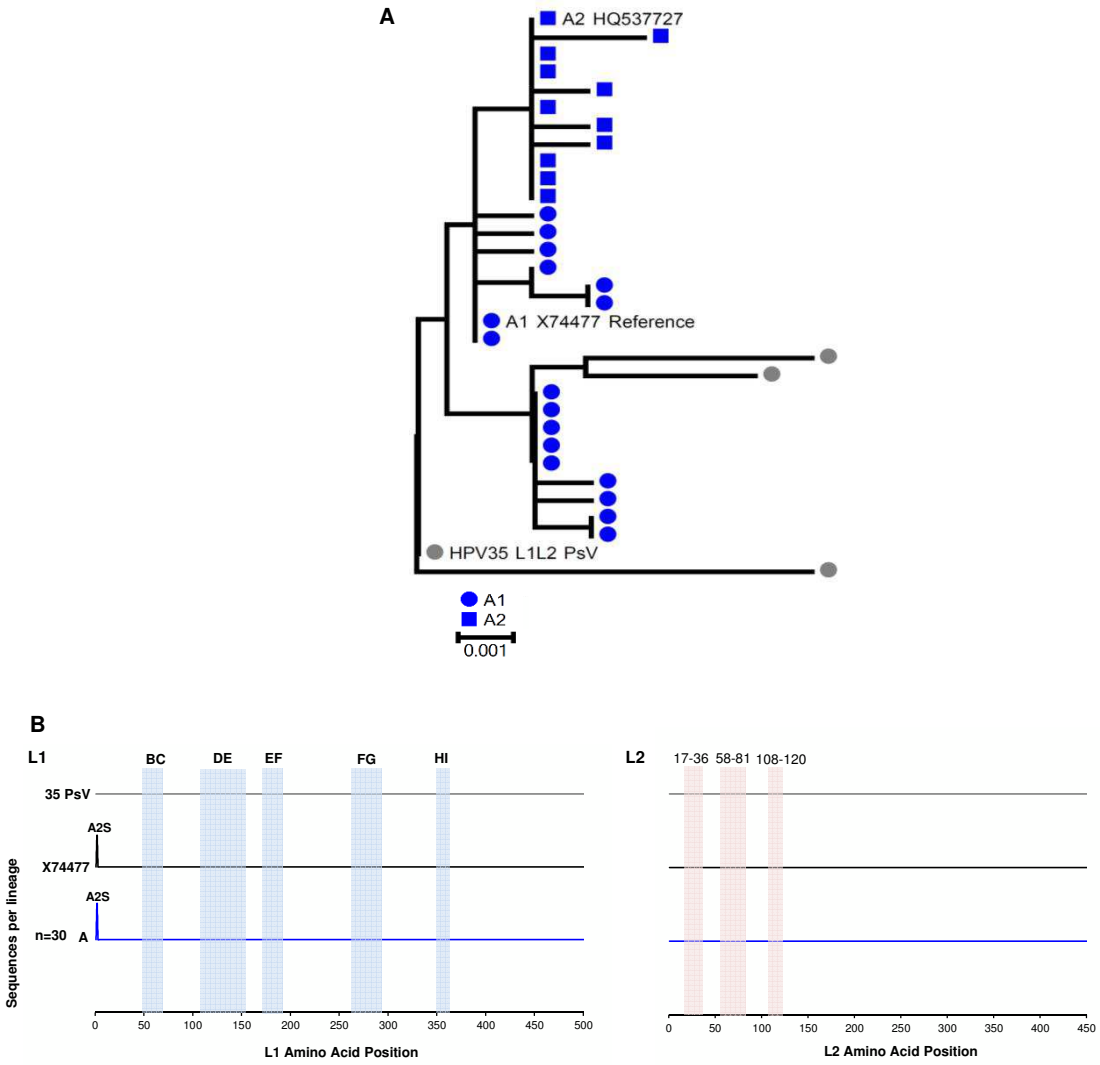
Figure 18



Figures 18 HPV33 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV33 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A, 0/15 = 0%; B, 6/6 = 100%; C, 1/1 = 100%. L2 sequences represented by lineage consensus: A, 3/15 = 20%; B, 3/6 = 50%; C, 1/1 = 100%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

Figure 19

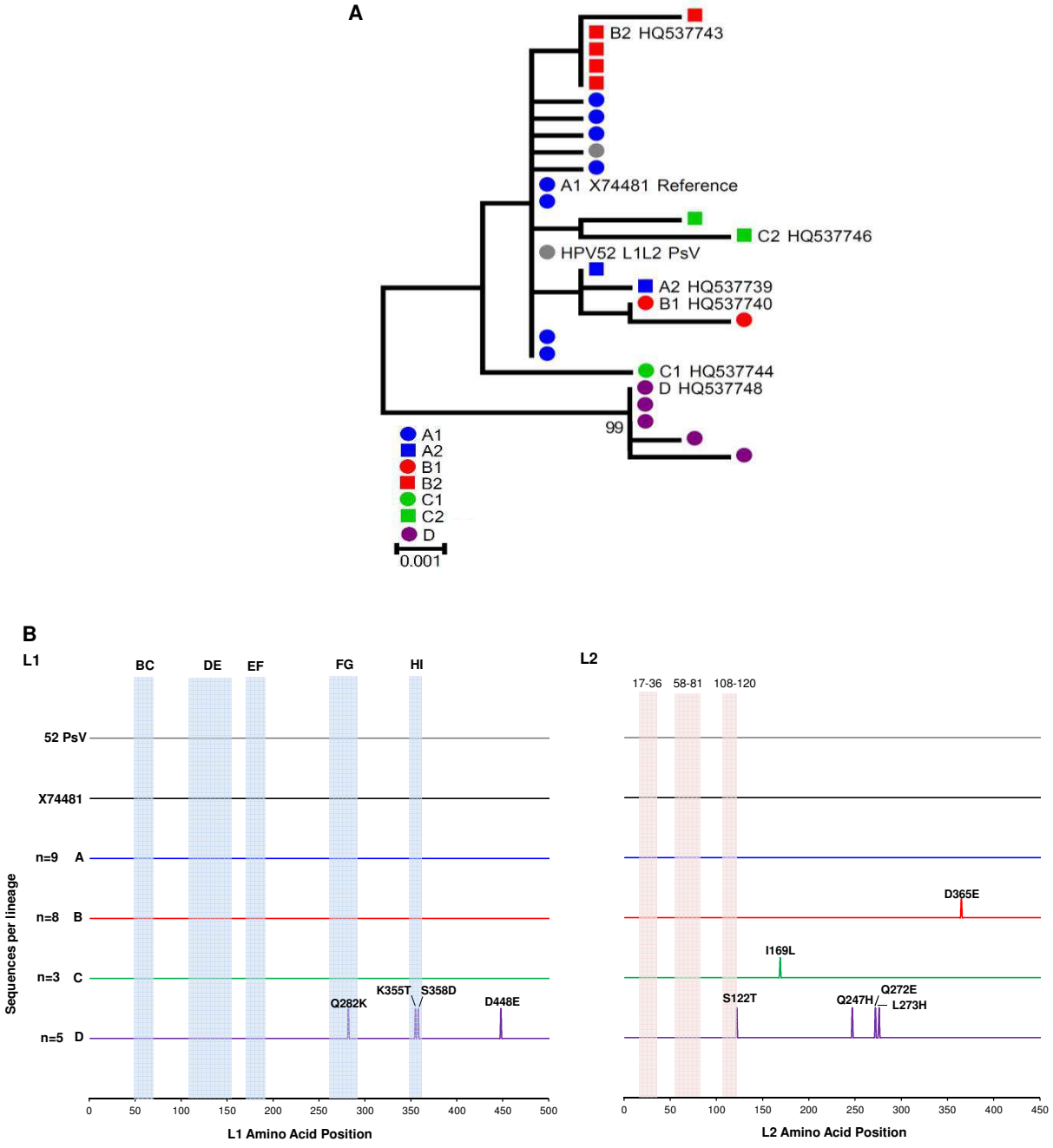
HPV35



Figures 19 HPV35 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV35 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A,15/30 = 50%. L2 sequences represented by lineage consensus: A, 2/30 = 7%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

Figure 20

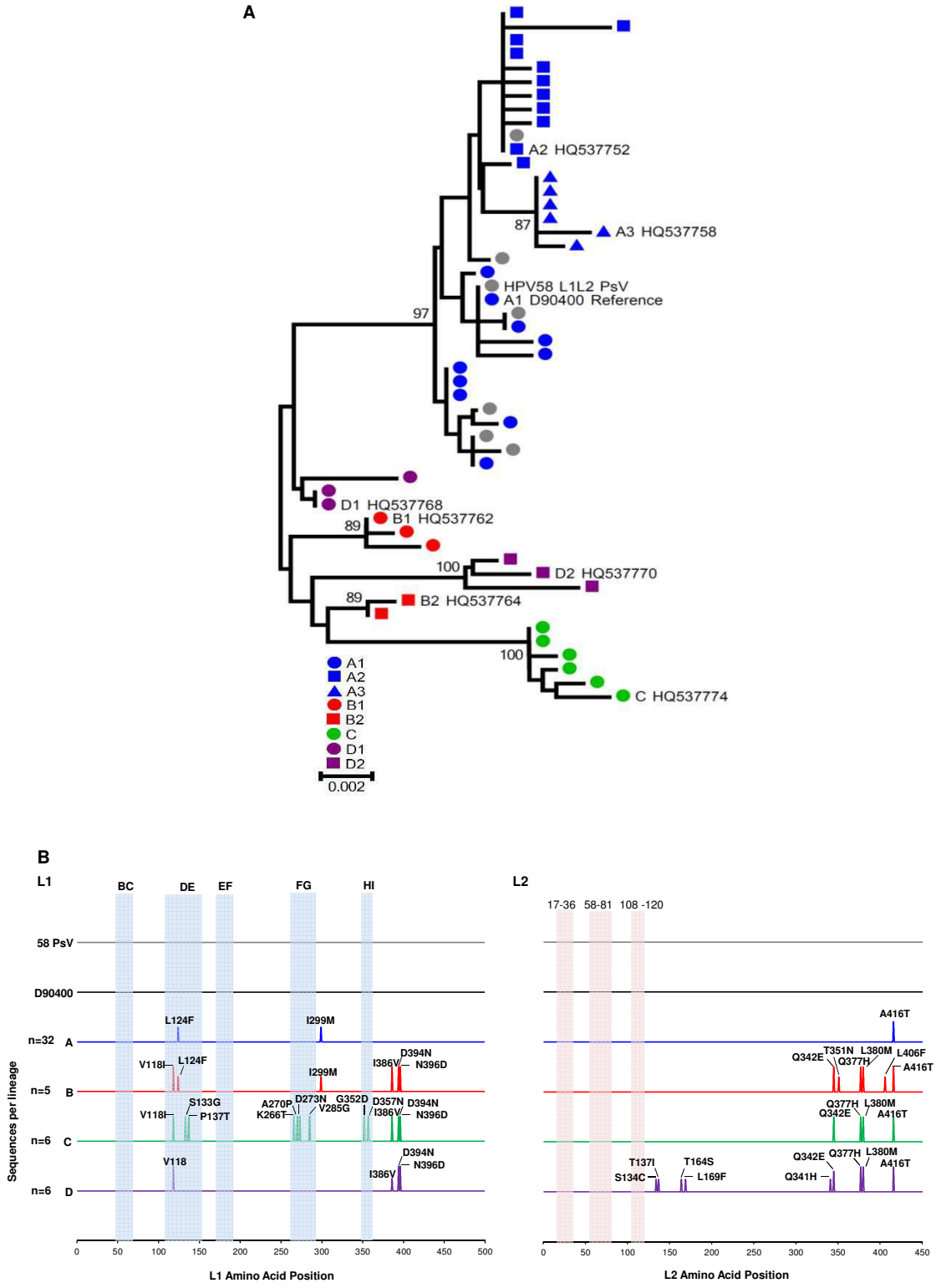
HPV52



Figures 20 HPV52 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV52 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A, 7/9 = 78%; B, 5/8 = 63%; C, 1/3 = 33%; D, 2/5 = 40%. L2 sequences represented by lineage consensus: A, 5/9 = 56%; B, 4/8 = 50%; C, 0/3 = 0%; D, 3/5 = 60%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

Figure 21

HPV58



Figures 21 HPV58 L1 and L2 sequence diversity from PsV. (A) Phylogenetic tree constructed from concatenated L1 and L2 amino acid sequences supported by bootstrap values ≥80% (n= 500 iterations), including representative sequences from variant sublineages and the L1L2 PsV sequence. Variant lineages are represented by colours and sublineages by colour-filled shapes. Grey filled circles represent sequences which do not have a sublineage designation. (B) The HPV58 L1 and L2 amino acid sequences of the reference and variant lineages (derived consensus) were compared against L1L2 PsV. L1 sequences represented by lineage consensus: A, 16/32 = 50%; B, 2/5 = 40%; C, 5/6 = 83%; D, 3/6 = 50%. L2 sequences represented by lineage consensus: A, 5/32 = 16%; B, 2/5 = 40%; C, 3/6 = 50%; D, 2/6 = 33%. Divergent amino acid positions are indicated by peaks representing the percentage of sequences within the variant lineages which have the variable residue. The L1 surface exposed loop regions (light blue shading) and characterised L2 neutralising antibody epitopes (light pink shading) are also indicated.

A2, A3 or A4 designated by WGS analysis) this level of segregation was no longer supported by bootstrap values (**Figure 16A**). There were also instances where sequences from one sublineage clustered with sequences of a neighbouring sublineage, for example the representative sequence of HPV16 variant sub-lineage D1 (HQ644257) clustered with D2 sequences.

The L1L2 PsV representing each A9 genotype demonstrated the closest relationship, based upon L1 and L2 amino acid identity, to the variant lineage A sequences of their respective genotype with sequence diversity from the L1L2 PsV generally increasing in a stepwise manner in the subsequent variant lineages (**Figure 22**). For example, the variant lineage A of HPV31 demonstrated a sequence diversity of 0.15% compared to the HPV31 L1L2 PsV with sequence diversity increasing to 0.21% in variant lineage B and 0.62% in variant lineage C. The HPV52 L1L2 PsV was the most representative of its genotype demonstrating a median inter-lineage diversity of 0.10% (IQR, 0.10 to 0.41) whilst the HPV33 L1L2 PsV was the least representative (0.73%; IQR, 0.10 to 0.88). Overall, the L1 and L2 amino acid diversity from the A9 genotypes compared to their respective L1L2 PsV was low (<2%).

3.2.4.2 A9 intra-genotype L1 and L2 amino acid diversity

The consensus L1 and L2 amino acid sequence of each variant lineage were determined and compared, alongside the reference sequence, to the L1L2 PsV sequence for that particular genotype in order to identify major positions of amino acid variation within the L1 and L2 proteins (**Figures 16B** to **21B**). The L1L2 PsV representing HPV16 and HPV35 demonstrated a small degree of sequence diversity compared to their respective reference sequences whereas the L1L2 PsV representing HPV31, HPV33, HPV52 and HPV58 had a 100% L1 and L2 amino acid sequence identity to their respective reference sequence. The PsV of HPV35 differed from the HPV35 reference sequence (M12732) at a single L1 amino acid position, Ala^{2} (**Figure 19B**). The HPV16 PsV differed from the HPV16 reference sequence (K02718) at five L1 positions, Asp^{202}, Ala^{266}, Thr^{422}, Ser^{423} plus a deletion at

Figure 22

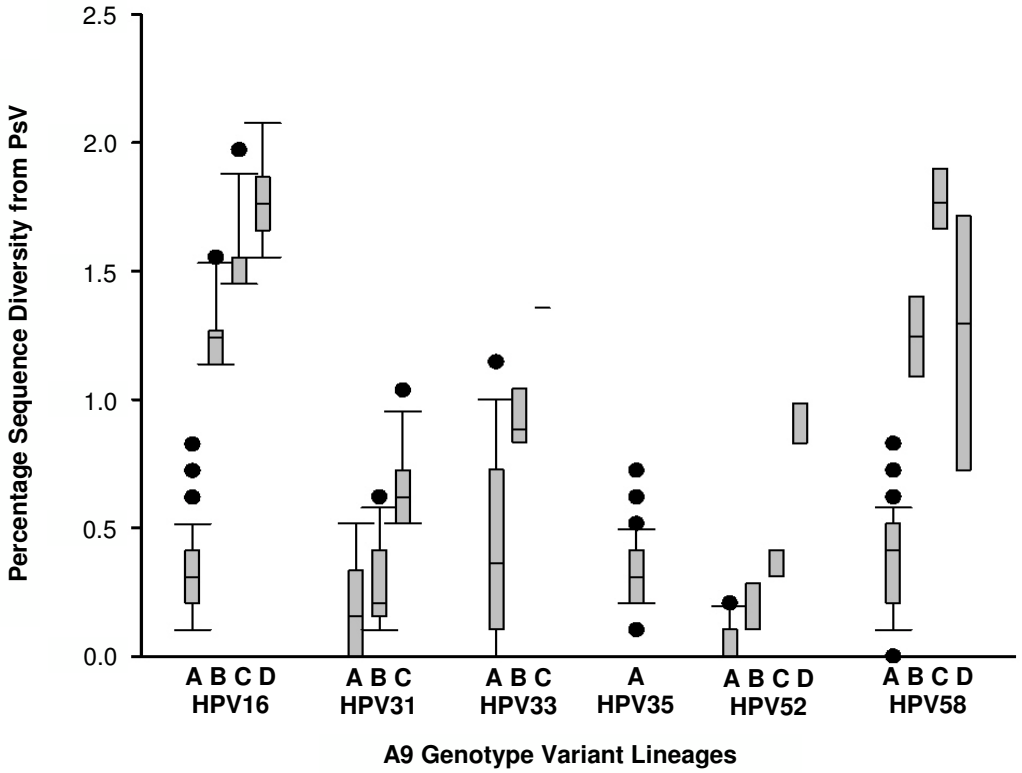


Figure 22 A9 variant lineage diversity from PsV. A9 variant lineage L1 and L2 amino acid sequence diversity from representive L1L2 PsV. Plots show box (median, IQR), whisker (± 1.5 IQR) and outliers (>1.5 IQR).

position 440 and at a single position within the L2 at Asp^{43} (**Figure 16B**). The L1 and L2 consensus sequences represented each variant lineage to different degrees. For example, the consensus L1 amino acid sequence for HPV16 variant lineage A represented 61% (n=42) of the sequences within this lineage indicating that the consensus represented the majority of circulating sequences (**Figure 16B**). In contrast, the consensus L1 amino acid sequence for HPV16 variant lineage D represented the minority of sequences (n=3; 10%) and whilst the consensus did represent circulating sequences, the lower number of those sequences demonstrated the increased diversity within this lineage.

The comparison of the L1L2 PsV against the consensus L1 and L2 sequences derived for each variant lineage re-affirmed that all the A9 L1L2 PsV shared the highest degree of amino acid similarity with variant lineage A of their respective genotypes. The level of amino acid sequence diversity between the variant lineages and their representative L1L2 PsV differed between A9 genotypes but was generally low. The L1 consensus sequences representing HPV16 variant lineage A (**Figure 16B**) and HPV52 variant lineages A, B and C (**Figure 20B**) had a 100% amino acid identity to their respective PsV. The L1 consensus of HPV58 variant lineage C was the most diverse, with twelve amino acid positions which varied from the L1 sequence of HPV58 PsV (**Figure 21B**). The L2 consensus sequence of HPV16 variant lineage D contained the highest level amino acid diversity compared to the L2 of its representative PsV, with eleven positions of amino acid variation (**Figure 16B**). In contrast the L2 consensus of HPV31 variant lineages A and B (**Figure 17B**), HPV35 variant lineage A (**Figure 19B**) and HPV52 variant lineage A (**Figure 20B**) had a 100% amino acid identity to their respective PsV.

It was apparent that variant lineage L1 amino acid diversity from the L1L2 PsV could be located to the surface exposed loops regions (BC, DE, EF, FG and HI). For example, of the eleven positions of amino acid variation within the L1 of HPV58 variant lineage C, nine variant positions fell within loop regions with the DE loop harbouring three (V118I, S133G

and P137T), the FG loop four (K266T, A270P, D273 and V285G) and HI loop two (G532D and D357N) (**Figure 21B**). Amino acid diversity within the L1 loops was also observed in variant lineages which exhibited lower levels of amino acid variation compared to their respective L1L2 PsV. For example, the HPV33 variant lineage B had four amino acid positions of variation within the L1, three of which were located in loop regions (BC: T56N; DE: G133S; FG: T266K) (**Figure 18B**). The C-terminal portion of the L2 protein harboured regions of amino acid variation between variant lineages and their respective L1L2 PsV which was particularly apparent for the variant lineages B, C and D of HPV16 and HPV58 (**Figures 16B and 21B**). For HPV16, this corresponded to *ca.* 90% of all L2 variation within lineages B, C and D being located in the C-terminal portion of the L2 protein.

3.2.4.3 HPV31 intra-genotype L1 and L2 amino acid variation

Despite the low level of L1 and L2 amino acid diversity between intra-genotype variant lineages it is unclear the potential impact that such variation may have upon capsid recognition by antibodies. The L1 consensus sequence of HPV31 variant lineages B and C demonstrated diversity from variant lineage A in the FG loop (**Figure 17B**). The HPV31 L1L2 PsV and variant lineage A sequences have Thr residues at FG loop positions 267 and 274 whilst in variant lineages B and C position 274 is an Asn and in variant lineage C position 267 is an Ala (**Figure 23A**). The L2 amino acid sequences of variant lineages A and B have a 100% sequence identity to the HPV31 L1L2 PsV; however, variant lineage C has three amino acid substitutions within the L2 at positions 115 (V115I), 270 (I270M) and 377 (V377L). HPV31 is a genotype for which a degree of vaccine-induced cross-protection has been demonstrated and cross-neutralising antibodies which recognised the HPV31 L1L2 PsV are commonly detected in the sera of vaccinees, therefore it was reasoned that variation within the FG loop region may result in differential variant lineage susceptibility to cross-neutralisation.

Figure 23

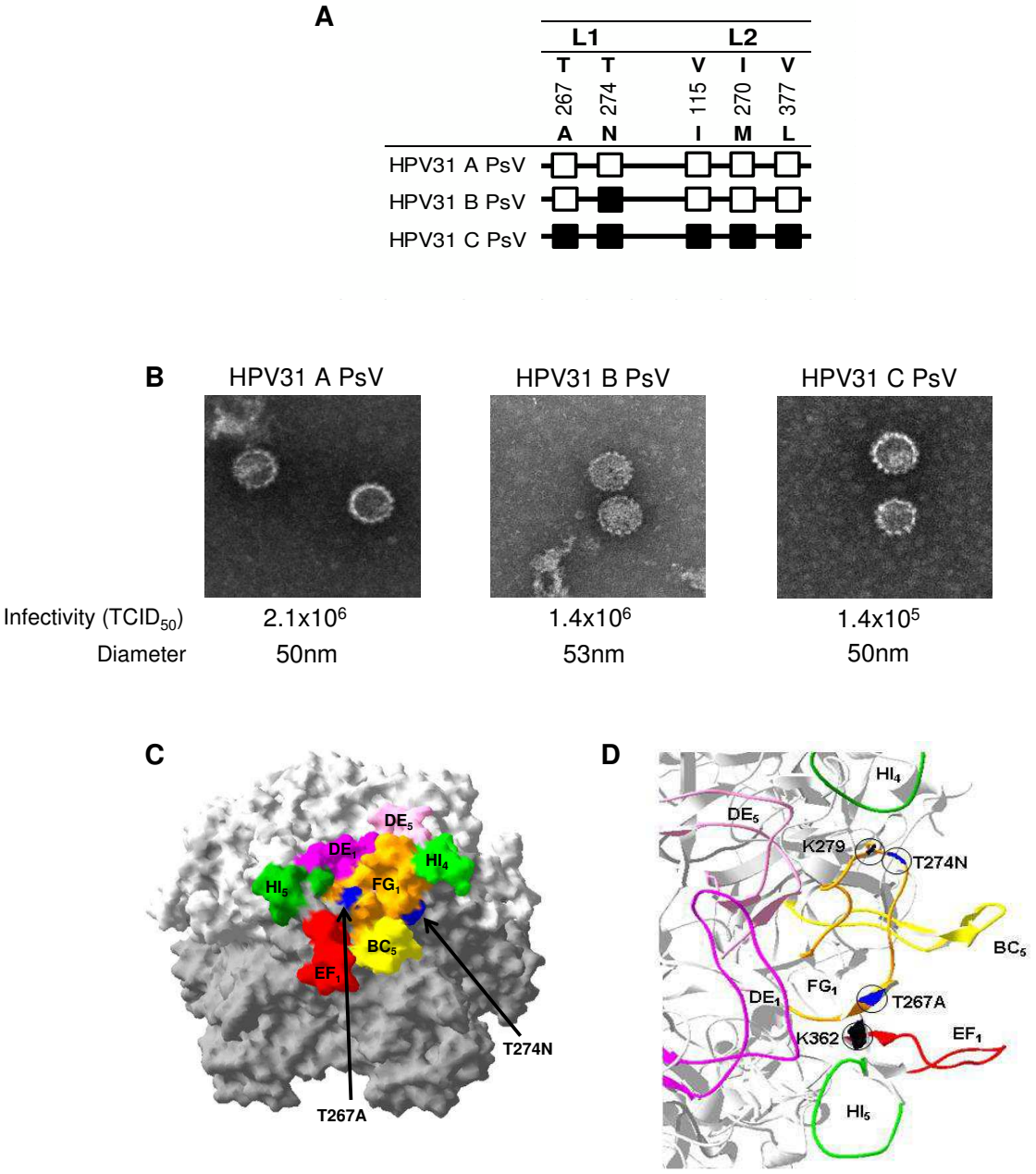


Figure 23 HPV31 L1 and L2 variants. (A) Graphical representation of L1 and L2 variant protein combinations. (B) Negatively stained EM images of HPV31 variant L1L2 PsV preparations. Infectivity represented by the $TCID_{50}$ and particle diameters are indicated for each variant. (C) Side view (C) and top view (D) highlighting loops in close proximity to FG loop variant residues 267 and 274 highlighted in blue. The FG loop of monomer 1 (FG_1) is coloured orange and neighbouring loops on the same (DE_1 - dark pink; EF_1 - red) or adjacent monomers (HI_4 - light green; BC_5 - yellow; DE_5 - light pink; HI_5 - dark green) are indicated. The remaining surface exposed regions of the capsomer are coloured in light grey and core regions are coloured in dark grey. Lys279 and Lys362 are highlighted in black (D only). Bissett *et al*, J Virol, 89:7748 (2015).

L1L2 PsV representing the HPV31 variant lineages A, B and C were generated in order to investigate the impact of amino acid sequence variation within the HPV31 FG loop. The three variant PsV, herein referred to as HPV31 A PsV, HPV31 B PsV and HPV31 C PsV produced similarly sized particles (*ca.* 50nm) which were infectious demonstrated by their individual $TCID_{50}$ (**Figure 23B**). Modelling of the L1 variant amino acid positions onto the L1 crystal structure demonstrated that the FG loop of monomer 1 (FG_1) is adjacent to the BC_5, DE_1, DE_5, EF_1, HI_4 and HI_5 loops within the capsomer (**Figure 23C**). Positions 267 and 274 of FG_1 are also within close proximity (within 10 Å) to residue positions predominantly within the adjacent BC_5, FG_1 and HI_5 loops including Lys^{279} within the FG loop and Lys^{362} within the HI loop (**Figure 23D**).

The HPV31 variant PsV were tested against HPV vaccine serum from Study-02 (Cervarix® n = 22; Gardasil® n = 24) and both the HPV31 B and C PsV were more susceptible to cross-neutralisation by vaccine-induced antibodies than the HPV31 A PsV (**Table 9**). The HPV31 B PsV displayed a median 1.7-fold (IQR, 1.1 to 2.4 fold; Wilcoxon paired signed-rank test, p <0.001) increased sensitivity to cross-neutralising antibodies compared to that of the HPV31 A PsV, while the HPV31 C PsV displayed a 1.4-fold (IQR, 1.1 to 1.6 fold; p <0.001) increased sensitivity compared to that of the HPV31 A PsV. The increased sensitivity of the HPV31 B and C PsV to cross-neutralising antibodies was independent of the HPV vaccine received.

3.2.5 Discussion

L1 and L2 amino acid sequences of the A9 HPV PsV were compared to available L1 and L2 sequences to determine how representative these PsV were of circulating HPV sequences. HPV genotypes can be divided into variant lineages which can then be further subdivided into sublineages based upon whole genome sequence (Burk et al., 2013). This segregation within a genotype is based upon the analysis of lineage-specific single nucleotide polymorphisms (Chen et al., 2011, 2013). The phylogenetic analysis of the A9 sequences

Table 9. Neutralisation sensitivity of variant HPV31 L1L2 PsV to HPV vaccine-induced antibodies

		Median (IQR) neutralisation titres against indicated HPV31 PsV variants				
		HPV31 A PsV	HPV31 B PsV		HPV31 C PsV	
Vaccine	n	Titre[a]	Titre	Fold Difference[b]	Titre	Fold Difference
Cervarix®	22	1,026 (646 – 1,543)	1,469 (1,260 – 2,582)**	1.8 (1.1 – 2.5)	1,180 (923 – 1,721)*	1.3 (1.0 – 1.7)
Gardasil®	24	712 (382 – 1,363)	1,016 (759 – 1,435)**	1.5 (1.1 – 2.3)	968 (659 – 2,249)***	1.4 (1.1 – 1.6)
All	46	885 (499 – 1,435)	1,273 (973 – 2,253)***	1.7 (1.1 – 2.4)	1,096 (763 – 2,216)***	1.4 (1.1 – 1.6)

[a] Neutralisation data presented as the median (inter-quartile range, IQR) of the 80% antibody neutralisation titres generated from the 2-5 data sets per serum. The Wilcoxon paired signed-rank test was used to compare neutralisation titres of the HPV31 B PsV and C PsV compared to the HPV31 A PsV. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

[b] Median fold difference (IQR) in the neutralisation titres of the HPV31 B PsV and C PsV compared to the HPV31 A PsV

conducted here demonstrated that there were sufficient diagnostic lineage-motifs within the L1 and L2 amino acid sequences to support segregation at the level of variant lineage; however, the further segregation into variant sublineage was not supported. The L1L2 PsV were classified as belonging to variant lineage A of their respective genotypes. This is not unexpected since the L1L2 PsV are either identical to (HPV31, HPV33, HPV52 and HPV58) or closely represent (HPV16 and HPV35) the reference sequence of their respective genotype. The reference will generally have been the first genome sequenced and as a consequence resides in the variant lineage A of a given genotype (Chen et al., 2011, 2013).

The consensus sequence of each variant lineage highlighted the intra-genotype L1 and L2 amino acid diversity in comparison with the representative L1L2 PsV; however, the majority of variant lineage consensus sequences represented only a proportion of circulating sequences. The representativeness of these lineage consensus sequences will be informed as additional sequence data becomes available. The number of L1 and L2 amino acid sequences representing a genotype and consequently the variant lineages within a genotype was disproportionate across the A9 genotypes. HPV16 was represented by the highest number of sequences and HPV33 was represented by the lowest number. HPV33 variant lineage C was represented by a single sequence. Disproportionate representation of sequences inevitably introduces bias into this kind of assessment; however, the number of sequences used was sufficient to highlight variant lineage-specific diversity in comparison with the L1L2 PsV of that respective genotype. The intra-genotype diversity of HPV31, HPV35 and HPV52 was low for both the L1 and L2 indicating that at the level of amino acid sequence the L1L2 PsV were generally representative of their respective genotypes. In contrast, HPV16, HPV33 and HPV58 demonstrated increased intra-genotype diversity across the L1 and L2 compared to their respective L1L2 PsV; however, the difference was of a relatively low magnitude.

Divergent L2 amino acid positions in the variant lineages B, C and D of HPV16 and HPV58 were predominantly located in the C-terminal portion of the protein. An L1-binding domain is located at positions 396 to 439 within the L2 protein of HPV11 (Finnen et al., 2003). The corresponding amino acids in HPV16 suggest that a putative L1 binding domain spans positions 412 to 455 of the L2 protein and would encompass the variable positions 420, 424 and 443 within the HPV16 variant lineages B, C and D. Further studies are required to determine whether residue variation at these amino acid positions could potentially impact upon L1-L2 protein interactions.

Intra-genotype variation within the L1 protein is mostly located in the surface exposed loop regions (Ahmed et al., 2013) and this was also observed when the consensus L1 sequences of each variant lineage were compared to the L1 sequences of their representative L1L2 PsV. Variation in these L1 regions has the potential to alter loop surface topography between different variant lineages resulting in differential recognition by L1 antibodies. Consequently, a single L1L2 PsV may not be sufficient to represent the diversity within a genotype. The potential antigenic impact of variation within the L1 loops was investigated for the three variant lineages of HPV31 (A, B and C) that have amino acid polymorphisms within the FG loop, which is known to contain residues which contribute to type-specific neutralising MAb epitopes (Fleury et al., 2009).

All three variant HPV31 L1L2 PsV were susceptible to vaccine-induced cross-neutralising antibodies with HPV31 B and C PsV demonstrating increased sensitivity compared to HPV31 A, although the difference between the three variants was of a low magnitude. An Asn^{274} is common to both variants B and C; however, the substitution from Thr to Asn is a relative subtle change as both amino acids have polar uncharged side chains and therefore it is unlikely that an Asn residue in itself has a critical role within this cross-neutralising epitope. The change of residue at position 274, near the tip of the FG loop, may result in local structural changes which increase recognition of more distal epitope residues. The

inter-genotype comparison of L1 pentamer crystal structures has previously demonstrated that a single residue difference between genotypes can shift loop structures by a few angstroms resulting in the altered presentation of L1 antigenic determinants between genotypes (Bishop et al., 2007). The variant positions 267 and 274 are in close proximity to charged residues located in the BC, FG and HI loops. The corresponding residues of HPV16 are involved in HPV binding to heparin sulfate which is an essential step for a successful HPV infection (Richards et al., 2013) and cross-neutralising antibodies may function by abrogating this virus-host interaction.

The precise criterion used to designate serotypes differs between virus families but is generally based upon an fold difference in antibody-mediated neutralisation titres between viral types: Adenovirus 8- to 16-fold (Heemskerk et al., 2005), Rotavirus ≥20-fold (Wyatt et al., 1982), Polyomavirus 4- to 100-fold (Pastrana et al., 2013). There are no currently defined criteria with which to designate HPV L1 serotypes. Geographical variants of HPV16 belong to a single serotype based upon a ≤4-fold difference in neutralisation titre between variants (Pastrana et al., 2001). Under this criterion the HPV31 variants lineages A, B and C should probably be considered as belonging to a single serotype since the significant differences in cross-neutralisation antibody titres observed between HPV31 variants lineages were of a low magnitude (<2-fold). This implies that for the testing of cross-neutralising antibodies, a single L1L2 PsV should be sufficient to represent HPV31. In a recent study, a single amino acid position within the HI loop of HPV45 appeared to influence the increased sensitivity to cross-neutralisation of L1L2 PsV representing the variant sublineages A2, A3 and B1 in comparison with variant sublineages A1; however, although the differences were significant they were again of a low magnitude (*ca.* 3-fold) (Godi et al., 2015b). Whether L1 polymorphisms have a greater impact upon the antigenicity of other genotypes is unclear.

Overall, these date demonstrate that there is L1 and L2 amino acid sequence diversity between the A9 genotype variant lineages and their respective PsV. Despite this, L1L2 PsV

are relatively representative of a genotype based upon the available sequence data. These data also inform our understanding of the antigenicity of the HPV structural proteins demonstrating that the HPV31 variants belong to a single L1 serotype based upon recognition by vaccine-induced cross-neutralising antibodies.

3.3 Cross-neutralising antibodies recognise an L1 domain incorporating amino acid residues from the DE and FG loops of a single monomer

3.3.1 Background

Inter-genotype amino acid sequence variation is mostly concentrated on the surface exposed L1 loop regions (Carter et al., 2003; Chen et al., 2000) and appears to dictate the predominantly type-specific nature of the L1 neutralising antibody response (Bishop et al., 2007). Both type-specific HPV16 natural infection antibodies and the majority of MAbs which neutralise HPV16 infectivity bind to one or more of these surface exposed loops (Carter et al., 2006; Christensen et al., 2001). L1 cross-neutralising antibodies which differentially recognise L1L2 PsV representing the non-vaccine A9 genotypes (Draper et al., 2011; Einstein et al., 2011a; Kemp et al., 2011) are generated in response to the HPV16 L1 VLP within the vaccine preparations and represent a minor component of the total HPV16 antibody response (see Section 3.1.4.4) (Bissett et al., 2014). These data indicate that the L1 of HPV16 harbours immunogenic domains which share sequence and/or structural homology with the L1 proteins of closely related A9 genotypes. The identification of these common domains should inform the design and generation of chimeric PsV in order to test specific L1 domain recognition by cross-neutralising antibodies.

3.3.2 Aim of chapter: To delineate the L1 domains recognised by inter-genotype cross-neutralising antibodies

3.3.3 Specific objectives

1. Examine the L1 amino acid sequence diversity between PsV representing HPV16 and the non-vaccine A9 genotypes using appropriate bioinformatic tools

2. Model the L1 amino acid sequence diversity between the PsV representing HPV16 and the non-vaccine A9 genotypes on the pentameric L1 crystal structure of HPV16
3. Design and generate PsV to test cross-neutralising antibody recognition of L1 domains

3.3.4 Results

3.3.4.1 L1 amino acid diversity of A9 L1L2 PsV

The L1 sequences of the non-vaccine A9 L1L2 PsV were analysed for amino acid identity, hydrophobicity, molecular weight and charge compared to the L1 sequence of HPV16 PsV, in order to identify L1 regions of inter-genotype diversity. Alignment of the L1 sequences (**Figure 24**) demonstrated that the level of non-vaccine A9 amino acid diversity from HPV16 PsV was higher in the surface exposed loop regions, with a median diversity from HPV16 of 35% (IQR, 29 to 36), compared to the α-helices and β-sheets contained in the L1 backbones which had a median diversity from HPV16 of 19% (IQR, 14 to 19). L1 amino acid insertions and deletions in the non-vaccine A9 L1L2 PsV compared to HPV16 PsV were observed, with positions harbouring insertions/deletions restricted to the loop regions and the L1 C-terminus. The HPV52 L1L2 PsV had three amino acid insertions within the BC loop (Ser^{57}, Gly^{58} and Gly^{60}) whilst the PsV representing HPV31 (Pro^{60}), HPV33 (Ala^{60}) and HPV58 (Asn^{60}) had single amino acid insertions in the BC loop compared to the HPV16 PsV. Both HPV33 and HPV58 L1L2 PsV had a single amino acid deletion within the EF loop at Gly^{186}. The HPV35 L1L2 PsV had a two amino acid deletion within the FG loop (Ser^{283} and Gly^{286}) whilst the HPV52 PsV had a two amino acid insertion (Asn^{284} and Ser^{285}) compared to the HPV16 PsV. The PsV representing HPV33, HPV52 and HPV58 all had single amino acid deletions within the HI loop at Thr^{355}. In addition, amino acid deletions within the C-terminus were also apparent for HPV31 (Pro^{494}) HPV33 (Thr^{486}, Leu^{487}, Gly^{488} and Pro^{494}) HPV52 and HPV58 (Thr^{486}, Leu^{487}, Gly^{488}, Pro^{494} and Thr^{496}) compared to the HPV16 PsV.

Figure 24

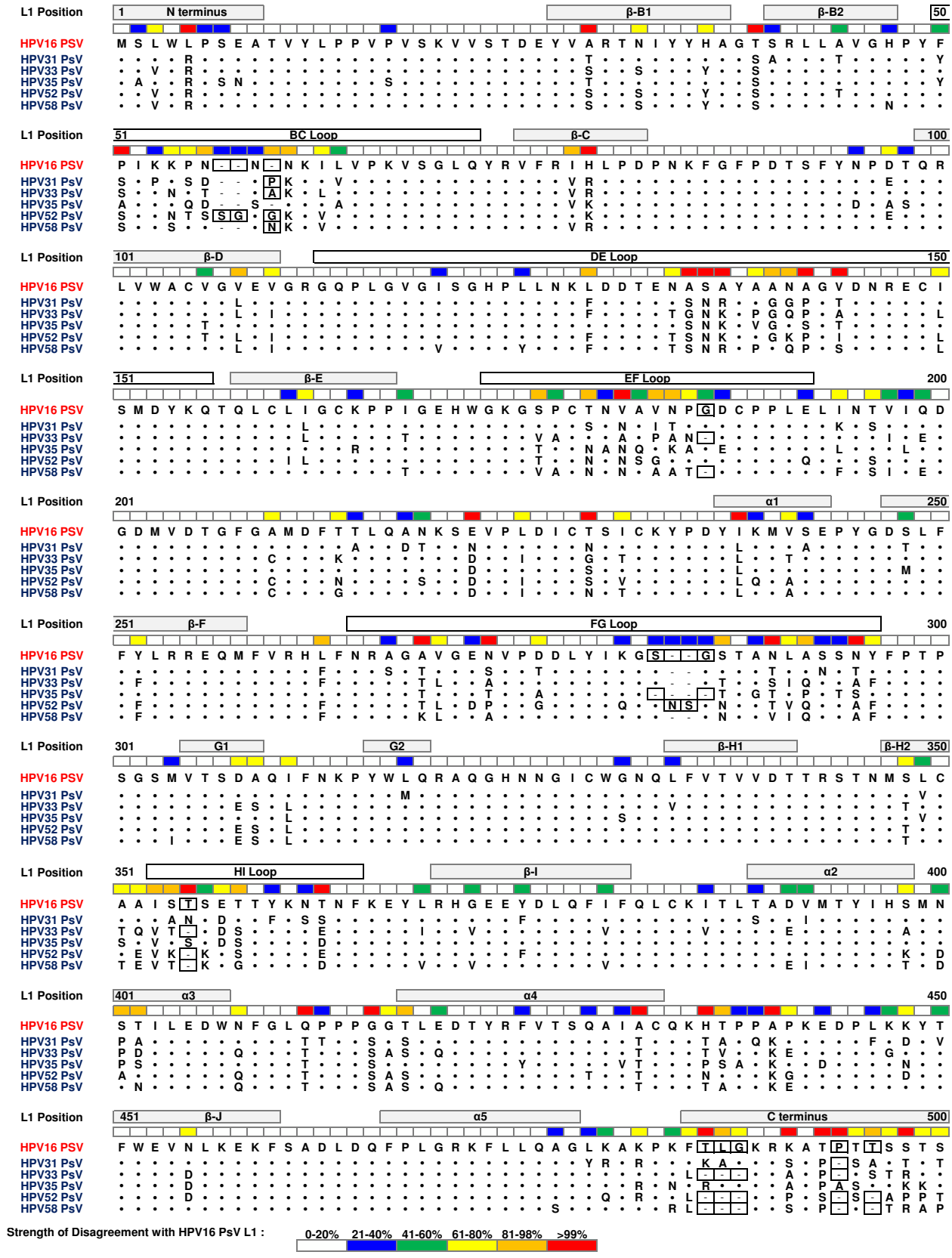


Figure 24 L1 amino acid alignment of A9 L1L2 PsV. The L1 sequences of the non-vaccine A9 genotypes were aligned against HPV16 using MegAlign (DNASTAR). A colour code represents the strength of disagreement between the non-vaccine genotypes and HPV16. L1 amino acid positions with residue insertion or deletions compared to HPV16 are boxed. L1 structural and loop regions are also indicated.

The L1 loop sequences were analysed further to see whether the differences in amino acid sequence correlated with differences in charge, hydrophobicity and/or molecular weight between the L1L2 PsV representing the non-vaccine A9 genotypes and HPV16. The amino acid charge within the L1 loops was calculated using EMBOSS Pepinfo (www.ebi.ac.uk/Tools/seqstats/emboss_pepinfo/) and the differences in charge profiles compared to HPV16 were plotted onto the top view of the L1 capsomer (**Figure 25A**) using a colour-coded scale (**Figures 25B** to **25F**). All of the non-vaccine A9 L1L2 PsV had a net positive charge in the DE loop compared to HPV16 due to additional positively charged residues: Arg^{135} (HPV31), Lys^{135} (HPV33), Lys^{134} (HPV35), Lys^{137}, Lys^{141} (HPV52) and Arg^{135} (HPV58) (**Figure 24**). The net charge within the other L1 loop compared to HPV16 differed between the non-vaccine A9 genotypes. For example, the net charge of the HPV33, HPV52 and HPV58 BC loops did not differ from HPV16 as the positively charged Lys^{54} of HPV16 was matched by the downstream Lys^{59} (HPV33), Lys^{61} (HPV52) and Lys^{59} (HPV58); however both HPV31 and HPV35 had a net negative charge compared to HPV16 in the BC loop due to an additional negatively charged Asn^{56} (**Figure 24**).

The relative hydrophobicity score of the amino acids within the L1 loops were calculated by ProtScale software (web.expasy.org/protscale/) using the hydrophobicity scale determined by Eisenberg (Eisenberg et al., 1984). Differences in the hydrophobicity profiles of the L1L2 PsV representing the non-vaccine A9 genotypes compared to HPV16 were plotted onto the top view of the L1 capsomer (**Figure 26A**) using a colour-coded scale. The hydrophobicity of the BC and HI loops differed between the non-vaccine PsV relative to HPV16 whilst all the DE and EF loops were hydrophilic and all the FG loops were hydrophobic (**Figures 26B** to **26F**). Significant differences in hydrophobicity relative to HPV16 were only observed for the DE loop of HPV58, the EF loops of HPV31, HPV35 and HPV52, the FG loop of HPV35 and the HI loop of HPV52 (**Table 10**). For example, the significant hydrophilic nature of the HPV58 DE loop relative to HPV16 appears to be due to the combined effects of two hydrophilic residues, Arg^{135} and Gln^{139} (**Figure 24**).

Figure 25

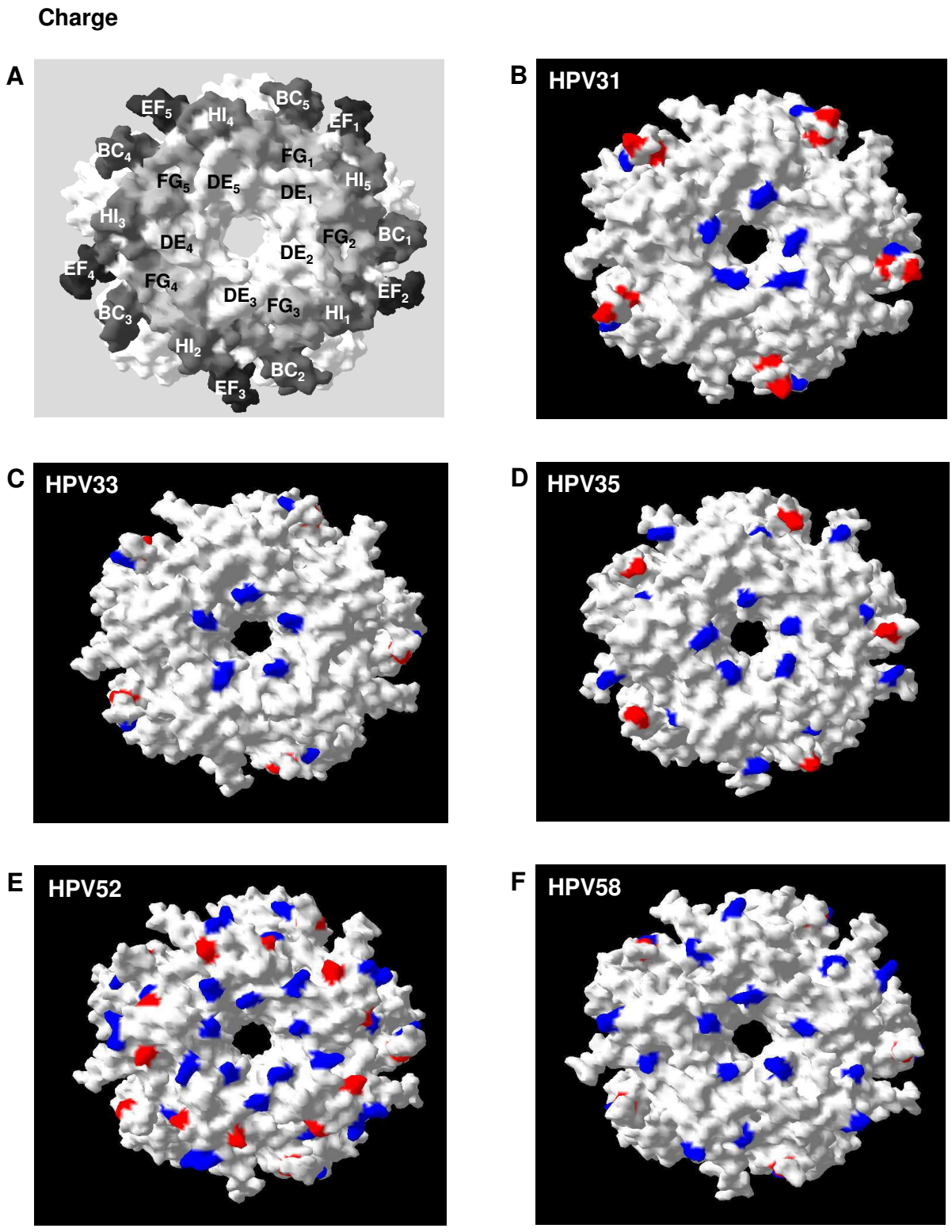


Figure 25 L1 loop amino acid charge profiles of non-vaccine A9 L1L2 PsV. (A) Top view of L1 capsomer with loop regions indicated for reference compared to the top view of non-vaccine A9 capsomers with divergent residues compared to HPV16 colour-coded. The L1 amino acid charge for the A9 L1L2 PSV were calculated using EMBOSS Pepinfo (www.ebi.ac.uk/Tools/seqstats/emboss_pepinfo/) using a window size of 9, HPV31 (B), HPV33 (C), HPV35 (D), HPV52 (E) and HPV58 (F). L1 loop positions are coloured-coded where the non-vaccine genotype contains an amino acid residue which has a positive (blue) or negative (red) charge relative to the HPV16 residue in the corresponding position. Neutral residue positions are colour-coded in grey.

Figure 26

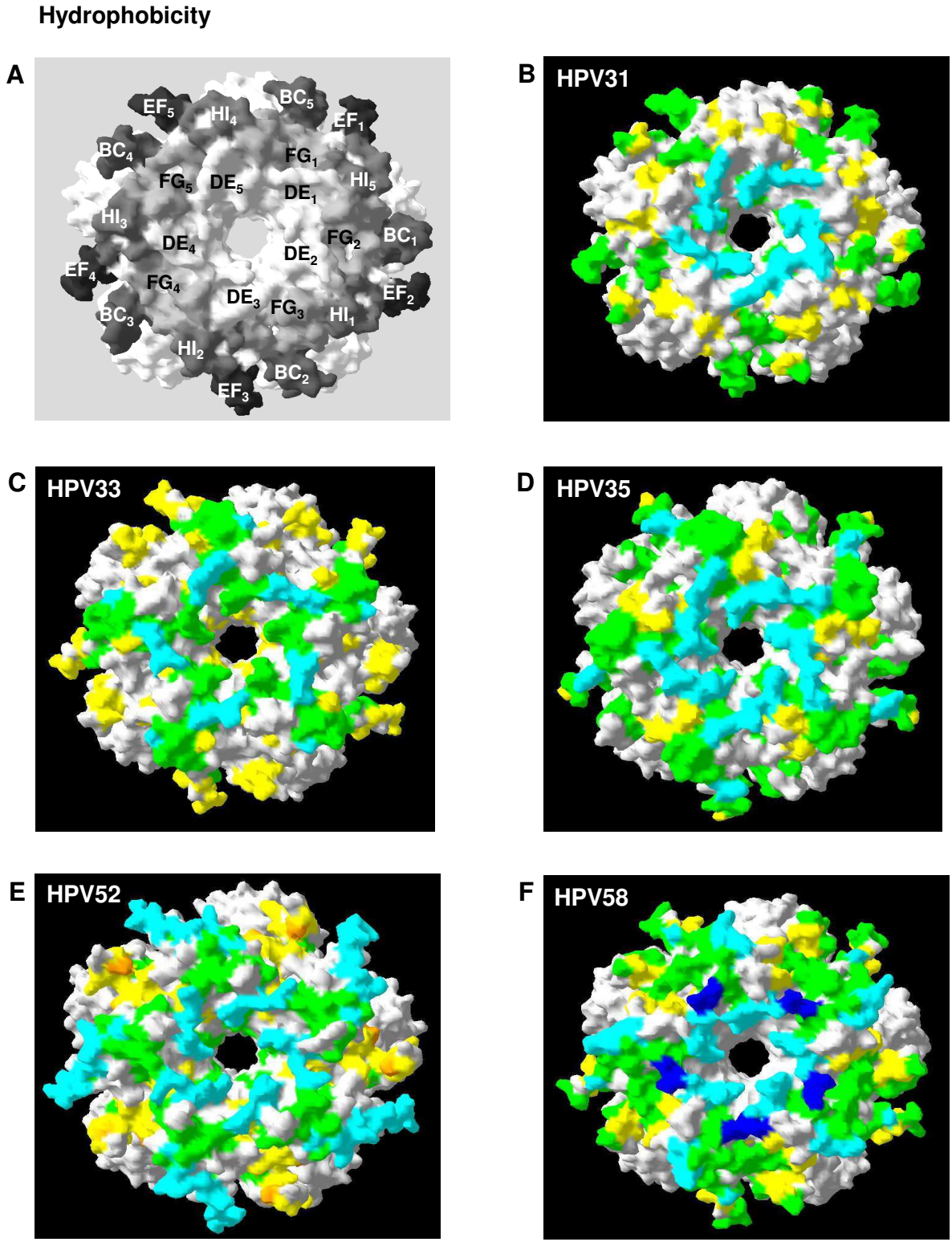


Figure 26 L1 loop amino acid hydrophobicity profiles of non-vaccine A9 L1L2 PsV. (A) Top view of L1 capsomer with loop regions indicated for reference compared to the top view of non-vaccine A9 capsomers with divergent residues compared to HPV16 colour-coded. The L1 hydrophobicity scores for the A9 L1L2 PSV were determined using ProtScale software (web.expasy.org/protscale/) with a window size of 9 and the scale normalised from 0 to 1, HPV31 (B), HPV33 (C), HPV35 (D), HPV52 (E) and HPV58 (F). Colour-coded scale is based upon the difference in L1 hydrophobicity score between HPV16 and the non-vaccine genotypes with positive (hydrophilic – dark blue, light blue and green) and negative (hydrophobic – red, orange and yellow) values proportionately ranked. Residue positions with equivalent hydrophobicity to HPV16 are colour-coded in grey.

Table 10. A9 PsV L1 loop amino acid hydrophobicity and molecular weight

		Hydrophobicity			Molecular weight		
Loop	L1L2 PsV	Median score[a] (IQR)	Relative to HPV16	*p* value[b]	Median weight[c] (IQR)	Relative to HPV16	*p* value[d]
BC	HPV16	0.636 (0.597 – 0.675)	-		0.445 (0.408 – 0.463)	-	
	HPV31	0.621 (0.600 – 0.662)	Hydrophilic	0.948	0.422 (0.401 – 0.445)	Lower	0.151
	HPV33	0.650 (0.609 – 0.663)	Hydrophobic	0.584	0.412 (0.388 – 0.445)	Lower	**0.085**
	HPV35	0.609 (0.577 – 0.665)	Hydrophilic	0.490	0.430 (0.375 – 0.463)	Lower	0.401
	HPV52	0.648 (0.611 – 0.663)	Hydrophobic	0.543	0.351 (0.321 - 0.444)	Lower	**0.002**
	HPV58	0.623 (0.589 – 0.670)	Hydrophilic	0.725	0.425 (0.404 – 0.444)	Lower	0.114
DE	HPV16	0.628 (0.592 – 0.694)	-		0.369 (0.305 – 0.455)	-	
	HPV31	0.603 (0.556 – 0.672)	Hydrophilic	0.087	0.403 (0.310 – 0.480)	Higher	0.276
	HPV33	0.601 (0.570 – 0.672)	Hydrophilic	0.125	0.389 (0.331 – 0.461)	Higher	0.243
	HPV35	0.601 (0.560 – 0.671)	Hydrophilic	0.069	0.399 (0.332 – 0.459)	Higher	0.170
	HPV52	0.599 (0.570 – 0.672)	Hydrophilic	0.105	0.411 (0.338 - 0.473)	Higher	0.123
	HPV58	0.580 (0.547 – 0.652)	Hydrophilic	**0.005**	0.434 (0.354 – 0.480)	Higher	**0.032**
EF	HPV16	0.684 (0.647 – 0.698)	-		0.328 (0.296 – 0.364)	-	
	HPV31	0.653 (0.633 – 0.676)	Hydrophilic	**0.031**	0.329 (0.304 – 0.364)	Higher	0.829
	HPV33	0.683 (0.671 – 0.693)	Hydrophilic	0.855	0.314 (0.269 – 0.373)	Lower	0.518
	HPV35	0.635 (0.615 – 0.667)	Hydrophilic	**0.006**	0.350 (0.339 – 0.378)	Higher	0.123
	HPV52	0.626 (0.587 – 0.649)	Hydrophilic	**<0.001**	0.340 (0.297 - 0.378)	Higher	0.776
	HPV58	0.679 (0.649 – 0.689)	Hydrophilic	0.298	0.311 (0.289 – 0.373)	Lower	0.844
FG	HPV16	0.669 (0.651 – 0.687)	-		0.345 (0.288 – 0.399)	-	
	HPV31	0.683 (0.671 – 0.702)	Hydrophobic	0.143	0.352 (0.291 – 0.400)	Higher	0.836
	HPV33	0.679 (0.675 – 0.702)	Hydrophobic	0.090	0.357 (0.289 – 0.388)	Higher	0.871
	HPV35	0.696 (0.680 – 0.712)	Hydrophobic	**0.006**	0.363 (0.307 – 0.390)	Higher	0.629
	HPV52	0.682 (0.648 – 0.722)	Hydrophobic	0.386	0.348 (0.296 - 0.387)	Higher	0.693
	HPV58	0.681 (0.652 – 0.702)	Hydrophobic	0.355	0.368 (0.310 – 0.401)	Higher	0.391
HI	HPV16	0.566 (0.554 – 0.676)	-		0.441 (0.379 – 0.504)	-	
	HPV31	0.595 (0.580 – 0.683)	Hydrophobic	0.118	0.391 (0.351 – 0.454)	Lower	0.209
	HPV33	0.552 (0.529 – 0.592)	Hydrophilic	0.142	0.447 (0.412 – 0.524)	Higher	0.568
	HPV35	0.542 (0.522 – 0.643)	Hydrophilic	0.101	0.417 (0.331 – 0.503)	Lower	0.608
	HPV52	0.528 (0.509 – 0.557)	Hydrophilic	**0.007**	0.506 (0.477 - 0.525)	Higher	0.128
	HPV58	0.556 (0.517 – 0.579)	Hydrophilic	0.142	0.456 (0.434 – 0.493)	Higher	0.463

IQR, inter-quartile range

[a] The L1 loop hydrophobicity scores for the A9 L1L2 PsV were determined using ProtScale software (web.expasy.org/protscale/) with a window size of 9 and the scale normalised from 0 to 1

[b] *p* values obtained using the Mann-Whitney test represent difference in hydrophobicity scores of indicated non-vaccine A9 genotype compared to HPV16 with significant differences highlighted in bold

[c] The L1 loop molecular weight scores for the A9 L1L2 PsV were determined using ProtScale software with a window size of 9 and the scale normalised from 0 to 1

[d] *p* values obtained using the Mann-Whitney test represent difference in molecular weight scores of indicated non-vaccine A9 genotype compared to HPV16 with significant differences highlighted in bold

The ProtScale software was also used to determine the relative molecular weight scores of the PsV L1 loops with differences in molecular weight profiles compared to HPV16 plotted onto the top view of the L1 capsomer (**Figure 27A**) using a colour-coded scale. The molecular weight of the EF and HI loops increased or decreased relative to HPV16 dependent upon the non-vaccine PsV whilst all the DE and FG loops were of a higher weight and all the BC loops were of a lower weight (**Figures 27B** to **27F**). Significant differences in molecular weight relative to HPV16 were only observed for the BC loop of HPV33 and HPV52, and the DE loop of HPV58 (**Table 10**) which has a stretch of residues, Ser^{133} to Pro^{140}, which are of a higher molecular weight then the HPV16 residues at the corresponding positions (**Figure 24**).

3.3.4.2 Modelling of L1 loop amino acid diversity between HPV16 and non-vaccine A9 PsV

To determine whether the differences in amino acid profiles of the L1 loops between the non-vaccine A9 genotypes were predictive of structural changes compared to HPV16, homology models of the L1 capsomer representing the L1 amino acid sequence of each PsV were created for subsequent pairwise modelling, using SWISS MODEL (http://swissmodel.expasy.org/). The crystal structure of the HPV16 L1 capsomer (PDB code: 2R5H) was first used to create a homology model of the HPV16 PsV L1. The L1 sequence of the HPV16 PsV differed from that of the crystal structure by three amino acids at positons Q177N and Q181N within the EF loop and position A266T within the FG loop but these did not adversely impact upon the quality of the predicted model which had a maximum GMQE score of 1.00. The HPV16 L1 capsomer crystal structure was subsequently used to make homology models from the L1 amino acid sequences represented in the non-vaccine A9 PsV, to which the L1 loop amino acid positions divergent from HPV16 were modelled (**Figures 28A** to **32A**). The crystal structure of the HPV35 L1 capsomer (PDB code: 2R5J) has also been resolved and was used to evaluate the structural accuracy of the HPV35 PsV L1 homology model based upon the HPV16 crystal structure.

Figure 27

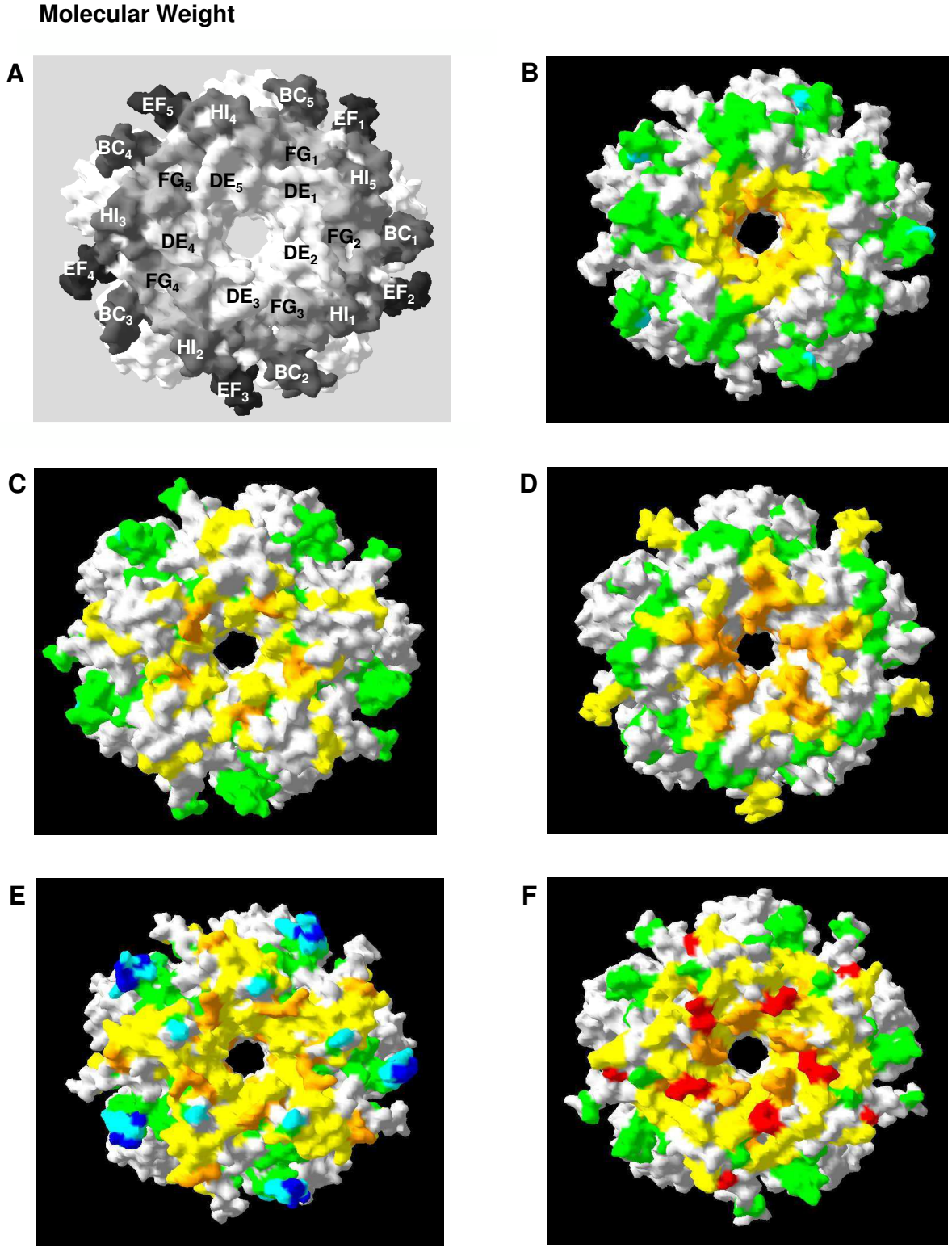


Figure 27 L1 loop amino acid molecular weight profiles of non-vaccine A9 L1L2 PsV. (A) Top view of L1 capsomer with loop regions indicated for reference compared to the top view of non-vaccine A9 capsomers with divergent residues compared to HPV16 colour-coded. The L1 molecular weight scores for the A9 L1L2 PSV were determined using ProtScale software (web.expasy.org/protscale/) with a window size of 9 and the scale normalised from 0 to 1, HPV31 (B), HPV33 (C), HPV35 (D), HPV52 (E) and HPV58 (F). Colour-coded scale is based upon the difference in L1 molecular weight score between HPV16 and the non-vaccine genotypes with higher (red, orange and yellow) and lower (dark blue, light blue and green) molecular weight values proportionately ranked. Residue positions with equivalent molecular weight to HPV16 are colour-coded in grey.

Figure 28

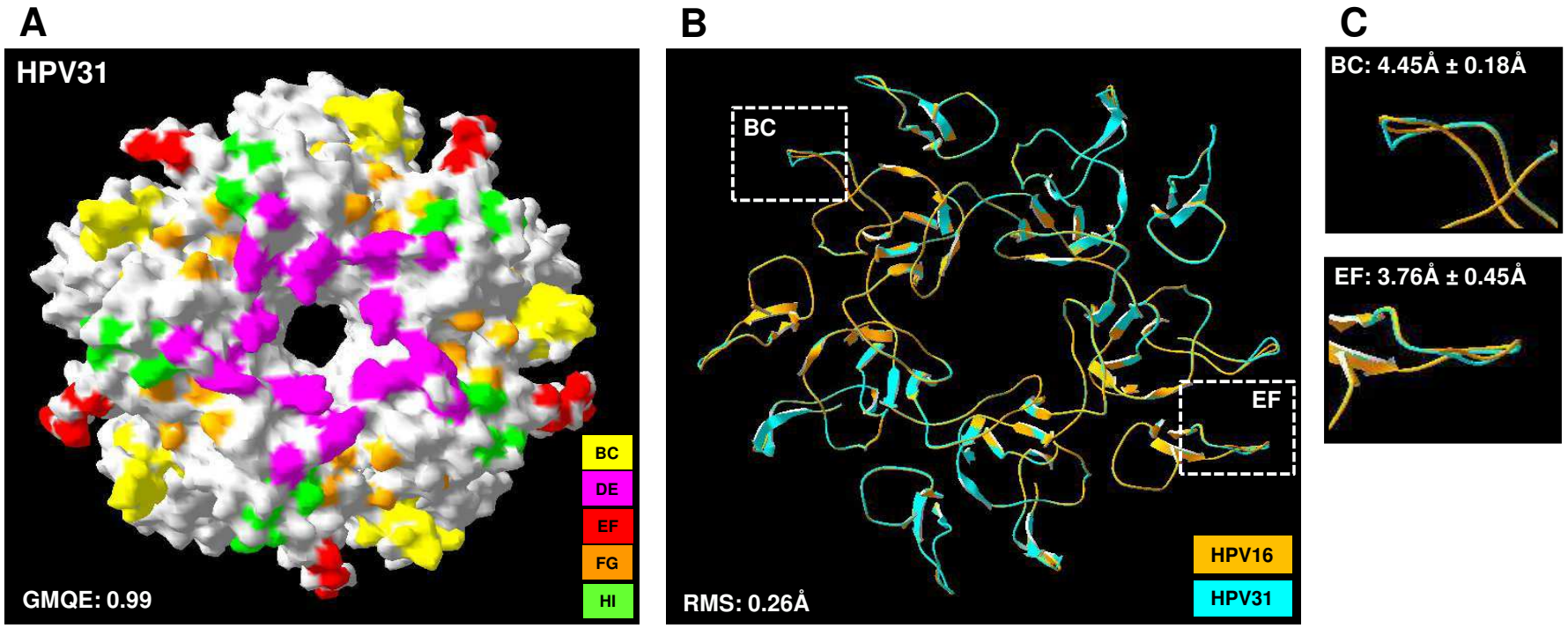


Figure 28 L1 homology model highlighting L1 loop amino acid diversity between HPV16 and HPV31 L1L2 PsV. (A) Top view of HPV31 L1 homology model created using the crystal structure of the HPV16 L1 pentamer (Bishop et al., 2007) with amino acid residues different from HPV16 highlighted in the BC (yellow), DE (pink), EF (red), FG (orange) and HI (green) loops. The GMQE score is indicated (Biasini et al., 2014). (B) Top view of pairwise model generated by the superimposition of the L1 ribbon structure from HPV31 (blue) onto the HPV16 L1 ribbon (orange) with RMS deviation value indicated. Loops with predicted structural differences between HPV31 and HPV16 are highlighted. (C) Expanded view of predicted structural differences between loops with the mean and standard deviation in Å between the loops indicated.

Figure 29

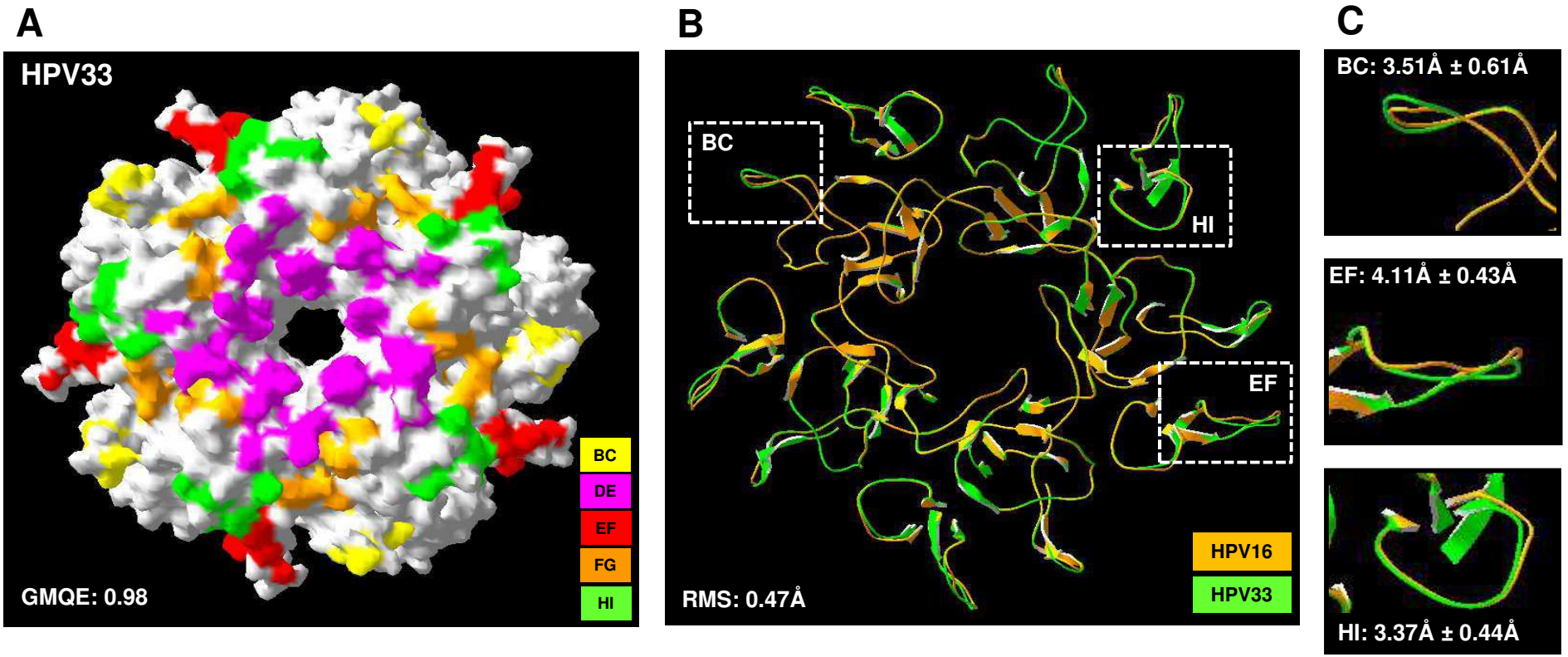


Figure 29 L1 homology model highlighting L1 loop amino acid diversity between HPV16 and HPV33 L1L2 PsV. (A) Top view of HPV33 L1 homology model created using the crystal structure of the HPV16 L1 pentamer (Bishop et al., 2007) with amino acid residues different from HPV16 highlighted in the BC (yellow), DE (pink), EF (red), FG (orange) and HI (green) loops. The GMQE score is indicated (Biasini et al., 2014). (B) Top view of pairwise model generated by the superimposition of the L1 ribbon structure from HPV33 (green) onto the HPV16 L1 ribbon (orange) with RMS deviation value indicated. Loops with predicted structural differences between HPV33 and HPV16 are highlighted. (C) Expanded view of predicted structural differences between loops with the mean and standard deviation in Å between the loops indicated.

Figure 30

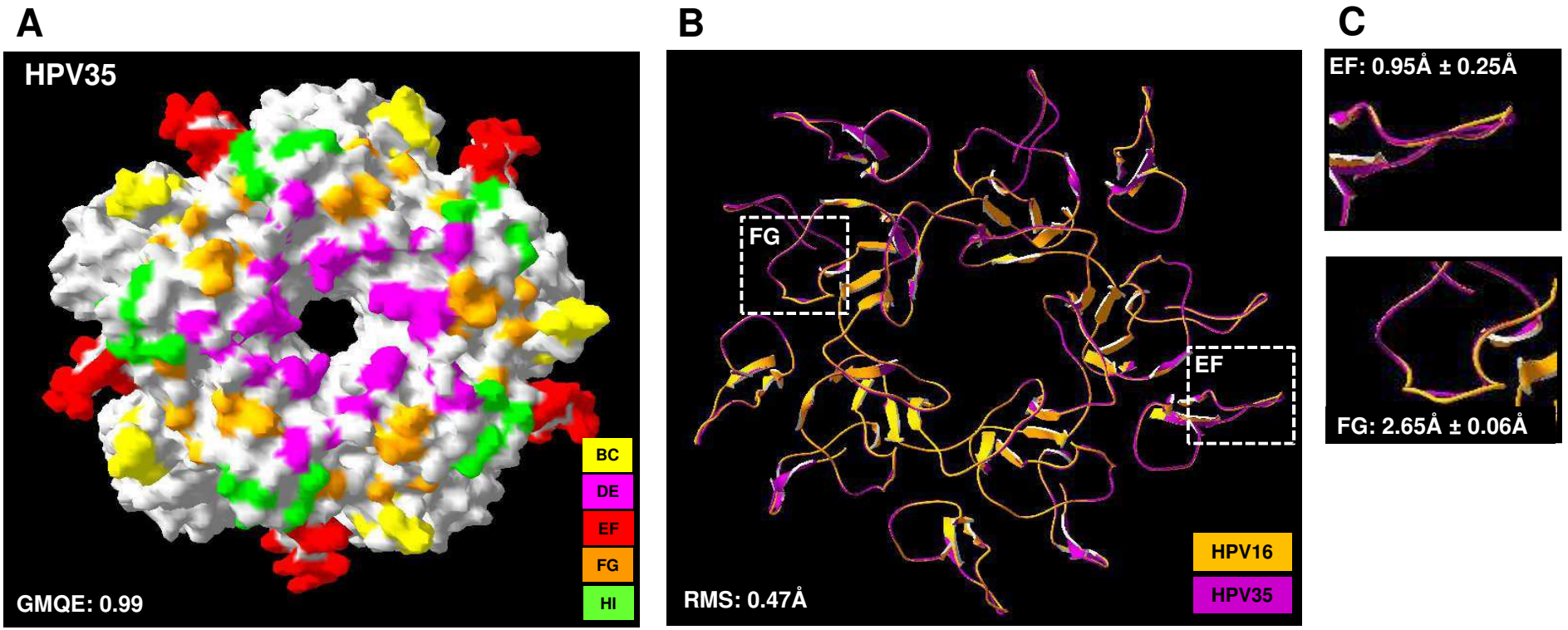


Figure 30 L1 homology model highlighting L1 loop amino acid diversity between HPV16 and HPV35 L1L2 PsV. (A) Top view of HPV35 L1 homology model created using the crystal structure of the HPV16 L1 pentamer (Bishop et al., 2007) with amino acid residues different from HPV16 highlighted in the BC (yellow), DE (pink), EF (red), FG (orange) and HI (green) loops. The GMQE score is indicated (Biasini et al., 2014). (B) Top view of pairwise model generated by the superimposition of the L1 ribbon structure from HPV35 (purple) onto the HPV16 L1 ribbon (orange) with RMS deviation value indicated. Loops with predicted structural differences between HPV35 and HPV16 are highlighted. (C) Expanded view of predicted structural differences between loops with the mean and standard deviation in Å between the loops indicated.

Figure 31

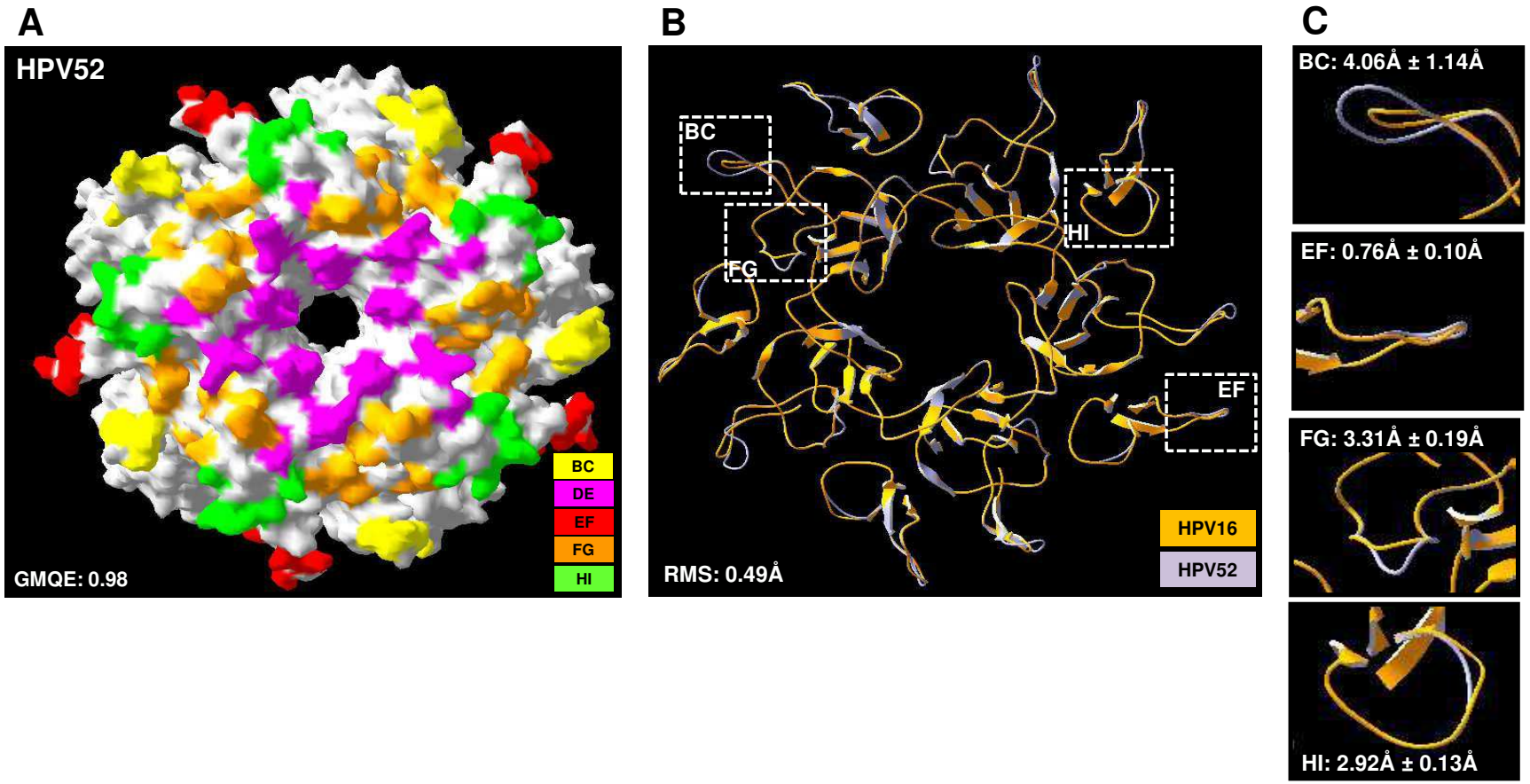


Figure 31 L1 homology model highlighting L1 loop amino acid diversity between HPV16 and HPV52 L1L2 PsV. (A) Top view of HPV52 L1 homology model created using the crystal structure of the HPV16 L1 pentamer (Bishop et al., 2007) with amino acid residues different from HPV16 highlighted in the BC (yellow), DE (pink), EF (red), FG (orange) and HI (green) loops. The GMQE score is indicated (Biasini et al., 2014). (B) Top view of pairwise model generated by the superimposition of the L1 ribbon structure from HPV52 (grey) onto the HPV16 L1 ribbon (orange) with RMS deviation value indicated. Loops with predicted structural differences between HPV52 and HPV16 are highlighted. (C) Expanded view of predicted structural differences between loops with the mean and standard deviation in Å between the loops indicated.

Figure 32

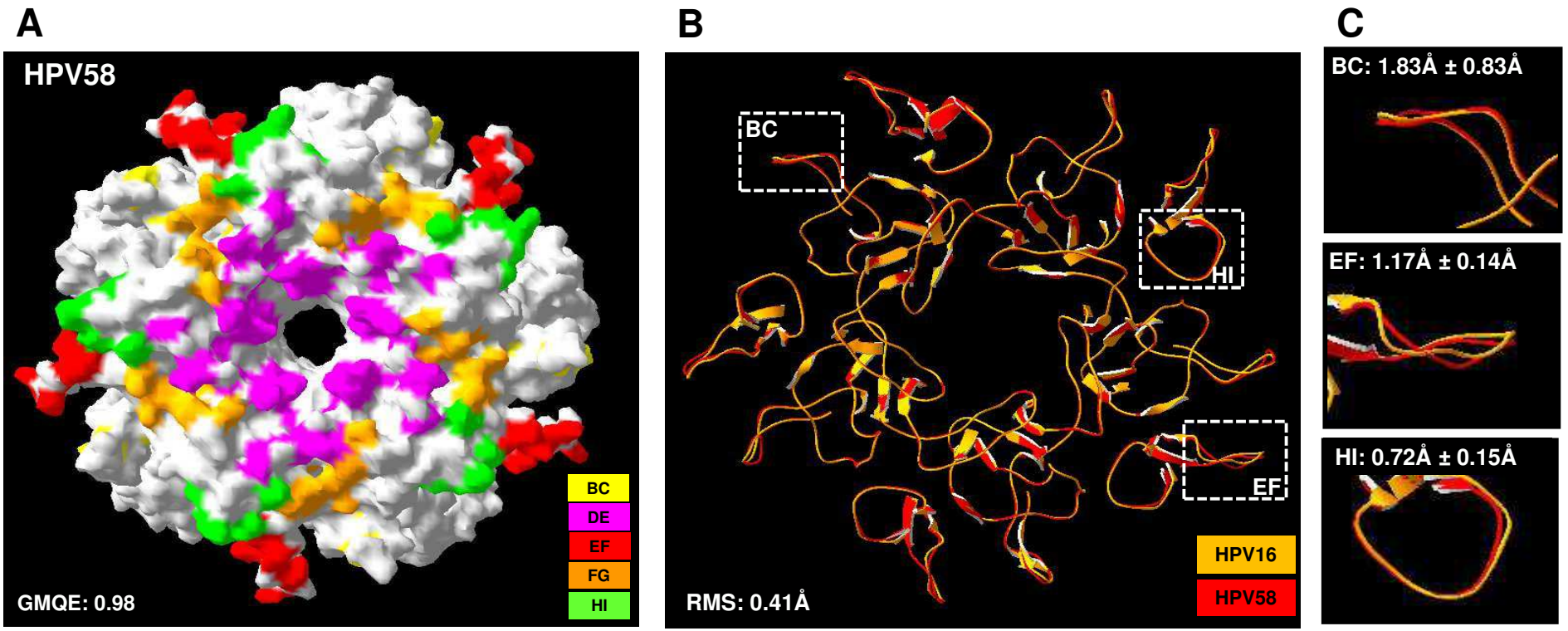


Figure 32 L1 homology model highlighting L1 loop amino acid diversity between HPV16 and HPV58 L1L2 PsV. (A) Top view of HPV58 L1 homology model created using the crystal structure of the HPV16 L1 pentamer (Bishop et al., 2007) with amino acid residues different from HPV16 highlighted in the BC (yellow), DE (pink), EF (red), FG (orange) and HI (green) loops. The GMQE score is indicated (Biasini et al., 2014). (B) Top view of pairwise model generated by the superimposition of the L1 ribbon structure from HPV58 (red) onto the HPV16 L1 ribbon (orange) with RMS deviation value indicated. Loops with predicted structural differences between HPV58 and HPV16 are highlighted. (C) Expanded view of predicted structural differences between loops with the mean and standard deviation in Å between the loops indicated.

The pairwise comparison of the homology model on to the crystal structure by superimposition demonstrated a RMS deviation of 0.68Å which indicated that the HPV35 L1 homology model was structurally similar to HPV35 crystal structure. Pairwise model comparisons between the L1 homology model of the HPV16 PsV and the L1 homology models of each individual non-vaccine A9 PsV were performed by superimposition and generated a RMS deviation value in Å (**Figures 28B – 32B**). Changes in loop structure between the two models which were greater than the RMS value were considered to be more accurate predictions whilst changes less than the RMS value were considered less precise.

The BC and EF loops which are positioned on the outer rim of the capsomer were predicted to have the greatest degree of structural diversity between the non-vaccine A9 PsV and the HPV16 PsV. Structural changes in the BC loop were predicted for all non-vaccine genotypes except HPV35, with the shift in the non-vaccine BC loops compared to the HPV16 BC loop ranging from a mean 1.83 ± standard error 0.83Å for HPV58 (**Figure 32C**) to 4.45 ± 0.18Å for HPV31 (**Figure 28C**). These predicted structural changes may be attributable to the insertion of an additional amino acid into the BC loop of HPV31 (Pro^{60}), HPV33 (Ala^{60}), HPV52 (Gly^{60}) and HPV58 (Asn^{60}) in comparison to HPV16 (**Figure 24**). Structural changes in the EF loop were predicted for all non-vaccine A9 genotypes compared to HPV16 and for HPV33 and HPV58 these changes may be attributable to a single deletion at Gly^{186} resulting in mean EF loop shifts compared to HPV16 (**Figure 24**) of 4.11 ± 0.43Å and 1.17 ± 0.14Å, respectively (**Figures 29C & 32C**). These single amino acid deletions coincide with both HPV33 and HPV58 having lower molecular weight EF loops relative to HPV16 (**Table 10**). The structural changes predicted for HPV31, HPV35 and HPV52 are not attributable to either insertions or deletions and may result from differences in amino acid characteristics which render their EF loops more hydrophilic and of a higher molecular weight in comparison with the EF loop of HPV16 (**Table 10**).

Structural changes in the FG loop were only predicted for HPV35 and HPV52 with a mean FG loop shift compared to HPV16 of 2.65 ± 0.06Å and 3.31 ± 0.19Å, respectively (**Figures 30C – 31C**). Both these genotypes had either a two amino acid deletion (HPV35: Ser^{283} and Gly^{286}) or insertion (HPV52: Asn^{284} and Ser^{285}) within the FG loop compared to HPV16 (**Figure 24**). No amino acid residue insertions or deletions were apparent in the FG loops of HPV31, HPV33 and HPV58 and the L1 sequence difference between these genotypes did not impact upon FG loop structure compared to HPV16. Structural changes within the HI loop were predicted for HPV33, HPV52 and HPV58 resulting in mean HI loop shifts compared to HPV16 of 3.37 ± 0.44Å, 2.92 ± 0.13Å and 0.72 ± 0.15Å, respectively (**Figures 29C, 31C & 32C**). These structural changes may be attributable to a single deletion at Thr^{355} within the HI loops of HPV33, HPV52 and HPV58 compared to HPV16 (**Figure 24**). No structural changes were predicted for the DE loops of the non-vaccine genotypes despite L1 sequence diversity which altered charge (**Figure 25**), hydrophobicity (**Figure 26**) and molecular weight (**Figure 27**) relative to DE loop of HPV16. Additionally, no amino acid residue insertions or deletions were apparent in the DE loops of the non-vaccine genotypes relative to HPV16 (**Figure 24**). Taken together these data demonstrated that the majority (11 out of 14) of predicted structural differences between the L1 loops of the PsV representing HPV16 and the non-vaccine A9 were due to either insertions and/or deletions of amino acid residues within the loop regions compared to HPV16.

3.3.4.3 Design and generation of chimeric PsV

In section 3.1.4.3 the hierarchical clustering of serological data derived from testing Cervarix® vaccine antibodies in the L1L2 PsV neutralisation assay permitted ranking of the target A9 L1L2 PsV antigens. This demonstrated that the antigenic relationship between the HPV16 L1L2 PsV and the non-vaccine A9 PsV, based upon recognition by vaccine-induced cross-neutralising antibodies, was ranked as 31>33>58>52>35 with HPV31 ranked as the nearest antigenic relative to HPV16 and HPV35 as the farthest. This functional recognition profile was combined with the predicted structural shifts in the L1 loops between the PsV

representing HPV16 and the non-vaccine A9 PsV, in order to inform the design of chimeric PsV with inter-genotype loop swaps for testing the recognition of cross-neutralising antibody specificities (**Figure 33**). Prior to this analysis it was apparent that the DE loop was a candidate for further investigation due to the predicted structural similarity between the DE loop of HPV16 and DE loops of all the non-vaccine A9 genotypes. However, this analysis also demonstrated that HPV31, HPV33 and HPV58, the three closest antigenic relatives to HPV16, shared the commonality of having no predicted structural differences in the FG loop compared to HPV16, highlighting the FG loop as another candidate for further investigation. HPV31 has the closest antigenic relationship with HPV16 and these two genotypes share predicted structural similarity in the DE, FG and HI loops. These data taken together with the close proximity of the HI loop to the DE and FG loops on the apex of the capsomer (**Figure 25A**) supported the additional selection of the HI loop for further investigation.

A panel of chimeric L1L2 PsV with inter-genotype DE, FG and HI loop swaps in isolation or combination were designed and generated. HPV31 was used as the cross-neutralising antibody target. The choice of background control target was initially HPV51, an oncogenic HPV genotype from the A5 species group; however, whilst the inter-genotype FG loop switches between HPV51 and HPV31 resulted in L1 protein expression, no infectious PsV particles were formed. The A9 genotype HPV35 was subsequently chosen as the background control, since cross-neutralising antibodies which recognise HPV35 are sparse resulting in a distant antigenic relationship to HPV16 (**Figure 33**). The number of amino acid residues which required switching between HPV31 and HPV35 differed between the three loops (**Figure 34A**). The DE loop, which is the longest L1 loop spanning forty-four amino acids, only had five positions where the residue varied between HPV31 and HPV35 whilst in the HI loop nine of its sixteen amino acids were variable between the two genotypes. The FG loop varied at twelve amino acid positions, including a two amino acid deletion within the FG loop of HPV35 which corresponded with a Ser^{281} and Gly^{282} in the FG loop of HPV31. The L1L2 PsV with either a HPV31 or HPV35 backbone and single (DE, FG & HI) double

Figure 33

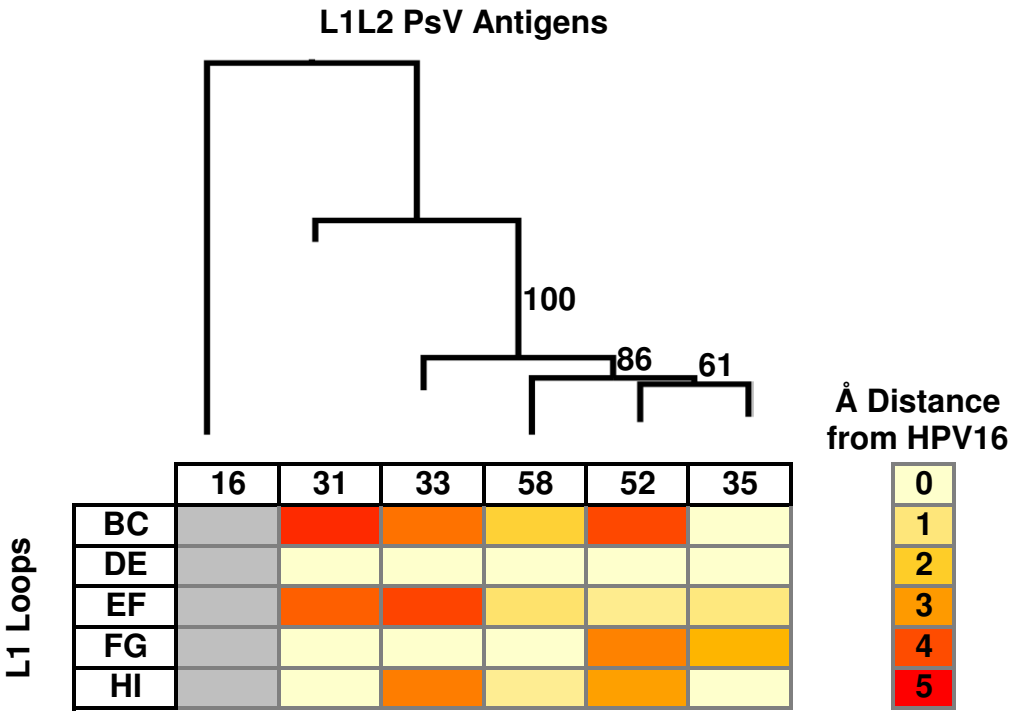


Figure 33 Antigenic relationship between A9 L1L2 PsV in combination with L1 loop distance between HPV16 and non-vaccine A9 genotypes. The A9 L1L2 PsV antigen dendrogram, supported by bootstrapping of 500 pseudoreplicates, was derived from the hierarchical clustering of neutralisation data from the testing of 69 sera from Cervarix® vaccine recipients. The heatmap represents the predicted distance (mean Å) between the L1 loops of HPV16 and the non-vaccine A9 genotypes. Key indicates heatmap gradient of Å distance (0 to 5) from HPV16.

Figure 34

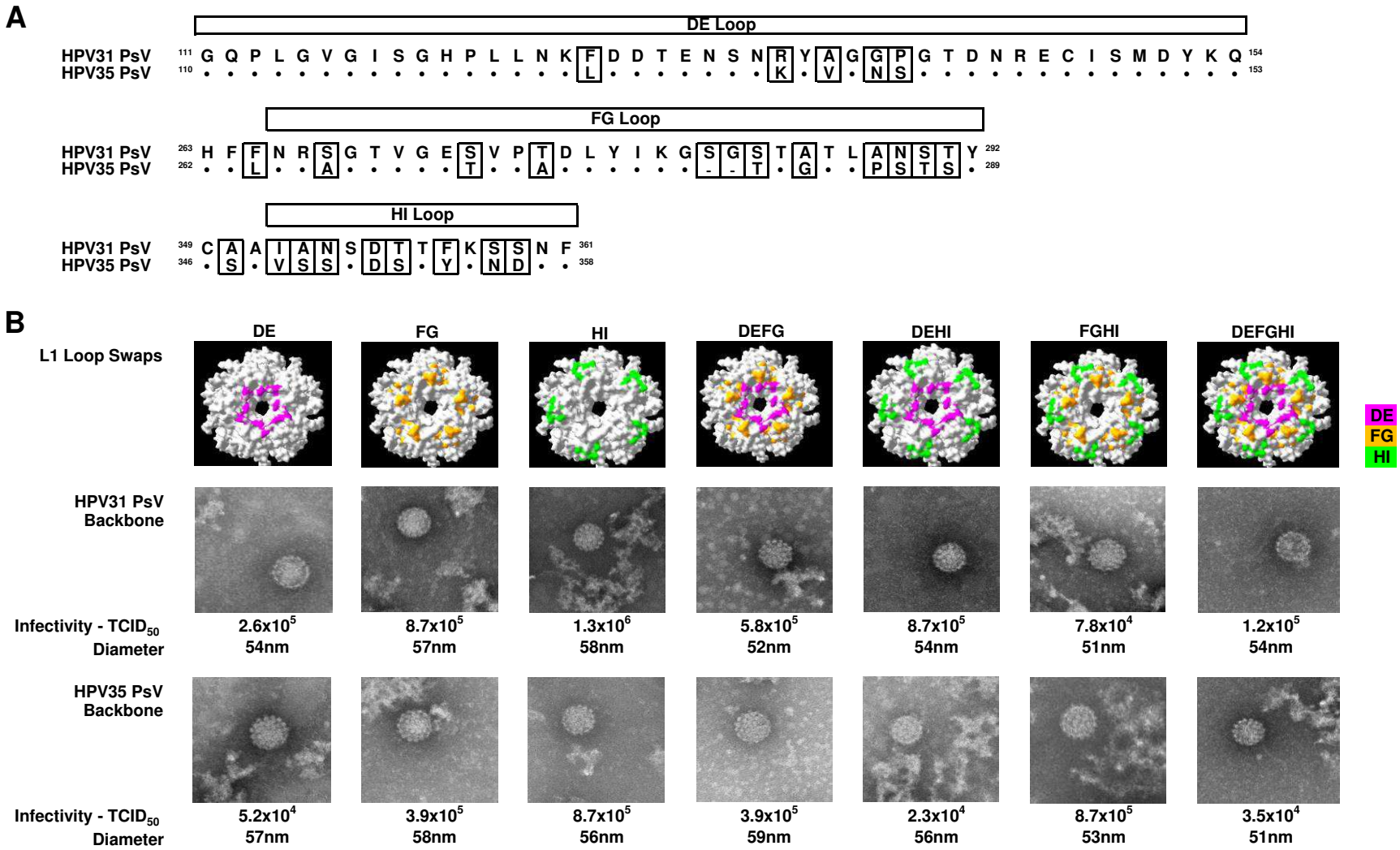


Figure 34 Chimeric L1L2 PsV (A) Amino acid alignment of HPV31 and HPV35 DE, FG and HI loops with variable positions between the two genotypes indicated. (B) Top view of L1 capsomer with variable positons between HPV31 and HPV35 within the DE (pink), FG (orange) and HI (green) loops highlighted. EM images of chimeric L1L2 PsV preparations, infectivity represented by the $TCID_{50}$ and particle diameters are indicated.

(DEFG, DEHI & FGHI) or triple (DEFGHI) loop swaps produced similarly sized particles which were infectious as demonstrated by their individual $TCID_{50}$ (**Figure 34B**).

3.3.4.4 Cross-neutralising antibody recognition of specific L1 domains

The chimeric L1L2 PsV were tested against HPV vaccine sera from Study-02 (Cervarix® n = 19; Gardasil® n = 17) alongside the wild-type HPV31 and HPV35 PsV, with differences in the neutralisation titres generated against the chimeric PsV compared against the wild-type PsV with the corresponding backbone (**Figure 35**).

The replacement of the HPV31 DE loop with that of HPV35 (**Figure 35A**) reduced cross-neutralising antibody recognition of HPV31 by a median 4.2-fold (IQR, 2.0 to 6.5-fold; Wilcoxon paired signed-rank test, $p < 0.001$) with the combination of the HPV35 DE and FG loops having the greatest effect, resulting in a median fold decrease in cross-neutralising antibody recognition of 12.2-fold (IQR, 5.1 to 40.5-fold; $p < 0.001$) compared to the wild-type HPV31 PsV (**Table 11**). The replacement of the HPV35 DE loop with the HPV31 DE alone had no significant effect on cross-neutralising antibody recognition of HPV35, producing a GMT of 25 (IQR, 21 to 31; $p = 0.637$) compared to wild-type HPV35 PsV titre of 22 (IQR, 18 to 25) (**Table 12**). Only when the HPV31 DE loop was in combination with the FG loop of HPV31, with or without the HPV31 HI loop, did the cross-neutralisation titre reach wild-type HPV31 PsV levels (**Figure 35A**).

The replacement of the HPV31 FG loop with the FG loop of HPV35, either alone or in combination with the DE and/or HI loops of HPV35, significantly reduced cross-neutralising antibody recognition of HPV31 PsV (**Figure 35B**) with the replacement of the FG loop alone resulting in a median fold decrease in cross-neutralising antibody recognition of 20.1-fold (IQR, 8.3 to 41.4-fold; $p < 0.001$) compared to the wild-type HPV31 PsV (**Table 11**). Conversely, the introduction of the HPV31 FG loop into the HPV35 backbone significantly increased cross-neutralising antibody recognition of the HPV35 PsV and this effect was most

Figure 35

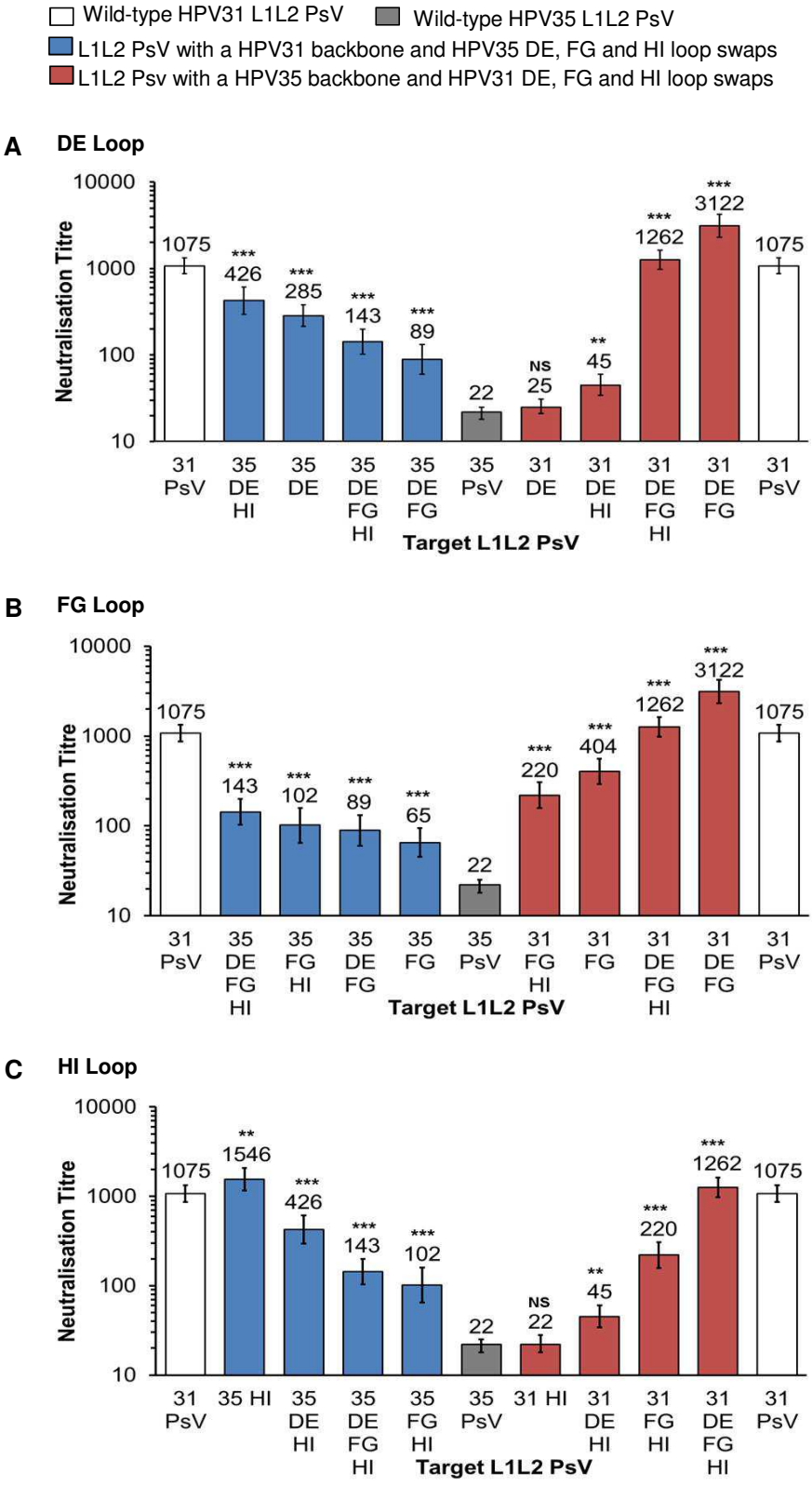


Figure 35 Neutralisation sensitivity of chimeric L1L2 PsV to HPV vaccine-induced antibodies. Colour-code indicates L1L2 PsV constructs: HPV31 wild-type (white), HPV35 wild-type (grey), HPV31 backbone with HPV35 loop switches (blue) and HPV35 backbone with HPV31 loop switches (red). Bar graphs representing the geometric mean neutralisation titre of n=36 HPV vaccine serum (Cervarix® n=19; Gardasil® n=17) against the (B) DE loop swap, (C) FG loop swap and (D) HI loop swap PsV. Error bars represent neutralisation titre 95% confidence intervals. *p* values obtained using the Wilcoxon paired signed-rank test represent differences in neutralisation titre for loop swap PsV constructs compared to wild-type PsV. The presentation of neutralisation data is arranged by decreasing and increasing titers, with the neutralisation data against certain PsV constructs necessarily reproduced within the graphs and across graphs A, B and C.

Table 11. Neutralisation sensitivity of HPV31 L1L2 PsV with HPV35 DE, FG and HI switches

Vaccine	Loop Swaps	Neutralisation titre GMT (95% CI)[a]	*p* value[b]	Fold-decrease from wild-type Median (IQR)[c]
Cervarix	-	1,237 (942 – 1,624)	-	-
Gardasil	-	920 (653 – 1,295)	-	-
All	-	1,075 (868 – 1,332)	-	-
Cervarix	DE	264 (161 – 433)	**<0.001**	5.3 (4.5 – 9.2)
Gardasil		310 (228 – 421)	**<0.001**	2.3 (1.5 – 3.6)
All		285 (214 – 379)	**<0.001**	4.0 (2.0 – 6.5)
Cervarix	FG	58 (35 – 96)	**<0.001**	17.8 (8.2 – 39.8)
Gardasil		75 (42 – 133)	**<0.001**	20.1 (10.8 – 40.5)
All		65 (45 – 94)	**<0.001**	19.0 (8.3 – 41.4)
Cervarix	HI	1,319 (898 – 1,937)	0.091	0.7 (0.5 – 1.2)
Gardasil		1,848 (1,153 – 2,962)	0.075	0.7 (0.5 – 0.9)
All		1,546 (1,156 – 2,070)	**0.002**	0.7 (0.5 – 1.0)
Cervarix	DEFG	118 (70 – 199)	**<0.001**	12.2 (6.9 – 24.3)
Gardasil		65 (35 – 121)	**<0.001**	11.2 (2.1 – 42.1)
All		89 (60 – 132)	**<0.001**	11.7 (5.1 – 40.5)
Cervarix	DEHI	489 (267 – 893)	**0.002**	3.2 (1.5 – 8.0)
Gardasil		365 (234 – 570)	**0.016**	2.0 (1.0 – 2.9)
All		426 (295 – 614)	**<0.001**	2.1 (1.4 – 4.8)
Cervarix	FGHI	87 (51 – 147)	**<0.001**	10.5 (4.8 – 24.1)
Gardasil		122 (55 – 271)	**<0.001**	9.5 (3.2 – 24.5)
All		102 (65 – 159)	**<0.001**	10.0 (3.4 – 25.2)
Cervarix	DEFGHI	173 (112 – 267)	**<0.001**	7.7 (3.2 – 14.6)
Gardasil		117 (70 – 195)	**<0.001**	8.5 (2.2 – 21.0)
All		143 (103 – 199)	**<0.001**	8.5 (2.6 – 17.7)

[a] Data presented as the geometric mean titres (GMT) and 95% confidence intervals (95% CI) of the antibody neutralisation titre generated by serum samples from HPV vaccine recipients (Cervarix n=19; Gardasil n=17; All n=36)

[b] *p* values obtained using the Wilcoxon paired signed-rank test represent differences in neutralisation titre for loop swap PsV constructs compared to HPV31 wild-type PsV with significant differences highlighted in bold

[c] Median and IQR fold-decrease in neutralisation titre for loop swap PsV constructs compared to HPV31 wild-type PsV

Table 12. Neutralisation sensitivity of HPV35 L1L2 PsV with HPV31 DE, FG and HI

Vaccine	Loop Swaps	Neutralisation titre GMT (95% CI)[a]	*p* value[b]	Fold-increase from wild-type Median (IQR)[c]
Cervarix	-	22 (18 – 27)	-	-
Gardasil	-	21 (16 – 26)	-	-
All	-	22 (18 – 25)	-	-
Cervarix	DE	30 (22 – 40)	0.304	1.0 (1.0 – 1.4)
Gardasil		21 (17 – 27)	0.477	1.0 (0.8 – 1.2)
All		25 (21 – 31)	0.637	1.0 (0.8 – 1.4)
Cervarix	FG	291 (190 – 447)	**<0.001**	13.8 (9.0 – 19.0)
Gardasil		582 (366 – 928)	**<0.001**	36.5 (10.8 – 63.6)
All		404 (293 – 557)	**<0.001**	17.2 (9.9 – 47.6)
Cervarix	HI	19 (16 – 23)	0.546	1.1 (0.8 – 1.3)
Gardasil		26 (16 – 43)	0.461	1.0 (0.9 – 1.9)
All		22 (18 – 28)	0.322	1.1 (0.9 – 1.5)
Cervarix	DEFG	4,603 (3,349 – 6,325)	**<0.001**	239 (118 – 330)
Gardasil		2,024 (1,255 – 3,264)	**<0.001**	94.4 (28.6 – 229)
All		3,122 (2,310 – 4,219)	**<0.001**	156 (49.7 – 288)
Cervarix	DEHI	50 (31 – 80)	**0.048**	1.4 (0.9 – 3.0)
Gardasil		40 (28 – 57)	0.055	1.6 (0.9 – 3.8)
All		45 (34 – 60)	**0.005**	1.5 (0.9 – 3.3)
Cervarix	FGHI	170 (108 – 268)	**<0.001**	10.5 (3.6 – 22.2)
Gardasil		294 (180 – 480)	**<0.001**	14.8 (9.7 – 25.8)
All		220 (158 – 306)	**<0.001**	13.2 (4.8 – 23.5)
Cervarix	DEFGHI	1,457 (1,024 – 2,074)	**<0.001**	82.9 (34.9 – 183)
Gardasil		1,075 (738 – 1,567)	**<0.001**	69.3 (17.5 – 135)
All		1,262 (979 – 1,627)	**<0.001**	71.3 (19.7 – 151)

[a] Data presented as the geometric mean titers (GMT) and 95% confidence intervals (95% CI) of the antibody neutralisation titre generated by serum samples from HPV vaccine recipients (Cervarix n=19; Gardasil n=17; All n=36)

[b] *p* values obtained using the Wilcoxon paired signed-rank test represent differences in neutralisation titre for loop swap PsV constructs compared to HPV35 wild-type PsV with significant differences highlighted in bold

[c] Median and IQR fold-increase in neutralisation titre for loop swap PsV constructs compared to HPV35 wild-type PsV

dramatic when the FG loop was in combination with the DE loop of HPV31, resulting in a GMT of 3,122 (IQR, 2,310 to 4,219; p <0.001) (**Table 12**).

The replacement of the HI loop within the HPV31 backbone did not reduce cross-neutralising recognition and the replacement of the HI loop within the HPV35 backbone did not significantly increase recognition, compared to the respective wild-type PsV (**Figure 35C**). A significant decrease in HPV31 PsV recognition was only observed when the HPV35 HI loop was in combination with the HPV35 DE and/or FG loops, with the combination of the HI and FG loops resulting in a median fold decrease in cross-neutralising antibody recognition of 10.5-fold (IQR, 3.4 to 25.2-fold; p <0.001) compared to the wild-type HPV31 PsV (**Table 11**). Cross-neutralising recognition of the chimeric HPV35 PsV only reached wild-type HPV31 PsV levels when the HI loop of HPV31 was in combination with both the HPV31 FG and DE loops, producing a GMT of 1,262 (IQR, 979 to 1,627; p <0.001) (**Table 12**) compared to wild-type HPV31 PsV GMT of 1,075 (IQR, 868 to 1,332) (**Table 11**).

The cross-neutralising antibody recognition of the chimeric PsV with HPV31 backbones and HPV35 inter-loop swaps in comparison with wildtype HPV31 PsV was independent of the HPV vaccine received (**Table 11**). For example, the replacement of the HPV31 FG loop resulted in a median 17.8-fold (IQR, 8.2 to 39.8-fold; p <0.001) decrease in Cervarix® sera recognition and a median 20.1-fold (IQR, 10.8 to 40.5-fold; p <0.001) decrease in Gardasil® sera recognition (**Table 11**). Cross-neutralising antibody recognition of the chimeric PsV with HPV35 backbones and HPV31 inter-loop swaps was also independent of the HPV vaccine received (**Table 12**). For example, the replacement of the HPV35 HI loop by the HPV31 HI had no significant effect on recognition by either Cervarix® sera (GMT 19; IQR, 16 to 23; p = 0.546) or Gardasil® sera (GMT 26; IQR, 16 to 43; p = 0.461) compared to wild-type HPV35 PsV (GMT 22; IQR, 18 to 25) (**Table 12**).

3.3.4.5 Predicted epitope footprint of cross-neutralising antibodies

The data generated from testing the chimeric PsV indicated that the FG loop is necessary for cross-neutralising antibody recognition and that the DE loop enhances this recognition. In order to predict the residues within the DE and FG loops which may be involved in the epitope footprint recognised by cross-neutralising antibodies induced against HPV16 L1 VLP, the amino acid sequences of the DE and FG loops of HPV16, HPV31 and HPV35 were aligned (**Figure 36A**). Amino acid positions for which HPV16 (vaccine type) and HPV31 (cross-neutralising antibody target) shared the same residue but HPV35 (background control) did not, were identified in the DE (Ala^{137}) and FG loops (Ser^{281}, Gly^{282}, Ser^{283}, Ala^{285}, Ala^{288} & Ser^{290}). Mapping of the residues onto the HPV31 L1 homology model (**Figure 36B**) demonstrated that these seven residues from a single monomer were in close proximity to each other within a domain on the capsid surface. Additionally, the positions of these residues were mapped alongside DE and FG loop residues which contribute towards a common epitope footprint recognised by type-specific, neutralising HPV16 MAbs (**Figure 36C**) and the FG loop residue, Lys^{279}, which mediates primary binding in HPV infection (**Figure 36D**).

3.3.5 Discussion

The L1L2 PsV representing the non-vaccine A9 genotypes harbour L1 antigenic domains which are recognised by cross-neutralising antibodies (Draper et al., 2011; Einstein et al., 2011a; Kemp et al., 2011). It was reasoned that these antigenic domains would likely have similar conformational structure to HPV16, and that domains which differed from HPV16 are less likely to be targets for such antibody specificities. The amino acid alignment of the L1 proteins from the non-vaccine A9 PsV against the HPV16 PsV L1 protein demonstrated, as expected, that the greatest degree of inter-genotype diversity from HPV16 was located within the surface exposed loops regions (Carter et al., 2003; Chen et al., 2000); however, the degree to which sequence diversity altered the predicted structure of the non-vaccine A9

Figure 36

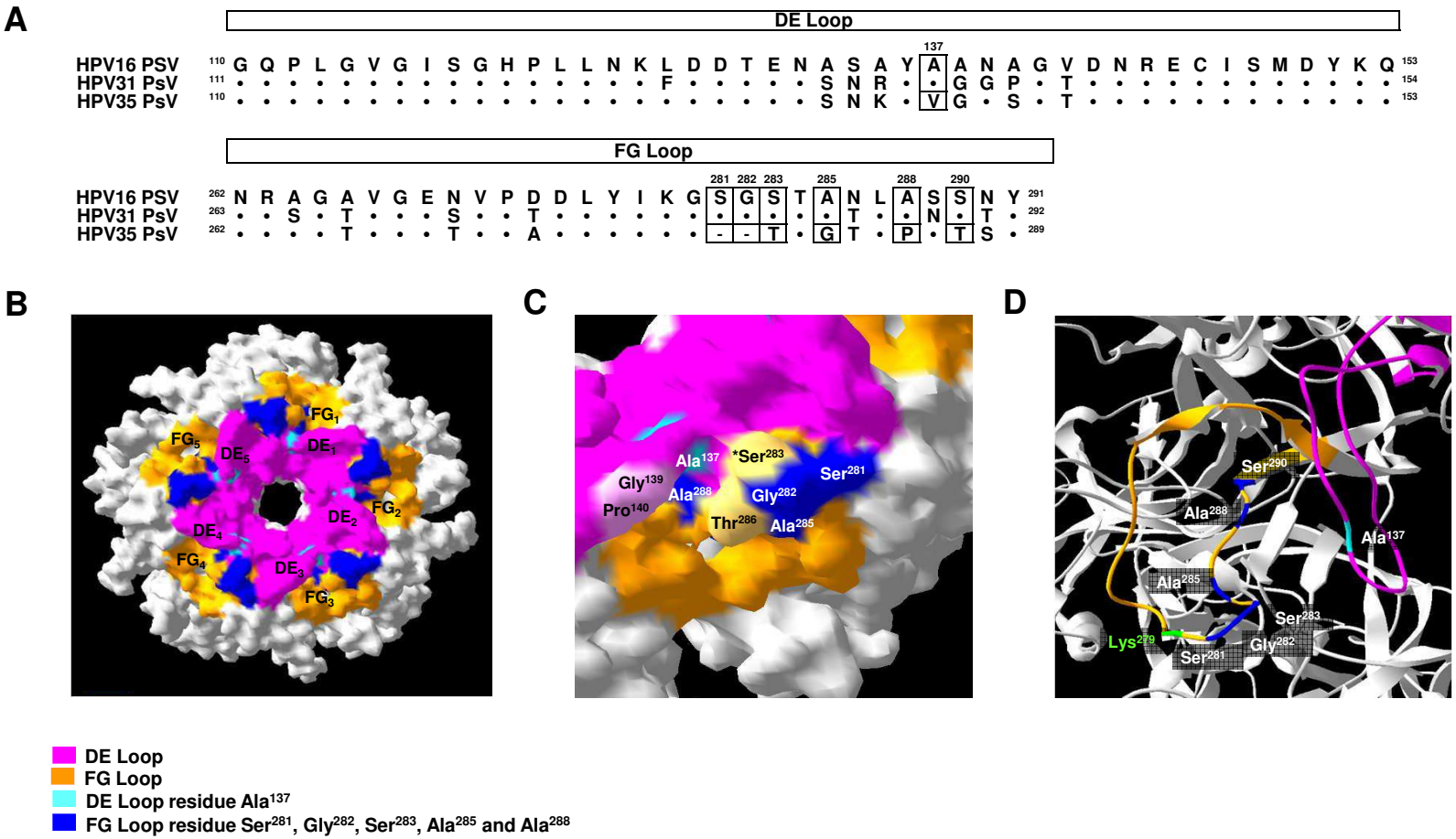


Figure 36 Predicted L1 epitope footprint of cross-neutralising antibodies. (A) Amino acid alignment of HPV16, HPV31 and HPV35 DE and FG loops with identical residue positions between HPV16 and HPV31 but different in HPV35 highlighted. Top view of HPV31 L1 homology model with (B) DE and FG positions with identical residues between HPV16 and HPV31 indicated and (C) in context of four DE and FG residue positions (labelled in black) which correspond to HPV16 L1 positions which contribute to common type-specific epitope, numbered according to HPV31. Asterisk indicates residue position overlap between epitope footprints. (D) Top view of HPV31 L1 DE and FG loop ribbons, positions with identical residues between HPV16 and HPV31 are labelled alongside lysine^{279}.

loops in comparison with the HPV16 loops differed quite widely for each individual loop and between genotypes.

The BC and EF loops are both located on the outer rim of L1 capsomer and whilst the BC loop takes up a discrete position lower down the capsomer stem, the EF loop is in close proximity to the HI loop (Bishop et al., 2007). The prediction of structural differences in the BC and EF loops of the non-vaccine genotypes, compared to HPV16, were common so it was reasoned that these two loops were unlikely to play a major role in a L1 domain recognised by cross-neutralisation antibodies. The greater degree of predicted structural similarity between HPV16 and the non-vaccine A9 genotypes observed for the DE, FG and HI loops, which are in close proximity on the apex of the capsomer (Bishop et al., 2007), indicated that these three loops are more likely to contribute towards a cross-neutralising antibody footprint. The HI loop which takes up a more peripheral position on the apex of the capsomer was predicted to be structurally different in three non-vaccine genotypes (HPV33, HPV52 and HPV58) whilst the FG loop was predicted to be structurally different in two non-vaccine genotypes (HPV35 and HPV52). No structural differences were predicted between HPV16 and any non-vaccine genotypes for the DE loop which is centrally positioned, encircling the lumen of the capsomer.

The predicted differences in L1 loop structure between HPV16 and non-vaccine A9 genotypes have to be interpreted with the caveat that these are predictions derived from the pairwise comparisons of L1 homology models. These models were created by the modelling of the L1 amino acid sequence from the A9 PsV onto the crystal structure of the HPV16 L1 capsomer and whilst the individual A9 homology models were supported by a quality score (GMQE), they still represent models rather than the experimentally resolved crystal structures. Nevertheless homology modelling is a standard approach and has been used for H1N1 pandemic flu (Igarashi et al., 2010), HBV (Langley et al., 2007) and HIV (Kwong et al., 2000) in order to predict the location of antigenic domains and sites of protein-protein

interactions. The crystal structure of HPV35 L1 capsomer was resolved alongside the crystal structure of HPV16 L1 capsomer and the superposition of HPV35 crystal on to that of HPV16 crystal identified structural differences in all the L1 loops between the two genotypes, with the change between the FG loops being the most dramatic (Bishop et al., 2007). Within this present study, the pairwise comparison between L1 homology models of HPV16 and HPV35 predicted a pronounced FG loop shift between the two genotypes, in accordance with the data derived from the comparison of the HPV16 and HPV35 crystal structures, implying that the L1 homology models have utility for the identification of potential structural differences.

The distance between the L1 loops of HPV16 and the non-vaccine A9 genotype PsV were measured and used in conjunction with the L1 antigenic relationship between A9 genotypes, based upon recognition by vaccine-induced cross-neutralising antibodies, to inform the design of chimeric L1L2 PsV with inter-genotype loop switches. The L1 loops of HPV16 have been investigated extensively using L1 VLP as target antigens for type-specific antibody recognition. The insertion of foreign B-cell epitopes from HIV and HBV into individual L1 loops identified the FG and HI loops as immunogenic regions of the L1 capsid (Carpentier et al., 2005; Sadeyen et al., 2003; Slupetzky et al., 2001). L1 VLP with point mutations and complete inter-genotype loop switches have identifying residues which contribute to the epitope footprints recognised by neutralising murine MAbs (Carter et al., 2003; Roth et al., 2006; Ryding et al., 2007). However, the use of functional chimeric L1L2 PsV, which measure antibody specificities capable of neutralising PsV infectivity, has been limited to a single HPV16 construct with a HPV33 BC loop swap which was used to map the epitope of a HPV33 L1 MAb (H33.J3) (Roth et al., 2006).

The novel chimeric L1L2 PsV constructs utilised in this study consisted of DE, FG and HI inter-genotype loop swaps between a cross-neutralising antibody target, HPV31, and a background control, HPV35. The choice of background control target was initially the A5

genotype HPV51 but no infectious PsV particles were formed following inter-genotype FG loop switches. This indicated that whilst the L1 loop regions may be fairly tolerant of manipulation, the impact of such changes upon regions involved in L1 protein interactions, and L1 and L2 protein interactions, is less well tolerated. By using two genotypes from within the A9 group (HPV31 and HPV35) to carry out inter-genotype loop swaps, infectious chimeric L1L2 PsV particles were successfully formed for the testing of cross-neutralising antibody recognition of specific L1 domains.

The chimeric L1L2 PsV demonstrated that the FG loop is necessary and sufficient for the epitope footprints of a significant proportion of cross-neutralising antibody specificities. The DE loop enhances this recognition whilst the HI loop was not necessary for cross-neutralising antibody recognition. The FG loop appeared to be the primary antigenic target of both type-specific natural infection and MAbs which target a variety of HPV genotypes (Carter et al., 2006; Christensen et al., 2001; Fleury et al., 2009; Ludmerer et al., 2000; Orozco et al., 2005; Roth et al., 2006). The epitopes of L1 MAbs, H16.J4 and 31.D24, which demonstrate cross-binding but not cross-neutralisation between HPV16 and HPV31 have also been mapped to the FG loop (Christensen et al., 1996a; Fleury et al., 2009).

The structure of the FG loop can be divided into proximal and distal regions, with the early region (HPV16 numbering: Ala^{264} to Lys^{278}) in close proximity to the peripheral BC and EF loops whilst the late region (Gly^{279} to Ser^{288}) has a more central position, inserting between the DE and HI loops (Bishop et al., 2007). The FG loop contains a Lys^{278} which is conserved among all the A9 genotypes, except HPV52. It has been demonstrated for HPV16 that Lys^{278}, alongside Lys^{361} from the HI loop, mediates primary binding to HSPG, the initial step required for successful HPV infection (Richards et al., 2013). Residues within the predicted footprint recognised by cross-neutralising antibodies, particularly Ala^{285}, are in close proximity to Lys^{279} of HPV31 which corresponds to HPV16 Lys^{278}. *In vivo*, the passive transfer of vaccine-induced L1 type-specific antibodies neutralised HPV16 PsV by

preventing this primary interaction between L1 and HSPG (Day et al., 2010) providing a possible mechanistic explanation of the antigenic targeting of the FG loop independent of genotype.

The majority of L1 epitopes that have been identified to date are made up of residues from two or more L1 loops, although there are instances where a single loop has supported an epitope footprint; for example the BC loop alone is targeted by some HPV6, HPV33 and HPV16 L1 MAbs (Christensen et al., 2001; McClements et al., 2001; Roth et al., 2006). Two recent studies used cryo-electron microscopy to identify the precise epitope footprints of four neutralising HPV16 MAb, H16.V5, H16.1A, H16.14J and H263.A2, all of which were known to target the FG and HI L1 loops (Guan et al., 2015; Lee et al., 2015). These analyses demonstrated for the first time that residues from the DE loop constituted the core of each epitope, with additional residues contributed from the FG and HI loops and a minor number from the BC and EF loops. In this present study, the DE loop enhances cross-neutralising antibody recognition of the FG loop within the HPV35 L1 backbone. This enhancement may be due to DE loop interactions which support the correct presentation of the FG loop or may result from DE loop residues functioning as part of the epitope footprint.

The HPV16 MAbs H16.V5, H16.1A, H16.14J and H263.A2 recognise unique epitope footprints; however, the positive overlap between footprints allowed the identification of residues from the DE (HPV16 numbering: Asn^{138} and Ala^{139}), EF (Gln^{181}), FG (Gly^{281}, Ser^{282} and Asn^{285}) and HI (Ile^{348} and Lys^{361}) loops common to all four footprints suggesting that a common epitope is shared by all four MAbs (Guan et al., 2015). The DE and FG residues predicted to contribute towards the cross-neutralising epitope footprint are located in the same region as DE and FG residues which contribute towards the common HPV16 type-specific epitope footprint. Cross-neutralising antibodies display specificities that recognise a single non-vaccine genotype or multiple non-vaccine genotypes (see Section 3.1.4.4) (Bissett et al., 2014). It is feasible that these cross-neutralising antibody specificities target

distinct epitopes which share common amino acid residues thereby facilitating the recognition of multiple non-vaccine A9 genotypes.

In this study, the L1 domains recognised by inter-genotype cross-neutralising antibodies have been delineated as the DE and FG loops and amino acid residues which potentially contribute to the cross-neutralising antibody epitope footprint have been identified. The cross-neutralising antibodies which target such L1 domains may play a fundamental role in HPV vaccine-induced cross-protection.

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distinct epitopes which share common amino acid residues thereby facilitating the recognition of multiple non-vaccine A9 genotypes.

In this study, the L1 domains recognised by inter-genotype cross-neutralising antibodies have been delineated as the DE and FG loops and amino acid residues which potentially contribute to the cross-neutralising antibody epitope footprint have been identified. The cross-neutralising antibodies which target such L1 domains may play a fundamental role in HPV vaccine-induced cross-protection.

4. Final Discussion

The HPV vaccines Cervarix® and Gardasil® have been licensed in over 100 countries since 2006, following successful clinical trials which demonstrated efficacy against the development of cervical cancer precursors and other HPV-attributable diseases (Markowitz et al., 2012). Today at least 49 countries use the HPV vaccines as part of national immunisation programmes (Regan and Hocking, 2015) and the population level impact in early uptake countries is becoming apparent with Australia, the USA and the United Kingdom all reporting a significant reduction in vaccine type infections in vaccinated cohorts (Drolet et al., 2015; Kavanagh et al., 2014; Markowitz et al., 2013; Mesher et al., 2013; Tabrizi et al., 2012). Significant reductions in high-grade cervical abnormalities have also been reported in vaccinated cohorts in Australia and Scotland whilst in the USA a reduction in the detection of HPV16 and HPV18 in CIN2+ lesions has been observed in women who received at least one dose of the HPV vaccine (Brotherton et al., 2011; Hariri et al., 2015; Pollock et al., 2014). These population-based data support the findings from the vaccine efficacy trials. The duration of vaccine-induced protection is unknown but if it extends up to 20 years it is estimated that a reduction in cervical cancer incidence of 82% is potentially achievable (Choi et al., 2010).

No immune correlate of protection has been identified for the HPV vaccines, since vaccine type efficacy is very high and vaccine type breakthrough infections will be rare (Stanley et al., 2012). Neutralising antibodies which target the L1 are assumed to be the immune effectors of vaccine type protection. This is based upon evidence from passive transfer studies in animal PV models and the murine CVC model (Breitburd et al., 1995; Longet et al., 2011; Suzich et al., 1995), and through the observations that protection in human vaccinees is coincident with the detection of L1 neutralising antibodies in serum and cervicovaginal secretions (Einstein et al., 2009; Kemp et al., 2008). Neutralising antibodies that can block infection or subsequent viremia and bacteraemia, are the correlates or surrogates of protection for almost all prophylactic viral and bacterial vaccines (Plotkin, 2010).

The characterisation of vaccine-induced antibody responses and their antigenic targets contributes to our understanding of vaccine-induced protection and is important for vaccine monitoring and the prediction of vaccine efficacy. The prophylactic HBV vaccines comprise recombinant hepatitis B surface antigen (HBsAg) (Coleman, 2006) and a serum antibody response (anti-HBs) of ≥10 IU/mL post-vaccination has been found to correlate with T helper cell mediated memory B cell induction upon challenge (Plotkin, 2010; Tuaillon et al., 2006). The anti-HBs response primarily targets epitopes within the immunodominant region of the HBsAg termed the "a" determinant. A glycine to arginine switch at position 145 of the "a" determinant has been found to alter the antigenicity of the HBsAg, reducing recognition by polyclonal anti-HBs and contributing to the emergence of HBV vaccine escape mutants in immunised individuals (Carman, 1997). Influenza vaccines contain either inactivated or live attenuated influenza virus and elicit neutralising antibodies which recognise the haemagglutinin (HA) (Reber and Katz, 2013). A serum HA antibody inhibition titre of 1:40 has been found to correlate with a ≥50% reduction in the risk of acquiring influenza (Plotkin, 2010). The majority of influenza neutralising antibodies are thought to target conformational epitopes in the Sa and Sb antigenic sites of the HA and amino acid changes in these regions results in escape from antibody-mediated neutralisation (Martinez et al., 2009). Monitoring the HA antigenicity of circulating influenza viruses contributes to the detection of antigenically novel viruses which informs the choice of vaccine strain (Gerdil, 2003).

The HPV vaccine trials also demonstrated the partial efficacy of Cervarix® and Gardasil® against non-vaccine genotypes HPV31, HPV33 and HPV45, an additional benefit to the expected vaccine type protection (Brown et al., 2009; Schiller et al., 2012; Wheeler et al., 2012). Cross-neutralising antibodies which target non-vaccine genotypes can be found in the sera of HPV vaccine recipients from clinical trials (Draper et al., 2013; Einstein et al., 2011a; Kemp et al., 2011; Toft et al., 2014) and national immunisation programmes (Barzon et al., 2014; Draper et al., 2011). Furthermore, antibodies which recognise HPV31 and HPV45 can be detected in genital samples taken from the site of infection (Draper et al., 2013). This

latter study also demonstrated a strong positive association between the prevalence of cross-neutralising antibodies in its study population and cross-protection against persistent infection and CIN2+ attributable to non-vaccine genotypes from vaccine efficacy trials (Draper et al., 2013). This coincidental relationship between cross-protection and the detection of cross-neutralising antibodies in vaccinees led to the working hypothesis that cross-neutralising antibodies mediate cross-protection.

In this present study, cross-neutralising antibodies which recognise non-vaccine A9 genotypes were characterised as being a minor component of the total HPV16 antibody response, rather than a predominantly type-specific antibody specificity which exhibits a reduced recognition for non-vaccine genotypes. Cross-neutralisation appears to be mediated by antibody specificities which recognise single and multiple non-vaccine genotypes with equivalent recognition for HPV16. A greater breadth of cross-recognition tended to be associated with a higher magnitude of HPV16 neutralising antibody titre, supporting previous observations that the magnitude and breadth of the cross-neutralising antibody response generally increased in line with the vaccine type neutralising response (Draper et al., 2011; Draper et al., 2013; Kemp et al., 2011). If this small pool of cross-neutralising antibody specificities potentially contributes to vaccine-induced cross-protection then factors which effect vaccine immunogenicity such as age at the time of vaccination, reduction in vaccine dose or longevity of vaccine-induced antibodies, may impact upon this clinically beneficial effect.

The long-term vaccine type immunogenicity of the HPV vaccines has been monitored in vaccine efficacy trial participants. Sustained immunogenicity of the Cervarix® vaccine has been reported up to 9.4 years post-vaccination in 15-25 year old women, with all vaccinees seropositive for HPV16 and HPV18 neutralising antibodies which was coincident with sustained vaccine efficacy against CIN1+ and CIN2+ (Naud et al., 2014). Seropositivity and vaccine type antibody titres remained high up to 9 years following vaccination with Gardasil®

in 16-23 year old women from the FUTURE II trial (Nygard et al., 2015), which demonstrated vaccine efficacy against CIN2+ and CIN3+ (Munoz et al., 2010). Cross-neutralising antibodies which target HPV31 and HPV45 have also been detected up to 24 months post-vaccination in 18-45 year old women (Einstein et al., 2011a) and the detection of HPV31 cross-neutralising antibodies has subsequently been reported up to 48 months post-vaccination (Safaeian et al., 2013b). These data indicate that despite cross-neutralising antibodies representing a minor proportion of vaccine-induced antibodies their longevity is sustainable through several years of follow-up post-vaccination.

Expanded licensure of the HPV vaccines to younger adolescents (9-15 years) was based upon immunogenicity bridging studies whereby the vaccine-induced immunogenicity of a younger age group was compared to the immunogenicity of 15-26 year old women for which efficacy had been demonstrated. Immunological non-inferiority is now recognised as a primary end-point for HPV vaccine immunobridging studies based upon the rationale that non-inferior immunogenicity of the younger age group will correlate with non-inferior efficacy (Lowy et al., 2015). The long-term follow up of females vaccinated with Cervarix® between the ages of 10-14 years not only demonstrated sustained vaccine-type immunogenicity up to 6 years post-vaccination but also a higher magnitude of antibody response compared to females vaccinated between the ages of 15-25 years (Schwarz et al., 2014). Sustained vaccine-type antibody titres have also been demonstrated in 10-15 year old girls and boys up to 8 years post-vaccination with Gardasil® (Ferris et al., 2014), with age-related increases in vaccine immunogenicity compared to 16-23 year old women also reported (Block et al., 2006). Data on the longevity of the cross-neutralising antibody response in adolescents is limited and does not extended past the detection of HPV31 and HPV45 cross-neutralising antibodies at 6 months post-third vaccine dose (Draper et al., 2013). The vaccine-type immunogenicity data suggest that the age at which the HPV vaccine is received impacts upon vaccine immunogenicity. The increased vaccine immunogenicity observed in younger

adolescents compared to women should result in the increased longevity of the cross-neutralising antibody response in this group, the target population for vaccination.

Reduced dose HPV vaccination schedules have recently been adopted by the national immunisation programmes of several countries including the United Kingdom (Donken et al., 2015) following immunobridging studies which compared the vaccine immunogenicity of 2 doses in girls versus 3 doses in women. Girls (9-14 years old) who received 2 doses of Cervarix® at months 0 and 6 demonstrated non-inferior vaccine-type antibody responses at month 7, 1 month post final dose, compared to women (15-25 years old) who received 3 doses (Romanowski et al., 2011). The non-inferior vaccine immunogenicity of the younger age group extended up to at least 24 months post first dose. Vaccine-type antibody responses were non-inferior at month 7 in girls (9-13 years old) who received 2 doses of Gardasil® at 0 and 6 months compared to women (16-26 years old) who received 3 doses (Dobson et al., 2013). However, antibody responses were inferior for HPV18 at 24 months when compared to girls who had received 3 doses. The clinical efficacy of a reduced dose schedule of Cervarix® in women (18-25 years old) demonstrated that protection against incident HPV16 or HPV18 infection was independent of vaccine dose, suggesting that 2 doses or even 1 dose may be as protective as 3 doses against vaccine type infection (Kreimer et al., 2015).

Limited data are available on the impact of a reduced dose schedule on the cross-neutralising antibody response and cross-protection. In one study, seropositivity for HPV31 cross-neutralising antibodies at 4 years post-vaccination decreased from the 3-dose group to the 1-dose group (Safaeian et al., 2013b). Another study found that the cross-protective clinical efficacy of Cervarix® observed for 3 doses vs. 2 doses against incident HPV31, HPV33 and HPV45 infections combined, was similar when the 2 doses were delivered 6 months apart; however, vaccine efficacy was lost in the groups which received 2 doses delivered 1 month apart or a single dose (Kreimer et al., 2015). Both these studies were

carried out in women (18-25 years old) and it is possible that the increased vaccine immunogenicity of the younger target group for vaccination may go some way to counteract the impact of a reduced dose schedule on the generation and maintenance of vaccine-induced cross-neutralising antibodies. However, no data are available at present to support this assumption.

Broadening the protection of HPV prophylactic vaccines has been an objective since the successful clinical trials of the Cervarix® and Gardasil® vaccines (Bosch, 2009), with the aim of turning the partial vaccine-induced cross-protection into complete vaccine type protection as observed for HPV16 and HPV18. Different approaches have been taken including the development of multivalent L1 VLP vaccine formulations, and the use of alternative L1 antigens such as capsomers, L2-based vaccines and chimeric VLP (Chatterjee, 2014). A 9-valent L1 VLP-based vaccine formulation (Gardasil®9) manufactured by Merck has recently completed successful clinical trials (Joura et al., 2015) and subsequently received approval from the US Food and Drug Administration (FDA) (Kirby, 2015). Gardasil9® contains L1 VLP representing the oncogenic genotypes HPV31, 33, 45, 52 and 58 in addition to the L1 VLP represented in the original Gardasil® vaccine (HPV16 and HPV18), plus genotypes HPV6 and HPV11 which cause the development of anogenital warts (Lacey et al., 2006). This vaccine formulation has the potential to prevent *ca.* 90% of cervical, vulvar, vaginal, and anal cancers (Li et al., 2010).

The adoption of Gardasil9® by existing HPV immunisation programmes will be contingent upon its cost-effectiveness compared to Cervarix® or Gardasil® (Van de Velde et al., 2012). The manufacture of multivalent L1 VLP vaccines, both first and second generation formulations, is a complex and costly approach, factors which are prohibitive to their widespread introduction in low- and middle-income countries (Markowitz et al., 2012). A 2-dose schedule for Cervarix® or Gardasil® has cut the cost of establishing and maintaining HPV immunisation programmes. It is not known whether a reduced-dose schedule of

Gardasil9® will be an effective alternative to the 3-dose schedule for which efficacy, safety and immunogenicity have recently been reported (Joura et al., 2015; Van Damme et al., 2015). If Gardasil9® does supersede the current HPV vaccines, it will be at a point where several birth cohorts worldwide will have already received either Cervarix® or Gardasil®, therefore a better understanding of the cross-protection and the cross-neutralising antibody response induced by the current HPV vaccines remains of importance.

HPV genotypes can be segregated into variant lineages and then further into sublineages based upon single nucleotide polymorphisms identified across the whole HPV genome (Chen et al., 2011, 2013). These variant lineages appear to exhibit differences in the risk of disease development following infection (Chen et al., 2014a; Chen et al., 2014b; Schiffman et al., 2010; Xi et al., 2014; Xi et al., 2012). Vaccine-induced cross-neutralising antibodies recognise antigenic sites on the L1 protein and variant-specific polymorphisms located within these sites have the potential to alter surface topography between different variant lineages. Consequently, differential L1 recognition by cross-neutralising antibodies may result in differential vaccine efficacy against non-vaccine A7 and A9 variants, altering the circulating viral variants in the post-vaccine era.

In this present study the individual A9 genotypes have been represented by a single L1L2 PsV and therefore a single L1 amino acid sequence. The potential antigenic impact of L1 variation was investigated for HPV31, which is closely related to vaccine type HPV16 within the A9 species group. The partial vaccine efficacy against HPV31, which is coincident with the detection of HPV31 L1 cross-neutralising antibodies in the sera of vaccine recipients (Brown et al., 2009; Draper et al., 2013; Einstein et al., 2011a; Wheeler et al., 2012), has the potential to reduce the *ca.* 3.8% of cervical cancer cases attributable to HPV31 worldwide (Li et al., 2010). The variant lineages of HPV31 (A, B and C) demonstrate differential natural histories, with infection attributable to variant lineages A and B associated with an increased

risk of developing CIN2/3 whilst paradoxically variant C infections persist for longer (Xi et al., 2012).

The FG loop of HPV31 is an antigenic target for both type-specific and cross-reactive L1 MAbs (Carpentier et al., 2005; Fleury et al., 2009) and contains two amino acid positions where the residues differed between variants A, B and C. The L1L2 PsV representing the three variants were all susceptible to neutralisation by vaccine-induced cross-neutralising antibodies. Variants B and C demonstrated increased sensitivity to cross-neutralisation compared to variant A, although this difference was of a low magnitude (<2-fold difference in neutralisation titre). A recent study examining Cervarix® cross-protection against variants of non-vaccine genotypes found no difference in vaccine efficacy against transient infection between HPV31 variants A/B and C (Harari et al., 2015). However, vaccine efficacy against persistent infection and/or CIN 2/3 progression was only demonstrated for variant C. The persistent infections and/or CIN 2/3 progression attributable to HPV31 variants A and B were pooled together for this analysis so it is unclear of the contribution of each variant lineage to these clinical events.

The antigenic impact of naturally-occurring variation within the capsid proteins of HPV45 has recently been investigated and found that residue differences at a single amino acid position within the HI loop contributed to significantly different susceptibilities to cross-neutralisation (Godi et al., 2015b). L1L2 PsV representing variant sublineages A2, A3 and B1 exhibited increased sensitivity to cross-neutralisation (*ca.* 3-fold) whilst variant sublineage B2 displayed a slight decrease in sensitivity compared to variant sublineage A1. Additionally, variant sublineages A2, A3 and B1 were more sensitive to cross-neutralisation by Cervarix® vaccine sera compared to Gardasil®, suggesting that there may be differences in the cross-neutralising antibody specificities generated in response to Cervarix® compared to Gardasil®. No variant-specific differences in Cervarix® vaccine efficacy were observed for cross-protection against transient or persistent infection, and/or CIN 2/3 progression, attributable to

HPV45 variant lineages A and B (Harari et al., 2015). It is as yet unclear as to whether the differential sensitivity of the L1L2 PsV representing variants of HPV31 and HPV45 to *in vitro* cross-neutralisation will have an impact at the population level following the introduction of the HPV vaccines.

Cross-neutralising antibodies which recognised non-vaccine A7 and A9 genotypes originate from the vaccine type to which they have the closest genetic relationship (Scherpenisse et al., 2013). These data together with the observation that A9 cross-neutralising antibodies have equivalent recognition for HPV16 (Bissett et al., 2014) indicate that the L1 proteins of vaccine types harbour immunogenic domains which share sequence and/or structural similarity with the L1 proteins of non-vaccine genotypes. Studies of HPV31 and HPV45 L1 variants indicate that amino acid residues in the FG and HI loops, respectively, influence cross-neutralising antibody recognition and may contribute to the epitope footprint recognised by these antibody specificities (Bissett et al., 2015; Godi et al., 2015b). The precise L1 epitope footprints recognised by several HPV16 type-specific MAbs have recently been identified using cryo-electron microscopy (Guan et al., 2015; Lee et al., 2015). For the first time, residues in the DE loop were identified as having a major contribution towards the footprint, alongside FG and HI loop residues, with minor contributions from residues in the BC and EF loops. No HPV L1 MAbs exist which have the potential to cross-neutralise A9 genotypes so at present this approach cannot be used for the mapping L1 cross-neutralising epitopes.

An alternative approach was utilised within this current study whereby the L1 proteins of the vaccine type HPV16 and the non-vaccine A9 genotypes were characterised in an attempt to identify regions recognised by cross-neutralising antibodies. Antigenic regions within viral proteins, such as the envelope protein of dengue virus or gp41 of HIV-1, have been characterised by the analysis of amino acid sequence properties such as charge and hydrophobicity which impact upon protein structure and stability (Bryson et al., 2009;

Fibriansah et al., 2015). The amino acid sequence diversity between HPV16 and the non-vaccine A9 genotypes located within the L1 surface exposed loop regions was underpinned by differences in the charge, hydrophobicity and molecular weight. However, it was residue deletions and insertions within the amino acid sequences of the loops which had the biggest impact upon the predicted loop structures of the non-vaccine A9 genotypes in comparison with HPV16.

The nearest antigenic relative to HPV16 within the A9 species group, based upon recognition by L1 cross-neutralising antibodies, is HPV31 whilst HPV35 is the farthest (Bissett et al., 2014). This differential recognition was exploited in the design of novel chimeric L1L2 PsV with HPV31 or HPV35 L1 backbones and inter-genotype loop swaps. These chimeric L1L2 PsV demonstrated that the DE and FG loops, which were predicted to be the most structurally similar between HPV16 and the non-vaccine A9 genotypes, are L1 antigenic targets for cross-neutralising antibodies which recognise non-vaccine A9 genotypes. Furthermore, specific residue differences between the DE and FG loop amino acid sequences of HPV16, HPV31 and HPV35 allowed the epitope footprint of cross-neutralising antibodies to be postulated. The majority of residues predicted to contribute towards the cross-neutralising antibody footprint were located in the late region of the FG loop (HPV16 numbering: Gly^{279} to Ser^{288}) and direct overlap exists with residues which contribute towards HPV16 type-specific footprints, including a recently characterised neutralising HPV16 human MAb (Xia et al., 2016) and several type-specific HPV16 murine MAbs (Guan et al., 2015). The generation of L1 MAbs which have the potential to cross-neutralise A9 genotypes would further our understanding of these L1 regions recognised by cross-neutralising antibodies and their relationship to type-specific epitopes in the context of the L1 capsid.

The clinical trials of Cervarix® and Gardasil® which demonstrated partial vaccine efficacy against non-vaccine genotypes were completed just under a decade ago (Schiller et al.,

2012). A recent systematic review has suggested that cross-protection is occurring at the population level following the introduction of HPV vaccination programmes, demonstrated by the significant reduction in infections due to HPV31, HPV33 and HPV45 in vaccinated girls aged 13-19 years (Drolet et al., 2015). Furthermore, HPV31 infections in women vaccinated with Cervarix® has been found to be associated with a reduced detectability of HPV31 cross-neutralising antibodies leading to the suggestion that the partial vaccine efficacy against HPV31 may be mediated by these antibody specificities (Safaeian et al., 2013a). In this study the DE and FG loops have been identified as the antigenic targets recognised by L1 cross-neutralising antibodies which potentially have a role in HPV vaccine-induced cross-protection. The monitoring of these antibody specificities in vaccine recipients may be useful at the population level for defining a correlate or surrogate of cross-protection.

Understanding the immune system mechanisms which underpin vaccine-induced protection generally results from the accumulation of data from multiple studies which contribute towards the establishment of a scientific consensus. The identification of the L1 regions recognised by HPV vaccine-induced cross-neutralising antibodies contributes towards this understanding by identifying viral antigenic targets recognised by the host immune system.

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Appendices

Supplementary Table 1. Primer sequences

Name	Target	Application	Sequence (5' to 3')
16 VLP L1 FOR NotI	HPV16 L1	PCR	GCGGCCGCATGTCTCTTTG
16 VLP L1 REV NotI	HPV16 L1	PCR	GCGGCCGCTTACAGCTTAC
16 VLP L1 FOR1	HPV16 L1	SEQ	GCTGGTTTGGGCCTGTGTAG
16 VLP L1 FOR2	HPV16 L1	SEQ	CAGAACCATATGGCGACAGC
16 VLP L1 FOR3	HPV16 L1	SEQ	TCCAGCACCTAAAGAAGATCC
16 VLP L1 REV1	HPV16 L1	SEQ	AGCCGCTGTGTATCTGG
16 VLP L1 REV2	HPV16 L1	SEQ	GAATATTTGGGCATCAGAGGTAAC
16 VLP L1 REV3	HPV16 L1	SEQ	GTGCTGGAGGTGTATGTTTTTG
31 VLP L1 FOR EcoRI[a]	HPV31 L1	PCR	GAATTCATGTCTCTGTGGCGGC
31 VLP L1 REV SphI[a]	HPV31 L1	PCR	GCATGCTTACTTTTTAGTTTTTTTACG
31 VLP L1 FOR1[a]	HPV31 L1	SEQ	CTGTGTTGGTTTAGAGGTAGGTC
31 VLP L1 FOR2[a]	HPV31 L1	SEQ	AAGATGGGGATATGGTTGAT
31 VLP L1 REV3[a]	HPV31 L1	SEQ	CTCCAAAGCCTGTATCAACCA
31 VLP L1 REV4[a]	HPV31 L1	SEQ	TAAAAATTTGTGCATCTGAAGTAAG
31 VLP L1 REV5[a]	HPV31 L1	SEQ	GAGGGAGGTGTGGTCAATC
FastBac FOR	pFastBac 1	PCR	AACCATCTCGCAAATAAATAAGTA
FastBac REV	pFastBac 1	PCR	GGGGAGGTGTGGGAGGTT
M13 Forward (-40)[b]	Bacmid	PCR	GTTTTCCCAGTCACGAC
M13 Reverse[b]	Bacmid	PCR	CAGGAAACAGCTATGAC
35 L1 FOR	HPV35 L1	PCR	GGATCCATGGCTCTGTGGCG
35 L1 REV	HPV35 L1	PCR	GGATCCTTAACTTTTTACTTTTCTACG
35 L2 FOR	HPV35 L2	PCR	TCTAGAATGCGACACAAAAGGTCTAC
35 L2 REV	HPV35 L2	PCR	CTCGAGTTAGACCGCCACAGAGAC
35 L1 FOR1	HPV35 L1	SEQ	GGTCAGCCATTAGGAGTAGGTA
35 L1 FOR2	HPV35 L1	SEQ	CGGGGACATGGTAGACACAGG
35 L1 FOR3	HPV35 L1	SEQ	TAACCTCCGATGCACAAATATTT
35 L1 FOR4	HPV35 L1	SEQ	CCTTACACCACCGCCTTCTG
35 L1 REV1	HPV35 L1	SEQ	GACCACGACCTACTTCAACTCC
35 L1 REV2	HPV35 L1	SEQ	ACCATGTCCCCGTCTTGTAGT
35 L1 REV3	HPV35 L1	SEQ	AAATATTTGTGCATCGGAGGTTA
35 L1 REV4	HPV35 L1	SEQ	CAGAAGGCGGTGGTGTA
35 L2 FOR1	HPV35 L2	SEQ	ACGACCCCCTGTAACTGTG
35 L2 FOR2	HPV35 L2	SEQ	TTATGAAGAAATCCCTATGG
35 L2 FOR3	HPV35 L2	SEQ	ATAGTAGAGTAGGTAATAAAC
35 L2 FOR4	HPV35 L2	SEQ	AACAGCAGGGCCAGACATTG
35 L2 REV1	HPV35 L2	SEQ	AACAGGGGCACCAGACTCAA
35 L2 REV2	HPV35 L2	SEQ	TTAGTTATATTATTGCTGTCTGTG
35 L2 REV3	HPV35 L2	SEQ	GCCCCTATAGCTTTTCCACTTC
35 L2 REV4	HPV35 L2	SEQ	AATGTCTGGCCCTGCTGTTAT
L1 PsV FOR	psheLL	SEQ	GTTTTGACCTCCATAGAAGACA
L1 PsV REV	psheLL	SEQ	TGTCCAGACTCATCAGCCTAAG
L2 PsV FOR	psheLL	SEQ	CTTAGGCTGATGAGTCTGGACA
L2 PsV REV	psheLL	SEQ	CATAGCGTAAAAGGAGCAACATAG
FG PsV FOR	PsV L1	SEQ	GCTTCGGCGCCATGGACTTCAC
FG PsV REV	PsV L1	SEQ	GCCCCAGCAGATGCCGTTGTTGT
HI PsV REV	PsV L1	SEQ	CGCTGAACTTCTCCTTCAGGT
31 PsV L1 A267T FOR	HPV31 L1	Mutagenesis	TTCACCAGGAGCGGCACCGTGGGCGAGAGCG
31 PsV L1 A267T REV	HPV31 L1	Mutagenesis	CGCTCTCGCCCACGGTGCCGCTCCTGTTGAA

[a] Designed by Dr Eve Draper
[b] Designed by Thermo Fisher Scientific

Supplementary Table 2. Accession numbers and source references

HPV	Accession number(s)	Reference
16	K02718	(Seedorf et al., 1985)
	NC_001526	(Kennedy et al., 1991)
	EU118173	(Kirnbauer et al., 1993)
	U37217	(Icenogle et al., 1995)
	AF125673	(Flores et al., 1999)
	AF402678	Direct Submission (2001)
	AF472508 - AF472509	Direct Submission (2002)
	AF534061	Direct Submission (2002)
	AF536179 - AF536180	Direct Submission (2002)
	AY686579 - AY686584	(Chen et al., 2005)
	FJ006723	Direct Submission (2008)
	FJ610146 - FJ610152	(Lurchachaiwong et al., 2009)
	EU918764	(Wu et al., 2009b)
	HM057182	Direct Submission (2010)
	HQ644234 - HQ644299	(Smith et al., 2011)
	JQ004092 - JQ004099	Direct Submission (2011)
	JQ067943 - JQ067944, JN565302 - JN565303	(Sabol et al., 2012)
	AB818687 - AB818693, AB889488 - AB889494	(Kukimoto et al., 2013)
	KF880690	Direct Submission (2013)
	KF954093	Direct Submission (2013)
31	J04353	(Goldsborough et al., 1989)
	U37410	(Icenogle et al., 1995)
	HQ537666 - HQ537687	(Chen et al., 2011)
	KJ754561 - KJ754580	(Bissett et al., 2015)
33	M12732	(Cole and Streeck, 1986)
	EU918766	(Wu et al., 2009b)
	HQ537688 - HQ537707	(Chen et al., 2011)
	KF436865	(Burk et al., 2013)
35	X74477	(Delius and Hofmann, 1994)
	HQ537708 - HQ537730	(Chen et al., 2011)
	JN104062 - JN104067	(Draper et al., 2011)
	JX129485 - JX129488	(Marincevic-Zuniga et al., 2012)
52	X74481	(Delius and Hofmann, 1994)
	GQ472848	(Wu et al., 2010)
	HQ537731 - HQ537751	(Chen et al., 2011)
	AB819272 - AB819274	(Kukimoto et al., 2013)
58	D90400	(Kirii et al., 1991)
	FJ385261 - FJ385268, FJ407192, FJ407194 - FJ407195, FJ407199 - FJ407201	(Wu et al., 2009a)
	EU918765	(Wu et al., 2009b)
	GQ472850	(Wu et al., 2010)
	HQ537752 - HQ537777	(Chen et al., 2011)
	AB819275 - AB819279	(Kukimoto et al., 2013)

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Figure 3A

Molecular biology of human papillomavirus infection and cervical cancer

John Doorbar

Clinical Science May 01, 2006, 110 (5) 525-541

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Figure 3B

Papillomavirus genome structure, expression, and post-transcriptional regulation
Zhi-Ming Zheng, Carl C. Baker
[Frontiers in Bioscience, Landmark, 11, 2286 - 2302, September 1, 2006]

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Figure 6A

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Author:	Christopher B. Buck, Naiqian Cheng, Cynthia D. Thompson et al.
Publication:	Journal of Virology
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Figure 7

Neutralization of non-vaccine human papillomavirus pseudoviruses from the A7 and A9 species groups by bivalent HPV vaccine sera

Original Research Article

Vaccine, Volume 29, Issue 47, 3 November 2011, Pages 8585-8590

Eve Draper, Sara L. Bissett, Rebecca Howell-Jones, Debbie Edwards, Graham Munslow, Kate Soldan, Simon Beddows

Title: Cross-neutralizing antibodies elicited by the Cervarix® human papillomavirus vaccine display a range of Alpha-9 inter-type specificities

Author: Sara L. Bissett,Eve Draper,Richard E. Myers,Anna Godi,Simon Beddows

Publication: Vaccine

Publisher: Elsevier

Date: 26 February 2014

Figure 23, Table 9 and Full Article

Title:	Naturally Occurring Capsid Protein Variants of Human Papillomavirus Genotype 31 Represent a Single L1 Serotype
Author:	Sara L. Bissett, Anna Godi, Maxime J. J. Fleury et al.
Publication:	Journal of Virology
Publisher:	American Society for Microbiology
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P-27.15

ANTIGENIC RELATIONSHIP BETWEEN A9 HPV TYPES DEFINED BY VACCINE ANTIBODIES

S Bissett, *Health Protection Agency, London, UNITED KINGDOM*
R Myers, *Health Protection Agency, London, UNITED KINGDOM*
E Draper, *Health Protection Agency, London, UNITED KINGDOM*
A Godi, *Health Protection Agency, London, UNITED KINGDOM*
S Beddows, *Health Protection Agency, London, UNITED KINGDOM*

Background: Understanding the antigenic relationship between vaccine-incorporated and closely-related oncogenic HPV types is a key element in determining the limitation of the current vaccines and informing second generation vaccine development.
Objectives: To use data derived from seroreactivity to the L1 protein by bivalent vaccine sera in pairwise distance matrix algorithms to estimate the antigenic relationship between A9 types
Methods: Sera were collected from 69 girls aged 13-14 years, within 6 months of receiving their third bivalent vaccine dose. Neutralisation assays (purified L1L2 pseudoviruses) and direct binding assays (purified L1L2 pseudoviruses and purified baculovirus-derived L1 VLP) were carried out using antigen targets derived from the A9 HPV types 16, 31, 33, 35, 52 and 58. Pairwise euclidean distances were calculated for all serological data and then used to generate distance matrices which were clustered using the neighbour-joining algorithm. Resulting serological and viral dendrograms, supported where possible by bootstrapping, were combined with heat maps to define the antigenic relationships between A9 HPV types.
Results: There was a stepwise decrease in seropositivity between the L1 binding assay, the L1L2 binding assay and the L1L2 pseudovirus assay; for example, HPV31 percent positive 100%, 93% and 88%, respectively. This is further exemplified by, for example, the titers of HPV31 antibody reactivity as a function of the HPV16 type-specific response: L1 VLP binding, median 1.15% (IQR, 0.65-2.13%), L1L2 binding 0.86% (0.49-1.80%), L1L2 neutralisation 0.50% (0.24-0.96%); analysis by trend $p<0.001$. Distance matrices and the resulting dendrograms based on these three serological checkerboards, demonstrated different antigenic relationships between the A9 types (supported by bootstrap values ≥80%) depending on the context of the L1 protein.
Conclusions: These data further define the specificity of antibodies elicited to the bivalent HPV vaccine and the antigenic inter-relationship of oncogenic HPV types within the A9 species group.

Declaration of interest
None declared

OC 13-7

NATURALLY OCCURRING FG LOOP VARIANTS OF HPV31: IMPLICATIONS FOR CURRENT AND NEXT GENERATION L1-BASED PROPHYLACTIC VACCINES

Bissett S, Godi A and Beddows S

Virus Reference Department, Public Health England, London, UK

An analysis of available full length HPV31 L1 sequences (n=95) identified two major variant residues within the FG loop compared to the reference sequence: a Thr to Asn at position 274 (T274N) in 28% of sequences and a dual variant containing Thr to Ala at position 267 (T267A) alongside T274N in 49% of sequences. The FG sequence of the reference was present in only a minority of L1 sequences (15%). The epitopes of several neutralising monoclonal antibodies have been mapped to a region encompassing residue 267 and adjacent to 274 suggesting that changes in this region may alter antibody recognition.

Objectives: To evaluate whether L1 proteins with the single or dual FG loop variant residues differ from the L1 protein representing the reference in their ability to elicit type-specific neutralising antibodies (NAb) and in their sensitivity to both type-specific and cross-reactive antibody (Ab) mediated neutralisation.

Methods: HPV31 L1 virus-like particles (VLP) were generated containing the single T274N or dual T267A/T274N variant residues. These VLP were used in animal immunisations alongside VLP based upon the reference L1 sequence to generate type-specific NAb. Cervarix® and Gardasil® vaccinee sera were used as a source of cross-reactive HPV16/HPV31 NAb. The potential of the sera to neutralise HPV31 L1L2 pseudovirions (PsV) containing the single or dual variant residues were evaluated by neutralisation assay, alongside the PsV based upon the reference.

Conclusions: Overall, little difference was observed between the neutralisation potency of type-specific and cross-reactive Ab targeting the single or dual PsV compared to the reference PsV. For example, type-specific Ab raised against the reference L1 VLP (n=7) demonstrated no significant difference in NAb titre against the dual PsV compared to the reference PsV [geometric mean fold-difference 1.09 (95% CI: 0.45-2.62; p 0.612)]. Cross-reactive Ab in HPV vaccinee sera (n=17) did demonstrate borderline significantly higher NAb titres against the dual PsV compared to the reference PsV [1.35 (1.10-1.65; p=0.019)]. Naturally occurring polymorphisms in the FG loop of HPV31 are unlikely to have a major impact on the recognition of NAb elicited by the current or next generation L1 VLP-based vaccines.

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VACCINE-INDUCED CROSS-NEUTRALIZING ANTIBODIES TARGET COMPLEX EPITOPES ON THE ALPHA-9 MAJOR CAPSID PROTEIN

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Abstract Text

Objectives: To use chimeric pseudoviruses to investigate the antigenicity of the major capsid protein (L1) of HPV31, a non-vaccine genotype related to HPV16, in order to improve our understanding of vaccine-induced cross-protection.

Methods: L1L2 pseudoviruses were generated using HPV16 (vaccine type), HPV31 (non-vaccine type) or HPV35 (background control) backbones and inter-genotype loop swaps, in isolation or combination. Vaccine sera were then used against these chimeric L1L2 pseudoviruses in a neutralization assay and the contribution of different loops to L1 antibody recognition was evaluated by fold-differences in neutralization titers.

Results: A fold-reduction in neutralization titer of 3.8 (IQR 2.6 – 5.5) against HPV16:35FG compared to wild-type HPV16 indicated that the majority, but not the entirety, of the vaccine-type response appeared to target epitopes located in or around the FG loop. This region also appeared to be the target for a significant proportion of the cross-neutralizing antibody response since the neutralization titer against HPV31:35FG demonstrated a fold-reduction of 13.9 (IQR 7.9 – 29.0) compared to wild-type HPV31. The DE loop, whilst not appearing to be a target for cross-neutralizing antibodies, did enhance recognition of its homologous FG loop within a heterologous backbone.

Conclusion: The L1 loops of HPV31 work in concert by either presenting residues involved in cross-neutralizing epitope footprints and/or by stabilising the presentation of epitope residues on adjacent loops. These data provide insight into the complex interactions between the L1 loops and demonstrate that chimeric L1L2 pseudoviruses are appropriate tools with which to address differential inter-genotype antigenicity.

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Cross-neutralizing antibodies elicited by the Cervarix® human papillomavirus vaccine display a range of Alpha-9 inter-type specificities

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ABSTRACT

The highly efficacious human papillomavirus (HPV) vaccines contain virus-like particles (VLP) representing genotypes HPV16 and HPV18, which together account for approximately 70% of cervical cancer cases. Vaccine-type protection is thought to be mediated by high titer, type-specific neutralizing antibodies. The vaccines also confer a degree of cross-protection against some genetically-related types from the Alpha-9 (HPV16-like: HPV31, HPV33, HPV35, HPV52, HPV58) and Alpha-7 (HPV18-like: HPV39, HPV45, HPV59, HPV68) species groups. Cross-protection is coincident with the detection of low titer serum responses against non-vaccine types by vaccinees. Such antibodies may be the effectors of cross-protection or their detection may be useful as a correlate or surrogate.

This study evaluated whether cross-neutralization of HPV types from the Alpha-9 species group is mediated by antibodies with a predominantly type-restricted specificity for HPV16 that nevertheless exhibit low affinity interactions with non-vaccine types, or by antibody specificities that demonstrate similar recognition of vaccine and non-vaccine types but are present at very low levels.

Antibodies generated following Cervarix® vaccination of 13–14 year old girls were evaluated by pseudovirus neutralization, VLP ELISA and by enrichment of target antigen specificity using VLP-immobilized beads. Two-dimensional hierarchical clustering of serology data demonstrated that the antibody specificity profile generated by VLP ELISA was both quantitatively and qualitatively different from the neutralizing antibody specificity profile. Target-specific antibody enrichment demonstrated that cross-neutralization of non-vaccine types was due to a minority of antibodies rather than by the weak interactions of a predominantly type-restricted HPV16 antibody specificity. Furthermore, cross-neutralization of non-vaccine types appeared to be mediated by multiple antibody specificities, recognizing single and multiple non-vaccine types, and whose specificities were not predictable from examination of the serum neutralizing antibody profile. These data contribute to our understanding of the antibody specificities elicited following HPV vaccination and have potential implications for vaccine-induced cross-protection.

1. Introduction

The human papillomavirus (HPV) vaccines, Cervarix® and Gardasil®, comprise virus-like particles (VLP) based upon the major capsid protein, L1, of HPV16 and HPV18. Both vaccines are highly efficacious at preventing persistent infection and more progressive disease associated with HPV16 and HPV18 [1,2]. Antibodies capable of neutralizing pseudoviruses representing HPV16 and HPV18 can be detected in the serum and cervicovaginal secretions of vaccinees [3–5]. Together with passive transfer studies demonstrating that immune sera, purified IgG or monoclonal antibodies (MAbs) can protect animals against papillomavirus challenge [6–8], has led to the reasonable assumption that vaccine-induced type-specific protection is mediated by neutralizing antibodies [9,10].

A degree of cross-protection has also been demonstrated against some closely-related types within the Alpha-papillomavirus species groups, Alpha-9 (HPV16-like: HPV31, HPV33, HPV35, HPV52, HPV58) and Alpha-7 (HPV18-like: HPV39, HPV45, HPV59, HPV68) [1,2]. Cross-protection is coincident with the detection of cross-neutralizing antibodies against these types in the serum

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and cervicovaginal secretions of vaccinees [4,11–13]. Whether such antibodies are effectors, or their detection has some utility as a correlate or surrogate of vaccine-induced cross-protection is uncertain.

The antibody response following VLP immunization has been measured using a VLP enzyme-linked immunosorbent assay (ELISA) [14], a pseudovirus-based neutralization assay [15] and a competitive Luminex® immunoassay (cLIA) [16]. Different antibody specificities are measured by each of these assays but the nature of any potential discrepancies are not fully understood [9,11]. The cLIA assay uses the type-restricted murine MAb H16.V5 [17], whose human homologue appears to be the majority specificity generated during natural infection [18] and is assumed to constitute a high proportion of the antibodies elicited during vaccination.

The magnitude and breadth of the vaccine-induced serum neutralizing antibody response against non-vaccine types generally increases with the vaccine-type response [4,12,13]. It is unclear whether cross-neutralization within the Alpha-9 group is facilitated by antibodies other than the H16.V5-like human homologue or that this antibody exhibits some degree of cross-recognition not present in the murine version.

In this study we attempted to dissect the serum antibody response generated against non-vaccine types from the Alpha-9 group following Cervarix® vaccination in order to further describe the antibody specificities responsible for cross-neutralization.

2. Material and methods

2.1. Study samples

Serum samples ($n = 69$) were collected from 13 to 14 year old girls a median 5.9 months following their third dose of Cervarix® [12].

2.2. L1L2 pseudovirus neutralization assay

L1L2 pseudoviruses representing vaccine-relevant Alpha-9 types (HPV16, HPV31, HPV33, HPV35, HPV52 and HPV58) and carrying a luciferase reporter were expressed from transiently transfected 293TT cells, purified and characterized as previously described [12]. The equivalent of a Tissue Culture Infectious Dose 50% ($TCID_{50}$) was estimated using the Spearman–Karber equation and a standardized input of 300 $TCID_{50}$ was used for all pseudoviruses [12,15]. Serum samples were subjected to 4-5 serial dilutions and the 80% reciprocal neutralization titer estimated by interpolation. A panel of six serum samples were retested against the six pseudoviruses ($n = 36$; Pearson's $r = 0.976$; $p < 0.001$) and demonstrated good inter-assay reproducibility.

2.3. L1 VLP ELISA

L1 VLP were expressed using the Bac-to-Bac® Baculovirus System (Life Technologies), as previously described [20], wherein the L1 genes shared 100% amino acid sequence identity with the L1 genes of the Alpha-9 pseudovirus clones [12]. The L1 VLP were used as target antigens in a ELISA, as previously described [4]. Serum samples were subjected to 4–5 serial dilutions and the 50% reciprocal binding titer estimated by interpolation. Good inter-assay reproducibility was demonstrated by retesting a panel of six serum samples against the six L1 VLP ($n = 36$; Pearson's $r = 0.947$; $p < 0.001$).

2.4. Hierarchical clustering of serology data

Serological and viral dendrograms were generated by calculating the pairwise Euclidean distances for the Log_{10}-transformed pseudovirus neutralization assay and VLP ELISA data, generating distance matrices that were then clustered using a neighbor-joining algorithm (http://evolution.genetics.washington.edu/phylip.html). The resulting viral dendrograms were bootstrapped by resampling the sera data to generate 500 pseudoreplicates. Dendrograms were viewed using FigTree 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). The serological data were then represented by a heat map ordered according to the resulting serological and viral dendrograms.

2.5. Antibody adsorption and elution from L1 VLP

VLP (HPV16 10 μg; non-vaccine type 5 μg) were coupled to magnetic sepharose beads (GE Healthcare) overnight at 4 °C. Antibody adsorption and elution were performed as described elsewhere [21,22] with minor modifications. Sera for adsorption were diluted five-fold in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) (Life Technologies) and incubated with beads for 1 h at room temperature. The post-adsorption serum fraction was separated from the beads using a magnetic rack before being subjected to a second round of adsorption using a freshly coupled bead set. Both bead sets were then washed three times in DMEM containing 10% FBS. No residual antibody activity was detectable in the final washes. Antibodies were eluted using 0.1 M glycine–HCl (pH 2.9–1.9) and neutralized with 1 M Tris–HCl, pH 9 (GE Healthcare). The pooled eluted antibody fractions were concentrated using Vivaspin 500 columns (GE Healthcare). Each serum was also subjected to two rounds of adsorption on, and elution from, beads coupled with 10 μg BSA which was used as a control for non-specific activity; when eluted fractions were tested against the HPV16 pseudovirus they were found to have levels of neutralizing antibody below the detection threshold.

2.6. Statistical methods

Pearson's correlation was used to evaluate the relationship between HPV16 antibody titers. Fisher's exact test was used to determine whether the proportion of sera reactive against a particular non-vaccine type differed between the two assay systems. Tests were 2-tailed where appropriate and performed using Stata 12.1 (Statacorp, College Station, TX).

3. Results

Sixty nine serum samples from Cervarix® vaccinees, previously tested in the pseudovirus neutralization assay against vaccine-relevant Alpha-9 types [12] were tested against VLP representing the same HPV types by ELISA.

3.1. Antibody titers measured in pseudovirus neutralization assay and VLP ELISA

As in the pseudovirus neutralization assay [12], all sera ($n = 69$, 100%) tested positive for HPV16 antibodies by VLP ELISA. A significant correlation was observed between the antibody titers generated by the pseudovirus neutralization assay (median 19,258 [inter-quartile range, IQR, 11,730–28,132]) and VLP ELISA (9279 [7290–44,719]) (Pearson's $r = 0.833$; $p < 0.001$).

For non-vaccine types, there were differences between antibody titers generated in the VLP ELISA and the pseudovirus neutralization assay. While the number of samples positive for HPV31 antibodies in the VLP ELISA ($n = 58$; 84%) and pseudovirus neutralization assay ($n = 60$; 87%) were similar ($p = 0.810$), antibody titers of sera positive in both assays were higher in the VLP ELISA (median 651 [IQR 576–771]) than in the pseudovirus neutralization

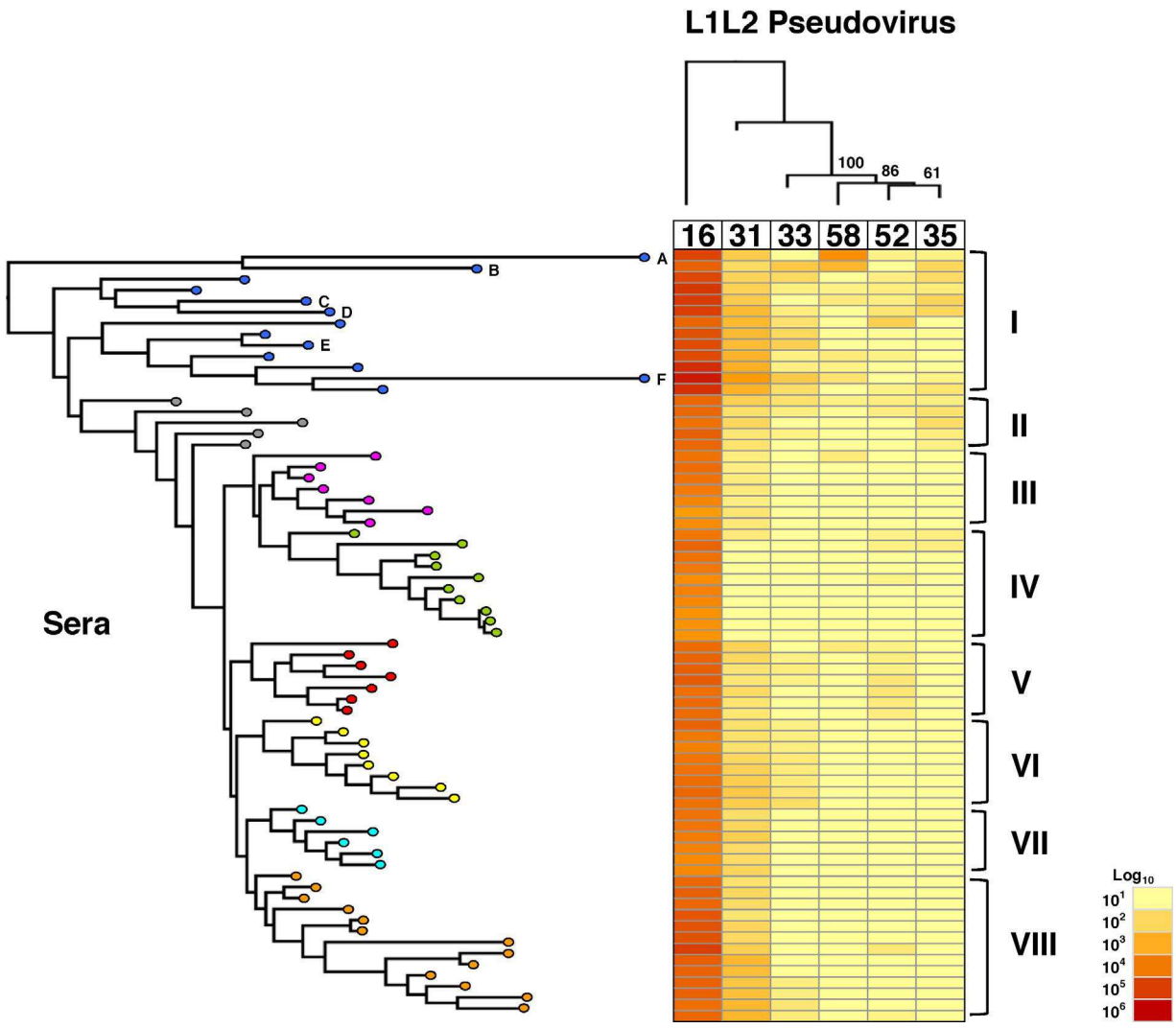

Fig. 1. Hierarchical clustering of L1L2 pseudovirus neutralization data.
Log10-transformed pseudovirus neutralization data (centre, heat map) were subjected to two-dimensional hierarchical clustering and re-ordered according to serological (left) and pseudovirus (top) dendrograms constructed from the resulting distance matrices. The serological dendrogram is labeled I-VIII based upon intuitive clustering of serological data whereas the pseudovirus target dendrogram clusters are supported by bootstrapping of 500 pseudoreplicates.

assay (96 [50–203]) ($p < 0.001$). More serum samples were positive for HPV33 antibodies by VLP ELISA ($n = 47$; 68%) than by the pseudovirus neutralization assay ($n = 29$; 42%; $p = 0.003$) with dual positive titers higher in the VLP ELISA (600 [374–735]) than in the pseudovirus neutralization assay (29 [25–54]) ($p < 0.001$).

These data suggest that there were quantitative differences between the pseudovirus neutralization assay and VLP ELISA and/or target antigens, particularly for non-vaccine types. We next sought to evaluate whether these data also reflected qualitative differences.

3.2. Hierarchical clustering of serological data

Two-dimensional antigenic dendrograms were constructed by hierarchical clustering of the pseudovirus neutralization assay (Fig. 1) and VLP ELISA (Fig. 2) data. The target antigens (L1L2 pseudovirus or L1 VLP) were clustered horizontally while the sera were clustered vertically against a heat map representing the Log_{10}-transformed antibody titer data. This approach allowed us to sort the pseudovirus neutralization and VLP ELISA data into clusters of sera displaying similar antigenic profiles.

The magnitude and breadth of the individual serum neutralizing antibody responses against vaccine and non-vaccine types permitted intuitive clustering (Fig. 1). Serum samples in Cluster I displayed the highest HPV16 neutralization titers and the broadest coverage of non-vaccine types, while Cluster VI included samples that had intermediate HPV16 neutralization titers and whose breadth of reactivity extended to HPV31 and HPV33 (Table 1). These data support a generally quantitative relationship between the level of antibodies in vaccinee sera against HPV16 and an ability to recognize non-vaccine types. However, there also appeared to be a number of antibody specificities displayed. Samples within Clusters II, V and VI for example exhibited differential neutralization of HPV33, HPV35 or HPV52, in addition to HPV31 despite similar HPV16 antibody titers.

The serological dendrogram based upon VLP ELISA binding titers (Fig. 2) permitted the formation of branches but the ordering of individual sera bore little relation to the arrangement in the serological dendrogram based upon the pseudovirus neutralization data.

The hierarchical clustering of antibody responses also permitted the ranking of the target antigens. Pseudoviruses HPV31 and HPV33 were the nearest antigenic relatives to HPV16 followed by HPV58 (Fig. 1). HPV52 and HPV35 pseudoviruses clustered together suggesting a close antigenic relationship between these types. The antigenic dendrogram based upon VLP ELISA data (Fig. 2) was

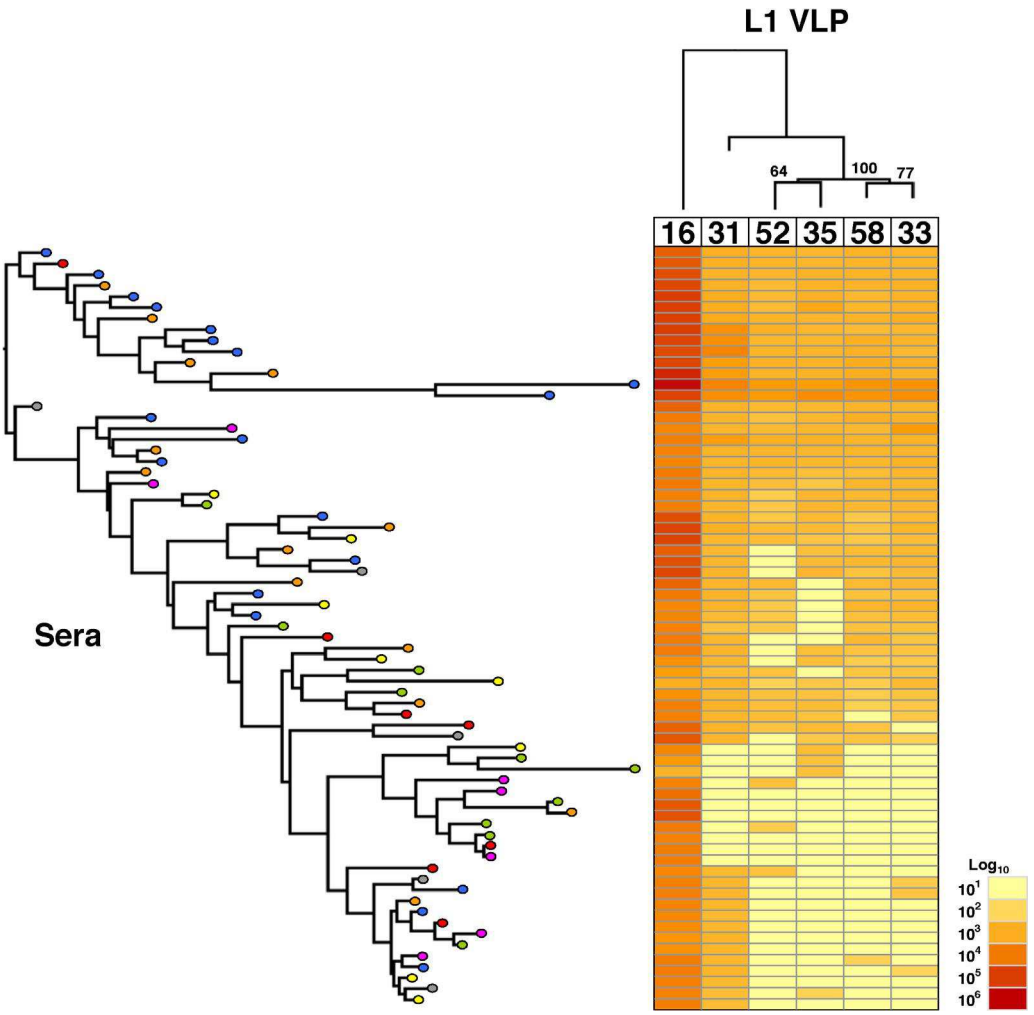

Fig. 2. Hierarchical clustering of L1 VLP binding data.
Log10-transformed VLP binding data (centre, heat map) were subjected to two-dimensional hierarchical clustering and re-ordered according to serological (left) and pseudovirus (top) dendrograms constructed from the resulting distance matrices. VLP target dendrogram is supported by bootstrapping of 500 pseudoreplicates.

broadly similar such that the nearest antigenic relative to HPV16 was HPV31, followed by two separate clusters of HPV33 and HPV58, and HPV35 and HPV52. These inter-type antigenic relationships had good bootstrap support and differed somewhat from the inter-type genetic distances based upon L1 amino sequence (Fig. 3).

3.3. Enrichment of vaccine and non-vaccine antibody specificities

Potential differences in cross-neutralizing antibody specificity were addressed by adsorption on, and elution from, individual non-vaccine type VLP. We reasoned that if cross-neutralization was due to antibodies that constitute a minor fraction of the total vaccine antibody repertoire, such an approach should enrich for these specificities in preference to type-specific HPV16 antibodies. Six serum samples (A–F) were selected from Cluster I (Fig. 1) for enrichment and the neutralization titers against pseudoviruses HPV16, HPV31 and another relevant type were determined prior to and post enrichment. Antibodies enriched on non-vaccine type VLP displayed a range of different cross-neutralizing specificities (Fig. 4).

The enrichment of sera A–D on a particular non-vaccine type VLP did not also enrich for antibodies against another non-vaccine type. Enrichment of serum A on HPV31 or HPV58 VLP yielded antibodies

Table 1
Differential pseudovirus neutralization responses informed by hierarchical clustering.

Cluster	*n*	Median (IQR) serum neutralization titers against indicated HPV pseudovirus[a]					
		HPV16	HPV31	HPV33	HPV35	HPV52	HPV58
I	13	74,295 (55,880–122,896)	482 (195–665)	54 (24–87)	22 (10–68)	21 (10–25)	20 (10–32)
II	5	20,556 (20,032–20,559)	58 (51–98)	23 (10–27)	27 (25–49)	10 (10–25)	10 (10–10)
III	7	9721 (5959–12,954)	31 (27–33)	10 (10–10)	10 (10–10)	10 (10–10)	10 (10–10)
IV	10	6953 (4366–11,584)	10 (10–10)	10 (10–10)	10 (10–10)	10 (10–18)	10 (10–10)
V	7	18,351 (17,026–25,055)	45 (42–84)	10 (10–21)	10 (10–10)	28 (25–35)	10 (10–10)
VI	8	13,302 (11,612–17,578)	108 (57–166)	30 (25–37)	10 (10–10)	10 (10–10)	10 (10–10)
VII	6	8275 (6386–11,407)	87 (70–107)	10 (10–10)	10 (10–10)	10 (10–10)	10 (10–10)
VIII	13	25,962 (21,195–40,113)	152 (90–399)	10 (10–26)	10 (10–10)	10 (10–10)	10 (10–10)

[a] Median (IQR, interquartile range) neutralizing antibody titers of sera within indicated intuitive clusters against indicated HPV pseudoviruses.

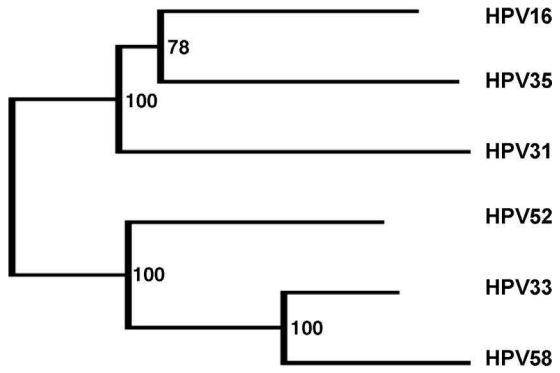

Fig. 3. Distance matrix based upon L1 amino acid sequence used for both Alpha-9 pseudoviruses and VLP, generated using a neighbor-joining algorithm and supported by bootstrap values.

capable of recognizing HPV16 and only the type used for enrichment. For example, the pre-treatment titers against HPV31 and HPV58 were 211 and 2696, respectively. Enrichment on HPV58 VLP increased the titer against HPV58 to 6188 but no HPV31 antibody reactivity was detectable. Serum B which demonstrated post-enrichment neutralization activity against HPV31, HPV33, HPV35 and HPV58 appeared to comprise multiple antibody specificities that recognized HPV16 and only the indicated non-vaccine type. Enrichment of sera C and D on HPV35 VLP yielded antibodies capable of recognising HPV16 and HPV35, but not HPV31.

Antibodies enriched from serum E and F exhibited cross-recognition of more than one non-vaccine type. The enrichment of serum E on HPV31 or HPV33 VLP yielded antibodies capable of recognizing HPV16, HPV31 and HPV33 pseudoviruses. Serum F when enriched on HPV31, HPV33 and HPV58 demonstrated neutralization of HPV31 pseudovirus to a comparable level, and serum F antibodies enriched on HPV31 or HPV33 VLP had similar titers against HPV33.

The HPV16 titer dropped by a median 1.8 Log_{10} (IQR 1.7–2.8; $n = 13$) fold following enrichment on non-vaccine VLP. Enriched antibody titers against HPV16 were similar to the titers observed against the type used for enrichment, for example antibodies in serum A when enriched on HPV31 VLP neutralized HPV16 and HPV31 at titers of 861 and 795, respectively.

Antibodies enriched from serum samples A–F, were also tested against L1 VLP representing the same HPV types (Supplementary material S1). Antibody binding titers further confirmed the observations that non-vaccine type antibodies are a minority species which display similar reactivity against HPV16 and non-vaccine types and again highlighted discrepancies between binding and neutralizing antibody specificity.

4. Discussion

We undertook a proof of concept study to investigate the cross-neutralizing antibody specificities generate in response to HPV vaccination. Cross-neutralizing antibodies are elicited in response to both licensed vaccines, Cervarix® and Gardasil® [4,11–13] and this is coincident with differential degrees of vaccine-induced cross-protection [1,2], although a direct link between the two observations has not been established. The characterisation of the cross-neutralizing response beyond antibody titer has been limited to studies of avidity [23] and the vaccine-type specificity of cross-neutralizing antibodies [24]. Sera from Cervarix® vaccinees were chosen since it is this vaccine that appears to elicit the broadest cross-neutralization of non-vaccine types [4].

In the present study, sera from Cervarix® vaccinees were shown to have high antibody titers with broad reactivity against L1 VLP with homologous L1 sequences to those of the pseudoviruses. HPV16 neutralizing antibody titers were similar to those generated in the VLP ELISA corroborating observations in other studies [5,25]. Agreement between antibody reactivity against L1L2 pseudoviruses and L1 VLP representing non-vaccine HPV types was weaker with VLP ELISA antibody titers generally an order of magnitude higher than the corresponding pseudovirus neutralizing titers [4,26].

To examine the discrepancy between cross-reactive antibody profiles, both sets of serological data were subjected to hierarchical clustering. This approach has been used for the evaluation of HIV [27–30], foot and mouth disease virus [31] and H5N1 avian Influenza virus [32] antibody specificities, but we believe this is the first time that this approach has been used to examine HPV vaccine antibody specificity. Differences between pseudovirus neutralizing and VLP binding antibody profiles were stark. There are likely several confounding factors that contribute to this outcome including technical differences between the assays and differences between the range of binding and neutralizing antibody specificities generated. Thus, while L1 VLP binding may be a useful surrogate for type-specific vaccine antibody responses [25] they may not be a similarly useful surrogate for neutralizing antibody reactivity against non-vaccine types.

A number of murine MAbs are capable of binding L1 VLP but lack the ability to neutralize the homologous L1L2 pseudovirus [17,33–35]. For example, MAb H16.J4 cross-reacts with L1 VLP representing various HPV types by ELISA [17], cross-neutralizes HPV31, HPV33 and HPV58 in an L1-based reporter transduction assay [36], but poorly recognizes its epitope on HPV16 L1L2 pseudoviruses [34,35]. Conversely, the neutralizing type-specific MAb H16.V5 appears to recognize its epitope on L1 VLP and L1L2 pseudoviruses to a similar extent [35]. It is reasonable to assume, therefore, that the majority of non-neutralizing antibodies in vaccine sera that recognize VLP representing non-vaccine types, bind to portions of the L1 protein not involved in (pseudo)virus entry or to domains that become altered when L2 is incorporated into the capsid.

There was some agreement in the antigenic inter-type ranking of target HPV types. For both L1 VLP and L1L2 pseudovirus antigens, HPV31 was ranked as the nearest relative to HPV16, and both HPV33/HPV58 and HPV35/HPV52 appeared to share some antigenic similarity, at least based upon reactivity of antibodies generated against the archetypal Alpha-9 group type, HPV16. Some of these antigenic similarities could have been predicted from the distance matrix based upon the L1 amino acid sequence (HPV33 and HPV58), while some could not (HPV35 and HPV52).

Hierarchical clustering of the pseudovirus neutralization data also suggested that Cervarix® vaccination elicits multiple cross-reactive antibody specificities. The underlying basis for individuals generating such a range of cross-reactive antibody specificities is unclear. There may be a genetic component [37] that could impact on an individual's ability to process certain immunogenic epitopes displayed on the vaccine antigens but identifying such contributing factors is challenging. In an attempt to examine the multiplicity of this cross-neutralizing response, we performed antibody enrichment of sera using L1 VLP immobilized onto beads and then tested the eluted fractions against relevant pseudoviruses. The enrichment of antibody specificities using this approach appears to suggest that cross-reactive antibodies formed a distinct, minority specificity within the vaccine-induced antibody repertoire and were not a consequence of a low affinity interaction of an otherwise predominantly type-specific antibody.

The enriched fractions displayed a range of cross-neutralizing antibody specificities including those that recognize multiple non-vaccine types and those that recognize only single non-vaccine types. The cross-neutralizing specificities of the enriched antibody

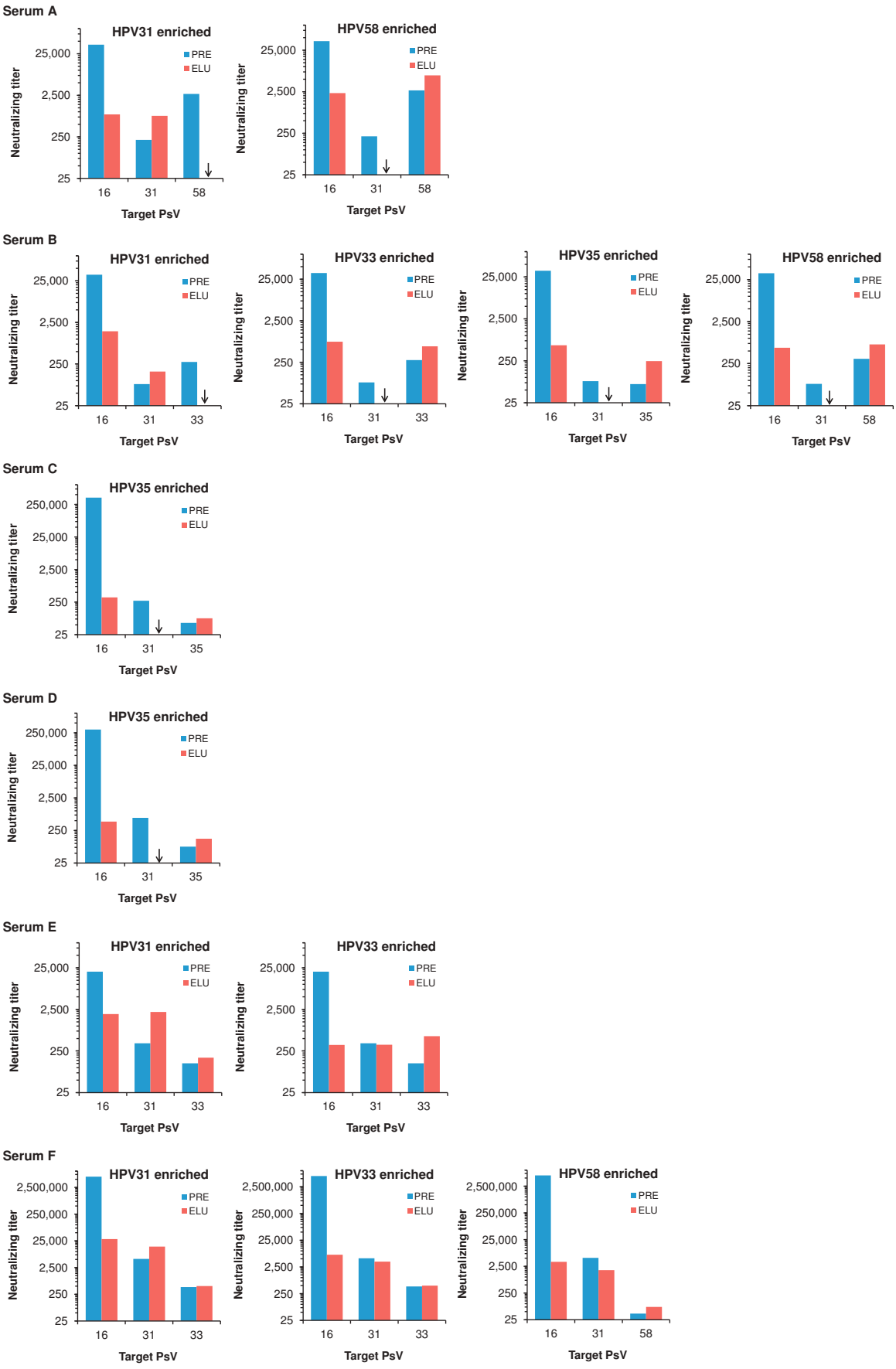

Fig. 4. L1L2 pseudovirus neutralization titers prior to and post antibody enrichment on non-vaccine L1 VLP.
Serum samples (A–F) were enriched on VLP representing non-vaccine A9 types. The neutralization titer against pseudoviruses (PsV) representing HPV16, HPV31 and another relevant type were determined for the serum samples prior to and post enrichment.

fractions could not have been predicted from the neutralization profile of the source serum. These data suggest that there are multiple immunogenic sites on the surface-exposed domains of the HPV16 L1 protein that share sequence and/or structural homology with other Alpha-9 types. These regions may include the variable loops DE, FG and HI that appear to be common target domains of antibodies generated by natural HPV16 infection [38].

There are several potential shortcomings to this work. Only six sera were evaluated from individuals given Cervarix® vaccine. Caution should therefore be employed when attempting to extrapolate these findings to the majority of HPV vaccinees. Extending this work to include sera from both Cervarix® and Gardasil® vaccinees will support a more robust evaluation. The target antigens for the enriched antibodies were L1L2 pseudoviruses whereas the antigens used for the enrichment were L1 VLP which may have introduced some bias in the antibody specificities being measured. This approach was used for two reasons. First, in our hands, the expression and purification of L1 VLP generates purer populations of antigen than the corresponding purification of L1L2 pseudoviruses. Second, the immunogens used in the HPV vaccines are L1 VLP and so the use of L1 VLP as the immobilized antigen should have allowed capture of the majority of L1-specific antibodies able to recognize a particular HPV type. The recovery of high titer cross-neutralizing antibodies following enrichment on non-vaccine VLP appears to support the maintenance of some VLP conformational integrity following bead immobilisation.

If cross-neutralizing antibodies form a tiny minority of the antibodies elicited following HPV vaccination it is possible that their generation and maintenance is more precarious than those of vaccine type antibodies. HPV31 cross-neutralizing antibody can be detected at 18 months after the third Cervarix® vaccine dose suggesting some degree of stability in this regard [26]. A two-dose schedule may also be an issue for the generation and maintenance of a sizeable cross-neutralizing antibody fraction. While HPV16 antibody titers following a two dose schedule appear to be non-inferior to those following a three dose schedule [19], the impact on the generation of antibodies to non-vaccine types is unclear. Understanding the potential impact of prior infection on vaccine antibody responses [23] and differences between the specificities of antibodies generated following vaccination and during natural infection will also be important.

Overall, these data support the notion that antibody neutralization of non-vaccine types by Cervarix® vaccine sera is due to a small fraction of antibodies exhibiting different but overlapping specificities, rather than a predominantly type-specific antibody specificity that nevertheless exhibits a small degree of cross-recognition of non-vaccine types. Identifying the HPV16 L1 domains responsible for their generation and perhaps improving HPV16 VLP immunogenicity toward the generation of such antibodies will be important if the development of high titer neutralizing antibodies targeting non-vaccine types is considered to be a desirable outcome of HPV vaccination.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material S1 L1 VLP binding titers prior to and post antibody enrichment on non-vaccine L1 VLP.

Serum samples (A–F) were enriched on VLP representing non-vaccine A9 types. The binding titer against VLP representing HPV16, HPV31 and another relevant type were determined for the serum samples prior to and post enrichment.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.01.008.

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Naturally Occurring Capsid Protein Variants of Human Papillomavirus Genotype 31 Represent a Single L1 Serotype

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ABSTRACT

We investigated naturally occurring variation within the major (L1) and minor (L2) capsid proteins of oncogenic human papillomavirus (HPV) genotype 31 (HPV31) to determine the impact on capsid antigenicity. L1L2 pseudoviruses (PsVs) representing the three HPV31 variant lineages, variant lineages A, B, and C, exhibited comparable particle-to-infectivity ratios and morphologies. Lineage-specific L1L2 PsVs demonstrated subtle differences in susceptibility to neutralization by antibodies elicited following vaccination or preclinical L1 virus-like particle (VLP) immunization or by monoclonal antibodies; however, these differences were generally of a low magnitude. These data indicate that the diagnostic lineage-specific single nucleotide polymorphisms within the HPV31 capsid genes have a limited effect on L1 antibody-mediated neutralization and that the three HPV31 variant lineages belong to a single L1 serotype. These data contribute to our understanding of HPV L1 variant antigenicity.

IMPORTANCE

The virus coat (capsid) of the human papillomavirus contains major (L1) and minor (L2) capsid proteins. These proteins facilitate host cell attachment and viral infectivity and are the targets for antibodies which interfere with these events. In this study, we investigated the impact of naturally occurring variation within these proteins upon susceptibility to viral neutralization by antibodies induced by L1 VLP immunization. We demonstrate that HPV31 L1 and L2 variants exhibit similar susceptibility to antibody-mediated neutralization and that for the purposes of L1 VLP-based vaccines, these variant lineages represent a single serotype.

Human papillomaviruses (HPVs) have a double-stranded DNA genome of approximately 8 kb which is replicated via host cell polymerases with an error rate of ca. 2×10^{-8} base substitutions per site per year (1), substantially lower than that found in the majority of single-stranded RNA viruses (ca. 1×10^{-3} base substitutions per site per year) (2). Despite the low evolutionary rate of the HPV genome, variants have arisen over time, leading to the generation of distinct intragenotype lineages classified by a sequence difference of 1 to 10% across the whole genome (3). The single nucleotide polymorphisms (SNPs) that allow segregation of these variants into distinct lineages can be found in each gene/region, with the highest number accumulating in the noncoding regions (NCR1, NCR2, and URR) and the lowest number accumulating in structural (L1 and L2) genes (4).

The HPV structural genes encode the major (L1) and minor (L2) proteins that form the nonenveloped icosahedral viral capsid, which comprises 72 pentameric L1 capsomers, and each capsomer has an upper estimate of one L2 protein (5). The L1 protein mediates attachment to host cells (6), while the L2 protein is essential for subsequent viral infectivity (7).

The humoral immune response following natural HPV infection predominately targets conformational epitopes on the surface-exposed loop regions of the L1 protein (8, 9). Seroconversion generally occurs 6 to 18 months after infection, with low levels of L1 antibodies being detected in 50 to 70% of individuals (10, 11). It is not clear whether antibodies induced by natural infection protect against subsequent reinfection by the same HPV genotype, but increasing evidence indicates that high antibody titers can be associated with a reduced risk of reinfection (12–15).

The L1 protein can self-assemble into virus-like particles (VLPs), which are the basis of the current prophylactic HPV vaccines, Cervarix and Gardasil (16). Clinical trials have demonstrated the high degrees of efficacy of both vaccines against infection and cervical disease associated with vaccine genotypes HPV genotype 16 (HPV16) and HPV18. A degree of vaccine-induced cross-protection against closely related genotypes, particular HPV31, HPV33, and HPV45, has also been demonstrated (16–18). HPV vaccine type-specific protection is assumed to be mediated by L1-neutralizing antibodies, which can be detected in the serum and cervicovaginal secretions of vaccinees (16, 19–22). The role of L1-neutralizing antibodies in mediating cross-protection is less clear, although a recent study reported an association between the presence of HPV31 cross-neutralizing antibodies and a reduced risk of HPV31 infection (23). Next-generation L1 VLP-based vaccines aim to extend the breadth of coverage by incorporating an increased number of L1 VLPs (24, 25).

Intragenotype variation within the L1 protein is generally localized to the surface-exposed loop domains (26), akin to the majority of intergenotype variation (27, 28). Data informing the po-

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tential impact of such variation on L1 antigenicity are limited to HPV16, where L1L2 pseudoviruses (PsVs) representing lineage-specific L1 variants were neutralized to a similar extent by antibodies elicited against a single L1 VLP (29). However, the impact of a common Thr-to-Ala switch at amino acid residue position 266 within the FG loop of HPV16 was not evaluated, nor was variation within the corresponding L2. Another study found that an FG loop-specific neutralizing monoclonal antibody (MAb), H16.E70, had reduced recognition for HPV16 L1 VLP bearing a Thr at amino acid residue 266, indicating that variation within this region can impact antigenicity (30).

HPV31 is closely related to HPV16 within the alpha 9 species group and is associated with ca. 3.8% of cervical cancer cases worldwide (31). The full-genome sequence analysis of HPV31 has led to the delineation of three distinct variant lineages: A, B, and C (4). Infections due to HPV31 lineage variant A or B have been associated with an increased risk of development of cervical intraepithelial neoplasia grades 2 and 3 (CIN2/3), yet, somewhat paradoxically, infections with lineage variant C appear to persist for longer periods (32, 33). Differences in the natural history between variants of other HPV genotypes have also been observed (34, 35). The relative infectivity of variants or the ability of variants to differentially disrupt cellular differentiation is a possible virological factor which can contribute to the disparities in variant pathology (3). One study suggested that the genetic background of the host may also play a role, since African-American women were found to be less likely to clear a HPV31 lineage variant C infection than a lineage variant A infection, yet there was no difference in the likelihood of clearing a lineage variant A over C infection in Caucasian women (33).

Two nonsynonymous, lineage-specific SNPs within the L1 of HPV31 are located within the FG loop at positions 267 and 274 (26). The FG loop of HPV31 has been shown to be an important antigenic domain targeted by both type-specific and cross-reactive L1 MAbs (36, 37). In the present study, we generated additional HPV31 L1 and L2 sequences and synthesized representative antigens in order to investigate the potential impact of this variation. Such data should improve our understanding of the potential biological impact of naturally occurring HPV31 variation.

MATERIALS AND METHODS

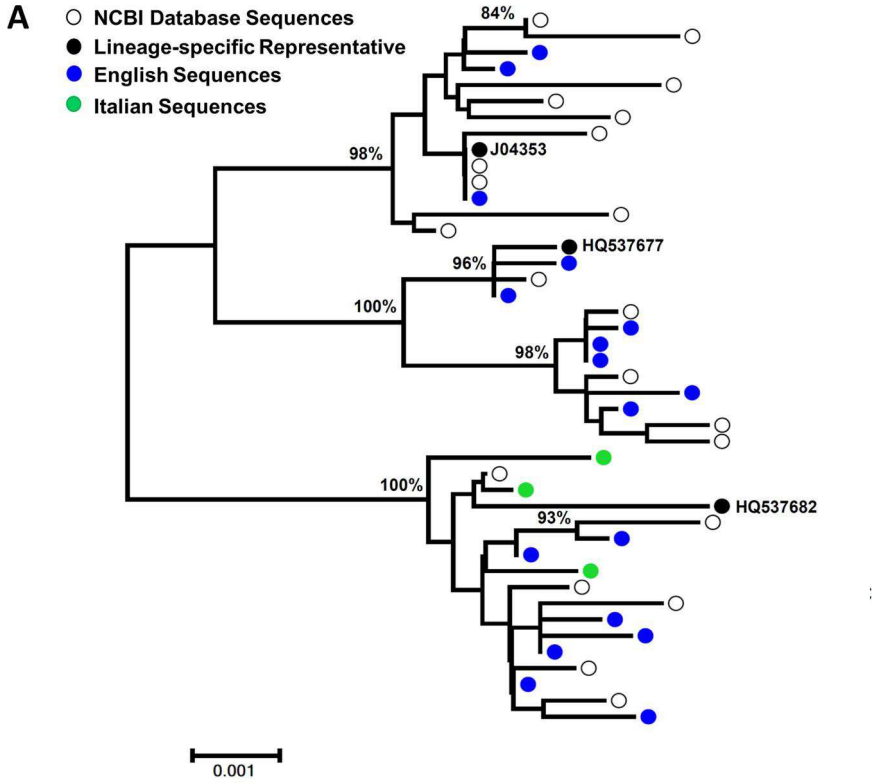
Study samples. Residual vulva-vaginal samples that had been collected from 16- to 24-year-old females who were undergoing chlamydia testing in England and that had previously been confirmed to be HPV31 DNA positive using the Hybrid Capture 2 HPV DNA test (Qiagen) and the Linear Array HPV genotyping test (Roche) (38) were selected for L1 and L2 sequencing. Cervical cell samples from HPV31 DNA-positive women (ages, 19 to 76 years) attending gynecological care at the San Gerardo Hospital (Milan, Italy; ethical approval study code 08/UNIMIB-HPA/HPV1) following a cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS) or low-grade squamous intraepithelial lesions (LSILs) were available for L1 and L2 sequencing. Serum samples were available from HPV31 DNA-positive women within this cohort. Serum samples were available from 12- to 15-year-old girls 1 month after receiving three doses of Cervarix or Gardasil HPV vaccine (20). A panel of HPV31 MAbs was available as either ascitic fluid or tissue culture supernatant (39).

Sequencing of HPV31 capsid genes. The L1 gene (bp 5443 to 7119 [40] or the FG loop region from bp 6141 to 6476; numbered according to the HPV31 reference sequence with GenBank accession number J04353) and the L2 gene (bp 3921 to 5725) were amplified with Platinum *Taq* high-fidelity DNA polymerase (Life Technologies) and sequenced using an ABI 3730 genetic analyzer. Sequence data were collated using DNASTAR Lasergene (v9.0) software (DNASTAR, Inc.). Additional HPV31 L1 and L2 sequences were downloaded from the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/; GenBank accession numbers HQ537666 to HQ537687 [4], U37410 [41], and J04353 [42]) and analyzed using the neighbor-joining tree algorithm, with bootstrap values ($n = 500$ iterations) being generated using the MEGA (v6) program (43). HPV31 L1 variant residues were mapped to the surface of the HPV16 capsomer crystal structure (PDB accession number 2R5H) and analyzed using the Swiss-PDP viewer algorithm (v4.0; Deep View) (44).

L1 VLPs. HPV31 L1 VLPs were expressed using a Bac-to-Bac baculovirus system (Life Technologies) and purified on an iodixanol (Sigma-Aldrich) gradient as previously described (45). The L1 protein was visualized by SDS-PAGE, the gel was stained with SimplyBlue SafeStain (Life Technologies), and the L1 protein concentration was determined by comparison with a standard curve derived from known input concentrations of bovine serum albumin. Gel analysis was carried out using ImageJ software (U.S. National Institutes of Health; http://imagej.nih.gov/ij) to determine the L1 concentration of the gradient fractions. VLP formation was confirmed by electron microscopic analysis of negatively stained particles. The HPV31 L1 VLPs shared a 100% amino acid sequence identity with the amino acid sequence of the L1 protein of the HPV31 reference sequence (GenBank accession number J04353) of lineage variant A. Site-directed mutagenesis with a QuikChange kit (Stratagene) was employed to generate L1 sequences representing lineage variants HPV31 B and HPV31 C. The L1 VLPs were used as target antigens in an enzyme-linked immunosorbent assay (ELISA), as previously described (20, 45). The panel of HPV31 MAbs was tested at a standardized input concentration of 250 μg/ml of mouse IgG for all MAbs except 31.D24, for which the starting input concentration was 20 μg/ml. The MAbs were subjected to serial dilutions, the IgG concentration which resulted in a 50% maximal binding optimal density (OD) was estimated by interpolation, and the results are presented as the 50% binding concentration.

Mouse immunizations. VLPs were adsorbed onto aluminum hydroxide (Alhydrogel; Brenntag Biosector) before addition of the monophosphoryl lipid A (MPL)-based Sigma adjuvant system (Sigma-Aldrich). BALB/c mice were injected intramuscularly with 2 μg of VLPs on day 0 and day 14, before a terminal blood sample was taken at day 21. Pretreatment blood samples were taken from all mice prior to the initial immunization. A total of 10 mice were immunized with either HPV31 A VLPs, HPV31 B VLPs, or HPV31 C VLPs over three separate immunization schedules. All animal husbandry and procedures were carried out in strict accordance with United Kingdom Home Office guidelines, were governed by the Animals (Scientific Procedures) Act of 1986, and were performed under licenses PPL 70/7412 and 70/7414.

L1L2 pseudoviruses. A bicistronic psheLL vector (46) containing codon-optimized HPV31 L1 and L2 genes from the HPV31 reference sequence (GenBank accession number J04353) of lineage variant A was expressed and purified on an iodixanol (Sigma-Aldrich) gradient as previously described (47). The L1 and L2 genes from lineage variants HPV31 B and HPV31 C were either synthesized by GeneArt (Life Technologies) or generated by site-direct mutagenesis with a QuikChange kit (Stratagene). Particle formation and particle size were determined by electron microscopic analysis of negatively stained particles. The L1 concentrations of PsV stocks were estimated by semiquantitative L1 Western blot analysis using CamVir-1 antibody (Abcam, United Kingdom), and the 50% tissue culture infective dose ($TCID_{50}$) was estimated using the Spearman-Karber equation as previously described (47). Particle-to-infectivity (PI) ratios were determined on the basis of an estimated particle amount of 3×10^7 particles per ng L1 protein (http://home.ccr.cancer.gov/lco/production.asp), with the ratio being normalized for the input volume and the $TCID_{50}$. The presence of the L2 protein and the reporter gene (luciferase) in purified PsV stocks was confirmed by qualitative L2 Western blot analysis using HPV16 L2 antipeptide-containing sera (amino



B

	L1				L2							
aa	194	267	274	432	115	269	270	362	377	N	%	Lineage
HPV31 J04353	S	T	T	T	V	N	I	T	V			
	.	.	.	.	.	.	.	.	.	7	16%	A
	.	.	.	S	.	.	.	.	.	6	14%	A
	.	.	.	S	.	.	M	.	.	1	2%	A
	.	.	N	.	.	.	.	A	.	5	11%	B
	.	.	N	.	.	.	.	.	.	4	9%	B
	T	.	N	.	.	.	.	.	.	3	7%	B
	T	.	N	A	.	.	.	.	.	1	2%	B
	.	A	N	.	I	.	M	.	L	12	27%	C
	.	A	N	.	I	D	M	.	L	5	11%	C

FIG 1 HPV31 L1 and L2 variation. (A) Phylogenetic tree constructed from concatenated L1 and L2 nucleotide sequences, including representative sequences from lineages A (J04353), B (HQ537677), and C (HQ537682). (51) and bootstrap values of >95%. (B) Site-specific amino acid (aa) covariation within the L1 and L2 proteins. N, number of sequences in the phylogenetic tree represented by each L1 and L2 combination.

acids 17 to 36) and qualitative PCR (bp 1222 to 1641; pGL4.51; Promega) following DNA extraction (QIAamp DNA blood minikit; Qiagen), respectively. The PsV neutralization assay was performed as previously described (48) with minor modifications (47). A standardized input of 100 $TCID_{50}$s was used for all PsVs, and samples were subjected to serial dilutions, with the antibody titer or concentration resulting in an 80% reduction of the luciferase signal (in relative light units) produced by the control wells containing PsV only being estimated by interpolation. HPV antibody control reagents were included in each assay run (49) alongside heparin (H-4784; Sigma-Aldrich), which was used as a positive inhibitor control. The median neutralization titer and interquartile range (IQR) for the positive-antibody-control reagent (high-titer HPV16/18) were as follows: for HPV31 A PsVs, 231 (IQR, 173 to 337; $n = 14$); for HPV31 B PsVs, 462 (IQR, 387 to 671; $n = 10$); and for HPV31 C PsVs, 500 (IQR, 354 to 589; $n = 12$). The negative-antibody-control reagent (HPV negative) had a titer of <40 in all assays ($n = 42$).

Statistical analysis. The Wilcoxon paired signed-rank test was used to compare neutralization titers using Stata (v12.1) software (StataCorp, College Station, TX).

Nucleotide sequence accession numbers. The HPV31 L1 and L2 sequences generated in this study were assigned the following GenBank accession numbers: KJ754561 to KJ754580.

RESULTS

HPV31 L1 and L2 amino acid variation. Full-length HPV31 L1 and L2 sequences were represented by contemporary English ($n = 17$) and Italian ($n = 3$) sequences, in addition to available NCBI database sequences ($n = 24$). Analysis of the aligned, concatenated L1 and L2 sequences demonstrated three distinct clusters consistent with the lineages HPV31 A, HPV31 B, and HPV31 C (Fig. 1A). Lineage A contained 14 sequences, including the

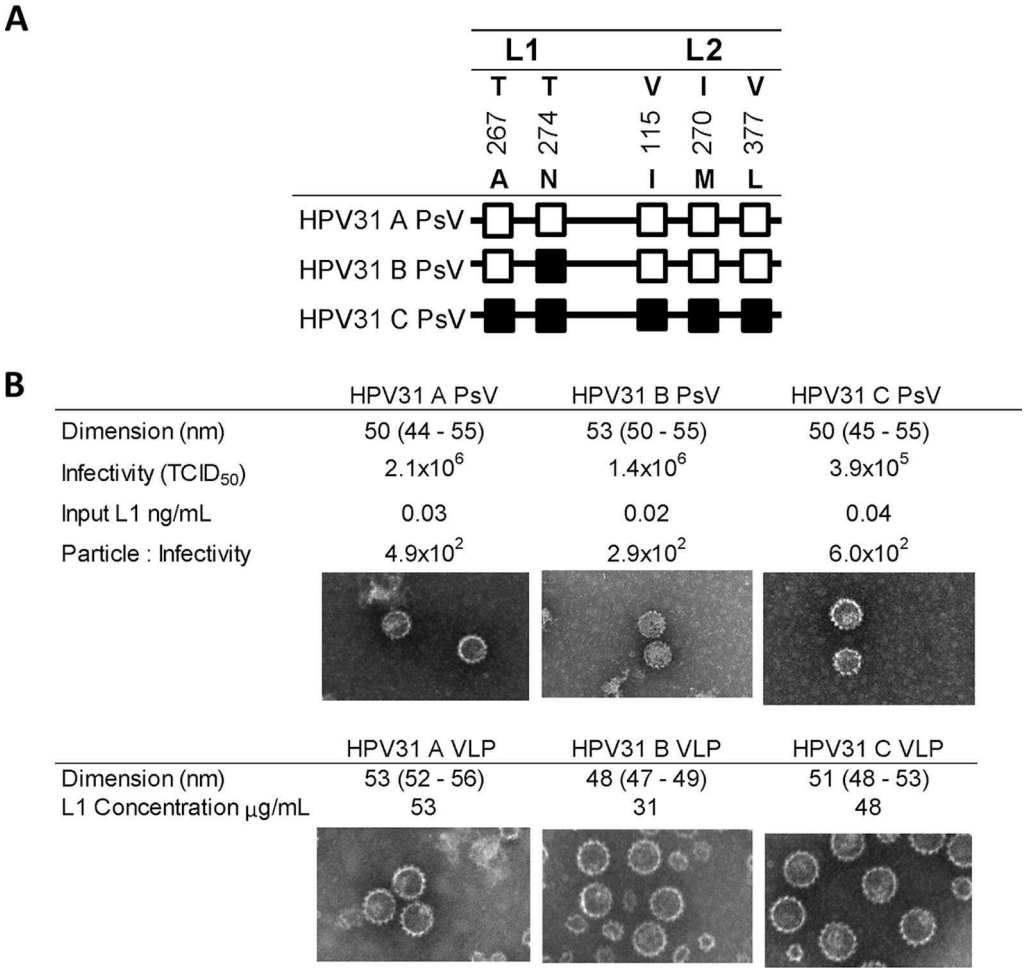


FIG 2 HPV31 L1 and L2 variants. (A) Graphical representation of L1 and L2 variant protein combinations. Site-specific amino acid alterations from the reference (HPV31 A; top sequence, open squares) are indicated using the residue position and resulting amino acid sequence code (filled squares). (B) L1L2 pseudovirus preparation characterized for the median (IQR) particle dimension, infectivity, and L1 concentration. (C) Median (IQR) L1 VLP dimensions (for particles >40 nm in diameter) and L1 concentrations.

HPV31 reference sequence (GenBank accession number J04353), and 7 of these demonstrated variation from the reference sequence in L1 (T432S) and a single sequence demonstrated variation within L2 (I270M) (Fig. 1B). All 13 sequences in lineage B demonstrated variation from the reference sequence at L1 amino acid position 274 (T274N) within the FG loop, while 4 sequences also varied at position 194 (S194T) and 1 varied at position 432 (T432A). Five sequences exhibited variation from the reference sequence at L2 position 362 (T362A). Lineage C contained 17 sequences, all of which demonstrated variation from the reference sequence at L1 amino acid positions 267 (T267A) and 274 (T274N) within the FG loop and L2 positions 115 (V115I), 270 (I270M), and 377 (V377L); 5 sequences also varied from the reference sequence at L2 amino acid position 269 (N269D).

Sensitivity of variant HPV31 L1L2 PsVs to antibody-mediated neutralization. L1L2 PsVs representing lineage variants HPV31 A (GenBank accession number J04353), HPV31 B, and HPV31 C were generated from the consensus L1 and L2 sequences representing each lineage and bore the major L1 variant residues (at positions 267 and 274) and L2 variant residues at positions 115, 270, and 377 (Fig. 2A). All three lineage variant PsVs, here referred to as HPV31 A PsVs, HPV31 B PsVs, and HPV31 C PsVs, generated similarly sized PsV particles of about 50 nm and produced comparable PI ratios of ca. 10^2 (Fig. 2B). The PsV preparations also contained the L2 protein and the luciferase reporter plasmid (data not shown).

The HPV31 variant PsVs were tested against sera from girls who received either the Cervarix or the Gardasil ($n = 46$) HPV vaccine (Table 1). Both HPV31 B and C PsVs were more sensitive to neutralization than HPV31 A PsVs. HPV31 B PsVs displayed a median 1.7-fold (IQR, 1.1- to 2.4-fold; Wilcoxon paired signed-rank test, $P < 0.001$) increased sensitivity to vaccine-induced cross-neutralizing antibodies compared to that of HPV31 A PsVs, while HPV31 C PsVs displayed a 1.4-fold (IQR, 1.1- to 1.6-fold; $P < 0.001$) increased sensitivity compared to that of HPV31 A PsVs. The increased sensitivity of HPV31 B and C PsVs to cross-neutralizing antibodies was independent of the HPV vaccine received (Table 1).

All three HPV31 variant PsVs were susceptible to neutralization by a small panel of longitudinal serum samples (collected at 0, 6, 12, and 18 months) from women naturally infected with HPV31

TABLE 1 Neutralization sensitivity of variant HPV31 L1L2 PsVs to HPV vaccine-induced antibodies

Vaccinee group	No. of serum samples	HPV31 A PsV titer[a]	HPV31 B PsVs		HPV31 C PsVs	
			Titer	Fold difference[b]	Titer	Fold difference
Cervarix vaccinees	22	1,026 (646–1,543)	1,469 (1,260–2,582)[c]	1.8 (1.1–2.5)	1,180 (923–1,721)[d]	1.3 (1.0–1.7)
Gardasil vaccinees	24	712 (382–1,363)	1,016 (759–1,435)[c]	1.5 (1.1–2.3)	968 (659–2,249)[e]	1.4 (1.1–1.6)
All vaccinees	46	885 (499–1,435)	1,273 (973–2,253)[e]	1.7 (1.1–2.4)	1,096 (763–2,216)[e]	1.4 (1.1–1.6)

[a] Neutralization titer data are presented as the median (IQR) 80% antibody neutralization titers generated from the 2 to 5 data sets per serum sample.
[b] Median (IQR) fold difference in the neutralization titers for HPV31 B PsVs and HPV31 C PsVs compared to the neutralization titer for HPV31 A PsVs.
[c] $P < 0.01$ using the Wilcoxon paired signed-rank test.
[d] $P < 0.05$ using the Wilcoxon paired signed-rank test.
[e] $P < 0.001$ using the Wilcoxon paired signed-rank test.

(Table 2). Median antibody neutralization titers against HPV31 A, B, and C PsVs were 576 (IQR, 391 to 1,144), 839 (IQR, 587 to 1,899), and 882 (IQR, 337 to 1,895), respectively.

Immunogenicity of variant HPV31 L1 VLPs. HPV31 L1 VLPs bearing L1 variant residues T267A and T274N (Fig. 2A), here referred to as HPV31 A, HPV31 B, and HPV31 C VLPs, were expressed and used to immunize BALB/c mice. All three preparations contained VLPs of various sizes, ranging from ca. 20 nm to up to 60 nm in diameter, with VLPs of >40 nm in diameter constituting 30% of the HPV31 B VLP preparations, 37% of the HPV31 A VLP preparations, and 57% of the HPV31 C VLP preparations (Fig. 2C).

The three variant PsVs demonstrated differential susceptibility to neutralization by mouse polyclonal serum containing antibodies to HPV31 VLPs (Fig. 3). Both HPV31 B PsVs (median $\log_{10}$ neutralization titer, 4.27; IQR, 3.75 to 4.55; Wilcoxon paired signed-rank test, $P = 0.008$) and HPV31 C PsVs (median $\log_{10}$ neutralization titer, 4.18; IQR, 3.86 to 4.49; $P = 0.007$) were more sensitive to neutralization by sera containing antibodies to HPV31 A VLPs than the homologous HPV31 A PsVs were (median $\log_{10}$ neutralization titer, 4.09; IQR, 3.54 to 4.26). HPV31 A PsVs were less sensitive to neutralization by sera containing antibodies to HPV31 B VLPs (median $\log_{10}$ neutralization titer, 3.74; IQR, 3.53 to 4.07; $P = 0.031$), while HPV31 C PsVs demonstrated increased sensitivity (median $\log_{10}$ neutralization titer, 4.12; IQR, 3.96 to 4.53; $P = 0.028$) compared to that of HPV31 B PsVs (median $\log_{10}$ neutralization titer, 3.98; IQR, 3.69 to 4.22). Both HPV31 B and C PsVs had similar sensitivities to neutralization by sera containing antibodies to HPV31 C VLPs (for HPV31 B PsVs, median $\log_{10}$ neutralization titer, 4.42 [IQR, 4.04 to 4.35]; for HPV31 C PsVs, median $\log_{10}$ neutralization titer, 4.34 [IQR, 4.08 to 4.55]), while HPV31 A PsVs demonstrated a reduced sensitivity (median $\log_{10}$ neutralization titer, 4.01; IQR, 3.84 to 4.14; $P = 0.021$). There were also differences in the magnitude of the antibody response on the basis of reactivity against homologous PsVs, with HPV31 C VLPs (median $\log_{10}$ neutralization titer, 4.34; IQR, 4.08 to 4.55) being slightly more immunogenic than HPV31 A VLPs (median $\log_{10}$ neutralization titer, 4.09; IQR, 3.54 to 4.26; $P = 0.018$) and HPV31 B VLPs (median $\log_{10}$ neutralization titer, 3.98; IQR, 3.69 to 4.22; $P = 0.006$).

TABLE 2 Neutralization sensitivity of variant HPV31 L1L2 PsVs to serum antibodies induced by natural infection

Sample	Detected variant	Time point (mo)	Neutralization titer[a]		
			HPV31 A PsVs	HPV31 B PsVs	HPV31 C PsVs
P1	HPV31 A	0	3,987	11,084	5,591
		6	1,363	2,284	2,870
		12	1,144	2,057	1,895
		18	391	2,616	401
P2	HPV31 C	0	—	—	—
		6	148	113	104
		12	68	54	58
		18	106	106	93
P3	HPV31 C	0	—	—	—
		6	1,890	661	882
		12	637	587	542
		18	337	377	232
P4	HPV31 C	0	486	1,357	337
		6	549	727	893
		12	1,497	620	2,378
		18	NA	NA	NA
P5	HPV31 C	0	638	1,899	679
		6	837	667	2,193
		12	511	839	1,208
		18	576	982	1,235

[a] —, neutralization titers of <50 were assigned a value of 25 for calculation purposes; NA, not available.

Antigenicity of variant HPV31 L1 VLPs and L1L2 PsVs. HPV31 L1 MAbs against immunogens representing the HPV31 reference sequence (GenBank accession number J04353) were previously generated (39). The majority of MAbs were raised against L1L2 VLPs; the exceptions were MAbs 31.D24 and 31.A19, where L1 VLP immunogens were used. Generally, all the type-specific MAbs bound L1 VLPs and L1L2 PsVs representing the lineage variants A, B, and C at similar 50% binding concentrations (in micrograms per milliliter) by ELISA; the exceptions to this were MAb 31.F16, which demonstrated a higher binding concentration against the HPV31 B PsVs than the HPV31 A PsVs, while both MAb 31.H12 and MAb 31.H17 bound L1 VLPs representing HPV31 lineage variants B and C at lower concentrations than L1 VLPs representing HPV31 lineage variant A. Although the cross-reactive MAbs (MAbs 31.D24, 31.B5, 31.C19, and 31.E22) bound the L1 VLPs, they did not bind or neutralize the L1L2 PsVs (Table 3). All type-specific MAbs were able to neutralize the three variant PsVs to similar orders of magnitude (Table 3). HPV31 C PsVs demonstrated an increased sensitivity, ca. 3.5-fold, to neutralization by FG loop MAb 31.F16 and a ca. 4.0-fold increase in sensitivity to neutralization by MAb 31.H17 (epitope unknown) compared to the sensitivity of HPV31 A and B PsVs (Table 3).

The three neutralizing FG loop MAbs (MAbs 31.B1, 31.F16,

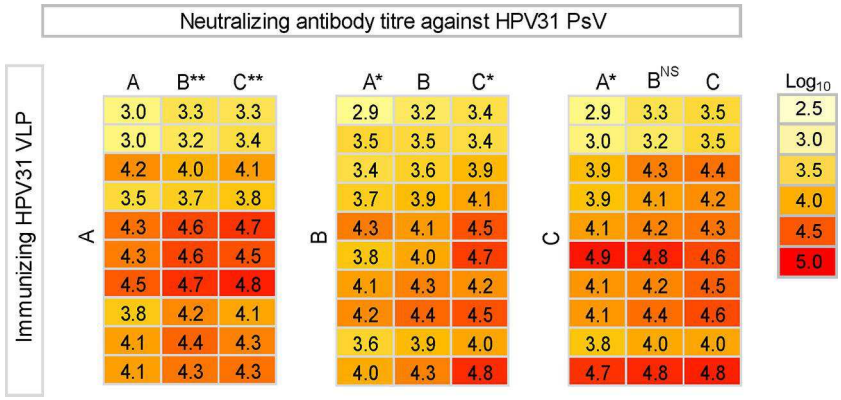


FIG 3 Heat maps representing the potential of serum from mice immunized with variant HPV31 L1 VLPs to neutralize variant HPV31 L1L2 PsVs. The $\log_{10}$ neutralization titers of sera from BALB/c mice ($n = 10$) following variant HPV31 VLP immunization carried out over three separate schedules are presented as the averages for two data sets per sample. The key on the right indicates the $\log_{10}$ heat map gradient. *P* values, obtained using the Wilcoxon paired signed-rank test, represent differences in median neutralization titers from homologous variant VLP and PsV pairs. *, $P < 0.05$; **, $P < 0.01$; NS, no significant difference ($P > 0.05$).

and 31.H12) recognize conformational epitopes which encompass variant amino acid position 267 and are adjacent to amino acid 274 (Fig. 4A). The FG loop of monomer 1 (FG_1) is adjacent to the BC_5, DE_1, DE_5, EF_1, HI_4, and HI_5 loops within the capsomer (Fig. 4B), and residues 267 and 274 are within close proximity (within 10 Å) to residue positions predominantly within the adjacent BC_5, FG_1, and HI_5 loops (Fig. 4C and D). These include lysine residues at position 279 within the FG loop and position 362 within the HI loop.

DISCUSSION

This study attempted to evaluate the potential impact of nonsynonymous SNPs within the HPV31 L1 and L2 genes on capsid protein antigenicity and immunogenicity. We generated additional HPV31 L1 and L2 sequences to supplement those already available and created L1L2 PsVs and L1 VLPs representing lineage variants A, B, and C to evaluate lineage-specific immunogenicity and antigenicity, including susceptibility to antibody-mediated neutralization.

All three variant L1L2 PsVs were susceptible to neutralization by vaccine-induced cross-neutralizing antibodies. HPV31 B and C PsVs demonstrated an increased sensitivity to neutralization compared to that of HPV31 A PsVs, but the difference was of a low magnitude. The cross-protection afforded by the current prophylactic vaccines is an unexpected additional benefit, although no correlate of protection has been defined (10). If cross-neutralizing antibodies are determined to be the immune effectors of vaccine-induced cross-protection, it is important to demonstrate that the contemporary circulating HPV31 variants, represented by L1L2 PsVs, do not exhibit resistance to cross-neutralization by such antibody specificities.

TABLE 3 Sensitivities of variant HPV31 L1 VLPs and L1L2 PsVs to MAbs[a]

					MAb IgG concn[b] (μg/ml)								
					L1 VLP ELISA			L1L2 PsV ELISA			L1L2 PsV neutralization		
Epitope	MAb	Structure	Specificity	Neutralizing	A	B	C	A	B	C	A	B	C
FG loop	31.B1	C	TS	Yes	1.01	0.49	0.53	4.48	4.85	3.78	9.48	7.17	6.34
	31.D24	L	XR	No	0.041	0.016	**0.013**	—	—	—	—	—	—
	31.F16	C	TS	Yes	0.050	0.038	0.035	0.0075	**0.11**	0.0061	0.31	0.35	**0.090**
	31.H12	C	TS	Yes	0.93	**0.28**	**0.29**	1.09	1.78	1.37	0.25	0.52	0.58
Unknown	31.A19	C	TS	Yes	1.63	1.89	1.57	1.65	4.27	3.28	3.71	3.59	1.44
	31.B5	L	XR	No	28	26	21	—	—	—	—	—	—
	31.C19	C	XR	No	25	15	25	—	—	—	—	—	—
	31.C24	C	TS	Yes	19	18	16	23	37	38	31	38	11
	31.E16	C	TS	Yes	12.00	5.99	7.28	7.70	8.16	12.00	79	104	66
	31.E22	L	XR	No	207	167	153	—	—	—	—	—	—
	31.H17	C	TS	Yes	0.26	**0.068**	**0.089**	0.37	0.72	0.41	0.204	0.156	**0.051**

[a] Epitope location, structure (C, conformational; L, linear), specificity (TS, type specific; XR, cross-reactive), and MAb neutralizing potential were taken from the work of Fleury et al. (39). —, MAbs for which binding or neutralization concentrations could not be determined at the highest input concentrations (by ELISA, 250 μg/ml for all MAbs except 31.D24, for which the highest input concentration was 20 μg/ml, and by neutralization assay, 125 μg/ml for all MAbs except 31.D24, for which the highest input concentration was 10 μg/ml). Concentration values in bold indicate a ≥3-fold difference from the concentration obtained for the antigen representing HPV31 variant A for a single MAb within an assay format.

[b] For the L1 VLP ELISA and L1L2 PsV VLP ELISA, the 50% binding concentration; for L1L2 PsV neutralization, the average 80% neutralization concentration. All results are averages from 2 to 3 experiments per assay format.

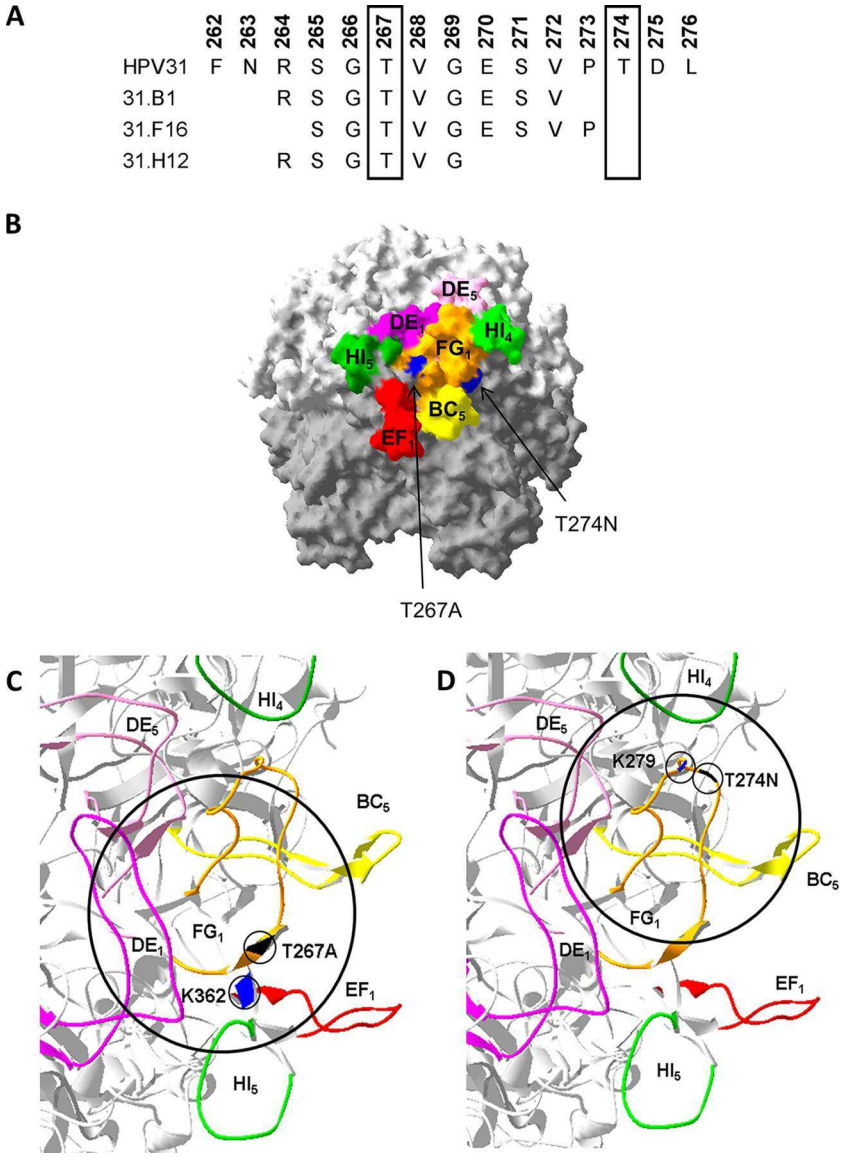


FIG 4 Crystal model surface highlighting HPV31 FG loop variant residue locations. (A) Linear amino acid epitope footprint of HPV31 FG loop MAbs. (B) Side view highlighting loops in close proximity to FG loop variant residues 267 and 274. (C and D) Top view of loop ribbons. Circled areas indicate regions within a 10-Å radius of residues 267 (C) and 274 (D), as determined by the Swiss-PDP viewer algorithm. Blue, lysine residues at positions 279 and 362; orange, FG loop of monomer 1 (FG_1); black, residues 267 and 274. Neighboring loops on the same monomer (dark pink, DE_1; red, EF_1) or adjacent monomers (dark green, HI_4; yellow, BC_5; light pink, DE_5; light green, HI_5) are indicated. The remaining surface-exposed regions of the capsomer are colored in light gray, and core regions are colored in dark gray.

The increased sensitivity of HPV31 B and C PsVs to cross-neutralization suggests that the Asn residue at position 274, which is common to both variants, enhances the recognition of HPV31 L1 epitopes by cross-neutralizing antibodies produced against vaccine HPV16 L1 VLPs. It is unlikely that the Asn residue in itself has a critical role within a cross-neutralizing epitope, since the switch from Thr and Asn is a relatively subtle change, as both amino acids have polar uncharged side chains. However, the change of residue at position 274, near the tip of the FG loop, may result in local structural changes which increase the level of recognition of more distal epitope residues.

Subtle differences in variant antigenicity were identified when the activities of a panel of HPV31 MAbs against the HPV31 lineage variants was tested in a neutralization assay. HPV31 C PsVs demonstrated increased sensitivity to neutralization by the FG loop MAb 31.F16 in comparison to that of both HPV31 A and B PsVs, indicating that the double residue switch at positions 267 (T267A) and 274 (T274N) impacts MAb 31.F16 epitope recognition. It has previously been demonstrated by comparison of L1 pentamer crystal structures from different genotypes (HPV11, HPV16, HPV18, and HPV35) that L1 antigenic determinants can be altered by a shift of a few angstroms within the loop as a result of a single residue substitution (28).

In contrast, the other two FG loop MAbs (MAbs 31.B1 and 31.H12) neutralized all variants to a similar extent. These data corroborate previous data from a bacterial cell surface display

model which demonstrated that these three MAbs recognize overlapping, yet distinct, FG loop epitopes (36). Residues 267 and 274 are in close proximity to the Lys279 and Lys362 residues or near the Lys54, Asn57, Lys60, and Lys367 residues. The corresponding residues of HPV16 are involved in HPV binding to heparin sulfate (50), which is an essential step for a successful HPV infection, and the FG loop MAbs may neutralize by abrogating this virus-host interaction.

The panel of HPV31 MAbs bound all three variant L1 VLPs, when used as target antigens in an ELISA format, indicating that the residues at positions 267 and 274 were not critical in the epitope footprints recognized by this panel of MAbs. However, when variant L1L2 PsVs were used as the target antigens, the 50% binding concentration of the four cross-reactive MAbs (MAbs 31.D24, 31.B5, 31.C19, and 31.E22) could not be determined due to the reduction in epitope recognition. This observation implies that inclusion of the L2 protein within the PsV capsid alters L1 epitope exposure and therefore impacts L1 protein antigenicity. It has been reported that a subset of HPV16 MAbs demonstrated reduced binding to L1L2 PsVs compared to L1 VLPs, with the differential binding being thought to be as a result of L2 altering the conformation or availability of L1 epitopes (51). These findings imply that the capsids of native HPV virions, represented by L1L2 PsVs and the L1-only VLPs within the prophylactic vaccine preparations, differ in their L1 antigenicity.

All three variant L1L2 PsVs were susceptible to neutralization by the HPV31 VLP antibodies generated in mice; however, differences in neutralization sensitivity were evident. Both HPV31 B and C PsVs demonstrated increased sensitivity to antibody-mediated neutralization in comparison to HPV31 A PsVs, irrespective of the variant L1 VLPs used as the immunogen. These findings are in line with those of a previous study of HPV16 variants which demonstrated that sera containing antibodies raised against an HPV16 European variant were able to neutralize pseudoviruses representing a range of geographical variants of HPV16, with a $\leq$4-fold difference in neutralization titer between the homologous and heterologous types being detected, leading to the conclusion that HPV16 variants belong to a single serotype (29).

The criterion used to designate serotypes is generally based upon a fold difference in antibody-mediated neutralization titers between viral types, which differ in magnitude and range between virus families: for adenovirus, 8- to 16-fold (52); for rotavirus, $\geq$20-fold (53); and for polyomavirus, 4- to 100-fold (54). For HPV there are no currently defined criteria with which to designate L1 serotypes. It is reasonably clear that HPV genotypes induce high-titer, type-specific neutralizing antibody responses which represent different serotypes (55–57). However, for lineage variants, the relationship between L1 sequence and antigenicity is less clear (29, 58).

Although HPV31 lineage variants demonstrated differences in susceptibility to neutralization by antibodies elicited by vaccination or preclinical L1 VLP immunization and MAbs, the difference was $<$4-fold, and under this criterion, as defined for HPV16 (29), HPV31 variants should be considered to belong to a single serotype. This implies that the choice of a representative HPV31 L1 sequence for VLP-based vaccines is not critical.

Given the relatively low prevalence of HPV31 (59), only a small panel of serum samples from HPV31 DNA-positive women was available. These data suggest that all three HPV31 lineage variants were susceptible to neutralization by antibodies derived from natural infection. However, further work will be required to address this issue appropriately by utilizing a larger panel of samples with an equal representation of variant lineages.

There are potential shortcomings to this work. L1 VLP immunizations were carried out using a relatively small number of animals, and while all three constructs induced neutralizing antibodies against all three variant PsVs, the variability inherent in using small groups of animals may have concealed subtle differences in immunogenicity. Although HPV L1L2 PsVs have been used widely to monitor antibody responses to vaccines and natural infection (22, 47, 48, 60), as well as elucidate steps in the entry process (61–64), there are likely to be some differences between how these behave *in vitro* and how authentic HPV31 lineage variants behave *in vivo*, although this is a limitation of most PsV-based systems.

Despite these caveats, these data suggest that HPV31 lineage variant PsVs display similar sensitivities to recognition by antibodies elicited following vaccination with the current HPV vaccines and after preclinical HPV31 L1 VLP immunization, indicating that HPV31 variants belong to a single L1 serotype. Such data may be useful to guide modeling of the impact of the current L1 VLP vaccines and informing postvaccination surveillance programs. These data also inform our understanding of the antigenicity of the HPV structural proteins.

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We declare no conflicts of interest.

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