Circulating HPV DNA as a Biomarker for Early Invasive Cervical Cancer

A thesis submitted for the degree of
Doctor of Medicine (MD)

By

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I, Dr Stacey Bryan confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

High risk HPV infection is responsible for >99% of cervix cancers. HPV enters epithelial cells and integrates with the host genome, causing viral infection and replication. In persistent infections that lead to cancer, the tumour breaches the basement membrane releasing HPV DNA into the bloodstream.

A next generation sequencing assay (NGS) for detection of plasma HPV circulating DNA (HPV cDNA) has been developed and demonstrates 88% sensitivity and 100% specificity in patients with locally advanced cervix cancers undergoing radical chemoradiation. We hypothesise that HPV cDNA is detectable in early invasive cervical cancers but not pre-invasive lesions.

We recruited two cohorts of patients: those with high-grade CIN and those with early invasive carcinoma of the cervix (1A-1B). A blood sample was taken prior to treatment and again at follow-up. DNA extraction from plasma followed by NGS were used for detection of HPV cDNA.

We recruited 52 patients, 40 (77%) with high grade lesions and 12 (23%) with early invasive tumours. None of the patients with pre-invasive lesions were positive for HPV cDNA. Two of the samples from the invasive cancers were found to be stage 2 at follow up and therefore excluded from the results. Of the remaining 10 invasive tumours, 1 (10%) reached the threshold of positivity for HPV cDNA in plasma. Re-calculating the thresholds for positivity did not increase the detection of HPV cDNA.

We have confirmed that HPV cDNA is absent in CIN. In early cervical tumours, there was low detection of HPV cDNA. This may be explained by small tumour size, poorer access to lymphatics and the circulation, and therefore little shedding of HPV cDNA in plasma at detectable levels. The detection rate of HPV cDNA in patients with early invasive cervix cancer using even the most sensitive of currently available technologies is sub optimal.

Impact Statement

Cervical cancer is a disease of younger women. It is the 14th most common cancer in women and people with a cervix, in the UK. The well-established cervical screening program allows detection of pre-invasive cells which can be treated before they progress to cancer. We know that HPV is responsible for the majority of cervical cancers and pre-invasive lesions and therefore its detection and eradication is central to treatment. Whilst the introduction of HPV vaccination gives hope that this disease will be eradicated within the next 10 years it is still important that women engage with the cervical screening program as well as follow up after treatment of pre-invasive disease and invasive cancers. There are many barriers to access to cervical screening including physical access issues, ethnicity and language barriers, lack of knowledge and fear and anxiety surrounding the examination and results. Every year almost 200,000 women are referred to colposcopy services with abnormal smears or symptoms of cervical disease, with only a very small proportion being diagnosed with cervical cancer. It is important that we try to improve access to, and education surrounding, detection, management and follow up of cervical diseases.

There has been much work on improving uptake for cervical screening, and with the introduction of HPV testing, discussions surrounding non-invasive testing have come to the forefront, including self-swabs and urine tests for HPV. There has also been some work surrounding early detection and follow up of treated cervical disease with circulating DNA playing a

promising role. However, these studies have a tendency to include advanced cancer rather than early invasive cancers.

This project has looked into the use of plasma HPV DNA testing in early cervical cancers, and although does not avoid cervical assessment, may go some way to providing an alternative to investigating pre-invasive and invasive cervical diseases. In an academia, this project has contributed an early invasive cervical cancer cohort to aid in the validation of a next generation sequencing assay for circulating HPV, which has shown high sensitivity and specificity in advanced cervical cancers. If the sensitivity and specificity of this test can be replicated or even improved, then cHPV DNA can be used to improve diagnosis, guide treatment, improve compliance and enhance monitoring of cervical disease.

This study has shown a high specificity for cHPV DNA which suggests that it can be used to rule out cancer in those patients who present with cervical abnormalities. In a clinical setting this may translate to its use as a triage tool to reduce referrals to colposcopy units, and particularly if turnaround times can be reduced, allaying anxieties in waiting for results from cervical cytology. In the future and after further studies, a blood test for HPV may be more acceptable than a speculum examination and therefore help to improve compliance - whether that be seeking initial medical advice when presenting with symptoms or follow up of treated disease - cHPV DNA may prove to be useful in both settings.

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Acknowledgements

The work for this thesis would not be possible without the teams at both UCLH, UCL and The Institute of Cancer Research.

I would like to thank Dr Shree Bhide who was pivotal to the collaboration between the ICR and UCLH. I am indebted to my supervisors Miss Adeola Olaitan, Prof Martin Widschwendter, and Mr Adam Rosenthal for their guidance and support in the execution of this study, both clinically and in supervising this thesis. The clinical work and recruitment of patients took place in the Gynaecology Oncology clinic at UCLH, and the Colposcopy clinic, and I thank the respective teams for signposting and allowing me access to patients for this study.

I am grateful to Professor Widschwendter and his laboratory team at the Translational Research Lab, UCL - Allison Jones and Richard Gunu, for carrying out the initial plasma extraction and storage from patient samples. Without their patience, understanding and willingness to help, this project would not have been possible. The rest of the laboratory work was performed by me at the Institute of Cancer research under the supervision of Jen Lee using the Targeted therapy Team lab and I would therefore like to express my thanks to Jen, Professor Kevin Harrington, and Isaac Garcia-Murillas for their technical support. In addition, I am grateful to the Tumour Profiling Unit, and to Ros Cutts who performed the subsequent statistical analysis for this study.

I would like to thank the patients who agreed to take part in this study, for their time and co-operation during what was most likely an anxious time for them.

Lastly, I am grateful to my family for their support, understanding and encouragement in completing this research.

Chapter 1 BACKGROUND

Worldwide the prevalence of cervical cancer in 2018 was 570,000, with 311,000 deaths occurring in the same year due to disease [1]. It was the fourth most common cause of cancer in women worldwide with an incidence of 13.1 per 100,000 globally. It was the leading cause of cancer related death in all parts of Africa accept the North in 2018, as well as 42 other lower resource countries. China and India made up more than 30% of the global burden of cases in the same year. The average age of death from cervical cancer globally is 59 years [1]. The variation in cervical cancer incidence and survival across the globe is thought to be related to inequalities in access to healthcare i.e., primary and secondary prevention of human papillomavirus (HPV) infection, adequate screening programs and high-quality treatment facilities [1].

In the UK, cervical cancer is the 14th most common cancer in females. In 2016 – 2018 there were 3200 new cases in the UK, with around 850 deaths from cervical cancer within the same time period. The highest incidence occurs in the 30–34-year age group and in those from deprived areas - with an approximate 65% higher incidence in the most deprived quintile compared to the least deprived in England. A woman in the UK has a lifetime risk of 1 in 142 of developing cervical cancer. Overall five-year survival is approximately 61% but dependent on stage at diagnosis, with 96% of women surviving the disease for a year or more if diagnosed early, versus 50% who are diagnosed at a later stage [2]. Equally, age at diagnosis plays a role, with around 90% of women diagnosed between ages

15-39 surviving for 5 years or more, compared to 25% of those aged 80 or over.

The cervical cancer five-year survival rates in England, Scotland and Wales are slightly lower than that of the European average (60% vs 62%) with Norway having the highest survival rate at 71%. There have been postulations with regards to the cause of this, including, socioeconomic status and sexual behaviours, access to screening (both organised and opportunistic) and diagnosis with subsequent high-quality care, differences in cancer biology, stage at diagnosis, and data collection and recording – with some places in Europe recording deaths from cervical cancer as uterine corpus or unspecified uterine cancer [3] [4] [5].

1.1 Risk factors

Cancer Research UK (CRUK) have posited that 99.8% of cervical cancer cases in the UK are preventable [2] [6]. Risk factors for cervical cancer are multiple. Green *et al* conducted a UK national case-control study of cervical cancer in women aged 20-44 years to identify the prevalent risk factors [7]. This included increasing lifetime number of sexual partners (>5 carrying a significant risk compared to one partner). Women who experienced an earlier age at first intercourse (<17yrs vs >20yrs) had a 2-3-fold higher risk of developing cervical cancer. Similarly, those who gave birth at an earlier age (<19yrs) had an increased risk, in addition, women with high parity (7 or more full term pregnancies) had a 4-fold increased risk of developing cervical cancer in comparison to women who were nulliparous or had low parity [8]. Some suggestions for the cause of this include the exposure to

oestrogens and progestogens in pregnancy, coupled with the related cervical trauma caused by childbirth, leading to cervical ectopy and therefore greater susceptibility to infections. In addition, the relative immunodepression of pregnancy would render the immune system inadequate to clear any infections [9].

It has been described that male sexual behaviours also plays a role in the development of cervical cancer in their partners, with an increased lifetime number of sexual partners, and participating in sexual intercourse with sex workers, being a key determinant [10] [11].

Long term use of oral contraceptives (OC) is also associated with almost twice the increased odds of developing cervical cancer compared to those who had never used contraceptives. This odds risk increases to almost 3 times for 5-9 years of use and 4.5 times for over 10 years use but reverts to average after cessation of oral contraceptive of 5-10 years [8]. The combination of the use of OC for longer than 5 years and having had more than 5 full term pregnancies, increases the risk by 11-fold [8].

Smoking significantly increases the risk of squamous cell cancer (but not adenocarcinoma), by approximately 2-fold - with a significant dose response - when compared to women who have never smoked [7] [8]. Women who are long-term smokers (> 20yrs), smoke at a high intensity or are recent or current smokers, have the highest risk of developing cervical cancer [7] [12]. It is hypothesised that tobacco and its metabolites cause a direct carcinogenic effect and an indirect immunosuppressive effect which would put a woman at increased risk of cervical cancer [8].

Cervical cancer is recognised as one of the AIDS defining illnesses in HIV positive women [8]. HIV and specifically immunocompromise, reduces the immune system's ability to fight pre-cancerous cells [13]. Immunosuppression, as measured by low CD4 count in HIV positive patients, appears to have a greater correlation with cervical (and anal) pre-invasive disease, with a faster progression to high grade disease. This correlation using CD4 count is stronger than that of viral load and does not seem to apply to cervical cancer, with other factors such as genetic changes having a greater influence on progression from high grade disease to cancer [14] [15]. In developing countries, the rate of cervical cancer is higher in HIV negative women than HIV positive women which may reflect the close monitoring of HIV positive women vs lack of screening to detect pre-invasive disease in asymptomatic HIV negative women [14].

Co-existing, sexually transmitted infections, namely *Chlamydia Trachomatis* and Herpes Simplex Virus type 2, have been said to confer a two-fold increased risk of squamous cell carcinomas and adenocarcinomas of the cervix, respectively. This is thought to be due to the cervical inflammation which occurs during these infections and therefore increasing the risk of development and progression of pre-invasive disease [8].

There was no association found between weight and cervical cancer risk [7]. Garcia- Closas *et al* attempted to identify dietary components which may have a protective effect against cervical cancer and identified diets rich in Vitamin C, Vitamin E, folate, retinol, and B12 as well as diets rich in fruit and vegetables, as possible protective factors [16]. This was supported by

Koshiyama, with the explanation that fruit, vegetables and vitamins act as antioxidants which prevent DNA damage and reduce the proliferation of cancer cells [17]. The evidence, however, was not convincing and required further larger studies.

Professor Harald zur Hausen was awarded the Nobel prize in Physiology or Medicine in 2008 for discovering the role between human papillomavirus (HPV) and the development of cervical cancer. It was in fact between 1974 and 1976 that the link between HPV infection and cervical cancer was made, with isolation and cloning of HPV 16 and HPV 18 by zur Hausen and his team in 1983 and 1984 respectively [18] [19] [20]. In the first study, it was demonstrated that 60% of cervical tumour samples from German patients contained HPV 16 DNA, compared to only 6% in genital warts – which had an abundance of HPV 6 or 11 [19]. In the second study it was shown that 15% of cervical tumours from German patients and 25% of tumours from Kenyan, Brazilian, and Ugandan patients, contained HPV18 DNA – with absence of HPV DNA in dysplasia, genital warts and normal cervical tissue [20]. The multiple studies performed subsequent to these findings have shown that high-risk human papillomavirus (HRHPV) is detected in 99.7% of cervical cancers, and has consequently been listed as a carcinogen by the International Agency for Research on Cancer (IARC) [2] [21].

1.2 Human Papillomavirus

HPV is a member of the *Papillomaviridae* family which is divided into 39 genera [22]. So far there have been 205 different types of HPV that have

been classified. They comprise of five evolutionary groups – *Alphapapillomaviruses*, *Betapapillomaviruses*, *Gammapapillomaviruses*, *Nupapillomaviruses* and *Mupapillomavirus*, with the largest group being the *Gammapapillomaviruses* [22] [23]. The *alpha-papillomaviruses* include the low-risk mucosal types responsible for genital warts or oral focal epithelial hyperplasia [22] (HPV 2, 4, 6, 11) and the high-risk mucosal types which cause cervical neoplasia and cancers (HPV 16 and 18). The difference in infectivity of the various HPV types is thought to be due to the different strategies of transmission and subsequent interaction with the immune system [23]. The first HPV types to be associated with cancer were HPV 5 and 8 (of the beta group) found in those with warts and those diagnosed with Epidermodysplasia verruciformis – a rare, genetic lifelong dermatological disorder, characterised by the development of widespread flat, wart-like lesions which develop in childhood and can undergo malignant progression [22] [24]. Cutaneous lesions caused by low-risk alpha, beta, gamma, and mu subtypes may be particularly difficult to manage in patients who are immunocompromised and can therefore lead to papillomatosis and some cancers i.e., non-melanoma skin cancer [23].

The World Health Organisation (WHO) have recognised 12 high-risk cancer-causing HPV subtypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59) with additional possible cancer-causing types (68 and 73). These are not only associated with cervical cancer, but also anal (95%), penile (35%), vaginal (60%), vulval (50%), tonsillar/oropharyngeal and base of tongue cancers (70%) – with HPV 16 being the most prevalent subtype [22]. HPV has an affinity for squamous epithelium. The cervical and anal

transformation zones are most at risk. Infectivity does not equate to disease in the vast majority of individuals [25].

The structure of HPV is that of a non-enveloped icosahedral capsid containing a molecule of double stranded circular DNA. Although this DNA contains 7906 base pairs, only a single strand of the genome is used for transcription. There are 3 genomic regions with 10 open reading frames (ORFs) within the HPV DNA coding strand. The early regions (E) contain seven ORFs (E1, E2, E4, E5, E6, E7, E8) and the late regions (L) encode for the two viral capsid proteins (L1 and L2). The third genomic region contains sequences which control viral replication and transcription and is known as either the long central region (LCR), the upstream regulatory region (URR) or the noncoding region (NCR) [22].

Each ORF plays a key role in the infectivity of the virus. E1 is known as the ATPase -dependent origin binding helicase involved in replication of the genome. E2 regulates transcription and is associated with E1. E4 acts as a fusion protein with E1 causing destabilisation of the cytokeratin network and therefore aiding virus release and transmission. E5 is a transmembrane protein which activates tyrosine kinase receptors including epidermal growth factor (EGF) and platelet derived growth factor (PDGF) receptors. It also plays a role in apoptosis and immune evasion. E6 and E7 collectively drive genome amplification in upper epithelial layers via cell cycle entry. E8 is part of a fusion protein with E2 and acts to repress transcription and replication during the viral life cycle [22].

High risk HPV can persist for many years and drive cell proliferation at the basal and parabasal cell layers. The transformation zone of the cervix is a site of increased cellular metaplasia allowing easy access to basal cells. The viral L1 protein is the primary structural protein whilst the L2 protein is a minor component [26]. Infection occurs when there has been damage to the epithelial layer, through disruption to skin and/or mucosa. Binding of HPV capsids to heparin sulphate proteoglycans (HSPGs) in the cell surface is predominantly mediated by the L1 protein [22] [26] [27]. This interaction leads to conformational changes in L1 and L2 proteins with the critical cleavage of L2 by furin convertase causing internalisation of the viral genome to the host cytoplasm. Studies have shown that the additional extracellular matrix (ECM) protein laminin 332, plays an important role as an attachment receptor for HPV 11 and 16 but not HPV18 [27]. Once the viral genome is internalised, uncoating of the capsid occurs and L1 is released from L2 protein with the majority degraded by lysosomes. The L2/viral genome complex remains and evades lysosomal degradation by L2s ability to penetrate the host intracellular membranes and become a transmembranous protein which interacts with factors within the cytosol, required for genome transfer. In this aspect, the L2 protein is crucial for infection [27]. It is then stored as stable episomes [26] causing infection of the dividing basal epithelial cells, which consists of stem cells [22]. The internalisation of the capsids is asynchronous and can take between two to four hours, and a delay of one to three days can occur between this process and viral transcription [22]. HPV genomes replicate during S phase of the cell cycle and it is in this latent phase that the virus has the ability to persist

for years to decades in the basal layer of the epithelial cells, evading the immune system [22]. The viral replication proteins E1 and E2 play an important role in viral DNA replication, acting as transcription factors and regulators of the expression of the oncogenes E6 and E7 using host DNA synthesis and replication machinery [22]. The mechanism by which high risk HPV is thought to cause cancer is by dysregulation of the cell cycle via its E6 and E7 oncoproteins. The E7 protein targets the retinoblastoma tumour suppressor (pRB) causing degradation and avoiding G1 cell cycle arrest which gives a net effect of cell proliferation [22]. The E6 protein inactivates the tumour suppressor TP53, preventing apoptosis and leading to hyperproliferation of host cells [28]. With the inactivation of pRB the tumour suppressor p16 (cyclin dependent kinase inhibitor), is no longer inhibited, increasing its expression which can be detected by immunohistochemistry. This phenomenon can be used as a marker for HRHPV infection [29]. Low risk HPV and beta HPV types do not bind TP53 or target the same pathways as the high-risk HPV types and therefore do not have the same infectivity potential [22]. The increased genetic error in the infected cell makes it easier for integration of virus into the host chromosomes [23]. After viral genome replication and amplification, cell cycle exit must occur [22]. Infectious particles are created with accumulation of the capsid proteins L1 and L2 and assembly of virions in the upper epithelial layers [26].

It is believed that the levels and therefore activity of E6 and E7 increase the greater the degree of cervical intraepithelial neoplasia i.e., CIN 1 to CIN 3 [22]. As HRHPV triggers genomic instability via the expression of E6 and E7, it is plausible that the higher the degree of CIN, the higher the likelihood

of progression to malignancy [22]. CIN1 has a low level of basal cell proliferation, with the HPV life cycle largely being kept intact. The low activity of E6 and E7 in these lesions confers insufficient compromise of cellular function to facilitate progression to cancer [30]. These lesions are often flat and can resemble warts.

The genetic changes that occur in CIN2+ can be brought about by hormonal changes or viral epigenetic modifications [23]. Oestrogen increases the rate of cell replication in the S phase of the DNA cycle. It also causes proliferation of ectocervical squamous epithelial cells, but not endocervical cells, whilst progesterone increases the proliferation of endocervical columnar epithelial and reserve cells. Any defects on the cells of the ectocervix becomes re-epithelialized under the influence of oestrogen. This process lends itself to the development of precancerous lesions in a small number of cases [31].

Cytosine bases that are located 5' to a guanosine base, form CpG dinucleotides. Short regions of these are known as CpG islands and are underrepresented in the genome. In normal mammalian cells the CpG islands are unmethylated and found at the proximal promoter regions of the genes. Various studies have now shown that hypermethylation of these promoter regions is found in tumours and is at least as common as the classic mutation in tumour suppressor genes [32]. DNA methylation has the ability to silence tumour suppressor genes whilst maintaining their stability during cell division [33]. Furthermore, a tumour suppressor gene may have one stably mutated allele and one hypermethylated allele which

cumulatively causes inactivation of that gene. Methylated CpGs also act as preferred binding sites for carcinogens such as those found in tobacco smoke [32] therefore perpetuating cell damage.

In the context of cervical cancer, methylation of p16 can cause overexpression, which is necessary for the survival of HPV infected cells which express E7 [34]. The hypermethylation of other genes such as FHIT (fragile histidine triad gene), CCNA1 (Cyclin A1), DAPK1, and the Ras family – all important for cell cycle regulation, apoptosis and repair – has also been implicated in the development of cervical cancer with the degree of methylation directly correlated to the progression of disease [34].

1.2.1 HPV transmission and infection

Transmission of HPV is largely via sexual activity and in particular skin to skin contact, however there are other documented methods of transmission. A recent review paper suggests that HPV transmission can be vertical i.e., from mother to baby [35]. Some studies in the review have even suggested that infection can occur at the time of fertilisation, and also during the pregnancy via the placenta or 'micro tears' in the fetal membrane. It has been well established that women with a history of genital warts in pregnancy can pass on the virus at the time of vaginal delivery due to exposure of the fetus to the genital tract. This can then manifest as laryngeal papillomatosis in the newborn, a benign neoplasm caused by HPV 6 and 11. It is estimated that the concordance of HPV infection between mother and child is 39% [35]. Vertically acquired HPV infection in newborn babies usually resolves within 6 months to 2 years [36] [37]. There has

been no correlation between breast feeding and transmission through breast milk. Autoinoculation with low-risk HPV, can also occur with finger-to-genital contact such as when changing nappies and with oral contact such as kissing by family members.

HPV can be transmitted via fomites, although its role in the development of active infection is not well established. It has been shown that HPV can be detected on transvaginal ultrasound probes and colposcopes, despite sterilisation, and therefore this is a potential transmission route [35].

HPV infection is largely asymptomatic and as much as 80% of sexually active adults show evidence of HPV infection. Approximately 80% of these infections will spontaneously regress within 18 months, with reinfection by the same HPV type being uncommon [26]. Infection of host with HPV causes recruitment of dendritic and Langerhans cells which present antigen to CD4+ and CD8+ T cells. This activation of CD4+ and CD8+ cells produce a cascade of responses including cytokine production which helps to clear HPV infection [38]. As there is no viraemia, no viral cell death, and shedding of virus occurs at the surface of the epithelium where there is poor lymphatic and vascular supply; the humoral response to HPV infection is weak [39]. Thus, failure to mount an effective cell mediated immune response leads to persistence of infection [23]. Clearance is therefore significantly impaired in women with HIV/AIDS, or those who are immunosuppressed such as renal transplant patients [13]. Total clearance of HPV was higher in those over 30 years whilst persistence of infection occurred more frequently in women less than 30 years [40]. Less than 10% of persistent HPV infections

progress to carcinoma in situ, although persistent infection is a strong risk factor for the development of cancer. It can take over 15 years for cancer to develop in women with normal immune systems, (5-10 years in those with a weakened immune system) [25].

As previously stated, the life cycle of the virus is wholly intraepithelial with no systemic shedding and therefore no associated inflammation or symptoms. In this manner, it is typical for HPV infection to evade recognition by the innate immune system, leading to persistence of infection. HPV 16 has a longer length of persistence in comparison to other HPV types, which may account for its increased cancer risk [23]. It is frequently associated with squamous cell carcinoma of the cervix (up to 63%) whilst HPV 18 (and 45) is associated with adenocarcinoma of the cervix (37-41%) [26].

1.3 HPV associated Head and Neck Cancers

Head and neck squamous cell cancers (HNSCC) have a worldwide incidence of approximately 600,000 per year with a 40-50% mortality [41]. They originate from epithelial cells of the oropharynx, larynx, and hypopharynx. The most common risk factors are smoking and alcohol use, however, an increasing subset of tumours caused by HPV has emerged particularly in the Western world [42]. HPV related HNSCC seems to have an affinity for the oropharynx - tonsils and base of tongue in particular - with HPV 16 being identified as the most common subtype found in up to 90% of oropharyngeal squamous cell carcinoma (OPSCC) [42]. HPV positive HNSCC have a more favoured outcome than HPV negative HNSCC and

the TNM staging has been changed in 2017 to reflect this, with the addition of testing for p16 [41].

In the United States it is estimated that approximately 7% of the population have oral HPV infection with 1% of these being HRHPV subtypes. Infection alone is asymptomatic, and usually cleared within 2 years, however persistence occurs with smoking. Similar risks factors of increased sexual partners and in particular oral sex activity, increases the risk of infection. There is currently no effective screening for HPV associated OPSCC partly due to the lack of a pre-invasive phase and also due to lack of easy access to cells – with OPSCC arising deep in the tonsillar crypts [42].

There is a paucity of studies on the pathogenesis of HPV driven OPSCC. The tonsillar crypts are made up of reticulated lymphoepithelium which expresses PDL-1, a protein which regulates and thereby supresses T-cell responses to HPV infection. This provides an ideal site for viral infection [42]. In a similar fashion to cervical HPV infection, a break in the epithelium – often caused by chronic inflammation – gives the opportunity for HPV to gain access to the basal cells [43].

Delay in diagnosis can occur as tumours are often small and asymptomatic, surrounded by an abundance of tonsillar tissue and therefore difficult to detect on examination. In those who develop enlarged cervical lymph nodes, these are often cystic and fine needle aspiration can be non-diagnostic. An ultrasound guided aspiration with p16 immunohistochemistry may increase the detection and raise suspicion of a tumour, with CT/MRI and PET being imaging modalities that are helpful in

localising tumour. Excision of the lesion i.e., tonsillectomy, is preferable over multiple biopsies. The histology of HPV related OSCC tends to be a poorly differentiated, non-keratinizing basaloid type with an abundance of lymphocytes [42].

Treatment includes surgery, radiotherapy +/- chemotherapy and is aimed at reducing locoregional spread. Ideally single modality treatment is offered in HPV related OSCC to minimise toxicity, although combination therapies are advised for advanced disease. In approximately 10-25% of patients, disease progression occurs within 3 years, with the majority locoregional recurrences in the first-year post treatment. The lung is the most common site of distal relapse. In either instance of recurrence, the presence of p16 still confers a good prognosis compared to its absence [42].

1.4 Cervical Screening

The cervical screening programme is a population-based programme which was officially started in England in 1988 and has been estimated to have prevented up to 70% of cervical cancer deaths since implementation; projected 82.9% if everyone eligible for screening in England attended [44]. The aim of the cervical screening programme is to reduce the incidence and mortality from cervical cancer. Currently the screening protocol for women in England is that they are invited into the programme at the age of 24.5 such that by the age of 25 they have undertaken their first smear. Between the ages of 24.5 to 49 screening occurs every 3 years. From the age of 50 to 64 this increases to every 5 years, stopping at 64 unless a prior test had been reported as abnormal. Liquid based cytology has previously been the

standard for cervical screening as it has been shown to be more sensitive and reduce the number of inadequate smears in comparison to the older Papanicolaou tests [45]. However primary HPV screening has now been shown to be more sensitive than liquid-based cytology at detecting pre-invasive diseases (40% greater for CIN3) and cervical cancer (30% greater), with a resultant reduction in adeno- and squamous carcinomas [46]. As such, since January 2016 it has been recommended by the UK National Screening Committee that primary HRHPV screening should be rolled out nationally.

As HPV testing confers advanced sensitivity, it has been suggested that the screening intervals can be safely extended from 3 to 5 years in those who are HRHPV negative, and particularly those who have been vaccinated [47]. Although the sensitivity of the test is high in comparison to cytology, the specificity is low due to the high prevalence of HRHPV. This means that any positive HRHPV results will be reflex tested with liquid-based cytology and the current guidelines have been changed to reflect this. [47]

Cytology describes the presence of dyskaryotic cells with raised nuclear: cytoplasmic diameter ratios, the degree of which provides a classification of the grade. Cells with a nuclear: cytoplasmic ratio of less than 50% are classified as low grade dyskaryosis, whilst those with a ratio above 50% are classified as high grade dyskaryosis. Where dyskaryosis cannot be identified with certainty, this is classified as borderline change in squamous or endocervical cells - which may be due to inflammation, hormonal or a metaplastic process. Samples reported as ?glandular neoplasia of the

endocervical type suggest features in keeping with cervical glandular intraepithelial neoplasia (CGIN) or endocervical adenocarcinoma – often with overlapping features. Lastly samples which are reported as ?glandular neoplasia of non-cervical type are often an incidental finding of a non-cervical cancer and should prompt referral to a gynaecologist rather than colposcopy. HRHPV is also used as a test of cure for those women who have undergone treatment for pre-invasive lesions [47].

1.4.1 Management post screening

Women with negative HRHPV tests are returned to routine recall with the interval for screening determined by age as outlined above.

Women with positive HRHPV tests undergo triage via cytology with abnormal cytology prompting a referral to colposcopy. Those with negative cytology are recalled for a repeat HRHPV test at 12 months. Those with persistent HRHPV but with negative cytology should have a repeat test at 24 months. At this time if the HRHPV is negative they are returned to routine recall, however if it is positive then cytology is performed and referral to colposcopy should be made [47].

1.5 Pre-invasive Disease

Cervical Intraepithelial Neoplasia (CIN) refers to dysplasia at the transformation zone of the cervix as a result of infection by HPV. The pathological identification of CIN relies on the presence of nuclear abnormalities throughout the epithelium, with assessment of the extent and location of cytoplasmic maturation. CIN 1 describes a full thickness nuclear

abnormality of the epithelium with atypical nuclei prominent in the basal third, and cytoplasmic maturation confined to the upper one third of the epithelium [48]. CIN 1 also typically shows koilocytosis – where the epithelial cells take on an irregular size, shape, and colour which is pathognomic of HPV infection [49]. CIN 2 is a continuum of CIN 1 and describes atypical nuclei largely throughout the epithelium, and cytoplasmic maturation confined to the upper third of the epithelium, with increasing mitotic figures. When cytoplasmic maturation is absent and nuclear atypia is severe and present throughout the full thickness of the epithelium – but with an intact basement membrane - then this is termed as CIN 3 [48].

In 2012 the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology, developed a consensus group – The Lower Anogenital Squamous Terminology (LAST) Project – in order to unify the terminology used to describe HPV related squamous lesions of the lower genital tract [50]. The new terminology is based on the knowledge that HPV interacts with the squamous epithelium to cause either a transient infection (low grade squamous intraepithelial lesion [LSIL]), or a persistent infection where viral oncogenes drive cell proliferation (high grade squamous intraepithelial lesion [HSIL]). LSIL encompasses CIN 1 and HSIL encompasses CIN2/3. If HSIL is left untreated then approximately 30% - 50% will progress to invasive cancer within 30 years. If LSIL is left untreated then less than 10% will progress to invasive cancer [51], over 50% will regress over 22 months [47]. Treatment of high-grade lesions is usually surgical and involves a large loop excision of the transformation zone (LLETZ), knife cone biopsy or laser conisation, under local anaesthetic if

tolerated. Alternatives include cryotherapy, laser ablation, cold coagulation, and radical diathermy. Women who are treated for high grade lesions are 2-5 times more likely to develop cervical cancer compared to the general population and therefore follow up is important. The current NHS screening programme utilises HPV test of cure at 6 months, to ensure that treatment removes HPV thereby reducing the risk of recurrence and progression to cancer [47]. In those who are HRHPV negative following treatment, repeat screening is performed at 3 years (12 months if treatment was for CGIN). In those who are HRHPV positive following treatment, they are referred back to colposcopy for further management which is determined by the type of lesion and completeness of excision.

In 2015-2016, 188,179 women in the UK were referred to colposcopy clinics either due to abnormal screening results or clinical indications i.e., symptoms of cervical disease. A small proportion (approx. 1.7%) were diagnosed with invasive cervical cancer, with the majority being diagnosed with CIN 3 (44.8%) [52].

1.6 Cervical Cancer

Cervical cancer usually arises from pre-invasive disease and is most commonly squamous cell carcinoma (70-80%). The rest are rarer tumour types such as adenocarcinomas (25%), adenosquamous carcinomas, and neuroendocrine tumours (5%) – the latter occurring in younger patients. Adenocarcinomas have a poor prognosis as they arise from the endocervical canal where the efficacy of cervical cytology is less and

therefore diagnosis occurs later. They are more aggressive and tend to be associated with HPV 18 and a younger age demographic [53].

Early cervical cancer could be completely asymptomatic i.e., discovered on a LLETZ sample prompted by a routine smear test, or may present with irregular vaginal bleeding, and/or a suspicious appearance to the cervix. In more advanced disease other symptoms may include an offensive discharge, backache, leg pain, haematuria, bowel changes, weight loss and even acute renal failure. Diagnosis is usually by biopsy, with MRI, CT and /or PET scans used to assess the extent of local spread and lymph node involvement [54]. An examination under anaesthesia including cystoscopy, and sigmoidoscopy can also add further information to aid clinical staging of the disease.

1.6.1 Management

Management of cervical cancer is dependent on stage at diagnosis (Table 1), the presence or absence of lymphovascular space invasion (LVSI), and increasingly, the desire for fertility. Early invasive disease (1A1) can be treated with LLETZ or cone biopsy alone - if clear margins are obtained and there is no LVSI. If margins are involved, then a repeat cone could be performed if there is a desire for fertility. If a woman has completed her family, then a hysterectomy could be recommended, with pelvic lymphadenectomy if LVSI present [54]. For those with 1A2/1B disease who would like to preserve their fertility, then a radical trachelectomy with pelvic lymph node sampling can be performed, as there is a small risk of lymph node metastases. In stage 1B to 2 tumours, the size of the lesion, lymph

node positivity and local spread are high risk factors which places a patient at risk of recurrence [54]. The smaller tumours with minimal spread within this group should be treated by radical hysterectomy with pelvic lymph node dissection. The ovaries can be spared in premenopausal women as spread to the ovaries is rare. In those tumours which are larger (>4cm) and/or LVSI present, adjuvant pelvic radiotherapy is recommended and has been shown to improve progression free survival [55]. Radiotherapy can be given as either external beam therapy or brachytherapy and may also be given in conjunction with chemotherapy as the primary treatment for stage 3-4 tumours. Radiation may also be used in the palliative setting particularly where there is pelvic pain, vaginal bleeding, and discharge in advanced disease [54].

In addition to surgery and chemo/radiotherapy, antiviral and immune modulatory agents have been investigated as a treatment of cancer. Cidofovir is an antiviral traditionally used in the treatment of CMV infections and has been shown to inhibit HPV infected cell proliferation in in vitro studies [56]. Podophyllin is a cytotoxic agent usually used to treat genital warts. It has also been shown in studies to sensitise HPV infected cells to apoptosis [56].

The 5-year survival of stage 1 disease is approximately 95%, declining to 5% for stage 4 disease. The majority of patients present at stage 1 (47%) with 4% presenting at stage 4 [2].

1.7 HPV Vaccine

It has already been described that the natural immune response to HPV infection is cell mediated. In animal studies, this is closely followed by seroconversion and production of antibodies to L1 protein, however, the concentrations are low and not every woman will seroconvert. Due to the absence of viraemia in natural infections, the virus particles that are shed from the squamous epithelia have poor access to the vascular and lymphatic system where usually immune responses are initiated. The time taken for seroconversion is thus typically 9 months after first detection of HPV DNA on smear [39].

Over 70 years ago, studies in rabbits had shown that serum neutralising antibodies to virus capsid protein is effective for prophylaxis [57]. The same theory was used to develop the HPV vaccine. The prophylactic HPV vaccine was developed in 2006 to prevent primary infections with the oncogenic HPV subtypes. These vaccines are composed of recombinant HPV L1 capsid proteins that assemble into virus like particles (VLPs) and induce high level production of B cell mediated neutralizing antibodies [22]. The vaccine is delivered intramuscularly which results in rapid access to local lymph nodes and activation of both innate and adaptive immune responses [39]. This potent and sustained immunological response post vaccination leads to high clinical protection, close to 100% in those with no prior exposure to HPV [13].

The mechanism by which the HPV vaccine is effective against a wholly intraepithelial virus has been explored. The squamo-columnar junction of

the cervix is bathed in cervical mucous secretions which contains antibody. It could therefore be argued that surface neutralisation by antibodies in these secretions could be one mechanism of protection. Another hypothesis is that of microabrasion of the epithelium – resulting in full thickness loss of the epithelium but with an intact basement membrane – induces serous exudation into the now wound bed which is rich in serum proteins, phagocytes, IgG and B cells. This therefore prevents virus entry into the cell basement membrane [39].

Gardasil was the first vaccine to be approved – with protection against the LRHPV 6 and 11 as well as the HRHPV 16 and 18. Cervarix is another vaccine which protected against HPV 16 and 18 only and was used in the UK until 2012 [58]. The newest vaccine to be approved, Gardasil 9, protects against HPV 6,11,16,18,31,33,45,52, and 58 – the latter five HPV types accounting for up to an additional 20% of cervical cancers [22]. The added benefit in that it protects against anogenital warts.

Worldwide, approximately 100 countries have introduced HPV vaccination programmes – as of October 2019 [59]. The UK school based national vaccination programme started in 2008 with vaccine being offered to all girls aged 12-13 years with the aim of preventing 2/3rd of cervical cancers in women under 30 years by 2025. The vaccine is administered as two injections, the second being given 6-12 months after the first dose (three doses over 6 months if over the age of 15) and is said to provide long term immunity for at least 10 years [60]. To be effective it must be administered prior to exposure to HPV.

The vaccine is also licensed for use in adolescent boys and recent policy change from the Joint Committee on Vaccination and Immunisation (JCVI), has meant that since September 2019, 12-13-year-old boys have been included in the vaccination programme in the UK, making it now gender neutral [61] [62] [63]. The JCVI state the benefit of vaccinating boys to include protection against HPV associated genital warts, penile, anal and oropharyngeal cancers [61]. Those who miss the vaccination during the school's programme are eligible for the vaccine up until the age of 25.

Both vaccine types have a very good safety profile and are highly efficacious against the respective HPV infections – 91.6% for the bivalent vaccine, 98% for the quadrivalent vaccine and 97.4% for the 9-valent vaccine [64] [65] [66]. They also convey limited cross protection from other HPV types not covered in the vaccines. The induction of herd immunity may also be achieved providing the uptake and coverage rates are high. Many studies have already demonstrated a reduction in the incidence of pre-invasive lesions, genital warts and HPV infections since the introduction of the vaccine [22]. One such meta-analysis reports an 83% decrease in the prevalence of HPV 16 and 18, 54% decrease in HPV 31, 33, and 45, and a 67% reduction in anogenital warts amongst adolescent girls 5-8 years post vaccination [67]. Amongst boys of similar ages, the effect of herd immunity was evident with the prevalence of anogenital warts decreased by 48%. In addition, CIN2 reduced significantly by 51% after 5-9 years of vaccination [67]. It is estimated that 64,000 cervical cancers and 50,000 other cancers will have been prevented by vaccination by 2058 [58].

1.8 The Future

There is a move towards non-invasive HPV testing to primarily increase the uptake of cervical screening [68] [69] [70]. If a second line relatively non-invasive diagnostic blood test could be developed and implemented at the point of referral to colposcopy following a HRHPV positive cervical smear test, it may provide a tool which would aid in the diagnosis of invasive disease and therefore direct patients into the biopsy and cancer pathways with minimal delay. Those who test negative and therefore do not have invasive disease, could not only have their anxieties allayed, but also be confidently managed in line with the cervical screening program, with the possibility of minimising interventions. A triage tool such as this would also be useful in reducing the burden of the large number of 2 week wait referrals to colposcopy clinic, of those patients who are low risk for invasive disease.

Table 1 - Staging of cervical cancer – FIGO 2018

Stage	Description
I	*Carcinoma strictly confined to the cervix (extension to the uterine corpus should be disregarded*
IA	Invasive carcinoma that can be diagnosed only by microscopy, with maximum depth of invasion <5mm
IA1	Measured stromal invasion <3mm in depth
IA2	Measured stromal invasion ≥3mm and <5mm in depth
IB	Invasive carcinoma with measured deepest invasion ≥5mm, lesion limited to the cervix
IB1	Invasive carcinoma >5mm depth of stromal invasion, and <2cm in greatest dimension
IB2	Invasive carcinoma ≥2cm and <4cm in greatest dimension
IB3	Invasive carcinoma ≥4cm in greatest dimension
II	*The carcinoma invades beyond the uterus, but has not extended onto the lower third of the vagina or to the pelvic wall*
IIA	Involvement limited to the upper 2/3rd of the vagina without parametrial involvement
IIA1	Invasive carcinoma <4cm in greatest dimension
IIA2	Invasive carcinoma >4cm in greatest dimension
IIB	With parametrial involvement but not up to the pelvic wall
III	*The carcinoma involves the lower 1/3rd of the vagina and/or extends to the pelvic wall and/or causes hydronephrosis or non-functioning kidney and/or involves pelvic and/or para-aortic lymph nodes*
IIIA	The carcinoma involves the lower 1/3rd of the vagina, with no extension to the pelvic wall
IIIB	Extension to the pelvic wall and/or hydronephrosis or non-functioning kidney (unless known to be due to another cause)
IIIC	Involvement of the pelvic and/or para-aortic lymph nodes, irrespective of tumour size and extent (with r and p notations)
IIIC1	Pelvic lymph node metastases only
IIIC2	Para-aortic lymph node metastasis
IV	*The carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the mucosa of the bladder or rectum.*
IVA	Spread to adjacent pelvic organs
IVB	Spread to distant organs

Chapter 2 LITERATURE REVIEW

In order to understand the potential role of circulating HPV DNA in cervical invasive and pre-invasive disease, a literature review was undertaken to determine the origins of circulating DNA, its clearance mechanisms, and its role in cancer in particular. A systematic search of the literature was performed and included historic as well as current studies, systematic reviews, review articles, cohort studies, and case-controlled studies, with a strategy to review the best available evidence. Studies included all in relation to circulating DNA then were narrowed down to circulating tumour DNA, viral DNA, and then further refined to HPV DNA in the context of cervical cancer and pre-invasive disease. The studies relating to HPV DNA in the context of cervical cancer also included its role in diagnosis, monitoring, and detection of relapse.

Searches were carried out in 2018 via PubMed with terms including HPV DNA OR Human Papillomavirus OR cell free DNA OR tumour free DNA AND cervical cancer OR cervical intraepithelial neoplasia, with no defined date limit.

2.1 Circulating DNA

The concept of circulating free DNA (cfDNA) was first described in 1948 when Mandel and Metais discovered the presence of nucleic acid in serum [71]. It wasn't until twenty years later that Tan *et al* described the presence of DNA in the blood of patients with systemic lupus erythematosus (SLE) which led to the formation of anti-dsDNA antibodies and complexes leading to the organ damage that is pathognomic of the disease [72].

In 1977, an attempt was made at the quantification of free DNA in the serum of patients diagnosed with cancer [73]. This early study by Leon *et al* compared the concentration of free DNA in the serum of patients with

cancer to those without and found higher concentrations in patients with most notably uterine, head and neck, lung, breast, and ovarian cancers. In addition, it was found that those patients with fast growing, poorly differentiated tumours and metastatic disease had even higher levels of free DNA in the serum. Furthermore, after radiation therapy, the majority of patients showed a response with decreased or unchanged DNA levels, which appeared to correlate with treatment effectiveness [73].

Further supporting the hypothesis of circulating DNA in patients with cancer, Stroun and Anker in 1989 demonstrated that cfDNA had the same double stranded instability as original tumour DNA [74], and Vasioukhin in 1994 discovered that plasma cfDNA had the same point mutations found in tumour cells of patients with myelodysplastic syndrome [75]. This, and other studies, spawned the idea of a “liquid biopsy’ [76].

Other areas of interest of circulating DNA arose from a study by Lo *et al* in 1997 where it was demonstrated that fetal DNA could be found circulating in maternal blood [77]. This led to the application of circulating fetal DNA in the identification of fetal anomalies including Down’s Syndrome – avoiding the need for invasive procedures such as amniocentesis. Analysis of circulating fetal DNA from maternal blood can also determine Rhesus status, and fetal sex [78].

2.1.1 Properties of circulating DNA

The properties of DNA are such that in the circulation, nucleic acids form complexes with proteins and lipids or are shielded within membranes, thus

evading recognition from the immune system and protecting DNA from degradation by nucleases [79]. One such complex is the nucleosome which is composed of a histone octamer and dsDNA turned about the protein complex and stabilised by histone H1. The binding of DNA around the octamer occurs by electrostatic interaction – DNA being negatively charged. In addition, nucleosomes are attached to each other via linker DNA which are approximately 20-90 base pairs; this helps to stabilise the DNA further and protect it from enzymatic degradation in the circulation [78].

Circulating DNA may exist as cell free or cell surface bound – such as those associated with the surface of blood cells [79]. cfDNA of tumour origin is more fragmented than cfDNA deriving from healthy cells. Holdenrieder confirmed the presence of nucleosomes in the circulation of cancer patients and to a lesser extent in healthy individuals [80]. Some theories for the origins of circulating DNA (cfDNA) include release from apoptosis, necrosis, phagocytosis, oncosis, and active secretion [78].

2.1.2 Apoptosis

The cell regulated process of apoptosis is required to maintain cell homeostasis. Oxidative stress and DNA damage can trigger an imbalance in homeostasis and initiate the apoptotic pathway which includes inhibition of the inflammatory response, enzyme degradation of cell components, fragmentation of DNA and packaging and release into extracellular vesicles (EVs) [81]. During this process DNA is degraded into large fragments which are cleared into nucleosome units [82]. Degradation occurs by inter-nucleosomal cleavage of chromatin at spacer regions [82] [83]. A large

proportion of degraded DNA arises from lymphocytes, monocytes, and granulocytes. This produces debris which is usually cleared by macrophages [84], as dying cells express phosphatidylserine (PS) which acts as a signal for engulfment by macrophages [82]. These engulfed cells enter the lysosome of the macrophage where lysosomal proteases digest these to proteins and DNase II in turn degrades them into nucleotides [82]. A small proportion of DNA escapes this process, especially when there are too many dead cells or phagocytic activity is reduced. This results in an increase in circulating nucleosomal DNA [82] [85]. Apoptotic DNA fragments form a distinct ladder pattern after electrophoresis [86]. Fournié *et al* found a correlation between the quantity of DNA measured in serum of patients with lung cancer and the activity of cell death markers [83].

It has been thought for a number of years that apoptosis was the primary source of cfDNA. However, apoptosis is dysregulated in malignant cells with a resultant increase in the anti-apoptotic pathway, hence proliferation of cells [82]. Therefore, apoptosis is unlikely to be the only cause for DNA release into the serum.

2.1.3 Necrosis

A second theory is that the DNA in serum arises from tumour necrosis. Necrosis, in contrast to apoptosis, is the unprogrammed cell death that occurs in response to cell injury, inflammation, infection, and toxins [86]. Necrotic cell death occurs more rapidly than apoptosis resulting in slower removal of debris and longer resulting DNA fragments, with the number of fragments extracted dependent on the type of necrosis inducing event [87].

At the end of necrosis, in contrast to apoptosis, the cell membrane structure is not intact and therefore intracellular substances (including chromatin) are released into the interstitial space. This is difficult to digest by phagocytes and therefore leads to inflammation of tissues [82].

As previously mentioned, circulating DNA levels were significantly reduced following radiotherapy, which in itself causes tumour necrosis [73]. In this situation there would be expected to be an increase in cfDNA, however, the reduction may be explained by the effects of radiation on inhibiting tumour growth outweighing the tumour necrosis effect [86], and also the short half-life of cfDNA.

2.1.4 Phagocytosis

Phagocytosis plays an important role in the clearance and digestion of dead cells such that DNA is not detectable in blood with apoptotic/necrotic cells unless macrophages are present [86]. Not only do phagocytes ingest and cleave DNA fragments, but their own cell death releases their DNA and that of the ingested cells into the bloodstream [88].

The process of erythrocyte enucleation results in the formation of reticulocytes and pyrenocytes. These pyrenocytes are readily phagocytosed due to signals on their surface, with their engulfed nuclei subsequently being digested by DNase II in lysosomes. There is very little evidence for erythroblast enucleation being a source of cfDNA, with some studies stating that it represents <30% of plasma cfDNA [81].

2.1.5 Oncosis

Oncosis refers to a non-apoptotic, accidental cell death process which is often seen in ischaemia such as myocardial ischaemic cell death [89]. The process itself involves cell membrane injury leading to increase in cell permeability, and then subsequent swelling and disruption of the cell – as compared to apoptosis which causes shrinkage of the cell. Oncosis can occur in the presence of apoptosis and lead to necrosis [89]. There is a characteristic fragmentation pattern of DNA seen following cell death due to oncosis involving ischaemia [78].

2.1.6 NETosis

NETosis is an innate cell death program by neutrophils activated by pathogenic agents and leading to release of Neutrophil Extracellular DNA traps (NETS). This is different and independent to apoptosis and necrosis [81]. NETS contains disintegrated chromatin which acts as a binding matrix for antimicrobial proteins that kill entrapped bacteria. There are two forms of NETosis 1) suicidal NETosis where there is slow release of proteins leading to lytic cell death, and 2) vital NETosis where there is rapid release of chromatin and peptides. In chronic inflammation, the lytic cell death mechanisms can be pro-inflammatory and therefore detrimental to organisms. NETs have also been shown to reactivate dormant cancer cells in mice following inflammatory reactions. Persistence of NET products and structures in the bloodstream can lead to vascular occlusion. Removal of NET structures is also dependant on degradation by proteases and engulfment by macrophages [81]. Several disease processes have been

shown to have irregularities in the degradation pathway. These include SLE, antiphospholipid syndrome, and lupus nephritis. An increase in cfDNA following NETosis has been described in these diseases.

NETS therefore constitute one of the origins of cfDNA and they possess biological and pathophysiological effects in autoimmune pathologies, cancer, sepsis, thrombotic illnesses and the inflammatory response [78].

2.1.7 Active secretion

Gahan *et al* in 2010 demonstrated active secretion of DNA from dividing and differentiated cells into the bloodstream. In this paper they describe the discovery of the virtosome – a DNA/RNA lipoprotein complex common to eukaryotic cells and found to be spontaneously released from living cells but not dead or dying cells [90]. Virtosomes contain newly synthesised products which are released in a regulated manner, and extracellular concentrations cannot be exceeded once equilibrium is reached [86]. The released virtosome has the ability to readily enter other cells (acting as an intercellular messenger) avoiding lysosomal degradation, modify the biology of the recipient cell, and cause immunological changes which can lead to transformation to cancer [78] [90] [91]. This may be an alternative mechanism for the initiation of metastases [78].

An abnormally high number of microvesicles are secreted and found in the blood of cancer patients and constitute a key structure of cDNA [78]. Exosomes are microvesicles secreted by most cells and are composed of mostly lipids which encapsulate RNA, mRNA, microRNA, ssDNA,

mitochondrial DNA and genomic DNA [78] [82]. Tumour derived DNA has recently been characterised from exosomes [92] and in a similar fashion to virtosomes, has been found to serve as a communication between cancer cells, affecting the tumour microenvironment and facilitating metastases [82]. It has been described that exosomal EGFR from gastric cancer can be found in liver specific metastases [82].

2.1.8 Others

Autophagy is a regulated mechanism of a cell that removes unnecessary or dysfunctional components. It regulates apoptosis and therefore cDNA release [78]. Hypoxia is a proposed major process involved in tumour growth, invasion, and metastasis, with malignant cells activating autophagy as a survival mechanism. In this way it generates an alternate energy source, and this theory could be used to explain why there is a high level of malignant derived cDNA. Autophagy is not activated in cells from the microenvironment [78]. cDNA has been found to be dependent on hypoxic conditions, hence in pancreatic cancer, extreme hypoxic signatures have been detected and are being proposed as a marker for screening and surveillance of pancreatic cancer [78].

Mitochondrial derived cDNA is released following mitophagy cell death – degradation of mitochondria by autophagy. They are either bound to internal and external mitochondrial membrane fragments or remain intact as cell-free mitochondrial DNA [78].

2.2 The fate of circulating DNA

There are limited publications available on the fate of cfDNA – some have shown that it is rapidly degraded by nucleases present in the blood and rapidly metabolized and eliminated by the liver and kidneys [87]. The estimated half-life of cfDNA is said to be anywhere between several minutes to 2 hours [87]. Lo *et al* state the estimated half-life of fetal DNA in maternal blood to be 16.3 minutes [77] with Yu *et al* describing a biphasic nature to clearance – the first rapid phase of between 10 minutes and 1 hour, and a second slow phase of approximately 13 hours [93]. Diehl *et al* determined a half-life of tumour ctDNA of 114 minutes after surgery in a single colorectal cancer patient [94].

The half-life of cfDNA is determined by the type of DNA complexes, origin of cfDNA and any treatment given. Degradation is largely mediated by enzymes such as deoxyribonuclease (DNase I) and proteases [87].

The liver is the main organ for nucleosome clearance with the majority cleared by 10 mins [87]. Macrophages in the spleen have also been shown to be involved in DNA and nucleosome clearance, with clearance rate through the glomeruli of the kidneys dependent on DNA fragment size [87]. Urine has high DNase I activity and therefore low DNA concentration with high DNA fragmentation [95].

2.3 Circulating DNA and Cancer

In a similar fashion to cfDNA, circulating tumour DNA (ctDNA) is released into the bloodstream during tumour necrosis and apoptosis (as

nucleosomes), secretion by living tumour cells (lymphocytes), as well as active secretion by circulating tumour cells [96] [97]. The tumour microenvironment such as stromal/endothelial cells, lymphocytes and other immune cells, also make up a potential source of ctDNA release and has been found to be related to tumour progression [78]. The fragments of ctDNA are said to be shorter than those of cfDNA, and they make up only a small fraction of total cfDNA (<1%) [97].

The amount of circulating tumour DNA (ctDNA) in blood differs according to the tumour type. Higher levels of ctDNA have been found in bladder, colorectal and pancreatic cancers, and lower levels in thyroid tumours and gliomas [73] [96] [98]. In addition, studies have shown that ctDNA increases with tumour cell number increase – as is the case in advanced and metastatic cancers where higher levels are found compared to early cancers [78]. Spindler *et al* demonstrated the prognostic value of total cfDNA concentration and mutant ctDNA concentration in a cohort of metastatic colorectal patients. High levels of total cfDNA and mutant ctDNA, high mutation load and ctDNA fragmentation level, were strongly correlated with poor overall survival [99].

The majority of research relating to circulating DNA is in the field of oncology, with its clinical relevance in non-invasive diagnosis, monitoring of treatment effect, recurrence and prognosis [78]. It has been described that ctDNA possesses cancer associated characteristics such as single nucleotide mutations, methylation changes and cancer derived viral

sequences, which may be important in the development of detection assays used in cancer diagnostics [96].

As the sequencing of plasma DNA has advanced, so has the development of various mutation specific testing for cancer [97]. There are two approaches to analysis of ctDNA,

1) Tumour guided analysis of plasma DNA - where knowledge of the specific mutation, such as from a biopsy, can be looked for using probes. A technique useful to detect relapse and treatment effect.
2) Tumour independent analysis – where there is no prior knowledge of the original tumour mutation, and a broad panel of commonly mutated genes is used to determine ctDNA. This technique is commonly used in diagnostics [97].

The use of ctDNA to monitor disease processes, prognosis, relapse and treatment effect has been studied in melanoma, lymphoma, prostate, liver, gastric, head and neck, gynaecological and amongst other cancers [100].

2.3.1 Detection of relapse

Studies have shown that ctDNA is useful in the prediction of chemotherapy resistance and detection of residual disease as well as relapse. Scholer *et al* demonstrated that increased ctDNA detected post colorectal surgery predicted relapse, with none of the patients who had undetectable ctDNA post-surgery experiencing a relapse. In addition, this prediction occurred 9.4 months earlier than detected by imaging, namely CT [101] .

There is a subset of patients with non-small cell lung cancer who have epidermal growth factor receptor (EGFR) mutations which increases intrinsic kinase activity. These patients benefit from treatment with specific tyrosine kinase inhibitors, and therefore detection of this EGFR mutation in ctDNA can help to target treatment and improve outcomes in these patients. The cobas® EGFR mutation test is such a test that has been developed and approved for diagnostics and the decision for targeted therapies [100] [102].

2.3.2 Other biomarkers

Although conventional biomarkers do not require the same processing as ctDNA analysis and are less expensive, they are known to have low sensitivity and specificity. Biomarkers such as CA125, CEA and PSA can be found in other non-cancer diseases, are not always elevated in advanced cancer and therefore cannot be used confidently for diagnostic purposes. In contrast, ctDNA is not present unless there is a cancer and strongly correlates with disease burden. The half-life of ctDNA is 15 mins to 3hours and therefore gives an accurate reflection of the current disease state, in comparison to other biomarkers which may still be raised long after treatment [100].

2.3.3 Other biological fluids

Owing to the blood-brain barrier, ctDNA released from brain tumours would be difficult to detect in the blood, however, has been detected in cerebrospinal fluid. A lumbar puncture to assess for ctDNA would be less invasive than a brain biopsy and therefore provides an alternative.

Urine ctDNA has been detected in patients post resection of bladder cancer and has been shown to be a good predictor of recurrence. In the case of lung cancers, salivary ctDNA has shown concordance with tumour samples and has been flagged as a potential in head and neck cancers. Lastly a stool analysis for ctDNA may be a viable alternative to a blood test for colorectal cancer [100].

2.3.4 Early detection of disease

It is well known that early diagnosis of cancers increases survival as they are treated earlier, and advanced disease is prevented. Tumours secrete DNA before becoming visible on imaging or causing any symptoms and it is this factor that could be utilised for early detection of disease. Pancreatic cancer has a slow development phase of approximately 11.7 years, however, is detected late with over 80% of patients being diagnosed at an advanced stage. This then amounts to a poor 5-year survival of 4-7%. Mutations in KRAS, TP53, CDKN2A and SMAD4 have been linked to the development of pancreatic cancer and widely studied in this field. A liquid biopsy in this context could save many lives [100].

Due to the various structures involved in cfDNA, it is difficult to determine the proportion of each structure, their stability and fate. Such information would be crucial for tumour biology and distinguishing between primary tumour and metastases [78]. It may also be useful in determining tumour aggressiveness in order to then tailor treatment. Being able to extract ctDNA based on their particular compartments or molecular structure should in theory increase the diagnostic power. In a similar fashion, quantitative

determination of malignant versus non-malignant derived cfDNA in cancer patients may also improve diagnostic power [78].

2.4 Circulating HPV

Tumours exhibit genetic heterogenicity which means that no one standard marker can be used to identify ctDNA. Viral ctDNA however, has been found to be a sensitive and specific marker for viral associated cancers as the viral genome is vastly different to the human genome, exhibiting multiple copies per tumour genome and therefore holding a greater advantage over single nucleotide detection [103] [104].

Epstein-Barr virus (EBV) infects B-lymphocytes and has been implicated in the development of nasopharyngeal cancer [103] [104]. The detection of circulating EBV DNA in nasopharyngeal cancer has been described, with one study reporting detection in asymptomatic patients with early-stage disease [105]. Plasma EBV is derived from tumour and is resistant to DNase treatment. Mechanisms for release of EBV DNA include cell death, episomal release, and lytic replication. Circulating viral genome has been found in other cancers such as Hepatitis B virus where elevated levels have been found to be a strong predictor of the development of hepatocellular carcinoma (HCC) [106].

Plasma HPV DNA is also thought to be derived from tumour however, the mechanism of release is controversial as there are differing results from various studies with some suggestions that about 20% arise from episomal DNA – although not replicated in other studies. Both the plasma and

tumour DNA are integrated into the host genome which means there may be a low incidence and copy number in the circulation and less efficient mechanisms for release in comparison to other viral genomes [104]. Another hypothesis is that of release during lysis of circulating cancer cells or in micrometases shed by tumour [104] [107].

Pornthanakasem *et al* looked into the diagnostic and prognostic potential of circulating HPV DNA in patients with cervical cancer [107]. They collected tissue from 63 patients with cervical cancer and also obtained blood samples from the same cohort and from 20 healthy blood donors. Only 6/50 patients with HPV associated cervical cancer had circulating DNA, indicating low sensitivity. However, circulating DNA was not found to be present in normal controls or in HPV negative cervical cancer indicating a high specificity. Furthermore, HPV genome from both the tumour and the plasma matched, indicating that the circulating viral DNA was derived from the cancer cells. This study also reported that HPV in the plasma was associated with distant metastases, recurrence within 1 year and therefore poor prognosis [107].

Sathish *et al* also looked at circulating DNA in cervical cancer patients. They collected cervical biopsies and plasma samples from 58 women with invasive cervical cancer. In addition, they also collected samples from 10 women with pre-invasive disease and 30 control women matched for age. All paired samples for the control group were negative for HPV DNA, however only 8 (11.8%) were positive for plasma HPV DNA in the cervical cancer group. In the CIN group all 10 patients were positive for HPV in

cervical biopsies but none were positive in the plasma samples. Of the 8 patients who were plasma DNA positive, 7 were HPV 16 positive and 6 were at stage 3-4 cancer. Cervical tissue and plasma pairs correlated on sequencing [108].

Widschwendter *et al* proposed from their study that HPV DNA may be a useful marker for early detection of recurrence in cervical cancer patients [109]. In this study they recruited 94 patients with invasive cervical cancer and 20 patients with non-HPV associated tumours and tested serum for HPV DNA. These samples were taken at the date of diagnosis and before initial treatment and then follow up samples taken after initial treatment. Forty-two out of 94 (45%) cases were HPV DNA positive with HPV 16 being the predominant type. In 36/42 cases the HPV DNA type in the serum sample correlated with the tissue type. There were 4 cases of HPV negative cancer showing HPV positivity in the serum, prior to treatment. HPV DNA detection seemed to increase in advanced disease. All patients with non-HPV associated tumours were HPV DNA negative.

In the follow up samples all patients without recurrence were HPV DNA negative in the sera. In the patients with recurrence after treatment the HPV DNA was negative in 11 cases after treatment and re-detected in 12/13 cases during follow up. HPV DNA was positive a median 72 days before clinical diagnosis of recurrence [109].

HPV plasma ctDNA has also been detected in HPV positive head and neck cancers, with many of the studies employing PCR methods which have a

sensitivity of 19-79% in overt disease. Often a combination of saliva and blood samples are required to increase the sensitivity. The use of digital droplet PCR (ddPCR) using probes for HPV 16 and 33, has been shown to increase the detection of HPV ctDNA in OSCC to almost 96%. In addition, ctDNA has been detected in over 85% of anal SCC. The use of ddPCR gave a specificity of 100% amongst negative controls [103].

There has been limited success in detecting HPV ctDNA for cervical cancer diagnosis due to lack of sensitivity of traditional tests [110].

2.5 Next Generation sequencing HPV assay

Next generation sequencing (NGS) was developed within the last decade and allows simultaneous sequencing of DNA fragments without prior knowledge of the sequence. Earlier and more traditional methods such as Sanger sequencing, which was developed in 1997, utilised amplification of one or a few relatively short segments of DNA by PCR. Sequencing was then performed on specific regions for specific samples. With NGS the complete genome can be sequenced in a few days with less cost [111].

Viral detection strategies include sample collection followed by nucleic acid isolation. There are two strategies for detecting viruses: -

1) Isolate and sequence the nucleic acid from the sample then use computational analysis methods to detect viral sequences
2) If the characteristics of the virus are already known, then processing of the sample further to enrich for known virions.

Some strategies include a PCR amplification step to enrich for viral sequences. PCR primers are designed to detect the specific type or family of viruses. Analysis is then performed by comparing results to existing viral sequencing in order to identify the virus [112].

Traditionally, HPV genotyping involved amplification of a region of the L1 gene with NGS using specific primers. Lippert *et al* designed a targeted NGS panel which included specific regions of the L1 gene as well as the E6 and E7 oncogenes, of the HRHPV subtypes of interest. They tested this panel in biopsy samples and plasma and had near equal correlation in terms of detection and genotyping of HPV, as compared to a more commonly used assay [113].

Lee *et al* developed an amplicon based next generation sequencing assay to detect circulating plasma viral DNA ('*HPV16-detect*'), in order to assess the response to chemoradiotherapy in locally advanced head and neck cancer [114]. This novel NGS assay comprised a 39-amplicon single pool panel which covered 34 distinct regions of the HPV 16 genome, as well as 5 amplicons of human reference genes. In their cohort, the assay demonstrated 100% sensitivity and 93% specificity in the detection of HPV 16 positive head and neck cancers at diagnosis. In addition, by tracking HPV through and post chemoradiation, they were able to predict response and residual disease, demonstrating that plasma HPV DNA can be used as a marker of response to treatment.

In their other study including a cohort of HPV related anal squamous cell carcinoma patients post chemoradiotherapy, Lee *et al* expanded the novel NGS assay to include HPV subtypes 16, 18, 31, 33, 35, 45, 52 and 58 (*panHPV-detect*) [115]. This included two primer pools covering distinct regions (single nucleotide polymorphisms) of the aforementioned HRHPV genome, in order to detect cHPV DNA. In this study they demonstrated a 100% sensitivity and specificity of *panHPV-detect* in diagnosing the HRHPV subtypes prior to treatment of anal cell carcinoma. Furthermore, and in agreement with their previous study, *panHPV-detect* was able to pick up those patients who either had residual disease post treatment or relapsed a few months after [115].

In line with the previous studies, this thesis will also aim to explore the role of *panHPV-detect* as a non-invasive tool in the diagnosis, post treatment follow-up and monitoring of early invasive cervical cancers.

2.6 Aim

In this prospective cohort study, which is an extension to the Lee *et al* study, we aimed to investigate the use of this highly sensitive assay - *panHPV-detect*, for the detection of HPV circulating DNA in both pre-invasive cervical lesions and early invasive cervical cancer. As HPV is a mucosal virus and does not enter the blood stream in its infective phase, we hypothesized that HPV-DNA would not be detected in pre-invasive cervical disease but would be detectable in early-stage cancers.

2.7 Hypotheses

1. HPV cDNA can be detected in early invasive cervical cancers but not pre-invasive disease
2. We expect *panHPV-detect* to demonstrate high sensitivity and specificity in the detection of HPV cDNA in cervical cancer
3. HPV cDNA should be undetectable post treatment for cervical lesions
4. *panHPV-detect* can be used as a non-invasive triage tool for diagnosis, follow up and monitoring of early invasive cervical cancer

Chapter 3 MATERIALS AND METHODS

3.1 Research Design

We designed a prospective cohort study - in line with similar studies found from the literature review - as this allowed us to follow up the two research groups (pre-invasive vs invasive disease) both at diagnosis and then after their respective treatments. A negative control group was not required as we were determining the presence or not of circulating HPV in HPV related disease.

We recruited patients who were referred to the colposcopy clinic at UCLH with a cervical cytology diagnosis of high grade dyskaryosis, or moderate dyskaryosis and HPV positive (recruitment occurred prior to recent change to primary HPV testing). Patients were identified from clinic letters confirming the above and attending for cervical treatment in the form of LLETZ or cone biopsy. On arrival to clinic, patients were informed regarding the study and an information leaflet was provided. Written, informed consent was taken for participation in the study and for venepuncture. A venous blood sample, 20mL collected in EDTA tubes, was taken prior to treatment and then at the next appointment following treatment, to assess for HPV cDNA. The colposcopic and biopsy findings were correlated with the *panHPV-detect* result. Women who could not tolerate treatment in the outpatient setting or were undergoing general anaesthetic for treatment to a larger lesion, were seen in clinic at the time of consent for their surgery

and recruited to the study. A venous blood sample was then taken on the morning of surgery.

A second cohort of patients with biopsy proven early stage (1A/1B) invasive disease, were recruited from the UCLH Gynaecological Oncology clinic. These patients were recruited to the study in the out-patient clinic, based on radiological staging, and prior to definitive treatment, which was typically a simple or radical hysterectomy.

In the head and neck cancer pilot study, HPV 16- detect demonstrated 100% sensitivity and 93% specificity in detecting HPV16 positive cancers at diagnosis. To prove 85% sensitivity assuming that the true sensitivity is 99% and to have 80% power with a 2-sided type 1 error of 0.05, we calculated that at least 19 HPV positive, invasive cancer patients were required to be recruited. As approximately 95% of cervical cancer patients are HPV positive, we aimed to recruit 22 patients with early invasive carcinoma. To prove 80% specificity for pre-invasive disease, assuming that the true specificity is 94%, we required 37 patients with high grade squamous intraepithelial lesions (HSIL) to be recruited.

An audit on referral and treatment outcomes of cervical cancer patients at UCLH between April 2015 and March 2016, showed that 46 patients had been seen and treated for cervical cancer, therefore extrapolating this data, we would expect to recruit the required number of patients.

3.2 Primary end point

The sensitivity of panHPV-detect for the detection of plasma cHPV DNA at baseline in patients with early-stage invasive cancer, and the specificity of panHPV-detect for proving the absence of plasma cHPV DNA in patients with pre-invasive disease.

3.2.1 Inclusion criteria

- Patients with HPV positive HSIL referred for colposcopic treatment
- Age >18 years
- Able to give informed consent

3.2.2 Exclusion criteria

- Unable to give informed consent
- Previous diagnosis of cancer
- Other pre-invasive HPV associated lesions e.g., VIN/VAIN/AIN
- Previous CIN treatment

3.3 SCIENTIFIC METHODS

3.3.1 Whole blood sampling and plasma extraction

After confirmation of patient details and consent to study, blood was collected via venous puncture from the antecubital fossa, directly into 2 x 10ml EDTA tubes. These were pre- labelled with trial ID numbers only and no patient identifiers, such that the laboratory staff were blinded to the origin of the sample. The tubes were inverted 3-4 times following the blood draw

and stored in an upright position, prior to transfer to the Translational Research Laboratory at University College London. The blood collection tube was then centrifuged at 1600g for 10 minutes at room temperature, and repeated – as per Institute of Cancer Research (ICR), Chester Beatty Laboratories (CBL), standard operating procedures (SOP). This step was necessary to separate whole blood into a top layer of plasma, middle layer of buffy coat containing leucocytes and platelets, and a bottom layer of erythrocytes. The top layer of plasma was taken off and then aliquoted into 2mL Cryogenic Vials, labelled with a unique barcode identifier and the trial ID. Approximately 8mls of plasma was obtained per 20mls whole blood sample. All samples were then immediately stored in a -80C freezer, recording the transfer in storage in a computerised system.

3.3.2 Extraction of cfDNA from Plasma samples using Qiagen QIAamp circulating nucleic acid kit

Free circulating nucleic acids are usually bound to proteins, lipids or vesicles in biological fluid; therefore, an efficient lysis step is required to release nucleic acids. The Qiagen QIAamp circulating nucleic acid kit was used for this purpose.

All extractions were carried out in a DNA free area at Chester Beatty Lab at the Institute of Cancer Research. The plasma was thawed on ice prior to extraction and then equilibrated to room temperature. In order to remove contaminating proteins and enzymes – particularly DNAses and RNAses - Proteinase K 200μl was added to a 50ml centrifuge tube. As there was a good yield across all samples, 5mls of plasma was then added to the 50ml

tube. Lysis Buffer ACL with carrier RNA dissolved in buffer AVE, was then added to this mixture and pulse-vortexed for 30 seconds to help lyse the cells and release DNA into the solution. The mixture was then incubated at 60C for 30 minutes to further aid denaturation. ACB lysate was then added to this mixture to allow later optimal binding of nucleic acids to a silica membrane during the elution step and mixed thoroughly by pulse-vortexing for 15-30 seconds before being incubated on ice for 5mins.

The QIAvac 24 Plus system was used to adsorb the nucleic acids from the above mixture onto a small silica membrane. The vacuum system assists in drawing the lysates through a QIAamp Mini column which contains the silica membrane. Washer buffer ACW1 600μl was then added to the column and drawn through under vacuum pressure followed by ACW2 750μl. After all of the buffer ACW2 was drawn through then 750μl of ethanol (96-100%) was added to the column and drawn through under vacuum pressure. The purpose of this process is to wash away any contaminants whilst the nucleic acids remain bound to the silica membrane. The QIAamp Mini columns were then placed in 2ml collection tubes, centrifuged at 20,000 x g; 14,000 rpm for 3 mins and then incubated at 56C for 10 minutes to dry the membrane completely.

In order to elute the DNA from the membrane the QIAamp Mini columns were transferred to a 1.5ml elution tube and 25μl of Buffer AVE was added to the centre of the membrane – this volume chosen as it has been shown to give highly concentrated nucleic acid elutes. This was then incubated at room temperature for 3 mins prior to centrifugation at full speed (20,000 x

g; 14,000 rpm) for 1 min to elute the nucleic acids. This process was repeated such that the final volume of elute was approximately 50μl for elute 1, again repeated for elute 2. The extracted nucleic acids were then stored at -20C.

3.3.3 Quantification of plasma extracted cfDNA by ddPCR using RNase P assay

The yield of free circulating nucleic acids from biological samples can be low, and highly fragmented. The two methods described for quantification of nucleic acids are spectrophotometry and UV fluorescence tagging. It would be difficult to determine the quantity of DNA within a sample with techniques such as spectrophotometry as this technique is non-specific for dsDNA. Spectrophotometry works by measuring absorbance of ultraviolet light in a solution at a wavelength of 260nm for DNA and RNA. The absorbance of light being directly correlated to the nucleic acid concentration in the sample. This method would therefore detect RNA as well as other free nucleotides giving a falsely high reading.

The alternative method of UV fluorescence tagging utilises a fluorescent dye which is tagged to the sample and fluoresces when bound to nucleic acids. The intensity of this fluorescence is then measured, and as such the sensitivity is superior to spectrophotometry in the context of quantifying DNA.

The more superior techniques utilise amplification as well as quantification, using PCR. Real time quantitative PCR (qPCR) measures the intensity of fluorescence at specific times during the reaction to determine the relative

amount of target molecule. Digital droplet PCR involves partitioning the solution into Nano litre sized droplets in which a separate PCR reaction can take place. After multiple PCR cycles the droplets are checked for fluorescence and relative quantification can occur. In this study we used Thermo Fisher's RNase P Taqman assay with ddPCR to quantify the extracted DNA. All work was carried out in a DNA free area of the CBL.

A reaction mix of RNase P Primer Probe mix, Master Mix and water was prepared according to the number of samples to be processed, including a non-targeting control. The reaction mix was aliquoted into 0.2 ml PCR tubes, DNA added, vortexed and spun down. In order to aid quantification of the DNA, the PCR mixture (20μl) was then made into droplets with the use of the automated droplet generator. A final droplet mixture of 40μl was collected. The droplets were then transferred onto a PCR plate, sealed and then run on a Thermal cycler in a 96 well PCR plate as per protocol. After the PCR cycle was run, data was analysed using QuantSoft software.

The software analysis then produces a graph with a two-channel split – Channel 1 represents empty droplets; Channel 2 represents VIC positive droplets. Data was analysed and exported to Excel. The concentration obtained is the number of RNase P copies/μl of reaction, this was multiplied by 20 to obtain the total number of RNase P copies on starting DNA. This was further calculated to obtain the ng/μl such that 3ng of DNA could be used for library preparation.

3.3.4 Preparation of DNA for library prep

Next generation sequencing (NGS) allows preparation of DNA into a form which can be sequenced. An NGS library is a collection of DNA fragments which are similarly sized and have known adaptor sequences attached to the 5' and 3' ends. Multiple libraries can be pooled and sequenced in the same run owing to their unique adaptors.

The general steps involved in NGS library preparation include:

1. Fragmenting or sizing the target sequences to a desired length
    a. This may be achieved by physical (acoustic shearing) or enzymatic methods (endonucleases) – fragment libraries
    b. Alternatively, if the sequence of the target is known then PCR amplification may be used to produce DNA amplicons of the desired size – Amplicon libraries
2. Attaching oligonucleotide adaptors to the ends of target fragments
    a. Specific adaptors (commonly 20 – 40 bp which contain known sequences) are annealed to the 5' and 3' ends of the fragmented DNA. Two different adaptors may be used, the first containing the primer annealing site for the sequencing primer, the second used to anchor the DNA fragment to a surface such as beads, for sequencing
3. Size selection of fragments
    a. This can be performed by either gel electrophoresis or a bead-based method where magnetic beads are used with varying

concentrations of buffer to isolate the DNA fragments size of interest

4. Quantifying the library product for sequencing
    a. This is performed either by a bioanalyser which gives library concentration and fragment size or by qPCR which is more accurate with regards to quantification as it only measures amplifiable library fragments. However, it lacks information on library size.

We used Ion Torrent AmpliSeq Gene panel library preparation protocol for 2 custom primer pools (8 HPV).

After drying and dilution with nuclease free water, a uniform starting concentration of 3ng of DNA was transferred to 0.2ml PCR tubes, on ice. A mix of x5 Ion AmpliSeq HiFi Mix, x 2 Ion AmpliSeq Primer Pool was added to the DNA making a library of 10μl per sample – performed in duplicate for primer pools 1 and 2. The PCR tubes with the mix were centrifuged at 1,000 rpm for 20 seconds to remove any bubbles. The tubes were then placed into the thermal cycler and the PCR program run according to protocol in order to produce the DNA amplicons.

After PCR amplification the two pools from each sample were combined, vortexed for 20 seconds and centrifuged at 1000 rpm for 20 seconds to collect any droplets. Of these pooled samples, 10μl was used for further preparation. In order for the adaptors to anneal successfully to the amplicons, partial digestion of the primer sequences is needed. FuPa reagent was used for this purpose, with 1μl added to each amplified sample.

This was thoroughly mixed, and libraries placed in the thermal cycler and run according to the PCR program protocol for approximately 40mins to aid digestion.

Once digestion occurred the samples were removed from the thermal cycler and 1μl of unique Ion Xpress Barcode Adaptor was added to the individual solutions. In order to ligate the adaptors to the digested primers, 2μl of Switch Solution and 1μl of DNA Ligase was added to each sample. The solutions were mixed thoroughly and returned to the thermal cycler for another approximately 40 minutes.

Purification of the sample was performed using AMPure XP beads. After removal from the thermal cycler 15μl of nuclease free water was added to each library followed by 45μl of Agencourt AMPure XP beads and mixed thoroughly. The tubes were placed in a 96 Ring Magnet plate and incubated for 6 minutes. This allowed the bead bound DNA to form a pellet at the bottom of the tube with the DNA free supernatant on top which was discarded. In order to purify further, 150μl of freshly prepared 70% ethanol was added to each library, mixed thoroughly, and the solution returned to the magnet and incubated for 1 minute. The supernatant was removed, and the ethanol wash step repeated. Excess ethanol droplets were removed with a fine pipette and the libraries were then dried on a thermal cycler at 37°C for 8 minutes or once visible cracks were noted in the pellet (indicating that the samples were dry). This step ensures that all ethanol has been removed prior to elution as this results in poor library quality. Once all samples were dry, 50μl of low TE buffer was added to the tubes in order to

resuspend the now purified DNA samples. This was homogenised, centrifuged at 1,000 rpm for 1 second and incubated for 3 minutes at room temperature. The tubes were then placed back in the magnet and the supernatant containing the now unbound target DNA with their unique barcode adaptors, was transferred to new PCR tubes.

3.3.5 Ion Torrent qPCR Quantitation

We used the Ion Torrent Library Quantitation Kit to quantify the sample library. In order to generate a standard curve, three 10-fold serial dilutions of a control library were prepared (E. coli DH10B) at 6.8 pM, 0.68 pM and 0.068pM. Each library is quantified as a 1:400 dilution and was prepared as per steps in appendix.

A master mix of TaqMan qPCR mix and TaqMan quantitation assay was made such that 11μl was added to each library in a 96 well plate. The control library was prepared in triplicate and the samples in duplicate. The plate was then sealed and centrifuged and run in the qPCR StepOnePlus machine for quantitation. The resulting data was read, and standard and amplification curves produced. At the end of the PCR quantification the molarity in picomolar, of each library was recorded.

In order to standardise volumes, nuclease free water was added, and the two pools were then combined. The resultant library was sent to the Tumour Profiling Unit at the Institute of Cancer Research, where sequencing was performed using the Ion Proton sequencer.

Plasma sample processing was performed in batches and in stages in order to ensure accuracy. Samples from patients who potentially had cancer or pre-invasive disease, were run together as blinding was in process. Whole blood samples were taken into barcoded EDTA tubes which were delivered to the lab at UCL with no patient identifiers. Plasma was extracted at the UCL lab and aliquoted into smaller 2ml vials which also carried their own unique barcodes but linked to the donor patient. These vials were then delivered to ICR and it was therefore not possible to know who the samples came from and whether they had CIN or invasive disease. It was only after all samples were run and results obtained, that the barcode numbers were linked by the researcher to the patient. As the samples were run together it is possible that contamination could occur between the two groups, particularly when plating the extracted DNA prior to PCR runs. However, the internal control processes during the PCR quantitation steps – including the analysis of a control library and running samples in duplicate – meant that it was easy to see from the resultant amplification steps whether results from neighbouring wells were the same, suggesting contamination. Where similar amplification curves were noted, the samples were repeated to ensure different samples were being read.

Chapter 4 RESULTS

We recruited 52 patients into this study between November 2018 and January 2020. The patients were recruited from a combination of the colposcopy clinic and the gynaecological oncology clinic at UCLH. Owing to the Covid 19 pandemic recruitment of new patients and follow up of already recruited patients was stopped prematurely in January 2020. At this point we recruited 87% of the target population. Of note none of the recruited patients had a history of prior HPV vaccination. Patient demographics can be found in Table 2.

Table 2: Patient demographics

Demographics	
Age	N (%)
25 - 35	34 (65)
36 – 45	13 (25)
46 – 55	3 (6)
>55	2 (4)
Ethnicity	
White British	32 (62)
European	9 (17)
Asian	5 (10)
Black	2 (4)
Other	4 (7)
Smoker	
Previous	11 (21)
Current	16 (31)
No	25 (48)
HPV Subtype	
16	8 (40)

18	3 (15)
Non 16/18	9 (45)

4.1 Detection of cHPV in Baseline Samples

Of the 52 patients recruited, 12 had early invasive cervical cancer and 40 had pre-invasive disease (Table 3). The mean age of participants with cancer was 39 and with pre-invasive disease 35. At the time of baseline blood collection, the final histology was not known; therefore, all samples were included for analysis. Analysis was performed blind to the HPV status on referral cytology. An adequate quantity of DNA could be extracted from 50 of the 52 patients, with two samples (9 and 11) failing the library preparation stage due to insufficient volume of DNA despite PCR amplification.

Table 3: Stage of cancer and degree of CIN

Results	Stage	Number of patients	Total
Cancers	1A1	6	12
	1B1	4	
	2B	2	
Pre - invasive	CIN 1	9	40
	CIN 2	17	
	CIN 3	11	
	CGIN	3	

The HPV assay that was used has a threshold for positivity of HPV DNA set at 10 reads in 7 different HPV amplicons of the different subtypes. This was

based on ROC analyses which gave the greatest sensitivity and specificity (based on head and neck cancers). Using this threshold, only 2 of the 50 baseline samples were HPV positive. HPV reads were detected in 15 other samples but in no more than 6 amplicons per HPV subtype and therefore did not reach the threshold for positivity (Table 4).

4.1.1 Detection of cHPV DNA in early invasive cervical cancer

Final histology was available after all plasma samples were analysed and DNA sequencing performed (including follow up samples). Out of the 12 cancer samples, 2 were found to be at a more advanced stage (FIGO 2B) than the inclusion criteria and therefore had to be excluded from this study (1 of these reached the threshold for positivity for HPV). Of the remaining 10 samples, only one sample was positive for HPV as per threshold criteria. This sample came from a patient who had a stage 1A1 cervical cancer, and was found to be HPV18 positive, which correlated with the HPV subtype on referral cytology (Table 4). This patient was an ex-smoker, under 30 years old and had a small tumour. The other 5 patients whose histology indicated FIGO stage 1A1 tumours, did not reach the threshold for HPV positivity in plasma. None of the 4 1B1 tumours had HPV in the plasma at levels which reached the threshold for positivity.

4.1.2 Detection of cHPV DNA in pre-invasive cervical disease

None of the plasma samples from patients with final histology showing CIN 1-3 or CGIN, reached threshold for positivity of HPV (Table 4). In 10 of these samples, HPV amplicons were detected but at numbers far less than the threshold. Combining the results from detection of cHPV DNA in pre-

invasive and invasive disease gives a sensitivity of 10% (95% CI, 0.24-44.5%) and a specificity of 100% (95% CI, 91.2-100%). There was no correlation between the number of amplicons detected and the grade of CIN or stage of cancer.

Table 4: Stage and Grade of diagnosis with HPV subtype (where known) and number of amplicons read. *Excluded from study

Sample no.	Diagnosis	HPV type cytology	HPV type plasma	Number of amplicons >6	Pos/neg
42	1A1	-	16	1	NEG
44	1A1	-	52	1	NEG
52	1A1	-	-	0	NEG
24	1A1	16	-	0	NEG
25	**1A1**	**18**	**18**	**11**	**POS**
35	1A1	16	16	1	NEG
41	1B1	-	-	0	NEG
10	1B1	-	16	1	NEG
23	1B1	-	31	2	NEG
2	2B	18	18	79	POS*
4	2B	NON 16/18	-	0	NEG*
5	CGIN	-	16	1	NEG
31	CGIN	-	-	0	NEG
40	CGIN	-	-	0	NEG
49	CIN 1	16	-	0	NEG
1	CIN 1	-	-	0	NEG
8	CIN 1	NON 16/18	16	3	NEG
17	CIN 1	18	33	1	NEG
21	CIN 1	-	-	0	NEG

22	CIN 1	-	-	0	NEG
28	CIN 1	16	-	0	NEG
37	CIN 1	-	-	0	NEG
38	CIN 1	-	-	0	NEG
43	CIN 2	-	-	0	NEG
45	CIN 2	-	-	0	NEG
46	CIN 2	-	16	1	NEG
47	CIN 2	-	-	0	NEG
51	CIN 2	-	-	0	NEG
3	CIN 2	NON 16/18	-	0	NEG
6	CIN 2	NON 16/18	-	0	NEG
7	CIN 2	16	-	0	NEG
12	CIN 2	NON 16/18	-	0	NEG
18	CIN 2	-	-	0	NEG
19	CIN 2	-	16	2	NEG
20	CIN 2	NON 16/18	-	0	NEG
29	CIN 2	NON 16/18	-	0	NEG
34	CIN 2	-	16	1	NEG
36	CIN 2	-	33	0	NEG
39	CIN 2	-	-	0	NEG
48	CIN 3	-	-	0	NEG
50	CIN 3	-	-	0	NEG
13	CIN 3	16	16	1	NEG
14	CIN 3	16	-	0	NEG
15	CIN 3	16	-	0	NEG
16	CIN 3	-	-	0	NEG
26	CIN 3	-	-	0	NEG
27	CIN 3	-	16	0	NEG

30	CIN 3	-	-	0	NEG
32	CIN 3	-	-	0	NEG
33	CIN 3	-	16	0	NEG

4.2 Adjusting thresholds

As this test had not been used for early cervical cancers, we performed a ROC analysis based on our data (Figure 1 + 2). The best performing threshold was that of 3 reads per amplicon, giving a sensitivity of 90% and specificity of 55% with an overall accuracy of 74.3% (57.1% - 91.5%) AUC. Using this adjusted threshold, sample 13 from a pre-invasive disease would have been classed as positive with >3 reads in 13 amplicons; however, this is and would be a false positive result. Similarly, the other lowered thresholds did not detect any further HPV positive cancers.

Figure 1: ROC Analysis

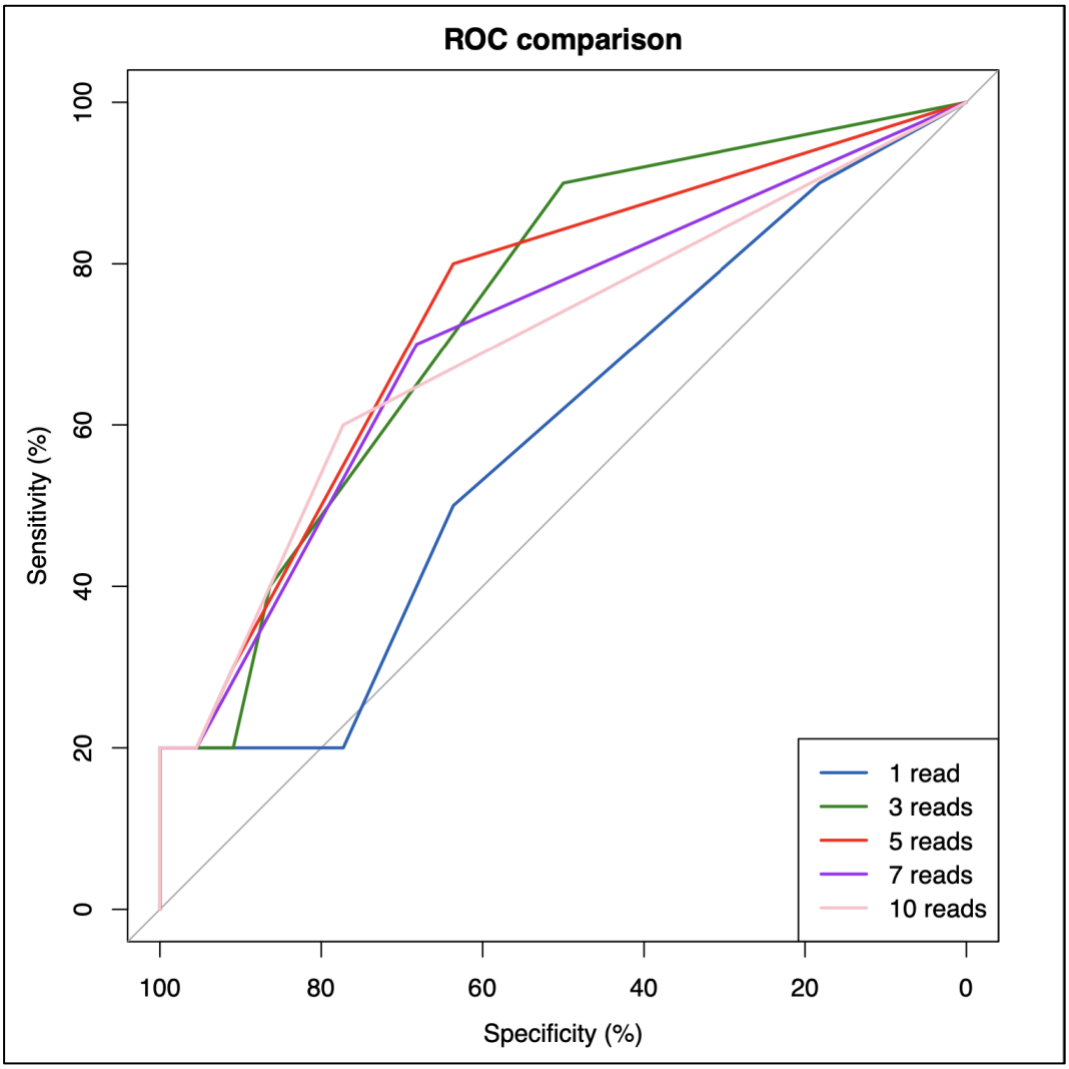
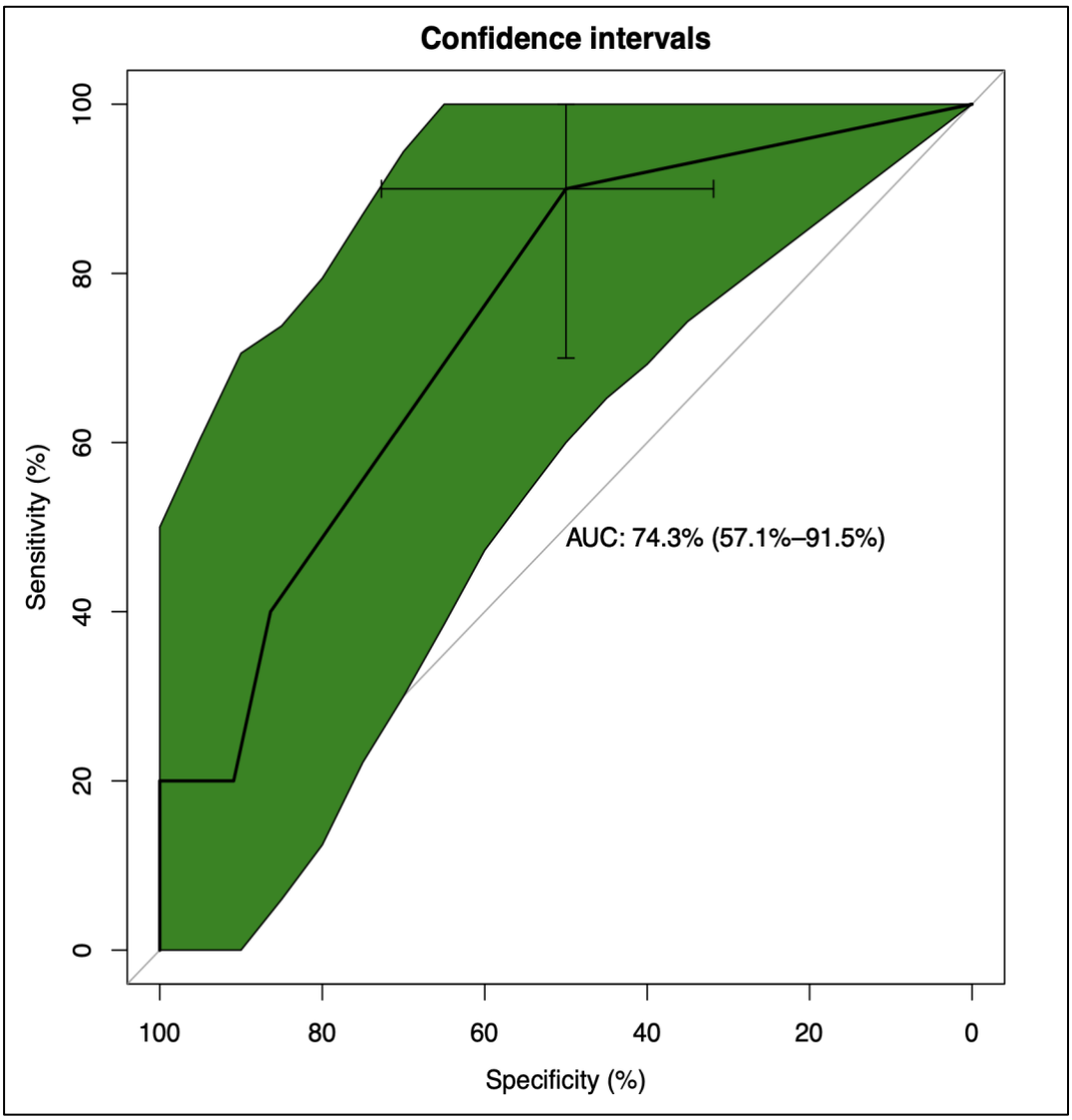


Figure 2: AUC for threshold of 3 amplicons



4.3 Detection of cHPV DNA in Follow- up samples

Only eighteen patients out of the cohort of 50 were followed up prior to the Covid-19 pandemic. Two of the patients (9 and 11) had follow up samples analysed despite baseline samples not being available (all samples were blinded and run on the same chip, regardless of follow up or baseline). It was not known at the time of blood draw that the baseline plasma had failed the PCR and library preparation stage. Of these 18 follow-up samples, 10 had HPV reads detected but none >6 amplicons and therefore none of the follow up samples reached thresholds for positivity (Table 5). Sample

number 25 which was the only positive baseline sample - detecting HPV 18 prior to treatment - was negative at follow up.

Of the 10 samples that had HPV reads at follow up only 2 correlated with the baseline HPV read – in one case there was no baseline sample to compare. Seven samples detected HPV types which were either new or did not correlate with the baseline HPV types (Table 5).

Table 5: Follow up data

Sample No.	Diagnosis	HPV type BL sample	HPV type f/u	Number of amplicons >6	Pos/Neg
44	1A1	52	-	0	NEG
3	CIN 2	Non 16/18	16	2	NEG
5	CGIN	16	-	0	NEG
6	CIN 2	-	16	1	NEG
7	CIN 2	-	-	0	NEG
8	CIN 1	16	16/18	3	NEG
9	1B1	No BL sample	18	3	NEG
10	1B1	16	16	1	NEG
11	CIN 2	No BL sample	-	0	NEG
12	CIN 2	-	-	0	NEG
13	CIN 3	16	-	0	NEG
16	CIN 3	-	33	1	NEG
18	CIN 2	-	33	1	NEG
21	CIN 1	-	-	0	NEG
23	1B1	31	18	1	NEG
24	1A1	-	18	1	NEG
25	**1A1**	**18**	**16**	**1**	**NEG**
31	CGIN	-	-	0	NEG

4.4 Clinical outcomes of participants

Although the majority of participants could not be followed up with a blood test, review of the clinical notes was undertaken in order to gather data on the test of cure results. In line with the NHSCSP guidance, women treated with LLETZ procedure should be followed up 6 months post procedure with a test of cure (TOC) which involves cervical sampling for HPV. Some women experienced a delay in follow up due to the pandemic but the majority (39/50, 78%) had a TOC within 1 year of treatment. Two patients were persistent non-attenders, 1 patient fell pregnant and therefore test will be performed post-partum, 1 is yet to have the TOC, 7 had hysterectomy and are being followed up as per cancer pathways. Of the 39 women who had TOC within 1 year, 29 (74%) were found to be HPV negative on smear and therefore returned to normal routine recall - interval dependent on age. The remaining 10/39 (26%) tested positive at the 6 months to 1 year smear and therefore undergoing yearly follow-up. None of these 10 were included in the follow up data presented.

Chapter 5 DISCUSSION

The aim of this project was to determine whether *panHPV-detect* could detect HPV DNA in the plasma of patients with early-stage cervical cancers, as it has previously been shown to have high sensitivity and specificity for locally-advanced HPV associated cervical, head and neck, and anal cancers. Using the thresholds of positivity for head and neck, and anal cancers, this study has demonstrated that none of the patients with pre-invasive disease had HPV DNA in their plasma (specificity 100%) – which is compatible with the first of our study hypotheses; that pre-invasive disease does not shed detectable levels of HPV DNA into the circulation. However, we demonstrated an assay sensitivity of only 10% for early-stage invasive disease . This patient underwent a laparoscopic hysterectomy and HPV cDNA was subsequently found to be negative. The low sensitivity in this study correlates with other similar studies as previously described.

In order to determine possible reasons for low sensitivity, it is important to explore the different steps and stages required for DNA analysis. There were two samples that failed the library prep stage, one was that of a 1B1 cancer and the other CIN 2. Both failed due to insufficient volumes of DNA extracted from plasma. We have already discussed that the yield of free circulating nucleic acids from blood samples can be low and highly fragmented. In addition, ctDNA only represents a small fraction of cfDNA especially in the early stages of cancer, which may make it difficult to detect.

A systematic review by Trigg *et al* looked into the possible factors that influence the quality and yield of circulating free DNA [116]. The factors

evaluated included the specimen type, time to processing, centrifugation protocols and methods of cfDNA isolation and quantification. They reported that total cfDNA levels were found to be 1.6 to 11-fold higher in serum than plasma in both cancer patients and healthy controls. In addition, cfDNA from serum had a significantly higher integrity than plasma which was thought to be due to high molecular weight DNA from contaminating hematopoietic cells in serum. With regards to ctDNA in particular, sensitivity (31% vs 25%) and specificity (97% vs 100%) were similar for plasma and serum respectively. It was therefore recommended that plasma is preferable where detection of tumour specific DNA may be masked by wild type DNA [116]. The decision to analyse plasma rather than serum in this study, was made to help reduce the chance of detection of non-HPV DNA.

The time between venepuncture and centrifugation of blood plays a role in the levels of cfDNA obtained, with a delay increasing the levels [117]. This is largely due to lysis of leucocytes which subsequently release DNA and RNA contributing to the total DNA yield and causing background interference which can skew further analyses. To overcome this, there are specialised blood collection tubes containing preservatives which help to stabilise leucocytes from whole blood and can delay centrifugation by several days without detriment. In this study we used standard EDTA tubes which has shown stability of 6 to 24 hours in the systematic review by Trigg et al [116]. Standard EDTA tubes were chosen for this study, as it was ensured that samples were taken directly to the laboratory immediately after venepuncture and therefore processed on average in under 2 hours. One of the samples fell outside of this parameter and was taken late in the

afternoon, delivered to the lab after closing, and therefore processed the next day (<18 hours). However, this sample was not any of the two failed samples, and the yield of cfDNA for this particular sample, was not affected when compared to the yield obtained from other samples.

The systematic review also looked at the centrifugation protocols - in particular the speed and number of spins – in relation to DNA yield. The consensus was that in all studies examined, the speed of centrifugation over 10mins did not result in significantly different DNA yields. The addition of a second centrifugation step helps to minimise contamination from cells in the buffy coat layer which may have been disturbed by removal of plasma in the first step. In all but one study, this had no significant effect on DNA yield. Our protocol included 2 spins of 10 mins each at 1600g.

Lastly the methods of cfDNA isolation were examined, with the QIAamp Circulating Nucleic Acid Kit (Qiagen) – used in this study - being the most widely used and leading to the highest yield of cfDNA. When assessing the best method for subsequent quantification of DNA, qPCR, ddPCR and fluorometry performed better than UV spectrophotometry [116]. Based on the results of this systematic review, our protocols followed the recommendations suggested in order to maximise the yield of DNA from plasma. Of the two samples with insufficient DNA, the extractions were repeated but gave the same results, suggesting a true deficit in cfDNA in those samples rather than an error in technique.

In their paper investigating HPV cDNA in head and neck cancers, Lee et al set the thresholds of HPV cDNA positivity based on ROC analyses, as 10

reads in 7 amplicons. This was said to give the greatest combined sensitivity and specificity and was based on human DNA reads in plasma of 57,000 to 3,000,000 [114]. The same thresholds were therefore used for this study in order to standardise processes when calculating the sensitivity and specificity of the novel NGS assay for the different tumour types.

As this was the first study using this novel NGS assay in cervical cancer, which also included the other high-risk subtypes of HPV, a ROC analysis was performed specific to the data from this study. The question to be answered being how good the test is at picking up cancer from pre-invasive disease. This demonstrated that a lower threshold of 3 reads per amplicon had the greatest sensitivity but a low specificity (90% sensitivity, 55% specificity). As such, when applying this lower threshold to the data, only one further sample would have been reported as positive and this was in fact a pre-invasive sample and therefore would have resulted in a false positive result – as commonly seen with tests with low specificity. It is also important to note when setting thresholds for a PCR based assay that false positives can arise from amplification of unwanted/untargeted PCR products i.e., noise. We can conclude from this that lowering the threshold of positivity for cervical cancer would not have improved the accuracy of the test, and therefore the detection of HPV cDNA.

We have caried out a similar study (in preparation), which evaluated *panHPV-detect* in later stage cervical cancers, in patients undergoing radiotherapy; this found high sensitivity in terms of HPV cDNA identifying primary and recurrent cancer. This suggests that stage or volume of tumour

plays a role in the amount of circulating tumour DNA that may be released into the plasma. As per the FIGO 2018 revised staging for cervical cancer, a Stage 1A1 tumour is diagnosed microscopically with a maximum depth of stromal invasion of <3mm. A stage 1B1 tumour is classified by a depth of invasion of >5mm but with the lesion limited to the cervix and <2cm in greatest diameter. It is interesting to note from the findings in this study that none of the four 1B1 tumours were positive for HPV. The excluded 2B tumour had been positive and by inference, it may be that the parametrial and/or vaginal involvement increases the spread of tumour cells rather than the size of the tumour, or depth of invasion, which is limited to the cervix and therefore would have poor lymphatic and vascular access.

Gu *et al* published a recent meta-analysis of circulating HPV DNA as a biomarker for cervical cancer [118]. They found 10 studies published between 2001 and 2018, which met their eligibility criteria, and covered 684 patients with cervical cancer. Six studies were performed in Asian countries, two in France and two in America. Patients with primary or metastatic cervical cancers were included, at stages 1 – 4 and who were receiving all modalities of treatment. Tumour types were both squamous and adenocarcinomas with a greater proportion being squamous cell tumours. Six studies extracted DNA from plasma whilst four studies extracted DNA from serum. All studies used PCR to detect cHPV DNA with two studies using ddPCR specifically. None of the control or pre-invasive disease groups had positive HPV cDNA in the serum. The overall pooled sensitivity and specificity was 0.27 (95% CI 0.24 – 0.30) and 0.94 (95% CI 0.92 – 0.96) respectively. The pooled positive likelihood ratio and negative likelihood

ratio suggests that patients with cervical cancer were 7 times more likely to be HPV cDNA positive than normal controls. Further analysis suggested that HPV cDNA in plasma or serum had a high accuracy for the diagnosis of cervical cancer. The use of ddPCR increases the diagnostic value of HPV cDNA when compared to qPCR [119].

On further analysis of each study, the detection of HPV cDNA in plasma in early-stage cervical cancer showed mixed results with two studies showing no detection at stage 1B [107] [120], 3 studies with <10% detection at stage 1B [121], [108], [122], and another 3 studies showing detection between 24-100% [123], [119], [110]. The studies which described high levels of HPV cDNA in stage 1 tumours, also noted that the viral DNA load was directly proportional to clinical stage; the patients also tended to be high risk i.e., lymphovascular space invasion (LVSI), had deep stromal invasion, and tumour size >20mm. In one study a couple of the samples were taken soon after biopsy for diagnosis, which the authors acknowledge may play a role in disseminating HPV into plasma. This may go someway to explain why many of the early-stage cancers in this study were negative for HPV cDNA.

Fiala *et al* described the challenges in using ctDNA for early-stage cancer diagnosis [100]. As aforementioned, there is usually not enough ctDNA present in such cancers to allow diagnosis which makes it difficult to design highly sensitive assays. Small tumours i.e., <5mm are unlikely to cause symptoms, unlikely to be detected on imaging, have less chance of progressing and easier to cure when detected early. They also reported and estimated that a tumour of $1cm^3$ or 12.5mm in diameter, will release

enough ctDNA to represent 0.01% of all circulating DNA [124] [125]. They concluded therefore that ctDNA could be used to detect tumours of approximately 10mm but not smaller.

The described meta-analysis and reviews above included studies in centres outside of the UK, patients with all stages of cervical cancer, and used more conventional PCR techniques for HPV analysis, which have had limited success. This strength of this study is that it focuses on early-stage cervical cancer only, using a novel NGS assay for HPV, with proven utility in head and neck and advanced cervical cancer. It contributes to the knowledge domain, a UK based, early invasive cervical cancer cohort, therefore aiding the use and validation of this novel assay. We used the existing literature to ensure that the sample collection and processing was optimised, and therefore expected to achieve a greater sensitivity for the detection of disease. The low sensitivity for the detection of HPV cDNA in early-stage cervical cancer may be explained by not only the small size of the tumour but also the inherent difficulties in design of a sufficiently accurate HPV assay. The high specificity, however, does give some assurance that a positive test is more likely to be a true positive.

The limitation of this study is that we were unable to recruit the desired number of patients particularly to the early cervical cancer cohort, due to the coronavirus pandemic, which also made follow-up difficult. The number did not meet that required by the power calculation. However, as only one cervical cancer sample was positive for HPV cDNA, this likely would not have made a statistical difference. Another inherent limitation of this

prospective cohort study was the difficulty with follow-up post treatment. Again, as all but one of the baseline samples were negative, follow up of these patients is unlikely to have added a significant finding to this study. Of the patients that were followed up, there were no discrepancies between baseline and follow up samples i.e., none became positive after treatment having had a negative baseline result. Most importantly, the sole positive patient was followed up and was negative after treatment.

5.1 Conclusion

Based on this study we cannot recommend the use of *HPVdetect* as a biomarker for diagnosis of early-stage cervical cancers, given the low sensitivity demonstrated. However, we have shown that circulating HPV is not detectable in pre-invasive disease, supporting the hypothesis that such lesions do not shed DNA into the circulation. Patients with pre-invasive disease have an effective screening tool, based on high-risk HPV detection and cytology in cervical samples. Whilst work is ongoing on the use of *HPVdetect* as a potential biomarker in monitoring treatment effectiveness and predicting recurrence in locally-advanced cervical cancers, there is still a knowledge gap on the best biomarker for diagnosis and detection of early disease, and further research is needed to establish whether any cDNA assay has sufficient sensitivity for reliable detection of early-stage cervical cancer.

Chapter 6 Bibliography

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LIST OF TABLES & FIGURES

APPENDIX

SOPs submitted separately