The evaluation of the feasibility and clinical utility of liquid based cytology, human papillomavirus testing and high-resolution anoscopy to screen for anal intraepithelial neoplasia in high-risk groups

A thesis submitted to The University of Manchester for the degree of Doctor of Medicine in the Faculty of Medicine and Human Sciences

School of Medicine

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ANALOGY (ISRCTN: 69055668)
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III. ABBREVIATIONS

ACPGBI The Association of Coloproctology of Great Britain and Ireland
AKT Protein Kinase B (PKB)
AIDS Acquired Immunodeficiency Syndrome
AIN Anal intraepithelial Neoplasia
AJCC American Joint Committee on Cancer
AMS Alice Martha Schofield
ANOVA Analysis of variance
ASCJ Anal Squamocolumnar Junction
ASCRS American Society of Colon and Rectal Surgeons
ASCC Anal Squamous Cell Carcinoma
ASCCP American Society for Colposcopy and Cervical Pathology
ASC-H Atypical cells cannot exclude High Grade Disease
ASCUS Atypical Cells of Uncertain Significance
ASIL Anal Squamous Intraepithelial Neoplasia
ATZ Anal Transitional Zone
CAP College of American Cytopathologists
CAP-ASCCP College of American Cytopathologists - American Society for Colposcopy and Cervical Pathology
CI Confidence Interval
CIN Cervical Intraepithelial Neoplasia
CMFT Central Manchester Foundation Trust
CRF Clinical Research Form
CSCC Cervical Squamous Cell Carcinoma
CT Computerised Tomography scanning
DNA Deoxyribonucleic Acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>DRE</td>
<td>Digital Rectal Exam</td>
</tr>
<tr>
<td>DPCP</td>
<td>Detectable Pre Clinical Phase</td>
</tr>
<tr>
<td>ECM</td>
<td>Extra Cellular Matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine Tetracetic acid</td>
</tr>
<tr>
<td>EUA</td>
<td>Examination Under Anaesthetic</td>
</tr>
<tr>
<td>FLOQ™</td>
<td>Nylon® fibre tipped swab for collecting anal liquid based cytology</td>
</tr>
<tr>
<td>GUM</td>
<td>Genito Urinary Medicine</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haemytoxylin and Eosin staining</td>
</tr>
<tr>
<td>HAART</td>
<td>Highly Active Anti-Retroviral Therapy</td>
</tr>
<tr>
<td>HG</td>
<td>Henry Galletta</td>
</tr>
<tr>
<td>HGAIN</td>
<td>High Grade Anal Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>HGCIN</td>
<td>High Grade Cervical Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>HIM</td>
<td>The Natural History of HPV Infection in Men Study</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HPF</td>
<td>High Power Field</td>
</tr>
<tr>
<td>HPV</td>
<td>Human Papilloma Virus</td>
</tr>
<tr>
<td>HR-HPV</td>
<td>High Risk Human Papilloma Virus</td>
</tr>
<tr>
<td>HRA</td>
<td>High Resolution Anoscopy</td>
</tr>
<tr>
<td>HSIL</td>
<td>High Grade Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>HSPG1</td>
<td>Heparin Sulphate Proteoglycans Receptors</td>
</tr>
<tr>
<td>H-SCORE</td>
<td>Standardised Scoring System for Histopathology Staining</td>
</tr>
<tr>
<td>ICER</td>
<td>Incremental Cost Effectiveness Ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>-IN</td>
<td>Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>JCVI</td>
<td>Joint Committee on Vaccination and Immunisation</td>
</tr>
<tr>
<td>Ki67</td>
<td>Antigen, nuclear protein for cellular proliferation. Associated with ribosomal RNA transcription</td>
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LAT Lower Anogenital Tract
LAST Lower Anogenital Tract Squamous Terminology project
LBC Liquid Based Cytology
LGAIN Low-grade Anal Intraepithelial Neoplasia
LLETZ Large Loop Excision of the Transformation Zone
LR-HPV Low Risk Human Papilloma Virus
LSIL Low-grade Squamous Intraepithelial Lesion
MCSH Manchester Centre for Sexual Health
MNA Mahshid Nikkhoo Amiry
MRI Magnetic Resonance Imaging
MSM Men Who Have Sex with Men
MSW Men Who Have Sex with Women
NATSAL National Attitudes to Sex and Lifestyle 1, 2 & 3
NBF Neutral Buffered Formalin (aldehyde)
NHS National Health Service
NHIS National Health Interview Survey
NILM Negative for Anal Invasive Malignancy
NMGH North Manchester General Hospital
OPD Out Patient Department
p16 Gene, Cyclin Dependent Kinase Inhibitor
PAIN Peri-anal Intraepithelial Neoplasia
PCR Polymerase Chain Reaction
PeIN Penile Intraepithelial Neoplasia
PDL Pectinate Dentate Line
PIK3CA Phosphatidylinositol-4, 5-bisphosphate 3-kinase
pRb Retinoblastoma Protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ProExC™</td>
<td>Immunoperoxidase Biomarker used in immunohistochemistry containing antibodies against topoisomerase II alpha and minichromosome maintenance 2 proteins</td>
</tr>
<tr>
<td>Rb</td>
<td>Retinoblastoma Gene</td>
</tr>
<tr>
<td>RMcM</td>
<td>Raymond McMahon</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Controlled Trial</td>
</tr>
<tr>
<td>SCJ</td>
<td>Squamocolomnar Junction</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SPANC</td>
<td>Study into the Prevention of Anal Cancer (Australia)</td>
</tr>
<tr>
<td>SIL</td>
<td>Squamous Intraepithelial Lesion</td>
</tr>
<tr>
<td>SIR</td>
<td>Standardised Incidence Rate</td>
</tr>
<tr>
<td>STI</td>
<td>Sexually Transmitted Infection</td>
</tr>
<tr>
<td>TBS</td>
<td>The Bethesda System</td>
</tr>
<tr>
<td>Tp53</td>
<td>Tumour Protein 53</td>
</tr>
<tr>
<td>TR</td>
<td>Transplant Recipients</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>UCSF</td>
<td>University of California San Francisco</td>
</tr>
<tr>
<td>UICC</td>
<td>Union Internationale Controle Cancer</td>
</tr>
<tr>
<td>US</td>
<td>United States</td>
</tr>
<tr>
<td>VaIN</td>
<td>Vaginal Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>VIN</td>
<td>Vulvar Intraepithelial Neoplasia</td>
</tr>
<tr>
<td>WHIS</td>
<td>Women’s Interagency HIV Study</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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IV. ABSTRACT

The evaluation of the feasibility and clinical utility of liquid based cytology, human papillomavirus testing and high-resolution anoscopy to screen for anal intraepithelial neoplasia in high-risk groups.

Dr Alice Martha Schofield: The University of Manchester for the degree of Doctor of Medicine, January 2016.

Background: The increased incidence and natural history of anal cancer in high-risk groups, provides a screening opportunity to detect precancerous lesions, anal intraepithelial neoplasia (AIN), as well as early invasive lesions. The ANALOGY study was performed to strengthen the evidence base required to determine the case for anal screening in terms of the feasibility and clinical utility of liquid based cytology (LBC), high-risk human papillomavirus (HR-HPV) testing and high-resolution anoscopy (HRA) in high-risk groups.

Methods: This prospective study offered screening to four cohorts aged over 25 at varying but elevated risk; human immunodeficiency virus (HIV) positive and negative men who have sex with men (MSM), HIV positive women with prior history of abnormal cervical cytology or anogenital warts, HIV negative women who practice anoreceptive sex and transplant recipients (TR). Recruitment commenced in March 2013 and concluded in December 2014, with follow-up until March 2015. All participants underwent testing for HR-HPV, LBC and had HRA performed, sites of abnormality were biopsied. Participants were seen at initial consultation and at a second visit six months later. Immunostaining with Ki67 and p16 antibody was performed on 100 anal tissue biopsies. The cellular positivity of each biomarker were scored by automated and manual methods. H-SCORES of p16 biomarker and block positive staining of AIN2 were quantified and analysed.

Results: 409 participants were recruited; 284 MSM (203 HIV positive, 81 HIV negative), nine HIV positive women, four HIV negative women and 112 TR. HR-HPV was highly prevalent in anal samples from MSM (HIV positive 88.0% and HIV negative, 77.8%) and much less so in HIV positive and negative women and TR (19.3%). Despite the high prevalence of cytological abnormality in MSM, almost half of AIN of all grades was associated with negative cytology. AIN3+ on biopsy was found in 4.4% (18/409) of participants; three HIV positive MSM had cancer. One new case of AIN3 was identified at the second visit. Low-grade disease (AIN1/2) was highly prevalent in all groups. Ki67 and p16 biomarker expression increase as the grade of anal disease increased when scored manually. AIN2 histology samples, which demonstrate block positive staining, have an association with an increased H-SCORE.

Conclusions: Anal screening in some high-risk groups is clinically feasible in terms of diagnostics with evidence of significant disease prevalence particularly amongst MSM. The high prevalence of HR-HPV infection and frequency of false negative cytology indicates that in terms of sensitivity and specificity, HRA would be the best primary screening tool. The use of Ki67 and p16 in the identification of anal disease appears to have clinical utility, especially in the detection of AIN2; with the majority of samples displaying block p16 staining that corresponded with an increased H-SCORE. The prevalence of AIN3+ in HIV positive MSM lends support for a policy of screening in this group, however limitations of treatment, as well as highly prevalent low-grade lesions of dubious significance, require careful consideration.
V. DECLARATION

I declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other institute of learning. I independently conducted all of the clinical screening procedures as well as the immunohistochemistry readings and generated the entire dataset for analysis.

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VIII. THE AUTHOR

The author graduated M.B. Ch.B. from the University of Manchester in 2006 and completed Foundation Training at Salford Royal Foundation Trust. A fourth year placement as a student doctor in Obstetrics and Gynaecology sparked interest in the specialty. Following Foundation training, the author secured a training number in Obstetrics and Gynaecology in the North Western Deanery. The desire to train as a Gynaecological Oncologist prompted a postponement in general training. A year was spent demonstrating anatomy at the University of Manchester to further understanding of the structure and function of the human body whilst gaining valuable teaching experience of medical and dental students. In 2009 the author commenced general training in Obstetrics and Gynaecology. In 2012, as specialty training registrar, interest in human papilloma virus driven anogenital disease and precancerous screening led the author to be appointed as Gynaecological Clinical Research Fellow at the University of Manchester. The author has been lead clinician for the ANALOGY study (ANAL cytology and HPV testing in the prevention of anal cancer) for the past three years. In this time the author has also trained as a Forensic Physician at St Mary’s Sexual Assault Referral Centre, sat on the undergraduate medical education committee for Obstetrics and Gynaecology at Central Manchester Foundation Trust and been a mentor for undergraduate student doctors.

In April 2015 when the ANALOGY study closed, the author returned to the North West Deanery for one day a week and for a further ten months completed onwards referrals, discharges, data completion for the study and wrote this thesis. The author will return to full time training in February 2016.
IX. DEDICATION

For Jordan,
My family and
My Nan,
Monica I miss you every day.
CHAPTER 1

INTRODUCTION
1 INTRODUCTION

The United Kingdom (UK) National Screening Committee has suggested that screening for the precursor lesion of anal squamous cell carcinoma (ASCC), anal intraepithelial neoplasia (AIN), in populations at high-risk of would be of benefit. The ANALOGY study was established to evaluate the feasibility and effectiveness of liquid based cytology, human papilloma virus (HPV) testing, high-resolution anoscopy (HRA) and anal tissue biopsy in the detection of AIN of all grades. The ANALOGY study sought to establish a baseline prevalence of disease in high-risk groups and permitted comparison of incidence between high-risk groups in the UK. All other significant data surrounding screening for AIN has been produced in other national healthcare systems. The ANALOGY study required the development of a new service within Central Manchester Foundation Trust (CMFT) for the provision of an anal screening service. This encompassed a multidisciplinary team from many different specialties in order to provide the service.

Screening high-risk groups for AIN may reduce the incidence of anal cancer (Smyczek et al., 2013). Currently there is no established national screening programme in the UK or indeed worldwide.

1.1 The Anus

Anatomical or histological landmarks can define the anal canal. Understanding the embryological development and the formation of anal canal anatomy provides insight into the development of anal neoplasia.

1.1.1 Anal embryology

By completion of the fourth week of embryological development, the gut tube, which is derived from endoderm, differentiates into foregut, midgut and hindgut (Pokorny WJ, 1996) see Figure 1. During embryological development, endodermal and ectodermal germ cell layers fuse to form the anal canal (Tench, 1936). The upper two thirds of the anal canal is derived from hindgut (endoderm) and the lower third from the anal pit (ectoderm) (Moore K, 2007). The junction of the anal pit and the hindgut is identified by a macroscopic landmark, the pectinate dentate line (PDL) (Pansky, 1982). The PDL is at the level of the anal or semi-lunar valves, separating the upper two-thirds and lower one-third of the anal canal (Pansky, 1982). The PDL is also the site of the anal membrane (Moore K, 2007). The PDL also overlies the anal transitional zone (ATZ) and is the site of the anal squamocolumnar junction (ASCJ) where the columnar epithelium of the rectum is replaced by transitional epithelium of the anus (Martin et al., 2009). The anus, like the cervix, undergoes remodelling as the result of metaplasia. There is replacement of the glandular epithelium by squamous epithelium, sometimes known as transitional epithelium. The histology of the anus is similar to that of the cervix. The transition in the anus from squamous epithelium to the columnar epithelium of the rectum is where most malignancies are identified. The squamocolumnar junction (SCJ) can be seen clearly in Figure 2 (a) in the anus and 2 (b) the cervix.
Figure 1 Development of the hindgut, Chapter 86 (Pansky, 1982)
Figure 2 a & b Haemotoxylin and eosin staining of the anal and cervical squamocolumnar junctions

2a Anal squamocolumnar junction x4 magnification

Image used with permission from Dr Ray Mcmahon, CMFT, Manchester, UK

2b Cervical squamocolumnar junction x4 magnification

Image used with permission from Dr Rhona McVey, CMFT, Manchester, UK

Figure 2a & 2b: The squamocolumnar junction has a step in the tissue architecture due to the change between squamous and columnar epithelium. Stratified squamous epithelium are flat and scale-like, it is generally multi-layered. The basal layer is in contact with basal lamina (the collagen membrane between the epithelia and capillaries) and is selectively permeable. Columnar epithelium is composed of a single layer of tall cells with dark-staining nuclei close to the basement membrane.
1.1.2 Anal anatomy

There has been much confusion about the boundaries of the anal canal. Examples of terminology used are the surgical or functional anal canal along with the embryological, histological or anatomical anal canal (see Figure 3). The surgical canal has been described as 4cm length from the anal verge to the ano-rectal ring, conversely the embryological or anatomical anal canal boundaries were described as from the anal verge to the PDL; this is 2cm shorter (Corman, 2004).

It is now the consensus that the anal canal extends from PDL to the anal verge (the ASCJ with the perianal skin), measuring 5cm in length (Minsky, 2010). The American Society of Colon and Rectal Surgeons (ASCRS) introduced this definition in 2007. This is supported by both the American Joint Committee on Cancer (AJCC) in its staging manual, and the Union Internationale Contra le Cancer (UICC) (Welton ML, 2007). The ASCRS standardisation has clarified the anal canal boundaries.

The anal canal is defined anatomically where the rectum enters the pelvic floor through the levator ani muscle, puborectalis and it lies inferiorly to the pelvic floor (Mahadevan, 2011, Moore KL, 2010). This makes the anal canal completely extraperitoneal. Proximal to the anus is the rectum; distal to the anus is the anal verge and the apex of the anal sphincter complex is where the distal rectum meets the anus at the ano-rectal junction. The anal canal is surrounded by the sphincter ani-externus and the ischio rectal fossae laterally and has no peritoneal covering (Standring, 2008) (Moore, 2010). The anal canal blood supply, innervation and lymphatic drainage all reflect the embryological origin (Moore, 2010).

The ATZ is located 6-12mm proximal to the PDL as seen in Figure 4, and is 4-5mm in length (Fenger, 1987). Proximal to the PDL, the anal canal is relatively insensitive to pain. The tissue is comprised of crypts, columnar and goblet cells. The epithelium, distal to the PDL, is non-keratinised stratified squamous, is pain sensitive and appears smooth in texture. There are no sweat glands; it is devoid of epidermal appendages such as hair follicles and apocrine glands, however the tissue contains melanocytes. At the anal verge the squamous mucosa merges with the peri-anal skin (true epidermis) and there are hair follicles, keratin and apocrine glands (Corman, 2004) as seen in Figure 4.

The anal sphincter complex can be palpated clinically by digital rectal examination (DRE) roughly 1-2cm above the PDL and can be palpated along all of its length (Standring, 2008). The distal end of the canal at the anal verge is defined as a palpable groove between the lower edge of the internal sphincter and the subcutaneous part of the external sphincter (Fenger, 1987).
Figure 3 A picture demonstrating the differences in boundaries of the anal canal

Figure 4 A picture illustrating the histological boundaries of the anal canal

A: Columnar epithelium

B: Anal transitional zone (ATZ): squamous metaplasia

C: Squamous epithelium

D: Keratinised squamous and skin appendages
1.1.3 Anal disease

1.1.3.1 Anal intraepithelial neoplasia pathology

Fenger and Bichel first described dysplastic changes in the anal canal in 1981. In this study areas of dysplasia were found adjacent to the majority of ASCC (Fenger and Bichel, 1981). Anal intraepithelial neoplasia (AIN) was the term coined by Fenger and Neilson in 1986 to describe these dysplastic changes. Since this time the classification of anal cytology and histology samples has evolved dramatically. Cyto and histopathologists are able to report liquid based cytology and anal tissue biopsy samples respectively in the identification of anal dysplasia. Pre-cancer and cancer of the anal canal in association with high-risk human papilloma virus (HR-HPV) infection has become plausible since the link between persistent HR-HPV 16 infection and cervical disease was made (Parkin and Bray, 2006). Documented clinical cases involving HR-HPV infection with oncogenic properties in both pre-invasive and invasive anal disease has now cemented this association (Hoots et al., 2009). This link has been acknowledged by the International Agency for Cancer in 1995 (Durst et al., 1983, IARC, 1995). The precancerous phase in the development of ASCC is termed high-grade anal intraepithelial neoplasia (HGAIN) or more recently high-grade squamous intraepithelial lesion (HSIL) (Darragh et al., 2012b). These lesions can be further sub-classified into AIN grades 2 and/or 3. Like CIN3, AIN carries an increased risk of progression to invasive disease (Palefsky et al., 1998).

To date, routine screening for anal cancer (Mani and Aboulafia, 2013) or its supposed precursor HGAIN is not yet recommended, even in high-risk groups. The management of low-grade lesions LGAIN1 / low-grade squamous intraepithelial lesion (LSIL) is also not standardised.

For the purpose of this thesis the terminology high-grade and low-grade AIN will be used.

1.1.3.2 Prevalence of anal intraepithelial neoplasia

The prevalence of AIN of all grades is highest in human immunodeficiency virus (HIV) positive men who have sex with men (MSM) (Denny et al., 2012), followed by HIV negative MSM (Chin-Hong et al., 2008). Studies of AIN in women are limited, they predominantly focus on HIV positive cohorts only (Denny et al., 2012). A study from the United States (US) in 2009 concluded that the prevalence of AIN in HIV positive women was high and significantly increased above a comparison group of HIV negative women (Hessol et al., 2009). In the transplant population, data are also limited and only a handful of studies have used cytology and or histology samples in the detection of AIN with or without HPV testing. One study demonstrated that a significant proportion of transplant recipients (TR) had AIN (Ogilvie et al., 2008). Forty TR participated, 15 were women and 25 were men; their median duration of immunosuppression was 5.6 years. Of all cytology specimens, 88% (35/40) had sufficient cells for interpretation and 6% (2/40) demonstrated dysplasia. Biopsies were performed in 11 patients; six had dysplasia (four low-grade, two high-grade). Normal anal cytology was seen in five patients. The sensitivity of cytology to predict histologic abnormality was 17% (Ogilvie et al., 2008). Overall, 18% (7/40) of patients had anal dysplasia detected by cytology or histology; 5%
(2/40) had high-grade dysplasia (Ogilvie et al., 2008). Another study conducted in a cohort of TR in a London hospital (Patel et al., 2010) used anal cytology and polymerase chain reaction (PCR) to assess anal HPV disease. One hundred and eight patients participated, 68 men and 40 women. The median duration of immunosuppression was 75-months. Patel et al demonstrated that anal dysplasia was associated with anal HR-HPV infection ($p \leq 0.001$), previous genital warts ($p = 0.018$), duration of immunosuppression ($p \leq 0.050$), and receptive anal intercourse ($p \leq 0.013$) (Patel et al., 2010). Other factors were also recorded in this study; 4.6% (5/108) had engaged in anoreceptive sex; all were women. Genital warts were reported by 8.3% (9/108) of the cohort (six men and three women). Five patients (4%; three men and two women) recalled a previous sexually transmitted infection (STI) (Patel et al., 2010). A meta-analysis comparing immune deficiency due to HIV infection and organ transplantation demonstrated that anal cancer standardised incidence rate (SIR) was much higher in HIV positive individuals (Grulich et al., 2007).

1.1.3.3  **Clinical presentation of anal intraepithelial neoplasia**

AIN is usually asymptomatic and is detected during HRA. The clinical appearance of AIN is heterogenous and may include whitish, scaly, erythematous, papillomatous, papular or fissured plaques (Zbar et al., 2002, Abbasakoor and Boulos, 2005). The application of acetic acid allows the visualisation of lesions to be seen more clearly (see Figure 5) (Palefsky, 2012).

**Figure 5 Photograph of AIN during HRA**

![Photograph of AIN during HRA](image)

*With permission from Dr Mayura Nathan, Homerton University Hospital, London*

Figure 5: The ASCJ after the application of 3% acetic acid. There is intense acetowhite uptake at the 12 o'clock position with striations. This is an area of suspicion and requires biopsy to exclude high-grade disease.
1.1.3.4 The prevalence of HPV infection in patients with anal intraepithelial neoplasia

Hoots et al completed a systematic review of HR-HPV types found in anal cancer and high-grade and low-grade squamous intraepithelial lesions (HSIL and LSIL) (Hoots et al., 2009). This study demonstrated HR-HPV infection was identified in up-to 88% of LSIL (AIN1) and 91% of HSIL (AIN2/3) (Hoots et al., 2009). HR-HPV positivity was dependant on the method of DNA detection method used. Individuals who had AIN and tested HR-HPV positive were infected with HR-HPV types 16 and/or 18 in up to 27% of LSIL (AIN1) and 69% of HSIL (AIN2/3) (Hoots et al., 2009).

1.1.3.5 Similarities between anal and cervical intraepithelial neoplasia

Anal and cervical cancers both develop in a region of immature metaplastic epithelium, the anal transitional zone (ATZ) and cervical transformation zone (CTZ). At these sites there is active metaplasia, where columnar epithelium meets squamous epithelium. Metaplasia in the cervix is accelerated by vaginal acidity and in the anus by trauma repair associated with ano-receptive intercourse (Darragh, 2004). Both the ATZ and CTZ are particularly susceptible to HR-HPV infection with oncogenic potential and may well result in dysplasia that can progress to invasive disease (Palefsky, 1994). It is considered the majority of AIN can be identified microscopically at HRA using an anoscope to access the anal canal and a colposcope to visualise epithelial changes. ASCC can be identified macroscopically or microscopically in close proximity to or at the ATZ (Goldstone et al., 2001). One small study of TR (n=50) demonstrated that not all lesions detected during HRA and biopsied from the ATZ in displayed AIN (Tramujas da Costa e Silva et al., 2008). Cervical intraepithelial neoplasia (CIN) is easily identified at the cervical transformation zone (CTZ) using a speculum and a colposcope. Figure 6 summarises the similarities between identification and detection of cervical and anal precancerous lesions.

1.1.3.6 Malignant potential of cervical intraepithelial neoplasia

The potential for progression of CIN is well documented in an unethical policy completed between 1955-1976, in a large cohort of New Zealand women. The definitive treatment of CIN3 was withheld in this cohort, and the cumulative incidence of cervical or vaginal vault cancer at 30-years was 31% compared to 0.7% who received adequate treatment (McCredie et al., 2008). This kind of study cannot be duplicated in the anus due to ethical reasons because it is widely accepted that AIN can progress to cancer.
Figure 6: Anogenital intraepithelial neoplasia is the result of HR-HPV infection. In both the cervix and the anus, the transformation zone is most common site of infection. At the transformation zone columnar and squamous epithelium meet. As columnar epithelium is only one cell thick, trauma is common and a break in the basement membrane permits infective entry of HR-HPV. If the infection persists and is not cleared, this then becomes the site of abnormality. Squamous intraepithelial neoplasia can develop in both anal and cervical tissue. In order to visualise the transformation zone in both the anus and the cervix a clear plastic anoscope or vaginal speculum must be used. Once inserted acetic acid can be applied which identifies abnormality that is visible using a microscope. Any area of abnormality can then be biopsied for histopathological analysis.
1.2 Anal cancer

1.2.1 Epidemiology

Anal squamous cell carcinoma (ASCC) is rare in the general population affecting less than 2 cases per 100,000 per year (Grulich et al., 2012). The incidence of ASCC in the general population is increasing in both sexes (Nielsen et al., 2012). Over the past 30-years the incidence of ASCC has increased substantially in certain high-risk groups, accounting for a significant amount of disease burden (Johnson et al., 2004, Jin et al., 2011, Joseph et al., 2008). This rising trend has been observed in the UK, with women having a greater rise 1960 to 2004 (Robinson et al., 2009). A twofold increase has been recorded in Scotland between 1975 to 2002 in both sexes (Brewster and Bhatti, 2006). In the UK during 2011 there were 1175 new cases of ASCC, 414 men and 761 women. Incidence in women who live in the UK continues to be higher than in men; 2.4 per 100,000 compared to 1.3 per 100,000 population. The number of deaths from anal cancer in the UK during 2012 was 307 persons; 115 men and 192 women (ONS, 2013).

Patients who are at a higher risk than the general population of developing ASCC are immunosuppressed due to HIV infection or chronically immunosuppressed due to solid organ transplantation (Arends et al., 1997, Roka et al., 2004, Sillman et al., 1984, Sillman et al., 1997, Sillman and Sedlis, 1991). A recent meta-analysis reported that anal cancer incidence was 5.1 per 100,000 in MSM (Machalek et al., 2012a). HIV positive MSM risk of developing anal cancer is increased up-to 100-fold (Silverberg et al., 2012). HIV negative MSM risk is documented as 14 per 100,000 years, the observed and expected numbers of anal cancer in one study were 15 and 0.6 producing a SIR of 24.2 (CI 13.5-39.9) (D’Souza et al., 2008, Koblin et al., 1996). In HIV positive women, the risk of developing anal cancer is 14-times higher than in HIV positive women who have been diagnosed with acquired immune deficiency syndrome (AIDS) (Chaturvedi et al., 2009). The risk of anal cancer for solid organ TR has been estimated at six to 14-times higher when compared with the general population (Grulich et al., 2007, Sunesen et al., 2010).

It is considered that persistent infection of the anal canal with HR-HPV, particularly types 16 and/or 18, is necessary for the development of AIN and ASCC. Until 1996 there were no effective treatment modalities for HIV positive patients. The introduction of highly active antiretroviral treatment (HAART) does not appear to have any impact on reducing the incidence of anal cancer in HIV positive MSM (Silverberg et al., 2012, D’Souza et al., 2008). Also, before the therapeutic use of HAART, HIV positive MSM died from AIDS before they developed anal cancer.

Patients who are immunosuppressed and also practice anoreceptive sex are at highest risk of developing ASCC as there is an increased likelihood of acquiring anal HR-HPV infection that persists (Palefsky, 1999). Attempting to estimate the proportion of the male population who practice anoreceptive sex is not straightforward. Recent data collected in the UK suggests that
6% of men aged 15-60-years of age report a same sex experience; this equates to approximately 1-million men (Mercer et al., 2013). It is estimated that 43, 500 MSM in the UK are HIV positive, (Aghaizu A, 2013).

1.2.2 Risk factors for the development of anal intraepithelial neoplasia and anal cancer

1.2.2.1 The practice of anoreceptive sex and the acquisition of human papillomavirus infection

In the latter half of the 20th century sexual behaviours have changed. Since the 1970’s the rising incidence of anal cancer of roughly 2% a year in both men and women is in part attributable to the exposure to HPV in the anal canal. Anoreceptive sex is the likely mode of anal HPV infection but is not essential in the acquisition of anal HPV (Ong et al., 2015a, Roka et al., 2004, Nyitray, 2012). In 2010 McBride and Fortenberry reviewed the prevalence and frequency of heterosexual anal intercourse in the US. They concluded that a lifetime prevalence of heterosexual anal intercourse was between 6-40% with 10% heterosexuals reporting at least one instance of anal intercourse within the past year (McBride and Fortenberry, 2010). Data from the third National Survey of Sexual Attitudes and Lifestyle (NATSAL-3) in Britain found that anal sex is infrequently reported but practice of anoreceptive sex had increased in men and women between NATSAL-1 and NATSAL-2, and between NATSAL-2 and NATSAL-3 (Mercer et al., 2013). A large meta-analysis on gender differences surrounding sexual practices found 72-articles published between 1993 and 2007 reporting incidence or frequency of receptive or insertive heterosexual anal intercourse. Men were found to be more likely to report anal intercourse compared with women (Petersen and Hyde, 2010). It is likely that anoreceptive sex is prevalent but not reported.

Any individual in a high-risk group who practices anoreceptive sex further increases his or her risk of developing AIN and therefore ASCC. Sex workers and recreational drug users also practice anoreceptive sex frequently (Powis et al., 1995), and these groups probably also have an increased lifetime risk of developing anal cancer which may be attributable to HPV acquisition by the practice of anoreceptive sex. To date no data has been collected to prove or disprove these hypotheses. Although mainly transmitted by sexual intercourse (Kjaer et al., 2001), HPV infection can be acquired through non-penetrative sexual contact between the female anus and male scrotum, along with female hand and male genital contact (Hernandez et al., 2008, Widdice et al., 2010). This suggests that the acquisition of anal HPV does not appear to require anoreceptive sex (Goodman et al., 2010).

1.2.2.2 The prevalence of anal HPV in anal cancer

In a global review, overall HPV infection has been demonstrated in up to 71% of ASCC depending on the method of deoxyribonucleic acid (DNA) detection method. Oncogenic HPV types 16 and/or 18 infection was seen in 72% of ASCC (Hoots et al., 2009). The data from this review demonstrates a type 16 and/or 18 prevalence of 70%, similar to that found in CSCC.
In another study, 80% of ASCC were caused by infection with HR-HPV (De Vuyst et al., 2009).

### 1.2.2.3 Anal HPV prevalence in HIV positive and negative MSM

When comparing the high-risk populations at risk of developing ASCC, HIV positive MSM have the highest proportion of anal HPV infection (Denny et al., 2012). Detectable type-specific anal HPV infection in one study was 21.3 per 100 person-years [95% confidence interval (CI) 17.7-25.4] (Hernandez et al., 2014). In the same study infection with oncogenic HPV types was 13.3 per 100 person-years [95% confidence interval (CI) 10.5-16.6] (Hernandez et al., 2014). In a recent study of the prevalence of anal HPV, overall 93.3% and 72.4% of HIV positive and negative MSM respectively were infected. Multiple HPV infections were evidenced in 48.2% of the HIV negative and 76.1% of the HIV positive MSM (Latini et al., 2014).

### 1.2.2.4 Anal HPV prevalence in HIV negative men who have sex with women

There are few studies reviewing the natural history of men who have sex with women (MSW) and anal HPV infection. One study recruited men in Brazil (São Paulo), Mexico (Cuernavaca), and the US (Tampa) for the HPV in Men (HIM) Study, a cohort study of the natural history of anogenital HR-HPV (Nyitray et al., 2010). Men who completed their initial HIM Study visit between July 2005 and February 2007 were included in the MSW study (n=1392). HIV negative MSW were found to have substantially lower prevalence of anal HR-HPV infection, 12%. In this cohort 7% of MSW had at least one oncogenic HPV type (Nyitray et al., 2010). In men, a factor contributing to increased risk of any anal HPV infection is lifetime number of female sex partners (Moscicki et al., 2014).

### 1.2.2.5 Anal HPV prevalence in HIV positive and HIV negative women

Studies regarding the prevalence of anal HPV infection in women are limited. One of the first studies comparing women involved HIV positive and HIV negative women who were at high-risk of contracting HIV infection (Moscicki et al., 2014). Several studies demonstrate that the rates of anal HPV infection are equal to or more common in these women than cervical HPV infection (Moscicki et al., 2004, Palefsky et al., 2001). A study conducted amongst healthy women in Hawaii demonstrated a prevalence of anal HPV comparable to cervical HPV infection at 27% and 29% respectively (Shvetsov et al., 2009). Prospective follow-up of these women displayed half of them had an incident anal HR-HPV infection, of which 58% cleared the infection at a one-year follow-up (Shvetsov et al., 2009). Observed factors contributing to persistent anal HR-HPV infection include tobacco smoking, douching, anoreceptive sex and risk taking behaviour that was alcohol induced (Moscicki et al., 2014).

### 1.2.2.6 Anal HPV prevalence in transplant recipients

Oncogenic anal HPV infection in renal TR has generally been documented when studying AIN. The prevalence of HPV when collecting anal cytology was 21.3% (23/108) in TR (Patel et al., 2010). HPV typing was possible in 95.8% (23/24) of samples collected. Patients who had anal
HPV infection had a longer duration of immunosuppression (Patel et al., 2010). In a study investigating the prevalence of anal HR-HPV in liver TR, active hepatitis B infection was associated with an increased prevalence of any HPV infection (Grat et al., 2014). Grat et al concluded that the presence of any HPV infection was significantly associated with a higher number of sexual partners (p=0.020) and a younger age of sexual debut (time of first sexual intercourse). There were no differences in anal HPV detection rates with respect to the type of immunosuppressive medication (Grat et al., 2014).

1.2.2.7 Men who have sex with men

The association between MSM and anal cancer has been known since the 1970s from a series of published cases (Daling et al., 1982). The highest incidences of ASCC are seen in HIV infected MSM, where the incidence is as high as 60-112 per 100,000 (D'Souza et al., 2008, Scholefield et al., 2005, Piketty et al., 2008). This is comparable with the rates of cervical cancer prior to the introduction of Papanicolau screening cytology (Chiao et al., 2006). There is a higher incidence of anal neoplasia in men who were never married with a single marital status at the time of tumour registration (as a marker of MSM) compared with married heterosexual men (Scholefield et al., 1990).

1.2.2.8 Immunosuppression

A recent meta-analysis by Grulich et al. demonstrated that 20 of 28 cancer types studied had an increased incidence in patients with HIV/AIDS or TR, suggesting that immunodeficiency may increase risk (Grulich et al., 2007). Most of the cancer in the analysis had an infectious cause, such as HPV related cancers, Hodgkins lymphoma, liver and stomach cancers (Grulich et al., 2007). Interestingly, a recent meta-analysis observed an association between HR-HPV infection and HIV acquisition (Lissouba et al., 2013). Incident HIV infection was significantly associated with HR-HPV infection in five out of six studies reviewed (Lissouba et al., 2013). Oral HR-HPV infection is common amongst HIV positive men and women (Kreimer et al., 2004), demonstrating a lack of clearance in immunosuppressed individuals.

1.2.2.9 Immune response to human papillomavirus infection

The failure of a normal immune response allows the persistence of HR-HPV infection. Most papillomavirus infections are cleared within 12-months in an immune competent individual (Crosbie et al., 2013b), by cell-mediated immunity. Cell mediated immune deficiency due to HIV infection can lead to florid LR-HPV lesions and an increased risk of progressive disease (Moscicki et al., 2004; Palefsky and Holly, 2003). In an immune competent individual, L1 antibody major viral capsid proteins can still be detected six months after infection (Carter et al., 2000) and a level can still be measured in individuals who have cleared a HR-HPV infection (Wang et al., 2004). Individuals who do not successfully clear a HR-HPV infection are at increased risk of developing malignancy (Hathaway, 2012). Immune compromised individuals who fail to amount an appropriate cell mediated response have persistent HR-HPV infections.
Persistent HR-HPV infection in the anal canal increases the probability of progression from HGAIN to invasive anal cancer (Doorbar et al., 2012). In immune competent individuals, further study of HPV, host protein interaction and steps in oncogenic transformation are needed to ascertain why some patients develop malignancy and others clear the virus without issue.

1.2.2.10 Human immunodeficiency virus

HIV positive patients are immunosuppressed. Any individual who has a CD4+ count below 200 cells/µl and an active HR-HPV types 16 and/or 18 infection, are considered to be at greater risk of developing of HGAIN and ASCC (Northfelt, 1996). More advanced immunosuppression is suspected to be associated with increased HPV replication. This is reflected by a low CD4+ and an increased rate of abnormal anal cytology in HIV positive patients (Scott et al., 2008).

1.2.2.11 HIV and highly active antiretroviral treatment

HIV is now a very treatable disease. The advent of HAART in 1996 has changed prognosis and mortality for HIV infected individuals, with AIDS defining conditions being seen less frequently. The restoration of immune function in HIV positive patients is successfully achieved by HAART, however anal cancer in HIV positive MSM still continues to rise (Piketty et al., 2008, Powles et al., 2009). This is thought to be due to improved survival of HIV infected patients and sufficient amount of time for HPV associated AIN to develop into malignancy (D’Souza et al., 2008). One study showed combination HAART does not prevent anal cancer (Piketty et al., 2008), however conflicting data from another study demonstrated that patients receiving greater than four years of the same HAART regimen have a reduced risk of HGAIN (de Pokomandy et al., 2011).

HIV positive individuals commence HAART when CD4+ count falls below 350cells/µl (Kober et al., 2012), and it is possible that maintaining a higher CD4+ level by commencing earlier treatment with HAART may reduce risk of ASCC in this patient group (Chao et al., 2012). Immune function would then be normalised and able to surmount an appropriate response to clear any HR-HPV infection therefore reducing oncogenic potential. A recent study in HIV positive MSM demonstrated the time from HGAIN to invasive cancer was shorter if CD4+ nadir was very low in the pre HAART era (Duncan et al., 2015). A CD4+ nadir of less than 100cells/µl and a longer duration of immunosuppression with a CD4+ of less than 100cells/µl were associated with a shorter time frame in the development of anal cancer (Duncan et al., 2015). HAART studies involving cervical disease have shown reduced progression to high-grade CIN (HGCIN) along with regression of CIN to normal epithelium (Heard et al., 2004), though this is not consistent in all studies as there is still marked progression to HGCIN in a proportion of these patients, but it does appear that HAART has a limited ability to clear HPV infection (Palefsky, 2003).
1.2.2.12 **Solid organ transplantation**

Solid organ transplantation is a risk factor for ASCC in both sexes due to iatrogenic immunosuppression (Tramujas da Costa e Silva et al., 2008, Ogunbiyi et al., 1994). Renal transplant recipients have an increased incidence of anal cancer at 14 per 100,000 population in comparison to the general population (Patel et al., 2010). Long-term survival after solid organ transplantation with immunosuppressive medication is now associated with the rising propensity in the development of certain types of cancers (Euvrard et al., 2003). Post-transplant malignancy has become an important cause of mortality and it is thought that in the next 20 years it will be the leading cause of death (Buell et al., 2005). It has been observed that anogenital cancers are typically diagnosed seven years after transplantation (range nine-months to 17-years) (Penn, 1986).

1.2.2.13 **Other risk factors**

AIN and anal cancer are associated with previous intraepithelial neoplasia at another anogenital site. Women with HGCIN or vulval intraepithelial neoplasia (VIN) (Scholefield et al., 1989) along with current or previous endometrial, cervical, vulval and vaginal cancer are also at increased risk of developing ASCC (Tseng et al., 2003, Ryan et al., 2000). The development of ASCC increases with cigarette smoking (Daling et al., 1992), physical inactivity and social deprivation (Brewster and Bhatti, 2006) and these risk factors have been observed in both sexes. Previous or current infection with gonorrhoea (*Neisseria gonorrhoea*), herpes simplex type 2 and syphilis (*Treponoma pallidum pallidum*) in heterosexual men and chlamydia (*Chlamydia trachomatis*) in women have all been associated with an increased risk of ASCC (Tseng et al., 2003, Daling et al., 1992).

1.2.3 **Clinical presentation of anal cancer**

ASCC symptoms are variable and non-specific. Most commonly they include anal pain, itching, general discomfort and per-rectum bleeding (Glynne-Jones and Renehan, 2012), and very rarely faecal incontinence and ano-vaginal fistula can be seen at presentation. Other findings may be a significant change in bowel habit, diarrhoea or constipation, weight loss, vaginal, abdominal or inguinal mass and abdominal pain (Khatri and Chopra, 2004). However Fleshner has reported that only 20% of patients are symptomatic at the time of diagnosis (Fleshner et al., 2008). ASCC presents in different ways and can be easily confused with a large number of benign disorders such as hemorrhoids, fissures, ano-rectal fistula and dermatitis (Crooms and Kovalcik, 1985). This often results in late presentation and can be due to earlier misdiagnoses. If a patient is immunosuppressed, a later diagnosis can have a significant reduction in life expectancy. Diagnosis requires full inspection of the external anus, including palpation of the inguinal lymph nodes and digital rectal examination. Ultimately diagnosis relies on histopathology confirmation of cellular change on tissue biopsy. Computed Tomography (CT) scanning is used to ascertain any metastatic spread to the abdomen, chest and pelvis.
Magnetic Resonance Imaging (MRI) of the pelvis is used to establish spread of disease. ASCC often presents late with symptoms coinciding with invasive disease (Edwards et al., 1991, Gallen et al., 1997). The treatment options that are available can be radical and very unpleasant for the patient as disease is often diagnosed at a late stage (Gallen et al., 1997). Chemoradiation is the standard treatment for anal cancer, which allows preservation of anal sphincter function.

1.2.4 Progression of anal intraepithelial neoplasia to anal cancer

The natural history and rate of progression of high-grade anal precancerous lesions, unlike in the cervix, is not well studied or understood (Nathan, 2013). The international community are making steps to understand more thoroughly the natural history of anal disease. There have been a few studies demonstrating that high-grade biopsied lesions progress to anal cancer in HIV positive MSM. In one study of 138 HIV positive MSM (Berry et al., 2014), there was direct progression observed in both anal canal AIN2/3 and perianal AIN2/3 to invasive cancer in an area of previously biopsied AIN2/3 in 27 men. Time to progression to cancer from the initial diagnosis of AIN2/3 was variable (Berry et al., 2014).

Data from a French research group studying the malignant progression of AIN3 in both men and women aged over 18-years with no previous history of anal cancer or AIN3 (NCT01877135) will hopefully provide natural history to support or dispute the findings made by Berry (Berry et al., 2014). Several studies have estimated the risk of AIN3 progressing to ASCC is 1:633 per year in HIV positive MSM, 1:377 in the HAART era and 1:4196 per year in HIV negative MSM (Machalek et al., 2012a). The often-late presentation of ASCC like cervical squamous cell cancer (CSCC) poses consideration for the potential earlier detection of a precancerous tissue change. It is therefore considered that early identification of AIN, as with CIN, via a screened cohort of patients could be beneficial to disease prevention and outcome. One study has described progression of HSIL on anal cytology alone in HIV positive patients (Cachay et al., 2015). In this study HIV positive patients with baseline HSIL anal cytology had a five-year cumulative incidence of anal cancer of 1.65% (Cachay et al., 2015). Other studies demonstrating AIN2/3-lesion progression are from surgical cohorts in mostly symptomatic patients. One study recruited HIV positive men (n=40), all of whom had gross and histological evidence of AIN in the anal canal or at the anal margin. In this study three patients developed anal cancer after a median of 16-months follow-up (Devaraj and Cosman, 2006). In a second study that followed 55 patients who had a baseline AIN2/3, eight patients (14.5%) developed anal cancer after a median time of 42-months. The patients had unknown HIV serostatus and the majority of them were women (Watson et al., 2006). Another study that also included mostly women and no HIV infected individuals concluded that three of 35 patients (8.5%), who were all immunosuppressed due to other medical conditions, progressed to anal cancer after a median of 60-months of follow-up (Scholefield et al., 2005). All of these studies, involving symptomatic patients having surgical management, have a considerable potential bias for more advanced disease.
1.2.4.1 Regression of anal intraepithelia neoplasia

There are no studies demonstrating the spontaneous regression of histologically confirmed high-grade lesions in HIV positive or HIV negative MSM (Machalek et al., 2012a). Similarly in patients who have solid organ transplantation there are no studies documenting AIN regression.

1.3 Epidemiology of human papillomavirus

HPV is the most common sexually transmitted infection worldwide (Bouvard et al., 2009, Veldhuijzen et al., 2010), and HPV driven lower anogenital tract neoplasia contributes up-to 5.2% of the global cancer burden (Tota et al., 2011). It is thought that most men and women who engage in sexual activity will acquire HPV infection at some point during their lifetime, with increased risk being proportional to the number of sexual partners (Baseman and Koutsky, 2005, Karlsson et al., 1995). Mucosal HPV types are categorised into low-risk (LR-HPV) and high-risk (HR-HPV) dependant on their oncogenic association with cervical cancer (Humans, 2007). There appears to be a geographical variation for genotypes of HPV, and black women have a higher incidence of all HR-HPV types. HR-HPV types such as 16 and 18 predispose normal tissue to oncogenic changes whilst LR-HPV types (6, 11, 40, 42, 43 and 44/45) are rarely found in cancer and are categorised to reflect this association (Martin et al., 2009, Pirotta et al., 2009). LR-HPV infections are associated with genital warts and have a massive psychological, social and negative impact on quality of life for patients with the infection (Woodhall et al., 2008, Pirotta et al., 2009). Several other cancers are associated with HPV infection including vaginal (70%), penile (50%), vulval (43%) and oropharyngeal cancer (26%) (de Martel et al., 2012).

1.3.1 The structure and function of human papillomavirus

Papillomaviruses are an ancient family of pathogens that have a diverse range of hosts, including various species of animals and man. HPV is a small non-enveloped double stranded DNA virus with a genome of about 8000 base pairs (Scheurer et al., 2005). The genome is enclosed in an icosahedral capsid shell comprised of major and minor capsid proteins, which in turn encodes for E1-E7 early proteins and L1 and L2 late structural proteins (zur Hausen, 2009). E6 and E7 are thought to be the oncogenes responsible for malignant transformation (zur Hausen, 2009). Papillomavirus isolates are described as “types” (de Villiers et al., 2004) of which more than 100 have been identified and 13-types are categorised as high-risk. These are associated with increased oncogenic potential (zur Hausen, 2009). In humans HPVs are most commonly known for their benign and neoplastic diseases of the lower anogenital tract (Chow et al., 2010) and the development of epithelial malignancies (Alani and Munger, 1998). Almost all HPV are strictly specific to their chosen host and do not infect even closely related species (Chow et al., 2010).
1.3.2 Transmission of human papillomavirus

HPV is spread from skin to skin contact and genital HPV is associated with sexual activity; both vaginal and anal intercourse (Ho et al., 1998, Kjaer et al., 2001). In women, penetrative vaginal intercourse is not required for transmission of HPV infection (Winer et al., 2003). Although HPV is seen rarely in cervical samples taken from women before their sexual debut, it is possible that alternative routes of transmission such as hand contamination, in utero transmission or sex toys infect those who have never had intercourse (Pakarian et al., 1994, Sedlacek et al., 1989, Melbye et al., 1996). A study of patients infected with genital warts highlighted that greater than a quarter of patients had matching HPV DNA types on genital, fingertip and fingernail samples (Sonnex et al., 1999). This demonstrates that non-intercourse sexual behaviours such as anal digitation should be considered as an independent risk factor. This is in keeping with other sexually transmitted infections (STI’s) such as chlamydia (Chlamydia trachomatis) and gonorrhoea (Neisseria gonorrhoea) and genital warts (Jin et al., 2007b) (Jin et al., 2007a).

1.3.3 Mechanism of human papillomavirus infection

There have been substantial advances into the understanding of the infectious entry pathway of HPV into cells, although many details remain to be clarified (Horvath et al., 2010). The papillomavirus lifecycle takes roughly two to three weeks (Crosbie et al., 2013a). This begins with infection with HPV that only occurs in the basal epithelial layer where it is an obligatory intracellular parasite. The infection process starts when the virus enters via micro-abrasions in the skin or other types of epithelial trauma (Schiller et al., 2010) and internalisation of the virus then only takes several hours. HPV delivers its accessory proteins and genome into host cells where viral replication is completed by making use of the host’s biosynthetic cellular machinery (Horvath et al., 2010).

HPV primary attachment is L1 dependent resulting in binding to heparin sulphate proteoglycan (HSPG1) receptors in the extra cellular matrix (ECM). The capsids are then transferred to a secondary HSPG2 binding site on the cell surface (Sapp and Day, 2009). This causes exposure of the L2 terminus which in turn, via a series of unconfirmed receptor interactions, triggers endocytosis of the HPV into the cell (Horvath et al., 2010). The HPV genome encodes for nine viral proteins, the early E region encodes for regulation, transformation and replication of the viral genes that are then expressed in un- to moderately differentiated basal replicating cells (Horvath et al., 2010). The role of L2 is a multifunctional protein involved in genome encapsidation and L1 interaction (Okun et al., 2001, Finnen et al., 2003). It also promotes capsid stabilisation (Finnen et al., 2003, Ishii et al., 2005), virion escape by endosomes for infectious entry of the virus into new host cells (Horvath et al., 2010) and the nuclear transport of HPV genome (Kamper et al., 2006).

Early HPV genes E1, E2, E4, E5, E6 and E7 are expressed after infection and the viral DNA replicates from episomal DNA (Woodman et al., 2007). The HPV genome is replicated further in
the upper layers of the stratified epithelium (the midzone and superficial zone) where the expression of late genes L1 and L2, as well as E4 occurs (Johansson and Schwartz, 2013). Encapsulation of the viral genome by L1 and L2 occurs in the most superficial layers of the epithelium allowing viral assembly to take place then resulting in newly assembled infectious viral particles being shed from the epithelial surface (Hinten et al., 2012).

1.3.4 Malignant transformation

HPV proteins E6 and E7 are known oncogenes (Yim and Park, 2005). E6 inactivates the tumour suppressor p53 (wild type p53) and E7 binds to the retinoblastoma tumour suppressor (Rb) causing chromosomal instability, increased cell growth and reduced apoptosis, and these cells then never mature (Hinten et al., 2012). Wild type p53 arrests the cell cycle when there is DNA damage to allow repair and causes apoptosis (Cox and Lane, 1995). E6 protein prevents this resulting in cellular genomic instability, as well as alterations to the cellular DNA and allows genetically unstable cells to replicate. Malignant potential of the cell infected with HPV is then propagated and likely to develop into invasive cancer (Tommasino et al., 2003). E7 binds to Rb1, RbL1 and RbL2 contributing to oncogenesis. E7 proteins target, bind to and degrade genes, which control cell cycle entry in the basal layer, promoting DNA synthesis and stimulate host genome instability (McLaughlin-Drubin and Munger, 2009) (Horvath et al., 2010). It is generally considered that levels of E6 and E7 expression increase with CIN1-3, although this has not been assessed at other sites of infection (Middleton et al., 2003). High-risk E5 supports E6 and E7 to promote cellular proliferation (Maufort et al., 2010).

1.3.5 The natural history of human papillomavirus infection

The majority of HPV infections are transient and eliminated over time by immunocompetent individuals, yet they tend to persist in immunosuppressed individuals (Kreuter et al., 2008). Most infections with HPV are cleared via a cell-mediated immune response (Evander et al., 1995, Moscicki et al., 1998). If the HPV infection does not persist it will not cause deregulation in gene expression and genetic errors (Doorbar et al., 2012). HPV evades innate immune recognition, as its viral lifecycle is entirely intraepithelial. There is no viraemia; replication and release are not associated with any kind of inflammatory process as there is no viral induced cytolysis, cell death or necrosis (Frazer, 2009). Superficial epithelial cells do express viral antigens but they are destined for desquamation, they escape immunological surveillance and this allows the virus to remain undetected for a long period of time (Doorbar et al., 2012, Frazer, 2009).

The evasion by HPV of innate immune recognition results in little to no cell mediated response to infection. Inside the infected keratinocyte, HPV down regulates signalling pathways of the innate immune system. Pro-inflammatory cytokines, such as type I interferons are not released. Furthermore, the signals for Langerhan cell activation, migration and recruitment of dendritic cells and macrophages are either absent or inadequate (Kanodia et al., 2007). Circulating immune cells are in the basal layer of the epithelium but HPV infected cells which express viral
proteins are shed at the epithelial surface, further evading innate detection (Doorbar et al., 2012). High-risk HPV infections have complex mechanisms for immune evasion; HPV oncoproteins reduce levels of E-Cadherin, a calcium dependant cell-cell adhesion molecule in epithelial tissue (Pecina-Slaus, 2003), which lowers the quantity of Langerhans cells surrounding the site of HPV infection (Matthews et al., 2003, Caberg et al., 2008). HPV E7 also interferes with the activation of the interferon regulatory-1 factor (Perea et al., 2000) and E6 affects STAT, a cytokine receptor system, by interfering with tyrosine kinase 2 function (Li et al., 1999), reducing its role in anti-viral immunity.
1.4 Prevention, treatment and screening of anal precancerous lesions

1.4.1 Human papillomavirus vaccination of MSM

The most common types of HPV worldwide are 16 and 18, both of which are now preventable by vaccination in adolescence (Giraldi and De Luca d’Alessandro, 2012). Vaccination as a preventative measure should be considered for preventing AIN and ASCC (Sendagorta et al., 2011). Vaccination against HPV 16 and 18 for prevention of genital warts and anal cancer in MSM is thought to be cost effective (Kim, 2010). Vaccination schedules would have to incorporate both girls and boys. It is considered that therapeutic vaccination against HPV is not effective in treating existing abnormalities (de Melker et al., 2012).

There are two prophylactic HPV vaccines available: bivalent Cervarix and quadrivalent Gardasil. Cervarix is effective against HPV types 16 and 18 and Gardasil is effective against HPV types 6, 11, 16 and 18 (Schiller et al., 2012). In October 2009 the US Food and Drug Administration approved the qHPV vaccine for the prevention of condylomas caused by HPV 6 and 11 in males aged nine to 26-years of age. Australian and US Health Authorities now recommend the vaccination of boys; this is thought to be most effective if it is administered before sexual debut (Sendagorta et al., 2011). Australia is the first country to publically fund a quadrivalent HPV (qHPV) vaccination program for both sexes, young boys in 2013 and girls in 2008 aged 12-13 years (Poynten et al., 2013) for anogenital disease. Identifying young MSM at risk for anal cancer prior to sexual debut would be challenging, if not impossible. It is plausible that blanket vaccination of both preadolescent boys and girls is an appropriate preventative measure in HPV associated disease.

It is thought that the vaccination of boys will have an effect on the incidence of HPV related anal cancer. In November 2014 the Joint Committee on Vaccination and Immunisation (JCVI) published an interim recommendation for the qHPV vaccine for MSM aged 16–40-years, to be implemented in sexual health clinics, providing the vaccines and programme can be delivered at a cost-effective price (JCVI, 2014). Vaccination for primary prevention of anal cancer in MSM still has significant challenges such as the implementation and uptake of a vaccination schedule, along with considerations regarding its cost effectiveness. A US study examining parental awareness and qHPV vaccine uptake among nine to 17-year olds males during the first year of the recommendation (2009) highlighted the challenges faced by vaccinating adolescent males (Laz et al., 2013). Laz et al collected data from the 2010 National Health Interview Survey (NHIS) in order to assess the vaccination status (n = 2973) of nine to 17-year old males (Winer et al., 2003, Laz et al., 2013). Correlates of parental awareness and uptake of the HPV vaccine were performed using a univariate logistic regression analysis. Overall, 55% of parents were aware of the HPV vaccine. Only 2.0% of nine to 17-year old males initiated greater than one dose and 0.5% completed greater than three doses the vaccine series, respectively. Laz et al demonstrated that very few nine to 17-year old males received any doses of qHPV vaccine during the first year in the US following its recommendation for this gender. Therefore,
Interventional programs are needed to improve vaccine uptake among adolescent males (Laz et al., 2013). Cost-efficacy studies have suggested that vaccination of males to prevent cervical cancer in females would not be cost efficient if high levels of coverage were achieved in the female population (Kim and Goldie, 2009). US data regarding vaccination coverage of the female population ranging in age from 13 to 17-years is lower than that used in the cost-efficacy model, with only 37% of women receiving greater than one dose and 18% receiving all three doses, therefore vaccination of males is likely more cost-effective than current estimates suggest (CDCP, 2008). All previous cost efficacy analyses did not take into account the vaccine’s potential impact on anal cancer amongst MSM. This is a factor that should be considered in deciding whether vaccination of males is warranted. Another possible limitation is the vaccine’s efficacy against AIN and anal cancer; this will depend on the timing of its delivery and the levels of penetration achieved in the target population of MSM. If amongst MSM low levels of vaccination are achieved or vaccination occurs after the acquisition of anal HPV, then the vaccine may not have a great impact on the incidence of anal cancer in MSM (Park and Palefsky, 2010). Studies evaluating immunogenicity of the qHPV vaccine in HIV-infected individuals are underway. HPV vaccination studies with qHPV in HIV negative MSM are showing promising results regarding the prevention of development of HGAIN (LARSEN, 2013).

1.4.2 Treatment of anal intraepithelial neoplasia

The aim of treating a precancerous lesion is to prevent progression to invasive cancer. The treatment of AIN is not currently standardised; indeed optimal management of AIN is extremely challenging. Large data series comparing treatments, with prolonged follow-up are lacking.

There are a variety of treatment modalities being used in the management of AIN. These include immunomodulation therapy involving the topical application of imiquiod 5% or cidofovir 1%, photodynamic treatments and ablative therapies. Ablative therapies include CO\textsubscript{2} laser, cryotherapy and electro cautery. Many of these treatments suffer from high recurrence rates and significant morbidity. Surgical excision of HGAIN is widely practiced, but has a high reoccurance rate, particularly in HIV positive MSM (Abbasakoor and Boulos, 2005). Radical treatment in the way of excision of AIN3 is considered to be excessive, whilst more conservative excision, ablation or indeed non-surgical treatments all carry a high-risk of reoccurrence.

A Cochrane review completed in 2012 concluded that “there was an absence of reliable evidence for any of the interventions used in the treatment of AIN, and therefore precludes any definitive guidance or recommendations for clinical practice” (Macaya et al., 2012). The international community are striving to find an appropriate treatment of AIN. A recent study conducted in the Netherlands (Richel et al., 2013) between August 2008 and December 2010 investigated several different methods of treatment. All of the participants were HIV positive MSM with histologically confirmed AIN. This study provided more evidence that treatments of AIN to date do not provide cure. 388 HIV-positive MSM were screened by HRA and 63% (246/388) of them had AIN. In total 63% (156/246) were randomly assigned to receive treatment by imiquimod (54 patients), topical fluorouracil (48 patients), or electrocautery (46 patients).
following withdrawing of consent by eight patients (Richel et al., 2013). Treatment schedules in Richel et al.'s study were 16-weeks of imiquimod three times a week, 16-weeks of topical fluorouracil twice a week or monthly electrocautery for four months. All patients were assessed by HRA four weeks after commencing treatment. Responding patients returned for follow-up at 24, 48 and 72-weeks after treatment. The primary endpoint was the histological resolution of AIN measured four weeks after treatment and AIN reoccurrence at 24, 48 and 72-weeks after treatment (Richel et al., 2013). The side effects of each treatment was also graded, zero being no side effects and four being severe. A complete response was seen in 13 (24%, 95% CI 15-37) imiquimod treated patients, eight (17%, 95% CI 8-30) fluorouracil treated patients, and 18 (39%, 95% CI 26-54) electrocautery treated patients. When reviewed at week-24, 22% (11/50) of patients who had responded to treatment had reoccurrence; at week-48, 46% (22/48) had reoccurred; and at week-72, 67% (30/45) had reoccurred. The large proportion of patients (71%; 10/14) treated with imiquimod had reoccurrence at 72-weeks. In patients treated with fluorouracil and electrocautery, reoccurrence at 72-weeks was observed in 58% (7/12) and 68% (13/19) respectively (Richel et al., 2013). Grade three and four side-effects were noted in 43% (23/54) of patients in the imiquimod group, 27% (13/48) of patients in the fluorouracil group, and 18% (8/46) patients in the electrocautery group (p=0.019). The most common reported side effects were pain, bleeding, and itching. This study concluded that electrocautery is better than imiquimod and fluorouracil in the treatment of AIN but the reoccurrence rates and side effects of treatment are substantial (Richel et al., 2013).
1.5 Screening

1.5.1 The principles of screening

Commissioned by The World Health Organisation, Wilson and Jungner in 1968 published “Principles and Practice of Screening for Disease” (Wilson and Jungner, 1968). This publication defined screening criteria, as we know it today, and it has subsequently become a public health classic. It highlights selecting the appropriate condition suitable for screening, the capacity to detect the condition at an early stage and the availability of an acceptable treatment (Wilson and Jungner, 1968). Wilson and Jungner screening criteria can be seen in Figure 7.

Screening is defined by the UK National Screening Committee (England, 2015) as “testing people who do not have or have not recognised the signs or symptoms of the condition being tested for, either with the aim of reducing risk of an adverse outcome, or with the aim of giving information about risk”.

**Figure 7 Wilson and Jungner screening criteria (Wilson and Jungner, 1968)**

<table>
<thead>
<tr>
<th>The condition sought should be an important health problem</th>
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<tbody>
<tr>
<td>There should be an accepted treatment for patients with recognised disease</td>
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<tr>
<td>Facilities for diagnosis and treatment should be available</td>
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<tr>
<td>There should be a recognisable latent or early symptomatic stage</td>
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<tr>
<td>There should be a suitable test or examination</td>
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<td>The test should be acceptable to the population</td>
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<tr>
<td>The natural history of the condition, including development from latent to declared disease, should be adequately understood</td>
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<tr>
<td>There should be an agreed policy on whom to treat as patients</td>
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<tr>
<td>The cost of case finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole</td>
</tr>
<tr>
<td>Case finding should be a continuing process and not a “once and for all” project</td>
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1.5.2 Screening practice in the detection of anal intraepithelial neoplasia

A recent study (Patel et al., 2014) published in 2014 reviewed worldwide HRA practices in the detection of AIN. Screening appeared to be widespread; 82 providers from 80 worldwide clinics completed an online questionnaire. The majority of clinics offered anal cytology (98%) and HRA (99%) but only 59% of clinics offered HPV testing. Over a third of clinics did not restrict access to screening; in the rest, eligibility was most commonly based on HIV status and abnormal anal cytology results (Patel et al., 2014). There was no clear risk stratification for screening eligibility. Of the clinics surveyed, 53% would perform HRA in an asymptomatic patient if they had abnormal anal cytology, of these 74% used a result of Atypical Cells of Uncertain Significance (ASCUS) or worse to trigger HRA. The most common modality of treatment for internal AIN was infra-red coagulation (IRC) (61%), followed by topical imiquimod application (54%) and then bi/trichloroacetic acid (51%). High-grade (AIN2/3) external lesions were most commonly treated with imiquimod (49%). In the US, private insurance provided payment for screening services, in Canada and other countries the service was publically funded (Patel et al., 2014). A study published in Australia reported the views of 20-HIV physicians (infectious diseases, immunology, sexual health, and general practice) in different settings (hospital, sexual health centres, and general practice). This study concluded that HIV physicians felt that anal cancer was a significant health issue and that HIV positive MSM should be screened if a valid method is available. Physicians at this time were ambivalent regarding the most effective modality to screen for anal cancer and that more research was needed to address their concerns before any service could be implemented (Ong et al., 2015b). An additional review supports anal screening in high-risk groups, particularly HIV positive MSM (Smyczek et al., 2013).

1.5.3 Routine screening for anal intraepithelial neoplasia

It is clear that there is an association between the development of anal cancer and certain high-risk groups (Palefsky, 1994, Patel et al., 2007). Those most at risk are HIV positive MSM. The identification of MSM is not straightforward as patients; even if they practice anoreceptive sex with other men, sometimes do not identify as being MSM. Screening and treatment of anal disease has not been routinely adopted by any major healthcare system to date for several reasons. As discussed earlier, there is still very limited data as to the complete natural history and rate of progression from HGAIN to ASCC and or regression of anal disease in high-risk population groups. There are still several issues in the detection of disease. Although the advent of LBC has improved reporting of anal cytology due to faecal debris being filtered out, it is still not as sensitive in comparison to cervical LBC. Anal cytology to date therefore cannot be used effectively to stratify risk and offer HRA to those with high-grade abnormalities on LBC sampling. It is possible this is because the ATZ cannot be seen clearly at the time of sample collection. Infection with HR-HPV is abundant in HIV positive and negative MSM and therefore cannot be used as a triage system for HRA. HRA appears to be the gold standard in the detection of precancerous changes in the anal canal (de Ruiter et al., 1994, Goon et al., 2015).
The entire canal can be visualised microscopically and tissue biopsy can be taken if disease is suspected (Palefsky, 2012). Once a biopsy is taken, there are no specialised tests to aid the diagnosis of low and high-grade anal disease.

To date, the major downfall in a potential screening service in the detection of AIN would be in its treatment and management. There has been no effective treatment of AIN, with high levels of reoccurrence in disease of all grades. The management of AIN is not straightforward, as the incidence of anal cancer is not accurately known. Once established, this will enable understanding of the natural history assisting in the prediction of the potential workload from new cases and follow-up of patients detected with AIN.

Responsibility for the management of patients with AIN would need to be established and standardised in the UK. These clinicians will need training in the identification of AIN by HRA and a training course will need to be established. Histopathologists would also need to be trained in reading biopsied anal specimens. There would need to be new specific collaborations between clinicians with large AIN practices and local genitourinary medicine (GUM) and HIV clinicians (Scholefield et al., 2011).

From the literature reviewed, anal screening in other healthcare economies appears to be warranted in high-risk groups. Data from an Australian prospective Study of the Prevention of Anal Cancer (SPANC; NCT02007421; HREC/09/SVH/168) is long awaited. SPANC commenced recruitment in September 2010 and closed recruitment in June 2015 with follow-up until December 2018 had a recruitment target of 600 participants. SPANC has studied the epidemiology of LR and HR-HPV infection and related cytological and histological abnormalities in HIV negative and HIV positive MSM aged 35-years and over. Primary outcomes from this natural history study are to inform the potential usefulness of a cytology/HPV/HRA based screening program and determine the prevalence, incidence and risk factors for type-specific HPV (37 types). SPANC has also collected data on the impact of anal screening on men's physical and psychological well-being and quality of life. The study group aims to use these data to assess the benefits and potential harm of MSM having the screening test (Machalek et al., 2013). This study will provide much needed evidence collected in a healthcare economy similar to the UK about screening a high-risk population for anal precancer.

As the treatment of AIN is still suboptimal, further research is needed in the form of randomised controlled trials (RCT). There are two important trials of treatment at an early stage. The first is a large US based randomised phase III trial investigating Anal Cancer HSIL (High-grade Squamous Intraepithelial Lesion) Outcomes Research (ANCHOR; NCT02135419). ANCHOR will compare topical and ablative treatments with active monitoring in preventing anal cancer in patients with HIV and HGAIN (AIN2/3). Men and women aged 35-years and older with a HIV positive serostatus are eligible to participate. Participants must have no previous history of treatment or excision of HGAIN or anal cancer, along with no history of penile, cervical, and
vaginal or vulvar cancer. All patients must have a biopsy proven HGAIN at baseline visit. A recruitment target of 5058 has been established. The primary objective is the time from randomisation to the diagnosis of cancer, up to five years. Other outcome measures are incidence of adverse events at each treatment; quality of life assessed using Functional Assessment of Incontinence Therapy – Faecal (FAIT-F) questionnaire along with behavioural risk actors. HR-HPV testing will be performed and biopsies containing HPV type 16 will be analysed to ascertain if the locus of HPV in invasive disease differs from HGAIN. The other is a large UK based RCT that will investigate the effectiveness of Laser ablation versus Observation to Prevent Anal Cancer (LOPAC; TA: 11/92/01, LOPAC Trial., Nathan M et al, UKCRN ID 19230) in men with HIV who have HGAIN, defined as AIN2/3. A recruitment target of 660 men has been established with follow-up for six years. Patients in the untreated group will be offered six monthly active observation. Data will be collected regarding the impact of HIV positive patients’ immune status measured by CD4 count, CD4 nadir, viral load, duration of HAART and duration of HIV positive status. Cost effectiveness along with quality of life outcomes are also going to be measured in this study. In order for an effective screening programme to be conducted the natural history of the condition, including development from latent to declared disease, should be adequately understood (Wilson and Jungner, 1968). Hopefully these trials will provide much needed evidence regarding treatment, quality of life, cost effectiveness of treatment and progression of precancerous anal disease.

### 1.6 Summary

The true prevalence of AIN in high-risk groups in the UK is unknown. As the incidence of ASCC rises year on year predominantly in high-risk populations, the need to ascertain if there is an identifiable and treatable precancerous lesion is of high importance. Management of AIN poses great difficulty for any clinician treating it effectively, due to its uncertain risk of progression to anal cancer and ineffective treatment for cure. HGAIN has a concrete association with HR-HPV infection.

In the future, the incidence of HPV associated lower anogenital tract disease is going to be affected by HPV vaccination in women. There will hopefully be a reduction in lower anogenital tract disease at all sites other than the cervix. As there is no vaccination schedule for HIV positive MSM, presumably this group still remains most at risk of AIN. HIV positive MSM have the highest risk of developing HR-HPV related ASCC.
1.7 Laboratory diagnosis of anal intraepithelial neoplasia

1.7.1 Terminology

The cytological and histological terminology used to describe AIN is confusing. A US steering group recently convened and recommended a unified terminology to classify lower anogenital tract neoplasia. The Lower Anogenital Squamous Terminology (LAST) Standardisation Project (2012) details recommendations for a histopathological nomenclature system by the College of American Pathologists and the American Society for Colposcopy and Cervical Pathology (Darragh et al., 2012a). The LAST project now allows clear communication between different specialties at an international level.

Up until the publication of LAST there had been no guidelines for the classification of anal, perianal, penile and vaginal pre invasive HR-HPV related disease. This is partially due to the vast overlap of disease at each site. The anatomical demarcation of the peri-anus and the perineum in both men and women is difficult to define. In women the anatomical boundaries are further complicated as there is also vulvar tissue to be considered. Before the LAST publication, these lesions were variously called Bowen’s disease, Carcinoma In Situ or Perianal Intraepithelial Neoplasia grades 1, 2 and 3. The LAST project attempts to combine cytological and histological classification systems to minimise confusion.

Recommendations from the LAST publication are as follows for lower anogenital tract neoplasia (Darragh et al., 2012a):

1. A unified histopathological nomenclature with a single set of diagnostic terms for all HPV related pre invasive lesions of the lower anogenital tract.

2. Reporting should be via a two tiered nomenclature for non-invasive HPV squamous related disease of the lower anogenital tract. This should be further qualified with the appropriate intraepithelial neoplasia (–IN) with the addition of the appropriate location, see Table 1.

3. The recommended terminology for HR-HPV associated lesions of lower anogenital tract is low-grade squamous intraepithelial lesions (LSIL) and high-grade intraepithelial neoplasia (HSIL). This may be further categorised as –IN when describing histology reports to avoid any confusion between cytopathological and histopathological description of disease.
Roberts and Ekman (2012) support the two-tiered reporting system proposed by LAST outlined in the Table 2. Rather than categorising histological abnormalities as AIN1, 2 and 3, the two-tiered nomenclature identifies anal ‘pre-cancer’ as comparable to cervical ‘pre-cancer’ which may not represent a stepwise disease process. Low-grade histological change is usually self-limiting whilst high-grade disease undergoes active processes placing the individual at risk of developing neoplasia (Roberts and Ekman, 2012). A diagnosis of intraepithelial neoplasia (–IN) 2 reflects an intermediate category that is thought to represent a mixture of high and low-grade lesions (Roberts and Ekman, 2012). These lesions may have the potential to develop into invasive cancer. Furthermore, intermediate lesions may be difficult to reliably distinguish from low or high-grade disease by pathologists (Stoler and Schiffman, 2001, Castle et al., 2007). Finally, the LAST project concluded that a two tiered reporting system leads to a more consistent diagnosis (Darragh et al., 2012a).

Table 1 Terminology of site dependant intraepithelial neoplasia

<table>
<thead>
<tr>
<th>SITE</th>
<th>Intraepithelial Neoplasia Terminology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anus</td>
<td>AIN</td>
</tr>
<tr>
<td>Cervix</td>
<td>CIN</td>
</tr>
<tr>
<td>Penis</td>
<td>PeIN</td>
</tr>
<tr>
<td>Perianus</td>
<td>PAIN</td>
</tr>
<tr>
<td>Vagina</td>
<td>VaIN</td>
</tr>
<tr>
<td>Vulva</td>
<td>VIN</td>
</tr>
</tbody>
</table>
Table 2 Comparison of histology and cytology reporting systems for HPV related anal disease (Roberts and Ekman, 2012)

<table>
<thead>
<tr>
<th>Proposed histology terminology</th>
<th>Bethesda / LAST terminology cytology</th>
<th>AIN grade</th>
<th>Cytology grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-grade anal intraepithelial neoplasia (LGAIN)</td>
<td>Low-grade squamous intraepithelial lesion (LSIL)</td>
<td>Anal intraepithelial neoplasia 1 (AIN1)</td>
<td>Mild dysplasia</td>
</tr>
<tr>
<td>High grade anal intraepithelial neoplasia (HGAIN)</td>
<td>High grade squamous intraepithelial lesions (HSIL)</td>
<td>Anal intraepithelial neoplasia 2 (AIN2) Anal intraepithelial neoplasia 3 (AIN3)</td>
<td>Moderate dysplasia Severe dysplasia</td>
</tr>
</tbody>
</table>

1.7.1.1 Anal cytology terminology

Anal cytology is now classified as: negative for intraepithelial lesion or malignancy (NILM), atypical squamous cells of undetermined significance (ASCUS), atypical squamous cell cannot exclude HSIL (ASC-H), low-grade squamous intraepithelial lesions (LSIL), high-grade squamous intraepithelial lesions (HSIL) and anal squamous cell carcinoma (ASCC) (Darragh TM, 2004a). The Bethesda System (TBS) is used worldwide to report lower anogenital tract cytology except for in UK, where this system is not yet in widespread use (Darragh et al., 2013). All of the anal cytology samples collected during the ANALOGY study were reported using TBS.

1.7.1.2 Anal histology terminology

The nomenclature used to describe the histopathological appearances of lower anogenital tract neoplasia remains confusing. For the purpose of this thesis the anus and the peri-anus AIN will be categorised as low-grade (LGAIN) AIN1 and/or Ungraded AIN, or high-grade (HGAIN) AIN2 and/or AIN3.

LGAIN consists of the low-grade spectrum of lesions: condyloma, mild dysplasia, AIN1 and ungraded AIN. Ungraded AIN an indeterminate histology reading, the epithelium in the sample is very thin. The epithelium may only be a few cells thick and although the presence of nuclear abnormalities is unequivocal, confident grading by the histopathologist is not. In CIN reporting ungraded specimens are generally considered to be grade 1-2 unless there is an increased degree of nuclear atypia, suggesting that it is CIN2 to 3 rather than CIN1 to 2. Ungraded anal specimens would then have to re-graded using LAST terminology which states morphologically, these are immature intraepithelial lesions less than 10 cells thick (Darragh et al., 2012b). If a lesion is unequivocal squamous –IN with significant immature abnormal basal proliferation or mitosis above the basal cells, it is designated as HSIL (AIN 2/3) (Darragh et al., 2012b). If there
is doubt about the nature of the proliferation (for example, immature metaplasia vs. -IN) then p16 staining can be used. The LAST guidelines recommend any specimen that on H&E has a morphologic differential diagnosis between precancer (–IN2 or –IN3) and a mimic of precancer should have p16 immunohistochemistry (IHC). Any specimen with a “strong and diffuse block positive p16 result” supports a categorisation of precancerous disease. Strong and diffuse block positive p16 is defined as “continuous strong nuclear or nuclear plus cytoplasmic staining of the basal cell layer with extension upwards involving at least one third of the epithelial thickness” (Darragh et al., 2012a). The LAST group states that the height restriction (one third of epithelial thickness) is arbitrary but adds specificity to the grading of the specimen. Also, a specimen that demonstrates full thickness p16 expression or extension into the upper third or upper half is specifically not required to call a specimen positive (Darragh et al., 2012a). HGAIN comprises lesions previously referred to as severe dysplasia, carcinoma in situ, and AIN2, AIN2, or AIN3 (Darragh, 2011).
1.7.1.3 Cytological appearance of lower anogenital tract neoplasia

Abnormal anal cytology displays the following features: high mitotic activity, a high nuclear to cytoplasmic ratio and cellular immaturity as seen in Figure 8. Darragh et al state that nuclear changes in squamous intraepithelial lesions (SIL) are frequently noted throughout the full thickness of the epithelium irrespective of the lesions severity and this is reflected in cytological sampling of the superficial layers, as it will detect both high and low-grade lesions (Darragh TM, 2004a). Cytologically ASCC is challenging to diagnose. Features of invasion can be very difficult to distinguish due to faecal material (Darragh, 2011).

Figure 8 Anal liquid based cytology high-grade squamous intraepithelial lesion (comparable to AIN3) x600 magnification

![Image used with permission from Dr Mina Desai, CMFT, Manchester, UK](image)

Figure 8: High-grade squamous intraepithelial lesions have a high nuclear chromatic ratio. This can be seen in aggregates or in individual cells. There is nuclear hyperchromasia, chromatin clumping and nuclear membrane irregularities (Bean and Chhieng, 2010).
1.7.1.4 **Histological appearance of lower anogenital tract neoplasia**

In the UK, AIN is graded by a similar system that is used for cervical disease into three grades of dysplasia. The grade represents the severity of observed atypia and in the cervix, their potential for malignant transformation, AIN1, AIN2 and AIN3. In the anus, there are fewer onuses on AIN2 and its malignant potential as it is in cervical reporting. SIL are defined by abnormal cellular proliferation with nuclear enlargement, atypia, pleomorphism, chromatin texture change and nuclear border irregularities. When these changes are confined to the lower third of the epithelium, the lesion is classified as LSIL / LGAIN (1). When seen in throughout the epithelium the changes are consistent with HSIL / HGAIN (2 & 3) (Darragh et al., 2012a).

Diagnostic features of atypia seen in AIN include abnormal sized nuclei, increased mitotic activity, hyperchromatic nuclei and a distinct increase in nuclear cytoplasmic ratio. Nucleoli are usually absent and spongiosis is not usually seen.

**Histological features of AIN grades 1-3 (Abbasakoor and Boulos, 2005)**

- **AIN1** is defined histologically as a proliferation of dysplastic squamous cells confined to the lower third of the anal squamous epithelial lining on haematoxylin and eosin (H&E). Minimal cytoplasmic maturation is seen in the lower third of the epithelium on H&E, maturation begins in the middle third and is relatively normal in the upper third. Mitotic figures are also limited to the lower third of the epithelium (Abbasakoor and Boulos, 2005).

- **Histologically, AIN2** is defined as a proliferation of dysplastic squamous cells confined to the lower two-thirds of the anal squamous epithelium. Minimal cytoplasmic maturation is seen in the lower two-thirds of the epithelium, with maturation beginning in the upper third (Abbasakoor and Boulos, 2005).

- **AIN3** is defined as a proliferation of dysplastic squamous cells involving the full thickness of the anal squamous epithelium on H&E. In the squamous epithelial lining little or no cytoplasmic differentiation is seen. Mitotic figures are not confined to the lower third, and may be found as high as the upper third of the epithelium (Abbasakoor and Boulos, 2005). AIN3 was previously known as carcinoma in-situ, however, this term is now not encouraged.

Biopsy tissue specimens taken from the anal canal are likely to display changes that are the result of viral infection. A “koilocyte” is a squamous epithelial cell that has undergone a number of structural changes as a result of infection of the cell by a virus. The following are terms used in histology and cytology to describe the presence of koilocytes within a specimen; “koilocytosis”, “koilocytic atypia” or “koilocytic atypia”. The term “koilocyte”, from the Greek word for “empty space” or “hollow cell”, was first used in 1956 to describe cells with ballooned cytoplasm, and in 1976 was linked to HPV (Hajdu, 2006). Koilocytes have a clear area around
the nucleus; they are vacuolated cells with clear cytoplasm. These findings are often described as “perinuclear halo” or “perinuclear clearing” and have also been termed “halo cells” (Krawczyk et al., 2008). Koilocytosis is generally observed in the uppermost, well differentiated layers of stratified squamous epithelium. The higher the degrees of dysplasia, fewer koilocytes are seen, though it has been documented in both low and HR-HPV infection (Krawczyk et al., 2008). The presence of koilocytes in tissue specimens is a diagnostic feature of HPV infection. Most HPV testing is now performed on LBC samples using detection systems such as Roche Cobas 4800®.

1.7.2 Classification of AIN and implications in clinical management

There is a global consensus that AIN3 requires treatment, but disparity amongst HRA practitioners as to whether AIN2 can be observed or treated. There is interobserver and intraobserver variability with respect to the classification of AIN, particularly AIN2. There are several studies published demonstrating discrepancy of the diagnostic histology reporting of anal biopsies (Carter et al., 1994, Costa e Silva et al., 2011). However, this is also seen in the cervix and vulva. Various recommendations have been made recently in the UK by the Association of Coloproctology of Great Britain (ACPGBI) and US College of American Pathologists – American Society for Colposcopy and Cervical Pathology (CAP–ASCCP) to reclassify premalignant anal lesions into a two-tiered rather than three-tiered system in agreement with the LAST guidelines (Darragh et al., 2012b). Reporting as either low-grade or high-grade dysplasia is considered to reduce intraobserver and interobserver disagreement. The aim is an improved agreement among pathologists leading to a more consistent grading of specimens. The US therefore reports all -IN2 and/or 3 as high-grade. In the UK, -IN is generally reported as grade 1, 2 or 3. It has been considered for some time that there is poor agreement in the diagnosis of -IN2 using three grade system H&E morphology. Robertson et al reported fair strength of kappa agreements in benign tissue (kappa 0.52) and CIN1 (kappa 0.24), and poor agreement in CIN2 (kappa 0.20). A good strength of agreement was only seen in CIN3+ (kappa 0.61) (Robertson et al., 1989).

According to the ACPGBI in the UK, AIN3 and multicentric (at more than one site) -IN need specialist management and follow-up, AIN1 and AIN2 do not require any long-term follow-up. The most recent guidelines in the UK state that AIN1 and 2 are considered to be low-grade lesions and AIN3 represents a high-grade lesion warranting treatment (Scholefield et al., 2011).

1.7.2.1 Collection of anal cytology specimens

Cytological specimens are either liquid based or conventional smears. LBC in cervical screening has reduced the number of inadequate samples and therefore pressure on the workforce collecting and processing the specimens and results (Harrison et al., 2007). This has in turn saved the screening service money and reduced anxiety in women having a cervical
smear due to reduction in repeat tests. The LBC samples can be processed more quickly than conventional smears (Moss S.M, 2004).

Anal cytology is a simple, non-invasive technique. HPV testing can be performed on LBC (Sendagorta et al., 2011) and sample collection does not require the use of an anoscope. LBC is preferred over a conventional smear for evaluation of anal cytology due to its tendency to collect an increased cellular yield with improved cellular preservation. A reduction in faecal material and bacteria that can obscure cellular detail is seen in LBC anal samples allowing for easier interpretation, coupled with the elimination of the air-drying and mechanical artefacts that are commonly encountered with conventional anal smears (Darragh TM, 2004b). Parameters for an adequate cellularity on anal cytology were not defined before 2004. Anal LBC typically consist of nucleated squamous cells, anucleated squames, squamous metaplastic cells, and rectal columnar cells. A good specimen should contain 2000 to 3000 nucleated squamous cells, for LBC 3-6 nucleated squamous cells per high power field (HPF) for Surepath™ and 1-2 nucleated squamous cells/HPF for Thinprep® (Darragh, 2004).

Sensitivity and specificity rates for the detection of anal dysplasia are comparable with those observed in cervical cytology. Anal LBC gives sensitivity in the range of 69-92% and a specificity of 32-59% (Berry et al., 2009, Salit et al., 2010, Membrilla-Fernandez et al., 2009, Chiao et al., 2006) but the diagnostic accuracy of anal cytology has still not been subjected to randomised control trials (Mallari et al., 2012).

1.7.2.2 Concordance between anal cytology and histology

In the cervix there is good concordance between LBC and histology, which has been further enhanced of late by the use of HR-HPV testing in screening for high-grade CIN in need of treatment in the prevention of cancer. In the detection of anal disease the evidence thus far is not as concrete. An audit conducted by Williams et al, Australia, paired anal cytology and histopathology outcomes in patients referred to a public sexual health clinics. The authors reported that biopsies taken from an area of obvious disease at HRA are highly specific for defining grade of anal abnormality (Williams et al., 2010). Williams et al concluded that anal cytology in the detection of abnormal squamous cells is highly sensitive, while anal cytology has low specificity for predicting the grade of abnormality compared with biopsy outcome (Williams et al., 2010). A Spanish study concluded that anal biopsies taken simultaneously with anal cytology have a high degree of concordance for dysplasia. HGAIN showed 94% agreement between cytology and biopsy however there was only a 50% concordance in LGAIN (Repiso Jimenez et al., 2013). In comparison, a recent study in Australia showed poor correlation between anal cytology and histology for all grades of AIN. HRA was performed on patients if abnormal cytology was detected after self-collected cytology sampling (Botes et al., 2013). Mathews et al. (2011) found anal screening to be less discriminating for detecting histological HGAIN than cervical screening is for detecting histological CIN2+ on punch biopsy in a meta-analysis of 33 cervical and 11 anal screening studies (Mathews et al., 2011).
1.8 The application of immunohistochemistry in the detection of anal intraepithelial neoplasia

Immunohistochemistry (IHC) may assist in the diagnosis and classification of AIN. Interpretative and diagnostic variation due to the subjectivity of the histopathologist could be reduced in part by reducing the number of grades used to report AIN and also by using the biomarker, p16. Histology reporting of HPV related anogenital disease remains disparate partly because of different classification systems, which can result in inconsistency. Clinicians need reliable reporting as the basis of disease management, so it is key that lesion classification is as robust as possible. This was the rationale behind the development of the LAST guidance in the US (Darragh et al, 2012). Lesions that are reported as AIN3 can be regarded as high-grade, however lesions reported as AIN2 probably include both low and high-grade disease. When the LAST guidance is applied to AIN2, the purpose was to define lesions that could be considered potentially preinvasive, as opposed to those that were more likely to be simply HPV infection, and therefore transient lesions. This requires staining with p16, the presence of which as strongly positive is classified as high-grade disease. The addition of single or multiple biomarkers to aid diagnosis of disease would be extremely beneficial. It is common practice to label using one single antigen, but it is possible to stain tissue sections with multiple markers and therefore preserve precious tissue samples (Stack et al., 2014). IHC permitting the differentiation between low and high-grade disease would also aid potential patient follow-up and possible selection of patients for treatment of high-grade disease.

1.9 Biomarkers used in the grading of AIN

There have been several biomarkers thought to be able to assist histopathologists in the classification of AIN. However, to date there is no evidence to support one combination of markers to improve reporting performance. The use of p16 IHC has been reported as being useful as a marker of HR-HPV infection (Sano et al., 1998). Other studies support dual staining with p16 and Ki67 as a marker of cellular proliferation and therefore dysplasia (Costa e Silva et al., 2011). It has been reported that the use of p16 and Ki67 enhances the detection of AIN and p16 in the reporting of disease (Pirog et al., 2010, Bean et al., 2009). It has been documented that p16 can be used to triage patients with p16 positive cytology has a greater specificity for high-grade disease (Arora et al., 2014).

The use of a specific biomarker has to be considered in conjunction with the grade of AIN. The LAST study states that if the histopathologist is entertaining a H&E morphologic interpretation of -IN grade 2 (in this study AIN2), then p16 IHC is recommended to help clarify the grade of disease. The LAST guidance states that “strong and diffuse block positive p16 results support a categorisation of precancer”, high-grade disease (Darragh et al., 2012a). Negative or non-block positive staining strongly favours an interpretation of low-grade disease (AIN1) or non-HPV-associated pathology (Darragh et al., 2012a). The reason for this recommendation is that p16
can be used if the differential diagnosis is between precancer (AIN2 or 3) or a mimic of precancer (e.g. squamous metaplasia) on H&E. IHC with p16 biomarker is not recommended as a routine adjunct to histological assessment of biopsy specimens, where the diagnosis includes benign tissue such as AIN1 or AIN3 (Darragh et al., 2012a). This is due to normal mucosa being p16 negative and the natural history of p16 positive AIN1 and p16 negative AIN3 is uncertain. If p16 is the appropriate biomarker in the detection of HGAIN this could assist in the selection of patients that needed treatment.

1.9.1 The normal cell cycle

The cell cycle is divided into four major phases (Figure 9). During cellular replication (somatic cells only), chromosomes are duplicated during the S (synthesis) phase. At the end of S phase the cells enter G2 with twice the amount of genetic material as they did entering S phase and G1; this is 4n in diploid organisms (Lodish H, 2000). The end of G2 is marked by the onset of mitosis, the M phase, which leads to cellular division. Following mitosis (M), daughter cells contain 2n chromosomes in diploid organisms and 1n chromosomes in haploid organisms (Lodish H, 2000). Lordish states that in cells that proliferate, G1 phase is the time frame between the “birth” of the replicated cell following mitosis and the initiation of DNA synthesis, the S phase. The G1, S, and G2 phases are collectively referred to interphase the period between one mitosis and the next (Lodish H, 2000).

Figure 9 The normal cell cycle

Figure 9: The cell cycle is continuous. Resting cells G0 that are large enough and contain all relevant cellular material go into G1. Cells that are sufficiently grown and contain all of the appropriate proteins then progress into synthesis, S phase. If the DNA has been replicated correctly, the cell progresses into mitosis, M phase. At the M checkpoint, if mitosis is complete, the cell can divide and the cycle repeats.
1.9.2 Tumour suppressor genes

The molecular biology of ASCC is still poorly understood. It is unclear whether the mutational pathways seen in cervical squamous cell carcinoma (CSCC) are similar or parallel to those seen in ASCC. Deregulated cellular proliferation and inhibition of apoptosis that occurs in relation to oncogenic HPV infection are thought to be insufficient for causing ASCC (Stelzer et al., 2010). Tumour suppressor genes and their corresponding proteins mediate control over the cell cycle. These proteins restrict cellular growth and division and they promote apoptosis (Sherr, 2004). This is best exemplified by tumour protein (TP53) gene, which is mutated in the majority of human cancers (Liu and Chen, 2006, Vogelstein and Kinzler, 2004). TP53 plays a critical role in maintaining G1 into S phase of the cell cycle, its role is a gatekeeper (Levine, 1997). The inactivation of TP53 allows cellular division even in the presence of DNA damage (Chow, 2010); this is demonstrated in Figure 10.

Cells that grow out of control often exhibit reduced expression or alternatively produce mutated forms of tumour suppressor proteins, which can promote carcinogenesis. Retinoblastoma protein (pRb) is another tumour suppressor gene and pRb1 stops the progression of the cell into S phase of the cell cycle. The inactivation of pRb allows for uncontrolled cell division (see Figure 10) (Chow, 2010).

Figure 10 Cell cycle control by tumour suppressor and oncogenes (Chow, 2010)

Figure 10: Checkpoints are depicted as thick red bars. The stages of the cell cycle (G1: Gap 1, S: DNA synthesis, G2: Gap 2, and M: mitosis) are indicated. Tumor suppressors act to maintain checkpoints (arrows) whereas oncogenes allow for checkpoints to be overcome (stop lines) (Adapted from Kopnin 2000) Nature Education.
1.9.3 Tumour protein 53 (TP53)

Tumour protein 53 (TP53) is a phosphoprotein made of 393 amino acids and has been mapped to chromosome 17. TP53 was first discovered by its ability to interact with the large T antigen produced by the transforming polyoma virus SV40 and it is now well established that the HPV E6 protein also interacts with TP53 in order to facilitate its degradation (Lane et al., 1985). Indeed many transforming viruses target TP53 in order to promote their own replication. In response to various signalling it regulates cell cycle arrest, cellular senescence, DNA repair, apoptosis or changes in metabolism (Liu and Chen, 2006). When a cell is subjected to oxidative stress, TP53 is activated to play an antioxidant role (Sablina et al., 2005). Once TP53 gene is damaged the cell will begin uncontrolled division, resulting in cancer.

1.9.4 Tumour suppressor gene p16

p16 is a tumour suppressor gene product; it is a cyclin-dependant kinase inhibitor that regulates the transition from G1 to S phase of the cell cycle (Ortega et al., 2002). The p16 gene product is up-regulated and overexpressed in high-grade dysplasia. There is a recognised association between p16, HR-HPV and anogenital disease in the cervix (Sano et al., 1998) and vulva (Riethdorf et al., 2004). Histopathologists use p16 as an IHC biomarker in the identification of cervical cancer and CIN from tissue biopsies (Walboomers et al., 1999, Schiffman et al., 1993). It is therefore likely that p16 could be a useful biomarker in anal disease. The LAST project states that p16 is useful for determining low-grade and high-grade lesion. The use of p16 stain is recommended by the LAST group, as discussed earlier, strong and diffuse block positive p16 results support a high-grade disease categorisation (Darragh et al., 2012b). As the natural history of AIN1 is uncertain, p16 is only recommended to determine a low-grade or high-grade precancerous lesion. The LAST project concluded that further work was needed to make a determination of no –IN versus AIN1 using p16 staining. It is also recognises that a positive p16 stain is seen in –IN1. It is reported that at least 30.0% of CIN1 cases are p16 positive, hence currently LAST guidance states that p16 should not be used on specimens that, on H&E alone, would have been reported as negative or –IN1 (Darragh et al., 2012b).

The interpretation of p16 IHC is controversial; defining the boundaries between strong, intermediate and weak staining can be problematic. Weak staining can sometimes be considered to be a positive or negative result (Murphy et al., 2003, Milde-Langosch et al., 2001). There is inconsistency between authors with respect to whether only nuclear staining of p16 is relevant (Geradts et al., 1995). Some believe p16 staining in both the nucleus and the cytoplasm is important (Murphy et al., 2003, Milde-Langosch et al., 2001). In a recent study, p16 overexpression did not always correlate with HR-HPV infection. Some HR-HPV lesions did not express p16, which means that limiting IHC screening to p16 alone would exclude a cohort of patients who were harbouring HR-HPV infection from any long term follow up (Samama et al., 2006). The same study concluded that screening for p16 overexpression in anogenital lesions allowed for discrimination. This was observed between HPV-integrated lesions and those infected with episomal (cytoplasmic) HPV or no HPV at all (Samama et al., 2006). Pirog et al
demonstrated that the p16 stain was only present in HGAIN histopathology samples. A positive result was defined as diffuse, moderate-to-strong cytoplasmic and nuclear staining (Pirog et al., 2010). A negative result was defined as no staining or staining of isolated cells/clusters. This study concluded that p16 staining in HGAIN is sensitive and specific (1.0 and 1.0 respectively) therefore p16 is a reliable marker for HGAIN (Pirog et al., 2010).

A study completed by Darvishian et al in 2006 highlighted that tissue that was p16 positive correlated with histological findings. Forty-three specimens were taken from 29 patients, 28 were p16 positive. Twenty-two had HGAIN, three LGAIN, two samples were negative for neoplasia and one inconclusive. The sensitivity and specificity of p16 immunoreactivity in the detection of HGAIN or ASCC were 72% and 71% respectively. The positive and negative predictive values of HGAIN or ASCC were 93% and 33% respectively (Darvishian et al., 2006). This supports that the presence of p16 immunoreactivity is a good predictor of anal dysplasia, although the sensitivity and specificity of this biomarker are not high. A recent study published in December 2015 wanted to determine the impact of implementing the LAST guidance of using p16 as a biomarker for high-grade disease. The group reviewed all cervical biopsy specimens diagnosed by two pathologists before and after the implementation of the LAST guidelines. They calculated the rate and reason for using p16 in all biopsy specimens along with HSIL detection and follow-up. In total 1,829 cervical biopsies that were initially read pre LAST guidance and 1,623 when the standards were in place were reviewed. They concluded that the overall use of p16 increased from 2.8% to 6.2% (p= < 0.001), p16 block positive sample’s in high-grade specimens increased from 1.4% to 2.3% (p= < 0.05) and the PPV of block positive p16 increased from 48% to 76% (p= < 0.05). This review demonstrates that new guidance resulted in a significant increase in the use of p16 biomarker along with an increase in the PPV of block positive samples ad high-grade disease (Clinton et al., 2015).

In another study p16 was compared to ProEx C and Ki67 in the evaluation of anal biopsy grading. In total 75 anal and perianal lesions from 65 different patients were analysed; 17 of the samples were non-dysplastic, 23 had LGAIN, 20 HGAIN and 15 invasive ASCC. These results were then further correlated with the presence of HR-HPV infection. Nineteen out of 20 HGAIN cases had p16 reactivity (18 strong and diffuse positive block, 1 focal strong). p16 was only negative in one block that contained HR-HPV 16. There was no p16 reactivity in the no-dysplastic cases (Bala et al., 2013). In this study p16 was the superior biomarker in identifying HGAIN, supporting its use for identification and diagnosing anal disease. In support of this finding, Bernard et al. also concluded that there was a correlation between AIN and p16, in this study with 100% of HGAIN positive for nuclear p16, but only 80% of LGAIN. Samples were collected from 128 anal biopsies and 25 hemorrhoidectomys (Bernard et al., 2008). This further supports the use of p16 IHC to help with diagnoses.
1.9.5 Ki67 protein

Ki67 protein is a marker of cellular proliferation; active infection with HPV has an association with increased proliferation in squamous epithelial cells. Ki67 is present during all active phases of the cell cycle, phases G1 to S, and is absent from cells at rest in G0 (Scholzen and Gerdes, 2000). The Ki67 antigen can be detected within the cell nucleus during interphase. During mitosis, most of the Ki67 protein relocates to the surface of chromosomes. Normal squamous epithelium displays Ki67 positivity confined to the parabasal layer. HPV associated lesions express Ki67 positivity in the parabasal, intermediate and superficial layers with increased cellular activity (Pirog et al., 2010). This is well documented by an increased labelling of Ki67, a marker of cellular proliferation (Lytwyn et al., 2005, Walts et al., 2006, Bean et al., 2007). Numerous studies define Ki67 positivity as the presence of a cluster of at least two strongly stained epithelial nuclei in the upper two thirds of the squamous layer (Pirog et al., 2010). The most recent recommendations from CAP-ASCCP concluded that evidence does not support any combination of biomarkers to improve performance when compared to using p16 alone, despite this the World Health Organisation (WHO) states that the expression of Ki67 can assist in grading. The LAST study does state that in cases where p16 is technically inadequate or inconclusive, Ki67 may be considered to assist in grading (Darragh et al., 2012a). IHC using Ki67 has been reported by Harber et al (Haber MM, 2004) as being useful in distinguishing dysplastic from non-dysplastic anal epithelium .

Dual staining with p16 and Ki67 biomarkers is thought to reflect HPV induced AIN better than Ki67 alone. Walts et al concluded that p16 and Ki67 dual staining showed improved concurrence with consensus diagnoses, reduced intra- and interobserver variability, and reduced two-step differences in diagnosis of AIN (Walts et al., 2008). HGAIN and negative diagnoses showed the most improvement in interobserver agreement levels (Walts et al., 2008). Pirog et al. concluded that using a combination of p16 and Ki67 biomarkers enhances the interpretation of anal biopsies. Ki67 labelling in this study detected anal HPV changes with a high degree of sensitivity and specificity (1.0 and 1.0 respectively) (Pirog et al., 2010). A positive result was defined as a cluster of at least two strongly stained epithelial cells in the upper two thirds of the epithelium, and a negative result had no staining present (Pirog et al., 2010).
1.10 Hypotheses

1.10.1 Clinical study Hypotheses

High-resolution anoscopy is the gold standard in the detection of AIN and provides means to evaluate the clinical utility of HR-HPV testing and anal cytology as screening tests in high-risk groups.

1.10.2 Immunohistochemistry laboratory hypotheses

This study will explore biomarkers in the identification of AIN in order to explore the relationship with disease progression.

- To ascertain the sensitivity of HR-HPV expressions by using p16 positivity scored by automated and manual methods and then compare them to the gold standard of HR-HPV detection Roche Cobas®.

- Anal biopsy specimens will display Ki67 and p16 positivity in relation to grade of anal disease. There will be an increase in positivity of Ki67 and p16 biomarkers as the grade of AIN increases.

- The modified H-SCORE of p16 stained tissue will be increased in tissue specimens with a higher grade of AIN.

- There will be a difference in the detection of Ki67 and p16 dependant on whether the slides are scored manually or by an automated system.

1.11 Aim of this study

The aim of this study was to evaluate the feasibility and clinical utility of liquid based cytology, human papilloma virus testing and high-resolution anoscopy to screen for anal intraepithelial neoplasia in high-risk groups.
CHAPTER 2

MATERIALS AND
METHODS
2 MATERIALS AND METHODS

2.1 Clinical study

2.1.1 Study design

ANALOGY is a prospective cohort study. All patients were offered two clinic appointments where they had anal cytology, HPV testing and HRA along with biopsy if required. The target for recruitment was 1000 patients with 250 patients in each of one of the four different groups. The study Protocol and Ethics (12/NW/0204) application can be seen in appendix i and ii.

2.1.2 Patient recruitment

Patients were screened for eligibility by the research team. The notes of patients who attended the Manchester Centre for Sexual Health (MCSH) and North Manchester General Hospital (NMGH) for HIV care or sexual health services were screened. If the patient was eligible to participate in the study, an ANALOGY sticker was inserted into the inside left leaf of the hospital notes along with a patient information leaflet (appendix iiia, iiib, iiic), which was specific to the patient group and location at which they were recruited. The patient information leaflet explained the background and purpose of the study, along with what will happen during both clinic appointments and information regarding onward referral if required. Medical and specialist HIV nurses in the MCSH and NMGH also recruited patients during routine HIV follow-up appointments, and a patient information leaflet was handed out during consultation. Patients who were HIV negative or unknown and were simply attending MCSH or NMGH for a sexual health screen did not have their notes reviewed prior to attending for an appointment. Patients attending follow-up appointments in transplant outpatient clinic at Manchester Royal Infirmary were also screened for eligibility to participate in the study. A screening log of eligibility was kept. Notes that were screened by the research team in transplant clinic also had a sticker inserted into the inside leaf of the notes with the date of review documented, the patient information leaflet inserted into them and then the transplant surgeons discussed the study with the patients during consultation. After this consultation, a member of the research team also discussed the study.

In transplant clinic a nurse specialist who performed annual reviews also assessed eligibility and issued interested patients with information leaflets. Initially patients who were interested in taking part in the study but had not seen the research team at the first point of contact were being lost, as there was no consent for contact by any modality. An amendment was submitted to ethics for an "expression of interest form", see appendix (iv). After this amendment all patients who were happy to be contacted by the research team were asked to complete an expression of interest form. The form had the patients preferred contact modality i.e., text, phone, or email and was signed by the patient. This enabled the research team to contact the patient after they had received the patient information leaflet. All of the health professional that assisted in recruitment onto the ANALOGY study after this amendment ensured that patients who wanted to participate completed an expression of interest form so the research team could
then contact them at a later date. The timeframe for contact had to greater than 24-hours after receiving information about the study. This ensured that recruited patients had a time to consider if they truly wanted to participate in the ANALOGY study and did not feel obligated to take part. It also ensured they could read and understand the patient information leaflet in their own time and contact the research team with any questions prior to taking part.

All patients were encouraged to discuss the study with family, friends and medical health care providers. Patient’s whose judgment appeared to be impaired were not included in the study. Patients without good understanding of English were recruited via an interpreter.

2.1.3 Inclusion criteria for participation

In order to participate in the ANALOGY study, all patients had to be over 25-years of age. The patients then had to belong to one of the four high-risk groups:

- HIV positive men who have sex with men (MSM)
- HIV positive women with previous cervical, vulval or vaginal dysplasia
- HIV negative men and women who have anoreceptive sex
- Renal transplant recipients who were immune suppressed

It was agreed that TR had to have a minimum duration of two years since their first transplant to take part in the study. The consultant transplant team felt that at two years post procedure the majority of patients would be stabilised on an appropriate immunosuppressive medication. There were patients attending transplant clinic who had not had a renal transplant, but another organ transplantation. The types of transplants were pancreas or islet cell and as long as they were two years post first procedure, these patients were deemed eligible to take part in the study as they were also immunosuppressed taking oral immunosuppressive medication.

2.1.4 Exclusion criteria

Patients who were screened for the ANALOGY study but had to be excluded from participating were those who were less than 25-years of age. Women who were pregnant were excluded. If any screened individual had a previous history of AIN or anal cancer they were also not eligible to participate in the study.

2.1.5 Recruitment targets

The initial recruitment target for the study was 1000 participants, with 250 individuals from four high-risk groups. It became clear that HIV positive and negative women were not interested in participating in this study. The objective was therefore to recruit as many participants as possible to the other groups that had expressed an interest i.e. HIV positive and negative MSM who practiced anoreceptive sex and TR.
2.2 Development of the clinical service

The development of a HRA service was based on experience gained both in the University of California San Francisco (UCSF) with Professor Joel Palefsky and his team. Along with time spent at Homerton University Hospital, London, during HRA training under the supervision of Dr Mayura Nathan.

2.2.1 High-resolution anoscopy training

I spent five days in San Francisco UCSF hospital in December 2012, working with Professor Joel Palefsky’s team who run an HRA service for screening and treatment of AIN. I observed first time consultations, HRA, infra-red coagulation treatments and follow-up appointments. I learnt what information was collected during a consultation and watched multiple HRA’s performed by three different physicians. Visualisation of the anal canal was made possible by the utilisation of a teaching arm on the colposcope and real time camera. All patients in San Francisco had HRA in the left lateral position on a bed fit for purpose.

I received further training from Dr Mayura Nathan in his HRA and laser clinic in Homerton University Hospital London, on a regular basis. I attended from December 2012 until I felt able to start a similar service in Manchester at the end of March 2013. Under Dr Nathans supervision I learnt how to perform an appropriate consultation, assess the peri-anus, take an anal cytology sample, perform HRA and take anal tissue biopsies.

2.2.2 Development of clinical research forms

Following the visits to San Francisco and time spent in London I designed two clinical research forms (CRF); one for sexual health and the other for transplant patients. The CRF design was based on experience at these two leading institutions (see appendix va, vb & vc). One CRF was designed for MCSH patients, encompassing three groups within the study; HIV positive MSM, HIV positive women and HIV negative men and women who receive anoreceptive sex. When a second site, NMGH, was included to recruit patients, the same CRF was used. The second CRF was designed for transplant patients. Discussions were also held with the Consultant team for the transplant service and the sexual health centre in Manchester so that essential information was added to each CRF to be collected during the study.

2.2.3 Establishing a service

A completely new service for HRA was established in MCSH, NMGH sexual health and the outpatient colposcopy suite in St Mary’s hospital CMFT. It took four months to put the service in place. By the end of the study there were four clinics a week until the study closed in March 2015.

The outpatient colposcopy department in St Mary’s hospital had rooms that were fit for a HRA clinic with a colposcope in each suite. It was decided that transplant men and women would attend the outpatient colposcopy suite as they did not feel comfortable attending appointments
held in sexual health clinics. Sexual health females were offered an appointment in outpatient colposcopy or in MCSH.

The HRA clinic in MCSH was set up with relative ease. There were two clinics a week, with one clinic running into the early evening. In order to accommodate patients who need an appointment outside of working hours.

In order to enhance recruitment, it was deemed necessary to screen patients and offer HRA at another site within the city. Patients who attended sexual health services at NMGH and the Infectious Diseases outpatient clinic were approached to take part in the study from March 20th 2014. A weekly Thursday clinic commenced at NMGH sexual health clinic until the study closed. The clinic at NMGH was conducted in the sexual health department and the colposcope used was loaned for the duration of the study from the Gynaecology team.

When cytology is collected from the cervix, the practitioner visualises the external os in order to collect the sample, anal LBC samples were collected blindly. The medium that allowed for cytology analysis and for HPV testing was SurePath™. The anoscope used to perform anoscopy was supplied by Pelican. There were two sizes of anoscopes, a medium that had a diameter of 18mm and larger sized anoscope that had a diameter of 22mm. Visual inspection of the anal canal after the application of 4% acetic acid was achieved using Carl Zeiss colposcope. Lugols’ iodine was applied to confirm areas suspicious of AIN. When a biopsy was required, 2.2ml of Citanest and Octapressin (Prilocaine hydrochloride and felypressin) 3% solution anaesthetic was inserted using a disposable dental syringe. “Baby” tischler forceps were used to collect the sample; they had a 2mm x 5mm bite. Specimens were fixed in neutral buffered formalin (NBF aldehyde). Silver nitrate and ferric subsulphate solution (monsels solution) was used to promote haemostasis. A 4-0 vicryl rapide suture was inserted if haemostasis was not achieved.

2.2.4 Patient position during high-resolution anoscopy examination

Patients who undergo HRA in San Francisco are examined in left lateral position on a bed fit for purpose and this position appeared to make the patients more comfortable, especially if they were male. In London, all patients are examined in lithotomy. The beds in MCSH, NMGH and outpatient colposcopy suite were designed for colposcopy and gynaecological examination. There were no beds that were the same as those beds used in San Francisco. It was therefore decided that all patients would be examined in lithotomy.
2.3 Consultations

2.3.1 Documentation of consultations

All consultations were completed on the study CRF. A written entry was also inserted in the patient notes under a Research heading.

2.3.2 Clinical consultations

Consultations generally lasted between 30-40 minutes. TR were seen in St Mary’s outpatient department (OPD) colposcopy suite. All other patients were seen in MCSH or NMGH. On arrival, reception staff issued all patients with a questionnaire investigating the patient’s preconceptions about participation in the study, HPV, anal cancer and precancerous cell changes, these data will not be discussed in this thesis. This was prior to seeing the research team for the HRA consultation.

2.3.3 Patient study number allocation

All patients were allocated a study number. Patients who are recruited from MCSH had a study number from 1000 upwards; transplant patients from 2000 upwards and patients from NMGH 3000 upwards for ease of group identification. Each group’s CRF’s are filed in separate folders in ascending order. The CRF’s are stored in accordance to Trust and University of Manchester policy and good clinical practice guidelines.

2.3.4 Consent process

I obtained written patient consent during the first clinical consultation. During the consent process patients were asked if they had any questions surrounding the study. A copy of the consent form can be seen in appendix (vi a-b). The consultation was then outlined, with an explanation that a digital rectal exam, anal smear for cytology and HPV testing would be taken followed by HRA with the possibility of anal biopsy if deemed necessary and only if the patient consented to having biopsies taken. At every consultation I had a detailed conversation with every patient regarding being infected with HR-HPV and if not cleared its potential effect on anal tissue. I also discussed at this current time there is no therapeutic treatment for HPV infection.

The patients were informed the delivery of results would be via telephone either by my research nurse or me. They were also informed that if high-grade precancerous change, AIN3 or invasive cancer were found in an anal tissue biopsy site that they would be referred on to Professor James Hill for examination under anaesthetic.

All of the patients who agreed to participate in the ANALOGY study then completed a consent form on the day of consultation in HRA clinic. In total three copies of the consent form were made. The original, this is filed with the CRF along with the participation questionnaire. A second copy is filed in the participant’s main notes along with a written consultation history and participant information leaflet under a research divider. The third copy was made for the patient.
Before the examination commenced I enquired about allergies to latex or other substances, if there was any history of bleeding disorders or if they were taking any anticoagulants. Patients taking sub-cutaneous thromboprophylaxis were re-scheduled for another appointment if a biopsy was needed following direct instruction from the haematology team.

2.4 Study procedures

A patient identification band was placed on the patient’s wrist for the entirety of the examination and removed once completed. All patients were then asked to undress behind a privacy curtain from the waist down and lie on the examination couch with their legs in lithotomy. A paper towel sheet was provided for modesty. Once in a comfortable position the assessment could begin. Patients then went on to have an external anal inspection followed by a digital rectal examination (DRE). I then performed a blind LBC anal cytology sample, collected using a FLOQTM swab. Following this, the HRA is performed, see Figure 11.

2.4.1 Anal cytology and reporting

Liquid based anal cytology taken during the ANALOGY study was preserved using SurePath™. This is the liquid based cytology medium used in Manchester, UK. See appendix (vii) for SurePath™ composition.

Before the sample was collected, the patient’s study number, name, date of birth and hospital numbers were recorded on the specimen pot. First the patient’s name and date of birth were confirmed before the examination commenced. With the patient in lithotomy position, a full inspection of the peri-anus was completed, including inspection of the perianal skin. A premoistened FLOQ swab was then inserted into the anal canal. This was achieved by exposing the anus with one hand and inserting the swab gently through the external anal sphincter with the other. The swab was inserted into the anal canal blindly past the anal verge to roughly a 5cm depth. Firm lateral pressure was then applied to the swab handle and it is rotated and withdrawn simultaneously, inscribing a cone shaped arc. The swab was then placed in a SurePath™ vial and agitated for a minute in order to maximise cellular harvest. A cytology request form was completed for each anal LBC sample, see appendix (viii). A summary of collecting anal cytology samples can be seen in Table 3 as per the Palefsky technique. All of the anal cytology samples were exclusively read and reported by Dr Mina Desai, who is an expert and very experienced cytopathologist. Dr Desai read all of the slides and then re-read them blinded to ensure quality of reporting. Cytology samples were reported according to the Bethesda System (TBS), the grades reported can be seen in Table 4.
Study population= aged 25 years + from high risk groups
HIV positive men who have sex with men
HIV negative men and women who practice anoreceptive sex
HIV positive women with a history of anogenital warts or intraepithelial neoplasia
Men & women who are transplant recipients & >2 years post transplantation

Consent to participate in the ANALOGY study

Liquid based anal cytology (SurePath™)
Anal HPV by Roche Cobas 4800® (incl. HPV16/18 +ve’s)
High-resolution Anoscopy

Negative anoscopy or AIN
grades 1/2

Review 6/12
Review 3/12*
*(after amendment)

AIN grade 3+

Refer to Colorectal Surgeon
Examination under anaesthetic
Treat according to management protocol

Negative anoscopy
No AIN detected
DISCHARGE TO GP

AIN grades 1/2
DISCHARGE TO COLORECTAL
Table 3 Steps for collecting anal cytology specimens (adapted from (Jay, 2011))

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Moisten synthetic swab (FLOQ) in SurePath™</td>
</tr>
<tr>
<td>2</td>
<td>Separate buttocks gently so that anal opening is clearly visible</td>
</tr>
<tr>
<td>3</td>
<td>Insert the swab slowly with gentle pressure until it bypasses the internal sphincter</td>
</tr>
<tr>
<td>4</td>
<td>Insert to 5-7cm (2-3inches) until resistance is met at distal rectal wall</td>
</tr>
<tr>
<td>5</td>
<td>Slowly remove swab in a spiral motion applying constant firm lateral pressure to sample all mucosa in the canal</td>
</tr>
<tr>
<td>6</td>
<td>Count to 10 slowly whilst removing the swab and collecting cellular sample</td>
</tr>
<tr>
<td>7</td>
<td>When reaching anal verge, let go of the buttocks so the verge is sampled</td>
</tr>
<tr>
<td>8</td>
<td>Transfer to LBC vial by vigorously swirling in fluid</td>
</tr>
</tbody>
</table>

Table 4 Cytology reporting terminology The Bethesda System for Anal reporting

<table>
<thead>
<tr>
<th>Grade of anal cytology</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative for intraepithelial lesion or malignancy</td>
<td>NILM</td>
<td>Negative for intraepithelial lesion or malignancy</td>
</tr>
<tr>
<td>Atypical cells of undetermined significance</td>
<td>ASCUS</td>
<td>Atypical cells of undetermined significance</td>
</tr>
<tr>
<td>Low-grade squamous intraepithelial lesion</td>
<td>LSIL</td>
<td>Low-grade squamous intraepithelial lesion</td>
</tr>
<tr>
<td>Atypical squamous cells, cannot exclude high grade lesion</td>
<td>ASCU-H</td>
<td>Atypical squamous cells, cannot exclude high grade lesion</td>
</tr>
<tr>
<td>High grade intraepithelial lesion</td>
<td>HSIL</td>
<td>High grade intraepithelial lesion</td>
</tr>
<tr>
<td>High grade intraepithelial lesion? Invasion</td>
<td>HSIL ? Invasion</td>
<td>High grade intraepithelial lesion? Invasion</td>
</tr>
</tbody>
</table>

2.4.2 Performing a digital rectal examination (DRE)

A DRE was then performed using lidocaine 5% anaesthetic ointment as a lubricant. This enabled assessment of the internal anus and permitted the identification of any palpable masses such as haemorrhoids, warts, fissures or suspicious palpable lesions.

2.4.3 Performing high-resolution anoscopy (HRA)

A disposable anoscope (Pelican) was used for each patient. The anoscope was lubricated then inserted into the anus, the obturator was removed and gauze soaked acetic acid swab was inserted into the anal canal via an anoscope and left in situ for a few minutes (Palefsky, 2012).
This is to maximize uptake of acetic acid into the epithelial tissue. Four per cent acetic acid was used to cause whitening of any suspicious mucosa that may require biopsy (Palefsky, 2012). The insertion of the gauze soaked swab permitted identification of abnormal epithelium in the anus.

After the gauze soaked swab was removed, the anoscope was lubricated again and reinserted into the anus, the obturator removed and the colposcope was focused on the anal mucosa on low magnification. On occasion after insertion of the anoscope the distal rectum was seen, the anoscope was then withdrawn to establish anatomical landmarks. The colposcope magnification was then modified to high power. The squamocolumnar junction (SCJ) and the anal transitional zone (ATZ) are the main focus of the examination and the canal is continually assessed for aceto-white change after application of acetic acid 4% via a large cotton swab. Interval reapplication of acetic acid is required for adequate visual assessment. The entire anal canal is viewed whilst simultaneously slowly withdrawing the anoscope whilst continually looking for vascular changes of punctuation and mosaicism. HGAIN is predominantly seen at the ATZ but LGAIN may be seen anywhere along the canal. Lugol's iodine stain was applied to highlight and define further suspicious lesions. If aceto-whitening is detected, AIN should be suspected. Also, if Lugols iodine stain is not taken up after aceto-white staining. Most HGAIN is non staining and LGAIN is variable after Lugols iodine application ranging from negative to partial light brown and positive dark brown (Palefsky, 2012). A full inspection of the anal canal was then performed. This was achieved by using a systematic method relating quadrants of disease to numbers on a clock face, see Figure 12. Areas of suspicion were documented on the CRF. Documenting the lesion site in a clock face manner permitted accurate identification at a second visit and at onward referral to the colorectal team.

**Figure 12 Documentation of anal canal examination at high-resolution anoscopy**

<table>
<thead>
<tr>
<th>Internal anal canal</th>
<th>External anal canal</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Right</strong></td>
<td><strong>Left</strong></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
<tr>
<td><strong>Right</strong></td>
<td><strong>Left</strong></td>
</tr>
<tr>
<td>12</td>
<td></td>
</tr>
</tbody>
</table>

![Diagram](image-url)
A full evaluation of the keratinised portion of the anal canal and the surrounding perianal skin was completed after application of acetic acid. Changes in the skin are similar to those seen on vulvar colposcopy and were noted at the end of the HRA.

2.4.4 Taking an anal biopsy during high-resolution anoscopy

HRA guided biopsy is the gold standard for diagnosing AIN. Anal tissue biopsy was taken when a suspicious area had been highlighted by the application of acetic acid. Depending on where the area of suspicion was in relation to the dentate line, anal verge and peri-anus, 2.2ml of Citanest and Octapressin (Prilocaine hydrochloride and felypressin) 3% solution anaesthetic was infiltrated. If the lesion was situated on the anal verge or perianally, infiltration was always required. If the area of suspicion was on the dentate line local Xylocaine 10mg was applied topically and administered by spray. Each spray delivered 10mg lidocaine base, ethanol, macrogol 400 and levomenthol. Elsewhere, it was not always required. If multiple biopsies were required local infiltration was always used. If multiple anal biopsies were needed, samples in the inferior field of vision were taken first so as not to obscure the view when taking superior samples.

“Baby” tischler forceps were used to take a small punch anal biopsy. Each biopsy was transferred to its own neutral buffered formalin (aldehyde; NBF) specimen pot and labelled according to location. All specimens were catalogued on a histopathology specimen request form, see Appendix (ix). Generally there was little bleeding from anal and peri-anal biopsy sites. Haemostasis was easily managed with silver nitrate stick application and ferric subsulphate solution (Monsels solution). In the event haemostasis was not achieved with silver nitrate, monsels and pressure, and a 4-0 vicryl rapide suture was inserted into the anal canal.

All patients were given verbal instruction after biopsy not to have anal intercourse or engage in any behaviour that may cause trauma to the anal mucosa for seven to 14 days afterwards dependant on number of samples taken. Advice about light diet and hydration to maintain a soft stool was also given. Patients were warned that they may experience light bleeding initially and then for several days with or just after a bowel movement. Instruction to seek medical attention from an Emergency Department if there was heavy bleeding was also delivered, along with reporting excessive pain not controlled by simple analgesia, fever, chills, swelling or discharge.

The “baby” tischler forceps are sent to for sterilising. All none disposable equipment was cleaned between each patient. Disposable equipment was sent for incineration as per Trust policy.

Any patients with an insufficient cytology or virology samples for HPV typing were invited back for a repeat sample.
2.5 Specimen transfer

All of the specimens collected were stored in a sealed transfer container. The research team transferred the specimens and handover of the specimens was logged. Liquid based cytology samples were sent with a cytology request form modified for purpose, Appendix (viii). These samples were processed at the Manchester Cytology Centre in Manchester Royal Infirmary. Once the cytology sample had been processed the residual aliquot was internally transferred to Virology Department for HPV typing. Anal tissue biopsies were transferred to the Histopathology Department in formalin for reporting. A standard CMFT histopathology form is used to send biopsy samples, Appendix (ix).

2.6 Clinical results management

Patients were asked during their first consultation to confirm how they would like to be contacted to receive their results. Patients were informed that a final summary of their results once the study was completed would be sent to them at an address they provided and their General Practitioner. Paper copies of results would only be sent out if the patient had provided consent. Results of anal cytology, high-grade HPV infection and histopathology samples were sent to the research team for collation. Results were documented in the patients CRF and stored in a locked cabinet on the CMFT hospital site.

2.7 Visit two

2.7.1 Consultation two

The purpose of a repeat consultation was to determine how much disease had been missed at the first visit. The second consultation was performed six months after the initial visit. At the second visit all patients had a repeated full consultation enquiring about any change in symptoms or circumstances. HPV infection and clearance were discussed with every patient. Cytology and virology samples were collected along with HRA and biopsies if needed. A second questionnaire was issued to the patient by reception staff; these data will not be discussed in this thesis. This was completed by the patient after the consultation, sealed and placed in a sealed box. The research team did not see the completed questionnaires they were given to Dr Laura Sadler, a social scientist for use in another study regarding the acceptability of anal screening. They were then stored separately from the CRF as per good practice.

An application to extend the recruitment timeframe was submitted and accepted along with a reduction in the time frame for consultation two from six to three months on October 1st 2014. This amendment permitted recruitment up-to 31st December 2014 and three month follow-up until March 31st 2015. The amendment can be seen in Appendix x. The amendment was made primarily to enhance recruitment to the ANALOGY study, but it was also decided that it would be more appropriate to give the patients their results face-to-face at visit two three months later after, in order to enable discussion about concerns engendered by their results.
At visit two I confirmed that patients were happy to have paper copies of their results sent to the address they had provided and their General Practitioner.

2.7.2 Data management and storage

All data were collated and then inputted into the spreadsheet package MS Excel. Mrs Linsey Nelson also inputted the full data set for each patient into an ACCESS database. At the time of data input every CRF was electronically scanned and stored.

A summary sheet was generated for the patient’s notes, results letters for the patients along with General Practitioner information summaries, as seen in Appendix xia (i-ii). The summary sheets were filed under the ANALOGY research divider in the hospital notes along with copies of all letters. If patients consented to be contacted by post, they were sent a letter informing them of their results and outcome of participation in the study, as seen in Appendix xib (i-vi). All of the letters used in the ANALOGY study can be seen in Appendix xi a & b (a i-ii, b i-vi).

2.7.3 Second consultation results and informing healthcare professionals

Patients were informed that one of the research team would call them with their results and management plan roughly six weeks after they had attended for their second visit. It was also reiterated by the research team on delivery of results via the telephone that a final summary of their results once the study was completed would be sent to them at an address they provided and their General Practitioner. Paper copies of results would only be sent out if the patient had provided consent and if they decided to not receive a summary once the study was completed, this was noted during the call.

2.8 Patients who failed to attend for clinic appointments

A log of patients who do not attend their clinic appointment was kept. An attempt is made by the research team to contact the patient to rebook the appointment. To try and reduce unattended clinic appointments, a member of the research team contacted all of the patients the day before the appointment by their chosen method of communication on the expression of interest form.

If a patient failed to attend three appointments he/she was then deemed lost to follow-up.

2.9 Onward referral of patients with high-grade results

2.9.1 Action following diagnosis of high-grade anal intraepithelial neoplasia

All patients with AIN3+ on biopsy were immediately referred to a colorectal Consultant, Professor James Hill, for examination under anaesthetic (EUA). The referral letter was emailed via a Trust account directly to Professor Hill and his secretary. A copy of the letter was filed in the patient’s main hospital notes. Initially the referred patients were seen as an out-patient by Mr Hill or a senior member of his team, and then booked for further management. It was decided in September 2013 that if a patient had AIN3+ then he/she would be booked for EUA and then seen by Professor Hill after the biopsy results were reported.
2.10 Clinical laboratory protocols

2.10.1 Cytology Protocol

All cytology samples were sent to the Cytology Centre based in Manchester Royal Infirmary. The SurePath™ standard operating procedure (SOP) for slide production can be seen in appendix (xii). Reporting of cytology followed the Bethesda System (TBS).

2.10.2 Virology protocol and human papillomavirus typing

Viral samples were tested in the Virology Department at Manchester Infirmary, Manchester, UK, using Roche Cobas 4800® testing protocol under the supervision of Dr Alex Sargent. Any sample that tested positive using Cobas 4800® was also run for a PapilloCheck® genotyping (see appendices xiii & xiv).

2.10.3 Roche Cobas 4800®

After cytological processing, 1ml of SurePath™ LBC was screened for HR-HPV using the Roche Cobas® 4800. The Cobas 4800® allows testing for 14-high-risk HPV types including simultaneous detection of types 16 and 18. Other high-risk types tested for are 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The Cobas 4800® features automated sample preparation combined with Real-Time PCR to detect HR-HPV. The PCR amplification and detection occur in a single tube. The identification of (i) HPV 16, (ii) HPV 18, separately along with (iii) 12 HR (31, - 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) as a pool. β-globin is used as the control for extraction and amplification adequacy of all samples. Results from the Cobas 4800® can be obtained roughly four hours after the sample has been received. HPV testing was performed in concordance with the department SOP and can be seen in Appendix (xiii).

2.10.4 PapilloCheck®

The PapilloCheck® is a microarray-based test for the qualitative detection and genotyping of HPV in DNA-preparations from human cervical smears. The test is not validated for other sample types however an internal control checked for adequacy of the sample. The assay itself is manual and processes 72 samples every 1.5 days. This includes sample preparation, extraction, amplification, detection of types and read out. Every chip contains 12 DNA-microarrays, allowing simultaneous detection of 10 samples plus a negative and positive control. The hybridisation and wash sequences are quick and the laser mediated readout is digitised to provide a genotype profile.

All samples that tested positive by Cobas 4800® were further genotyped. From the SurePath™ residue, a 500μl aliquot was centrifuged and the supernatant removed. The cell pellet was digested using Proteinase K lysis before being extracted. A 125μl of DNA was then extracted using the automated Nuclens easyMAG system (BioMerieux) rather than the manual oCheck extraction kit recommended by the manufacturers. PapilloCheck® (Greiner Bio-One GmbH, Frickenhausen, Germany) assay uses a fluorescent primer multiplex PCR. The primer amplifies a 350bp fragment of the HPV E1 gene. Within each fixed DNA chip there are 28 probes in five
replicate spots. To identify false negatives human ADAT1 gene is also co-amplified and used as an internal control. The hybridisation is performed on a microarray chip. The chip is automatically scanned and analysed using a CheckerScanner™ at 532nm and 635nm. The output is then reported by CheckReport™ software. Genotyping was completed using the PapilloCheck® as seen in Appendix (xiv).

PapilloCheck® tests for 24-types (18 high-risk and 6 low-risk) of HPV. The 18 high-risks types are 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82. The low-risk types are 6, 11, 40, 42, 43 and 44.

2.10.5 Histopathology specimen processing

All anal biopsies were sent to Dr Raymond McMahon, Consultant Pathologist at Manchester Royal Infirmary. Dr Ronah McVey, Consultant Pathologist, also reported all of the tissue specimens that required a second report for confirmation of the grade of disease and any disease that was suspected to be AIN3+. Both Consultants reported all of the tissue specimens without being aware of the cytology or HPV results. Sections were cut and then stained with H&E to confirm presence of AIN.

Specimens taken during HRA were fixed in NBF. All of the anal biopsy samples taken at HRA were transferred immediately to NBF, which then optimised the preservation of tissue architecture. A 10% NBF concentration was used for this study.

Tissue samples were then processed by the removal of water and then embedded in paraffin wax. This enabled thin cutting ribbons (3-5µm) to allow for microscopic examination. Paraffin processing enables preservation of cellular structures. Paraffin embedding allows infiltration down to the cellular level, and permits the tissue to have enough even rigidity to be thinly sectioned and placed on a slide for further analysis.

Tissue fixed in NBF is first dehydrated through graded alcohols (70%, 90%, and 95%). The tissue is then submerged in xylene. The final stage involves the tissue being impregnated with paraffin wax under a vacuum. The tissue is placed in molten paraffin wax and positioned in the mold. Once the mold has cooled, the tissue “block” is ready for cutting sections on a microtome.

The ribbon section once cut is floated on a warm water bath (47°C) to allow the paraffin to spread and remove any wrinkles caused by the cutting action. The ribbons were retrieved and placed on slides. The slides were then placed in an oven for 20 minutes at 70°C until the water evaporated and the paraffin melted. For staining of histopathology samples, a Combined Leica Autostainer XL and CV5030 coverslipper was used for all specimens. Haematoxylin and Eosin staining protocol for wax sections can be seen in Appendix (xv). The slides were then stored in relation to patient number and lab number.

Reporting of specimens was by grade of disease, AIN1, AIN2 or AIN3 and invasive disease see Figure 13. Any sample that was initially reported as “ungraded” AIN due to the tissue specimen having an epithelial depth of less than 10 cells was re-reported using LAST terminology.
Figure 13 Haemotoxylin and eosin slides of varying grades of AIN and invasive cancer

A) Shows an image with AIN2 and AIN3 at x4 magnification.
B) Shows an image with AIN2 and AIN3 at x10 magnification.
C) Shows an image with AIN2 and AIN3 at x40 magnification.
shows an image with invasive anal squamous cell carcinoma at x4 magnification.

E) Shows an image with invasive anal squamous cell carcinoma at x10 magnification.

2.10.6 Sample reporting problems

Some of the samples could not be processed by cytology, virology and or histopathology. They were categorised as insufficient if there was a cytological processing problem, and this was generally due to fecal debris or the cellular count not being sufficient for reading. Virology samples were categorised as inhibitory if there was a virology processing issue due to an internal control failure on the machine. Invalid samples were due to an issue testing for a HR-HPV type due to the PCR reaction failing or there being too few cells for reliable detection. These patients were recalled for a repeat sample. Biopsy samples that were not of a sufficient epithelial depth have been categorised as un-gradable.
2.11 Data analysis from clinical study

Matthew Gittins at the University of Manchester undertook formal statistical analysis using the statistical package STATA version 13. Standard descriptive statistics including frequency tables are reported to outline the characteristics of participants within the data. Due to limited recruitment causing sample size issues, parts of the analyses were only performed in the MSM subject group as comparisons with the non-MSM was not appropriate due to sample size.

The primary outcome of interest 'biopsy result' was dichotomised such that it represented two binary variables AIN2+ versus other, and AIN3+ versus other. The primary predictors of interest ‘cytology result’ and ‘HPV result’ were also dichotomised respectively to be Borderline versus Other, and HPV Positive versus Negative. Corresponding sensitivity and specificity associated with the two primary predictors and the outcome were calculated. Sensitivity and specificity represented the two tests ability to correctly identify a positive or a negative biopsy result, respectively. Reported as a percentage, the greater the percentage the greater the tests ability to identify a correct result.

A simple logistic regression model was performed to determine the predictive ability of the primary predictor of interest, cytology testing on the biopsy result. The model included covariates thought a priori to account for a large amount of confounding. These were; age, smoking status (Never, Previous, Current), alcohol status (Never, Previous, Current), HIV status (Positive versus Negative), and HPV status (Positive versus Negative). Results are reported in the form of an odds ratio along with corresponding 95% confidence intervals. Here an odds ratio represents the change in odds of a positive biopsy between the category and the reference category e.g. HIV positive vs HIV negative (base category). An odds ratio of 1 indicates no change, greater than 1 indicates an increase in the odds of a positive biopsy result, and 0-1 a decrease in the odds of a positive biopsy results.

A secondary analysis was performed to investigate the influence of time duration as a smoker, as sexually active, as anally active, and as HIV positive. In each case a time variable was generated to represent the time in years between first activity and test taken. The logistic regression model described above was repeated with time duration variables included. As the predictor is continuous, here the odds ratio represents the odds of a positive biopsy result as time increases by one unit e.g. for time as a smoker it represents a change in the odds for each year increase as a smoker.

No statistical analysis was performed on genotyping data.
2.12 Immunohistochemistry studies

Biomarkers in the reporting of anal disease are seldom used. Due to AIN being a HPV driven disease antibody labelling for Ki67 and p16 expression were selected. It is hypothesised that there will be a correlation between the grade of anal disease and the expression of Ki67 and p16.

A total of 789 specimens from 340 patients were collected over 24-months from March 2013 to March 2015 during the ANALOGY study. For this study 100 specimens were selected from the samples available in January 2014, Dr Ray McMahon (RMcM) and I (Dr Alice Martha Schofield AMS) reviewed all of the tissue slides together. At this time, it was decided all AIN3+ should be studied as currently in the UK the only treated grade of disease. It is still uncertain as to whether categorise AIN2 is high or low-grade disease. To ascertain if there was any difference observed in the specimens taken if Ki67 and p16 staining was inconclusive or technically inadequate; twice as many AIN2 samples were stained when compared to number of AIN3+ studied. The rationale for this was Ki67 and p16 expression along with HPV infection may vary by grade of disease and immune status, it is plausible that this could affect clinical management. The tissue selection can be seen in Table 5. In this table, specimens are categorised as they were reported. Biopsy samples were reported as normal (negative), condyloma, AIN1, AIN2, AIN3 or invasive cancer. In total seven samples were initially reported as “ungraded AIN”. These samples were reclassified using the LAST criteria. In accordance with LAST guidance, any lesion that is unequivocally SIL with mitosis above the basal cells or significant immature abnormal basal proliferation is graded as HSIL (AIN2+) (Darragh et al., 2012b). On the basis of these criteria, one sample was upgraded to AIN2 and six samples were graded as AIN1. All of the AIN2 samples stained with p16 were also reviewed to ascertain if they displayed block positivity (Darragh et al., 2012b) to ascertain high-grade disease.

In total 100 samples were selected from 87 patients. Seventy-seven specimens were collected from 65 different HIV positive MSM, 13 samples from the HIV negative MSM and 10 samples from nine different TR. The samples were heavily weighted towards HIV positive MSM, this was due to a higher proportion of this patient group participating in the study and requiring anal biopsies. There were 29 specimens that had been collected from the same 13 patients. This was due to the patients attending for a second visit and having AIN3+. A flow diagram demonstrating the origin of the anal specimens for this study can be seen in Figure 14.

Sections were already stained with H&E to confirm presence of AIN. This was completed independently to assessing HPV status, which was reported from Roche Cobas 4800® result.

All tissue used was registered, stored and documented in the University tissue bio bank in accordance with the Human Tissue Act 2004.

Table 5: Slide selection for immunohistochemistry studies by grade of disease and cohort group for Ki67 and p16 studies

<table>
<thead>
<tr>
<th>Grade of anal tissue biopsy</th>
<th>Patient Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV+ve MSM n=77 (%)</td>
</tr>
<tr>
<td>Normal</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>Condyloma</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>AIN1</td>
<td>15 (19.5)</td>
</tr>
<tr>
<td>AIN2</td>
<td>36 (46.7)</td>
</tr>
<tr>
<td>AIN3</td>
<td>20 (26.0)</td>
</tr>
<tr>
<td>Invasive cancer</td>
<td>2 (2.6)</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
</tr>
</tbody>
</table>

Table 5: This Table demonstrates which patient group the IHC samples were collected selected from for Ki67 and p16 staining. For the purpose of IHC studies, the specimens were categorised into <AIN2 (n=28) or > AIN2+ (n=72).
Figure 14 A flowchart demonstrating which anal specimens originated from the same patient by cohort group

In total 100 specimens were collected. 77 from HIV positive MSM, 13 from HIV negative MSM and 10 from transplant recipients. In total 29 of these specimens came from the same 13 patients, they were taken at a second visit, which was three to six months from the initial attendance. No HIV negative MSM had a repeat biopsy, all tissue specimens were from different patients.
2.12.1 Antigens

The antigens, site of staining, and tissue control used in this study are seen in Table 6. Immunohistochemistry analysis was performed using p16 and Ki67 as a dual stain. Hand staining was performed on 20-slides in order to learn how to prepare H&E, and Ki67 / p16 dual stain. I completed this under teaching and instruction from Ms Catherine Keeling, Advanced Biomedical Scientist in Histopathology.

Table 6 Antigen and site of staining within the cell and control tissue

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Site</th>
<th>Tissue control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 / p16</td>
<td>Intracellular</td>
<td>Tonsil</td>
</tr>
</tbody>
</table>

2.12.2 Dual Ki67 & p16 antibody staining and detection

The staining platform for Ki67 and p16 antibody was an automated Ventana BenchMark XT IHC /ISH Staining Module (Ventana Co., Tucson, AZ, USA).

The identification and detection of Ki67 antibody was performed using Ki67 MIB-1 mouse monoclonal antibody, 0.2ml Dako (M724029-2), RTU 50 test, Ventana, ULTRAview DAB Detection System, Ventana, 760-500 (Brown). The identification and detection of p16 CINtec antibody was performed using CINtec p16, 805-4713 Detection Systems Ventana, ULTRAview AP Detection System, Ventana, 760-501 (Red).

2.12.2.1 Protocol for dual staining and antigen retrieval

Tissue sections (4 µm) were deparaffinised and incubated in EZ Prep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based reaction buffer, pH 7.6. A heat-induced antigen retrieval protocol (30 minutes) was carried out using a TRIS–ethylenediamine tetracetic acid (EDTA)–boric acid pH 8 buffer (Cell Conditioner 1), (Ventana Co.). The slides were then incubated in antibody to Ki67 MIB-1 (Dako, M724029-2) at a concentration of 1:100 for 32-minutes at room temperature. Bound antibody was visualised using the ULTRAview DAB Detection System (Ventana Co.), producing a brown chromogen. The slides were then denatured at 95°C for four minutes and then incubated in antibody to CINtec p16 (Ventana Co, 805-4713) at a concentration of 3:1 (RTU antibody:diluent) for 40-minutes at room temperature. The bound antibody was visualised using the ULTRAview AP Detection System (Ventana Co.), producing a red chromogen. Slides were then counterstained in Haematoxylin II (Ventana Co.), which was applied for four minutes before an incubation of four minutes with Bluing Reagent (Ventana Co.). Slides were then removed from the machine and washed in warm soapy water. They were then dehydrated in three changes of 100% IMS,
followed by three changes of Xylene. They were then coverslipped using a xylene based mountant. A summary of the protocol for Ki67 and p16 antigen retrieval and dual staining can be seen in appendix xvi a-b.

A summary of antibody and antigen retrieval for Ki67 and p16 dual stain can be seen in Table 7.
Table 7 Antibody and antigen retrieval summary for Ki67 and p16 dual stain

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Company</th>
<th>Species</th>
<th>Antigen Retrieval</th>
<th>Antibody Dilution</th>
<th>Antibody Incubation</th>
<th>Detection Kit</th>
<th>Additional Steps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>MIB-1</td>
<td>Dako</td>
<td>Mouse mAb</td>
<td>30 min heat in CC1 buffer</td>
<td>1+100</td>
<td>32 min</td>
<td>Ultraview DAB</td>
<td>Ultra wash step</td>
</tr>
<tr>
<td>p16 CINtec</td>
<td>E6H4</td>
<td>Ventana</td>
<td>Mouse mAb</td>
<td>30 min heat in CC1 buffer</td>
<td>RTU 3: Diluent 1</td>
<td>40 min</td>
<td>Ultraview RED</td>
<td>Ultra wash step</td>
</tr>
</tbody>
</table>

The pre-treatment of fixed tissue specimen's enabled the effects of fixation to be reversed so that consistent staining could be achieved between samples. In turn this standardised result.
2.13 Tissue scoring of Ki67 and p16 biomarkers

In total 100 slides were stained and then scored for the identification of Ki67 and p16 antibody. Each tissue specimen selected for IHC studies also had a paired slide, which had been previously stained with H&E (n=100) for comparison. All samples had HR-HPV testing using Roche Cobas 4800®.

2.14 Manual tissue scoring for dual stain Ki67 and p16

Ki67 and p16 the tissue specimens were reviewed by three scorers who estimated the positivity of Ki67 stained cells and the positivity and intensity of p16 along with the proportion of positively stained cells by H-SCORE. The scorers’ (n=3) included one individual who was experienced in evaluating antibody labeling (RMcM) as well as two others who had little prior experience assessing percentages and intensity of stains (AMS, Mahshid Nikkho-Amiry MNA). Control staining was performed in tonsil for Ki67 and p16. Examples of Ki67 and p16 controls for staining can be seen in Figure 15.

All tissue samples that were AIN2 and p16 had additional analysis to ascertain if they were block positive for the biomarker.
Figure 15 Tissue control IHC for Ki67 / p16 dual stain in Tonsil

A) Positive control for Ki67 staining x10

B) Positive control for p16 staining x10

C) Positive control for p16 staining x20

Figure 15: Examples of staining in Ki67 and p16 positive control tissue x10 magnification (left) x20 magnification (right) in tonsil (Ki67 and p16). Images captured using Vectra multispectral camera (PerkinElmer, City, USA).
Detection of Ki67 used a Ki67 MIB-1 Mouse Monoclonal, 0.2ml Dako (M724029-2) and a Vetana autostainer (Roche). The location of the stain within the tissue was also documented but not analysed for this study. An index of positively staining cells was determined, and non-epithelial cells were ignored in the count (Garzetti et al., 1995). The slides were scored initially as positive or negative for each scorer estimated epithelial antibody staining then a percentage of positive cells. The average of the three scorers results were used for analysis.

Detection of p16INK4a used a CINtec histology kit (Roche, Basel, Switzerland) and a Vetana autostainer (Roche). Tissue was scored by each scorer who then produced a modified H-SCORE. The H-SCORE was the product of the proportion of cells staining positive in each grade of anal disease ranging from negative, AIN1, AIN2, AIN3 and invasive cancer. Positive staining was scored from 1-6 and staining intensity 1-3 (Detre et al., 1995, Leake et al., 2000), see Table 8 and Table 9. The overall H-SCORE is the product of the intensity score multiplied by the proportion of cells stained.

The maximum score for analysis was 18. The average score calculated from three independent scorers’ determined final values used for analysis. Any discrepancies were resolved with discussion. Examples of positive and negative tissue stains for Ki67 and p16 can be seen in Figure 16 and 17, along with the corresponding H-SCORE.
### Table 8 Calculation of the modified H-SCORE for the protein expression of immunohistochemical staining – staining intensity

<table>
<thead>
<tr>
<th>Staining intensity</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Mild</td>
<td>1</td>
</tr>
<tr>
<td>Moderate</td>
<td>2</td>
</tr>
<tr>
<td>Strong (Intensely positive)</td>
<td>3</td>
</tr>
</tbody>
</table>

### Table 9 Calculation of the modified H-SCORE for the protein expression of immunohistochemical staining – proportion of stained cells

<table>
<thead>
<tr>
<th>Proportion of stained cells</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 - 16.6%</td>
<td>1</td>
</tr>
<tr>
<td>16.7 - 33.3%</td>
<td>2</td>
</tr>
<tr>
<td>33.4 - 50.0%</td>
<td>3</td>
</tr>
<tr>
<td>50.1 - 66.6%</td>
<td>4</td>
</tr>
<tr>
<td>66.6 - 83.3%</td>
<td>5</td>
</tr>
<tr>
<td>83.4 - 100%</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 8 & 9: A modified H-SCORE representing protein expression was derived from subjectively assessing staining intensity and the proportion of stained cells. A maximum score of 18 was obtained. A score of greater than or equal to six was deemed positive.
Figure 16 Examples of positive and negative Ki67 staining in anal specimens x20 (A – B)

A) Negative Ki67 staining  
B) Positive Ki67 staining  

Figure 17 Examples of p16 staining in anal specimens x20 magnification (A – D)

A) Negative p16 staining H-SCORE 0  
B) Mild p16 staining H-SCORE 6  
C) Moderate staining H-SCORE 12  
D) Intense staining H-SCORE 18  

Figure 16 & 17: Examples of staining in Ki67 and p16 in anal biopsies. Ki67 positive and negative staining x20 magnification. In both p16 negative, mild, moderate and intense staining x20 magnification (Images captured using Vectra multispectral camera (PerkinElmer, City, USA).
2.15 Automated cell counts for dual stain Ki67 and p16

As a comparison to manual scoring, all of the selected slides were also processed and scored via an automated detection method. This was performed under the direction of Henry Galleta and Dr Kenneth Oguejiofor at the Patterson Research Institute, UK.

2.15.1 Multispectral imaging and cell counting

For each slide, Multispectral digital analysis was carried out using the Vectra multispectral camera (PerkinElmer, Waltham, MA, USA). This system accurately measures protein expressions and morphometric characteristics on one whole slide or in an area of particular interest. The system automatically loads the slides. Each section was imaged in its entirety. Averages of 10-images per patient were taken. Some samples were very small yielding only two high-powered (x20 magnification) images. Larger images yielded over 50-images per patient. An initial low powered scan was performed (x4 magnification) followed by a higher magnification (x20).

AMS and Henry Galetta (HG) reviewed all slides in order to estimate cellular positivity. The optical density was set based on the estimated visual threshold for each antibody. Areas that had captured artefacts or no tissue were discarded. Ki67 staining was either positive or negative, with a positive result having a threshold of greater than 0.09779. Identification of p16 was correlated to its H-SCORE. A training batch was selected, and thresholds were scored on the training batch of 50 images for both Ki67 and p16. This was then applied to the rest of the cohort. Optical density thresholds for Ki67 and p16 can be seen in Table 10. Examples of Ki67 and p16 staining can be seen Figure 18.

Table 10 Optical density thresholds for the automated identification p16 positivity in relation to modified H-SCORE (Quantified as optical density)

<table>
<thead>
<tr>
<th>Optical density</th>
<th>Colour</th>
<th>Stain intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤0.0200 (od)</td>
<td>Blue</td>
<td>NEGATIVE</td>
</tr>
<tr>
<td>0.0201 - 0.1500 (od)</td>
<td>Yellow</td>
<td>WEAK</td>
</tr>
<tr>
<td>1.501 - 0.1450 (od)</td>
<td>Orange</td>
<td>MODERATE</td>
</tr>
<tr>
<td>≥0.1451 (od)</td>
<td>Red</td>
<td>STRONG</td>
</tr>
</tbody>
</table>
The images were then analysed using InForm cell counting software (PerkinElmer, City, USA). This provided the number of cells positive for each chromogenic label, brown for Ki67 and red for p16. The software also counted the number of cells, which contained the nuclear counterstain (Haematoxylin). Images were then viewed on low x4 and high power x20 magnification.

Figure 18 Examples of Ki67 and p16 staining used for automated counting x20 magnification (A – B)

A Shows an image with a diffusely positive Ki67 brown chromogenic stain in the epithelial tissue along with a small area of p16 staining.

B Shows an image with intensely positive p16 staining in 2/3 of the epithelium.
2.15.2 Analysis in InForm

I reviewed every image using low power (four times magnification) and compared it to images of the same sections stained with H&E as a point of spatial reference. Images that did not represent any area of interest within the epithelium or the stroma were discarded. If there were large sections of sub-stromal compartments with no epithelium for comparison or smooth muscle aggregates, they were not used for the final analysis. By viewing all of the images in this manner, it enabled me to focus on the epithelium, which displayed various grades of AIN and invasive disease along with the underlying stroma.

2.15.3 Data analysis

The distribution of the majority of most marker scores was not normal. Analysis of non-parametric data were performed using semi continuous variable one way ANOVA and two tailed Mann Whitney U test. The test used was dependant on the amount of variables being analysed. Statistical significance was displayed using p values. The data output from Ki67 automated count was expressed as a percentage of positive cells. For the p16 data, the mean H-SCORE were calculated across three scorers. This mean was then converted from H-SCORE to percentages. In manual scoring, it was determined a negative sample had a percentage of less than 33.3% (H-SCORE of <6). A positive sample had a percentage of greater than 33.3% (H-SCORE of >6). This is different to (Singhi and Westra, 2010) who used a positivity percentage threshold of 70%. In automated scoring, a negative sample had a percentage of 0%. A positive sample had a percentage of >1%. In the comparison of AIN2 block positive and block negative staining, Mann Whitney test was applied to non-parametric data.
CHAPTER 3

CLINICAL RESULTS
3 CLINICAL RESULTS

3.1 Clinical data

In this section recruitment as well as demographic data, liquid based cytology, HPV testing and HRA biopsy results are described.

3.1.1 Expression of interest

Overall, 1050 expression of interest forms were collected from the three clinic locations (Table 11) and from these 409 participants were recruited.

Table 11 Expression of interest forms completed by recruitment site

<table>
<thead>
<tr>
<th>Site of recruitment</th>
<th>n=</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manchester Centre for Sexual Health</td>
<td>623</td>
</tr>
<tr>
<td>North Manchester General Hospital</td>
<td>174</td>
</tr>
<tr>
<td>Transplant clinic</td>
<td>253</td>
</tr>
<tr>
<td>Total</td>
<td>1050</td>
</tr>
</tbody>
</table>

3.2 Recruitment

Recruitment to the study commenced in March 2013 and concluded in December 2014, with the follow-up visits continuing until March 2015. The initial recruitment target had been 1000 to include 250 from each high-risk population group. The sample size was based on estimates from the literature of the likely prevalence of cytological, anoscopic and histological abnormalities, to allow estimation of population prevalence with 95% confidence and less than or equal to 6% margin of error in all participants and in each group of 250; and adequate comparison of differences between the three groups with 80% power and 95% confidence. For example, the study was originally powered to estimate population prevalence of 0.2 (20%) with 95% confidence interval (CI) of 0.15-0.25; of 0.1 (10%) with 95% CI 0.07-0.14; and of 0.01 (1%) with 95% CI of 0.004-0.04. Accrual was limited both by the interest shown by potential participants and the time available. HIV positive and negative women who had anoreceptive sex showed virtually no interest in the study. HIV negative MSM were less interested than their HIV positive counterparts as they were frequently attending sexual health clinics for a single check-up and were less inclined to engage.
In order to enhance recruitment another site was added. NMGH commenced recruitment in 20th March 2014. Recruitment until this time had been at MCSH and Renal OPD follow-up clinics. Recruitment by month can be seen in Figure 19. During the last nine months of recruitment 208, patients were recruited into the study. The addition of NMGH made a vital contribution to accrual. Although the sexual health centre at NMGH has a smaller cohort, staff had more time to explain the study to patients.

In total 409 participants were recruited; 284 MSM, of whom 203 were HIV positive and 81 HIV negative, as well as 112 TR of whom 66 were male and 46 female. None of the patients who had received an organ transplant and were also HIV positive. Additionally, nine HIV positive women and four HIV negative women who practiced anoreceptive sex were recruited.
Figure 19 A graph showing recruitment of patients by month to the ANALOGY study

The number of patients recruited by month to the ANALOGY study; from March 2013 to December 2014

The red line on the graph at month 12, March 2014 indicates the start of recruitment at NMGH
3.2.1 Patient characteristics

The median age range of HIV positive and negative MSM was 42-years (range 25-65) and 38-years (range 25-65) respectively. Male and female TR had a higher median age; 52.5-years (range 27-79) and 54.5-years (range 32-49) respectively. The median age of HIV positive women was 36-years (range 28-52) and HIV negative women 37-years (range 32-49) (Table 12).

Table 12 Median and range of age for recruited patients by study group

<table>
<thead>
<tr>
<th>Participant group</th>
<th>n=</th>
<th>Median age (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ MSM</td>
<td>203</td>
<td>42 (25-65)</td>
</tr>
<tr>
<td>HIV- MSM</td>
<td>81</td>
<td>38 (25-65)</td>
</tr>
<tr>
<td>HIV+ Women</td>
<td>9</td>
<td>36 (28-52)</td>
</tr>
<tr>
<td>HIV- Women</td>
<td>4</td>
<td>37 (32-49)</td>
</tr>
<tr>
<td>Male transplant</td>
<td>66</td>
<td>52.5 (27-79)</td>
</tr>
<tr>
<td>Female transplant</td>
<td>46</td>
<td>54.5 (29-73)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>409</strong></td>
<td></td>
</tr>
</tbody>
</table>

3.2.2 Ethnicity

The ethnicity of patients is shown in Table 13. The predominant ethnic group recruited was white British, 74% (151/203) of HIV positive MSM and 75% (61/81) HIV negative MSM. The other ethnic groups included; white other, white Irish and other ethnic groups. As seen in Table 13, TR were similarly predominantly White and the predominant ethnic group seen in HIV positive women was African. All HIV negative women identified as being white British.
Table 13 The ethnicity of recruited patients

<table>
<thead>
<tr>
<th>Ethnicity</th>
<th>Men who have sex with men (MSM)</th>
<th>Transplant recipients (TR)</th>
<th>HIV positive and negative women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV+ (n=203) n (%)</td>
<td>HIV- (n=81) n (%)</td>
<td>Total (n=284) n (%)</td>
</tr>
<tr>
<td></td>
<td>Male (n=66) n (%)</td>
<td>Female (n=46) n (%)</td>
<td>Total (n=112) n (%)</td>
</tr>
<tr>
<td></td>
<td>HIV+ (n=9) n (%)</td>
<td>HIV- (n=4) n (%)</td>
<td>Total (n=13) n (%)</td>
</tr>
<tr>
<td>White British</td>
<td>151 (74.4) 61 (75.3) 212 (75.0)</td>
<td>53 (80.3) 37 (80.0) 90 (80.4)</td>
<td>- 4 (100.0) 4 (100.0)</td>
</tr>
<tr>
<td>White Irish</td>
<td>12 (5.9) 1 (1.2) 13 (4.6)</td>
<td>- 2 (4.3) 2 (1.8)</td>
<td>- - -</td>
</tr>
<tr>
<td>White Other</td>
<td>14 (6.9) 10 (12.4) 24 (8.5)</td>
<td>3 (4.5) 1 (2.2) 4 (3.6)</td>
<td>1 (11.1) -</td>
</tr>
<tr>
<td>White &amp; Black Caribbean</td>
<td>1 (0.5) - 1 (0.4) 1 (1.5)</td>
<td>- - 1 (0.9)</td>
<td>- - -</td>
</tr>
<tr>
<td>White &amp; Black African</td>
<td>1 (0.5) - 1 (0.4)</td>
<td>- -</td>
<td>1 (11.1) -</td>
</tr>
<tr>
<td>Other Mixed Indian</td>
<td>2 (1.0) 2 (2.5) 4 (1.4)</td>
<td>- -</td>
<td>- - -</td>
</tr>
<tr>
<td>Other Asian Pakistani</td>
<td>4 (2.0) - 4 (1.4) 1 (2.2)</td>
<td>1 (0.9) -</td>
<td>- - -</td>
</tr>
<tr>
<td>Other Caribbean African</td>
<td>2 (1.0) 2 (2.5) 4 (1.4)</td>
<td>1 (1.5) -</td>
<td>1 (0.9) -</td>
</tr>
<tr>
<td>Other Chinese</td>
<td>1 (0.5) - 1 (0.4) 1 (1.5)</td>
<td>1 (2.2) 2 (1.8)</td>
<td>7 (77.7) -</td>
</tr>
<tr>
<td>Other Ethnic group</td>
<td>8 (3.9) - 8 (2.8) 1 (1.5)</td>
<td>-</td>
<td>- - -</td>
</tr>
<tr>
<td>Other Black Missing</td>
<td>- - -</td>
<td>-</td>
<td>- - -</td>
</tr>
<tr>
<td>Missing</td>
<td>3 (1.5) 1 (1.2) 5 (1.8)</td>
<td>2 (3.0) 1 (2.2) 3 (2.7)</td>
<td>- - -</td>
</tr>
</tbody>
</table>
3.2.3 Smoking status and Alcohol use

HIV positive MSM were the group with the highest proportion of current smokers 28.6% (58/203) compared with 21% (17/81) HIV negative, 12.1% (8/65) male TR, 6.5% (3/45) female TR and 25% (1/4) HIV negative women. HIV negative women had the highest percentage of never smoking 88.8% (8/9) followed by female TR 67.4% (31/46). There were similar proportions from all groups identifying as previously smoked. This number was highest in male TR 45.5% (30/66) these data can be seen in Table 14.

In both MSM and TR the majority of patients were currently alcohol drinkers. HIV negative women followed by HIV negative MSM had the highest percentage current use 100% (4/4) and 90.1% (73/81) respectively, as seen in Table 8. Only a small proportion in all groups stated they never drank any alcohol, this was higher in male and female TR and possibly due to their transplantation. Similar percentages were also seen in previous alcohol use throughout all groups apart from HIV positive and negative women as none of these women reported previous use.

3.2.4 Sexual orientation and relationship status

In total 93.1% (189/203) of HIV positive and 91.4% (74/81) of HIV negative MSM identified themselves as homosexual. Amongst MSM 5.9% (12/203) of HIV positive and 8.6% (7/81) of HIV negative MSM considered themselves to be bisexual along with one HIV negative women. As expected, 93.5% of TR identified themselves as being heterosexual with 98.5% (65/66) men and 93.5% (43/45) women from each group. One female TR identified herself as being lesbian. All of the HIV positive women identified themselves as being heterosexual along with 50% (2/4) of HIV negative women. One HIV positive man, one male TR, two female TR and one HIV negative women had missing data for this question.

Overall 52.7% (107/203) of HIV positive and 39.5% (32/81) of HIV negative MSM were in a current sexual relationship. In TR 84.8% of both men (56/66) and women (39/45) were in an active sexual relationship. All of the HIV negative women were in a current relationship along with 66.6% (6/9) of HIV positive women.
Table 14 Alcohol and smoking in recruited patients

<table>
<thead>
<tr>
<th></th>
<th>Men who have sex with men (MSM)</th>
<th>Transplant recipients (TR)</th>
<th>HIV positive and negative women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV+ (n=203) n (%) HIV- (n=81) n (%) Total (n=284) n (%)</td>
<td>Male (n=66) n (%) Female (n=46) n (%) Total (n=112) n (%)</td>
<td>HIV+ (n=9) n (%) HIV- (n=4) n (%) Total (n=13) n (%)</td>
</tr>
<tr>
<td>Alcohol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>174 (85.7) 73 (90.1) 247 (87.0)</td>
<td>49 (74.2) 34 (73.9) 83 (74.1)</td>
<td>6 (66.6) 4 (100.0) 10 (77.0)</td>
</tr>
<tr>
<td>Previous</td>
<td>22 (10.8)  6 (7.4)  28 (9.9)</td>
<td>9 (13.6)  4 (8.7) 13 (11.6)</td>
<td>0          0          0</td>
</tr>
<tr>
<td>Never</td>
<td>7 (4.45)  2 (2.5)  9 (3.2)</td>
<td>8 (12.1)  8 (17.4) 16 (14.3)</td>
<td>3 (33.3)    0 (0.0)   3 (23.0)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>58 (28.6) 17 (21.0) 75 (26.4)</td>
<td>8 (12.1)  3 (6.5) 11 (9.8)</td>
<td>0 (0.0)     1 (25.0)  1 (7.7)</td>
</tr>
<tr>
<td>Previous</td>
<td>63 (31.0) 27 (33.3) 90 (31.7)</td>
<td>30 (45.5) 12 (26.1) 42 (37.5)</td>
<td>1 (11.1)    2 (50.0)  3 (23.0)</td>
</tr>
<tr>
<td>Never</td>
<td>82 (40.4) 37 (45.7) 119 (41.9)</td>
<td>28 (42.4) 31 (67.4) 59 (52.7)</td>
<td>8 (88.8)    1 (25.0)  9 (69.3)</td>
</tr>
</tbody>
</table>
3.2.5 Years of anoreceptive sex

The duration of anoreceptive sex varied as seen in Table 15. The majority of HIV positive MSM had been practicing anoreceptive sex for greater than 21+ years 51.2% (104/203), in HIV negative MSM only 24% (20/81) had been having anal sex for this amount of time. In total one out of nine of HIV positive women and all four HIV negative women practiced anoreceptive sex. HIV negative women had been practicing anoreceptive sex for between 5 and 21 years. The singular HIV positive women did not stipulate the duration of time she had been practicing anoreceptive intercourse.

Table 15 Years of anoreceptive sex

<table>
<thead>
<tr>
<th>Time in years</th>
<th>HIV positive MSM (n=203) n (%)</th>
<th>HIV negative MSM (n=81) n (%)</th>
<th>HIV negative women (n=4) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 years</td>
<td>2 (1.0)</td>
<td>7 (8.6)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>6-10 years</td>
<td>20 (9.9)</td>
<td>18 (22.2)</td>
<td>1 (25.0)</td>
</tr>
<tr>
<td>11-20 years</td>
<td>75 (36.9)</td>
<td>36 (44.4)</td>
<td>2 (50.0)</td>
</tr>
<tr>
<td>21+ years</td>
<td>104 (51.2)</td>
<td>20 (24.7)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

3.2.6 HIV viral load and CD4 count at study baseline

An undetectable viral load is defined as fewer than 40 copies/per ml. The data for HIV viral load and CD4 count at baseline can be seen in Table 16. In total 8.4% (17) of HIV positive men and 11.1% (1/9) of HIV positive women had an undetectable viral load. The majority of both HIV positive men and women had fewer than 40,000 copies detected; 70.9% (144/203) and 77.7% (7/9) respectively. 12.3% (25/203) of HIV positive MSM had higher viral loads ranging between 40,000-168,000 copies/per ml. Data for viral load at baseline visit was not available for 17 (8.4%) of MSM and one woman.

The majority of HIV positive MSM and women had CD4 counts between 401-800 cells/mm³; 47.8% (97/203) of men and 55.5% (5/9) of women. Men and women had similar proportions of CD4 counts between 200-400 cells/mm³; 25.6% (52/203) and 22.2% (2/9) respectively. Amongst HIV positive MSM, 4.9% had a CD4 of less than 200 cells/mm³ at baseline visit, but no women. One HIV positive women had a CD4 count of greater than 800 cells/mm³ compared with 15.8% (32/203) of HIV positive MSM. Amongst HIV positive men 5.9% (12/203) and one woman 11.1% (1/9) had missing data for CD4 at baseline visit.
Table 16 HIV viral load and CD count at study baseline in HIV positive MSM and women

<table>
<thead>
<tr>
<th>HIV viral load at study baseline (copies per ml)</th>
<th>HIV positive MSM (n=203) n (%)</th>
<th>HIV positive women (n=9) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missing</td>
<td>17 (8.4)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>Undetectable (≤ 40 copies/ml)</td>
<td>17 (8.4)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>≤ 40,000</td>
<td>144 (70.9)</td>
<td>7 (77.7)</td>
</tr>
<tr>
<td>40,000 – 168,000</td>
<td>25 (12.3)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD4 count at study baseline (cells/mm³)</th>
<th>HIV positive MSM (n=203) n (%)</th>
<th>HIV positive women (n=9) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Missing</td>
<td>12 (5.9)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>≤200</td>
<td>10 (4.9)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>200 – 400</td>
<td>52 (25.6)</td>
<td>2 (22.2)</td>
</tr>
<tr>
<td>401 – 800</td>
<td>97 (47.8)</td>
<td>5 (55.5)</td>
</tr>
<tr>
<td>≥800</td>
<td>32 (15.8)</td>
<td>1 (11.1)</td>
</tr>
</tbody>
</table>

3.2.7 HAART

Overall 87.2% (177/203) of HIV positive MSM and 66.6% (6/9) of HIV positive women were taking HAART. The median duration of HAART in HIV positive men and women was 4-years (range 0-22) and 5-years (range 2-11) respectively. The three HIV positive women and 14 HIV positive MSM not taking HAART had adequate CD4 counts and viral loads. There was missing data for 5.9% of MSM (12/203).
3.2.8 Time since first transplantation

Table 17 displays the duration of immune suppression since organ transplantation. Around half of both male and female TR had a transplant for over 10-years, and around a third for less than five years.

Table 17 Duration of immune suppression post organ transplantation

<table>
<thead>
<tr>
<th>Time in years</th>
<th>Male (n=66) n (%)</th>
<th>Female (n=46) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 years</td>
<td>24 (36.4)</td>
<td>14 (30.4)</td>
</tr>
<tr>
<td>6-10 years</td>
<td>8 (12.1)</td>
<td>10 (21.7)</td>
</tr>
<tr>
<td>&gt;10 years</td>
<td>34 (51.5)</td>
<td>22 (47.8)</td>
</tr>
</tbody>
</table>
3.3 Baseline HPV data

High-risk (HR) HPV testing was performed using Cobas 4800®. If the sample failed to process due to technical issues or transportation and preservation of sample failure then a single repeat test was offered. All patients were offered HR-HPV testing, at the baseline visit at the follow-up visit.

3.3.1 Roche Cobas 4800® HPV testing

The Cobas 4800® allowed testing for 14 HR-HPV types including simultaneous read out of types 16 and 18. The other high-risk types tested for were 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68. The samples were reported as positive or negative for HR-HPV. If a sample was positive for HR-HPV it had a “type 16 and or 18” and or “other” readout. These data from the samples collected during the ANALOGY study can be seen in Table 18.

Amongst the MSM, 281 had a valid Cobas® result; 98.5% (200/203) of HIV positive and 100.0% (81/81) of HIV negative MSM. Amongst TR 97.5% (109/112) had valid results. All HIV positive and negative women had valid HPV results.

Amongst MSM, prevalence of HR-HPV was extremely high (85.4%; 240/281). The prevalence in HIV positive MSM was 88.0% (176/200) and 77.8% (63/81) in HIV negative MSM. The overall prevalence of HR-HPV in TR was much lower at 19.3% (21/109). In TR men the prevalence of infection was 10.9% (7/64) and TR women it was higher at 26.65% (12/45). Four out of nine HIV positive women were positive and three out of four HIV negative women.

The majority of infection in MSM involved types 16 and/or 18 and/or other. In total 45.3% (92/203) of HIV positive and 34.5% (28/81) of HIV negative MSM had multiple infections. This was not seen to the same extent in TR. No male TR tested positive for types 16 and/or 18 and/or other. One sample from a female TR participant tested positive for type 16 HR-HPV alone. This woman had been treated for CIN3 with a Large Loop Excision to the cervical Transformation Zone (LLETZ) in the preceding year. One out of nine HIV positive women had types 16 and/or 18 and two out of four HIV negative women, both of whom had genital warts.

Infection with types 16 and/or 18 alone was much less prevalent in all groups and was not seen at all in HIV positive and negative women. In MSM, only 1.0% (2/203) of HIV positive and 3.7% (3/81) HIV negative had only type 18 and/or 18 infection. In TR 1.52% (1/66) of men and 6.52% (3/45) women tested positive for types 16 and/or 18.

Similar proportions of HIV positive and HIV negative MSM were infected with non-16 and/or 18 genotypes, 40.39% (82/203) and 39.51% (32/81) respectively. The percentages in MSM are much higher when compared with TR; 9.09% (6/66) of males and 17.39% (8/45) of females were infected with non-16 and/or 18. In HIV positive women three out of nine and one out of four HIV negative women had non-18 and/or 18 infections.
Overall 80.45% (90/112) of TR had a negative Cobas 4800® test. Male TR had a higher percentage of negative tests 86.36% (57/66) when compared with women 71.74% (33/45). In comparison only 14.7% (42/284) of MSM had negative Cobas 4800® testing. HIV negative MSM had a higher number of negative tests compared with HIV positive MSM 22.2% (18/81) and 11.82% (24/203) respectively. These results are to be expected with a group who practice anoreceptive sex and a group who do not. In HIV positive and negative women 55.6% (5/9) and 25% (1/4) had a negative Cobas 4800® test.

### 3.3.2 PapilloCheck® genotyping

All of the Cobas 4800® samples that tested positive for HR-HPV were also further tested and genotyped using PapilloCheck® (Greiner Bio-one). Dr Alex Sargent in the Virology Department at Central Manchester Foundation Trust performed the PapilloCheck® testing. PapilloCheck® assay tests for 24-types (18-high-risk and 6-low-risk) of HPV. The 18-high-risks types detected are 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73 and 82. The low-risk types detected are 6, 11, 40, 42, 43 and 44/45.

Valid test results were obtained from 98.4% (260/265) of the Cobas 4800® positive results from HIV positive and negative MSM, HIV positive and negative women and TR (Table 18). All of the valid tests had detectable HPV that was either LR and/or HR. Of the samples that were tested by PapilloCheck®, 90.0% (238/265) tested positive for a Cobas 4800® target HR-HPV type. Two per cent (5/265) contained a non-target HR-HPV type and 8.3% (22/265) contained a LR-HPV type.

As can be seen in Table 19, there was a broad diversity of HR-HPV infection of the anal canal seen in the MSM population. In HIV positive MSM the four most prevalent types were 16 (28.1%), 51 (22.1%), 33 (16.8%) and 68 (15.9%). The most prevalent types of HR-HPV infection were similar in HIV negative MSM; type 16 (22.2%) and 51 (18.9%) had similar proportions of infection when compared to HIV positive MSM. Types 31 (17.7%) and 56 (17.7%) were also prevalent in HIV negative MSM. In HIV positive women types 16, 45, 52 and 53 were most prevalent. In TR and HIV negative women type 51 was the most prevalent type of infection.

HIV positive and negative MSM both had a high prevalence of multiple HR-HPV infections with 74.0% of infections displaying multiplicity. In total 41.2% of TR had multiple HR-HPV infection and these were all seen in women. TR had a higher prevalence of single HR-HPV infections than HIV positive individuals. HIV negative MSM had a single infection in 30.6% of cases, whereas HIV positive MSM showed a single type infection in 24.3% of infection of cases.
<table>
<thead>
<tr>
<th>HPV result</th>
<th>MSM n=284</th>
<th>Transplant n=112</th>
<th>Women n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV +VE</td>
<td>HIV -VE</td>
<td>TOTAL</td>
</tr>
<tr>
<td>n=203</td>
<td>n=81</td>
<td>n=284</td>
<td>n=66</td>
</tr>
<tr>
<td>HR 16 &amp;/or 18 &amp;/or Other</td>
<td>92 (45.32)</td>
<td>28 (34.57)</td>
<td>120 (42.2)</td>
</tr>
<tr>
<td>HR 16 &amp;/or 18</td>
<td>2 (0.99)</td>
<td>3 (3.70)</td>
<td>5 (1.7)</td>
</tr>
<tr>
<td>HR non 16 &amp;/or 18</td>
<td>82 (40.39)</td>
<td>32 (39.51)</td>
<td>114 (40.2)</td>
</tr>
<tr>
<td>Negative</td>
<td>24 (11.82)</td>
<td>18 (22.22)</td>
<td>42 (14.7)</td>
</tr>
<tr>
<td>Unsatisfactory / Invalid / Missing</td>
<td>3 (1.48)</td>
<td>0 (0.00)</td>
<td>3 (1.1)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>203 (100.00)</td>
<td>81 (100.00)</td>
<td>284 (100.00)</td>
</tr>
</tbody>
</table>
Table 19 Type specific prevalence of HR-HPV using PapilloCheck® assay by high-risk group

<table>
<thead>
<tr>
<th></th>
<th>16</th>
<th>18</th>
<th>31</th>
<th>33</th>
<th>35</th>
<th>39</th>
<th>45</th>
<th>51</th>
<th>52</th>
<th>53</th>
<th>56</th>
<th>58</th>
<th>59</th>
<th>66</th>
<th>68</th>
<th>73</th>
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<td>31</td>
<td>35</td>
<td>18</td>
<td>32</td>
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<td>(8.7)</td>
<td>(15.3)</td>
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<td>5</td>
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<td>(17.7)</td>
<td>(11.4)</td>
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</tr>
<tr>
<td>HIV positive women</td>
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<td>2</td>
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<td>2</td>
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<tr>
<td></td>
<td>(50.0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(25.0)</td>
<td>(50.0)</td>
<td>(25.0)</td>
<td>(50.0)</td>
<td>(50.0)</td>
<td>(0)</td>
<td>(25.0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td></td>
</tr>
<tr>
<td>HIV negative women</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(33.3)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(0)</td>
<td>(33.3)</td>
<td>(33.3)</td>
<td>(33.3)</td>
<td>(33.3)</td>
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<td>(0)</td>
<td>(0)</td>
<td></td>
</tr>
</tbody>
</table>
3.4 Baseline visit Cytology data

The cytology data for LBC samples are shown in Table 20. These represent the most severe grade of disease for any individual. Abnormal cytology was highly prevalent amongst MSM 41.9% (119/284), with 44.8% (91/203) and 34.6% (28/81) of abnormality seen in HIV positive and HIV negative MSM respectively. The large majority of changes were seen in low-grade disease but high-grade changes were seen in 10.6% (30/284) of MSM. This result was composed of a higher proportion of HIV negative MSM 12.35% (10/81) compared with 9.85% (20/203) of HIV positive MSM. The prevalence of cytological abnormality in HIV positive and HIV negative women was lower than in MSM with one third (4/13) having an abnormality. The prevalence of cytological abnormality in TR was much lower than all other groups; 14.2% (16/112), although five out of six high grade were found in females.

TR had the highest proportion of negative samples, (84.8%; 95/112). The percentage with negative cytology was also high in HIV positive and negative women, 69.2% (9/13). In total 55.9% (159/284) of HIV positive and negative MSM had negative samples.

Amongst MSM 1.77% (5/284) of samples had invalid testing, one sample taken from a TR was unsatisfactory. No samples taken for HIV positive or negative women had an invalid result.

3.4.1 Correlation of HPV genotyping and liquid based cytology with biopsy detected at baseline and second visit

These data can be seen in Table 21. At the baseline visit the majority of AIN3+ lesions were predominantly type 16 and/or 18 positive (61.1%, 11/18). Just over one third 33.3% (6/18) of AIN3+ lesions were non-16/18 positive. Only one AIN3+ lesion was HR-HPV negative. The majority of AIN2+ lesions were predominantly type 16 and/or 18 positive, 57.5% (46/80). AIN1 lesions were mainly non-16/18 positive (42.1%, 48/114). When compared with visit two the only additional AIN3 sample tested positive for type 16 and/or 18. AIN2+ was predominantly 16 and/or 18 positive 47.4% (9/19). AIN1 was again predominately HR-HPV negative 41.7% (32/48) and 35.4% (17/48) were non-16/18.

Baseline cytology did not appear sensitive with the majority of AIN3+ having either low-grade or negative results; this was also seen in AIN2+ samples. High-grade cytology was seen more frequently than expected in biopsy confirmed AIN1 13.4% (15/112). In AIN2+ and AIN3+ high-grade cytology was seen in 13.9% (11/79) and 17.6% (3/17) respectively. In AIN3+ samples this is much lower than expected. AIN1 samples had 59.8% (67/112) of samples reported as negative. In AIN2+ and AIN3+ cytology was not a good predictor of disease, with 40.5% (32/79) of AIN2+ and 29.4% (5/17) of AIN3+ having negative cytology.
Cytology results from AIN2+ samples at second visit were also predominantly low-grade or negative. The single AIN3+ sample also tested negative. In total high-grade cytology was seen in 10.6% (5/47) of AIN1 and only 15.8% (3/19) of AIN2+. 
<table>
<thead>
<tr>
<th>Cytology result</th>
<th>MSM n=284</th>
<th>Transplant n=112</th>
<th>Women n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV +VE</td>
<td>HIV -VE</td>
<td>TOTAL</td>
</tr>
<tr>
<td>High grade</td>
<td>n=203</td>
<td>n=81</td>
<td>n=284</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Low-grade</td>
<td>20 (9.85)</td>
<td>10 (12.35)</td>
<td>30 (10.56)</td>
</tr>
<tr>
<td></td>
<td>71 (34.98)</td>
<td>18 (22.22)</td>
<td>89 (31.34)</td>
</tr>
<tr>
<td>Ungraded</td>
<td>1 (0.49)</td>
<td>0 (0.00)</td>
<td>1 (0.35)</td>
</tr>
<tr>
<td>Unsatisfactory</td>
<td>4 (1.97)</td>
<td>1 (1.23)</td>
<td>5 (1.76)</td>
</tr>
<tr>
<td>Negative</td>
<td>107 (52.71)</td>
<td>52 (64.20)</td>
<td>159 (55.99)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>203 (100.00)</td>
<td>81 (100.00)</td>
<td>284 (100.00)</td>
</tr>
</tbody>
</table>
Table 21 Correlation of HPV genotyping and liquid based cytology at baseline in AIN detected at first visit and newly diagnosed at second visit in all groups

<table>
<thead>
<tr>
<th></th>
<th>AIN1 n (%)</th>
<th>AIN2+ n (%)</th>
<th>AIN3+ n (%)</th>
<th>AIN all grades n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First visit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Baseline HR-HPV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 16/18</td>
<td>36 (31.6)</td>
<td>46 (57.5)</td>
<td>11 (61.1)</td>
<td>82 (42.3)</td>
</tr>
<tr>
<td>Non 16/18</td>
<td>48 (42.1)</td>
<td>23 (28.8)</td>
<td>6 (33.3)</td>
<td>71 (36.6)</td>
</tr>
<tr>
<td>Negative</td>
<td>30 (26.3)</td>
<td>11 (13.8)</td>
<td>1 (5.6)</td>
<td>41 (21.1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>114</td>
<td>80</td>
<td>18</td>
<td>194</td>
</tr>
<tr>
<td><strong>Baseline LBC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>15 (13.4)</td>
<td>11 (13.9)</td>
<td>3 (17.6)</td>
<td>26 (13.6)</td>
</tr>
<tr>
<td>Low-grade</td>
<td>30 (26.8)</td>
<td>36 (45.6)</td>
<td>9 (52.9)</td>
<td>66 (34.6)</td>
</tr>
<tr>
<td>Negative</td>
<td>67 (59.8)</td>
<td>32 (40.5)</td>
<td>5 (29.4)</td>
<td>99 (51.8)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>112</td>
<td>79</td>
<td>17</td>
<td>191</td>
</tr>
<tr>
<td><strong>Second visit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HR-HPV</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 16/18</td>
<td>11 (22.9)</td>
<td>9 (47.4)</td>
<td>1 (100.0)</td>
<td>20 (29.8)</td>
</tr>
<tr>
<td>Non 16/18</td>
<td>17 (35.4)</td>
<td>5 (26.3)</td>
<td>0 (0.0)</td>
<td>22 (32.8)</td>
</tr>
<tr>
<td>Negative</td>
<td>20 (41.7)</td>
<td>5 (26.3)</td>
<td>0 (0.0)</td>
<td>25 (37.4)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>48</td>
<td>19</td>
<td>1</td>
<td>67</td>
</tr>
<tr>
<td><strong>LBC</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High grade</td>
<td>5 (10.6)</td>
<td>3 (15.8)</td>
<td>0 (0.0)</td>
<td>8 (12.1)</td>
</tr>
<tr>
<td>Low-grade</td>
<td>13 (27.7)</td>
<td>4 (21.0)</td>
<td>0 (0.0)</td>
<td>17 (25.8)</td>
</tr>
<tr>
<td>Negative</td>
<td>29 (61.7)</td>
<td>12 (63.2)</td>
<td>1 (100.0)</td>
<td>41 (62.1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>47</td>
<td>19</td>
<td>1</td>
<td>66</td>
</tr>
</tbody>
</table>

*First three LBC samples were insufficient.

**One second visit LBC were insufficient.
3.5 Baseline visit Histopathology data

The histopathology data are shown in Table 22. These data represent the most severe grade of seen in targeted anoscopic biopsies for any patient. Overall 71.8% (204/284) of MSM, 61.6% (69/112) of TR, 33.3% (3/9) of HIV positive women and three out of four HIV negative women underwent a biopsy because of an anoscopic abnormality at HRA.

Three microinvasive cancers were detected at HRA; all were in HIV positive MSM and all three of them were taking HAART. The prevalence of AIN3+ was 6.9% (14/203) and 3.7% (3/81) amongst HIV positive and HIV negative MSM respectively. Amongst TR the prevalence of AIN3 was much lower with only one lesion (0.89%, 1/112) being detected. No AIN3+ was identified in HIV positive and negative women. AIN grades 1 and 2 were highly prevalent in all groups. Grade AIN2 or worse (AIN2+) was present in 26.6% (54/203) and 21.0% (17/81) of HIV positive and HIV negative MSM respectively. The corresponding data for AIN1 were 32.0% (65/203) and 32.1% (26/81). Amongst HIV positive women AIN2+ and AIN1 were present in one and two out of nine respectively. HIV negative women had a higher prevalence of AIN2+ and AIN1 when compared with HIV positive women. Amongst HIV negative women, AIN2+ and AIN1 were present in 25.0% (1/4) and 50.0% (2/4) respectively. TR showed a low prevalence of both AIN2+ and AIN1, these lesions having been found in 7.14% (8/112) and 17.86% (20/112) of patients respectively.

Patients who had a negative HRA did not have a biopsy. This included 28.17% of MSM (80/284), 65.18% (73/112) of TR and 66.67% (6/9) of HIV positive women. Only one HIV negative women did not require a biopsy at visit one.

The majority of biopsies taken at HRA in all cohorts showed AIN. Only 3.9% (8/203) and 2.4% (2/81) of HIV positive and negative MSM respectively had negative biopsy results. In total 4.5% (3/66) male TR had a negative biopsy taken at HRA. All of the targeted biopsies taken in female TR, HIV positive and HIV negative women showed AIN.

Other HPV related anogenital disease such as condyloma or koilocytosis in H&E tissue reporting was seen most frequently in HIV positive MSM 13.79% (28/203). Overall 7.14% (8/112) of TR had other HPV related disease. This is not unsurprising as they are also immune compromised and would have a prolonged timeframe in order to clear HPV infection.
Table 22 Grade of anal disease on biopsy by patient group categorising AIN2+ as high-grade disease

<table>
<thead>
<tr>
<th>Biopsy result</th>
<th>MSM n=284</th>
<th>Transplant n=112</th>
<th>Women n=13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV +VE</td>
<td>HIV -VE</td>
<td>TOTAL</td>
</tr>
<tr>
<td></td>
<td>n=203</td>
<td>n=81</td>
<td>n=284</td>
</tr>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>Biopsy not taken</td>
<td>48 (23.65)</td>
<td>32 (39.51)</td>
<td>80 (28.17)</td>
</tr>
<tr>
<td>Invasive Cancer</td>
<td>3 (1.48)</td>
<td>0 (0.00)</td>
<td>3 (1.06)</td>
</tr>
<tr>
<td>AIN3+</td>
<td>14 (6.90)</td>
<td>3 (3.70)</td>
<td>17 (5.99)</td>
</tr>
<tr>
<td>AIN2+</td>
<td>54 (26.60)</td>
<td>17 (21.0)</td>
<td>71 (25.0)</td>
</tr>
<tr>
<td>AIN1</td>
<td>65 (32.02)</td>
<td>26 (32.1)</td>
<td>91 (32.04)</td>
</tr>
<tr>
<td>Negative (incl. inflammation &amp;)</td>
<td>8 (3.94)</td>
<td>2 (2.47)</td>
<td>10 (3.52)</td>
</tr>
<tr>
<td>HPV (incl. condyloma)</td>
<td>28 (13.79)</td>
<td>4 (4.94)</td>
<td>32 (11.27)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>203 (100.00)</td>
<td>81 (100.00)</td>
<td>284 (100.00)</td>
</tr>
</tbody>
</table>
3.6 Multiple biopsies

When during HRA the circumference of the anal canal was divided up into four quadrants in order to inspect for disease. Biopsies from three or more quadrants are suggestive of circumferential anal canal disease. As seen in Table 23, at baseline visit the majority of patients (95.2%; 237/249) had disease-requiring biopsy over two quadrants of the anal canal. Multiple biopsies from greater than three quadrants was required less frequently, (4.8; 12/249) with no HIV positive or negative women requiring biopsies from three or more quadrants of the anal canal. HIV positive MSM and negative were the groups of patients that required three or more biopsies most frequently.
Table 23 Biopsy at baseline visit by quadrants of disease in all groups

<table>
<thead>
<tr>
<th>Anoscopy</th>
<th>MSM (n=284)</th>
<th>TR (n=112)</th>
<th>Women (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HIV+ (n=203)</td>
<td>HIV- (n=81)</td>
<td>Male (n=66)</td>
</tr>
<tr>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
<td>n (%)</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-------------</td>
<td>---------</td>
</tr>
<tr>
<td>Within normal limits (i.e. biopsy not taken)</td>
<td>48 (23.6)</td>
<td>32 (39.5)</td>
<td>80 (28.2)</td>
</tr>
<tr>
<td>Biopsy taken</td>
<td>155 (76.4)</td>
<td>49 (60.5)</td>
<td>204 (71.8)</td>
</tr>
<tr>
<td>1 or 2 quadrants</td>
<td>147 (94.8)</td>
<td>46 (93.9)</td>
<td>193 (94.6)</td>
</tr>
<tr>
<td>3 or 4 quadrants</td>
<td>8 (5.2)</td>
<td>3 (6.1)</td>
<td>11 (5.4)</td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>81</td>
<td>284</td>
</tr>
</tbody>
</table>
3.7 Continued patient follow-up

Eighty-six patients did not attend a second visit. Female TR were the group with the highest rate of withdrawal (13%; 6/46), followed by HIV positive MSM who were more likely to be lost to follow-up (13%; 27/203) than actively withdraw (10.8%; 22/203). These data can be seen in Table 24.

Table 24 Patient withdrawal and those lost to follow-up at second visit

<table>
<thead>
<tr>
<th>Group</th>
<th>n=</th>
<th>Withdrawn (%)</th>
<th>Lost to follow-up (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ve MSM</td>
<td>203</td>
<td>22 (10.8)</td>
<td>27 (13.3)</td>
</tr>
<tr>
<td>HIV-ve MSM</td>
<td>81</td>
<td>7 (8.6)</td>
<td>10 (12.3)</td>
</tr>
<tr>
<td>HIV+ve Women</td>
<td>9</td>
<td>1 (11.1)</td>
<td>1 (11.1)</td>
</tr>
<tr>
<td>HIV-ve Women</td>
<td>4</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Male transplant</td>
<td>66</td>
<td>7 (10.6)</td>
<td>4 (6.1)</td>
</tr>
<tr>
<td>Female transplant</td>
<td>46</td>
<td>6 (13.0)</td>
<td>1 (2.2)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>43</strong></td>
<td></td>
<td><strong>43</strong></td>
</tr>
</tbody>
</table>
3.8 Histopathology follow-up data

At the follow-up visit when all screening tests were repeated (between 3-6 months), a further 68-AIN lesions of any grade were detected, this can be seen in Table 25. One new AIN3 lesion was identified in a HIV positive MSM. High-resolution anoscopy displayed a high sensitivity in the detection of AIN3 with 18/19 cases being identified at the initial visit. All three cancers were identified at visit one.

Table 25 Detection of new disease at a follow-up visit (3-6 months)

<table>
<thead>
<tr>
<th>Additional AIN at follow-up</th>
<th>2nd visit</th>
<th>1st visit</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIN 1</td>
<td>48</td>
<td>114</td>
</tr>
<tr>
<td>AIN 2+</td>
<td>20</td>
<td>103</td>
</tr>
<tr>
<td>AIN 3+ *(Cancer)</td>
<td>1</td>
<td>21 *(3)</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>216</td>
</tr>
</tbody>
</table>

*C Cancer

Examples of anal cytology samples collected during the ANALOGY study can be seen in the Cytology Image Library Figure 20 (A – G). Examples of H&E stained biopsy samples taken during the ANALOGY study displaying normal anal mucosa AIN1, AIN2 and AIN3+ can be seen in the Histology Image Library Figure 21 (images A – G) and Figure 22 (E - J)
3.9 Cytology and histology Image Library

3.9.1 Anal Cytology Image Library

All of the images were from specimens collected during the ANALOGY study. Cytopathology images collected from the ANALOGY study, used with permission from Dr Mina Desai, CMFT, Manchester UK

Figure 20 Cytopathology images collected from the ANALOGY study (A – G)

A) Normal squamous cells x20 magnification.

B) Normal glandular cells x40 magnification.

C) Unsatisfactory, poorly preserved cells with contaminants and debris x20 magnification.
Figure 20 Cytopathology images collected from the ANALOGY study (D – G)

D) HPV changes, koilocyte, mild dyskaryosis AIN1 (LSIL) x20 magnification.

E) HPV changes, koilocyte, mild dyskaryosis AIN1 (LSIL) x40 magnification.

F) Moderate dyskaryosis, AIN2 (HSIL) x60 magnification.

G) Moderate dyskaryosis, AIN2 (HSIL) in single cells x60 magnification.
3.9.2 Anal Histology Image Library

All of the images were from specimens collected during the ANALOGY study.

Histopathology images collected from the ANALOGY study, used with permission from Dr Raymond McMahon, CMFT, Manchester UK

Figure 21 Histopathology images collected from the ANALOGY study (A-J)

A) Invasive cancer and AIN3 (HSIL) x4 magnification.
B) Invasive cancer and AIN3 (HSIL) x20 magnification.
C) AIN2 and AIN3 (HSIL) x10 magnification.
D) AIN3 (HSIL) and active mitosis x60 magnification.
Figure 22 Histopathology images collected from the ANALOGY study (E-J)

E) AIN2 (HSIL) x10 magnification.

F) AIN2 (HSIL) x20 magnification.

G) AIN1 (LSIL) x20 magnification.

H) AIN1 (LSIL) x20 magnification.

I) HPV x4 magnification.

J) HPV x60 magnification.
CHAPTER 4

IMMUNO 
HISTOCHEMISTRY 
RESULTS
4 IMMUNOHISTOCHEMISTRY STUDY

This study focuses on anal tissue biopsies collected as part of the ANALOGY study. This section will report the results of the immunohistochemistry-staining methods that have been completed on anal tissue biopsies using Ki67 and p16 biomarkers. The anal tissue specimens had varying degrees of anal dysplasia ranging from normal to AIN3+.

All of the patients who participated in the ANALOGY study had Roche Cobas 4800® HR-HPV testing reported in Chapter 3. All of the positive Cobas 4800® samples were also genotyped using Grenier PapilloCheck® to ascertain multiplicity of HR-HPV infection. The Cobas 4800® HR-HPV status is used in this Chapter as a comparison between manual and automated detection methods of Ki67 and p16 biomarker detection. Ki67 is a marker of cellular proliferation and p16 is widely thought of as being the consequence of HR-HPV infection.

The objective of this study was to correlate the expression of Ki67 and p16 with the grade of anal intraepithelial neoplasia. It is considered AIN2 with strongly positive p16 staining should be classified as high-grade disease warranting treatment. Ki67 positivity can help with this histology diagnosis in the event p16 staining is inconclusive or technically inadequate (Darragh et al., 2012b). This would assist clinicians in stratification of patients for management of their disease.
4.1 RESULTS IMMUNOHISTOCHEMISTRY STUDIES

4.1.1 Specimens

During the ANALOGY study 789 specimens were collected from 340 patients. In this study 100 anal biopsy specimens were dual stained with Ki67 and p16 biomarkers, slide selection can be seen in Table 26. At the time of selection, all specimens with AIN3+ taken during the ANALOGY study were included in the IHC selection. The majority of samples had greater than AIN2+ (> AIN2+ = n=47 AIN2 and n=25 AIN3+). Seven specimens were originally reported as Ungraded AIN, all of these specimens were less than 10 cells thick. These specimens were reviewed and regraded according to the LAST criteria. In accordance with LAST guidance, any lesion that is unequivocally SIL with mitosis above the basal cells or significant immature abnormal basal proliferation is graded as HSIL (AIN2+) (Darragh et al., 2012b). After review only one specimen met this criterion. Six samples were categorised as AIN1. For ease of analysis biopsies were categorised as less than AIN2 or greater than or equal to AIN2+ (this included the two cancer specimens).

If automated detection of Ki67 and or p16 correlates with the grade of disease, then this would be a relatively easy method of detecting HGAIN. The quantification and detection methods of each of the antibody’s would have to be reliable in order for it to be used in clinical practice. Manual detection of antibody positivity would be time consuming with the possibility of interobserver bias and the need for specialist training in the reading of slides. HR-HPV is the driving force behind cellular change in squamous tissue. Roche Cobas® is a reliable test in the identification of HR-HPV positivity and type. Cobas 4800® is a sensitive test to compare Ki67 and p16 positivity in biopsies containing AIN. MSM have a known high prevalence of HR-HPV infection (as seen in Chapter Three). Data from the ANALOGY study suggests that testing for HR-HPV infection in MSM adds little clinical utility.

In total 100 specimens were selected from 87 patients: 65 HIV positive MSM, 13 HIV negative MSM and 9 TR. A total of 29 specimens came from the same 13 patients due to these patients having repeat biopsies and high-grade disease (Figure 14). For the purpose of this analysis the specimens will be considered independently as they were collected at different point in time with differing grades of disease.

Data tables containing each specimens grade of AIN, Cobas 4800® and PapilloCheck® result and high-risk group can be seen in appendix xvii, xviii and xix. Data for patients with more than one anal biopsy sample can be seen in appendix xx. These data will not be discussed in this Chapter and are for reference only.
Table 26 Slide selection for immunohistochemistry by grade of disease and cohort group for Ki67 and p16 biomarkers

<table>
<thead>
<tr>
<th>Grade of anal tissue biopsy</th>
<th>Patient Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV+ve MSM n=77 (%)</td>
<td>HIV-ve MSM n=13 (%)</td>
</tr>
<tr>
<td>&lt;AIN2</td>
<td>20 (26.0)</td>
</tr>
<tr>
<td>&gt;AIN2+</td>
<td>57 (74.0)</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
</tr>
</tbody>
</table>

Table 26: The tissue specimens selected for immunohistochemistry studies. In total 100 specimens were selected for Ki67 and p16 staining.
4.2 Detection of high-risk HPV in less than AIN2 and greater than or equal to AIN2+ anal tissue biopsies

4.2.1 HPV positivity by Roche Cobas 4800®

As reported in Chapter Three, of the specimens used in this study, 90% (90/100) of samples had positive Cobas 4800® testing. In the specimens analysed, this encompassed 93.0% (67/72) of greater than (> AIN2+ and in less than (<) AIN2 samples 82.1% (23/28) had positive Cobas 4800® testing respectively. In total 2% (2/100) samples were invalid for Cobas 4800® testing, these data can be seen in Table 27.

4.2.2 Automated and manual Ki67 positivity

Using automated and manual scoring analysis, all of the >AIN2+ and <AIN2 samples had a detectable output for Ki67. There were no negative samples in the entire cohort. Samples ranged in percentage positivity from 10% to 80% in manual scoring and 1.84% to 51.47% in automated scoring. In <AIN2 samples 3.6% (1/28) and in >AIN2+ 8.3% (6/72) had no automated detection data output for Ki67 staining, these data can be seen in Table 27.

4.2.2 Automated and manual p16 positivity

These data can be seen in Table 27. In total 44.0% (44/100) samples displayed p16 positivity when scored by the automated detection system. The comprised of 57.1% (16/28) of <AIN2 and 39.0% (28/72) of >AIN2+ samples. A higher proportion of >AIN2+ samples were negative for automated p16 detection 43.0% (31/72) compared with 35.7% (10/28) of <AIN2 samples. As the majority of high-grade anal disease is known to be HR-HPV positive this suggests that there is a possible threshold error in the detection of p16 by automated methods. Failure of automated detection of p16 was seen more frequently in >AIN2+ samples 18.0% (13/72).

Overall p16 positivity detected by manual scoring was 47.0% (47/100). In total 61.1% (44/72) of >AIN2+ samples were p16 positive when scored manually (H-SCORE >6), compared with 10.7% (3/28) of <AIN2. Samples with <AIN2 had a higher proportion of negative p16 staining, 89.3% (25/28). This is in keeping with the pathology of low-grade disease not being driven by HR-HPV types 16 and/or 18. AIN1 and negative tissue is generally HR-HPV negative and therefore usually also p16 negative.

The histogram (Figure 23) demonstrates the mean spread of H-SCORES used to manually score p16 biomarker. A score of greater than or equal to six was deemed as positive.
Table 27 Roche Cobas 4800® HR-HPV detection positivity along with manual and automated Ki67 and p16 cellular positivity detection in less than AIN2 or greater than AIN2+ with p values

<table>
<thead>
<tr>
<th>Test</th>
<th>Grade of Disease</th>
<th>Total n=100</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;AIN2 n=28 (%)</td>
<td>&gt;AIN2+ n=72 (%)</td>
<td></td>
</tr>
<tr>
<td>Positive Roche Cobas®</td>
<td>23 (82.1)</td>
<td>67 (93.0)</td>
<td>90</td>
</tr>
<tr>
<td>Negative Roche Cobas®</td>
<td>4 (14.3)</td>
<td>4 (5.6)</td>
<td>8</td>
</tr>
<tr>
<td>Failed Roche Cobas®</td>
<td>1 (3.6)</td>
<td>1 (1.4)</td>
<td>2</td>
</tr>
<tr>
<td>Positive automated Ki67</td>
<td>27 (96.4)</td>
<td>66 (91.7)</td>
<td>93 &lt; 0.8399</td>
</tr>
<tr>
<td>Negative automated Ki67</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0</td>
</tr>
<tr>
<td>Failed automated Ki67 Output</td>
<td>1 (3.6)</td>
<td>6 (8.3)</td>
<td>7</td>
</tr>
<tr>
<td>Positive manual Ki67</td>
<td>28 (100.0)</td>
<td>72 (100.0)</td>
<td>100 ≤ 0.0001</td>
</tr>
<tr>
<td>Positive automated p16</td>
<td>16 (57.1)</td>
<td>28 (39.0)</td>
<td>44 ≤ 0.0022</td>
</tr>
<tr>
<td>Negative automated p16</td>
<td>10 (35.7)</td>
<td>31 (43.0)</td>
<td>41</td>
</tr>
<tr>
<td>Failed automated p16 Output</td>
<td>2 (7.2)</td>
<td>13 (18.0)</td>
<td>15</td>
</tr>
<tr>
<td>Positive manual p16 (H-SCORE ≥6)</td>
<td>3 (10.7)</td>
<td>44 (61.1)</td>
<td>47 &lt; 0.0001</td>
</tr>
<tr>
<td>Negative manual p16 (H-SCORE ≤ 6)</td>
<td>25 (89.3)</td>
<td>28 (38.9)</td>
<td>53 &lt; 0.2428</td>
</tr>
</tbody>
</table>

Table 27: This Table provides a summary of positive and negative staining for Ki67 and p16 immunohistochemistry by patient group and detection method. Samples that had a failed output have also been included. H-SCORE positivity of p16 is defined as a score greater than or equal to 6. No manual p16 scoring had a failed data output. Statistical significance is demonstrated with a p value of less than 0.05. Statistical significance was observed using a two tailed Mann Whitney U Test in specimens scored manually expressing increased cellular positivity of Ki67 and p16 with an increasing grade of AIN and automated detection of p16. There was no statistical difference using a Mann Whitney U Test when comparing the expression of Ki67 by automated detection methods with the grade of anal biopsy.
Figure 23: Histogram of the mean H-SCORE for p16 expression

Figure 21: A H-SCORE of greater than 6 was considered as being p16 positive.
4.3 Block positivity of AIN2 specimens

A total of 47 AIN2 samples were initially used in this study, 59.5% (28/47) were p16 block positive as per the LAST criterion and 40.5% (19/47) were negative. The majority of samples had a H-SCORE of greater than six (53.2%; 25/47) and were p16 block positive. Only one sample had a high H-SCORE (12) and was negative for p16 block stain. The mean H-SCORE for p16 block positive tissue was 10.9 and block negative tissue was 2.7. A Mann Whitney Test was performed between block positive and negative samples, demonstrating a statistical significance of p=<0.0001. There is clearly an association between the H-SCORE of block positive and block negative p16 AIN2 samples. Therefore block staining could be used instead of H-SCORE when grading tissue presumed to be AIN2.

Once this staining had been completed and the ANALOGY study finished, it was decided that the remaining AIN2+ samples from visit one would be stained with p16 antibody and scored for block p16 positivity. A further 34 specimens were studied (AIN2 and AIN3+).

A total 63 AIN2 samples were stained. The staining results for block positive p16 in AIN2 (n=60) samples can be seen in Table 28, three specimens could not be scored due to the tissue being “cut out”. Overall block positivity was seen in 56.9% (34/60) of AIN2 samples taken at visit one. As a comparison, 18 AIN3+ were also scored, 88.8% (16/18) were p16 block positive. This is in keeping with AIN3+ having a strong association with HR-HPV.

Table 28 Block p16 positivity (as per LAST criterion) by group

<table>
<thead>
<tr>
<th>Group</th>
<th>p16 positivity</th>
<th></th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p16 positive n (%)</td>
<td>p16 negative n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV positive MSM</td>
<td>25</td>
<td>15</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>HIV negative MSM</td>
<td>8</td>
<td>4</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>HIV positive women</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>HIV negative women</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transplant Recipients</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
<td>26</td>
<td>60</td>
<td></td>
</tr>
</tbody>
</table>

Table 28: AIN2 specimens with block p16 positivity scoring in all high-risk groups. Three samples could not be scored due to “cut out” tissue being available.
4.4 Comparison of the sensitivity of HPV infection detection

Manual and automated p16 expression was compared against Roche Cobas 4800® in the detection of HR-HPV. Tissue that stains positively for p16 is considered to be the result of HR-HPV infection.

4.4.1 Manual and automated p16 scoring versus Roche Cobas 4800®

A total of 98 samples stained were eligible for comparison between manual p16 detection and Cobas 4800® in the detection of HR-HPV. A total of two Cobas 4800® samples were invalid. The prevalence of any inferred HR-HPV infection by manual p16 analysis was 65.3% (CI 54.9-74.4). The sensitivity of manual p16 scoring in the detection of HR-HPV was 93.7% (CI 83.9-97.9) and the specificity was 11.7% (CI 3.8-28.3). Manual p16 staining is sensitive but not specific in the detection of HR-HPV in anal biopsies, with a false negative rate of 50.0% (CI 17.4-82.5) these data can be seen in Table 29.

Table 29 Manual and automated p16 positivity scoring compared with Roche Cobas 4800® detection of HPV in anal biopsies

<table>
<thead>
<tr>
<th>HR-HPV positivity</th>
<th>p16 positive</th>
<th>p16 negative</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Manual (n=64)</td>
<td>Automated (n=45)</td>
</tr>
<tr>
<td>HPV +ve</td>
<td>60</td>
<td>39</td>
</tr>
<tr>
<td>HPV -ve</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>64</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 29: In total 98 samples could be compared for manual scoring, 83 automated samples were compared were eligible for analysis. Two samples had invalid testing by Roche Cobas 4800® and were excluded both in HIV positive MSM in both manual and automated detection. 15 automated samples had no data output.
4.4.2 Automated p16 versus Roche Cobas 4800®

A total of 83 samples stained were eligible for comparison between automated p16 detection and Cobas 4800® in the detection of HR-HPV. A total of 17 samples were invalid and could not be compared due to automated detection failure or invalid Cobas 4800® testing. The prevalence of any inferred HR-HPV infection was 54.2% (CI 42.9-65.0). The sensitivity of automated p16 in the detection of HR-HPV was 86.6% (CI 72.5-94.4) and the specificity was 5.2% (CI 0.9-19.0). Automated p16 staining is sensitive but not specific in the detection of HR-HPV in anal biopsies with a false negative rate of 75.0% (CI 35.0-95.5).

Roche Cobas 4800® is the test of choice to detect HR-HPV. From these data, manual detection of p16 is more sensitive than automated detection, although both lack specificity and have a high false positive rate in comparison with a gold standard test. It is thought that there is a difference in the relationship between <AIN2 and >AIN2+, being infected with HR-HPV and expressing p16 biomarker.

The majority of <AIN2 samples that were negative for Cobas 4800® testing and also had a H-SCORE of zero (75.0%; 3/4). One sample had a H-SCORE of five and was negative for Cobas 4800®. Three samples >AIN2+ that were p16 Cobas 4800® negative had a H-SCORE of four or less. Only one AIN2+ sample that was Cobas 4800® negative had a H-SCORE of greater than six (H-SCORE 13).

4.5 Comparison of detection manual and automated methods of Ki67 and p16 by grade of disease

Automated and manual staining detection methods of Ki67 and p16 were compared by grade of AIN (n=100). In total seven samples were removed due to “failed” automated Ki67 detection and 15 from the p16 cohort leaving n=93 Ki67 and n=85 p16. This was due to detection failure from the automated count.

4.5.1 Comparison of Ki67 positivity by automated and manual detection methods

Detection of Ki67 positivity by the automated system by grade of anal disease can be seen in Figure 24 and manual detection in Figure 25. In normal anal tissue Ki67 expression varied in both manual and automated detection methods. In automated scoring expression ranged from 3.6% to 46.7% and manual scoring 10.0% to 46.6%. Manual detection of Ki67 displayed a higher standard error of the mean (SEM) than automated detection (Figure 24 and 25). In automated detection the expression of Ki67 does not increase as the grade of anal disease increased. In contrast manual scoring of Ki67 displayed an increased expression as the grade of anal disease increased (Figure 25). There was no statistical difference using a Mann Whitney U test when comparing the expression of Ki67 by automated detection methods and grade of anal biopsy <AIN2 or >AIN2+ (Table 26). When manual scoring was compared there was a
significant difference between Ki67 expression and grade of disease <AIN2 or >AIN2+
p=0.0001 (Table 26).

As seen in Figure 24 automated scoring Ki67 was similarly expressed in AIN1 and AIN3+, with
a SEM of 17.9% and 16.5% of cells respectively. In AIN2 the SEM positivity was higher 20.1%.
This is not in keeping with the hypotheses.

When scored manually the expression of Ki67 increased with the grade of anal disease (Figure
25), with AIN1 having a mean positivity of 17.1%, AIN2 36.3% and AIN3+ 59.3%. This proves
the hypotheses that there would be a difference between the detection of positivity between
manual and automated scoring but also in manual detection there was a difference in positivity
between grades of anal disease.
Figure 24 & 25: Results of mean Ki67 staining of anal tissue biopsies in normal tissue and tissue reported as having anal intraepithelial neoplasia grade 1-3+ (including two cancers). Each marker on the graph represents one specimen stained with Ki67 and scored for positivity by automated detection Figure 24 and manual detection Figure 25. The red lines on the graph indicate the standard error of the mean and the black lines the interquartile ranges. There was statistical significance when Ki67 was scored manually against the grade of disease using a one-way ANOVA (p <= 0.0001).
4.5.2 Comparison of p16 detection by manual H-SCORE and automated methods by grade of anal disease

H-SCORE was used to estimate the intensity of the p16 stain along with the percentage of positive cells in each piece of tissue. In normal anal mucosa by manual scoring, detection of p16 biomarker was only positive (H-SCORE <6) in two specimens with SEM of 2.8 (H-SCORE). All four of the specimens analysed with automated p16 detection were negative. The expression of p16 positivity detected by manual scoring (H-SCORE) had a SEM value of 1.4 (range 0-10) in AIN1 specimens, 7.8 (range 0-16) in AIN2 and was highest in AIN3+ at 10.4 (range 0-15). When manual scoring (H-SCORE) was compared there was a significant difference between p16 expression and the grade of disease (<AIN2 or >AIN2+) as seen in Table 27. Statistical significance was also observed when comparing all grades of AIN in both manual and automated detection using a one way ANOVA (See Figure 27 and 27). A large proportion of automated p16 samples were negative (n=41) across all grades of disease. Statistical significance was observed when comparing the positivity of automated p16 detection in <AIN2 or >AIN2+ samples using a Mann Whitney U test (Table 27). From these data, manual scoring of p16 appears to be more reliable.

4.5.3 Summary

There is a clear trend in manual scoring in both Ki67 and p16 as the grade of anal disease increases so does the detection of the biomarker. Manual detection methods of Ki67 positivity and automated or manual p16 detection by H-SCORE, displayed statistically significant results when compared against differing grades of anal disease (<AIN2 and >AIN2+). Manual detection of Ki67 and p16 antibodies appears to be more sensitive than automated detection but lack specificity.
**Figure 26** Automated p16 positivity by grade of anal biopsy

Automated p16 positivity by grade of anal biopsy n=85

- **Negative**
- **AIN1**
- **AIN2**
- **AIN3+**

**Figure 27** Manual scored p16 positivity represented by H-SCORE & grade of anal biopsy n=100

- **Negative**
- **AIN1**
- **AIN2**
- **AIN3+**

**Figure 26 & 27**: Results of mean p16 staining of anal tissue biopsies in normal tissue and tissue reported as having anal intraepithelial neoplasia grade 1-3+ (including two cancers). Each marker on the graph represents one specimen stained with p16 and scored for positivity by automated detection Figure 26 and manual detection by H-SCORE Figure 27. The red bar lines indicate the standard error of the mean and the black lines the interquartile ranges. There was statistical significance when p16 was scored manually using H-SCORE and by automated detection against the grade of disease using a one-way ANOVA (p =< 0.0001).
CHAPTER 5

CLINICAL DISCUSSION
5 CLINICAL DISCUSSION

ANALOGY was the UK’s first prospective cohort study investigating whether high-resolution anoscopy is the gold standard in the detection of AIN, the precursor of ASCC in high-risk populations. This study has evaluated the clinical utility of HR-HPV testing and anal cytology as screening tests in these groups and has confirmed that:

- HIV positive MSM have a high prevalence of AIN3+.
- HIV negative MSM have a lower risk of AIN3+.
- There is a significant proportion of AIN2+ in all populations.
- HIV positive MSM exhibited a high prevalence of low grade anal neoplastic lesions.
- Microinvasive anal cancer is screen detectable with high-resolution anoscopy.
- HIV positive and negative MSM exhibited a high prevalence of HR-HPV infection.
- TR have a lower prevalence of high grade AIN and HR-HPV infection compared with MSM.
- HIV positive and negative women were disinclined to be screened in this study when compared with other MSM and TR.
- The high prevalence of HR-HPV and the poor sensitivity of cytology limit the utility of these as screening strategies.

It has been well documented that the incidence of HPV associated anal cancer is rising and has been for the last 30-years but it is still uncommon in the general population (Grulich et al., 2012, Brewster and Bhatti, 2006). It is also well known that the incidence in high-risk populations is greater than in the general population. The UK has a nationally standardised cervical screening programme for HPV related cervical precancer, however, it has been recognised for some time that universal screening protocols for HPV associated anal cancer are non-existent (Palefsky, 2009). Prior to the commencement of a cervical screening programme, the rate of cervical cancer was 14.2 per 100,000, but with the advent of such a service it has decreased to approximately 8 per 100,000 (Palefsky, 1999, Nanda et al., 2000). The ANALOGY study was the first study of its kind in the UK to evaluate the clinical utility of HR-HPV testing, LBC and HRA in the detection of anal precancerous disease in high-risk populations.

5.1 Challenges of the ANALOGY study

The ANALOGY study was challenging and required a number of hurdles to be overcome in order for the study to commence and be completed.
5.1.1  HRA training and disease identification learning curve

Training in anoscopy was needed in order to complete the study. This training was spread over visits to UCSF, US and Homerton University Hospital, London. The training received was thorough although acquired in very condensed periods of time and was also quite disjointed. There were many new clinical skills to learn bridged by large gaps in time. In total 50 non-study HRA were performed. Although similar in principle, HRA training and practice was very different to cervical colposcopy, primarily due to the invasive nature of HRA. The clinical research fellow, (AMS) is a gynaecologist in training who practices supervised cervical colposcopy, but not an accredited colposcopist.

Despite this professional background, it took seven months in total to feel competent in the detection of anal disease; three months of training at both centres of excellence and four months of practising HRA during the ANALOGY study. In total 722 HRA were completed during the ANALOGY study and in early months there was a distinct learning curve. When practising HRA, due to previous colposcopy training, there were minimal issues with learning to use three different colposcopes. There were no concerns with assessing the perianal skin, which does not involve depth perception, which is an issue with biopsies taken during HRA. Assessment of the anal canal and collection of biopsies was performed using the colposcope without any complications. A basis for pattern recognition of disease was established during training and honed during independent practice, as it is similar to colposcopy. Lesions seen at HRA are described using similar terms to cervical colposcopy and features such as contour (raised or flat), colour, vascular patterns (mosaic, punctuated, warty, atypical) and Lugols staining can be seen. Epithelial honeycombing is a pattern seen at HRA (Palefsky, 2012), but not at cervical colposcopy and may indicate HGAIN or atypical metaplasia. This was not recognised as quickly as other features that had greater similarity to cervical disease. The HRA learning curve is infrequently addressed in current literature. One small study documents that a sole HRA practitioner performed 200 anoscopies before there was consistent detection in anal disease (Richel et al., 2014). It is known that novice practitioners will rarely have the benefit of continued expert tuition (Palefsky, 2012). Discrepancies between histology and cytology results triggered concerns, especially if cytology is of a higher grade than the histology. In this instance it is acknowledged that disease has been missed, and recognising the findings at HRA may have been influenced by anoscopist inexperience.

During the ANALOGY study, a total of 789 specimens were collected from 340 patients; only 16 were negative for any disease (12 at visit one and four at visit two). Despite a limited amount of training, the detection of disease was high, yet it could be argued that disease was still missed due to inexperience in the practice of HRA. The learning curve of the HRA practitioner can be seen particularly in those that were negative for disease at baseline attendance but had AIN at visit two. Also at visit one 38 patients had HSIL on LBC sampling. During HRA 28.9% (11/38) were found to have AIN2+ on biopsy. It should be considered that those with AIN1 or indeed a negative screen requiring no biopsy, 71.1% (27/38) potentially had missed disease during HRA. These proportions did increase at visit two, 25 cytology samples were reported as HSIL. During
HRA 44% (11/25) had AIN2+. AIN cases being missed at baseline screening by the anoscopist (AMS) and the mismatch of LBC HSIL and HRA detected disease highlight anoscopist learning curve and initial inexperience. Similar experiences have been documented in a study in the Netherlands (Richel et al., 2014). The prevalence of AIN2+ detected by the HRA practitioner (AMS) over the four six month periods of the ANALOGY study (17.3%, 12.6%, 19.7% and 25.2%) differed. This supports a significant learning curve of the HRA practitioner during the study as high-grade disease was detected at higher percentages in the last 12-months of the study. Interestingly, if the detection of AIN2+ in MSM alone is considered over the four six month periods of the study (23%, 24%, 22%, 28%), there is little difference in the detection of disease. It is worth mentioning that during the ANALOGY study no patient had a palpable anal lesion on DRE that once biopsied was histologically anal cancer.

The high-prevalence of anal lesions detected in HIV positive MSM who participated in the ANALOGY study is consistent with studies worldwide (Gaisa et al., 2014) and the case for screening. The high prevalence of disease seen reflects the lack of prior screening, with an accumulation of AIN over a number of years. The three screen detected cancers found in the ANALOGY study were all microinvasive which have more favourable outcomes (Dalla Pria et al., 2014). They were all detected microscopically using acetic acid and a colposcope. It is recognised that DRE is a very important part of HRA, although cancer was not detected in this manner. During HRA training thickened anal masses had been palpated in other patients that were high-grade when biopsied. Performing DRE permitted assessment for any other pathology that warranted inspection during HRA, such as warts, haemorrhoids, fissures or scar tissue. DRE is therefore considered to be an essential part of HRA.

5.1.2 Establishing a service

The study was established in Manchester where no prior anoscopy service existed. New service establishment across several areas of two different Trusts was challenging. Running a new clinic around existing services was difficult, primarily due to research staff not belonging to any of the departments where clinics were conducted. Clinic establishment in sexual health was relatively easy when compared to clinics needed in order to screen TR within the main hospital. Within the sexual health service at both sites, MCSH and NMGH, there were dedicated rooms for the clinic to be conducted in and had equipment fit for purpose and a functioning colposcope, there was no such space at Manchester Royal Infirmary to see TR. In the early stages of the study it became apparent that TR were also not happy to be seen in the sexual health service due to presumed stigma of attending the clinic. This had an impact on recruitment as patients attended for appointments and then cancelled, as they did not want to come into the clinic. In order to accommodate these concerns, several months were spent trying to identify a suitable room in which TR could be seen. A clinic was finally established in OPD colposcopy suite at St Mary’s hospital, which fortuitously permitted the assessment of men as well as women as they already have a specialist faecal incontinence service catering for both sexes.
Specimen transfer and processing was time consuming and not without problems. The internal transfer of LBC medium from cytology to virology required the development of a protocol. This ensured correct and timely processing of specimens.

As per good clinical practice, each group of recruited patients required hard copy documentation of study results in their main patient notes. Once the study had finished and referrals made if required, GP and patient letters were written. These were all filed along with hard copies of results for each patient under a research divider. Due to the enhanced privacy of patient data at sexual health clinics, documentation and filing of results for the patients recruited from both MCSH and NMGH was complicated and time consuming. The correct filing of all this documentation took much longer than anticipated due to the complexity of differing information systems and information that patients had consented for the research team to share being checked meticulously, such as HIV status. Also, the majority of sexual health patients were not happy for communication about their participation to be sent to either a home address and/or to their GP. The research team had to review each patient’s wishes carefully to ensure no unwanted letters were sent out.

Onward referral to Professor Hill was completed with ease, although the scheduling of follow-up appointments particularly with sexual health patients was difficult. This was due to HIV positive patients not consenting to any home address mailing, phone calls or GP contact.

5.1.3 Recruitment of HIV positive and negative MSM

The group at most increased risk of anal cancer are HIV positive MSM, followed by HIV negative MSM. Both of these groups of men during the ANALOGY study proved very difficult to recruit and then retain on the study, with over 20.0% of recruited HIV positive and negative MSM either withdrawing or not attending a second visit (Table 23).

HIV positive MSM were easy to identify as they attend regular visits to sexual health services. These visits monitor their immune status (CD4, viral load and HAART medication) along with overall health and wellbeing. This provided the research team with multiple points of contact to deliver information about the study. Despite this, HIV positive MSM were an extremely difficult group of patients to recruit and retain on the study. The primary reason being that this patient group often would cancel appointments just before it was due to commence or not attend without informing the research staff. In the majority, they were also very difficult to contact in order to confirm clinic attendance. After several months, the research team commenced sending reminder texts, emails and telephone calls a week before and also 24-hours prior to the scheduled visit. This did improve clinic attendance but was extremely time consuming.

It appears from our data that MSM are much more likely to participate in anal screening when compared to other high-risk groups and speculation alone provides possible reasons. MSM may perceive themselves to be at the highest risk of developing anal cancer and precancer due to sexual practice and lifestyle choices and behaviour. Despite this, HIV negative MSM were under recruited during the study. Sexual health genitourinary medicine screening in HIV negative
patients at MCSH is historically 30.0% women and 70.0% men, with 30.0% of men identifying themselves as MSM or bisexual. HIV negative MSM were generally recruited when they attended sexual health services for a one off STI screen. Once the results of this screen were given to the patient, there was no incentive for them to return to MCSH or NMGH, unless they needed treatment or counselling. This is reflected in the numbers of HIV negative MSM recruited to the study and is considered to be the result of the point of recruitment generally being a one off point of contact. It could also be due to the health attitudes and beliefs of HIV negative MSM who possibly do not deem themselves at increased risk, or this cohort of MSM may not find HRA acceptable.

5.1.4 Recruitment of HIV positive and negative women

The lack of accrual of HIV positive women and HIV negative women who practiced anoreceptive sex is a significant finding from the ANALOGY study. In other studies, HIV positive women have participated in anal screening (Gaisa et al., 2014). Recruitment to of HIV positive and negative women is not reflective of those accessing services at MCSH or NMGH. In MCSH alone the number of HIV positive patients increased from 1692 in March 2014 to 1736 in September 2015. Of this cohort 25% were HIV positive women and historically 30% of the overall sexual health attendances are female. The data from the ANALOGY study in HIV positive women cannot be compared to studies such as Gaisa et al due to the fact that HIV positive women were disinclined to be screened. In Gaisa’s study, 2075 patients were screened and 27.0% (n=556) were HIV positive women of which 42.0% (n=233) had abnormal cytology and went on to have HRA. AIN3+ was found in 26.0% (n=45) of HIV positive women. The women in this study were more likely to undergo HRA, 73.0% (n=170) (Gaisa et al., 2014). There is evidence demonstrating that women readily accept they may need colposcopy after an abnormal smear and report pain that is proportional to the intensity if the treatment they are receiving (Kola-Palmer et al., 2015). It is therefore plausible that women would not participate in ANALOGY due to preconceived ideas about “pain” and the association with the practice of anoreceptive sex. Gaisa et al supports the screening HIV positive women in the detection of anal disease and unlike the ANALOGY study women participated in screening (Gaisa et al., 2014).

Other studies have shown that women are disinclined to have HRA. The Womens Interagency HIV study (WIHS) performed anal cytology and detected 31.0% of women had abnormal cytology. Less than half of the women with abnormal cytology underwent HRA (Hessol et al., 2009). Sadly there is no study confirming if anal screening is acceptable to this population. What also has to be recognised is the ethnic background from which the HIV positive women who participated in the ANALOGY study originated from; 77.7% (7/9) were African. Other studies also have a high number of black women participating, but they do not identify themselves as African (Hessol et al., 2009). It could be considered there is a cultural issue surrounding anal screening in women who do not practice anoreceptive sex; Gaisa does not report ethnicity for comparison.
Studies that have successfully recruited HIV positive women demonstrate that women with HPV-associated cervical disease are at increased risk for HGAIN and should be offered anal cancer screening (Heard et al., 2015). A recent study of 171 HIV positive women concluded that abnormal anal LBC and HR-HPV-16 infection testing performed best as a screening strategy for HGAIN+ histology (Heard et al., 2015). Heterosexual women who practice anoreceptive sex are known to have an increased risk of anal cancer (Frisch et al., 1997). This was demonstrated by Frisch et al from population control studies conducted in Denmark and Sweden. This study showed that that of the three groups involved in their research (one control group and two groups having anal cancer or carcinoma in situ), women with anal cancer were more likely to practice anoreceptive sex (Frisch et al., 1997). Yet both these cohorts of women were disinclined to be screened. In HIV positive women this is most surprising as they are subjected to enhanced cervical screening protocols due to their seropositive status.

Future work in the UK will have to factor in other international published studies when designing methodology for HIV positive women in the detection of AIN. The findings from Heard et al would suggest that HIV positive women could indeed be screened by LBC and HR-HPV type 16 rather than HRA. HRA could be the factor that reduced participation in this group. These women could then only be offered HRA with a combination of positive HR-HPV testing and not seen routinely as would best practice in HIV positive MSM. It is considered poor recruitment particularly in HIV negative women this is due to the association with the practice of anoreceptive sex in order to be eligible to be screened. The National Surveys of Sexual Attitudes and Lifestyles (NATSAL) interviewed 15,162 men and women aged 16-74 between September 2010 and August 2012. NASTAL-3 is the third survey of this kind. Although NATSAL-3 data does indicate although infrequent in heterosexual women when compared to vaginal sex there is still a proportion of women practicing anal sex. Similar percentages are seen in women under the age of 44. This confirms women are practicing anal sex but there is a possibility that there is still a social stigma attached to admitting this in order to be eligible for a research study. Further supporting the practice of anoreceptive sex in heterosexual relationships is anal sex with a female partner was the only sexual behaviour reported to increase in prevalence between NATSAL-2 to NATSAL-3 by men (Mercer et al., 2013). Unfortunately despite an additional drive amongst all staff to recruit women there was still minimal interest. It was decided that efforts would be focused on HIV positive and HIV negative MSM.

During ANALOGY women it was observed that found HRA more tolerable than men, even if they did not practice anoreceptive sex. A possible explanation for this is that all of the women on the study were over the age of 25, and in theory should have attended cervical screening or possibly had gynaecological examinations due to medical conditions or pregnancy. It is also feasible that the majority of women had previously or were currently practicing penile vaginal sex. Only one woman on the project identified herself as a lesbian and she had previously had cervical smear tests and therefore had a vaginal speculum passed. There was however no question in the CRF regarding current penile vaginal sex only previous and current penile
anoreceptive sex. It is possible women who had never had penile vaginal sex would have been more uncomfortable during HRA. It must also be considered that anatomically, women have a thin membrane between the anal canal and the vagina; therefore on introduction of the anoscope, there was “space” for it to be accommodated. In men, the area between the anus and the scrotum is made up solely of muscle.

5.1.5 Second visit challenges

What was surprising amongst MSM is HIV positive individuals were less likely to attend a second visit. These patients fell into two groups, those who scheduled an appointment but never re-attended and were lost to follow-up, 13.3% (27/203), and those who withdrew from the study, 10.8% (22/203). This group of MSM attend sexual health services regularly yet when compared to HIV negative MSM they did not attend a second visit as frequently. In total 12.3% (10/81) of HIV negative MSM were lost to follow-up and 8.6% (7/81) withdrew. This group of MSM generally only attend sexual health for STI and HIV testing. There appointments are not regular and they can go months, even years without re-attending the service. All of the HIV negative women attended a second visit. All of these women had previous anogenital warts that they were also seeking additional treatment for or were concerned about their practice of anoreceptive sex. Male and female TR were more likely to withdraw from the study than be lost to follow-up, the majority of the time it was documented that this was due to ill health. It is possible they did not find screening acceptable.

5.1.6 Other challenges

A significant portion of study time was devoted to counselling patients by the research team. It was not envisaged that the majority of the counselling and concerns from patients would be regarding HPV infection and not the detection of AIN. The majority of patients had many concerns about HPV infection. These concerns in the TR were mainly due to HPV being labelled a STI and the association with anoreceptive sex. Patients who did not practice anoreceptive sex were very concerned how they had acquired an anal HR-HPV infection. All patient groups expressed concerns that there was not therapeutic treatment for HPV at the time of the study. Despite counselling, the majority of patients struggled with the concept that any HPV infection can be acquired, persist and/or be cleared and that there was no test to ascertain the duration of infection. They also had concerns that their partners fidelity. A study based information leaflet about the acquisition, persistence and/or clearance and potential reinfection with HPV would have been extremely helpful in educating patients about HPV related anogenital disease.
5.2 Prevalence of HR-HPV in the ANALOGY cohort; implications for vaccination

5.2.1 HR-HPV prevalence

HR-HPV testing is clearly extremely sensitive in samples collected from the anal canal. The prevalence of HR-HPV seen in MSM who participated in the ANALOGY study was higher than reported in studies conducted in Brazil (40.7% for HIV positive MSM) (Guimaraes et al., 2011), China (61% for HIV positive and 40% for HIV negative MSM) (Hu et al., 2013), Taiwan (40.4% for HIV positive MSM) (Yu et al., 2013) and in Bangkok, Central Thailand (85% for HIV positive and 58.5% for HIV negative MSM) (Phanuphak et al., 2013). However, HR-HPV prevalence in HIV positive MSM was lower (88%, 176/200) than seen in northern Thailand. In this study 100% of HIV positive MSM were infected (Supindham et al., 2015). Prevalence in the HIV negative cohort was similar to ANALOGY, 70% testing positive for infection (Supindham et al., 2015).

HR-HPV testing lacks specificity in MSM due to an extremely high prevalence of positive samples seen in the ANALOGY study. It is considered that limiting HR-HPV testing to types 16 and/or 18 would add specificity, but this would only have identified 61.1% (11/18) of AIN3+. Female TR, a far smaller group, may warrant anal screening as 4/46 had HPV 16 and/or 18 thus increasing cost effective use of resources. The single female TR who had AIN3 had previously had HGCIN treated with a LLETZ. This patient tested positive for HR-HPV 16 by Roche Cobas® at the time of histology confirmed AIN3. Amongst TR the far lower prevalence of HR-HPV means that this could be used to triage for HRA. If anoscopy were restricted to those testing HR-HPV positive, it would only have been required in around 20% of patients. It therefore appears from the study at least that standalone HR-HPV triage lacks sensitivity and specificity; this is reflected in the standard logistic regression modelling which demonstrated non-significant increased odds ratio for HPV positive results that is due to the poor specificity of HR-HPV testing.

5.2.2 Genotyping and prophylactic vaccination

The genotyping performed by Dr Alex Sargent using Grenier PapilloCheck® on all Cobas 4800® positive samples showed a broad spread of genotypes. Prevalent strains in MSM were similar; in HIV positive MSM the four most prevalent strains included types 16, 51, 33 and 68 whereas HIV negative MSM types included 16, 51, 31 and 56. Type 16 is seen most frequently in the majority of studies as the most prevalent strain in MSM (Gao et al., 2010, Phanuphak et al., 2013). The prevalence of types 16 and/or 18 was higher using the Cobas 4800® compared with the full genotyping PapilloCheck® assay; it is known that PapilloCheck® is less sensitive in the detection of HR-HPV as reported in a large retrospective and prospective analysis of over 8,600 cervical samples by Crosbie et al (Crosbie et al, 2015). When compared to Hybrid Capture 2 (HC2) in the detection of CIN2+ samples, PapilloCheck® missed 11% (74/672) of lesions compared with 7% (44/672) by HC2 (Crosbie et al, 2015). In the ANALOGY study the discrepancy between the genotyping data generated by the Cobas 4800® and the
PapilloCheck® assay, was probably due mainly to inherent differences in sensitivity but could also have been contributed to by differences in stringency in terms of detecting sequence variations between types. It must also be considered that all Cobas 4800® testing was performed using fresh Surepath™ material. PapilloCheck® was tested towards the end of the study whilst the Surepath™ medium had been stored for up to a year before testing. Samples stored in Surepath™, which contains formalin, can inhibit PCR and may cause a reduction in HPV types detected. The MSM population in particular has a high level of mixed infections and there may have been competition with the other types when samples were harbouring low amounts of 16 or 18.

The strains seen in the ANALOGY study differ from other published work, in the 1990s a US study group reported in descending order; types 16, 18, 31 and 53 were most prevalent (Palefsky et al., 1998). Another study reports types 16, 58, 51 and 39 being the most prevalent strains in HIV positive MSM and types 16, 68, 58, 59 and 39 in HIV negative MSM (Supindham et al., 2015). It is clear that there is a difference in the prevalence of infective types geographically. Understanding the multiplicity of HR-HPV infection in the anal canal of all MSM has implications in potential future prophylactic vaccine development. Gardasil qHPV covers types 6, 11, 16 and 18; this would prevent the most prevalent strain, type 16, seen in HIV positive and negative MSM only. The nonovalent vaccine (types 6, 11, 16, 18, 31, 33, 45, 52, and 58) currently in clinical trials would prevent the three most prevalent strains seen in both HIV positive and negative MSM. In HIV positive women the most prevalent strains were 16, 45, 52 and 53. As in MSM, although the types differ, the most prevalent strains would be prevented with nonovalent vaccination in HIV positive women. The most prevalent strain in TR and HIV negative women was type 51; this would not be prevented by use of the nonovalent vaccine. Both of these groups had less multiplicity of HR-HPV infection when compared with other groups. The spread of genotyping seen in the ANALOGY study suggests that MSM and HIV positive women would benefit from vaccination with a nonovalent preparation rather than a quadrivalent preparation in order to prevent the most prevalent strains. Modelling studies would have to be conducted to see if this would be cost effective.

5.2.3 Prevention of disease - vaccination

Data regarding the prevention of HR-HPV infection by vaccination need to be considered along with outcomes from SPANC. The vaccination and therefore prevention of HPV associated anogenital disease is a multifaceted consideration. In the UK currently girls aged between 11 and 14 are offered vaccination with qHPV. Girls have two injections of the vaccine. The second injection is usually a year after the first but it can be any time between six to 24-months later. Recently there is pressure for boys and MSM to be vaccinated too (Baron et al., 2014). It is considered this may be cost effective (Baron et al., 2014). The JCVI issued an interim statement in November 2014 concluding that MSM aged 16-40 years of age should be vaccinated with qHPV subject to the programme being commissioned and implemented at a cost effective price (JCVI, 2014). A significant issue surrounding this statement is that if we use ANALOGYs and
other worldwide datasets for the prevalence of HR-HPV in MSM, a large proportion are already infected with high-risk strains.

If prophylactic vaccination were not to be effective for those that are already infected, then vaccination post treatment of HGAIN must be considered. A study conducted in NY, US, recruited 202 patients with a previous history of treated HGAIN; 88 patients were vaccinated with qHPV and 114 were not. The findings from this study demonstrated that vaccination after HGAIN had been treated significantly reduces reoccurrence amongst MSM. During 340.4 person year follow-up, 13.6% (12/88) of vaccinated patients and 30.7% of unvaccinated patients developed recurrent HGAIN. The study suggested that qHPV vaccination post HGAIN treatment may be an effective post adjuvant therapy, but a RCT is needed to confirm these results (Swedish et al., 2012). Further support of adjuvant qHPV vaccination in HIV positive MSM has been demonstrated in a study conducted in 2015 in the US. Deshmukh et al constructed a Markov model on anal histology samples in HIV positive MSM. They compared no qHPV vaccine after treatment for HGAIN versus qHPV vaccine after treatment for HGAIN along with the long-term clinical and economic outcomes. Deshmukh et al concluded that the use of qHPV vaccination decreased the lifetime risk of anal cancer by 63% when compared to no vaccination. Vaccination was also proven to be cost saving; decreasing lifetime costs by $419 whilst increasing quality adjusted life years by 0.16. The study demonstrated an incremental cost effectiveness ratio (ICER) of US $87,240 per quality-adjusted life-year (Deshmukh et al., 2014). When considering an ageing HIV positive population, those who have been HIV positive the longest and have survived the pre HAART AIDS era are probably most in need of qHPV vaccination. This group of men will be over the age of 40 and therefore will not benefit in the reduction of anogenital HPV related disease.

Along with prevention programmes, adjuvant treatment studies such as HPV Vaccination After Treatment of AIN (VACCAIN-P, NCT02087384) are now being conducted. VACCAIN-P is a RCT being conducted in the Netherlands evaluating vaccination with the qHPV versus placebo vaccination on prevention of HGAIN recurrence in HIV positive MSM who were successfully treated for HGAIN. The study commenced recruitment in March 2014 and is open until December 2017 and the estimated enrolment is only 125 participants. Another study also being conducted in the Netherlands is Therapeutic HPV-16 Vaccination for the Treatment of Anal Dysplasia (VACCAIN-T, NCT01923116). The estimated enrolment is only 45 participants but this study will be a starting point of possible adjuvant research. The study commenced recruitment in August 2013 and is open until October 2017. The objective of the study is to assess, in a phase 1/2 study, the safety and efficacy of this synthetic vaccine SLP-HPV-01® in HIV positive men with CD4 counts >350 cells/ul and HPV16-induced intra-anal HGAIN (grade 2/3) that failed on, or recurred after previous treatment. Both these studies are actively recruiting and will provide much needed data regarding the prevention of HR-HPV infection and the adjuvant treatment of HGAIN as to whether this reduces reoccurrence; it will be some time before these data are available.
The group in which there are minimal data in prevention against of HR-HPV infection is TR. A recent study in Alberta, US looked at the immunogenicity of qHPV vaccination in TR. Fifty adult solid organ TR aged 18-35 were recruited from 2008-2010 and they were at least three-months post-transplant on a stable immunosuppressive medication that had not changed in the preceding one-month. In total 47 patients were vaccinated with qHPV Gardasil at enrolment, two and six months. Using the IgG ELISA, the rate of seropositivity ranged from 52.6% to 68.4% depending on the HR-HPV type (Kumar et al., 2013). A recent RCT that recruited immunocompetent young men and women demonstrated that seropositivity after vaccination ranged from 97.0% to 99.0%, this is much higher than Kumar et al (Munoz et al., 2009, Giuliano et al., 2011). Kumar et al concluded that there were suboptimal responses in post-transplant recipients and that vaccination at a younger age may provide greater titers (Kumar et al., 2013). It is feasible all groups will benefit from the nonavalent vaccine for prevention of HR-HPV types 6, 11, 16, 18, 31, 33, 45, 52, and 58.

Primary prevention by HPV vaccination offers protection to uninfected individuals, but almost half of HIV positive MSM who participated in ANALOGY already have HPV 16 and/or 18 infections. Although vaccinating boys who are uncertain of their sexuality before their sexual debut this does not provide a solution to the problem amongst already sexually active MSM.

5.2.4 HR-HPV anal cancer

A large majority of anal cancers are positive for HR-HPV, mostly type 16. A recent systematic literature review included 992 cases of anal cancer and reported 72% positivity for HPV 16 and/or 18, as assessed by PCR or hybrid capture (Hoots et al., 2009). Of the three screen detected cancers found in the ANALOGY study, two out of three were HR-HPV type 16 and one type 18 positive. The patients whose cancers were type 16 positive by Roche® were also type 16 positive by PapilloCheck®. The patients in which the cancers were detected had a total of four HR-HPV strains when genotyped by PapilloCheck®. Even though the data set is smaller, the percentages of type 16 and/or 18 are similar to those seen in other studies. In other HPV associated cancers, HIV positivity not HPV positivity influences clinical outcome, although it is well known that OSPCC that are HR-HPV positive and show overexpression of p16 have been reported to have better outcomes following chemoradiation (Ukpo et al., 2011). Studies of ASCC treated with chemoradiation demonstrated disease reoccurrence more frequently in HIV positive patients. HIV positivity appeared to have an association with a higher recurrence rate and worse recurrence-free survival not HR-HPV positivity (Meyer et al., 2013). It is therefore considered that although HR-HPV drives precancerous change and the potential development of ASCC, being positive for HR-HPV at the time of treatment does not affect survival as having a HIV positive serostatus.
5.3 Cytology limitations and prevalence of AIN in the ANALOGY cohort

5.3.1 Cytology as poor predictor of anal disease

Data from the ANALOGY study suggested cytology was not sensitive enough to be considered as a stand-alone screening test as it is in the cervix. Developments in cytology specimen collection and the advent of LBC have improved anal cytology reading as faecal debris can be filtered out without the loss of cells. Despite this, a high proportion of AIN2+ was not detected by cytology in MSM. Using a standard logistic regression model, abnormal cytology increased the odds of underlying AIN2+ but the positive predictive value was not high. It is possible this was due to inadequate sample collection, although very few slides were classified as unsatisfactory; six in total. The sensitivity of anal cytology is at the lower range of other published worldwide data. It is known that anal LBC is not as sensitive as cervical LBC also that concordance between LBC and histology proven AIN is low.

A recent study conducted by Betancourt et al in Texas, US recruited 228 HIV positive men and women and yielded 318 LBC specimens. These patients underwent anal cancer screening investigating anal cytology as a predictor of AIN. The study demonstrated that the sensitivity of anal LBC in the detection of ASCUS, AIN1/3 or ASCC was 93.0%. Cytology was 88.0% sensitive for detecting LGAIN (AIN1), but the detection of HGAIN (AIN2/3) or ASCC was only 20.0% sensitive. Overall 32.5% (74) of patients had associated anal biopsies within a six-month period, with a total of 89 comparison cases. Six cases in total had negative cytology, all of which were associated with biopsy proven AIN there was therefore a false negative rate of 7.0%. The investigators concluded that anal LBC is a good predictor of AIN, as confirmed by the high degree of sensitivity (93%). It is clear that there is poor concordance between the cytological and histological grade of AIN, therefore LBC underestimates the grade of anal dysplasia compared to the corresponding biopsy (Betancourt et al., 2013).

There are differing sensitivities reported in the evidence when reviewing anal LBC. Mathews et al (Mathews et al., 2011) conducted a meta-analysis of 33-cervical and 11-anal screening studies. Mathews concluded, “anal LBC is somewhat less discriminating than cervical cytological screening”. There are two studies that suggest that anal LBC is comparable to cervical LBC in both respects. A study in 2006 found that sensitivity of anal LBC ranged from 69% to 93%, and the specificity from 32% to 59%, and concluded that anal LBC has a similar accuracy to a cervical LBC sample (Chiao et al., 2006). Bean et al., (2010) state that “the sensitivity and specificity of a single anal-rectal cytology specimen is comparable with that of a single cervical cytology test, but cytological interpretations do not always correlate with lesion severity” (Bean and Chhieng, 2010).

A study conducted recently by Nathan et al (2010) assessed cytology, using samples from 395 patients, 212 were HIV-positive. The sensitivity of cytology to detect any AIN was 70% and specificity 67% (based on 288 histology results). The sensitivity to detect HGAIN was 81% this is in line with other publications (Chiao et al., 2006). Results demonstrated that the sensitivity of
anal cytology was dependent on the area of documented disease identified at HRA (86% for two or more quadrants versus 69% for one or more quadrants, p=0.002). This study suggested that this could possibly explain previously reported sensitivity differences. It is therefore possible this also explains the sensitivity differences found during ANALOGY (Nathan et al., 2010).

The disparity between biopsy proven AIN and anal LBC is possibly due to the ATZ not being visualised during specimen collection, having a variable depth for collection dependant on the sex and size of the patient and due to the anal canal not being a fixed organ like the cervix, in-fact it has the propensity to rotate making sampling difficult. Also during HRA there may be a lesion seen clearly when the anal canal is visualised and the folds of the canal separated, but during blind LBC collection this could easily be missed. Other reasons a LBC sample does not detect a lesion in the anal canal are poor technique and the size of the size of lesion, which may be extremely small. If the lesion is very small, the Floq® swab may never brush across it, or a section of the lesion may be high-grade but only low-grade surrounding cells are sampled.

Collecting a cytology specimen required more vigour than when the “Palefsky technique” was taught at UCSF, US and honed at the Homerton, UK. Several of the first cytology specimens from the ANALOGY study were inadequate and had to be repeated. During HRA training, cytology sample collection was performed using a Dacron® swab and ThinPrep™ LBC medium. During the ANALOGY study, a Floq® swab was used to collect anal cytology and this was preserved in Surepath™. Evidence from a small study demonstrates that Floq® swabs yield more anal cells than Dacron® (Gage et al., 2011). It was decided Floq® swabs were suitable for cytology collection and Dacron® swabs were unavailable at the time the study commenced. Inadequate sampling was the result of the Floq® swab tip not making sufficient contact with the wall of the anal canal. This is due to insufficient pressure being applied on rotation through the anal canal and resulted in an inadequate cellular harvest. The cytology medium ThinPrep™ only requires 1-2 cells per HPF, whereas Surepath™ requires more cells in order for the sample to be adequate, 3-6 cells per HPF. The “Palefsky” technique learnt was appropriate when using Dacron® and ThinPrep™ but required modification when using a Floq® and Surepath™ to collect adequate samples. ThinPrep™, requires the Dacron® swab to be pre-moistened in sterile water before insertion into the anal canal. The Floq® swab used to collect anal LBC samples were not pre-moistened by sterile water as this was contraindicated when discussed with the manufacturer. After review by the cytopathologist, Dr Desai, the Floq® swab was pre-moistened in the SurePath™ vial, and improved anal cytology specimen collection.

There are several other reasons for poor cytology sampling others than those discussed above. In the early stages of the study when collecting anal cytology, the Floq® swab may have been inserted too distally into the anal canal up to the rectum. Adaptation had to be made for each individual due to a differing length of anal canal in proportion to his or her height and gender. Unlike cytology collected from the cervix, anal specimen collection is blind, accounting for areas of disease that were possibly not sampled. The research nurse who “agitated” the Floq® swab in the SurePath™ medium also had to learn how to do this correctly. The cells once inserted into
the SurePath™ medium needed at least a minute of vigorous “whisking” similar to when one beats an egg. After these issues had been modified, cytology sampling was improved. Despite improved LBC specimen collection, the cytopathologist reading the samples also had a marked learning curve in the identification of anal disease. Initially reports were using Bethesda terminology and mid-way through the study they were reported in concordance with the LAST terminology.

5.3.2 Prevalence of AIN

The high-prevalence of anal lesions seen in HIV positive MSM who participated in the ANALOGY study is consistent with studies worldwide (Gaisa et al., 2014). The prevalence data collected from HIV positive and negative MSM is similar to Machalek et al meta-analysis (Machalek et al., 2012b), this comprised of eight studies all controlled by anoscopy. There are no such meta-analyses for TR or HIV positive women. It should of course be considered that the high prevalence of disease seen in ANALOGY reflects the lack of prior screening. There has therefore been an accumulation of AIN over a number of years. The three screen detected cancers found in the ANALOGY study were all microinvasive and generally have more favourable outcomes. A recently reported study by Dalla Pria et al (2014) in a London HIV clinic showed prevalence of AIN3 of 13.0%, and an estimated five-year risk of anal cancer of 3.2% (95% C.I. 0-7.8%) (Dalla Pria et al., 2014), which is consistent with the systematic review by Machalek et al (Machalek et al., 2012b). Other studies have also demonstrated a high prevalence of lesions and indeed cancer in HIV positive women (Heard et al., 2015), and HIV positive heterosexual men (Wilkin et al., 2004). In one study, 728 HIV infected patients with abnormal anal cytology underwent HRA, 71.0% were MSM, 23.0% women, and 6.0% heterosexual men (Gaisa et al., 2014). The highest prevalence of HSIL/cancer was found seen in MSM, 32.0%, with similar percentages seen in women, 26.0%, and heterosexual men 23.0%. There were five cases of anal squamous cell carcinoma (0.7%), four in MSM and one in a heterosexual man demonstrating being HIV positive alone is also a significant risk factor (Gaisa et al., 2014). The data from the ANALOGY study in HIV positive women cannot be compared to Gaisa et al due to the fact that HIV positive women were disinclined to be screened, but there is prior evidence in support of screening HIV positive women in the detection of anal disease (Powles et al., 2009), but no study confirming if this is acceptable to this population.

All three of the screen-detected cancers were in HIV positive MSM taking HAART. Interestingly previous studies have documented that HAART does not seem to have an effect on the incidence of anal cancer in HIV positive MSM since the increased incidence rates have been reported in the HAART era when compared with the pre HAART era (Hessol et al., 2007). There is significant heterogeneity and there are limited data on the prevalence of AIN in HIV positive women. The ANALOGY study data set for HIV positive women is small; 88.9% (8/9) had negative cytology and one low-grade sample. Three women had biopsies and the majority of disease was low-grade AIN1, 33.3% (2/9) and only one sample having AIN2+.
5.4 Strengths and weaknesses

To-date the majority and most significant proportion of literature investigating anal screening and the detection of anal precancer has come from US based research groups. US based healthcare models differ greatly to those in the UK and are largely funded privately. A meta-analysis of anal HR-HPV infection and its associated neoplastic lesions in MSM concluded although lesions are common in MSM, rates of progression to cancer appear to be significantly lower than in similar lesions seen in the cervix (Machalek et al., 2012a). This analysis stated that large, well-designed cohort studies are needed to address these issues. To date worldwide there have been few studies that have prospective data in this field and they are often small in size and mainly comparing HIV positive and negative MSM (Critchlow et al., 1995). Therefore the ANALOGY study addresses these criteria. ANALOGY is a moderate sized study that successfully recruited 409 participants over 22-months and the majority of participants attended follow-up. It is the first UK study to recruit all high-risk groups simultaneously, routine anoscopy guided biopsy represents a gold standard against which cytology and HR-HPV infection can be compared. Previous impactful pieces of research in this area have been focused on one particular high-risk group such as HIV positive MSM or women, or have compared immunocompetent HIV negative MSM with immunocompromised HIV positive MSM. Along with MSM, ANALOGY recruited immunosuppressed TR who in the majority did not practice anoreceptive sex and comparatively had a decreased prevalence of AIN. Previous studies investigating the prevalence of disease in TR have only focused on those who are immune compromised and received organ transplantation. In these studies, no comparisons have been made between differing high-risk groups.

The ANALOGY study main strength is that it is one of only a small number of global cohort studies to perform LBC, HR-HPV testing and HRA screening as a gold standard for diagnostic comparison, on all participants at several time intervals. It has been previously documented that anal LBC and biopsy specimens under-diagnose HGAIN (Darragh and Winkler, 2011) therefore performing both at two separate visits permitted the most accurate research based estimate of the prevalence of disease in these high-risk populations along with checking the sensitivity of the initial screen. The second visit in the ANALOGY study was to ascertain disease missed at the initial visit, not to investigate disease progression, as the time interval was short, between three to six months. It has been documented as previously discussed from Berry et al that prevalent HGAIN on average progressed to anal cancer in HIV positive MSM over 57-months and it would therefore have been unlikely that a cancer would have developed at the site of previously biopsied HGAIN (Berry et al., 2014). The screen time interval makes the prevalence data from the ANALOGY study more reliable.

The ANALOGY study was a multidisciplinary collaboration amalgamating people with specialist skills sets from a clinician, nursing, cytopathology, histopathology, virology, colorectal surgery and social science research background. A single, initially inexperienced clinician (AMS) performed all HRA procedures, therefore eliminating variation in examination technique. Fifty
non-study HRA were performed prior to commencing HRA practice independently. The same highly trained specialists reported all of the cytopathology, histopathology and virology samples. They are considered experts in their fields of practice, but were inexperienced in anal disease detection and reporting. Initial inexperience in the detection and reporting of anal disease is a limitation for this piece of work. Single clinician HRA practice and reporting of cytopathology and histopathology therefore eliminated interobserver variability.

A strength of the ANALOGY study is that all of the biopsies collected were targeted. Although only 5.5% (14/252) of the visit one biopsies taken had no disease, control biopsies would have been useful for comparison of areas that were presumed to be negative. This would potentially highlight areas of missed disease by the practitioner and also confirm correct identification of normal tissue. It should have been mandatory for the HRA practitioner (AMS) to document the estimated grade of disease-identified anoscopically when taking the biopsy. The presumed grade of disease could then have been paired with the biopsy result. This would ascertain if the anoscopist (AMS) was identifying disease correctly and help to demonstrate HRA practice progression. Another option would be to have a control biopsy from each quadrant of the anal canal, yet ethically this would be unjustifiable, as it would cause increased and unnecessary pain and recovery time. Another consideration relates to the improvement of documentation of identified lesions. In total three different coloscopes were used for HRA during the ANALOGY study and they were all suitable to perform the procedure. It would have been useful for documentation and for this thesis to have images from the patients seen in clinic. The CRF permitted illustration of any lesion identified, but image capture would also allow easier comparison at a second visit to ascertain if there had been any change since the last HRA. It would also have assisted in documenting the size of the lesion along with the abnormalities seen within it.

Having another practitioner to perform HRA would have enabled more clinics and enhanced recruitment. Not diluting the observations between several interpreters is considered a strength of the study.

The development of a new HRA service in three clinics over two hospital sites demonstrated that this service could be established and that MDT approach is essential. Experienced cytopathologist, virologists and histopathologist are needed for this kind of service along with dedicated clinic space and anoscopists.

5.4.1 Other studies

Outcomes from “the Study of the Prevention of Anal Cancer”, SPANC, an Australian based prospective cohort study will provide comparative data for the HIV positive and negative MSM cohort recruited to ANALOGY. HIV positive and negative MSM aged over 35-years were recruited and had six visits over a three-year period from September 2010 with follow-up until mid-2018 with a total recruitment target of 600 MSM over 36-months, which has been met. Recruitment was from six different sites with 12 newly trained anoscopists performing HRA.
SPANC is investigating the epidemiology of anal HPV infection and anal intraepithelial abnormalities amongst MSM. The main study objectives were to investigate the following (Machalek et al., 2013):

- Epidemiology of anal HPV infection, determining the prevalence, incidence and risk factors in the acquisition of anal HPV
- Investigate the rates of clearance and persistence of anal HPV infection.
- Establish the rates of disease progression and regression of AIN.
- Assess the psychological and quality of life impact in screening MSM.

The final data from SPANC are due to be published in 2019 and will help to provide more robust evidence surrounding anal disease. It must be considered that there will be significant interobserver variability. Also, 12 anoscopists performed HRA on 600 patients and one anoscopist performed HRA on 409 patients with a total of 722 procedures, therefore the anoscopist on the ANALOGY study should have had more experience.
5.5 Criteria for an effective screening programme

The principles of screening laid out by Wilson and Jungner have to be addressed when a new screening programme is considered (Wilson and Jungner, 1968). Any anal screening service must meet these criteria. In Figure 26 the Wilson and Jungner criteria has been summerised in case for anal screening.

**Figure 28 Wilson and Jungner screening criteria applied to anal screening (Wilson and Jungner, 1968)**

| ✓ | The condition sought should be an important health problem |
| × | There should be an accepted treatment for patients with recognised disease |
| ✓ | Facilities for diagnosis and treatment should be available |
| ✓ | There should be a recognisable latent or early symptomatic stage |
| ✓ | There should be a suitable test or examination |
| ✓ | The test should be acceptable to the population |
| × | The natural history of the condition, including development from latent to declared disease, should be adequately understood |
| ✓ | There should be an agreed policy on whom to treat as patients |
| × | The cost of case finding (including diagnosis and treatment of patients diagnosed) should be economically balanced in relation to possible expenditure on medical care as a whole |
| ✓ | Case finding should be a continuing process and not a “once and for all” project |

Figure 26: A tick (✓) represents the criterion being met and a cross (×) indicates it is still outstanding and further work needs to be completed.

The first consideration regarding screening must be which group is regarded at sufficient risk to warrant being screened for the detection of a given disease. Anal cancer is considered to be an important health issue particularly in HIV positive individuals who are as a consequence of HAART are no longer dying from AIDS defining illness (Czoski-Murray et al., 2010) and findings from this study suggest supports the case for screening. Data from the ANALOGY study suggests that the prevalence of anal cancer in HIV positive MSM is significantly higher than other groups. Three anal cancers were identified in HIV positive MSM in a microinvasive stage;
this is rare in cancer of the anal canal as invasive disease is usually identified late resulting in a very poor prognosis with limited treatment options. Earlier identification in the detectable preclinical phase (DPCP) (Cole and Morrison, 1980) and treatment of invasive disease is synonymous with a better prognosis and chance of cure. In order to have an effective screening programme intervention has to be demonstrated to improve health outcomes; this can be seen from the data collected in the ANALOGY study as these cancers were treated at an early stage. HIV positive MSM only represent 5.0% of overall MSM, this would therefore be considered the most cost effective group to be screened by HRA and then placed under surveillance. Screening HIV positive MSM in this manner would histologically identify AIN3+. Worldwide the treatment of AIN3 is generally considered necessary because of its malignant potential. The estimated risk of progression at five-years has been documented in two recent studies of HIV positive MSM of the HAART era to be 3.2% and 1.7% for AIN3 and high-grade anal cytology (Dalla Pria et al., 2014, Cachay et al., 2015). Over a 20-year timeframe the risk of anal cancer would therefore be roughly 10.0% and cancer detected whilst being screened would probably be diagnosed at an earlier stage rather than with late presenting symptoms.

If screening were to be implemented in this group, the numbers of patients that would require screening needs to be considered, those already infected with HIV and future infection within MSM. It is currently estimated that 2.6% of the male population identify themselves as MSM (Mercer et al., 2013). The UK annual number of new HIV diagnoses in MSM continues to rise, placing more MSM at an increased risk due to immune suppression. Between 2011 and 2012 there was an increase in HIV diagnoses in the UK of 10% from 2,960 to 3,250 (Varney J, 2014). This is reflected over a nine-year period when the number of HIV positive MSM in the UK doubled from 16, 180 in 2003 to 33, 960 in 2012 (Varney J, 2014). In younger MSM (16-24 years) there was an observed increase by 30% from 340 in 2008 to 440 in 2012 (Aghaizu A, 2013), despite health campaigns in the practice safer sex. Currently in the UK, 6% (around 1 million) of men aged 15-60 years report same sex experience (Mercer et al., 2013); these men are at increased risk of HIV acquisition and also HR-HPV infection. Latest data estimates that 43, 500 HIV positive MSM were living in the UK at the end of 2012, but nearly one in five were unaware they were infected (Aghaizu A, 2013). This poses several issues, we do not know the true figure for HIV infected MSM, and also there is a significant proportion of MSM who do not know they are HIV positive. Therefore if a screening service were to be implemented, how would these MSM be identified?

It would appear that the model of screening we use to detect cervical disease may not be entirely appropriate for the anus. There is no highly effective treatment of cure for high-grade anal disease. Anal cytology does not perform as highly as cervical sampling in the detection of high-grade precancerous change disease, perhaps due to a “blind” procedure, and MSM have a very high prevalence of HR-HPV making this a unspecific triage test. Unlike the cervix, there is uncertainty regarding the management of AIN1/2, it is possible that these carry little risk of cancer, but in the ANALOGY study they have a sizeable prevalence in all groups and would be considered to be the majority of disease. Other healthcare economies classify AIN2 with AIN3;
this would increase the proportion of the patients who needed surveillance and or treatment of high-grade lesion. If those with an AIN2+ lesion were monitored over many years even if only AIN3 was treated this would accumulate a large amount of individuals under continued follow-up. There are several considerations to be made if this model were to be implemented, the anxiety of the patient being of primary concern, over time affecting quality of life, but also the provision and management of such a service. Standards as to who would be appropriate for discharge after a further round of screening, such as MSM with AIN1 would be necessary in order to reduce patient anxiety and ensure services were not overwhelmed. Discharging MSM with AIN1 may not be deemed “acceptable” to this patient group.

The time intervals between anal surveillance pose an issue. Women in the UK are routinely invited every three years for cervical screening between the ages of 25-49 and five yearly for women aged 50-64; these protocols are heavily evidence based (Sasieni et al., 2003, IARC, 1986). HIV positive women are currently advised to undergo annual cervical cytology as they are at an increased risk of false negative cytology and increased prevalence of squamous abnormality (Maiman et al., 1998, Mandelblatt et al., 1992, Smith et al., 1993, Wright et al., 1994, Schäfer A, 1991). Furthermore, low-grade lesions rarely regress in this cohort (Heard et al., 1995). Time intervals between screenings are well researched and the progression of cervical precancer into invasive disease is well understood. Recently there is evidence to suggest screen intervals for HIV positive women can be extended (Keller et al., 2012, Castle et al., 2012). Criterion for such a recommendation is based on persistently normal cytology results and consistent engagement with HIV and sexual health services along with a negative type 16 HR-HPV testing (Keller et al., 2015). HGCIN is treated at colposcopy with the aim of curing the precancerous disease detected.

Cervical and anal diseases are both HPV driven pathologies, the natural history of anal precancer is still not fully understood. This poses great difficulty in establishing a potential screen interval for those with detected disease. Progression of AIN3 and high-grade anal cytology cases to invasive disease at five years in HIV positive MSM has been reported to be between 1.7% and 3.2% (Dalla Pria et al., 2014, Cachay et al., 2015). Taking the incidence data from Berry et al, Cachay et al and Dalla Pria et al (Berry et al., 2014, Dalla Pria et al., 2014) into consideration, a screen interval of three years seems appropriate.

The age that anal screening should commence and terminate needs to be considered. Exposure and infection with HR-HPV coupled with persistence of infection due to immune suppression are key factors that would influence this decision. Anal cancer incidence is related to age; incidence in women is highest in those aged 60-64 and in men aged 65-69. During 2010 to 2012, 51% of cases of anal cancer were diagnosed in those aged over 65 in the general population (ONS, 2013). We have an aging HIV positive MSM population in the UK who are living longer due to HAART. These considerations would suggest that screening in HIV positive MSM should continue until 70 years of age. It must be reiterated that there are no data specific to anal cancer incidence, sexuality and sexual practice. Participation in the ANALOGY
study required patients to be over 25-years of age. Anal cancer incidence in the general population starts to rise in men aged 35-39 years of age (ONS, 2013). It is known that there has been a 30% increase in younger MSM (16-24) being infected with HIV (Aghaizu A, 2013). Data collected from ANALOGY demonstrates that risk taking sexual behaviour such as anoreceptive sex is widely practised. This places MSM at greater risk of infection, reinfection and persistence of HR-HPV infection causing disease. MSM are therefore becoming immune compromised younger and having a longer timeframe practising anoreceptive sex. The lifetime number of sexual partners also increases the risk of inoculation with HR-HPV infection. Data from the ANALOGY study demonstrated that around half of both HIV positive (43.8% 89/203) and negative (51.9% 42/81) MSM reported having greater than 15 sexual partners (personal communication Dr Laura Sadler). Young HIV positive MSM (16-24) could therefore warrant anal screening sooner than the documented rise in incidence seen in the general population at 35-39 years of age. There are no data on screening below the age of 25 therefore screening initiation HIV positive MSM could commence at 30. The initiation of HR-HPV vaccination in boys and HIV positive MSM would also influence recommendations.

5.5.1 Treatment limitations

In the UK we have a publically funded healthcare system, which would require robust data in order to justify a screening service for the detection of anal precancerous lesions. The ANALOGY study has provided data identifying and confirming the prevalence of cancer and high-grade disease. However studies demonstrating an effective treatment of precancerous disease are not yet available. There are currently no national or international standards on who to treat as the screened population, although is considered on the basis of the data collected from this study that it primarily should be HIV positive MSM. There is still currently no defined age range and screening interval in the screening of AIN in high-risk groups. There is much disparity globally between HRA practitioners treating AIN as to which lesions should be treated, along with the successful treatment of screen detected AIN3. A recent Cochrane review of interventions for anal canal intraepithelial neoplasia found only one small RCT reporting data on imiquimod versus a placebo treatment. There was no statistically significant difference in the risk of disease cure but there was a clear trend after the use of imiquimod to downgrade the AIN lesion (Macaya et al., 2012). The authors concluded at this time there was a distinct absence in reliable evidence for any intervention used to treat AIN and therefore precludes any definitive guidance or recommendations for clinical practice. The authors felt that well designed RCT’s were needed in order to make any recommendations (Macaya et al., 2012).

Treatment limitations of AIN have been recently been demonstrated in the Netherlands. This study compared treatment of histologically confirmed AIN in HIV positive MSM between August 2008 and December 2010. Richel et al screened 388 HIV-positive MSM by HRA and 63% (246/388) of them had AIN. In total 63% (156/246) were randomly assigned to receive treatment by imiquimod, topical fluorouracil, or electrocautery. The treatment schedules were 16-weeks of imiquimod (three times a week), 16-weeks of topical fluorouracil (twice a week) or monthly electrocautery for four-months. All patients were assessed by HRA four weeks after
commencing treatment, and those who responded returned for follow-up at 24, 48 and 72-weeks. Small numbers of patients had a complete response; 13 (24%, 95% CI 15-37) of those treated with imiquimod, eight patients treated with fluorouracil (17%, 95% CI 8-30), and 18 (39%, 95% CI 26-54) treated with electrocautery. Reoccurrence of disease at 72-weeks was high in all groups, 71% (10/14) of patients treated with imiquimod, 58% (7/12) of fluorouracil treated patients, and 68% (13/19) of patients treated with electrocautery. Side effects were common. This study concluded that electrocautery is better than imiquimod and fluorouracil in the treatment of AIN but the reoccurrence rates and side effects of treatment are substantial (Richel et al., 2013).

Radical treatment in the way of excision of AIN3 is considered to be excessive, whilst more conservative excision, ablation or indeed non-surgical treatments all carry a high-risk of reoccurrence. Effective treatment is limited in part by the added susceptibility of immune suppression. As previously discussed, insight into these issues comes from a follow-up of 138 HIV positive MSM in San Francisco; 72 MSM developed anal cancer, of which 27 whilst under surveillance were at the site of a previously biopsied and treated HGAIN lesion (Berry et al., 2014). This study provides powerful evidence of not only the malignant potential of AIN3 in HIV positive MSM but it also demonstrates limitations in its treatment.

### 5.5.2 Cost effectiveness

The cost effectiveness of any screening programme is a crucial consideration. The ANALOGY study did not collect any economic data and this is a limitation of the study. The management costs of anal cancer is considered to be similar to those of cervical cancer as demonstrated from a study conducted in France, costing 38 and 44 million Euros per year respectively (Abramowitz et al., 2010). If screening were to be introduced the cost of a service has to be considered. An outcome from the ANALOGY study is that HRA is the screening test of choice in the detection of anal lesions. In support of this from a cost based viewpoint, a study of 400 HIV positive MSM conducted in Canada using a decision analytical model concluded that despite higher unit costs, the limitations of both HR-HPV testing and LBC meant that HRA would be the most effective strategy. The costs ($ US) per procedure for HRA, LBC, and HPV testing were $193, $90, and $95, respectively. The direct use of HRA detected 98 individuals with AIN2/3 and had a cost-effectiveness of $809 per AIN2/3 case detected (Lam et al., 2011). A US based study had previously suggested that annual screening using cytology in MSM would be associated with an estimated cost per quality of life year saved $13, 000. Another study investigating anal cancer screening in HIV positive women concluded that biennial screening in women with CD4 counts less than 200 was cost-effective (Lazenby et al., 2012). The incremental cost-effectiveness ratio of biennial anal cancer screening compared to no screening was $34,763. The slow progression of AIN to anal cancer made annual screening not cost-effective (Lazenby et al., 2012).

The ANALOGY data suggest that screening by means of HRA would be sufficiently sensitive to allow less frequent screening. Another study found that the reference case cost-effectiveness...
model when screening MSM for anal cancer was unlikely to be cost-effective (Czoski-Murray et al., 2010). The HTA group performed a comprehensive literature review in order to estimate the cost-effectiveness of screening for anal cancer in the high-risk HIV positive population in particular MSM by developing a model that incorporated the national screening guidelines criteria. Published literature was identified and assessed by four reviewers. The inclusion criteria was as follows; data on population incidence, effectiveness of screening, health outcomes or screening and/or treatment costs; defined suitable screening technologies; prospectively evaluated tests to detect anal cancer. A total of 82 papers were included. The investigators felt that a majority of the criteria needed for a screening programme were not met and analyses showed little likelihood that screening any of the identified high-risk groups would generate cost-effective health improvements (Czoski-Murray et al., 2010).

5.5.3 Implications for developing a service

In any screening programme the test to detect disease must be sensitive and specific; this has been demonstrated using HRA as the screening test of choice in the ANALOGY study. As previously discussed MSM have a high incidence of HR-HPV and this would not be an appropriate triage system for the detection of a group requiring HRA, also anal cytology is not sensitive in the detection of high-grade disease. UK bases practice of LBC and HR-HPV triage in the detection of cervical disease leading to colposcopy is not a suitable management of anal abnormalities. Patients felt that screening was convenient and acceptable in this environment (Dr Laura Sadler, personal communication). Further work would need to be conducted in order to establish whether the benefit of anal screening outweighs harm in high-risk groups, along with an international consensus on how anal cancer prevention services should be implemented.

PHE has committed to make recommendations regarding anal cancer screening in HIV positive MSM based on the findings of the ANALOGY study due to the size of patient population an anal screening service could potentially benefit (Varney J, 2014). If surveillance or indeed screening by HRA was to occur in a selected high-risk groups such as HIV positive MSM this would require new service development. It is considered that such a service could be run within sexual health. This would require dedicated practitioners of HRA in areas with a high density of HIV positive MSM. HIV positive MSM who did not receive care at a HRA practicing sexual health centre would have to be invited elsewhere to be screened. This would be challenging in terms of monitoring patients and sharing information, but also the training of practitioners, accreditation and healthcare costs.

The practice of anal screening worldwide is relatively adhoc with no global training or standardisation. Screening for anal cancer only occurs in several specialised clinics in the UK and also internationally. HRA is currently being provided by a wide variety of differing practitioners from nurse led specialists to physicians from many differing specialities of medicine. No specialty in particular has ownership of a HRA service. It also needs to be recognised that the majority of clinicians who practice HRA worldwide provide a service from
private funding (Patel et al., 2014). Therefore, training and accreditation of anoscopists would need to be established in the practice of HRA. Establishing cytolopathology, histopathology and virology laboratory infrastructure was achieved with relative ease when the ANALOGY study commenced. For a screening service to exist, dedicated MDT members would be needed at each site with an anoscopist to practice HRA, along with cytopathologist and histopathologist trained in reading anal specimens. There would have to be a colorectal surgeon who was committed to seeing all those in need of treatment. If a national programme was to be created, standardisation in which LBC medium, if used, would need to be established. The reporting of cytopathology and histology samples would have to be uniform. It would be sensible to use the reporting standards defined in the LAST project (Darragh et al., 2012a). The threshold for treatment and what would be considered to be high-grade disease would therefore need to be established and national protocol would need to be implemented.
5.6 Immunohistochemistry, Ki67 and p16 biomarkers in anal intraepithelial neoplasia

The applications of Ki67 and p16 biomarkers to anal tissue biopsies containing varying degrees of AIN have demonstrated the following:

- The sensitivity of manual and automated p16 antibody testing is high but specificity is low when compared to Roche Cobas 4800® in the detection of HPV.

- There was a significant difference in the detection of Ki67 cellular positivity when scored by automated and manual methods. As the grade of anal disease increased the detection of cellular positivity was higher using manual detection methods.

- There was a difference in the detection of p16 positivity when scored by automated and manual H-SCORE.

- There was an increase in the proportion of positive cells detected when scored manually using both Ki67 and p16 antibody as the grade of anal disease increased.

- When block positive p16 compared to block negative p16 in AIN2 tissue, the former has a higher H-SCORE.

- AIN3+ samples are more likely to be block p16 positive.

In this study Ki67 and p16 biomarkers were evaluated, both of which have both been previously recommended in the diagnosis of cervical and anal disease (Padilla-Paz, 2005, Darragh et al., 2012a). In this study the detection of positivity was ascertained by automated and manual methods. It is known that nearly all cervical SCC’s and HGCIN are strongly p16 positive. p16 is a cyclin dependant kinase inhibitor regulating the transition from G1 to S phase of the cell cycle (Ortega et al., 2002). Normally p16 is not expressed in replicating tissue. Infection with HR-HPV enhances cellular turnover and therefore infection with HR-HPV should therefore yield and increased positivity when scored. In HR-HPV associated anogenital neoplasia, p16 is overexpressed due to the deregulated expression of viral oncogene E7 interfering with the cellular cyclin regulatory network (von Knebel Doeberitz, 2002). Statistical significance was seen in slides stained with p16 antibody that were scored by manual and automated methods as the grade of anal disease increased. Automated p16 positivity was only seen in 44.0% (44/100) specimens with a failure of detection and data output in 15.0% (15/100) of samples. This is higher than seen in the automated detection of Ki67 biomarker (7%: 7/100). All of the manually scored Ki67 samples had some degree of positivity, even those that were HR-HPV negative. Ki67 is a marker of cellular turnover and is expressed in all phases of the cell cycle apart from G0 (Indinnimeo et al., 2000). This does not reflect the prevalence of HR-HPV
infection by Cobas 4800® testing. Unless the biopsy specimens were tested for E6 and E7 there would be no accurate way of confirming if tissue was actively infected with HR-HPV. The majority of samples in this study (n=90) were Cobas 4800® HR-HPV positive. This hindered the study as there were very few negative specimens for comparison. It must also be considered that the thresholds set in automated scoring were possibly too high. Again, thresholds for the detection of positivity may not have been appropriately set.

Manual scoring of Ki67 and p16 positivity did demonstrate statistical significance as the grade of anal disease increased and therefore proved our hypotheses. When the histogram of manual H-SCORES for p16 expression is analysed, a score of zero has to be deemed negative and this was the most frequently seen score. These data were skewed and not normally distributed. The smallest and greatest H-SCORES were 0-16. A score of 16 was only seen in one AIN2 samples. Higher H-SCORES were generally seen with a high-grade of AIN. The mean H-SCORE from all samples was 6.87 (CI 5.7 – 7.9) and the mode 9. In AIN2 samples there appears to be two categories of AIN2, those with a negative or low H-SCORE in the detection of p16 and those with a score similar to those seen in AIN3+ disease. All of the AIN2 samples were analysed to ascertain if they had block positivity of p16 immunostaining. Block positivity confirms high-grade disease (Darragh et al., 2012b), 56.9% (34/60) of AIN2 specimens were block positive when stained. Block positivity was much higher in AIN3+ samples 88.8% (16/18), in keeping with high-grade disease being the result of active and persistent HR-HPV infection. There was a statistical difference (p=0.0001, Mann Whitney U Test) between AIN2 samples that were block negative and those that were block positive. The lowest H-SCORE in a block positive p16 AIN2 sample was 7. If H-SCORE were to be implemented as the quantification method of choice from these data it suggests that seven would correlate with high-grade disease and block positivity. It is plausible these specimens are indeed the grade of biopsy that should also be considered as high-grade and therefore in clinical practice referred for treatment.

In clinical practice manual scoring of both Ki67 and p16 to aid diagnosis of the grade of AIN would not be practicable and a suitable automated detection system ideally would need to be used. However, accurate classification of high-grade disease would help to establish which patients would be suitable for treatment. Another consideration is that all of the tissue in each slide was scored, the slide was categorised on the basis of the highest grade of disease found. Each slide will have had mixed grades of disease. Therefore, the positivity of all of the scoring must be questioned, as it is not specific to just the area displaying that grade of disease.

When automated and manual methods of p16 detection were compared as a marker of HR-HPV infection to Cobas 4800®, manual scoring was more sensitive 93.7% (CI 83.9-97.9) than automated scoring 86.6% (CI 72.5-94.4). This difference could be due to some samples not being included from the automated group due to failed output. This is more than likely due to threshold settings for positivity or inForm not detecting the p16 staining, also the automated system detecting p16 negative cells as being p16 positive.
Manual scoring demonstrated a better specificity when compared to automated scoring 11.7% (CI 3.8-28.3) and 5.2% (CI 0.9-19.0) respectively, but neither test was highly specific. Manual scoring had a lower false negative rate than automated scoring therefore overall is deemed to be the more sensitive test in the detection of p16 when compared to Roche Cobas 4800®. In clinical practice, scoring tissue manually for biomarker positivity is not efficient, however this would be the method recommended from these data.

The results reported should be interpreted with consideration of the study's limitations. At the time these investigations commenced, all of the available AIN3+ samples from the ANALOGY study were selected for Ki67 and p16 immunostaining. There were not an equal number of representative samples that were negative for any anal disease or those displaying AIN1 and AIN2 for comparison. Within this study as there were not equal numbers of specimens collected from each cohort group, the samples had to be analysed by grade of disease only. Also, there were samples (n=29) that were taken from the same 13 patients at two different visit attendances.
CHAPTER 6

FUTURE WORK
6 FUTURE WORK

6.1 Clinical research

Further research is required to improve understanding of anal cancer in the UK and worldwide. Research to date has focused on groups of a higher risk of anal cancer with less known about those that develop anal cancer in the general population.

The question as to whether high-risk individuals should be screened or placed under surveillance needs to be addressed. As the group most in need of screening is HIV positive MSM, it would imply that any such programme would be detecting precancer in a healthy population. As these individuals are immune compromised they are by definition not healthy and therefore any programme would be surveillance and not screening. Therefore future work in the UK would have to focus on improving the evidence base for surveillance. It would also have to establish an appropriate surveillance interval for those in need of monitoring. A ‘round table workshop’ at PHE was in July 2015 where the ANALOGY data and their implications were discussed. It was agreed that the high-risk group who could be considered for taking forward an NHS initiative were HIV positive MSM. They have the greatest prevalence of AIN3+ and the highest risk of disease progression. The agreed next step was to develop a concept for a larger, broader based pilot study. The panel decided that the pilot must be embedded in routine NHS practice and that a future workshop would need to invite a larger group of individuals who had the necessary range of specialist and public health expertise. A pilot of this kind would require health economics and modeling to determine cost per quality of life year saved.

In addition to surveillance of high-risk individuals, robust work needs to be completed to improve the treatment of HGAIN and the management of low-grade disease. Data from trials such as ANCHOR and LOPAC will provide much needed evidence in the treatment and management of anal disease. The use of HPV vaccination after treatment of HGAIN will also need to be studied more thoroughly. Provisional data suggests that HPV vaccination at the time of treatment is beneficial, but to date the studies completed are small. Once completed, internationally agreed standards will then need to be established in the detection, management and treatment of AIN along with training accreditation in the practice of HRA.

The issue of poor correlation between LBC and anal biopsy is a distinct finding in the ANALOGY study. A study comparing different cytology mediums in the collection and preservation of cellular harvest in high-risk groups with corresponding biopsy would help determine if there is a superior medium. Once a superior medium is identified, research needs to be conducted to correlate LBC with the grade of biopsied disease and the area of disease seen in the anal canal. Double reading of cytology specimens should also be considered to ensure that no cases of AIN2+ had been missed by the cytopathologist and may improve the concordance between cytology and HRA directed biopsy.
In a future study collection of such data would need to be optimised as duration of immune suppression is important in this high-risk cohort. The primary reason in difficulty of this data collection was due to patients being diagnosed and commenced on HAART at other sexual health clinics with poor onward documentation when care was transferred. Duration of HIV positivity, HAART, anoreceptive sex, smoking, alcohol were generated using year or age at diagnosis, i.e. each duration variable represents the number of years since event (e.g. years since HIV+ diagnosis). This data restriction means that in some cases it is possible that the time duration is out by up-to 12-months if, for example, HIV diagnosis was in January 2005 and the biopsy was December 2008 then the time would be 2008 – 2005 = three-years in reality it should be December 2008 - January 2005 = three-years and nine months. Accurate documentation of the day, month and year would be needed in future collection of such data with a more robust method of ensuring duration of any activity or treatment was correct.

The ANALOGY study collected data not generated by the author, which demonstrated the willingness of MSM and TR to attend for screening and whether HRA acceptable was an acceptable test. Review of this data and further research needs to be completed to ascertain why HIV positive and negative women will not be screened. HIV positive women particularly carry an increased risk of anal disease due to immune status and persistence of HIV infection. Methodologies from other larger studies that were successful in recruiting women need to be reviewed in order to review this issue. Any future study would also need to enhance recruitment of HIV negative MSM.

Any future study collecting anal tissue biopsies needs to collect presumed negative controls along with areas of suspicion. Also the HRA practitioner would need to document the presumed grade of the biopsied lesion and then compare it to the histology report. All future studies obtaining biopsied anal tissue would need to be scored using the LAST criteria and block p16 staining in AIN2 samples to determine high-grade disease. This would bring UK reporting in line with the US and also provide a clear distinction as to which patients need regular surveillance and possible treatment.

6.2 Immunohistochemistry

Future work using p16 IHC as a biomarker for anal precancerous disease would be needed with larger samples sizes and equal numbers from each cohort for comparison. Comparative samples sizes from HIV positive MSM and TR would also shed some lights as to whether being immune compromised by HIV or iatrogenic immunosuppressants displays an increased proliferation of biomarkers in anal disease. Ideally for all future work using manual scoring of cellular positivity in anal tissue, the scorers would ideally generate Kappa agreements when using H-SCORE in order to ensure that interobserver bias is minimised.

A similar sized study using HPV negative and HR-HPV positive samples with specimens representing negative tissue, less than AIN2 and greater than AIN2+ samples is required. This will provide information about HR-HPV infection and the biomarkers used in the detection of
The categorisation of anal disease as less than AIN2 and greater than AIN2+ would also bring reporting of p16 block positivity in line with worldwide practice and LAST terminology.

It would be interesting to ascertain if Cobas® negative AIN3+ had marked difference in p16 positivity and more importantly AIN2, as there is much debate as in the UK we should be treating this as high-grade disease. Using several methods of HR-HPV positivity, such as all participants having both Cobas 4800® and PapilloCheck® but as separate samples would provide data on positivity and also HR-HPV type in different grades of disease. It could then be ascertained if a differing grade of disease harbours an increased prevalence of a HR-HPV type. It would also be interesting to determine if tissue infected with low-risk and HR-HPV expressed biomarkers differently.

When scoring slides stained with Ki67 and p16, the majority of the time there was mixed disease displayed. The highest grade of disease was reported. All of the slides from H&E staining would need to be reviewed and the area of disease and the grade categorised before Ki67 and p16 staining. This would then give a more accurate quantification of the disease scored in relation to the biomarker proliferation.

Further work would require a single specimen from each patient. In this small study, there were multiple samples, with differing grades of disease collected at different time intervals from the same patient. Now that the ANALOGY study has closed it would be interesting to stain all of the available AIN3+ specimens to ascertain if they were p16 block positive or not. This would then provide further evidence in the support of accurate reporting of high-grade disease, as these are the patients that would require treatment. Staining all of the AIN2 specimens from the ANALOGY study would provide a larger data set for the analysis of p16 block positivity and the classification of high-grade disease.
CHAPTER 7

CONCLUSIONS
7 CONCLUSIONS

7.1 Clinical conclusions

High-resolution anoscopy is feasible, and the test of choice for surveillance of high-risk individuals in the detection of anal precancerous changes, along with targeted biopsy of suspicious lesions. HIV positive MSM by definition are not “healthy” and in this study have a prevalence of AIN3+ warranting disease surveillance, the extent to which lives would be saved by the prevention of cancer remains uncertain. There are no published data regarding interval cancers in the context of anal screening and HRA examination time intervals for individuals with anal precancerous lesions are yet to be determined. Cost effective intervals need to be researched, a large, multicentre, RCT of screening and treatment needs to be performed to acquire reliable data, and would require many years of patient follow-up.

In this study, HR-HPV is too prevalent, particularly in MSM (85-90%), to have value as a primary screening test. HR-HPV could be utilised as a triage in TR promoting further investigation-using HRA, this is due to the lower prevalence of disease of a lower grade detected in this group. In this study, cytology is associated with too high a proportion of false negatives to constitute as the primary screening test. Genotyping data suggests that primary prevention by HPV vaccination in boys; uninfected MSM and TR would offer protection and prevention of anal and indeed other anogenital disease. The prevalence of HR-HPV infection in MSM, much of which will be the result of persistent disease, suggests that these individuals would not benefit from vaccination. Research is required into the impact of HR-HPV vaccination on anal cancer rates in high-risk populations. With the advent of nonavalent vaccines, consideration also needs to be given to therapeutic vaccination at the time of treatment of HGAIN in high-risk groups.

Poor recruitment to this study of HIV positive and negative women demonstrates these groups of patients are disinclined to be screened. The reluctance of these women to be screened needs to be understood and further studies are needed into the prevalence of disease in these cohorts.

7.2 Immunohistochemistry

These data indicate that biomarkers such as Ki67 and p16 have the potential to be utilised in the detection of anal disease. Manual detection of both Ki67 and p16 cellular positivity increases as the grade of AIN increases. Manual detection of both Ki67 and p16 is more sensitive than automated methods but lacks specificity. When evaluating AIN2 samples, increased cellular positivity of p16 clearly has an association with a high H-SCORE and could be used in the absence of p16 block positive LAST criterion. It is considered that, as previously published; tissue positive for p16 block staining can determine high-grade disease; this could assist clinicians in making decisions as to which patients warrant treatment.
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APPENDICIES
8 APPENDICIES

i) Protocol for the ANALOGY study

ii) Ethics application

iii) Patient information leaflet
   a) Manchester Centre for Sexual Health
   b) North Manchester General Hospital
   c) Transplant Recipients from Manchester Royal Infirmary

iv) Expression of interest forms
   a) Central Manchester Foundation Trust
   b) North Manchester General Hospital

v) Clinical research forms
   a) Manchester Centre for Sexual Health
   b) North Manchester General Hospital
   c) Transplant Recipients from Manchester Royal Infirmary

vi) Consent forms
   a) Central Manchester Foundation Trust (MCSH and TR)
   b) North Manchester General Hospital

vii) SurePath™ components

viii) Cytology and virology request form

ix) Histology specimen request form

x) Amendment to recruitment for North Manchester General Hospital

xi) Communication with other health care professionals and patients
   a) General practitioner letters
      i. Letter to General Practitioner, MCSH and Transplant Recipients
      ii. Letter to General Practitioner NMGH
   b) Patient letters
      i. Negative cytology discharge to General Practitioner
ii. Negative cytology and biopsy discharge to General Practitioner

iii. Low grade cytology and negative biopsy discharge to General Practitioner

iv. Low grade cytology, no other abnormality: 12-month follow-up with colorectal

v. High grade cytology, no other abnormality; 12-month follow-up with colorectal

vi. High grade changes referral to colorectal

xii) SurePath™ Standard Operating Procedure

xiii) Roche Cobas 4800® Standard Operating Procedure

xiv) Papillocheck® Standard Operating Procedure

xv) Haemotoxylin and Eosin staining protocol

xvi) Ki67 and p16 protocols

a) Antigen retrieval

b) Ki67 and p16 dual staining protocol

xvii) HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas® and PapilloCheck®, (Tables a-f)

xviii) HIV negative MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas® and PapilloCheck® (Table a)

xix) Transplant Recipients specimens displaying grade of AIN, HR-HPV status by Roche Cobas® and PapilloCheck® (Table a)

xx) Repeat Biopsy specimens from HIV positive MSM displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck® from visit one and visit two attendances with the ANALOGY study (Table a-b)
8.1 Appendix i

Screening individuals at high risk for anal cancer in a clinical context: evaluation study


Clinical Lecturer:

Dr Sai Daayana

Clinical Research Fellow:

Dr Alice Martha Schofield

Research Nurse:

Ms Carol Shaw (NB Replace by Mrs Julie Burnett May 2014)

Summary

The UK National Screening Committee has suggested that screening for anal intraepithelial neoplasia in high-risk populations may be of benefit but that further information is needed. We propose to evaluate the feasibility, acceptability and efficacy of such screening in a clinical context using technologies now in routine use in the NHS Cervical Cancer Screening Programme, liquid-based cytology (LBC) and HPV testing.

Project aims

Anal cancer and its precursors (AIN 1-3) are linked to HPV infection in a similar way to cervical neoplasia. Screening for anal neoplasia by cytology and/or HPV testing may be appropriate for high-risk population groups such as HIV positive men and women and immuno-suppressed transplant recipients (Fox 2009; Palefsky, 2009; Ho and Cranston, 2010) but evidence on which to base informed decisions is limited (Chiao et al, 2006; Karnon et al, 2008). With the recent introduction within the NHS cervical screening programme of liquid-based cytology (LBC) and of HPV testing, both the technologies and the infrastructure needed for screening for anal neoplasia are potentially available. Conventional cytology was not well suited to anal sampling; whereas LBC is, by virtue of cleaning the preps of faecal debris.

We aim to evaluate the usefulness of LBC and HPV testing in screening for anal neoplasia in high-risk groups; to assess the uptake of screening through outpatient clinics and general practice of men and women at high risk of anal neoplasia for screening by liquid-based anal cytology and HPV testing; to estimate the prevalence of anal neoplasia and of HPV positivity in such population groups in the UK.
The project will provide the following outcome measures:

1. Uptake of screening and short-term follow-up: recruitment rates and reasons for refusal
2. Evaluation of LBC and HPV testing/typing as potential screening tests: test feasibility, adequacy, acceptability as well as sensitivity, specificity, and positive and negative predictive values for diagnosis of AIN
3. Reliable estimates of prevalence of anal cytological abnormality, HPV positivity by type, high resolution anoscopy and histologically confirmed anal neoplasia in relatively unselected UK high-risk patients including a HPV positive cohort who have sex with men, women who have anal intercourse and transplant recipients
4. Patient acceptability of screening for anal neoplasia
5. Identification of main resource implications for the NHS, including the screening costs of HPV testing, LBC and high resolution anoscopy.

The evaluation may identify areas where further research is needed to inform NHS policy decisions.

**Background and rationale**

Anal cancer and its precursors (anal intraepithelial neoplasia [AIN] 1-3) are uncommon in the general population (annual incidence rate of invasive anal cancer about 1-1.5 per 100,000; about 800 cases in England and Wales each year) but much more common in certain high-risk population subgroups such as HIV positive men and women, HIV negative men who have sex with men (MSM) and immunosuppressed transplant patients. In these groups the risk of developing anal cancer may be increased by 10 to 100-fold and is of the same order of magnitude as the incidence of cervical cancer in women in the general population. The natural history of anal intraepithelial neoplasia (AIN) appears to involve pre-invasive stages closely comparable to those seen in cervical cancer, and to bear a similar relation to infection with the human papillomavirus (HPV): hence the suggestion that it may be possible to screen for anal neoplasia using methods similar to those for cervical neoplasia. As well as HPV infection (and its main determinant, sexual behaviour), HIV-associated or other immunosuppression is a major risk factor (Fox 2006).

The incidence of anal cancer in the UK has risen by 2 fold since the advent of HIV and this trend is likely to continue, since antiretroviral therapy now in widespread use increases survival, often by many years, but does not reduce HIV-related anal cancer risk. Similarly, increasingly long survival after organ transplantation means that incidence of anal cancer in immunosuppressed transplant recipients is likely to be an increasing problem. Numbers in the high-risk groups in the UK are small in population terms (about 200,000 MSM not diagnosed with HIV; 50,000 diagnosed HIV positive men and women; 20,000 renal transplant recipients)
but within these groups the individual's risk of developing anal cancer may be appreciable and the available treatments are often invasive and unsatisfactory.

The UK National Screening Committee considered existing evidence for anal cancer screening in 2003 and 2005 (www.library.nhs.uk/SpecialistLibrarySearch/Download.aspx?resID=60464). The committee identified as priorities studies of the feasibility, acceptability and cost-effectiveness of screening and of the natural history of AIN 2/3 in both HIV-positive and high-risk HIV-negative subjects, as well as randomised trials of treatments for high-grade AIN. A recent HTA study of cost-effectiveness of screening in men who have sex with men (http://www.hta.ac.uk/project/1489.asp) reported that screening is unlikely to be cost-effective but that there remain major areas of uncertainty (Karnon et al, 2008). There are no adequate estimates of the current prevalence of AIN in relatively unselected high-risk populations in the UK. Most existing natural history studies are in highly-selected groups, and/or do not provide sufficient information on all study participants. Since 2004 several small clinic based screening studies have published preliminary results (eg Scott et al, 2008) but none has included a complete report of findings in all screened participants. There is no available evidence on anal cancer screening for immunosuppressed transplant recipients.

The conversion from Pap smear to liquid-based cytology within the UK cervical screening programme makes NHS screening for anal cancer a realistic possibility. The proposed evaluation project would be complementary to the HTA cost-effectiveness study in providing reliable information, currently unavailable, on the feasibility of screening and on UK prevalence of AIN in a range of high-risk groups.

**Project Setting**

Dedicated project clinic and specialist clinical laboratories (Central Manchester Foundation Trust), with recruitment through hospital outpatient and GUM clinics. Study co-ordination through the University of Manchester with evaluation at the University of Oxford Cancer Epidemiology Unit.

**Study population**

Men and women over age 25 in four groups at high-risk of anal neoplasia:

1. HIV positive men who have sex with men (MSM).
2. HIV positive women with a history of genital warts/abnormal cytology.
3. HIV negative men and women who have a history of anal sex.
4. Immunosuppressed transplant recipients (men and women) attending transplant follow-up clinics.

Patients with prior history of anal neoplasia will be excluded.
**Project design**

Anal cytology (LBC), HPV test and high resolution anoscopy will be carried out at recruitment and at 6 months for all participants. A diagnostic biopsy will be undertaken for all those with changes on anoscopy. Referral for treatment for those diagnosed with invasive anal cancer or AIN grade 3; observation with review by cytology and anoscopy at 6 months for those with AIN grades 1 and 2. If treatment is warranted, this will be offered, if not surveillance will be offered.

All participants will be offered testing for evidence of abnormality on anal cytology (LBC: SurePath) and for anal HPV DNA by Roche Cobas 4800. At the recruitment interview, participants would be asked to complete a brief questionnaire giving basic sociodemographic [age, sex, risk group] and medical [symptoms, past history] information and their perception of risk of anal pre-cancer and cancer attitude to screening and, if possible, reasons for deciding not to enter the study for those who choose not to take part. Cytology will employ a brush sampler inserted just into the anus and twisted to samples anal and perianal cells. High resolution anoscopy and biopsy of abnormal areas will be performed.

Those found to have invasive cancer or AIN grade 3 will be referred for immediate treatment. Those with AIN grades 1 and 2 will be kept under observation with the first review at 6 months, as will those with no abnormality detected at anoscopy. It is possible that the latter will include people with AIN and the study provides an opportunity to follow their subsequent progress. See attached Flow Chart.

**Assessment and follow-up**

Once the study is completed all participants diagnosed with anal neoplasia (low and high grades) will continue follow-up through the appropriate NHS clinic in accordance with routine clinical protocol, which usually is a six monthly appointment for digital rectal examination, anoscopy and anal cytology.

The project will not include screening for other ano-genital neoplasia (eg cervical, vaginal and vulval lesions in women; penile lesions in men). If such lesions are suspected as incidental findings, participants will be referred for appropriate clinical care.

**Sample size**

Total sample of 1000 participants, 250 from each target group. Sample size is based on best estimates from the literature of likely prevalences of cytological, anoscopic and histological abnormality, to allow estimation of population prevalence with 95% confidence and <=6% margin of error in all participants and in each group of 250; and adequate comparison of differences between the three groups with 80% power and 95% confidence. For example, the study is powered to estimate population prevalence of 0.2 (20%) with 95% confidence interval (CI) of 0.15-0.25; of 0.1 (10%) with 95%CI 0.07-0.14; and of 0.01 (1%) with 95%CI of 0.004-0.04).
Statistical analysis

Outcome prevalences with 95% confidence limits will be estimated using standard statistical methods, using the normal approximation to the binomial distribution, and Chi-squared tests used to obtain p-values for comparison between prevalences in the population subgroups.

Project timetable

Months 1-15: set-up, recruitment, screening; survey initial patient views, begin follow up.

Months 16-24: complete follow up, survey patient experience.

Months 25-30: clean data, analysis and writing up of results.

Study management and oversight

The applicants include senior clinical and academic staff with expertise in NHS cancer screening, study design and analysis, development and assessment of patient information, diagnosis and treatment of anal cancer, transplant medicine, and laboratory testing.

Regular meetings of investigators would be held to discuss study progress with one or more patient representatives invited to take part.

Ethical approval

The study has been reviewed and approval given by NRES Committee North West – Greater Manchester North, Reference 12/NW/0204.
References


Fox P. Human papillomavirus and anal intraepithelial neoplasia. Curr Opin Infect Dis 2006;19:62-


Evaluation of new technologies for screening for anal intraepithelial neoplasia in high-risk populations in the UK: FLOW CHART

Study population n=1000, aged 25 years + from high risk groups
250 HIV positive men who have sex with men
250 HIV positive women with a history of genital warts/abnormal cytology
250 HIV negative men and women who have anal sex
250 men & women who are organ transplant recipients
Recruited GUM/hospital clinics, CMFT

Consent to participate in anal screening

Liquid based anal cytology
Anal HPV by Roche Cobas 4800 (incl. HPV16/18 in+ve's)
Anoscopy

Negative anoscopy ± biopsy - AIN1
AIN grades 1/2
AIN3/ microinvasive
Anal Ca

Review 6/12
Review 6/12
Treat according to management protocol*
Treat according to management protocol*

Liquid based anal cytology
Anal HPV by Roche Cobas 4800 (incl. HPV16/18 in+ve's)
Anoscopy

As above

*Provisional management plan
AIN
Unifocal disease – excise
Anal cancer
Microinvasive – excision
Invasive - chemoradiation
<table>
<thead>
<tr>
<th>Applicants</th>
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| **Professor Julietta Patnick** | Director, NHS Cancer Screening Programmes  
Visiting Professor of Cancer Screening, University of Oxford |
| **Mr James Hill** | Consultant colorectal surgeon  
Central Manchester University Hospitals NHS Foundation Trust [CMFT] |
| **Dr Mina Desai** | Consultant cytopathologist, CMFT  
Director, NHS Cervical Cancer Screening Programme Manchester |
| **Professor Henry Kitchener (C.I.)** | Professor of Gynaecological Oncology, University of Manchester and CMFT |
| **Dr Ash Sukthankar** | Consultant in Genitourinary Medicine, Manchester Centre for Sexual Health |
| **Dr Ray McMahon** | Consultant pathologist, CMFT |
| **Dr Emma Crosbie** | NIHR Clinical Lecturer in Gynaecological Oncology, University of Manchester |
| **Dr Alex Sargent** | CMFT |
| **Dr Jane Green** | Clinical epidemiologist, University of Oxford |
| **Dr Laura Sadler** | Patient acceptability |
| **Rob Cookson** | Consumer rep (Lesbian and Gay foundation) |
8.2 Appendix ii

Welcome to the Integrated Research Application System

IRAS Project Filter

The integrated dataset required for your project will be created from the answers you give to the following questions. The system will generate only those questions and sections which (a) apply to your study type and (b) are required by the bodies reviewing your study. Please ensure you answer all the questions before proceeding with your applications.

Please enter a short title for this project (maximum 70 characters)
Evaluation of cytology and HPV testing for anal pre-cancer

1. Is your project research?
   - Yes   - No

2. Select one category from the list below:
   - Clinical trial of an investigational medicinal product
   - Clinical investigation or other study of a medical device
   - Combined trial of an investigational medicinal product and an investigational medical device
   - Other clinical trial to study a novel intervention or randomised clinical trial to compare interventions in clinical practice
   - Basic science study involving procedures with human participants
   - Study administering questionnaires/interviews for quantitative analysis, or using mixed quantitative/qualitative methodology
   - Study involving qualitative methods only
   - Study limited to working with human tissue samples (or other human biological samples) and data (specific project only)
   - Study limited to working with data (specific project only)
   - Research tissue bank
   - Research database

If your work does not fit any of these categories, select the option below:
   - Other study

2a. Will the study involve the use of any medical device without a CE Mark, or a CE marked device which has been modified or will be used outside its intended purposes?
   - Yes   - No

2b. Please answer the following question(s):
   a) Does the study involve the use of any ionising radiation?
      - Yes   - No
   b) Will you be taking new human tissue samples (or other human biological samples)?
      - Yes   - No
   c) Will you be using existing human tissue samples (or other human biological samples)?
      - Yes   - No

3. In which countries of the UK will the research sites be located? (Tick all that apply)
   - [ ] England

Date: 29/02/2012
3a. In which country of the UK will the lead NHS R&D office be located:

- England
- Scotland
- Wales
- Northern Ireland
- This study does not involve the NHS

4. Which review bodies are you applying to?

- NHS/HSC Research and Development office
- Social Care Research Ethics Committee
- Research Ethics Committee
- National Information Governance Board for Health and Social Care (NIGB)
- Ministry of Justice (MoJ)
- National Offender Management Service (NOMS) (Prisons & Probation)

For NHS/HSC R&D offices, the CI must create Site-Specific Information forms for each site, in addition to the study-wide forms, and transfer them to the PIs or local collaborators.

5. Will any research sites in this study be NHS organisations?

- Yes
- No

5a. Are all the research costs and infrastructure costs for this study provided by an NIHR Biomedical Research Centre, NIHR Biomedical Research Unit, NIHR Collaboration for Leadership in Health Research and Care (CLAHRC) or NIHR Research Centre for Patient Safety & Service Quality in all study sites?

- Yes
- No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP).

6. Do you wish to make an application for the study to be considered for NIHR Clinical Research Network (CRN) support and inclusion in the NIHR Clinical Research Network (CRN) Portfolio? Please see information button for further details.

- Yes
- No

If yes, NHS permission for your study will be processed through the NIHR Coordinated System for gaining NHS Permission (NIHR CSP) and you must complete a NIHR Clinical Research Network (CRN) Portfolio Application Form immediately after completing this project filter and before completing and submitting other applications.

7. Do you plan at any stage of the project to undertake intrusive research involving adults lacking capacity to consent for themselves?

- Yes
- No

Answer Yes if you plan to recruit living participants aged 16 or over who lack capacity, or to retain them in the study following loss of capacity. Intrusive research means any research with the living requiring consent in law. This includes use of
8. Do you plan to include any participants who are prisoners or young offenders in the custody of HM Prison Service or who are offenders supervised by the probation service in England or Wales?
   ☐ Yes  ☐ No

9. Is the study or any part of it being undertaken as an educational project?
   ☐ Yes  ☐ No

10. Will this research be financially supported by the United States Department of Health and Human Services or any of its divisions, agencies or programs?
    ☐ Yes  ☐ No

11. Will identifiable patient data be assessed outside the care team without prior consent at any stage of the project (including identification of potential participants)?
    ☐ Yes  ☐ No
Application to NHS/HSC Research Ethics Committee

The Chief Investigator should complete this form. Guidance on the questions is available wherever you see this symbol displayed. We recommend reading the guidance first. The complete guidance and a glossary are available by selecting Help.

Please define any terms or acronyms that might not be familiar to lay reviewers of the application.

Short title and version number: (maximum 70 characters - this will be inserted as header on all forms)
Evaluation of cytology and HPV testing for anal pre-cancer

Please complete these details after you have booked the REC application for review.

REC Name:
NRES Committee North West

REC Reference Number: 12/NW/0204
Submission date: 20/02/2012

PART A: Core study information

1. ADMINISTRATIVE DETAILS

A1. Full title of the research:
Evaluation of cytology and HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK

A3.1. Chief Investigator:

Title Forename/Initials Surname
Prof HC Kitchener

Post
Professor of Gynaecological Ccinology

Qualifications
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henry.kitchener@manchester.ac.uk

Date: 20/02/2012
A1. Who is the contact on behalf of the sponsor for all correspondence relating to applications for this project? This contact will receive copies of all correspondence from REC and R&D reviewers that is sent to the CI.

Title: Forename/Initials Surname
Dr Lynne Webster
Address: Head of Research Office, Research & Innovation Division
1st Floor, Postgraduate Medical Centre
St Mary’s Hospital, Oxford Road, Manchester
Post Code: M13 9WL
E-mail: lynne.webster@cmft.nhs.uk
Telephone: 01612763565
Fax: 01612765760

A3.1. Research reference numbers. Please give any relevant references for your study:

Applicant/organisation’s own reference number, e.g. R&D (if available):

Sponsor’s/protocol’s own reference number:
Protocol Version: Version 1
Protocol Date: 10/02/2012

Funder’s reference number:
Project website:
Registry reference number(s):
The Department of Health’s Research Governance Framework for Health and Social Care and the research governance frameworks for Wales, Scotland and Northern Ireland set out the requirement for registration of trials. Furthermore, Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that “every clinical trial must be registered on a publicly accessible database before recruitment of the first subject”, and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.

International Standard Randomised Controlled Trial Number (ISRCTN):
ClinicalTrials.gov identifier (NCT number):

Additional reference number(s):

Ref Number Description Reference Number

A3-2. Is this application linked to a previous study or another current application?

☐ Yes ☐ No

Please give brief details and reference numbers.

2. OVERVIEW OF THE RESEARCH

Date: 29/02/2012 5 96714/286444/1/447
A5.1 Summary of the study. Please provide a brief summary of the research (maximum 300 words) using language easily understood by lay reviewers and members of the public. Where the research is reviewed by a REC within the UK Health Departments’ Research Ethics Service, this summary will be published on the website of the National Research Ethics Service following the ethical review.

Anal cancer, like cervical cancer, involves infection with the human papilloma virus (HPV). Anal cancer is generally uncommon, but there are some people at increased risk. These include people whose immune system is impaired, for example because they have HIV infection, or have had a transplant and are taking drugs which suppress immunity; and men who have sex with men. It may be worthwhile offering such ‘high-risk’ people testing for anal cancer; this would involve a liquid-based cytology test for pre-cancerous abnormal cell changes similar to that now used for NHS cervical screening, and possibly a test for high risk HPV. Liquid-based cytology, because it filters out imurities, offers the ability to obtain a clear sample of cells from the anus. We do not yet have all the information needed to decide whether testing for anal cancer with the available technologies would be useful. This study is designed to make an initial evaluation of the utility of these tests; to test how easy it would be to recruit the at-risk population; to find out what patients feel about screening; and to obtain up to date information for the UK on the numbers of people likely to have an abnormal test result at each stage of the testing process. This initial study involves 1000 people and 4 high risk groups will be tested: HIV positive men; HIV positive men and women who have anal intercourse; kidney transplant patients; and men who have sex with men. Patients will be offered anal cytology and HPV tests, with further tests, including biopsy sampling of anal tissue for diagnosis, for those with abnormal cytology and/or positive HPV tests and, for comparison, for some with normal cytology and negative HPV tests. Those found to have abnormal cells would be offered treatment.

A5.2 Summary of main issues. Please summarise the main ethical, legal, or management issues arising from your study and say how you have addressed them.

Not all studies raise significant issues. Some studies may have straightforward ethical or other issues that can be identified and managed routinely. Others may present significant issues requiring further consideration by a REC, R&D office or other review body (as appropriate to the issue). Studies that present a minimal risk to participants may raise complex organisational or legal issues. You should try to consider all the types of issues that the different reviewers may need to consider.

There are no difficult ethical issues, because there is no potential disadvantage to participants.

3. PURPOSE AND DESIGN OF THE RESEARCH

A7. Select the appropriate methodology description for this research. Please tick all that apply:

- Case series/case note review
- Case control
- Cohort observation
- Controlled trial without randomisation
- Cross-sectional study
- Database analysis
- Epidemiology
- Feasibility/pilot study
- Laboratory study
- Meta-analysis
- Qualitative research
- Questionnaire, interview or observation study
- Randomised controlled trial
- Other (please specify)

Date: 29/02/2012
A10. What is the principal research question/objective? Please put this in language comprehensible to a lay person.

Whether the use of liquid-based cytology and/or HPV testing to test patients at high risk for anal neoplasia appears feasible in terms of recruitment, acceptability and test performance including sensitivity and specificity.

A11. What are the secondary research questions/objectives if applicable? Please put this in language comprehensible to a lay person.

Do the new technologies recently brought into use in the NHS Cervical Screening Programme facilitate screening for anal neoplasia in high risk groups? What is the prevalence of abnormal cytology, HPV positivity by type, and biopsy-confirmed AIN in the target groups? Identification of main resource implications for the NHS.

A12. What is the scientific justification for the research? Please put this in language comprehensible to a lay person.

Anal cancer and its precursors, anal intraepithelial neoplasia (AIN), are uncommon in the general population but much more common in certain high-risk population subgroups such as HIV-positive men and women, HIV negative men who have sex with men (MSM) and those who have undergone organ transplantation. In these groups, the risk of developing anal cancer may be increased by 10 to 160-fold. The development of anal intraepithelial neoplasia (AIN) appears to involve the same stages of development of those seen in cervical cancer, and to bear a similar relation to infection with the human papillomavirus (HPV). Hence, the suggestion that it may be possible to screen for anal neoplasia using methods similar to those for cervical neoplasia. The incidence of anal cancer in the UK has risen by 2-fold since the advent of HPV and this trend is likely to continue, since antiretroviral therapy now in widespread use increases survival, often by many years, but does not reduce HIV-related anal cancer risk. Similarly, increasingly long survival after organ transplantation means that incidence of anal cancer in these patients is likely to be an increasing problem. Numbers in the high-risk groups in the UK are small in population terms but within these groups the individual's risk of developing anal cancer may be appreciable and the available treatments are often invasive and unsatisfactory.

The UK National Screening Committee has considered existing evidence for anal cancer screening in November 2006. The committee identified as research priorities studies of the feasibility, acceptability and cost-effectiveness of screening and of the natural history of AIN 2a in both HIV-positive and high-risk HIV-negative subjects, as well as randomised trials of treatments for high-grade AIN. A recent HTA study of cost-effectiveness of screening in men who have sex with men found that estimation of cost-effectiveness depends heavily on the prevalence of AIN in the screening population. There is however no adequate estimates of the current prevalence of AIN in relatively unselected high-risk populations in the UK. Most existing natural history studies are in highly selected groups, and/or do not provide sufficient information on all study participants.

The recent conversion from Pap smear to liquid-based cytology within the UK cervical screening programme makes NHS screening for anal cancer a realistic possibility. The proposed study would provide reliable and independent information, currently unavailable, on the feasibility of testing on UK prevalence of AIN in a range of high-risk groups, and on performance of cytology and HPV testing in terms of specificity and sensitivity to detect AIN.

A13. Please summarise your design and methodology. It should be clear exactly what will happen to the research participant, how many times and in what order. Please complete this section in language comprehensible to the lay person. Do not simply reproduce or refer to the protocol. Further guidance is available in the guidance notes.

Study population
Men and women over age 25 in four groups at high-risk of anal neoplasia: HIV positive men, HIV positive men and women who have anal intercourse attending NHS genito-urinary medicine (GUM) clinics; men who have sex with men (MSM), recruited through GUM clinics and specialist general practices; immunosuppressed transplant recipients (men and women) attending transplant follow-up clinics. Patients with prior history of anal neoplasia will be excluded.

Planned interventions
All participants will be offered testing for evidence of abnormality including anal cytology (liquid based cytology) and for anal HPV testing involving a single anal swab sample for both tests as well as anoscopy in order to inspect the anus. The samples testing HPV positive will also be tested for the HPV type. At the recruitment interview, participants will be asked to complete a brief questionnaire giving basic sociodemographic (age, sex, risk group) and medical (symptoms, past history) information and their perception of risk of anal neoplasia, attitude to screening and, if possible, reasons for deciding not to enter the study for those who choose not to take part.

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A14.1. In which aspects of the research process have you actively involved, or will you involve, patients, service users, and/or their carers, or members of the public?

☐ Design of the research
☐ Management of the research
☐ Undertaking the research
☐ Analysis of results
☐ Dissemination of findings
☐ None of the above

Give details of involvement, or if none please justify the absence of involvement.
We will consult the lesbian and gay liaison group who are active and well organised in Manchester. They have already indicated their approval of this project.
A18. Give details of all non-clinical intervention(s) or procedure(s) that will be received by participants as part of the research protocol. These include seeking consent, interviews, non-clinical observations and use of questionnaires.

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days)
4. Details of who will conduct the intervention/procedure, and where it will take place.

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<thead>
<tr>
<th>Intervention or procedure</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
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<td>Research Nurse, CMFT clinic</td>
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<tr>
<td>Questionnaire</td>
<td>1</td>
<td>20</td>
<td></td>
<td>Research Nurse, CMFT clinic</td>
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</tbody>
</table>

A19. Give details of any clinical intervention(s) or procedure(s) to be received by participants as part of the research protocol. These include uses of medicinal products or devices, other medical treatments or assessments, mental health interventions, imaging investigations and taking samples of human biological material. Include procedures which might be received as routine clinical care outside of the research.

Please complete the columns for each intervention/procedure as follows:

1. Total number of interventions/procedures to be received by each participant as part of the research protocol.
2. If this intervention/procedure would be routinely given to participants as part of their care outside the research, how many of the total would be routine?
3. Average time taken per intervention/procedure (minutes, hours or days).
4. Details of who will conduct the intervention/procedure, and where it will take place.

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<td>3 mins</td>
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<tr>
<td>Anoscopy (HRA)</td>
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<td>10 mins</td>
<td>Clinical Research Fellow, CMFT clinic</td>
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<tr>
<td>Anal biopsy</td>
<td>1</td>
<td>5 mins</td>
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</table>

A20. Will you withhold an intervention or procedure, which would normally be considered a part of routine care?

- Yes
- No

A21. How long do you expect each participant to be in the study in total?

6 months

A22. What are the potential risks and burdens for research participants and how will you minimise them?

For all studies, describe any potential adverse effects, pain, discomfort, distress, intrusion, inconvenience or changes to lifestyle. Only describe risks or burdens that could occur as a result of participation in the research. Say what steps would be taken to minimise risks and burdens as far as possible.

The anal swab will cause only a scratch sensation for a few seconds. The anoscopy causes minimal discomfort. If a biopsy is warranted because of abnormal anoscopy, local anaesthetic will be provided. This causes a few seconds of discomfort. There may be a little bleeding at the time of the biopsy but this very rarely would require a stitch, as the biopsy is only 2mm diameter of depth.

Date: 29/02/2012
A23. Will interviews/questionnaires or group discussions include topics that might be sensitive, embarrassing or upsetting, or is it possible that criminal or other disclosures requiring action could occur during the study?

☐ Yes  ☐ No

A24. What is the potential for benefit to research participants?

They may be identified as having asymptomatic anal pre-cancer which if found would prevent cancer developing as a result of treatment or surveillance.

A25. What arrangements are being made for continued provision of the intervention for participants, if appropriate, once the research has finished? May apply to any clinical intervention, including a drug, medical device, mental health intervention, complementary therapy, physiotherapy, dietary manipulation, lifestyle change, etc.

Any patient with AN would be treated or followed up.

A26. What are the potential risks for the researchers themselves? (If any)

None.

RECRUITMENT AND INFORMED CONSENT

In this section we ask you to describe the recruitment procedures for the study. Please give separate details for different study groups where appropriate.

A27.1. How will potential participants, records or samples be identified? Who will carry this out and what resources will be used? For example, identification may involve a disease register, computerised search of GP records, or review of medical records. Indicate whether this will be done by the direct healthcare team or by researchers acting under arrangements with the responsible care organisation(s).

Men and women over age 25 will be recruited from the following established services:
HIV positive men, HIV positive men and women who have anal intercourse attending NHS genito-urinary medicine (GUM) clinics
Men who have sex with men (MSM), recruited through GUM clinics and specialist general practices
Immunosuppressed transplant recipients (men and women) attending transplant follow-up clinics.

These patients will be identified by the clinics. They will be recruited by a dedicated research doctor and nurse.

A27.2. Will the identification of potential participants involve reviewing or screening the identifiable personal information of patients, service users or any other person?

☐ Yes  ☐ No

Please give details below:

A28. Will any participants be recruited by publicity through posters, leaflets, adverts or websites?

☐ Yes  ☐ No

A29. How and by whom will potential participants first be approached?

They will be approached at clinics they are attending by the research staff.

A30.1. Will you obtain informed consent from or on behalf of research participants?

☐ Yes  ☐ No
If you will be obtaining consent from adult participants, please give details of who will take consent and how it will be done, with details of any steps to provide information (a written information sheet, videos, or interactive material). Arrangements for adults unable to consent for themselves should be described separately in Part B Section 6, and for children in Part B Section 7.

If you plan to seek informed consent from vulnerable groups, say how you will ensure that consent is voluntary and fully informed.

Consent will be obtained by the research nurse or research doctor both of whom will be trained with respect to the condition and procedures.

If you are not obtaining consent, please explain why not.

Please endorse a copy of the information sheet(s) and consent form(s).

A30. Will you record informed consent (or advice from consultees) in writing?

☐ Yes  ☐ No

A31. How long will you allow potential participants to decide whether or not to take part?

As long as they wish, but at least 24 hours.

A32. Will you recruit any participants who are involved in current research or have recently been involved in any research prior to recruitment?

☐ Yes  ☐ No  ☐ Not Known

A33. What arrangements have been made for persons who might not adequately understand verbal explanations or written information given in English, or who have special communication needs? (e.g. translation, use of interpreters)

An interpreter/link worker will be available for those patients who do not speak English. This is standard practice. If there are any doubts about the patient's understanding they will not be included in the study.

A34. What arrangements will you make to ensure participants receive any information that becomes available during the course of the research that may be relevant to their continued participation?

We will contact the patient directly should any information relevant to their participation become available during the course of the study.

A35. What steps would you take if a participant, who has given informed consent, loses capacity to consent during the study? Tick one option only.

☐ The participant and all identifiable data or tissue collected would be withdrawn from the study. Data or tissue which is not identifiable to the research team may be retained.

☐ The participant would be withdrawn from the study. Identifiable data or tissue already collected with consent would be retained and used in the study. No further data or tissue would be collected or any other research procedures carried out on or in relation to the participant.

☐ The participant would continue to be included in the study.

☐ Not applicable – informed consent will not be sought from any participants in this research.

☐ Not applicable – it is not practicable for the research team to monitor capacity and continued capacity will be assumed.

Further details:

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If you plan to retain and make further use of identifiable data/issue following loss of capacity, you should inform participants about this when seeking their consent initially.

CONFIDENTIALITY

In this section, personal data means any data relating to a participant who could potentially be identified. It includes pseudonymised data capable of being linked to a participant through a unique code number.

Storage and use of personal data during the study

A36. Will you be undertaking any of the following activities at any stage (including in the identification of potential participants)? (Tick as appropriate)

- Access to medical records by those outside the direct healthcare team
- Electronic transfer by magnetic or optical media, email or computer networks
- Sharing of personal data with other organisations
- Export of personal data outside the EEA
- Use of personal addresses, postcodes, faxes, emails or telephone numbers
- Publication of direct quotations from respondents
- Publication of data that might allow identification of individuals
- Use of audio/visual recording devices
- Storage of personal data on any of the following:
  - Manual files including X-rays
  - NHS computers
  - Home or other personal computers
  - University computers
  - Private company computers
  - Laptop computers

Further details:

A38. How will you ensure the confidentiality of personal data? Please provide a general statement of the policy and procedures for ensuring confidentiality, e.g. anonymisation or pseudonymisation of data.

Research data will be stored anonymised by trial number and patient initials. The file containing patient identifiers will be stored separately in a password protected file.

A40. Who will have access to participants’ personal data during the study? Where access is by individuals outside the direct care team, please justify and say whether consent will be sought.

The clinical research fellow, research nurse and data manager.

Storage and use of data after the end of the study

A43. How long will personal data be stored or accessed after the study has ended?

- Less than 3 months
- 3 – 6 months
- 6 – 12 months

Date: 20/02/2012
A6. Will research participants receive any payments, reimbursement of expenses or any other benefits or incentives for taking part in this research?  
☐ Yes ☐ No

A17. Will individual researchers receive any personal payment over and above normal salary, or any other benefits or incentives, for taking part in this research?  
☐ Yes ☐ No

A18. Does the Chief Investigator or any other investigator/collaborator have any direct personal involvement (e.g. financial, share holding, personal relationship etc.) in the organisations sponsoring or funding the research that may give rise to a possible conflict of interest?  
☐ Yes ☐ No

A19-1. Will you inform the participants’ General Practitioners (and/or any other health or care professional responsible for their care) that they are taking part in the study?  
☐ Yes ☐ No

If Yes, please enclose a copy of the information sheet/letter for the GP/health professional with a version number and date.

A19-2. Will you seek permission from the research participants to inform their GP or other health/ care professional?  
☐ Yes ☐ No

It should be made clear in the participant’s information sheet if the GP/health professional will be informed.

A20. Will the research be registered on a public database?  
The Department of Health’s Research Governance Framework for Health and Social Care and the research governance frameworks for Wales, Scotland and Northern Ireland set out the requirement for registration of trials. Furthermore, Article 19 of the World Medical Association Declaration of Helsinki adopted in 2008 states that “every clinical trial must be registered on a publicly accessible database before recruitment of the first subject”, and the International Committee of Medical Journal Editors (ICMJE) will consider a clinical trial for publication only if it has been registered in an appropriate registry. Please see guidance for more information.  
☐ Yes ☐ No

Please give details, or justify if not registering the research.  
http://www.controlled-trials.com/

Please ensure that you have entered registry reference number(s) in question A2-1.

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A31. How do you intend to report and disseminate the results of the study? Tick as appropriate:

- Peer reviewed scientific journals
- Internal report
- Conference presentation
- Publication on website
- Other publication
- Submission to regulatory authorities
- Access to raw data and right to publish freely by all investigators in study or by Independent Steering Committee on behalf of all investigators
- No plans to report or disseminate the results
- Other (please specify)

We will report to the National Screening Committee.

A32. Will you inform participants of the results?

- Yes
- No

Please give details of how you will inform participants or justify if not doing so. A summary of the findings will be produced and posted to participants who have previously indicated that they would like to receive this information.

5. Scientific and Statistical Review

A34. How has the scientific quality of the research been assessed? Tick as appropriate:

- Independent external review
- Review within a company
- Review within a multi-centre research group
- Review within the Chief Investigator’s institution or host organisation
- Review within the research team
- Review by educational supervisor

Justify and describe the review process and outcome. If the review has been undertaken but not seen by the researcher, give details of the body which has undertaken the review.

This study was reviewed by Joel Patefky and John Schofield who are leading experts in this area.

For all studies except non-doctoral student research, please enclose a copy of any available scientific critique reports, together with any related correspondence.

For non-doctoral student research, please enclose a copy of the assessment from your educational supervisor/institution.

A36. How have the statistical aspects of the research been reviewed? Tick as appropriate:

- Review by independent statistician commissioned by funder or sponsor
- Other review by independent statistician
- Review by company statistician
- Review by a statistician within the Chief Investigator’s institution
- Review by a statistician within the research team or multi-centre group
- Review by educational supervisor

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12/NI/0204  
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☐ Other review by individual with relevant statistical expertise

☐ No review necessary as only frequencies and associations will be assessed – details of statistical input not required

In all cases please give details below of the individual responsible for reviewing the statistical aspects. If advice has been provided in confidence, give details of the department and institution convened.

Title Forename/Initials Surname  
Dr Jane Green

Department  
Nuffield Department of Clinical Medicine

Institution  
University of Oxford

Work Address  
Richard Doll Building, Cancer Epidemiology Unit  
University of Oxford  
Oxford

Post Code  
OX3 7LF

Telephone  
01865 289859

Fax

Mobile  
jane.green@ceu.ox.ac.uk

E-mail

Please enclose a copy of any available comments or reports from a statistician.

A57. What is the primary outcome measure for the study?

Feasibility of liquid based cytology and/or HPV testing to test patients at high risk for anal neoplasia in terms of recruitment, acceptability and test performance.

A58. What are the secondary outcome measures? (if any)

Effectiveness of treatment in terms of recurrence/sexual disease after 6 months.  
Prevalence of abnormal cytology, HPV positivity by type, and biopsy-confirmed AIN in the target groups.

A59. What is the sample size for the research? How many participants/samples/data records do you plan to study in total? If there is more than one group, please give further details below.

Total UK sample size:  
1000

Total international sample size (including UK):  

Total in European Economic Area:

Further details:

1000 participants in total:

250 HIV positive men

250 HIV positive men and women who have anal intercourse

250 men who have sex with men (MSM)

250 immunosuppressed transplant recipients (men and women)

A60. How was the sample size decided upon? If a formal sample size calculation was used, indicate how this was done, giving sufficient information to justify and reproduce the calculation.

Sample size is based on best estimates from the literature of likely prevalences of cytological, anoscopic and histological abnormality, to allow estimation of population prevalence with 95% confidence and <=8% margin of error in all participants and in each group of 250; and adequate comparison of differences between the three groups with 80% power and 95% confidence. For example, the study is powered to estimate population prevalence of 0.2 (20%) with 95% confidence interval(CI) of 0.15-0.25; of 0.1 (10%) with 95% CI 0.07-0.14; and of 0.01 (1%) with 95% CI of 0.004-

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A61. Will participants be allocated to groups at random?

☐ Yes  ☐ No

A62. Please describe the methods of analysis (statistical or other appropriate methods, e.g. for qualitative research) by which the data will be evaluated to meet the study objectives.

Outcome prevalences with 95% confidence limits will be estimated using standard statistical methods, using the normal approximation to the binomial distribution, and Chi-squared tests used to obtain p-values for comparison between prevalences in the population subgroups.

6. MANAGEMENT OF THE RESEARCH

A63. Other key investigators/collaborators. Please include all grant co-applicants, protocol co-authors and other key members of the Chief Investigator’s team, including non-doctoral student researchers.

<table>
<thead>
<tr>
<th>Title Forename/Initials Surname</th>
<th>Post</th>
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<tbody>
<tr>
<td>Prof Julietta Patrick</td>
<td>Director, NHS Cancer Screening Programmes</td>
</tr>
<tr>
<td>Qualifications</td>
<td>CBE, BA(Hons), FFPH</td>
</tr>
<tr>
<td>Employer</td>
<td>NHS Cancer Screening Programmes</td>
</tr>
<tr>
<td>Work Address</td>
<td>Fulwood House</td>
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<tr>
<td></td>
<td>Old Fulwood Road</td>
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<td></td>
<td>Sheffield</td>
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<td>Post Code</td>
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<td>Telephone</td>
<td>01142711050</td>
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<td>Fax</td>
<td>01142711059</td>
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<tr>
<td>Mobile</td>
<td><a href="mailto:Julietta.Patrick@cancerscreening.nhs.uk">Julietta.Patrick@cancerscreening.nhs.uk</a></td>
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<tr>
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<td>Consultant Colorectal Surgeon</td>
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<tr>
<td>Qualifications</td>
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<tr>
<td>Employer</td>
<td>Central Manchester University Hospitals NHS Foundation Trust</td>
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<tr>
<td>Work Address</td>
<td>Department of General Surgery</td>
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<td></td>
<td>Manchester Royal Infirmary</td>
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<td>Oxford Road, Manchester</td>
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<tr>
<td>Mobile</td>
<td><a href="mailto:James.Hill@cmft.nhs.uk">James.Hill@cmft.nhs.uk</a></td>
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<tr>
<th>Title Forename/Initials Surname</th>
<th>Dr Mina Desai</th>
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<tr>
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<td>Manchester Royal Infirmary</td>
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<tr>
<td>Work Email</td>
<td><a href="mailto:mina.desai@cmft.nhs.uk">mina.desai@cmft.nhs.uk</a></td>
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<th>Consultant in Gynaecology Medicine</th>
</tr>
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<td>FRCGP, DGO, DFFP, MD (Mumbai), DNB (India), DVI (Mumbai), FCPS (Mumbai)</td>
</tr>
<tr>
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<td>Central Manchester University Hospitals NHS Foundation Trust</td>
</tr>
<tr>
<td>Work Address</td>
<td>Manchester Centre for Sexual Health</td>
</tr>
<tr>
<td></td>
<td>280 Upper Brook Street</td>
</tr>
<tr>
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<tr>
<td>Post Code</td>
<td>M13 GFH</td>
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<tr>
<td>Telephone</td>
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<tr>
<td>Work Address</td>
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<tr>
<td></td>
<td>Manchester Royal Infirmary</td>
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<tr>
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<td></td>
<td>Manchester Royal Infirmary</td>
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<td>M13 SWL</td>
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</table>

Date: 29/02/2012
A64. Details of research sponsor(s)

A44.1. Sponsor

Date: 29/02/2012  18  06714/296644/1/447
Lead Sponsor

Status:  
- NHS or HSC care organisation
- Academic
- Pharmaceutical industry
- Medical device industry
- Local Authority
- Other social care provider (including voluntary sector or private organisation)
- Other

If Other, please specify:

Commercial status:

Contact person

Name of organisation: Central Manchester University Hospitals NHS Foundation Trust

Given name: Lynne

Family name: Webster

Address: R&D, 1st Floor, Postgrad Centre

Town/city: Manchester Royal Infirmary

Post code: M13 9WL

Country: UNITED KINGDOM

Telephone: 01612765685

Fax: 01612765766

E-mail: lynne.webster@cmft.nhs.uk

Is the sponsor based outside the UK?
- Yes  ☐ No

Under the Research Governance Framework for Health and Social Care, a sponsor outside the UK must appoint a legal representative established in the UK. Please consult the guidance notes.

ASS. Has external funding for the research been secured?

☑ Funding secured from one or more funders
☐ External funding application to one or more funders in progress
☐ No application for external funding will be made

What type of research project is this?
- Standalone project
- Project that is part of a programme grant
- Project that is part of a Centre grant
- Project that is part of a fellowship/personal award/research training award
- Other

Other – please state:

Please give details of funding applications.

Date: 29/02/2012
A67. Has this or a similar application been previously rejected by a Research Ethics Committee in the UK or another country?

☐ Yes  ☐ No

Please provide a copy of the unfavourable opinion letter(s). You should explain in your answer to question A5-2 how the reasons for the unfavourable opinion have been addressed in this application.

A68. Give details of the lead NHS R&D contact for this research:

Title Forename/Initials Surname
Dr Lynne Webster

Organisation
Central Manchester University hospitals NHS Foundation Trust

Address
R&D, 1st Floor, Postgrad Centre
Manchester Royal Infirmary
Oxford Road, Manchester

Post Code
M13 9WL

Work Email
lynn.webster@cmft.nhs.uk

Telephone
01612763565

Fax
01612765768

Details can be obtained from the NHS R&D Forum website: http://www.rforum.nhs.uk

A69-1. How long do you expect the study to last in the UK?

Planned start date: 05/05/2012
Planned end date: 05/05/2014

Date: 26/02/2012
Total duration:
Years: 2 Months: 0 Days: 0

A70. Definition of the end of trial, and justification in the case where it is not the last visit of the last subject undergoing the trial (1)
The last follow-up visit of the last subject.

A71. Where will the research take place? (Tick as appropriate)

☑ England  
☐ Scotland  
☐ Wales  
☐ Northern Ireland  
☐ Other countries in European Economic Area

Total UK sites in study 1

Does this trial involve countries outside the EU?
☑ Yes  ☐ No

A72. What host organisations (NHS or other) in the UK will be responsible for the research sites? Please indicate the type of organisation by ticking the box and give approximate numbers of planned research sites:

☑ NHS organisations in England  1
☐ NHS organisations in Wales  
☐ NHS organisations in Scotland  
☐ HSC organisations in Northern Ireland  
☐ GP practices in England  
☐ GP practices in Wales  
☐ GP practices in Scotland  
☐ GP practices in Northern Ireland  
☐ Social care organisations  
☐ Phase 1 trial units  
☐ Prison establishments  
☐ Probation areas  
☐ Independent hospitals  
☐ Educational establishments  
☐ Independent research units  
☐ Other (give details)

Total UK sites in study:  1

A75.1. What arrangements will be made to review interim safety and efficacy data from the trial? Will a formal data monitoring committee or equivalent body be convened?

No, because these screening methods, diagnostic tests and treatments have all been widely used, if not in anal disease, then in cervical disease.

If a formal DMC is to be convened, please forward details of the membership and standard operating procedures to the Research Ethics Committee when available. The REC should also be notified of DMC recommendations and receive summary reports of interim analyses.

Date: 26/02/2012  21  06714/20164/1/447
A75. What are the criteria for electively stopping the trial or other research prematurely?
If accrual proved wholly inadequate for example, if accrual were less than 20%.

A76. Insurance/ indemnity to meet potential legal liabilities

**Note:** in this question to NHS indemnity schemes include equivalent schemes provided by Health and Social Care (HSC) in Northern Ireland

A76.1. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) for harm to participants arising from the management of the research? Please tick box(es) as applicable.

**Note:** Where a NHS organisation has agreed to act as sponsor or co-sponsor, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For all other sponsors, please describe the arrangements and provide evidence.

- [ ] NHS indemnity scheme will apply (NHS sponsors only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

Please endorse a copy of relevant documents.

A76.2. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of the sponsor(s) or employer(s) for harm to participants arising from the design of the research? Please tick box(es) as applicable.

**Note:** Where researchers with substantive NHS employment contracts have designed the research, indemnity is provided through NHS schemes. Indicate if this applies (there is no need to provide documentary evidence). For other protocol authors (e.g. company employees, university members), please describe the arrangements and provide evidence.

- [ ] NHS indemnity scheme will apply (protocol authors with NHS contracts only)
- [ ] Other insurance or indemnity arrangements will apply (give details below)

The University of Manchester will arrange insurance for research involving human subjects that provides cover for legal liabilities arising from its actions or those of its staff or supervised students, subject to policy terms and conditions.

Please endorse a copy of relevant documents.

A76.3. What arrangements will be made for insurance and/or indemnity to meet the potential legal liability of investigators/collaborators arising from harm to participants in the conduct of the research?

**Note:** Where the participants are NHS patients, indemnity is provided through the NHS schemes or through professional indemnity. Indicate if this applies to the whole study (there is no need to provide documentary evidence). Where non-NHS sites are to be included in the research, including private practices, please describe the arrangements which will be made at these sites and provide evidence.

- [ ] NHS indemnity scheme or professional indemnity will apply (participants recruited at NHS sites only)
- [ ] Research includes non-NHS sites (give details of insurance/ indemnity arrangements for these sites below)

Please endorse a copy of relevant documents.

A77. Has the sponsor(s) made arrangements for payment of compensation in the event of harm to the research participants where no legal liability arises?

Date: 29/02/2012
Part B: Section 5 – Use of newly obtained human tissue (or other human biological materials) for research purposes

1. What types of human tissue or other biological material will be included in the study?
   - Anal swab
   - Anal biopsy

2. Who will collect the samples?
   - A dedicated research nurse or research fellow

3. Who will the samples be removed from?
   - Living donors
   - The deceased

4. Will informed consent be obtained from living donors for use of the samples? Please tick as appropriate
   - In this research?
     - Yes
     - No
   - In future research?
     - Yes
     - No
     - Not applicable

6. Will any tissues or cells be used for human application or to carry out testing for human application in this research?
   - Yes
   - No

8. Will the samples be stored? [Tick as appropriate]
   - In fully anonymised form? (link to donor broken)
     - Yes
     - No
   - In linked anonymised form? (linked to stored tissue but donor not identifiable to researchers)
     - Yes
     - No
   - If yes, say who will have access to the code and personal information about the donor.
     - Code access and personal information will be stored by data manager.
   - In a form in which the donor could be identifiable to researchers?
     - Yes
     - No

9. What types of test or analysis will be carried out on the samples?
   - Anal swabs: HPV testing (positive or negative and type)
10. Will the research involve the analysis or use of human DNA in the samples?
   ☐ Yes ☑ No

11. Is it possible that the research could produce findings of clinical significance for donors or their relatives?
   ☐ Yes ☑ No

12. If so, will arrangements be made to notify the individuals concerned?
   ☐ Yes ☑ No ☐ Not applicable

13. Give details of where the samples will be stored, who will have access and the custodial arrangements.
    Samples will be stored in the Virology Department, access will be by Dr Sargent.

14. What will happen to the samples at the end of the research? Please tick all that apply and give further details.

   ☑ Transfer to research tissue bank
   (If the bank is in England, Wales or Northern Ireland the institution will require a licence from the Human Tissue Authority to store relevant material for possible further research.)

   ☐ Storage by research team pending ethical approval for use in another project
   (Unless the researcher's institution holds a storage licence from the Human Tissue Authority, or the tissue is stored in Scotland, or it is not relevant material, a further application for ethical review should be submitted before the end of this project.)

   ☐ Storage by research team as part of a new research tissue bank
   (The institution will require a licence from the Human Tissue Authority if the bank will be storing relevant material in England, Wales or Northern Ireland. A separate application for ethical review of the tissue bank may also be submitted.)

   ☐ Storage by research team of biological material which is not "relevant material" for the purposes of the Human Tissue Act
   ☐ Disposal in accordance with the Human Tissue Authority's Code of Practice
   ☐ Other
   ☐ Not yet known

Please give further details of the proposed arrangements:

Date: 26/02/2012
PART C: Overview of research sites

Please enter details of the host organisations (Local Authority, NHS or other) in the UK that will be responsible for the research sites. For NHS sites, the host organisation is the Trust or Health Board. Where the research site is a primary care site, e.g. GP practice, please insert the host organisation (PCT or Health Board) in the Institution row and insert the research site (e.g. GP practice) in the Department row.

<table>
<thead>
<tr>
<th>Research site</th>
<th>Investigator/ Collaborator/ Contact</th>
</tr>
</thead>
<tbody>
<tr>
<td>Institution name</td>
<td>Central Manchester NHS Trust</td>
</tr>
<tr>
<td>Department name</td>
<td>Manchester Royal Infirmary</td>
</tr>
<tr>
<td>Street address</td>
<td>Oxford Road</td>
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<td>Town/city</td>
<td>Manchester</td>
</tr>
<tr>
<td>Post Code</td>
<td>M13 8WL</td>
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<td></td>
<td>Henry</td>
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<td></td>
<td>Kitchenser</td>
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</table>
PART D: Declarations

D1. Declaration by Chief Investigator

1. The information in this form is accurate to the best of my knowledge and belief and I take full responsibility for it.

2. I undertake to abide by the ethical principles underlying the Declaration of Helsinki and good practice guidelines on the proper conduct of research.

3. If the research is approved I undertake to adhere to the study protocol, the terms of the full application as approved and any conditions set out by review bodies in giving approval.

4. I undertake to notify review bodies of substantial amendments to the protocol or the terms of the approved application, and to seek a favourable opinion from the main REC before implementing the amendments.

5. I undertake to submit annual progress reports setting out the progress of the research, as required by review bodies.

6. I am aware of my responsibility to be up to date and comply with the requirements of the law and relevant guidelines relating to security and confidentiality of patient or other personal data, including the need to register when necessary with the appropriate Data Protection Officer. I understand that I am not permitted to disclose identifiable data to third parties unless the disclosure has the consent of the data subject or, in the case of patient data in England and Wales, the disclosure is covered by the terms of an approval under Section 251 of the NHS Act 2000.

7. I understand that research records/data may be subject to inspection by review bodies for audit purposes if required.

8. I understand that any personal data in this application will be held by review bodies and their operational managers and that this will be managed according to the principles established in the Data Protection Act 1998.

9. I understand that the information contained in this application, any supporting documentation and all correspondence with review bodies or their operational managers relating to the application:

   - Will be held by the REC (where applicable) until at least 3 years after the end of the study, and by NHS R&D offices (where the research requires NHS management permission) in accordance with the NHS Code of Practice on Records Management.
   - May be disclosed to the operational managers of review bodies, or the appointing authority for the REC (where applicable), in order to check that the application has been processed correctly or to investigate any complaint.
   - May be seen by auditors appointed to undertake accreditation of RECs (where applicable).
   - Will be subject to the provisions of the Freedom of Information Acts and may be disclosed in response to requests made under the Acts except where statutory exemptions apply.

10. I understand that information relating to this research, including the contact details on this application, may be held on national research information systems, and that this will be managed according to the principles established in the Data Protection Act 1998.

11. I understand that the main REC or its operational managers may share information in this application or supporting documentation with the Medicines and Healthcare products Regulatory Agency (MHRA) where it is relevant to the Agency’s statutory responsibilities.

12. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named below. Publication will take place no earlier than 3 months after issue of the ethics committee’s final opinion or the withdrawal of the application.

Contact point for publication (Not applicable for R&D Forms)

NRES would like to include a contact point with the published summary of the study for those wishing to seek further information. We would be grateful if you would indicate one of the contact points below.

Date: 29/02/2012

Reference: 12/MW/204

IRAS Version 3.4
NHS REC Form

Reference:
12/MW/02/04

IRAS Version 3.4

[ ] Chief Investigator
[ ] Sponsor
[ ] Study co-ordinator
[ ] Student
[ ] Other – please give details
[ ] None

Access to application for training purposes (Not applicable for R&D Forms)
Optional – please tick as appropriate:

[ ] I would be content for members of other RECs to have access to the information in the application in confidence for training purposes. All personal identifiers and references to sponsors, funders and research units would be removed.

This section was signed electronically by Professor Henry Kitchener on 23/02/2012 13:48.

Job Title/Post: Professor of Gynaecological Oncology
Organisation: University of Manchester
Email: henry.kitchener@manchester.ac.uk
Signature: ........................................

Print Name: ........................................
Date: (dd/mm/yyyy) ........................................

Date: 29/02/2012

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96714/286644/1/447
D2. Declaration by the sponsor's representative

If there is more than one sponsor, this declaration should be signed on behalf of the co-sponsors by a representative of the lead sponsor named at A64-1.

I confirm that:

1. This research proposal has been discussed with the Chief Investigator and agreement in principle to sponsor the research is in place.

2. An appropriate process of scientific critique has demonstrated that this research proposal is worthwhile and of high scientific quality.

3. Any necessary indemnity or insurance arrangements, as described in question A70, will be in place before this research starts. Insurance or indemnity policies will be renewed for the duration of the study where necessary.

4. Arrangements will be in place before the study starts for the research team to access resources and support to deliver the research as proposed.

5. Arrangements to allocate responsibilities for the management, monitoring and reporting of the research will be in place before the research starts.

6. The duties of sponsors set out in the Research Governance Framework for Health and Social Care will be undertaken in relation to this research.

7. Where the research is reviewed by a REC within the UK Health Departments Research Ethics Service, I understand that the summary of this study will be published on the website of the National Research Ethics Service (NRES), together with the contact point for enquiries named in this application. Publication will take place no earlier than 3 months after issue of the ethics committee's final opinion or the withdrawal of the application.

This section was signed electronically by Dr Lynne Webster on 23/02/2012 10:30.

Job Title/Post: Head of the Research Office

Organisation: CMFT

Email: lynne.webster@cmft.nhs.uk

Date: 29/02/2012
8.3 Appendix iiia

Appendix va

Patient Information Sheet
Gentourinary clinic patients

A study of ANAL cytology and HPV testing in the prevention of anal cancer
(ANALOGY)

Chief Investigator: Professor Henry Kitchener

We would like to invite you to take part in our research study. Before you decide we
would like you to understand why the research is being done and what it would
involve for you. One of our team will go through the information sheet with you and
answer any questions you have.

Ask us if there is anything that is not clear.

What is the purpose of the study?
It is known that anal cancer can be caused by a persistent infection by the human
papillomavirus. This is the same virus that causes cancer of the cervix. It is known that
individuals who have anal sex are at increased risk of anal HPV infection and therefore
anal cancer. HIV positive individuals are also at increased risk because their immune
system is suppressed making them more susceptible to HPV infection in the anus. It is
now possible to screen individuals for HPV infection and abnormal cells in the anus,
simply by swabbing with a soft brush which loosens cells which can be used for
analysis. We can also inspect the anus painlessly using a magnifying instrument which
allows any abnormal areas to be seen.

The aim of the study is to evaluate whether these tests would be useful to recommend
screening of high risk groups. We will also be carrying out interviews to get a better
understanding of the views and opinions of participants of anal screening. We will ask
your permission to contact you to see if you would be interested in taking part in these
interviews.

Why have I been invited?
You have been asked to take part in this study as you have been identified as being at
high-risk of developing anal intraepithelial neoplasia which is a pre-cancer of the anus
which left untreated could develop into anal cancer.

Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through
this information sheet. If you agree to take part, we will then ask you to sign a consent
form. You are free to withdraw at any time, without giving a reason. This will not affect
the standard of care you receive.

Trial ID number: ANALOGY Patient Information Sheet (CUM)
Version 2, 10th July 2014
What will happen to me if I take part?
Your participation in the study will be explained to you by a member of the research team. You would be asked to complete a simple short questionnaire about your awareness of this health issue, attitude towards screening and any symptoms you have experienced. The doctor will then perform high resolution anoscopy. This procedure involves an external inspection of the anus followed by a digital inspection which will involve insertion of a finger into the anal canal using lubrication jelly. The doctor will then insert a plastic anoscope into the anal canal using lubrication jelly to allow visualisation of the internal tissue. A swab will be taken and then vinegar will be applied to the internal tissue to highlight any abnormalities using a microscope. This should take no longer than 5 minutes and is painless.

If an abnormality were identified you would be asked to agree to a biopsy. This would require the application of a local anaesthetic spray. In some cases this is also accompanied by an injection of local anaesthetic which stings very unpleasantly while it is being injected (5-10 seconds) and then the biopsy itself is painless. There may be some bleeding which usually settles with a dressing though occasionally a stitch is required.

If the biopsy confirmed changes that if left could lead to cancer in years to come you would be offered treatment which would be provided by an experienced consultant surgeon. The abnormality that would require treatment would be anal intraepithelial neoplasia (AIN). You will be asked to attend for a follow-up appointment 3 months later for a repeat high resolution anoscopy.

What are the alternatives for diagnosis or treatment?
Standard diagnosis and treatment for AIN will be used. If an abnormality is found this will be discussed with you and treatment options will be fully explained to you.

What are the possible disadvantages and risks of taking part?
The only disadvantage would be a biopsy that turned out to be negative. Otherwise the diagnosis of AIN could be seen as beneficial as a potential protective measure.

What are the side effects of any treatment received when taking part?
The treatment of AIN has some complications such as post excision infection.

What are the possible benefits of taking part?
Taking part in the research could lead to asymptomatic AIN being diagnosed and this then preventing anal cancer from developing.

What happens when the research study stops?
When the research stops, screening of these groups will stop. Any AIN will be treated and followed up as would routinely occur in the NHS.

What will happen if I don’t want to carry on with the study?
You are free to withdraw from this study at any time without your medical care being affected. If you do withdraw from the study, we would like to keep all of your samples.

Trial ID number: ANALOGY Patient Information Sheet (CUM)
Version 2, 10th July 2014
end to use the data collected up to the point of your withdrawal. However, if you wish, any stored samples already collected will be destroyed.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers directly who will do their best to answer your questions or contact the Chief Investigator, Professor Henry Kitchener on 0161 276 6461. If you remain unhappy and wish to complain formally, you can do this by contacting the Trust Patient and Advice Liaison Service (PALS) on 0161 276 8699 who will deal with your complaint or give you further advice.

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against Central Manchester University Hospitals Foundation NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate). In the event of harm where there has been no negligence, there are no special arrangements for compensation.

Will my taking part in this study be kept confidential?
If you do take part in the study, the data collected for the study may be looked at by authorised persons from Central Manchester University Hospitals NHS Trust who are sponsoring the study to ensure the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. All research data collected will be stored anonymously and securely and any paper records will be locked away. The researchers who have access to the data have been fully trained in handling confidential data.

Involvement of the General Practitioner/Family doctor (GP)
We will obtain your consent to contact your GP to notify him/her of your involvement in the study and send results of your tests to ensure your medical records are kept up to date. However, if you do not want us to contact your GP, or if you are not registered with a GP, this will not be a problem.

What will happen to any samples I give?
We will ask for your consent to store your tissue samples for future research. These samples will be stored anonymously and ethical approval will be obtained before the samples are used.

What will happen to the results of the research study?
The results of this study will be published in a scientific journal. You will not be identified in any publication reporting the results of this study. If requested, you will be sent a summary of the results of this study.

Who is organising and funding the research?
This study is funded by the Cancer Screening Programme. It is sponsored by Central Manchester University Hospitals NHS Trust who has reviewed the study and is happy.
for it to take place using its patients. The doctors and nurses involved in the study will not be receiving any payments for including you in this study.

Who has reviewed the study?
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Greater Manchester North Research Ethics Committee.

Further information and contact details
For further information please contact:
Chief Investigator Professor Henry Kitchener on 0161 276 6461 or email henry.kitchener@manchester.ac.uk

Research Nurse Julie Burnett on 0161 701 9119 / 07768 995 970 or email julieann.burnett@manchester.ac.uk

Research Fellow Dr Alice Schofield on 07768 995 970 or email alice.schofield@manchester.ac.uk

Thank you for taking the time to read this information and considering whether or not you would like to take part in this study.

Trial ID number: ANALOGY Patient Information Sheet (CUM)
Version 2, 10th July 2014
8.4 Appendix iiib

The Pennine Acute Hospitals NHS

Patient Information Sheet
Genitourinary clinic patients

A study of ANAL cytology and HPV testing in the prevention of anal cancer (ANALOGY)

Chief Investigator: Professor Henry Kitchener

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. One of our team will go through the information sheet with you and answer any questions you have.

Ask us if there is anything that is not clear.

What is the purpose of the study?
It is known that anal cancer can be caused by a persistent infection by the human papillomavirus. This is the same virus that causes cancer of the cervix. It is known that individuals who have anal sex are at increased risk of anal HPV infection and therefore anal cancer. HIV positive individuals are also at increased risk because their immune system is suppressed making them more susceptible to HPV infection in the anus. It is now possible to screen individuals for HPV infection and abnormal cells in the anus, simply by swabbing with a soft brush which loosens cells which can be used for analysis. We can also inspect the anus painlessly using a magnifying instrument which allows any abnormal areas to be seen.

The aim of the study is to evaluate whether these tests would be useful to recommend screening of high risk groups. We will also be carrying out interviews to get a better understanding of the views and opinions of participants of anal screening. We will ask your permission to contact you to see if you would be interested in taking part in these interviews.

Why have I been invited?
You have been asked to take part in this study as you have been identified as being at high risk of developing anal intraepithelial neoplasia which is a pre-cancer of the anus which left untreated could develop into anal cancer.

Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This will not affect the standard of care you receive.

Trial ID number: R01921

ANALOGY Patient Information Sheet (GUM)
Version 1.2, 20th November 2012

236
What will happen to me if I take part?
Your participation in the study will be explained to you by a member of the research team. You would be asked to complete a simple short questionnaire about your awareness of this health issue, attitude towards screening and any symptoms you have experienced. You would then undergo an anal swab which just takes a few seconds followed by a careful inspection of the anus by a trained doctor. The inspection involves only the external part of the anus and does not involve any inspection internally at the level of the sphincter (the muscle that squeezes the anus closed). The inspection would take 2-3 minutes and is painless.

If an abnormality were identified you would be asked to agree to a biopsy. This would require the injection of local anaesthetic which stings very unpleasantly while it is being injected (5-10 seconds) and then the biopsy itself is painless. There may be some bleeding which usually settles with a dressing though occasionally a stitch is required.

If the biopsy confirmed changes that if left could lead to cancer in years to come you would be offered treatment which would be provided by an experienced consultant surgeon. The abnormality that would require treatment would be anal intraepithelial neoplasia (AIN). You will be asked to attend for a follow-up appointment 6 months later for a repeat anal swab.

What are the alternatives for diagnosis or treatment?
Standard diagnosis and treatment for AIN will be used. If an abnormality is found this will be discussed with you and treatment options will be fully explained to you.

What are the possible disadvantages and risks of taking part?
The only disadvantage would be a biopsy that turned out to be negative. Otherwise the diagnosis of AIN could be seen as beneficial as a potential protective measure.

What are the side effects of any treatment received when taking part?
The treatment of AIN has some complications such as post excision infection.

What are the possible benefits of taking part?
Taking part in the research could lead to asymptomatic AIN being diagnosed and this then preventing anal cancer from developing.

What happens when the research study stops?
When the research stops, screening of these groups will stop. Any AIN will be treated and followed up as would routinely occur in the NHS.

What will happen if I don’t want to carry on with the study?
You are free to withdraw from this study at any time without your medical care being affected. If you do withdraw from the study, we would like to keep all of your treatment records as required by law.

Trial ID number: R01921
ANALOGY Patient Information Sheet (GUM)
Version 1.2, 20th November 2012
samples and to use the data collected up to the point of your withdrawal. However, if you wish, any stored samples already collected will be destroyed.

What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers directly who will do their best to answer your questions or contact the Chief Investigator, Professor Henry Kitchener on 0161 276 6461. If you remain unhappy and wish to complain formally, you can do this by contacting the Trust Research Office on 0161 276 3565 who will deal with your complain or give you further advice.

In the event that something does go wrong and you are harmed during the research and this is due to someone’s negligence then you may have grounds for a legal action for compensation against Central Manchester University Hospitals Foundation NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate). In the event of harm where there has been no negligence, there are no special arrangements for compensation.

Will my taking part in this study be kept confidential?
If you do take part in the study, the data collected for the study may be looked at by authorised persons from Central Manchester University Hospitals NHS Trust who are sponsoring the study to ensure the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. All research data collected will be stored anonymously and securely and any paper records will be locked away. The researchers who have access to the data have been fully trained in handling confidential data.

Involvement of the General Practitioner/Family doctor (GP)
We will obtain your consent to contact your GP to notify him/her of your involvement in the study and send results of your tests to ensure your medical records are kept up to date. However, if you do not want us to contact your GP, or if you are not registered with a GP, this will not be a problem.

What will happen to any samples I give?
We will ask you for your consent to store your tissue samples for future research. These samples will be stored anonymously and ethical approval will be obtained before the samples are used.

What will happen to the results of the research study?
The results of this study will be published in a scientific journal. You will not be identified in any publication reporting the results of this study. If requested, you will be sent a summary of the results of this study.

Who is organising and funding the research?
This study is funded by the Cancer Screening Programme. It is sponsored by Central Manchester University Hospitals NHS Trust who has reviewed the study and is happy

Trial ID number: R01921 ANALOGY Patient Information Sheet (GUM) Version 1.2, 20th November 2012
for it to take place using its patients. The doctors and nurses involved in the study will not be receiving any payments for including you in this study.

Who has reviewed the study?
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Greater Manchester North Research Ethics Committee.

Further information and contact details
For further information please contact:
Chief Investigator Professor Henry Kitchener on 0161 276 6461 or email henry.kitchener@manchester.ac.uk

Research Nurse Julie Burnett on 0161 701 9119 or email julie.burnett2@cmft.nhs.uk
Or Mobile 07768995070.

Thank you for taking the time to read this information and considering whether or not you would like to take part in this study.

Trial ID number: R01921
ANALOGY Patient Information Sheet (GUM)
Version 1.2, 20th November 2012
8.5 Appendix iiic

Patient Information Sheet
Transplant recipient patients

A study of ANAL cytology and HPV testing in the prevention of anal cancer
(ANALOGY)

Chief Investigator: Professor Henry Kitchener

We would like to invite you to take part in our research study. Before you decide we
would like you to understand why the research is being done and what it would
involve for you. One of our team will go through the information sheet with you and
answer any questions you have.

Ask us if there is anything that is not clear.

What is the purpose of the study?
It is known that anal cancer can be caused by a persistent infection by the human
papillomavirus. This is the same virus that causes cancer of the cervix. It is known
that individuals who have had a renal transplant are at increased risk of anal HPV
infection and therefore anal cancer. It is now possible to screen individuals for HPV
infection and abnormal cells in the anus, simply by swabbing with a soft brush which
loosens cells which can be used for analysis. We can also inspect the anus paniestes
using a magnifying instrument which allows any abnormal areas to be seen.

The aim of the study is to evaluate whether these tests would be useful to
recommend screening of high-risk groups. We will also be carrying out interviews to
get a better understanding of the views and opinions of participants of anal
screening. We will ask your permission to contact you to see if you would be
interested in taking part in these interviews.

Why have I been invited?
You have been asked to take part in this study as you have been identified as being
at high-risk of developing anal intraepithelial neoplasia which is a pre-cancer of the
anus which left untreated could develop into anal cancer.

Do I have to take part?
It is up to you to decide to join the study. We will describe the study and go through
this information sheet. If you agree to take part, we will then ask you to sign a
consent form. You are free to withdraw at any time, without giving a reason. This will
not affect the standard of care you receive.

Trial ID number: RO1921

ANALOGY Patient Information Sheet
(Transplant patients)
Version 1.2, 20th November 2012
What will happen to me if I take part?
Your participation in the study will be explained to you by a member of the research team. You would be asked to complete a simple short questionnaire about your awareness of this health issue, attitude towards screening and any symptoms you have experienced. You would then undergo an anal swab which just takes a few seconds followed by a careful inspection of the anus by a trained doctor. The inspection involves only the external part of the anus and does not involve any inspection internally at the level of the sphincter (the muscle that squeezes the anus closed). The inspection would take 2-3 minutes and is painless.

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If the biopsy confirmed changes that if left could lead to cancer in years to come you would be offered treatment which would be provided by an experienced consultant surgeon. The abnormality that would require treatment would be anal intraepithelial neoplasia (AIN). You will be asked to attend for a follow-up appointment 6 months later for a repeat anal swab.

What are the alternatives for diagnosis or treatment?
Standard diagnosis and treatment for AIN will be used. If an abnormality is found this will be discussed with you and treatment options will be fully explained to you.

What are the possible disadvantages and risks of taking part?
The only disadvantage would be a biopsy that turned out to be negative. Otherwise the diagnosis of AIN could be seen as beneficial as a potential protective measure.

What are the side effects of any treatment received when taking part?
The treatment of AIN has some complications such as post excision infection.

What are the possible benefits of taking part?
Taking part in the research could lead to asymptomatic AIN being diagnosed and this then preventing anal cancer from developing.

What happens when the research study stops?
When the research stops, screening of these groups will stop. Any AIN will be treated and followed up as would routinely occur in the NHS.

What will happen if I don’t want to carry on with the study?
You are free to withdraw from this study at any time without your medical care being affected. If you do withdraw from the study, we would like to keep all of your samples and to use the data collected up to the point of your withdrawal. However, if you wish, any stored samples already collected will be destroyed.

Trial ID number: RO1921

ANALOGY Patient Information Sheet
(Transplant patients)
Version 1.2, 20th November 2012
What if there is a problem?
If you have a concern about any aspect of this study, you should ask to speak to the researchers directly who will do their best to answer your questions or contact the Chief Investigator, Professor Henry Kitchener on 0161 276 6461. If you remain unhappy and wish to complain formally, you can do this by contacting the Trust Research Office on 0161 276 3565 who will deal with your complaint or give you further advice.

In the event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against Central Manchester University Hospitals Foundation NHS Trust but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you (if appropriate). In the event of harm where there has been no negligence, there are no special arrangements for compensation.

Will my taking part in this study be kept confidential?
If you do take part in the study, the data collected for the study may be looked at by authorised persons from Central Manchester University Hospitals NHS Trust who are sponsoring the study to ensure the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will do our best to meet this duty. All research data collected will be stored anonymously and securely and any paper records will be locked away. The researchers who have access to the data have been fully trained in handling confidential data.

Involvement of the General Practitioner/Family doctor (GP)
We will obtain your consent to contact your GP to notify him/her of your involvement in the study and send results of your tests to ensure your medical records are kept up to date. However, if you do not want us to contact your GP, or you are not registered with a GP, it is not a problem.

What will happen to any samples I give?
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What will happen to the results of the research study?
The results of this study will be published in a scientific journal. You will not be identified in any publication reporting the results of this study. If requested, you will be sent a summary of the results of this study.

Who is organising and funding the research?
This study is funded by the Cancer Screening Programme. It is sponsored by Central Manchester University Hospitals NHS Trust who has reviewed the study and is happy for it to take place using its patients. The doctors and nurses involved in the study will not be receiving any payments for including you in this study.

Trial ID number: RO1921

ANALOGY Patient Information Sheet
(Transplant patients)
Version 1.2, 20th November 2012
Who has reviewed the study?
All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. This study has been reviewed and given favourable opinion by Greater Manchester North Research Ethics Committee.

Further information and contact details
For further information please contact:
Chief Investigator Professor Henry Kitchener on 0161 276 6461 or email henry.kitchener@manchester.ac.uk
Research Nurse Julie Burnett on 0161 701 9119 or email Julie.burnett2@cmft.nhs.uk

Thank you for taking the time to read this information and considering whether or not you would like to take part in this study.
8.6 Appendix iva

Expression of interest to participate

ANALOGY study – Evaluation of cytology, HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK

Chief Investigator: Professor Henry Kitchener

Participant sticker:

I would like to be contacted by the research team

Yes [ ] No [ ]

If yes, I would like to be contacted by (tick those that apply)

[ ] Letter

[ ] Telephone Landline: _______________________ Mobile: _______________________

[ ] Text message Mobile: _______________________

[ ] Email Address: ______________________________

Signature: ______________________________ Date: ______________________________

Expression of interest form
V1, 20th November 2012
8.7 Appendix ivb

Expression of interest to participate:

ANALOGY study – Evaluation of cytology, HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK

Chief Investigator: Professor Henry Kitchener

Participant sticker:

I would like to be contacted by the research team: [ ] Yes [ ] No

If yes, I would like to be contacted by (tick those that apply)

[ ] Letter

[ ] Telephone
  Landline: ________________________ Mobile: ________________________

[ ] Text message
  Mobile: ________________________

[ ] Email
  Address: ________________________

Signature: ________________________ Date: ________________________

Expression of interest form
V1, 20th November 2012
Appendix va

CASE REPORT FORM (MCSH group)

A study of ANAL cytology and HPV testing in the prevention of anal cancer (ANALOGY)

Principal Investigator: Professor Henry Kitchener
St Mary's Hospital
Manchester, M13 9WL

Participant Study Number

Participant Initials

Demographic Data:

Date of Birth

Sex M F

Ethnic Group

Please select from the groups below

<table>
<thead>
<tr>
<th>White</th>
<th>A) White British</th>
<th>B) White Irish</th>
<th>C) White other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asian or Asian British</td>
<td>G) Indian</td>
<td>H) Pakistani</td>
<td>I) Bangladeshi</td>
</tr>
<tr>
<td>Black or Black British</td>
<td>J) Caribbean</td>
<td>K) African</td>
<td>L) Other Black</td>
</tr>
<tr>
<td>Other Ethnic Groups</td>
<td>M) Chinese</td>
<td>N) Any other ethnic group</td>
<td>P) Do not wish to answer</td>
</tr>
</tbody>
</table>

Employment Status: Currently the participant is:

Employed Unemployed Student

Full Time Parent Retired

Occupation (if applicable)

Level of Education:

GCE O-level /GCSE A-level or Equivalent

Undergraduate Postgraduate

Other (please Specify)

Postcode (first 4 characters) ___ ___ ___ ___
# STUDY CHECKLIST

<table>
<thead>
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<th>No</th>
<th>Date</th>
<th>Initials</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient information sheet given*</td>
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<tr>
<td>Written consent obtained*</td>
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<td>Questionnaire completed*</td>
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<td>First clinical assessment*</td>
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<tr>
<td>Cytology*</td>
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<tr>
<td>HPV testing*</td>
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<tr>
<td>Anoscopy*</td>
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</tr>
<tr>
<td>Biopsy taken</td>
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<tr>
<td>Second clinical assessment*</td>
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<tr>
<td>Cytology*</td>
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<tr>
<td>HPV testing*</td>
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<tr>
<td>Anoscopy*</td>
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<tr>
<td>Biopsy taken</td>
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<tr>
<td>Patient's GP informed at recruitment*</td>
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<tr>
<td>Patient's GP informed at 6</td>
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*(If answered ‘No’ to any of the questions marked with *, please specify why in the protocol deviation section on page 9)*

**Inclusion Criteria (tick):**
- Age 25+
- Written informed consent given
- Is the participant more than 2 years post transplant

**Exclusion Criteria (tick):**
- Current or Previous h/o AIN / anal cancer
- Participant is pregnant

---

2 Name: [Signature] Date:
Personal History *(please circle)*

Smoking
- Current
- Previous
- Never
- CPD
- Stopped smoking
- 

Alcohol
- Current
- Previous
- Never
- Units per week
- Started drinking
- 

Recreational drugs
- No
- Yes
- Current use
- 

Sexual History *(please circle)*

Heterosexual
- Homosexual
- Bisexual

Age first SI

In current relationship from

h/o receptive AI
- No
- Yes

Age first episode of receptive AI

Do you use protection
- Always
- Sometimes
- Never

h/o STI

h/o PIN (male) CIN, VIN (female)

Medical History *(use next page for transplant history)*

Surgical History

Drug History *(use next page for anti-retroviral medication)*

Is the participant on anti-coagulants
- Yes
- No

Name

Signature

Date
**Drug Allergy** (mention severity – mild / moderate / severe / life-threatening)

**HIV status**

<table>
<thead>
<tr>
<th>Positive</th>
<th>Negative</th>
<th>Unsure</th>
</tr>
</thead>
</table>

*If HIV positive: date of diagnosis

*Date of seroconversion (if +ve seroconversion)

Month: Year:

*Current viral load: Date:

*Current CD4 count: Date:

*Lowest CD4 count:

**Anti-retroviral therapy**

<table>
<thead>
<tr>
<th>Drug Therapy</th>
<th>Yes / No</th>
<th>Date Commenced</th>
<th>On-going? Yes/No</th>
<th>If not on-going, treatment duration</th>
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<tbody>
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**Other viral serology**

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<th>Positive</th>
<th>Negative</th>
<th>Don’t know</th>
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<tr>
<td>Hep B &amp; Ag</td>
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<tr>
<td>Hep C</td>
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<tr>
<td>EBV</td>
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<td>CMV</td>
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<tr>
<td>HIV</td>
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<td></td>
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<tr>
<td>Other</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

4 Name Signature Date:
FIRST CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe)

- Soreness
- Itching
- Discharge
- Bleeding
- Fistula
- Haemorrhoids
- Warts

Anal / Perianal / Genital

Other

Anoscopy Findings

Quadrants of Disease Anal Canal /4
Anal Biopsy YES / NO
Bx Site 1 Bx Site 2 Bx Site 3 Bx Site 4

Quadrants of Disease Perianal /4
Perianal Bx YES/NO
Bx Site A Bx Site B Bx Site C Bx Site D

5 Name Signature Date:
Results of cytology, biopsy

<table>
<thead>
<tr>
<th>Anal LBC Result</th>
<th>AIN I</th>
<th>AIN II</th>
<th>AIN III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEGATIVE</td>
<td>INSUFFICIENT</td>
<td></td>
</tr>
<tr>
<td>Anal Bx Results</td>
<td>Ex Site 1</td>
<td>Ex Site 2</td>
<td>Ex Site 3</td>
</tr>
<tr>
<td>Perianal Bx Results</td>
<td>Ex Site A</td>
<td>Ex Site B</td>
<td>Ex Site C</td>
</tr>
<tr>
<td>HPV Results</td>
<td>Negative</td>
<td>Positive</td>
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<td></td>
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<td>18</td>
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</table>

Has the participant been referred for further treatment? (Please specify)

No  Yes
SECOND CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe):

Soreness
Itching
Discharge
Bleeding
Fistula
Haemorrhoids
Warts Anal / Perianal / Genital
Other

Anoscopy Findings

Quadrants of Disease Anal Canal

Anal Biopsy

Bx Site 1   Bx Site 2   Bx Site 3   Bx Site 4

Quadrants of Disease Perianal

Perianal Bx

Bx Site A   Bx Site B   Bx Site C   Bx Site D

Name
Signature
Date:
Results of cytology, biopsy

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<thead>
<tr>
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<th>Bx Site B</th>
<th>Bx Site C</th>
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<tr>
<th>HPV Results</th>
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<th>Bx Site D</th>
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<tr>
<td>Positive</td>
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</table>

Has the participant been referred for further treatment? (Please specify)

No  Yes

In the last six months, have there been any changes to the medical / surgical / personal / sexual / drug / other history? (Please specify)

________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________

Please explain any deviation from protocol

________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________________

Name  Signature  Date:
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</table>

**Communication Sheet**

**Name**

**Signature**

**Date**
### CASE REPORT FORM (NMGH group)

**A study of ANAL cytology and HPV testing in the prevention of anal cancer (ANALOGY)**

**Principal Investigator:**
Professor Henry Kitchener  
St Mary’s Hospital  
Manchester, M13 9WL

**Participant Study Number**  
[ ] [ ] [ ]

**Participant Initials**  
[ ] [ ] [ ]

#### Demographic Data:

**Date of Birth**  
[ ] [ ] [ ] [ ] [ ] [ ]

**Sex**
- [ ] M  
- [ ] F

**Ethnic Group**  

<table>
<thead>
<tr>
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<th>Mixed</th>
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<tbody>
<tr>
<td>A) White British</td>
<td>D) White &amp; Black Caribbean</td>
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<tr>
<td>B) White Irish</td>
<td>E) White &amp; Black African</td>
</tr>
<tr>
<td>C) White other</td>
<td>F) Other Mixed</td>
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<table>
<thead>
<tr>
<th>Asian or Asian British</th>
<th>Black or Black British</th>
<th>Other Ethnic Groups</th>
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<tr>
<td>H) Indian</td>
<td>M) Caribbean</td>
<td>R) Chinese</td>
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<tr>
<td>J) Pakistani</td>
<td>N) African</td>
<td>S) Any other ethnic group</td>
</tr>
<tr>
<td>K) Bangladeshi</td>
<td>P) Other Black</td>
<td>T) Do not wish to answer</td>
</tr>
</tbody>
</table>

**Employment Status: Currently the participant is:**

- [ ] Employed  
- [ ] Unemployed  
- [ ] Student  
- [ ] Full Time Parent  
- [ ] Retired

**Occupation (if applicable)**  
---------------------------------------

**Level of Education:**

- [ ] GCE O-level /GCSE  
- [ ] A-level or Equivalent  
- [ ] Undergraduate  
- [ ] Postgraduate  
- [ ] Other (please specify)  
---------------------------------------

**Postcode (first 4 characters)**  
[ ] [ ] [ ] [ ]
## Study Checklist

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(If answered 'No' to any of the questions marked with *, please specify why in the protocol deviation section on page 9)

**Inclusion Criteria** (tick)
- Age 25+
- Written informed consent given
- Is the participant more than 2 years post transplant

**Exclusion Criteria** (tick)
- Current or Previous H/o AIN / anal cancer
- Participant is pregnant

---

2 Name
Signature
Date:
ANALOGY

Participant No: 3

DOB 

Personal History (please circle)

Smoking Current Previous Never
Started smoking CFD 
Stopped smoking

Alcohol Current Previous Never
Started drinking Units per week

Recreational drugs No Yes Current use

Sexual History (please circle)

Heterosexual Homosexual Bisexual

Age first SI

In current relationship from

h/o receptive AI No Yes

Age first episode of receptive AI

Do you use protection Always Sometimes Never

h/o STI

h/o PIN (male) CIN, VIN (female)

Medical History (use next page for transplant history)

Surgical History

Drug History (use next page for anti-retroviral medication)

Is the participant on anti-coagulants? Yes No

Name Signature Date: 3
**Drug Allergy** (mention severity – mild / moderate / severe / life-threatening)

**HIV status**  Positive  Negative  Unsure

*If HIV positive: date of diagnosis*

Date of seroconversion (if +ve seroconversion)
Month:............Year:.............

Current viral load:............Date:..................

Current CD4 count:............Date:..................

Lowest CD4 count:..................

### Anti-retroviral therapy

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### Other viral serology

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<tr>
<td>Other</td>
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</table>

4 Name  Signature  Date:
FIRST CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe)

Soreness

Itching

Discharge

Bleeding

Fistula

Haemorrhoids

Warts Anal / Perianal / Genital

Other

Anoscopy Findings

Quadrants of Disease Anal Canal

Bx Site 1  Bx Site 2  Bx Site 3  Bx Site 4

Anal Biopsy

Yes / No

Quadrants of disease Perianal

Bx Site A  Bx Site B  Bx Site C  Bx Site D

Yes / No

5 Name

Signature

Date:
Results of cytology, biopsy

<table>
<thead>
<tr>
<th>Anal LEC Result</th>
<th>AIN I</th>
<th>AIN II</th>
<th>AIN III</th>
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Has the participant been referred for further treatment? (Please specify)

No  Yes

6  Name  Signature  Date:
SECOND CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe)

Soreness
Itching
Discharge
Bleeding
Fistula
Haemorrhoids
Warts Anal / Perianal / Genital
Other

Anoscopy Findings

Quadrants of Disease Anal Canal

Anal Biopsy
Bx Site 1  Bx Site 2  Bx Site 3  Bx Site 4

Quadrants of disease Perianal

Perianal Bx
Bx Site A  Bx Site B  Bx Site C  Bx Site D

Name  Signature  Date:
## Results of cytology, biopsy

<table>
<thead>
<tr>
<th>Anal LBC Result</th>
<th>AIN I</th>
<th>AIN II</th>
<th>AIN III</th>
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</thead>
<tbody>
<tr>
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<table>
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<th>Bx Site 2</th>
<th>Bx Site 3</th>
<th>Bx Site 4</th>
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<table>
<thead>
<tr>
<th>Perianal Bx Results</th>
<th>Bx Site A</th>
<th>Bx Site B</th>
<th>Bx Site C</th>
<th>Bx Site D</th>
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<table>
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<th>HPV Results</th>
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<th>Positive</th>
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<tbody>
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</table>

Has the participant been referred for further treatment? (Please specify)

No  Yes

---

In the last six months, have there been any changes to the medical / surgical / personal / sexual / drug / other history? (Please specify)

---

Please explain any deviation from protocol

---

8 Name  Signature  Date:
### Communication Sheet

<table>
<thead>
<tr>
<th>Date</th>
<th>Signature</th>
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9. **Name**  
   **Signature**  
   **Date:**
CASE REPORT FORM (Transplant group)

A study of ANAL cytology and HPV testing in the prevention of anal cancer (ANALOGY)

Principal Investigator: Professor Henry Kitchener
St Mary's Hospital
Manchester, M13 9WL

Participant Study Number
Participant Initials

Demographic Data:
Date of Birth
Sex M F

Ethnic Group

Please select from the groups below

<table>
<thead>
<tr>
<th>White</th>
<th>A) White British</th>
<th>B) White Irish</th>
<th>C) White other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asian or Asian British</td>
<td>H) Indian</td>
<td>J) Pakistani</td>
<td>K) Bangladeshi</td>
</tr>
<tr>
<td>Black or Black British</td>
<td>M) Caribbean</td>
<td>N) African</td>
<td>P) Other Black</td>
</tr>
<tr>
<td>Other Ethnic Groups</td>
<td>R) Chinese</td>
<td>S) Any other ethnic group</td>
<td>Z) Do not wish to answer</td>
</tr>
</tbody>
</table>

Employment Status: Currently the participant is:
Employed ☐ Unemployed ☐ Student ☐
Full Time Parent ☐ Retired ☐

Occupation (if applicable)

Level of Education:
GCE O-level /GCSE ☐ A-level or Equivalent ☐
Undergraduate ☐ Postgraduate ☐
Other (please specify)

Postcode (last 4 characters) ___ ___ ___ ___
## STUDY CHECKLIST

<table>
<thead>
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<tbody>
<tr>
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<td>Written consent obtained*</td>
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<tr>
<td>Cytology*</td>
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<td>Patient’s GP informed at 6</td>
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</table>

(If answered ‘No’ to any of the questions marked with *, please specify why in the protocol deviation section on page 9)

### Inclusion Criteria (tick)

- Age 25+
- Written informed consent given
- Is the participant more than 2 years post transplant

### Exclusion Criteria (tick)

- Current or Previous h/o AIN / anal cancer
- Participant is pregnant

### Personal History (please circle)

- Name
- Signature
- Date:
Sexual History (please circle)
Heterosexual  Homosexual  Bisexual

Age first SI..................................................
In current relationship from......................................
h/o receptive AI  No  Yes
Age first episode of receptive AI.................................

Do you use protection? Always☐ Sometimes☐ Never☐
h/o STI................................................................
h/o PIN (male) CIN, VIN (female).................................

Medical History (use next page for transplant history)

Surgical History

Drug History (use next page for transplant medication)
Is the participant on anti-coagulants? Yes  No

Drug Allergy (mention severity – mild / moderate / severe / life-threatening)

3  Name  Signature  Date:
### Transplant status

**Organ(s) transplanted**

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<th>Number of transplants</th>
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<th>2</th>
<th>3</th>
<th>4</th>
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**Dates of transplant(s)**

<table>
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<tr>
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**Dialysis**

### Immunosuppression history

#### Anti Rejection Drugs

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<tr>
<th>Drug Therapy</th>
<th>Yes / No</th>
<th>Date Commenced</th>
<th>On-going? Yes/No</th>
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#### Other viral serology

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<th>Positive</th>
<th>Negative</th>
<th>Don't know</th>
<th>Donor</th>
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<th>Negative</th>
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</table>

4 Name __________________________ Signature __________________________ Date: ________
FIRST CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe)

Soreness........................................................................................................

Itching..............................................................................................................

Discharge.........................................................................................................

Bleeding...........................................................................................................

Fistula..............................................................................................................

Haemorrhoids.................................................................................................

Warts  Anal / Perianal / Genital........................................................................

Other..............................................................................................................

Anoscopy Findings

Quadrants of Disease Anal Canal

Anal Biopsy YES / NO

Bx Site 1  Bx Site 2  Bx Site 3  Bx Site 4

Quadrants of disease Perianal

Perianal Bx YES/NO

Bx Site A  Bx Site B  Bx Site C  Bx Site D

Name  Signature  Date:
Results of cytology, biopsy

<table>
<thead>
<tr>
<th>Anal LBC Result</th>
<th>AIN I</th>
<th>AIN II</th>
<th>AIN III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEGATIVE</td>
<td>INSUFFICIENT</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Anal Bx Results</th>
<th>Bx Site 1</th>
<th>Bx Site 2</th>
<th>Bx Site 3</th>
<th>Bx Site 4</th>
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<tbody>
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<thead>
<tr>
<th>Perianal Bx Results</th>
<th>Bx Site A</th>
<th>Bx Site B</th>
<th>Bx Site C</th>
<th>Bx Site D</th>
</tr>
</thead>
<tbody>
<tr>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HPV Results</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

Has the participant been referred for further treatment? (Please specify)

No  Yes
SECOND CLINICAL ASSESSMENT

Anal Symptoms (None / Mild / Moderate / Severe)
- Soreness
- Itching
- Discharge
- Bleeding
- Fistula
- Haemorrhoids
- Warts Anal / Perianal / Genital
- Other

**Anoscopy Findings**

Quadrants of Disease Anal Canal

- Quadrants of disease Perianal

Anal Biopsy

Bx Site 1   Bx Site 2   Bx Site 3   Bx Site 4

Perianal Bx

Bx Site A   Bx Site B   Bx Site C   Bx Site D

Name
Signature
Date:
Results of cytology, biopsy

<table>
<thead>
<tr>
<th>Anal LBC Result</th>
<th>AIN I</th>
<th>AIN II</th>
<th>AIN III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NEGATIVE</td>
<td>INSUFFICIENT</td>
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</tr>
<tr>
<td>Anal Bx Results</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx Site 1</td>
<td>Ex Site 2</td>
<td>Ex Site 3</td>
<td>Bx Site 4</td>
</tr>
<tr>
<td>Perianal Bx Results</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Bx Site A</td>
<td>Bx Site B</td>
<td>Ex Site C</td>
<td>Bx Site D</td>
</tr>
<tr>
<td>HPV Results</td>
<td>Negative</td>
<td>Positive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>16</td>
<td>18</td>
</tr>
</tbody>
</table>

Has the participant been referred for further treatment? (Please specify)

No    Yes

In the last six months, have there been any changes to the medical / surgical / personal / sexual / drug / other history? (Please specify)

..................................................................................................................................................................................
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Please explain any deviation from protocol

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8 Name  Signature  Date:
## Communication Sheet

<table>
<thead>
<tr>
<th>Date</th>
<th>Signature</th>
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</thead>
<tbody>
<tr>
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<td></td>
</tr>
</tbody>
</table>

**Name**

**Signature**

**Date:**
CONSENT FORM
Evaluation of cytology and HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK (ANALOGY)

Chief Investigator: Professor Henry Kitchener

1. I confirm that I have read and understood the ANALOGY information sheet dated 20th November 2012 (version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I consent to the storage of personal information (including electronic), for the purposes of this study. I understand that any information that could identify me will be kept strictly confidential and that no personal information will be included in the study report or other publication.

4. I understand that relevant sections of any of my medical notes and data collected during the study may be looked at by responsible individuals from the University of Manchester or from regulatory authorities or from the NHS trust as Trial Sponsor, where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

5. I agree to take part in the above study.

6. I wish to receive a copy of my results.

7. I agree to my test results being sent to my General Practitioner to ensure my medical records are updated.

8. I would like a copy of the final paper/summary of results. I therefore agree to my contact details being stored separately from the research data to enable a copy to be mailed to me.

9. I consent to my samples being stored and used in possible future research projects. I understand that these samples will be stored anonymously and that I will not be identified from any of the stored samples and that ethical approval will be obtained before these samples are used in research.

10. I consent to being contacted to discuss my participation in interviews to gain my views on anal screening.

Name of Participant __________________________ Date ____________ Signature __________________________

Name of person taking consent (if different from Principal Investigator) __________________________ Date ____________ Signature __________________________

Name of Principal Investigator __________________________ Date ____________ Signature __________________________

Thank you for agreeing to participate in this research
1 copy for participant; 1 copy for site file; 1 copy to be kept with hospital notes

Trial ID number: R01921 ANALOGY Consent Form Version 1.2, 20th November 2012
8.11 Appendix vib

CONSENT FORM
Evaluation of cytology and HPV testing for taring for anal intraepithelial neoplasia in high-risk populations in the UK (ANALOGY)

Chief Investigator: Professor Henry Kitchener

1. I confirm that I have read and understood the ANALOGY information sheet dated 20th November 2012 (version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

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10. I consent to being contacted to discuss my participation in interviews to gain my views on anal screening.

Name of Participant Date Signature

Name of person taking consent Date Signature
(if different from Principal Investigator)

Name of Principal Investigator Date Signature

Thank you for agreeing to participate in this research
1 copy for participant; 1 copy for site file; 1 copy to be kept with hospital notes

Trial ID number: ANALOGY Consent Form Version 1.2, 20th November 2012
8.12 Appendix vii

Source BioScience

Material Safety Data Sheet

Revision Date: 1/10/2009
Revision Number: 4
Material/Trade Name: SUREPATH PRESERVATIVE SOLUTION

1 - Substance Identification

Material/Trade Name: SurePath Preservative Fluid
Material type: Preservative
Company: Source BioScience
Address: 1 Orchard Place
Nettngaham Business Park
Nottingham
NG8 5PX
Telephone: +44 (0) 115 973 9012
Fax: +44 (0) 115 973 9013
Email: healthcare@sourcebioscience.com
Emergency Telephone: +44 (0) 7900 393013

2 - Composition

<table>
<thead>
<tr>
<th>Substance</th>
<th>% Wt</th>
<th>CAS No.</th>
<th>EC No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isopropanol</td>
<td>-24%</td>
<td>67-63-0</td>
<td>204-661-7</td>
</tr>
<tr>
<td>Distilled water</td>
<td>-24%</td>
<td>95-75-0</td>
<td>204-338-0</td>
</tr>
<tr>
<td>Methanol</td>
<td>-1%</td>
<td>67-56-1</td>
<td>209-659-6</td>
</tr>
</tbody>
</table>

3 - Hazard Identification

FLAMMABLE
IRRITATING TO EYES
VAPOURS MAY CAUSE DROWSINESS AND DIZZINESS
Keep away from sources of ignition – No Smoking
Take precautionary measures against static discharges
Do not breathe vapour
Avoid contact with eyes and skin

4 - First aid Measures

Inhalation: Remove to fresh air and rest
If recovery is not rapid call for prompt medical attention
Show this safety data sheet to medical personnel

Eyes: Irrigate with water for at least 15 minutes
Take care not to wash chemical from one eye to another
Get prompt medical attention

Skin: Remove contaminated clothing
Wash with soap/cleanser and rinse with plenty of water
If irritation persists, obtain medical attention
4 - First-aid Measures (Continued)

**Ingestion:**
- Do not induce vomiting
- Give plenty of water to drink
- Beware of aspiration if vomiting occurs
- Seek medical attention immediately

5 - Fire-fighting Measures

**Suitable Extinguishers:**
- Alcohol resistant foam
- Dry powder
- Carbon dioxide
- Water spray/tor

**Unsuitable Extinguishers:**
- Direct water jets

**Hazardous Decomposition:**
- Toxic fumes are produced in fire

**Special Procedure:**
- Do not breathe decomposition products and fumes
- Use approved self-contained breathing apparatus
- Wear fire resistant clothing
- Use water spray to cool containers
- Warning: Danger of flashback exists
- Use water fog to disperse vapours and leaks that have not ignited
- Prevent runoff from fire control from entering waterways
- Large fires should only be dealt with by trained personnel

6 - Accidental Release Measures

**Exposure Control:**
- Ventilate area
- Eliminate all sources of ignition
- Do not allow spill to enter drains and watercourses
- Mask area and warn all personnel

**Personal Protection:**
- Wear suitable respiratory protection for large spillages and in confined spaces
- Wear polythene or nitrile gloves
- Use eye protection such as goggles to BS EN 166 Chemical Grade

**Disposal Considerations:**
- Absorbing in inert material such as sand or absorbent granules
- Scoop up and place in plastic container to await transfer
- Spill is classed as special waste

7 - Handling and Storage

**Handling:**
- Avoid skin contact
- Avoid eye contact
- Avoid inhalation of vapour
- Ensure adequate ventilation
- Use local extraction equipment where possible
- Wear suitable protective clothing (see section 8)

**Storage:**
- Store in tightly closed labelled containers
- Store in a cool, dry, well-ventilated area
8 - Exposure Controls

Occupational Exposure Limit: 999mg/m³ 8hr TWA 125mg/m³ 15min STEL OES Prop-2-ol 1920mg/m³ 8hr TWA OES ethanol 268 mg/m³ 8hr TWA 33mg/m³ 15min STEL WEL S& MeOH

Wear polythene or nitrile gloves
Wear suitable overalls or aprons and change if contaminated
Wear suitable eye protection such as BS EN 166
Use in well ventilated areas
Use mechanical ventilation if possible
If excessive inhalation in a poorly ventilated area is likely then use a respirator with filter type A
After contact with skin wash off immediately

9 - Physical & Chemical Properties

Appearance: Clear colourless liquid
Odour: Mild alcoholic
pH: n/a
Boiling point/range: n/a
Melting point/range: n/a
Flash point: 30°C (Closed Cup)
Flammability: FLAMMABLE
Auto-flammability: n/a
Explosive properties: n/a
Oxidising properties: None
Vapour pressure: n/a
Relative density: <1
Solubility: Miscible in water
Vapour Density: n/a
Viscosity: n/a
Evaporation rate (B u A c = 1): n/a
(n/a = not established)

10 - Stability and Reactivity

Stable at normal temperatures
Materials to avoid: oxidising agents
Conditions to avoid: high temperature, sources of ignition & direct sunlight
No hazardous decomposition products when stored and handled correctly

11 - Toxicological Information

Routes of exposure: inhalation, skin absorption and ingestion
Acute effects from ingestion: Nausea, vomiting and stomach pain
Unconsciousness in severe cases
Health effects: Irritating to eyes, skin and respiratory tract
Repeated skin contact may cause dermatitis
Inhalation may cause depression of central nervous system
Chronic effects: May cause kidney & liver damage
12 - Ecological Information

Ecotoxicity: - Dangerous to aquatic life in high concentrations
Biodegradability: - Solvent is readily biodegradable
Bioaccumulative potential: - Not expected to bioaccumulate
Mobility: - Mobile, volatile liquid. May penetrate soil causing groundwater contamination

13 - Disposal Considerations

Waste materials must be treated as a fire hazard
Incinerate through a licensed site
Do not discharge into drains or watercourses
Dispose through properly licensed contractors
Dispose of in accordance with the Special Waste Regulations 1990

14 - Transport Information

<table>
<thead>
<tr>
<th>UN/NI Number:</th>
<th>1987</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMO:</td>
<td>Class 3</td>
</tr>
<tr>
<td>IATA/ICAO:</td>
<td>Class 3</td>
</tr>
<tr>
<td>ADR/RID:</td>
<td>Class 3</td>
</tr>
<tr>
<td>Proper Shipping Name (PSN):</td>
<td>ALCOHOLS, NOS (Ethanol/isopropanol/methanol solution)</td>
</tr>
<tr>
<td>HAZCHEM/Kenner Code:</td>
<td>3YE/30</td>
</tr>
</tbody>
</table>

15 - Regulatory Information

a) Risk & Safety

R10 FLAMMABLE
R36 IRRITATING TO EYES
R67 VAPOURS MAY CAUSE DROWSINESS AND DIZZINESS
S16 Keep away from sources of ignition – No Smoking
S33 Take precautionary measures against static discharges
S23 Do not breathe vapour
S24/25 Avoid contact with eyes and skin

b) Other Regulations:

Health & Safety at Work etc. Act 1974
Control of Substances Hazardous to Health Regulations 2002 as amended
Environmental Protection Act 1990
Special Waste Regulations 2005

c) Statutory risk phrases in section (2) and (3) of this Safety Data Sheet not appearing in final product classification

R11 Highly Flammable
R33/24/25 Toxic by inhalation, in contact with skin and if swallowed
R53/23/24/25 Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed

16 - Other Information

This information in this Safety Data Sheet should be provided to all who will use, handle, store, transport or otherwise be exposed to this product. This information is believed to be reliable and up-to-date at Revision Date, and represents the best information currently available and known by Source Bioclaim. However, Source Bioclaim makes no guarantees or warranty, express or implied, with respect to such information and we assume no liability resulting from its use. The information related herein is based on proper handling and anticipated use, and is for the material without chemical additions or alterations. Users should make their own investigations to determine the suitability of the information for their particular purposes. It is the responsibility of the user to undertake a suitable risk assessment COSHH assessment prior to using this material.
Appendix viii
### 8.14 Appendix xi

![Histopathology Request Form](image-url)

- **Please phone 0161 276 8808 for urgent requests**

#### CLINICAL PRESENTATION

- **ANATOMICAL SITE OF SPECIMENS**
  - [e.g. stomach, cervix etc.]

- **HOSPITAL NUMBER:**
  - **NHS NUMBER:**
  - **PATIENT TYPE:**
    - [NHS]
    - [IN-PATIENT]
    - [PRIVATE PATIENT]
    - [OUT-PATIENT]

- **PLEASE TICK IF WAITING LIST INITIATIVE**

#### PLEASE TICK IF THIS IS A KNOWN OR SUSPECTED HIGH-RISK SPECIMENS

- **DATE TAKEN:** ___/___/___
- **TIME TAKEN:** ___

#### CONSULTANT TO WHOM THE REPORT IS TO BE SENT

- **(Note: This must be the Consultant responsible for the overall management of the case)**

#### WARD/DEPARTMENT WHERE SPECIMENS TAKEN

- [e.g. Renal Unit, Gastroenterology, Wound Unit, Colposcopy clinic etc.]

#### PREVIOUS HISTOPATHOLOGY

- **DATE:**
- **LAB NUMBER:**

#### PREVIOUS CYTOLOGY

- **DATE:**
- **LAB NUMBER:**

#### FOR OBSTETRIC & GYNAECOLOGICAL CASES

- **LMP:** ___/___/___
- **PARITY:**

#### MENSTRUAL HISTORY

- **HORMONE THERAPY:** [Specific drugs used]

#### FOR RENAL CASES

- **CREATININE:**
- **IMMUNOLOGY:**

#### PRINT NAME

- **DATE:**

#### SIGNATURE

- **TELEPHONE/BLEEP:**

---

**N.B. FORMS MUST BE COMPLETED IN FULL. DOCTORS WILL BE REQUIRED TO ATTEND THE LABORATORY TO COMPLETE REQUESTS WITH INADEQUATE INFORMATION**
8.15 Appendix x

Health Research Authority
National Research Ethics Service
NRES Committee Northwest – Greater Manchester West
3rd Floor
Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7434

25 September 2014

Professor Henry C Kitchener
Institute of Cancer Sciences
University of Manchester/Central Manchester NHS Foundation Trust
St. Mary’s Hospital
Oxford Road
Manchester
M13 9WL

Dear Professor Kitchener

Study title: Evaluation of cytology and HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK
REC reference: 12/NW/0204
Amendment number: 7
Amendment date: 21 July 2014
IRAS project ID: 96714

- Bring follow up visit forwards from 6 months to 3 months.

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Notice of Substantial Amendment (non-CTIMP)</td>
<td>7</td>
<td>21 July 2014</td>
</tr>
<tr>
<td>Participant consent form</td>
<td>2</td>
<td>10 July 2014</td>
</tr>
<tr>
<td>Participant information sheet (PIS) [Transplant]</td>
<td>2</td>
<td>10 July 2014</td>
</tr>
<tr>
<td>Participant information sheet (PIS) [GUM]</td>
<td>2</td>
<td>10 July 2014</td>
</tr>
<tr>
<td>Research protocol or project proposal [Clean &amp;Tracked]</td>
<td>8</td>
<td>01 July 2014</td>
</tr>
</tbody>
</table>

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.
R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members’ training days – see details at http://www.hra.nhs.uk/hra-training/

12/NW/0204: Please quote this number on all correspondence

Yours sincerely

[Signature]

Dr Lorraine Lighton (Chair)
Chair

E-mail: nrescommittee.northwest-nwwest@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Mrs Lynne Webster (1), Central Manchester University Hospitals

NHS Foundation Trust
**NRES Committee North West - Greater Manchester West**

**Attendance at Sub-Committees by correspondence**

**Committee Members:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Profession</th>
<th>Present</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mr Ian Beaumont (Vice Chair)</td>
<td>Retired Pharmacist</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Dr Lorraine Lighton (Chair)</td>
<td>Consultant in Communicable Diseases</td>
<td>Yes</td>
<td></td>
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</tbody>
</table>

**Also in attendance:**

<table>
<thead>
<tr>
<th>Name</th>
<th>Position (or reason for attending)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miss Anna Barriester</td>
<td>REC Manager</td>
</tr>
</tbody>
</table>
Dear Dr,

Regarding your patient

Re: Evaluation of cytology and HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK (ANALOGY)

Your patient has agreed to take part in the above study of anal screening because of being identified as belonging to a group at increased risk of anal cancer. The screen consisted of anal liquid cytology, HPV testing and anoscopy with or without biopsy. The results are as follows:

<table>
<thead>
<tr>
<th>Cytology</th>
<th>Negative</th>
<th>Biopsy: AIN grade 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low grade</td>
<td></td>
<td>AIN grade 2</td>
</tr>
<tr>
<td>High grade</td>
<td></td>
<td>AIN grade 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HPV test</th>
<th>Negative</th>
<th>Anoscopy: Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td></td>
<td>Positive</td>
</tr>
</tbody>
</table>

The action based on these results is:

- [ ] Follow up at 6 months according to study protocol.
- [ ] Treatment for biopsy proven anal intraepithelial neoplasia (further details to

Enclosed is a copy of the patient information sheet. If you have any questions or any concerns regarding your patient and this trial, please do not hesitate to contact me on 0161 276 6461.

Yours sincerely

[Signature]

Professor Henry Kitchener
Chief Investigator of the ANALOGY study

ANALOGY® Letter Version 1.1, 16th April 2012
Dear Dr

Regarding your patient:

Re: Evaluation of cytology and HPV testing for testing for anal intraepithelial neoplasia in high-risk populations in the UK (ANALOGY)

Your patient has agreed to take part in the above study of anal screening because of being identified as belonging to a group at increased risk of anal cancer. The screen consisted of anal liquid cytology, HPV testing and anoscopy with or without biopsy. The results are as follows:

Cytology:  
- Negative  
- Low grade  
- High grade  

HPV test:  
- Negative  
- Positive  

Anoscopy:  
- Negative  
- Positive  

The action based on these results is:

- Follow up at 6 months according to study protocol.
- Treatment for biopsy proven anal intraepithelial neoplasia (further details to

Enclosed is a copy of the patient information sheet. If you have any questions or any concerns regarding your patient and this trial, please do not hesitate to contact me on 0161 276 6461.

Yours sincerely

[Signature]

Professor Henry Kitchener
Chief Investigator of the ANALOGY study
8.18 Appendix xi bi

Dear

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

Results

Cells - this is the cytology result from the swab sample.

High Risk HPV result - this can be positive (HPV detected) or negative (HPV not detected).

Biopsy result - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see from your negative cytology results, you do not require any follow up and you have been discharged to your GP.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Signature]

Professor Henry C Kitchener
Chief Investigator and Consultant Gynaecologist

To contact the ANALOGY team...
- Call/ Text 07768955970
- Email alice.schofield@manchester.ac.uk
Dear

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

**Results**

**Cells** - this is the cytology result from the swab sample.

**High Risk HPV result** - this can be positive (HPV detected) or negative (HPV not detected).

**Biopsy result** - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see from your negative biopsy and cytology results, you do not require any follow-up and you have been discharged to your GP.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Signature]

Professor Henry C Kitchener
Chief Investigator and Consultant Gynaecologist

---

To contact the ANALOGY team...
- Call/ Text 07758995970
- Email alice.schofield@manchester.ac.uk
Dear

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

Results

**Cells** - this is the cytology result from the swab sample.

**High Risk HPV result** - this can be positive (HPV detected) or negative (HPV not detected).

**Biopsy result** - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see from your results, there were low grade changes in your cytology sample, however the actual biopsy was negative and therefore you do not require any follow up and you have been discharged to your GP.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Karin C. Transmission]

Professor Henry C. Kitchener
Chief Investigator and Consultant Gynaecologist

To contact the ANALOGY team...
- Call/Text 07768995970
- Email alice.schofield@manchester.ac.uk
Dear

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

**Results**

**Cells** - this is the cytology result from the swab sample.

**High Risk HPV result** - this can be positive (HPV detected) or negative (HPV not detected).

**Biopsy result** - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see from your results, there were low grade changes in your cytology sample, suggestive of AIN 1. Because there were no other abnormal changes, this does not require immediate referral. You will receive a follow up appointment from Professor Hill, Consultant Surgeon, within 12 months’ time.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Signature]

Professor Henry C. Kitchener  
Chief Investigator and Consultant Gynaecologist

To contact the ANALOGY team...
- Call/Text 07768955970  
- Email alice.schofield@manchester.ac.uk
8.22 Appendix xi bv

25/06/2015

Dear

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

Results

Cells - this is the cytology result from the swab sample.

High Risk HPV result - this can be positive (HPV detected) or negative (HPV not detected).

Biopsy result - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see your cytology results show high grade cell changes. Because there were no other abnormal changes, this does not require immediate referral. You will receive a follow up appointment from Professor Hill, Consultant Surgeon, within 12 months' time.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Signature]

Professor Henry C Kitchener
Chief Investigator and Consultant Gynaecologist

To contact the ANALOGY team...
- Call / Text 07768995970
- Email alice.schofield@manchester.ac.uk
8.23 Appendix xi bvi

Dear,

Thank you for agreeing to take part in the ANALOGY study. Please find attached a summary of your clinical results.

**Results**

**Cells** - this is the cytology result from the swab sample.

**High Risk HPV result** - this can be positive (HPV detected) or negative (HPV not detected).

**Biopsy result** - sometimes more than one sample is taken, this is reported as one overall test result.

As you can see your biopsy results show high grade changes. You have been referred to Professor Hill, Consultant Surgeon and you will be seen in clinic for all further follow up appointments.

If you have any questions or would like to discuss your results further please contact the ANALOGY team.

Yours sincerely,

[Signature]

Professor Henry C Kitchener
Chief Investigator and Consultant Gynaecologist

To contact the ANALOGY team...
- Call/ Text 07768995970
- Email alice.schofield@manchester.ac.uk
ANALOGY Study Specimens

- This protocol has been compiled to meet CPA standard F Examination Procedures
- The document must be compiled with by all Staff

Checked by: Pamela Atkinson
Authorised by: Adanna Ehirin
Designation: Lead Biomedical Scientist

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0  Introduction

0.1  Rationale

The UK National Screening Committee has suggested that screening for anal intraepithelial neoplasia in high risk populations may be of benefit but further information is needed. The Analogue study proposes to evaluate the feasibility, acceptability and efficacy of such screening in a clinical context using technologies now in routine use in the NHS Cancer Screening Programme, liquid-based cytology (LBC) and HPV testing. The aim of the study is to evaluate the usefulness of LBC and HPV testing in screening for anal neoplasia in high risk groups; to assess the uptake of screening through outpatient clinic and general practice of high risk population groups for screening by liquid-based anal cytology and HPV testing; to estimate the prevalence of anal neoplasia and of HPV positivity in such population groups in the UK.

0.2  Responsibility and Personnel

Dr Mina Desai  
Chief/Senior Biomedical Scientists  
Medical Secretaries  
Medical Laboratory Assistants

0.3  Quality Control

Patient details on request form and specimen vial are checked prior to processing by MLA. The information entered onto the LIMS is checked with details on the request form by Dr Desai at reporting.  
Photocopied forms are matched with specimen vials in tray prior to sending to Virology for HPV testing.

0.4  Equipment Requirements

Access to LabCentre  
Barcode and print-readable labels  
SurePath processing system & designated consumables  
Specimen vials

0.5  Health and Safety

•  Howie style laboratory coat must be worn.
•  Disposable gloves must be worn when handling samples at processing.
[CYT COSHH 000] Specimen Reception
[CYT COSHH 002] Centrifugation
[CYT COSHH 007] SurePath sample processing
[CYT COSHH 008] Coverslipping – manual and automated
[CYT MHR 003] Computer workstation – specimen reception
[CYT MRH 008] Clinical waste disposal – preparation laboratory
[CYT RRA 004] Cytology specimen reception and preparation
[CYT RRA 008] Cytology preparation laboratory
[CYT RRA 013] Transport of waste, chemicals, samples and other consumables

Data Safety Sheet 1  Xylene
Data Safety Sheet 2  Industrial Methylated Spirit
Data Safety Sheet 3  Pertex mounting media
Data Safety Sheet 38  SurePath® preservative fluid
Data Safety Sheet 39  PrepStain Haematoxylin stain
Data Safety Sheet 40  PrepStain Tric Buffer concentrate
Data Safety Sheet 43  PrepStain Alcohol Blend Rinse
Data Safety Sheet 67  PrepStain EAOG stain
Data Safety Sheet 75  PrepStain Density Reagent

6.6 Reagents and Chemicals

- Xylene
- Industrial Methylated Spirit
- Pertex mounting medium
- SurePath® preservative fluid
- PrepStain Haematoxylin stain
• PrepStain Tris Buffer concentrate
• PrepStain Alcohol Blend Rinse
• PrepStain HA/OG stain
• PrepStain Density Reagent

1 Procedure

1. Patient details and specimen type of the analogy study specimen are entered onto LabCentre as a non-gynaecological cytology request and a unique laboratory number assigned. [CYT PREP NG 001] Receipt of non-gynaecological specimens

2. Photocopy request forms.

3. Take tray of specimens, original and photocopied request forms to the Preparation Laboratory.

4. Prepare specimens using the SurePath LBC processing system. [CYT PREP L 008] Preparation of SurePath LBC samples

5. After slide preparation, change tops of vials to new crowns.

6. Take vials and photocopies of request forms to Virology Reception.

7. Print labels for prepared slides.

8. Prepared slides and matching request forms are passed to Dr Desai for reporting. [CYT SCR NG 001] Screening and reporting of non-gynaecological cytology cases

9. Dr Desai’s hand written reports are passed to the medical secretaries for typing. [CYT SEC 001] Non-gynaec report entry (excluding synovial fluid specimens)

A hard-copy report is printed and sent to:
Dr A. Schofield
O/o Prof Kittscher
5th Floor (Research)
St Mary’s Hospital

10. Dr Desai matches request forms and slides and places these in designated non-gynaec filing tray, in the preparation laboratory.
2 References

[CYT COSHH 006] Specimen Reception
[CYT COSHH 008] Consent
[CYT COSHH 007] SurePath sample processing
[CYT COSHH 008] Coverslipping – manual and automated

[CYT MHR 003] Computer workstation – specimen reception

[CYT MHR 006] Clinical waste disposal – preparation laboratory

[CYT RRA 004] Cytology specimen reception and preparation
[CYT RRA 008] Cytology preparation laboratory

[CYT RRA 013] Transport of waste, chemicals, samples and other consumables

[CYT PREP NG 001] Receipt of non-gynaecological specimens

[CYT PREP G 006] Preparation of SurePath LBC samples

[CYT SCR NG 001] Screening and reporting of non-gynaecological cytology cases

[CYT SEC 001] Non-gynaecological report entry (excluding synovial fluid specimens)

3 Equality Impact Assessment

- This policy has not been equality impact assessed by the author using the Trust’s Equality and Diversity Impact Assessment (E&DA) framework
- This document is not required to be equality impact assessed as it is not in the required area for assessment
8.25 Appendix xiii

Cobas ® 4800 HPV Test

- This document has been compiled to comply with CPA standard
- This procedure should be adhered to by all staff

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See Q-Pulse for full distribution list
## Risk Assessments

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## COSHH Assessments

### General COSHH Assessments

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### Virology, Serology and Molecular Diagnostics COSHH Assessments

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<td>Use of Automated Analyzers</td>
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<td>Extraction of Nucleic Acid from BAL and Sputum</td>
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<td>DNA Extraction from Clinical Samples Living Qiang Columns</td>
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<td>2033</td>
<td>HRV DNA Extraction from Serum or Plasma</td>
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<td>Preparation of Suspension for Serogroup C Meningo - Control</td>
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<td>RNA Extraction for Roche Hepatitis C and HIV PCR</td>
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General Examination Procedures

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<td>1144</td>
<td>Fire Policy</td>
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<td>1164</td>
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<td>Retention of Clinical Specimens and Isolates</td>
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<td>Control of Process and Quality Records</td>
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<td>Customer Care Complaints Handling</td>
<td>1379</td>
<td>Reception of Foods and Waters for Onward Transmission to Preston Microbiology Lab</td>
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</tbody>
</table>

1.0 SAFETY CONSIDERATIONS

Any work performed during this procedure MUST be performed in accordance with the document “A Local Code of Practice for Working in Molecular Diagnostics (MMMP-MOL-PROC10).”

Additionally, the particular risks associated with the various steps and equipment used for this procedure MUST be read and understood before performing any testing using this EP. The relevant Risk assessments for this procedure include:

- Laboratory Robotic Instrumentation (MMMP-RISK-PROC70)
- PreservCyt® solution (Cytec Corp) and SurePath® preservative fluid (BD Diagnostics-TriPath) specimens (MMMP-RISK-PROC50)
- COSHH Assessment Cytology Reference CYT COSHH 007: SurePath sample processing (see appendix 4)
The work MUST be performed whilst wearing GLOVES, SAFETY SPECTACLES and appropriate lab COAT or GOWN (see White Coat Policy – MMMF-SAFETY-PROC8).

The main hazards that require consideration are:

- Contact with pathogenic Viruses and other infectious agents prior to nucleic acid extraction
- Exposure to toxic reagents e.g. Guanidium isothiocyanate
- Handling of flammable liquids e.g. ethanol
- Physical hazards associated with robotic instrumentation e.g. crush and puncture wounds or splashes caused by carelessness during loading
- Exposure to heated surfaces
- Potential exposure to laser beams

The above list is by no means exhaustive and the reduction of these risks is described within the individual documents described above. Liquid waste generated by the Cobas MUST be collected and transferred for storage in the flammable store in York Place to await authorised disposal.

Introduction

Persistent infection with human papillomavirus (HPV) is the principle cause of cervical cancer and its precursor cervical intraepithelial neoplasia (CIN). The presence of HPV has been implicated in greater than 90% of cervical cancers worldwide. There are > 100 different HPV types and approximately 40 different types can infect the human anogenital mucosa. However, only a subset of 13 to 15 of these types are considered high-risk for the development of cervical cancer and precursor high grade cervical lesions.

Although persistent infection with high risk (HR) HPV is a necessary cause of cervical cancer, a very small percentage of infections progress to cervical cancer. Sexual transmission with HPV is extremely common however. >90% of infected women will mount an effective immune response and clear the virus in 6 to 24 months without any long term health consequences.

In developed countries with cervical cancer screening programmes, the Pap smear has been used since the mid-1950s as the primary tool to detect early precursors of cervical cancer. Liquid based cytology (LBC) was introduced in the mid-1990s and is a refinement of the
conventional Pap smear. LBC preserves the integrity of the cellular material, slides are more homogeneous than the conventional smears and cellular debris, such as blood and mucus which may obscure cervical cells, is removed. In cervical primary screening there has been increasing interest for the use of HPV detection as a triage to cytology and in England HPV triage of women with borderline or mild cytology is currently being implemented.

HPV is extremely difficult to culture in vitro, and not all patients infected with HPV have a demonstrable antibody response. Therefore, nucleic acid (DNA) testing by PCR is a sensitive method for determining the presence of an active cervical HPV infection. Implementation of nucleic acid testing for HPV promises to increase the sensitivity and cost-effectiveness of the cervical screening programme by detecting high grade lesions earlier and reducing referral rates and unnecessary treatment at colposcopy.

Value of Testing for HR HPV by the Cobas 4800

The use of this test is indicated:
1. for the detection of 14 HR HPV types with simultaneous detection of HPV types 18 and 16
2. As a follow up test for women with borderline or mild cytological lesions in order to aid physician in patient management

The Cobas 4800 HPV DNA test should be used in conjunction with clinical information derived from other diagnostic and screening tests, physical examinations, and a full medical history in accordance with appropriate patient management procedures. Cobas 4800 HPV DNA test results should not be used as the sole basis for clinical assessment and treatment of patients.

Specimens

Both Thinprep (PreservCyt) and surepath (original sample only not the cell enriched) have been validated for this test.

Thinprep – may be processed directly out of the 20ml primary container with barcodes or a minimum aliquot of 1ml transferred to a barcoded 12ml round-based specimen tube and placed in
designated racks for the Cobas. Minimum volume 3ml from primary container or 1ml for secondary tubes. Samples are stable for up to 6 months at room temperature.

Surepath — A heat step process is available for samples that exceed 14 days from sample taken to tested. This however is not CE marked and the CSP have approved testing samples stored at room temp up to 28 days without this heat processing step.

A single run on the Cobas can have any combination of specimens.

Principles of the Procedure

1. Worksheet for the Cobas is MRCH
2. Vortex sample and transfer a minimum of 1ml into a sterile tube. NB. Only the original Thirteenth pots or standard tubes can be used on the Cobas.
3. Vortex samples for approx 30 seconds using the multivortex prior to decapping samples and loading into Cobas racks.
4. Test samples in batches of 22 or less.
5. Kits are stored in the HPV section at the back of the cold room. Bring kits to room temperature prior to use.
6. Turn on Computer, 2410 and 4810 in this order.
7. Place samples in sample rack (NB. Rack with sticker is for reagents only NOT samples)
8. On desktop open the Cobas 4810 work order editor
9. Under file — delete new
10. Run type — full
11. Test type — HPV workflow
12. Next (right hand side of screen)
13. Subset type — HPV HR (Hol + ST)
14. Media Type (enter SurePath for SurePath samples and Preservcyt for any other sample type)
15. Delete INSERTBARCODE
16. Scan the samples (Do not need to scan in the order of the worksheet. Number of samples scanned is shown in bottom right (22 samples/run) NB. Change the media type prior to scanning barcode as necessary if different sample types are included on the same run.)
17. Save
18. Workorders filename today's date and A for 1st run, B for 2nd etc
19. Save
20. File saved OK
21. Minimize screen
22. Click Cobas 4800 icon on desktop
23. User ID Cobas (lab manager)
24. Password Cobas (COBAS)
25. Maintenance status will show up in red (NB. The machine will not run if the service maintenance has not been carried out – keep an eye on the number of days until next service maintenance and arrange Roche to carry this out in advance so as not to delay testing. Serial number for 4800 5198; contacts for Roche see below)
26. Click on show details; a daily maintenance or weekly maintenance will be required depending on when the machine was last in use. Click run maintenance. This will take a couple of minutes.
27. Machine will ask you to check deck cleaning
28. To clean deck – use a small amount of Dacron Solarsep only (do not use bleach or media) and blue roll
29. Click ok after cleaning
30. Remove silver catch from waste and remove waste bag
31. Waste bags should be placed into autoclave bins
32. Empty the tip waste (this can be a little awkward)
33. Replace silver catch
34. Close door and click ok
35. Pressure drop timer will be checked
36. Click ok when maintenance is complete
37. To start a new run click ‘New run’ (right hand side of screen)
38. HPV workflow tick box ok
39. Follow instructions on screen
40. Load carriar – do not force at flashing green lights
41. Click load samples right hand corner
42. Choose workflow to process
43. Open worklist
44. Click now and load consumables
45. The deep well plate is loaded towards the back of the task and placed in position 1.6 on the Cobas
40. Loaded 1000ul tips in positions 11-10 (these must be full) and empty tips racks can be placed in positions 35-40
41. Press load consumables button
42. If barcodes are in wrong place machine will alert; unload carrier and correct then repeat and execute
43. Load reagents – scan wash bottle, large trough and wash buffer bottle and place into the 4th position on the carrier and load reagent
44. Continue to load remaining reagents – it does not matter what order reagents are loaded as long as they are in the correct position
45. Vortex Magnetic Glass Particles until bottom of vial is clean
46. Use rack with sticker for reagents only
47. Load positive control last and change gloves afterwards
48. Load reagents
49. No need to scan reagents this time
50. Start run
51. When run is complete you have 60 minutes to transfer plate to the 480.
52. When run is complete, press unload and be careful not to touch any racks unloading from the instrument as this will abort the run. Allow instrument to fully unload and then seal the MWP and load on the 480 instrument by pressing the button on the left hand side. Once the MWP is in position load the MWP by pressing the button again. The instrument will start automatically and takes approximately 2 hours.
53. Repeat all samples and remove consumables from the 480.
54. Refer to risk assessment for disposal of reagents.

Appendix

Projects using this test

Strategies to Increase Cervical Screening Uptake at First Invitation (STRATEGIC)

Brief outline
A research study which aims to find ways of improving cervical screening uptake amongst women receiving their first invitation from the NHS CEP to attend for cervical screening. Women
are offered HPV self sampling which is then sent to Virology for HR HPV testing. If the result of this test shows that the virus is not present the women is at very little risk of developing cervical changes within the next three years and can safely be recalled for cervical screening in three years. If the result shows that the virus is present the women will need to attend for a further cervical cytology test taken by the GP or practice nurse. The reason for this is that 25% of women aged 25 will test positive for HPV but only around half of those will have underlying cervical changes.

Specimen reception and planning of samples

Self samples from this study arrive in Virology self addressed envelopes with the accompanying consent/ request form. Samples are labelled up using 850000 numbers by the MLA in the HPV lab. Request forms are then booked in by specimen reception (see table below) and location added by the office. Request forms are then given to Andrea Reid and emailed via NISNET to the strategic office. The original request forms are stored in the Strategic box and taken over when convenient to the strategic office for storage.

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</tr>
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Sample processing

Delph Lavage samples

The lavage samples arrive in approx 2-3ml volume in universal provided with the kit. These tubes are not compatible for the Cobas. Transfer the entire sample fluid into a sterile test tube and store at -20°C (Freezer 39, room 317) until testing. The lavage may also be reused this can be discarded in an autoclave bin.
Evalyn brush samples

The Brush samples are received dry, a pink cap clicks onto the case covering the brush. Remove the pink case and carefully remove the brush using plastic tweezers (use a different set of tweezers for each sample) and place into a vial sealed tube containing 2.5mls of saline. Vortex sample leave at room temperature until the end of the day. Using a Pasteur pipette transfer saline sample to a new barcode labelled vial sealed tube leaving the brush behind. Discard sample tube with brush in autoclave bins and freeze tube containing sample at -20°C (freezer 39, room 317) until testing.

Reporting

All samples must be tested within three weeks of sample taken date. A maximum of 22 samples can be tested in a batch. All samples are tested using the Cobas 4800. Results are reported as High risk HPV detected (HRDD) or High risk HPV not detected (HRDN) and hard copy report is sent out to Samantha Fletcher at the strategic office.

Ordering of kits and consumables

Email Linsey.foison@manchester.ac.uk three weeks in advance with description and cat no. Keep all delivery notes and take over to Linsey when convenient.

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<tr>
<td>Cobas 4800 Liquid Cytology Preparation kit 240 (10x24) tests</td>
<td>06236812190</td>
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<tr>
<td>Cobas 4800 system HPV control kit</td>
<td>06236856150</td>
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<td>Cobas 4800 system extraction plate (50 plates)</td>
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<td>Code</td>
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<tr>
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<tr>
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<tr>
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<tr>
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</tr>
<tr>
<td>CO-RE tips (1ml with filter) 3040 pieces</td>
<td>0436842001</td>
</tr>
</tbody>
</table>

Contacts

Samantha Fletcher (Research Nurse)
Academic Unit of Obstetrics and Gynaecology
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M13 9WL
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St Mary's Hospital
Manchester
M13 9WL
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Jamie Oughton (Research Trial Coordinator)
Academic Unit of Obstetrics and Gynaecology
ANALOGY

Brief outline
This project is due to start at the beginning of 2013 and will be using the Cobas 4800 to screen samples for high risk HPV. This project is looking at anal screening for high risk populations to evaluate the feasibility, acceptability and efficacy of such screening in a clinical context using technologies now in routine use in the NHS cervical screening programme. High risk groups will include:

1. HIV positive men and women attending GUM
2. Men who have sex with men, recruited through GUM clinics
3. Immunosuppressed transplant recipients (men and women) attending transplant follow-up clinics

Approximately 250 will be recruited from each group

Specimen reception
Surepath samples for this study will arrive from Manchester Cytology clearly labelled Analogy study. Each sample will have an individual request form. Samples are labelled using 860000 numbers by the MLA in the HPV lab. Request forms are then booked in by specimen reception (see table below) and location added by the office. Request forms will be put into dart and original forms held in virology.
<table>
<thead>
<tr>
<th>Investigation</th>
<th>HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample type</td>
<td>VR3</td>
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<tr>
<td>Qualifier</td>
<td>LBCS</td>
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<tr>
<td>Lab procedure</td>
<td>HPV</td>
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<tr>
<td>Worksheet</td>
<td>MRCH</td>
</tr>
<tr>
<td>ILOG</td>
<td>1293022</td>
</tr>
<tr>
<td>Location Code</td>
<td>Reports sent to Prof Kitchener Research Floor St Mary's</td>
</tr>
</tbody>
</table>

Sample processing
Samples are taken in surepath. Transfer a minimum of 1ml of the original surepath sample into sterile vials and store at room temperature prior to testing. Samples must be tested within 28 days of the sample being taken.

Reporting
Contact Research nurse and Linsey nelson with regards to reporting

Ordering of kits
Email Linsey.nelson@manchester.ac.uk three weeks in advance with description and cat no.
Keep all delivery notes and take over to Linsey when convenient.

Contacts
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Research nurses
carol.shaw@omf.nhs.uk
julie.burnet@omf.nhs.uk
Tel: 0161 701 9119

**Hilary Powers study - Methyl donor status and oral HPV infection in young adults: importance in oropharyngeal cancer**

**Brief outline:**
This project is due to start at the beginning of 2013 and will be using the Cobas 4800 to screen samples for high risk HPV. This project is looking at oral high risk HPV infection associated with systemic folate status and buccal cell methyl donor status as a cause of oropharyngeal cancer.

The aim of the study is to conduct a screening study among 770 men and women between 20 and 40 years to determine the prevalence of oral high-risk HPV infection in this population. HPV testing will be carried out by Virology. The study will also investigate the concentration of folate in blood and methyl donor status in buccal cells which will be carried out by the Human Nutrition Unit, University of Sheffield.
Specimen reception

Specimens will be sent each month from the University of Sheffield in batches and clearly labelled for the attention of Rosemary Killough (oropharyngeal study – Prof Hilary Powers). Samples will arrive in saline solution and individually labelled with the trial number. Samples are labelled up using 800000 numbers by the MLA in the HPV lab. Request forms are then booked in by specimen reception (see table below) and location added by the office. Request forms will be put into dart and original forms held in virology.

<table>
<thead>
<tr>
<th>Investigation</th>
<th>HPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample type</td>
<td>VFL</td>
</tr>
<tr>
<td>Qualifier</td>
<td>MW</td>
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<td>Lab procedure</td>
<td>HPVP</td>
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<td>Worksheet</td>
<td>MRCH</td>
</tr>
<tr>
<td>ILOG</td>
<td>1353003</td>
</tr>
</tbody>
</table>

Sample processing

Samples will be put directly into sterilised tubes and sent to virology.

Reporting

Contact Hilary Powers with regards to reporting.

Ordering of kits

Email Linsey.nelson@manchester.ac.uk three weeks in advance with description and set no. Keep all delivery notes and take over to Linsey when convenient.

Contacts

Hilary J Powers
Professor of Nutritional Biochemistry
Department of Oncology
General notes for projects

1. We can only currently run up to 22 samples per run on the Cobas which should be sufficient for the number of samples we receive, if by any chance we start to receive more samples and doing multiple runs is a pain, 36 kits can be ordered instead. Contact Roche to arrange.
PapilloCheck test for genotyping HPV

- This document has been compiled to comply with CPA standard
- This procedure should be adhered to by all staff

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1.0 SAFETY

Printed

Valid on day of issue only
## Risk Assessments

<table>
<thead>
<tr>
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<tbody>
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<td>3001</td>
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<td>Disposal of Waste</td>
<td>3004</td>
<td>Storage of Bacterial, Fungal and Viral Cultures</td>
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<td>3005</td>
<td>Working in Containment Level 3</td>
<td>3006</td>
<td>Lona Working</td>
</tr>
<tr>
<td>3009</td>
<td>Working with Specimens that may contain TSE (Transmissible spongiform encephalopathies)</td>
<td>3010</td>
<td>Pregnancy: Safe working in the Laboratory</td>
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<tr>
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<td>Manual Handling</td>
<td>3012</td>
<td>Liquid Nitrogen</td>
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<tr>
<td>3013</td>
<td>Security</td>
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</table>

## COSHH Assessments

### General COSHH Assessments

<table>
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<th>Title of Assessment</th>
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<td>2001</td>
<td>Precautions for handling Blood and Serum</td>
<td>2002</td>
<td>Dry discarding of waste</td>
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<td>2003</td>
<td>Use of Wet Wipes</td>
<td>2005</td>
<td>Use of Sanichor</td>
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<td>2047</td>
<td>Opening Freeze Dried Cultures</td>
<td>2040</td>
<td>Transports Specimens</td>
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<td>2044</td>
<td>General Cleaning</td>
<td>2042</td>
<td>Leaking Specimens</td>
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### Virology, Serology and Molecular Diagnostics COSHH Assessments

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<th>Title of Assessment</th>
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</thead>
<tbody>
<tr>
<td>2020</td>
<td>Procedures for Mercury Vapour Lamps</td>
<td>2022</td>
<td>Immuno fluorescence of viral isolates</td>
</tr>
<tr>
<td>2023</td>
<td>Tissues / tissues for Viruses</td>
<td>2025</td>
<td>Complement Fixation Test</td>
</tr>
<tr>
<td>2027</td>
<td>Tracheal or bronchial Tissue</td>
<td>2026</td>
<td>Use of Automated Analysers</td>
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<td>2030</td>
<td>Extraction of Nucleic Acid from BAL and Sputum</td>
<td>2031</td>
<td>DNA extraction from clinical samples using qPCR columns</td>
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<tr>
<td>2032</td>
<td>Extraction of bacterial DNA using DNAZOL</td>
<td>2033</td>
<td>HBV DNA extraction from Serum or Plasma</td>
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<tr>
<td>2034</td>
<td>Preparation of Suspension for Serogroup C mening + control</td>
<td>2035</td>
<td>RNA extraction for dengue and HIV PCR</td>
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## 2.0 General Examination Procedures
### GENERAL EPS

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<td>1006</td>
<td>Safety Handbook</td>
<td>1008</td>
<td>Safe Handling of Chemicals</td>
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<tr>
<td>1009</td>
<td>Waste Disposal Policy (MRI)</td>
<td>1011</td>
<td>Centrifuge Policy</td>
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<td>1023</td>
<td>White Coat Policy (MRI)</td>
<td>1025</td>
<td>Security Policy</td>
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<td>1123</td>
<td>Immunization Policy</td>
<td>1125</td>
<td>Bulb Maintenance for the UV Microscope</td>
</tr>
<tr>
<td>1144</td>
<td>Electronic Communications – Guidance for Staff</td>
<td>1145</td>
<td>Fire Policy</td>
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<tr>
<td>1146</td>
<td>White Coat Policy (Wythenshawe)</td>
<td>1164</td>
<td>Retention of Clinical Specimens and Isolates</td>
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<tr>
<td>1177</td>
<td>Fixing Policy</td>
<td>1182</td>
<td>Telephone Policy</td>
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<td>1311</td>
<td>Actions Following Needlestick injuries</td>
<td>1314</td>
<td>Control of Process and Quality Records</td>
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<tr>
<td>1315</td>
<td>Use of Anemometer</td>
<td>1334</td>
<td>Waste Disposal Policy (Wythenshawe)</td>
</tr>
<tr>
<td>1352</td>
<td>Customer Care Complaints Handling</td>
<td>1379</td>
<td>Reception of Foods and Waters for Onward Transmission to Pession Microbiology Lab</td>
</tr>
</tbody>
</table>

**3.0 Introduction**

PapilloCheck test is intended to be used for the qualitative detection and differentiation of 24 types (high risk types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, 74, 82 and low risk types include 6, 11, 40, 42, 43, 44/56) of HPV in DNA preparations. Sample types tested using this test include biopsies, genital, oral, nasal, ocular swabs in viral transport medium, liquid based cytology samples and paraffin wax sections. The test contains an internal control that will check for adequacy of the sample. The assay itself is manual with 12 samples processable on a single plastic slide. The hybridisation and wash sequences are quick and the laser mediated readout is digitalised to provide a genotype profile. The turn around time for this test is two weeks. PapilloCheck results should be interpreted in conjunction with clinical findings.

**4.0 Assay Principle**

The PapilloCheck test is a microarray-based test kit for the detection and genotyping of a fragment of the E1 gene of the human HPV genome. Prior to the PapilloCheck analysis viral and human DNA is extracted using the BioMerieux EasyMag system or the Abbott M2000xp.
(N.B. 250ul of sample is required for the EasyMag and 500ul of sample is required for the Abbott M2000sp). Subsequently a DNA fragment of about 350bp of the viral E1 gene is amplified in the presence of HPV specific primers using PCR. The same primers also generate an internal control-template present in the PaqiloCheck MasterMix to monitor the performance of the PCR (PCR control). Additionally a fragment of the human ADAT1 (human tRNA-specific adenosine deaminase) gene is amplified to monitor the presence of human sample material in the sample (sample control). In addition the MasterMix contains dUTP. Thus, potential carry-over contamination from previous PCR reactions can be eliminated through the use of Uracil-N-Glycosylase (UNG). The PCR design results in the generation of single-stranded DNA fragments. The amplification products are then hybridized to complementary DNA probes fixed on the DNA chip. Every chip contains 12 DNA microarrays, allowing the simultaneous analysis of ten samples and two controls. Each HPV type is detected by a specific DNA probe present in replicates of 5 on each array. During hybridisation, the bound DNA is fluorescently labelled and subsequent washing steps remove unspecifically bound products or products in excess. The hybridisation efficiency is monitored (hybridisation control). The fluorescence light from the bound and labeled products is detected using the Chips-Scanner at excitation wavelengths of 532 and 635nm. Evaluation and analysis are carried out using the CheckReport analysis software. The report indicates the presence or absence of one or more of the 24 HPV types detectable.

5.0 Ordering information and checklist

<table>
<thead>
<tr>
<th>Description</th>
<th>Cat-no</th>
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<tbody>
<tr>
<td>Sample preparation reagents</td>
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<tr>
<td>10mM TRIS / 1mM EDTA (600ml)</td>
<td>SIGMA 95283</td>
</tr>
<tr>
<td>Triton X-100 (150ml)</td>
<td>SIGMA T8787</td>
</tr>
<tr>
<td>200ug/ml Protease K</td>
<td>SIGMA P2308</td>
</tr>
<tr>
<td>Cetane</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
</tr>
<tr>
<td>PaqiloCheck genotyping kit (test kit for 60 reactions)</td>
<td>Greiner Bio-one 405 000</td>
</tr>
<tr>
<td>HotStarTaq DNA Polymerase 5ul/* (4 per kit)</td>
<td>Qiagen 203265</td>
</tr>
<tr>
<td>Uracil DNA-Glycosylase fuM * (1 vial)</td>
<td>Fermentas (Thermo Scientific) EN0361</td>
</tr>
</tbody>
</table>

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### PapilloCheck kits
Kits by standing order each month (1 kit)
Not start by standing order on the Tuesday of the 3rd week of each month.
UNO - order when required
Consumables - order when required

### Specimen Transport and Storage
Samples for molecular HPV detection must be sent to the laboratory in accordance with postal or courier regulations. All samples (original and extract) are stored in accordance with the specimen retention policy and stored in freezer 20 Room 3.17.

### Specimen Reception
Non Cervical Screening Programme (CSP) samples often require a genotyping test rather than a high-risk HPV screen. Samples received include biopsies, Paraffin wax sections, swabs, Surepath and Thinprep. All samples except swabs taken in charcoal, blood and requests for cutaneous HPV typing can be tested for HPV using the PapilloCheck test.

### Other Urology requests which require HPV genotyping
- **Paraffin wax sections**
- **Biopsies**
- **Head and neck samples (throat swabs, oral swabs etc)**
- **Ocular samples**
- **Nasal samples**
- **Anal swabs**
- **Vulva swabs**
- **Vaginal swabs**
- **Penile swabs**

Clinical details ?? genital warts, ?? laryngeal papillomatosis
Sample Preparation

Specimen Processing Procedures

Octane, 85% ethanol and Proteinase K lysis buffer are stored in the -20°C freezer (no. 30) in room 3.17. Stocks of Octane and ethanol are stored in the flammable cabinet in room 3.15.

Preparation of Proteinase K lysis buffer stocks (when stocks are down to 1 vial let senior RMS know):

- 10mM Tris/1mM EDTA
- 0.5% Triton X 100
- 260 g/ml Pro K

Add 500ml 10mM Tris/1mM EDTA to 0.5ml of 0.5% Triton X 100 to 1ml of Pro K at 100mg/ml

A. Paraffin wax sections

- Wax sections should be approximately 4μ thickness with a minimum of 5 sections in a tube. (N.B. If wax blocks are received contact the sending laboratory and arrange for the sample to be returned in sections). Processing must be carried out in the cabinet in room 3.15.

- Switch on the hot block in room 3.17 and check temperature is set at 56°C
- Transfer sections to a 1.5ml tube (alternatively add octane directly to the sample and transfer using a pipette to a 1.5ml tube)
- De-wax sections by the addition of 1ml of Octane and 75ul of methanol (optional) and mix by inverting 5 times.
- After mixing, centrifuge at 7,000rpm for 30 sec.
- Remove the Octane and methanol layers with a fine-tipped Pasteur pipette being careful not to disturb the biopsy.
- Wash the tissue by the addition of 1ml of 0.6% ethanol
- Centrifuge at 7,000rpm for 30 sec
- Remove ethanol layers with a fine-tipped Pasteur pipette being careful not to disturb the biopsy
- Leave tube open at 56°C for 1 hour to dry the residual tissue pellet.
- Following this process digest the tissue in Proteinase K lysis buffer (volume of lysis buffer decided empirically depending on the size of the biopsy)
- Digest in a hot block for 48-72 hours.
- Extract DNA using the BioMerieux EasyMag system (MMMP-MOL-PROC26). Input volume 250ul and elution volume 100ul. Alternatively samples can also be extracted with the routine samples on the Abbott M2000sp (MMMP-MOL-PROC04): Input volume 500ul and elution volume 25ul. Once samples have been extracted on the Abbott system transfer extracted samples to 1.5ml tubes and store at -20°C in room 3.17 (to be tested genotyping tray).

b. Biopsy samples
Processing must be carried out in the cabinet in room 3.15
- Using disposable scissors (new scissors for each sample) cut a section from the biopsy and place into a 1.5ml tube.
- Digest the tissue in Proteinase K lysis buffer (volume of lysis buffer decided empirically depending on the size of the biopsy)
- Digest in a hot block for 48-72 hours.
- Extract DNA using the BioMerieux EasyMag system (MMMP-MOL-PROC26). Input volume 250ul and elution volume 100ul. Alternatively samples can also be extracted with the routine samples on the Abbott M2000sp (MMMP-MOL-PROC04): input volume 500ul and elution volume 25ul. Once samples have been extracted on the Abbott system transfer extracted samples to 1.5ml tubes and store at -20°C in room 3.17 (to be tested genotyping tray).

c. Swabs in transport medium

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250ul of transport medium is transferred directly to the EasyMag tray for extraction and eluted in 100ul (MMMP-MOL-PROC28); alternatively 500ul transferred to the Abbott tubes and put on the M2000sp instrument (MMMP-MOL-PROC04). Once samples have been extracted on the Abbott system transfer extracted samples to 1.5ml tubes and store at -20°C in room 3.17 (to be tested genotyping tray).

d. Dry swabs

Add 1ml of distilled water to a dry swab and treat as a swab in transport medium.

e. Thinprep

Vortex sample and add 250ul for extraction on the EasyMag and elute in 100ul (MMMP-MOL-PROC28); alternatively 500ul transferred to the Abbott tubes and put on the M2000sp instrument (MMMP-MOL-PROC04). Once samples have been extracted on the Abbott system transfer extracted samples to 1.5ml tubes and store at -20°C in room 3.17 (to be tested genotyping tray).

f. Serafale (one or post coat treated the same)

- Vortex sample and aliquot 500ul into a 2ml tube and spin down.
- Tip off supernatant
- Resuspend pellet in 500ul of PK lysis buffer
- Heat 60°C for 15-30 mins
- Add 250ul for extraction on the easyMag and elute in 100ul

Alternatively (preferred option) add 500ul to the Abbott tubes and put on the M2000sp instrument. Once samples have been extracted on the Abbott system transfer extracted samples to 1.5ml tubes and store at -20°C in room 3.17 (to be tested genotyping tray).

6.0 PeptideCheck Procedure

General information

- All components in the kit must be stored dry at 4-8°C and protected from light.
- Liquid components should be mixed well before use. Precipitation can occur in the Papillocheck Hybridization Buffer and Buffer B. To dissolve the precipitate, incubate the buffer at room temperature.
- DNA chips should be used in a dust-free environment. The deposition of dust and other particles on the chip surface must be prevented. Avoid direct skin contact with the hybridization zone on the chip surface.
- Do not use any marker pens for the identification of DNA chips as they lead to unspecific fluorescence on the chip. Store chips protected from light.
- Worksheet code MHVG (Check with senior DSM if all samples require genotyping)

1. Amplification – book thermocycler (#5) in advance

Thermocycler set up

The PapilloCheck test has been validated with the following thermal cyclers:
- GeneAmp PCR system 9700 (Applied Biosystems)
- Veriti 96-well Thermocycler (Applied Biosystems)

The thermal cycler program is summarised in Table 2

Table 2: Thermocycler program

<table>
<thead>
<tr>
<th>TIME</th>
<th>TEMP</th>
<th>NUMBER OF CYCLES</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 min</td>
<td>37°C</td>
<td>1</td>
</tr>
<tr>
<td>15 min</td>
<td>60°C</td>
<td>1</td>
</tr>
<tr>
<td>30 sec</td>
<td>55°C</td>
<td>40</td>
</tr>
<tr>
<td>25 sec</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>15 sec</td>
<td>55°C</td>
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</tr>
<tr>
<td>30 sec</td>
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<td>15</td>
</tr>
<tr>
<td>45 sec</td>
<td>72°C</td>
<td></td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td></td>
</tr>
</tbody>
</table>
In addition, the following run parameters must be set for each thermocycler.

**GeneAmp PCR system 9700** - Set the reaction volume at 26ul and the ramp speed to '1000'.

Veriti 96-well thermocycler – use the convenient tool of the Veriti 96-well thermocycler to enter the PipploCheck PCR program and choose the 0800 emulation mode. Set the reaction volume to 26ul and the temperature of the lid to 103°C.

7.0 PCR reaction setup

Special precautions must be taken to avoid contamination during this procedure. PCR is performed in a total volume of 26ul using 0.2ml thin walled PCR reaction tubes.

In the clean room the Taq polymerase and UNG are stored in freezer 3 shelf 4. Prepare the reaction mix for the required quantity of PCR reactions as outlined in Table 3. Use one vial of MasterMix for the reactions of one chip.

Mix the reaction mix thoroughly by either vortexing for 2 seconds and then spinning down or by pipetting up and down several times.

Aliquot the reaction mix by pipetting 21ul of the reaction mix for each PCR reaction into 8 x 0.2ml PCR strips.

**Table 3: Reaction set up**

<table>
<thead>
<tr>
<th></th>
<th>1 reaction</th>
<th>15 reactions (1 chip)</th>
<th>23 reactions (2 chips)</th>
<th>39 reactions (1 chip)</th>
<th>52 reactions (4 chips)</th>
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<tbody>
<tr>
<td>PapiloCheck MasterMix</td>
<td>10.8ul</td>
<td>257.4ul</td>
<td>514.8ul</td>
<td>772.2ul</td>
<td>1020.6ul</td>
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<td>HotStar Taq DNA polymerase (5 U/ul)</td>
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<td>2.0ul</td>
<td>5.2ul</td>
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<td>Urali DNA Glycosylase 1Ul (1:200 – 0.005 U/ul)**</td>
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<td>13ul</td>
<td>25ul</td>
<td>30ul</td>
<td>52ul</td>
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<td>Total volume per reaction</td>
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<td>27ul</td>
<td>27ul</td>
<td>27ul</td>
<td>27ul</td>
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</tbody>
</table>

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**To prepare UNG dilute 1:200 in PCR-grade water (2ul UNG + 198ul DW). Use a fresh UNG dilution for each papilloCheck PCR reaction set up. Do not reuse the diluted UNG. Mix the UNG dilution carefully by either vortexing for 2 seconds and then spinning down or pipetting up and down several times.**

Carry out the addition of the template DNA in room 3.17.
Add 5ul of DNA extract to each PCR reaction and mix by pipetting up and down several times. The total volume of one PCR reaction is 26ul.
Place the reaction tubes in the thermal cycler and start the PCR reaction using the thermal cycler program described in Table 2.
After the PCR has been completed, the amplification products must be stored in the dark at -20oC or hybridized directly.

8.0 Hybridisation and washing (Molecular lab)

**Preparation of washing buffer**

Hybridisation must be performed at room temperature (20-25oC).
To dissolve potential precipitates in the washing buffers, expose them to room temperature for 30 minutes and mix well before use.
Prepare the check Hybridisation Chamber; put a fresh wet paper towel into the hybridisation chamber and close the lid to create a humidity-saturated atmosphere. Incubate the required amount of chips in the prepared Hybridisation Chamber at room temperature for at least 10 minutes.
Prepare the washing solutions I, II and III appropriate for the number of chips being analysed as shown in Table 4

**Table 4 Preparation of the washing solution mix**

<table>
<thead>
<tr>
<th>components</th>
<th>1 chip</th>
<th>2 chips</th>
<th>3 chips</th>
<th>4 chips</th>
</tr>
</thead>
</table>

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Aliquot three equal volumes of the washing solution mix into three separate eCheck washers up to the correct engraved scale for the number of chips being tested (Figure 1).

Preheat washing solution II to 50°C in a temperature controlled water bath for at least 20 minutes prior to use. Ensure that the fill level of the water bath equals the fill level of the washing solution II.

5.4 Hybridisation

Briefly spin down the PCR products before use (if PCR products were stored at -20°C until hybridisation, first thaw PCR products before mixing).

Vortex the hybridisation buffer before use. Briefly spin down.

Mix 30ul of the hybridisation buffer in a fresh reaction tube of an 8x PCR strip with 5ul of the PCR product by pipetting up and down several times

Briefly spin down.

Transfer 25ul of the hybridisation mix into each chip well by using six channels of a multichannel pipette. Avoid air bubble formation. If bubbles do form leave until after hybridisation.

Incubate the chip for exactly 10 minutes at room temperature within the prepared hybridisation chamber in a dark, humidity-saturated atmosphere. Be careful not to move the hybridisation chamber during the hybridisation.

Figure 1
Washing and drying

Carefully remove the magnetic slideholder containing the hybridised slides from the hybridisation chamber.
Drop the slideholder containing the slides directly into the oCheck washbox containing washing solution 1. Ensure the magnetic slide is facing up (Figure 2).
Attach the oCheck handle to the slideholder and begin the first of three washing steps.
Wash the chip at room temp (20-25°C) in washing solution 1 by moving it quickly up and down for 10 seconds. The arrays must stay covered with washing solution at all time. NB: Avoid the chip surface to run dry!
Wash the chip for a further 60 seconds in washing solution II at 50°C by vigorously moving up and down.
Wash the chip in washing solution III at room temperature by moving it quickly up and down for 10 seconds.
Immediately remove any liquid from the chip surface by centrifugation using the special microcentrifuge for microarrays, centrifuge for 1 minute (Figure 3). The chip is now ready for scanning and should be scanned immediately. For cleaning of the oCheck washboxes, rinse several times with distilled water.
Figure 2

Figure 3

10. Scanning and evaluation
- Setting up the samplesheet

Click on 'Check report sample sheet' on desktop
Login (virology: virology1)
New
Enter barcode
Click on A1 and enter sample ID
Click on A2 and enter sample ID etc
For negative control tick the negative control box
In samplesheet functions save, print and close

- Scanning slides (Figure 4)

Turn on scanner
Click on 'Check report results' on desktop
Login (virology: virology1)
Place slide in scanner as shown in fig 4 (if scanner is not in the correct position for scanning turn scanner on and off)
Start scan

Figure 4
Generate report
Open tests
OK
Tick valid results
Create reports
Do you want to continue? Click yes
Print reports
Turn off scanner
Close down and exit
Technical Assistance

Dirk Leinberger
Greiner bio-one
Phone +49 (0) 7022-948-306
Dirk.Leinberger@gbo.com

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11. Andy Ford

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Mobile Phone +44 (0)7080967002
Switchboard +44 (0)1453 925255
Fax Number +44 (0)1453 820200
HAEMATOXYLIN AND EOSIN

For Frozen / Wax sections

Principles:
The haematoxylin stains the cell nuclei, while the eosin stains cell cytoplasm and most connective tissue fibres. For further information refer to Bancroft and Gamble.

Solutions
Both solutions are brought in commercially and need no preparation

Cellobiose Harris haematoxylin.
Lecia alcoholic Eosin

SAFETY PRECAUTIONS:
Haematoxylin Combustible. May evolve toxic fumes in a fire. May be harmful by ingestion. Stains. May irritate eyes and skin.
Eosin Combustible. May evolve toxic fumes in a fire. May be harmful by ingestion. Stains. May irritate eyes and skin.

If spillage occurs:- Use spillage granules and mop up cautiously with plenty of water and run to waste, diluting greatly with running water.

If contact occurs:-
Eyes Irrigate thoroughly with water for at least 10 minutes. Seek medical attention.
Lungs Remove from exposure. In severe cases seek medical attention.
Skin Drench the skin thoroughly with water. Remove contaminated clothing and wash before re-use. Seek medical attention.
Mouth Wash out thoroughly with water and drink excess water. Seek medical attention.

Protective measures
Ventilation: AFOS bench
Gloves: Nitrile
Others: Laboratory coat

A control is not required.

Method: For frozen sections
First fix sections in IMS for 1-2 min.
1) Wash in water
2) Sections stained in Haematoxylin approx. 30 sec
3) Wash in water
4) Blue in Scott’s tap water
4) Wash in water
5) Counter stain with Eosin solution approx. 15 sec
6) Wash in cold tap water
7) Dehydrate, clear and mount

Method: For wax sections
1) Place sections in xylen to remove wax upto 3 mins
2) Wash sections in IMS to remove xylen upto 1 min
3) Take sections to water
4) Stain sections in Haematoxylin 6 min
5) Wash in running water
6) Quickly differentiate background tissue in 1% Acid alcohol (control microscopically)
7) Wash in running water
8) Blue in Scott’s tap water 30 secs
9) Wash in running water
10) Stain sections in Eosin solution 30 secs-1 min

CONTROLLED DOCUMENT
PHOTOCOPIES ARE UNCONTROLLED
(check staining microscopically)

11) Wash in running water
12) Dehydrate, clear and mount

Results:
Nuclei—Blue/black
Cytoplasm—Varying shades of pink
Muscle fibres—Pink/red (deep)
Collagen—Pink/red (Pale)
RBC’s—Orange/red
Fibrin—Deep pink
ANTIGEN RETRIEVAL METHODS FOR MANUAL IMMUNOHISTOCHEMISTRY

PRINCIPLE:
Antigen retrieval methods unmask the antigens that have been concealed by the negative effects of formalin fixation. Formalin based fixatives in particular result in cross-links between proteins within the tissue which interferes with antigen-antbody binding in immunohistochemical staining techniques. Antigen unmasking is therefore essential to unmask the antigens by breaking these chemical cross links. Heating the tissue sections or enzyme digestion causes the break up of formalin cross links that are believed to interfere with antigen-antbody binding.

SAFETY PRECAUTIONS:
Spillages: see Departmental Safety Policy
Waste Disposal: see Departmental Waste Disposal Policy

PROTECTIVE MEASURES:
1. Appropriate PPE must be worn at all times.
2. Thermal gloves MUST be worn whenever hot solution(s) are being handled (thermal gloves are kept next to the microwave in research lab).
3. Metal objects MUST NOT be microwaved.

INSTRUCTIONS FOR PROTEASE DIGESTION ON SEQUENZA
1. Place sections in a black plastic staining rack and dewax in 3 changes of xylene, 5mins in each. Then rehydrate the sections by placing the rack in 3 changes of IMS (Industrial Methylated Spirit), 5mins in each and finally wash the rack of sections in running tap water for 2 mins.
2. Rinse sections in TBS, draining off any excess.
3. Load slides on the Sequenza (See Sequenza SOP).
4. Add 100µl of protease to each slide for 10 minutes.
5. Rinse off in buffer.
INSTRUCTIONS FOR MICROWAVE ANTIGEN RETRIEVAL:

1. Place sections in a black plastic staining rack and dewax in 3 changes of xylene, 5mins in each.
   Then rehydrate the sections by placing the rack in 3 changes of IMS (Industrial Methylated Spirit), 3mins in each and finally wash the rack of sections in running tap water for 2 mins.
2. Place 100ml of water into the appropriate glass Pyrex dish.
3. Add 10ml of Vector Antigen Unmasking Solution
4. Place sections into a plastic rack ensuring there is an empty space between each section and then place into the glass dish.
5. Place into the microwave. Press CLEAR button and input 25 min then press START.
6. Once complete open the door and leave to cool for 10min.
7. Once cooled, wearing thermal gloves move the dish to the sink and run under cold water for 2 min.
8. Slides are now ready to load onto the Sequenza (See Sequenza SOP).

INSTRUCTIONS FOR ANTIGEN ACCESS UNIT (AAU)

1. Place sections in black plastic staining rack and dewax in 3 changes of xylene, 5mins in each. Then rehydrate the sections by placing the rack in 3 changes of IMS (Industrial Methylated Spirit), 3mins in each and finally wash the rack of sections in running tap water for 2 mins.
2. Plug the Antigen Access Unit into appropriate power source.
3. Place the rubber reel into the groove around the top of the pan.
4. Place the pan into the Antigen Access Unit shelf (body), aligning their handles.
5. Pour 500ml of distilled water into the pan.
6. Pour 250ml of the desired antigen retrieval buffer into white plastic pots.
7. Place Tissue Tek racks (grey racks) into the white plastic pots.
8. Place slides into Tissue Tek racks (grey racks), do not let the slides to dry out.
9. Place the lid onto the pan by matching the word "OPEN" on the lid to the white dot on the left side handle of the unit.
10. Secure the lid by pressing the pan handle down whilst turning the lid clockwise until the word "CLOSED" is matched with the white dot on the side handle.
11. Ensure that the lid is fully turned and that the metal tabs on the lid are tightly seated against the pan's lid before proceeding.

12. Place the pressure weight in position onto the top of the pan.

13. Turn the instrument on by flipping the red toggle on the right of the control panel.

14. Then press the "Start/Stop" button to initiate the pre-set program.

15. The hot sticker will display a warning when the outside of the unit is hot.

16. Once the program is complete the unit will alarm and a "Cycle done" message will be displayed.

17. Observe that the pressure gauge red hand has reached the required pressure (15psi).

18. Visually confirm that the pressure has dropped to 0 psi.

19. Turn the unit off using the red toggle at the side of the control panel.

20. Ensure that the pressure petcock on the top of the unit is down; using forceps toggle this until it lowers if there is residual pressure.

21. Open the lid by applying weight and turning it left. Ensure that the steam is directed away from you.

22. Carefully remove the plastic pots from the unit quickly placing them straight to a bucket full of cold water to stop the retrieval.

23. Transfer the bucket with the pot to a sink and run cold water for 2 minutes.

24. The slides are now ready to be loaded onto the Sequenza (please see Shandon Sequenza SOP).
Antigen Retrieval Solution Options

Vector Antigen Unmasking Solution (High pH) – use at a 1+100 dilution

Vector Antigen Unmasking Solution (Low pH) (Citrate) – use at a 1+100 dilution

Access Super RTU - Purple High pH (9.0-10.0)
This has an inbuilt deparaffinising agent and so section deparaffinization can be ignored. It also contains a pH indicator turning from purple to gray. If the colour turns to yellow or red then this indicates that the pH of the solution is incorrect.

Access Revelation - Orange Lower pH (6.0) – used at a 1 in 10 dilution.
This has an inbuilt deparaffinising agent and so section deparaffinization can be ignored. It also contains a pH indicator. If the colour turns to deep yellow to indicate the pH is correct.
8.29 Appendix xvii

Ki67 / p16 Dual IHC Staining

Antibody Details

The identification and detection of Ki67 antibody was performed using Ki67 MIB-1 mouse monoclonal antibody, 0.2ml Dako (M724029-2), RTU 50 test, Ventana, ULTRAview DAB Detection System, Ventana, 760-500 (Brown). The identification and detection of p16 CINtec antibody was performed using CINtec p16, 805-4713 Detection Systems Ventana, ULTRAview AP Detection System, Ventana, 760-501 (Red).

Detection Systems

ULTRAview DAB Detection System, Ventana, 760-500 (Brown)

ULTRAview AP Detection System, Ventana, 760-501 (Red)

Staining Platform

Automated Ventana BenchMark XT IHC/ISH Staining Module (Ventana Co., Tucson, AZ, USA)

Protocol

Tissue sections (4 µm) were deparaffinised and incubated in EZ Prep Volume Adjust (Ventana Co.). At intervals between steps the slides were washed with a TRIS-based Reaction Buffer, pH 7.6. A heat-induced antigen retrieval protocol (30 min) was carried out using a TRIS– ethylenediamine tetracetic acid (EDTA)–boric acid pH 8 buffer (Cell Conditioner 1), (Ventana Co.). The slides were then incubated in antibody to Ki67 MIB-1 (Dako, M724029-2) at a concentration of 1:100 for 40 minutes at room temperature. Bound antibody was visualised using the ULTRAview DAB Detection System (Ventana Co.), producing a brown chromogen. The slides were then denatured at 95°C for 4 minutes and then incubated in antibody to CINtec p16 (Ventana Co, 805-4713) at a concentration of 3:1 (RTU antibody:diluent) for 40 minutes at room temperature. The bound antibody was visualised using the ULTRAview AP Detection System (Ventana Co.), producing a red chromogen. Slides were then counterstained in Haematoxylin II (Ventana Co.), which was applied for 4 min before an incubation of 4 min with Bluing Reagent (Ventana Co.). Slides were then removed from the machine and washed in warm soapy water. They were then dehydrated in three changes of 100% IMS, followed by three changes of Xylene. They were then coverslipped using a xylene based mountant.
8.30 Appendix xviii – xxb

### Appendix xvii

**Table a: HPV positive MSM specimens displaying grade of AIM, HR-HPV status by Roche Cobas 4800® and PapilloCheck®**

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Study no</th>
<th>Grade of disease</th>
<th>Cobas HPV status</th>
<th>Other</th>
<th>16</th>
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<th>PapilloCheck®</th>
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### Appendix xvii

**Table b:** HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck®

<table>
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<th>Sample no</th>
<th>Study no</th>
<th>Grade of disease</th>
<th>Cobas HPV status</th>
<th>Other</th>
<th>16</th>
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### Appendix xvii

**Table c: HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapiloCheck®**

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<tr>
<th>Sample no</th>
<th>Study no</th>
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<th>Cobas HPV status</th>
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### Appendix xvii d

#### Table d: HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck®

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## Appendix xvii

### Table e: HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800<sup>®</sup> and PapilloCheck<sup>®</sup>

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<tr>
<th>Sample no</th>
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<th>Cobas HPV status</th>
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<th>18</th>
<th>PapilloCheck&lt;sup&gt;®&lt;/sup&gt;</th>
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## Appendix xvii f

### Table: HIV positive MSM specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck®

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<th>Study no</th>
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Table 1: Transplant Recipient specimens displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck®

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<td>NEG</td>
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</table>

The highlighted first two rows on the Table demonstrate that two specimens came from one patient at two separate visits attendances with the ANALOGY study.
## Appendix xx

### Table a: Repeat Biopsy specimens from HIV positive MSM displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck®

from visit one and visit two attendances with the ANALOGY study

<table>
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<th>Cobas HPV status</th>
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</tr>
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<td>POS</td>
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Table b: Repeat biopsy specimens from HIV positive MSM displaying grade of AIN, HR-HPV status by Roche Cobas 4800® and PapilloCheck® from visit one and visit two attendances with the ANALOGY study

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<th>Study no</th>
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<th>Cobas HPV status</th>
<th>Other</th>
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<th>18</th>
<th>PapilloCheck®</th>
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<td>POS</td>
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<td>11,42</td>
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