HPV Primary Screening Pilot Study: molecular testing of potential triage strategies for HPV-positive women.

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Declaration

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Stephen Reynolds

For Tadhg Mac Giolla Riogh, Grandad Extraordinaire.

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Summary

Cytological based screening programmes are being phased out in favour of HPV-based screening both in Ireland and internationally. Infection with HPV is the primary aetiological agent in the development of cervical cancer. Screening for the virus through HPV testing is more sensitive than our current cytological approach. However, due to high rate of transient HPV infection, HPV DNA testing for cervical cancer or precancer suffers from a lower specificity. Several possibilities exist to improve specificity including the use of more specific tests such as HPV E6/E7 mRNA detection or the use of triage tests such as methylation testing of specific markers for HPV positive women. Evidence has showed that methylation testing may be a superior triage test compared to cytology triage.

The project aimed to assess the prevalence of HPV prevalence in the Irish population using the Cobas 4800 HPV DNA test and the Aptima HPV E6/E7 mRNA test on a population of over 12,000 women attending for their routine cervical smear test and compare both platforms for their overall performance in the context of screening this population. The population prevalence of HPV DNA was 15.98% compared to 13.22% for HPV mRNA. Both platforms had comparable sensitivities for CIN 2+ (96.26% and 96.55%), as well as a reasonable level of agreement between each other (kappa: 0.701). HPV mRNA however, had a higher specificity for detection of CIN 2+ (76.42%) in women <30 compared to HPV DNA testing (70.82%), due in part to the lower number of cytology normal women with a positive HPV mRNA test. Overall, both platforms were comparable and viable for a HPV primary screening population however, both would require adequate triage testing of HPV positive women. Cytology will likely be the initial triage test for HPV positive women. However, cytology will still suffer from a low specificity for detection of CIN 2+ as well as variability.

An alternative approach to triaging women using methylation markers was assessed in this study. The aim was to validate a three-marker methylation panel (CAD M1-M18, MAL M1 and hsa-mir-124-2), using standard qMSP techniques and determine a clinically relevant threshold for positive methylation for detection of CIN 2+. These individual methylation markers were investigated in several different and novel combinations. Two such combinations were termed the Total Methylation Score and the Paired Methylation Score. Cut-off points were determined for each individual methylation marker, as well as for the Total and Paired Methylation Score by ROC curve analysis. From this, the Total Methylation Score had a sensitivity and specificity of 78.57% and 80.00% for detection of CIN 2+. The Paired Methylation Score performed well with a sensitivity and specificity of 71.43% and 76.00%. Of the three individual methylation markers, hsa-mir-124-2 had the highest

individual performance with a sensitivity and specificity of 66.67% and 78.00% respectively. These three methylation approaches were brought forward to be assessed in a HPV positive triage population.

When applied to the HPV positive population, the Paired Methylation Score had the best performance with a sensitivity of 59.26% and specificity of 66.80%. The combination of HPV 16/18 genotyping with methylation testing of the other hrHPV types increased the sensitivity to 80.56% for both the Total and Paired Methylation Scores. Referral rates for each of the three methylation approaches were comparable to the referral rates of cytology (37.76% to 41.59% vs 38.27% respectively). The use of HPV 16/18 genotyping with methylation or cytology showed comparable referral rates also. Only the Paired Methylation Score had sufficient risk following a positive test to refer women to colposcopy in this study (>20%). However, in women <30 years the PPV increased sufficiently for all three approaches to safely refer women on to colposcopy. None of the methylation approaches with or without HPV genotyping were able to safely refer women back to the routine recall population. However, the Paired Methylation Score, in women >45, had a reduced negative triage test risk of 2.9%. Though no triage approach tested here reached the <2% cut off for the safe return to routine screening, the majority of other triage tests including cytology triage fail to do so also.

In conclusion, HPV DNA and mRNA have been shown to have both a comparable sensitivity, specificity and PPV with an NPV of >99%. However, in women <30 years, HPV mRNA testing is more specific than HPV DNA testing and overall refer fewer women for triage. Methylation triage testing has been shown to be both sensitive and specific for detection of CIN 2+ in a HPV positive population with comparable referral rates to cytology. The combination of HPV 16/18 genotyping with methylation testing greatly increases the sensitivity for detection of CIN 2+. In women <30 methylation testing has been shown to be more sensitive, while in women >45 a negative methylation test imparts a higher NPV which approaches the threshold for returning women safely to routine screening. The use of methylation testing shows great potential in the triage of HPV positive women.

Abbreviations

?SCC	Queried Squamous Cell Carcinoma (cytology)
°C	Degrees Celsius
A	HPV Amplification Reagent
A/D	Aspirate/dispense
ACTB	β-actin
AdoMet	S-Adenosylmethionine
AGC	Atypical glandular cells
AIS	Adenocarcinoma In Situ
AR	HPV Amplification Reconstitution Solution
ARTISTIC	A Randomised Trial in Screening to Improve Cytology
ASC-H	Atypical Squamous Cells but cannot exclude high grade
ASC-US	Atypical Squamous Cells of Undetermined Significance
ASR	The Age Standardised (World) Rate
ATHENA	Addressing the Need for Advanced HPV Diagnostics
AUC	Area Under the Curve
CAD M1-M18	Cell Adhesion Molecule (1/18)
CERVIVA	The Irish cervical screening Research consortium
cGIN	Cervical Glandular Intraepithelial Neoplasia
CI	Confidence intervals
CIN	Cervical Intraepithelial Neoplasia
CmPV1	Animal specific papillomavirus
CPV1	Animal specific papillomavirus
CWIUH	Coombe Women & Infants University Hospital
DAB	3,3′-diaminobenzidine tetrahydrochloride
DKA	Dual Kinetic Assay
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase Enzyme
dsDNA	Double stranded DNA
DWP	Deepwell Plate
E	Early Region
E5-AP	E6 associated protein
EB	Elution Buffer
EMT	Epithelial-mesenchymal transition
ER	Endoplasmic Reticulum
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration

GAST	German AHPV Screening Trial
H&E	Haematoxylin and eosin
HC2	Hybrid Capture 2
HDI	Human Development Index
HIQA	Health Information and Quality Authority
HPA	Hybridization Protection Assay
HPV	Human Papilloma Virus
HPV MMX	HPV Master Mix
hrHPV	High risk HPV
HSE	Health Service Executive
HSIL	High Grade SIL
HTA	Health technologies assessment
IARC	International Agency for Research on Cancer
IC	Internal Control
ICC	Invasive Cervical Cancer
ICGP	Irish College of General Practitioners
k	Kappa statistic
L	Late Region
LBC	Liquid Based Cytology
LCR	Long Control Region
LLETZ	Large Loop Excision of the Transformation Zone
LOD	Limit of Detection
LR	Likelihood Ratio
LSIL	Low Grade SIL
LYS	System lysis Buffer
MAL	Myelin lymphocyte proteolipid
MEM	Minimum Essential Medium
MGP	Magnetic Glass Particles
miRNA	micro RNA
MMLV	Moloney Murine Leukaemia Virus
mRNA	messenger RNA
MTU	Multi Tube Unit
n	Number
N:C	Nuclear to cytoplasmic ratio
NAD	No abnormality detected (cytology)
NC	HPV Negative Control
NcACTB	Non-Methylation Specific β-Actin
NCAL	HPV Negative Calibrator

NCBI	National Center for Biotechnology Information
NCRI	National Cancer Registry Ireland
NPV	Negative Predictive Value
NTCC	New Technologies in Cervical Cancer Trial
OR	Odds Ratio
ORF	Open Reading Frame
ORI	origin of replication
P	HPV Probe Reagent
p53	tumour protein 53
PAP	Papanicolaou test
PaVDaG	Papillomavirus Dumfries and Galloway
PBS	Phosphate-buffered saline
PC	HPV Positive Control
PCAL	HPV Positive Calibrator
PCR	Polymerase chain reaction
PK	Proteinase K
POBASCAM	Population-based Screening Study Amsterdam
PPV	Positive Predictive Value
PR	HPV Probe Reconstitution Solution
pRb	Phosphorylated Retinoblastoma protein
PSQ	Pyrosequencing
qMSP	Methylation Specific PCR
RCT	Randomised Control Trial
RLU	Relative Light Units
ROC	Receiver Operating Characteristic
S/CO	Signal to cut-off value
SCC	Squamous cell carcinoma
SCJ	Squamocolumnar Junction
SIL	Squamous Intraepithelial Lesions
SPSS	Statistical package for the social sciences
ssDNA	Single stranded DNA
TCR	Target Capture Reagent
TET	Ten-eleven translocation enzyme
TMA	Transcription mediated amplification
TOC	Test of cure
TS	Triage Strategy
TZ	Transformation Zone
URR	Upstream Regulatory Region

WB	System Wash Buffer
VM	Vasculogenic mimicry

Publications

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-Chapter 1-

General Introduction

1.1. Burden of Cervical Cancer and Pre-Cancer

Worldwide cervical cancer is the fourth most common cancer in women and being the eight most common cancer overall (Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, 2018). Though the incidence of cervical cancer is high, it varies widely amongst differing regions with the majority of cases occurring in poorly developed regions as shown in Figure 1.1.

In 2018, there were an estimated 311,365 deaths from cervical cancer worldwide. This accounted for 7.5% of all female cancer deaths and 3.3% of all cancer deaths. The Age Standardised (World) Rate (ASR) for mortality due to cervical cancer is proportionally higher in low Human Development Index (HDI) Countries (ASR per 100,000; 23.0) compared to Medium, High or Very High HDI rated countries (ASR per 100,000; 9.5, 4.9, 3.0). Naturally, mortality also varies between the different regions of the world, with rates ranging from less than 2 per 100,000 in Western Asia, Europe, Australia and New Zealand to greater than 10 per 100,000 in the Middle, Southern, Western and Eastern Africa (Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, 2018).

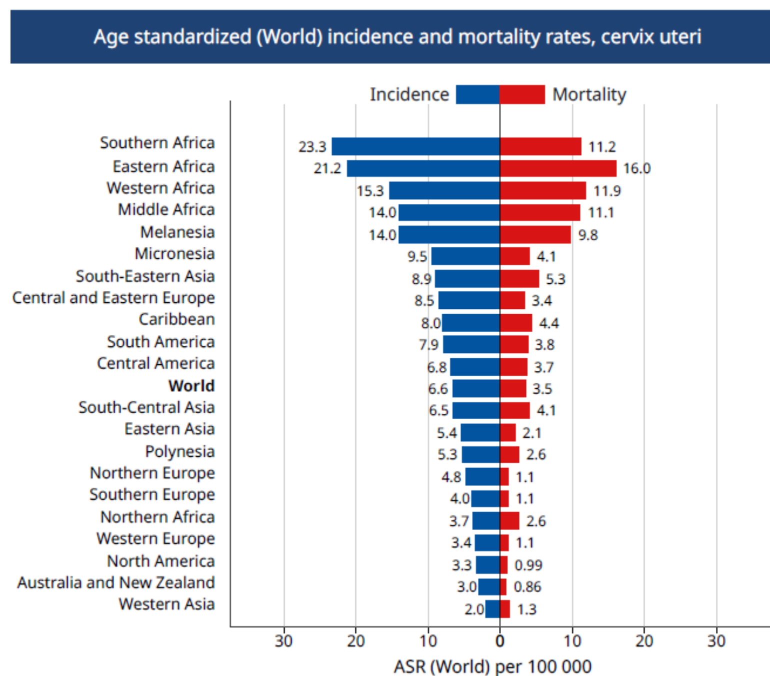

Figure 1.1: Estimate age standardised rates per 100,000 (Ferlay J, Ervik M, Lam F, Colombet M, Mery L, Piñeros M, Znaor A, Soerjomataram I, 2018)

In Ireland, according to the latest National Cancer Registry Ireland (NCRI) figures, the incidence rate of invasive cervical cancer is 11.5 per 100,000 cases and a rate of 115.1 per 100,000 cases for in-situ cancers with a 1 in 13 lifetime risk for an in-situ cancer (data collated from 2012-2014). Mortality rates in women with invasive cancer (2011-2013) is 3.8 per 100,000 cases (National Cancer Registry, 2017b). Overall, this sets invasive cervical cancer as the 8th most common cancer type in Ireland and accounts for 2.2% of all cancer deaths, the 12th most common cause in Ireland. The 5 year net survival (diagnosed: 2009-2013) is 61% (95% CI: 57.5-64.5%) (National Cancer Registry, 2017b). During the period

of 1994 to 2015, the proportions of invasive cervical cancers classified as squamous cell carcinoma was 77%, 16% were classified as adenocarcinoma with the remaining 7% consisting of other histological subtypes. During this period, the average age of diagnosis for carcinoma in-situ was between 25 and 29 years (incidence rate of 734 per 100,000), and 47% of invasive cervical cancers were diagnosed in women under the age of 45. The majority of cervical cancers are diagnosed as early stage cancers (stage 1) with 41% being stage 1 between 1994 to 1998. This increased to 47% between 2009 to 2013 which corresponds to the introduction of the National Cervical Sreening Service (National Cancer Registry, 2017a).

Cytological screening for cervical cancer was implemented in 2008 with CervicalCheck, The National Cervical Cancer Screening Service. Since then it has proven to be highly successful in reducing the incidence of cervical cancer in Ireland. As shown in Figure 1.2 since 2008, there has been a decrease in the rates of cervical cancer in Ireland. The initial spike in the incidence of cervical cancer in 2009 related to the increased rate of detection of cervical cancer not an increase in the actual cancer rate itself. Since 2009 onwards, however, we see the payoff from the screening programme as the incidence rate of cervical cancer decreased and has continued to decrease (National Cancer Registry, 2017a). While the rates of cervical cancer decline, the rates of non-invasive pre-cancerous lesions have increased since 2008 due, again, to the screening programme. From 2008 to its peak in 2011 high-grade precancer [CIN2+] was detected and treated before they could progress to an invasive cancer (Figure 1.3) as women participating in the programme had lesions detected earlier. Since 2011, the rates of both in-situ and invasive cancer has been steadily declining due to the screening programme.

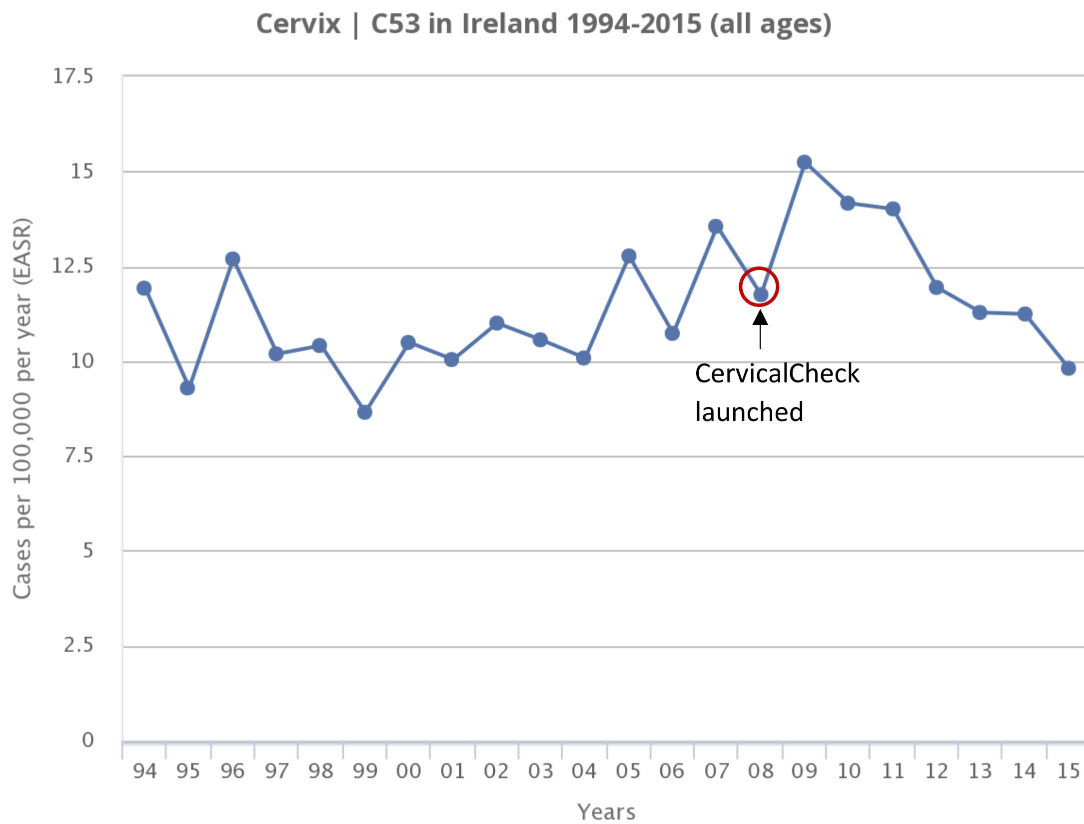

Figure 1.2: Cervical cancer incidence rates 1994-2015 (National Cancer Registry Ireland, 2018b)

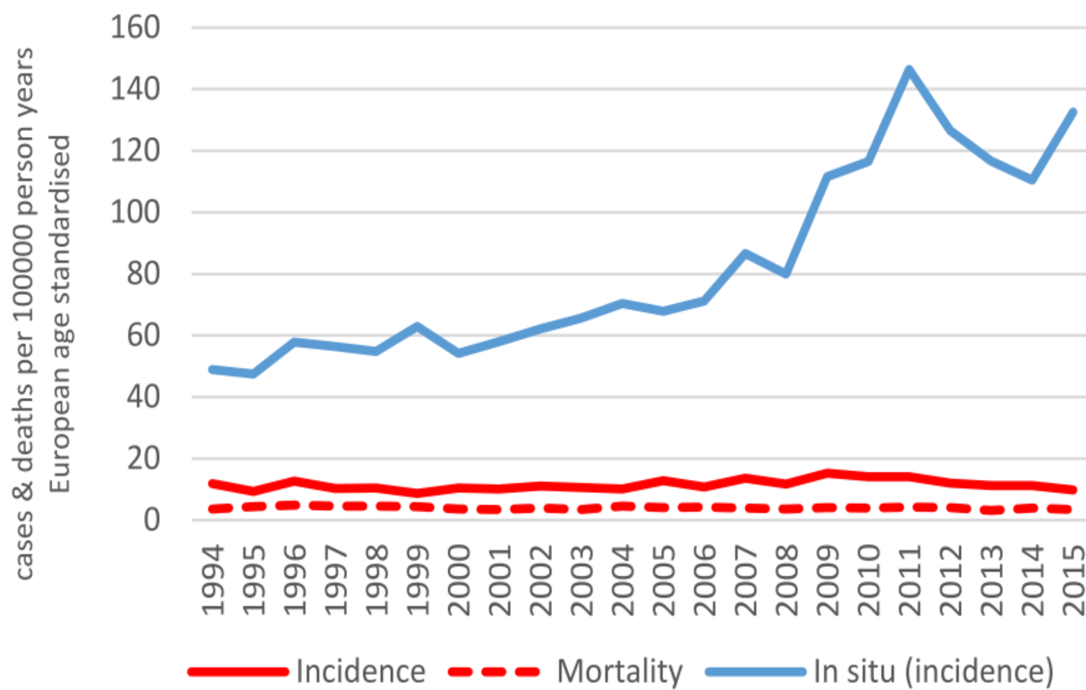

Figure 1.3: Trend in incidence and mortality for Ireland (National Cancer Registry Ireland, 2018a)

1.2. The Cervix

Normal cervical biology is well defined from an anatomical and biological viewpoint. The cervix is a narrow portion of the uterus projecting into the vaginal cavity. The cervix has an average measurement of approximately 3cm in length and 2.5cm in diameter. The anatomy of the cervix can be broken down into about 5 regions as seen in Figure 1.4. These five regions are the Ectocervix, Endocervix, Endocervical canal, Squamocolumnar Junction (SCJ) and the Transformation Zone (TZ).

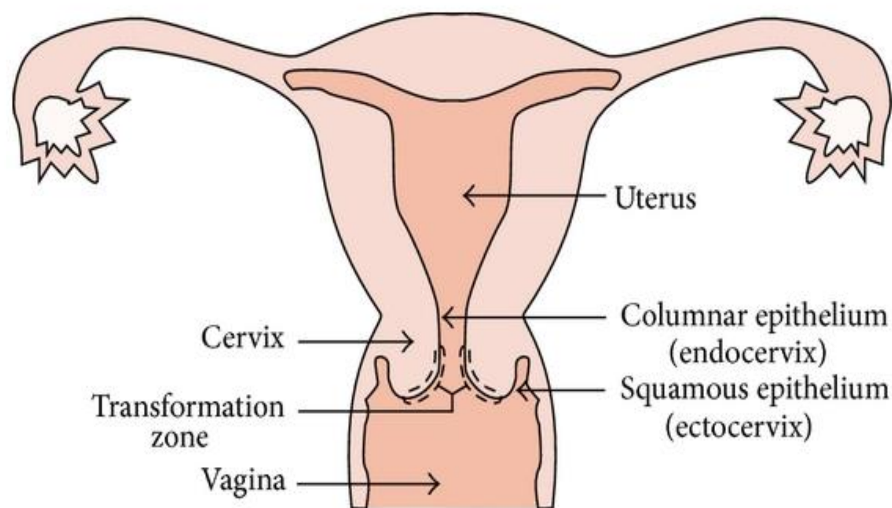

Figure 1.4: Uterine Anatomy. Conventional sampling is from both the Ectocervix and Endocervix with the overlap of the TZ (Bengtsson and Malm, 2014)

The Ectocervix is the outer most part of the cervix protruding into the vaginal cavity and is predominantly made up of non-keratinising stratified squamous epithelium. The Endocervix is the tissue that surrounds the Endocervical canal (lumen through the cervix into the uterus. This is made up of predominantly glandular (columnar) epithelium that can include crypts into the Endocervical stroma. The SCJ is the meeting point of both the Ectocervix and Endocervix. Anatomically there is usually an abrupt change from squamous to glandular epithelium that can be visualised on colposcopy. Depending on the woman's age (puberty, menopausal) and whether or not she has given birth the SCJ can vary both in location and cell type. Columnar epithelium can move out of the endocervical canal after puberty and during pregnancy but is followed by metaplasia of basal reserve cells to immature squamous epithelium. In women post menopause, the endocervical border of the SCJ is seen to retreat into the endocervical canal. The TZ is between the endo and ectocervix, during puberty the SCJ extends out into the ectocervix and this columnar epithelium becomes exposed to the acidic vaginal environment. This columnar epithelium undergoes

metaplasia and forms squamous epithelium: this new SCJ is termed the Transformation Zone. Histology shows immature squamous epithelium. This is also the region where most abnormalities are thought to arise from (Herbert, 2016). The primary role of the cervix is to act as a barrier between the environment of the vaginal cavity and the uterus. A complex of naturally forming acids and mucins in the endocervical canal alongside leukocytes, immunoglobulins, enzymes and other factors help form a plug that seals off the uterus from the vaginal cavity.

The Ectocervical non-keratinising stratified squamous epithelium can be subclassified as three regions as shown in Figure 1.5. Firstly, the Superficial Layer consists of mature superficial cells. These cells contain small nuclei and copious cytoplasm with a flattened appearance when sectioned. The cells in this region have stopped developing and maturing and are ready to be exfoliated off the cervix. They can act as a protective outer layer for the ectocervix. The second layer is the Intermediate Layer. Here the squamous cells are still maturing with a slightly larger nucleus than the superficial cells. The third layer is the Basal Cell layer. Compared to the other two layers this region is only a few cells thick but comprises of cells with large active nuclei and less cytoplasm. It can be subdivided into the parabasal and basal layers. The basal layer being those cells immediately adjacent to the basal lamina/basal membrane. Whilst the parabasal cells are those cells one or two layers above the basal layer that are moving from their metabolically active states towards the matured superficial layer, which includes the stratum granulousum and the stratum spinosum (Thomas, 2013). The endocervical columnar epithelium consists of a single layer of mucus producing cells. They have the classical picket fence appearance with the nucleus to the basal end of the cell. During mucus production for the endocervical plug, the nucleus can shift position in the cell. Ciliated cells are present to aid in mucus movement. These cells form crypts similar to that of the villi in the stomach and to a depth if around 0.5-1.0 cm's (Herbert, 2016). Each region working in conjunction with the normal hormonal cycle help form a healthy and normal cervix.

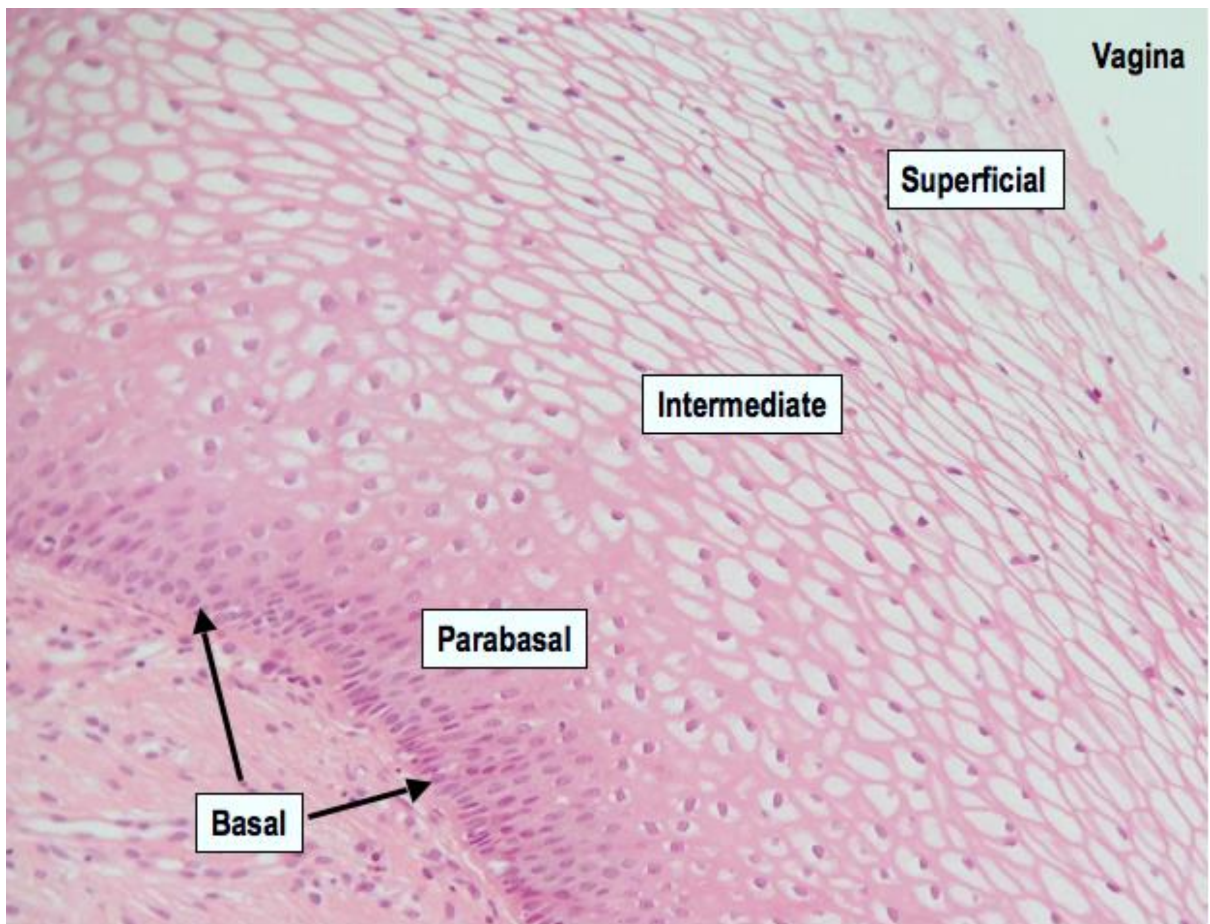

Figure 1.5: High Power H&E section of the normal cervical epithelium. From the Basal cells fixed on the basement membrane and below that the cervical stroma to then increasing in cellular age the parabasal up to the superficial layers in direct contact with the vaginal microenvironment (Thomas, 2013)

The histological subtypes of invasive cervical cancer generally reflect their point of origin and cellular type, either being squamous or glandular, which leads to the two primary histological findings that make up >90% of histological findings, Squamous cell carcinoma and Adenocarcinoma. Squamous cell carcinoma (SCC) typically arises from the immature (basal layer) cells from the transformation zone of the cervix compared to Adenocarcinoma which generally originates from the glandular cells in the endocervical canal. Of the two, SCC is the most commonly diagnosed histotype, with a diagnosis rate of between 75-90% of cervical cancers diagnosed in developing countries, Ireland as previously mentioned has a current SCC diagnosis rate of 77% (Bray, 2005; National Cancer Registry, 2017a). The rate of Adenocarcinoma however has been increasing over time possibly due in part to the difficulty in screening, as the endocervical canal is not well sampled by current methods (National Cancer Registry, 2017a). This generally leads to an adenocarcinoma being diagnosed more frequently through symptomatic indicators rather than screening in comparison to SCC.

SCC is generally preceded by a long pre-invasive stage termed Cervical Intraepithelial Neoplasia (CIN). It begins as an atypical cervical cell and progresses through worsening levels of dysplasia forming a pre-cancerous lesion before invading past the basal membrane and becoming an invasive cervical cancer. The progress of CIN is tightly related to Human Papilloma Virus (HPV) infection in respect to both the length of infection as well as other factors such as genomic integration, which will be discussed later. CIN is graded on histology from 1 to 3 with CIN 1 representing the first stage of the malignant progress with dysplastic cells occupying the lower third of the epithelium, moving towards CIN 3 which is a full thickness lesion without disruption of the basal membrane. This process from minor cellular dysregulation to CIN 3 and finally invasion can take up to 13-15 years to occur (Gustafsson and Adami, 1989; Melnikow *et al.*, 1998; Sankaranarayanan, 2003). The progression of CIN is not a linear process and at any stage of CIN the lesion can both progress or regress. However, progression and regression rates for any cancer are hard to accurately predict. Table 1.1 shows the current approximate outcomes relating to CIN classification. Few studies have looked at this due to the inherently unethical nature of allowing high grade CIN lesions to progress to cervical cancer in women, but it does show the high regression and low progression rates of CIN 1 lesions. Based on this knowledge, unlike CIN 2 and CIN 3 lesions, the treatment of CIN 1 can follow two paths, firstly immediate treatment in line with the other higher grade CIN lesions or alternately follow up over 18 to 24 months to determine if the lesion regresses, persists or progresses and treat accordingly (Sankaranarayanan, 2003).

Table 1.1: Progression and Regression Rates of CIN Grades

	CIN 1	**CIN 2**	**CIN 3**
Regression	57%	43%	32%
Persistence	32%	35%	56%
Progression	11%	22%	-

Outcomes of CIN classification (Modified from Östör 1993)

Adenocarcinoma involves the glandular cells of the cervix and have very similar risk factors to SCC. Of the subgroups of adenocarcinoma, endocervical type mucinous adenocarcinomas make up around 70% of adenocarcinomas (Tavassoli *et al.*, 2003). However a portion of the adenocarcinomas can be adenosquamous carcinomas (Sasieni, Castanon and Cuzick, 2009). Adenocarcinoma is generally preceded by Adenocarcinoma In Situ (AIS) or Cervical Glandular Intraepithelial Neoplasia (cGIN). These pre-cancerous cells are still contained by the basal membrane. cGIN can often be found with CIN which, is usually better detected during cytology based screening however cytology is less sensitive in detecting glandular based abnormalities (Östör et al., 2000; Talaat et al., 2012).

All stages of pre-cancer and cancer can regress or progress for a variety of reasons (Crosbie *et al.*, 2013) and can be dependent on other co-factors influencing the risk of cervical cancer. Known factors to date include sexual activity and the number of sexual partners, tobacco smoking, early life pregnancy and multiple pregnancies, immune incompetence or suppression, oral contraceptive use (type and duration dependant), poor diet, obesity and socio-economic status (Gadducci *et al.*, 2011). Other contraceptive measures such as condoms can offer a reduced risk (Hogewoning *et al.*, 2003). HPV however, remains the primary aetiological agent linked with the development of these lesions. This risk is type specific with SCC being firmly linked with HPV 16 infections whilst glandular based carcinomas seem to be more predisposed to the HPV 18 subtype.

1.3. Human Papillomavirus (HPV)

Human Papilloma Virus (HPV) are a large family of double stranded, circular, DNA viruses enclosed in an icosahedral capsid. Infection with this family of viruses is usually in the epithelial cells of the skin or the oral and genital mucosa. To date, over 150 HPV members have been isolated and fully sequenced but many more are still being researched and classified. One main issue around the classification of HPV is the method with which the virus is classified. Due to its circular nature, the point of cleavage and method of analysis, the HPV family can be grouped into several potential phylogenetic trees, though the current consensus is cleavage and analysis at the L1 capsid protein (Bernard *et al.*, 2010).

The papillomavirus family is an extensive family that have infect multiple mammalian species. Initially, papillomavirus were described in mammals such as horses, cows, dogs, cats, etc but also in other animals such as birds, snakes, fish and turtles (Bravo and Félez-Sánchez, 2015). Currently there are over 200 genotypes of papillomavirus distantly related to HPV. Many mammals, humans included, count papillomavirus as part of the normal skin microflora and it is really only a small fraction of the HPV family that have been shown to cause adverse effects in humans ranging from benign warts to a variety of cancerous and non-cancerous lesions. In the current phylogenetic tree from John Doorbar's working group, we can see the current classification of the known HPV family (Figure 1.6). The HPV family is classified into genera: Nu, Mu, Gamma, Beta and Alpha papillomavirus. HPV from the Alpha genus are often sub-classified as; Low-risk cutaneous (grey), Low-risk mucosal (orange) or High-risk (pink). Those Alpha HPV types that are highlighted in red text indicate confirmed human carcinogens from established epidemiological research (Egawa *et al.*, 2015). The remaining high-risk HPV's are classified as either probable or possible human carcinogens.

In each genera, there are many HPV sub-types. From the classification criteria an individual papilloma virus must be at least 10% divergent from other known HPV's in their L1 capsid sequence to be classified as a new subtype (Egawa *et al.*, 2015).
Though this is one method of classification and currently the preferred method, it is not ideal as many researchers admit that there should be inclusion of the papilloma virus's phylogeny, genome organization, biology and pathogenicity ,etc, instead of merely using genomic sequence analysis, or the analysis of HPV genomic fragments (Bernard *et al.*, 2010). Though our current methods produce reasonable classifications for many papillomaviruses' types, the detailed information regarding HPV's biology is poorly defined. Again, it is an important point to keep in mind that though we like to classify these viruses

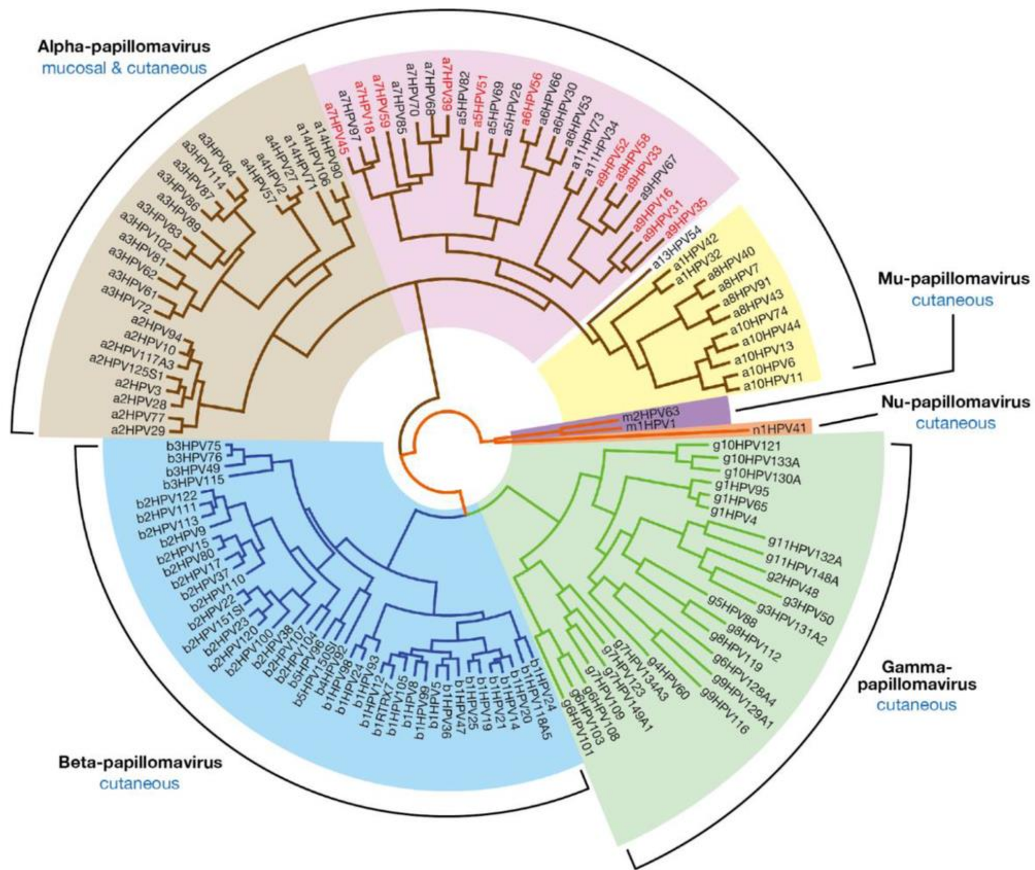

Figure 1.6: The HPV Phylogenetic Tree.
High risk HPV's highlighted red are confirmed "human carcinogens" from epidemiological data. The evolutionary tree is based on alignment of the E1, E2, L1, and L2 genes (Egawa et al., 2015)

to make the family manageable for us, the natural history of the virus and its properties may not match from one relative to the next perfectly.

At a general level the average person may be aware that HPV is carcinogenic and is strongly linked to cancers such as cervical cancer as well as head, neck, anal and penile cancer. However not all HPV types have oncogenic potential and of those high-risk HPV (hrHPV) types as shown in red in Figure 1.6, only a small fraction of people infected with a hrHPV type will ever develop a cervical pre-cancer or cancer. Of this large and diverse family, it was the work of Nobel prize winner Harald zur Hausen that brought the importance of specific HPV subtypes to light as a primary aetiological agent in cervical cancer, namely HPV 16 and HPV 18 (Gissmann and Hausen, 1980; de Villiers, Gissmann and zur Hausen, 1981). This has lead the way for the work of several groups such as IARC's Nubia Muñoz and the epidemiological studies that linked specific HPV types to invasive cervical cancer (ICC) (Munoz *et al.*, 1992). These studies have given us a strong indication of the potentially carcinogenic HPV types that currently exist. IARC, the International Agency for Research on Cancer, has classified these into 4 groupings as seen in Figure 1.7.

Group 1 Carcinogenic to Humans	Group 2A Probably Carcinogenic to Humans	Group 2B Possibly Carcinogenic to Humans	Group 3 Not classifiable	Group 4 Probably not Carcinogenic to Humans
Sufficient evidence of carcinogenicity in humans and in experimental animals	Limited evidence of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals	Limited evidence of carcinogenicity in humans and insufficient evidence of carcinogenicity in experimental animals	Inadequate evidence of carcinogenicity in humans and in experimental animals	Evidence suggesting lack of carcinogenicity in humans and in experimental animals
111 agents, including 8 biological agents: - Epstein-Barr virus - Helicobacter pylori (infection with) - Hepatitis B virus (chronic infection with) - Hepatitis C virus (chronic infection with) - Human immunodeficiency virus type 1 (infection with) - Human papillomavirus types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 - Human T-cell lymphotropic virus type I -Kaposi sarcoma herpesvirus	**65 agents, including 3 biological agents:** -Human papillomavirus type 68 -Malaria (caused by infection with Plasmodium falciparum in holoendemic areas) -Merkel cell polyomavirus	**274 agents, including 6 biological agents:** -BK polyomavirus -Human immunodeficiency virus type 2 (infection with) -Human papillomavirus types 5 and 8 (in patients with epidermodysplasia verruciformis) -Human papillomavirus types 26, 53, 66, 67, 70, 73, 82 -Human papillomavirus types 30, 34, 69, 85, 97 (Classified by phylogenetic analogy to the HPV genus alpha types classified in Group 1) -JC polyomavirus	**504 agents, including 5 biological agents:** -Human papillomavirus genus beta (except types 5 and 8) and genus gamma -Human papillomavirus types 6 and 11 -Human T-cell lymphotropic virus type II -SV40 polyomavirus -Hepatitis D virus	**1 agent, no biological agent**

Figure 1.7: Carcinogenicity classification based of IARC Monographs vol. 100B. HPV types are classified from Known, Probably, Possible, Not Classifiable and Probably Not Carcinogenic. Classification includes other Viral Families along with HPV as indicated through underlined text. (Bravo and Félez-Sánchez, 2015)

From epidemiological research, it was determined that the HPV subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 were carcinogenic termed Group 1 and commonly defined as the hrHPV group. HPV 68 has a probable high risk of being a carcinogen (Group 2A) whilst 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73 and 81 are typed as (Group 2B) commonly referred to as low risk HPV types. The other HPV types were classified Group 3/4 with either insufficient evidence for carcinogenesis or evidence proving lack of carcinogenesis. We can see that data like this has moved forward into the clinical field with testing platforms routinely detecting HPV 16 and 18 along with other hrHPV types as well as the development of HPV vaccines such as Gardasil 9 covering Types 16, 18, 6, 11, 31, 33, 45, 52, and 58 covering the majority of HPV types related to cancer.

HPV infections in general have many differing phenotypes that can culminate in short term or long-term productive infections to high grade lesions in infected mucosal tissue from the skin, oral cavity, anogenital tract and cervical tissues. These varying phenotypes all hinge on HPV's viral genome. HPV consists of a double stranded DNA episome ranging in length from 6953bp's to 8607bp's in animal specific papillomaviruses (CmPV1, CPV1) with HPV in around the 8000bp's length. As shown in Figure 1.8, there are 6 early stage genes (E1, E2, E4, E5, E6 and E7) and two late stage genes (L1 and L2) along with an Upstream Regulatory Region (URR) (also referred to as the Long Control Region (LCR) depending

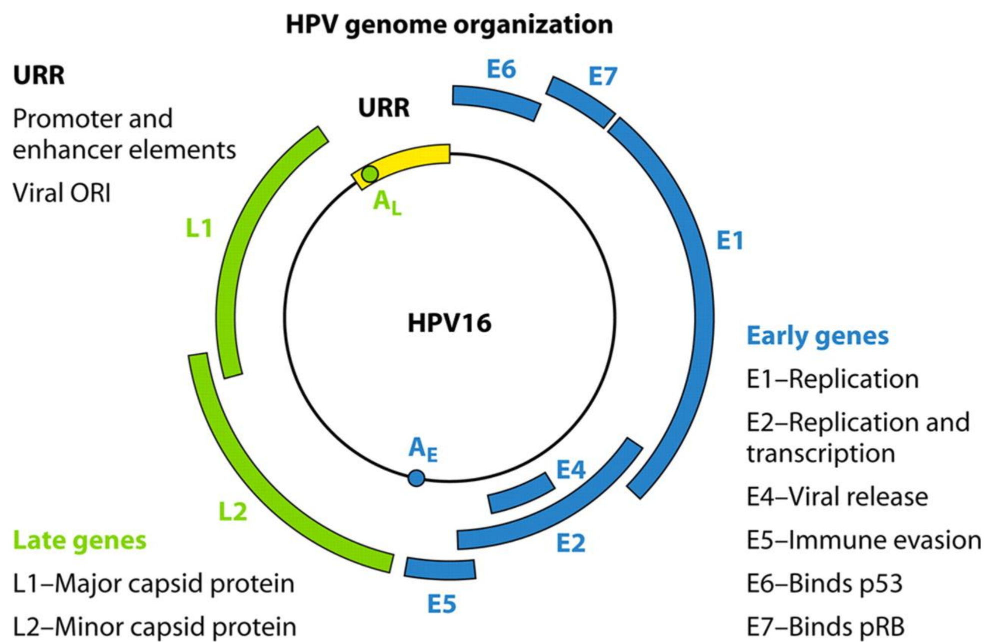

Figure 1.8: Illustration of HPV 16's genomic structure. The gene contains early (blue) and late (green) stage regions which relate to the timing of their expression in the viral life cycle. E1; Genome Replication; ATP dependant DNA helicase activity. E2; Genome Replication, transcription, segregation and encapsulation. E4; Remodels cytokeratin network. Cell cycle arrest and Virion assembly. E5; Control of cell growth and differentiation, immune modulation. E6; Oncoprotein. Inhibits apoptosis and differentiation. Regulates cell shape, polarity, mobility and signalling. E7; Cell cycle control. Controls centrosome duplication. L1; Major Capsid Protein. L2; Minor Capsid protein. Recruits L1 and viral assembly (Stanley, 2012)

on the paper). This is the common template around which the vast majority of HPV types adhere to and all HRHPV types follow. Each gene region plays a key functional role in the viral life cycle:

HPV E1 is necessary for viral replication as it binds to a specific E1 binding site in the viral origin of replication (ORI) and assembles into a hexameric complex with the aid of the E2 viral protein (Frattini and Laimins, 1994). This hexameric complex has a helicase activity that can allow the DNA to unwind before viral replication (Wilson *et al.*, 2002).

HPV E2 is exceedingly important in HPV replication and control. Depending on the expression level of E2 the protein can also control the E6 and E7 oncoproteins. High levels of E2 expression showed repression of E6 and E7 whilst low levels of E2 showed E6 and E7 being actively transcribed (Bouvard *et al.*, 1994).

HPV E4 gene overlaps the E2 gene but is transcribed in a different reading frame. It is expressed mainly at the later stages and becomes the most abundant viral protein expressed during a normal HPV life cycle. (Doorbar *et al.*, 1991, 1997; Roberts *et al.*, 1997). To support viral shedding, E4 interacts and disrupts the organisation and structure of the

intermediate filaments to possibly cause the host cell to rupture and release the cell contents and newly formed viral particles. (Raj *et al.*, 2004; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007).

HPV E5, not all HPV's contain an E5 Open Reading Frame (ORF). E5 has been shown to enhance the immortalisation potential of both E6 and E7 and in conjunction with E7 can stimulate proliferation of mouse primary cells (Animal cell model for human keratinocytes) (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007).

HPV E6 is the best known and most researched function of the E6 protein in hrHPV types is the ability to bind and degrade p53, the tumour suppressor protein, this is through the recruitment of the protein ubiquitin ligase (E6 associated protein (E6-AP)) which allows proteasomal degradation of p53 by targeting it for degradation by the 26S proteasome (Scheffner *et al.*, 1990).

HPV E7 is best known for its interaction with the Retinoblastoma (pRb) protein which functions as a tumour suppressor gene in the cell (Dyson *et al.*, 1989; Gage, Meyers and Wettstein, 1990). When E7 associates with pRb it promotes degradation of the pRb via a proteasome mediated pathway as with the E7:p53 complex (Gonzalez *et al.*, 2002). This disruption of pRb leads to the cells inability to inactivate E2F transcription factors and allow the cell to move from G1 to S phase of the cell cycle.

HPV L1 is the major structural protein of HPV. It should be noted that most research on L1 and its conformation has been based on the use of VLP's (empty capsids assembled in tissue cultures with either L1 or L2 alone or in combination). The capsids assemble into a regular 72-pentamer T=7 capsids with complex loops protruding from the surface of the structure (Zhou *et al.*, 1992; Chen *et al.*, 2000).

HPV L2 is the minor capsid protein of HPV, it contributes to the interaction of the virion with the cell wall in conjunction with L1. Regions of the N-terminal portion of L2 interact with the cell wall after the initial binding via L1(Kawana *et al.*, 2001; Yang *et al.*, 2003).

1.3.1. Pathogenesis and mechanisms of action of HPV in an oncogenic infection

Infection with HPV is the main risk factor for cervical cancer and pre-cancer. Over 90% of cervical cancers are caused by hrHPV types with HPV 16 and 18 being found in ~70% of cervical cancers (Walboomers *et al.*, 1999). HPV is also known to be associated with other cancers including; vulvar, vaginal, penile, anal and oropharyngeal cancers.

The initial infection requires access to the basal epithelial cells of the cervix. This is thought to be achieved through micro abrasions or trauma to the cervical epithelium allowing the viral particles access to the basal epithelium. Entry into the cell occurs through the L1 and L2 structural proteins binding cell surface receptors which package the HPV into carrier vesicles that delivers the virus to the host's lysosomes and endosomes. The viral particle can survive these environments thanks to viral proteins such as E5 (Straight, Herman and McCance, 1995) and eventually produce a productive infection. There is also a possibility that the particle is transported to the endoplasmic reticulum that is independent of lysosomes and endosomes (Smith, Campos and Ozbun, 2007). Once internalised, the viral genome makes its way to the host's nucleus and viral replication of HPV's episome is facilitated using the host's replication machinery. This pre-integration phase of infection can lead to cellular abnormalities as well as viral replication and propagation as seen in Figure 1.9. This initial infection stage is followed by the proliferative phase where there is an increase in the number of infected basal cells. The viral genome in this stage is maintained at a copy number of between 10 copies up to 200 copies per cell (De Geest *et al.*, 1993; Pyeon *et al.*, 2009). As the parent cell divides into daughter cells and move towards the surface epithelium E2 binds the host centrosomes and packages the viral genome into the newly forming cell (Van Tine *et al.*, 2004). As this occurs and subsequent divisions of the host migrates the cells towards the surface, the viral DNA shifts to the production of its late-stage genes including L1, L2 and E4 and form progeny viral particles which are packaged with the viral genome and subsequently shredded into the vaginal cavity to seed new infections in the host's cervix or to infect another host. The original basal cell remains in the cervix which acts as a repository of the viral genome allowing HPV to maintain a latent infection (Bosch and Iftner, 2005).

This style of infection is relatively common and in the vast majority of cases will be cleared by the host immune system eventually. It is the process of integration into the host cellular DNA that is the start of malignant transformation in the cell (Gómez and Santos, 2007). During viral replication where the episome has not integrated into the host's DNA, there is

expression of E2 which inhibits the LCR leading to suppression of both E6 and E7 (McBride and Warburton, 2017). Upon integration, there is a loss of E2 expression which releases the negative feedback loop leading to an increased level of expression of E6 and E7 oncogenes/oncoproteins. E6 interacts with p53 blocking apoptosis while E7 interacts with Rb.

Loss of p53 functions (via E6 binding) leads to loss of cell cycle control in the G1 phase, loss of DNA repair function and loss of the apoptotic function. Loss of pRB function (via E7) also impacts the cell cycle control in the G1 to S phase (Saavedra, Brebi and Roa, 2012). Both of these losses of function allow an unregulated cell proliferation to occur and initiate the oncogenic process in the affected cell. Further changes are required to lead to a precancerous lesion and cervical cancer, but this is the initial step required.

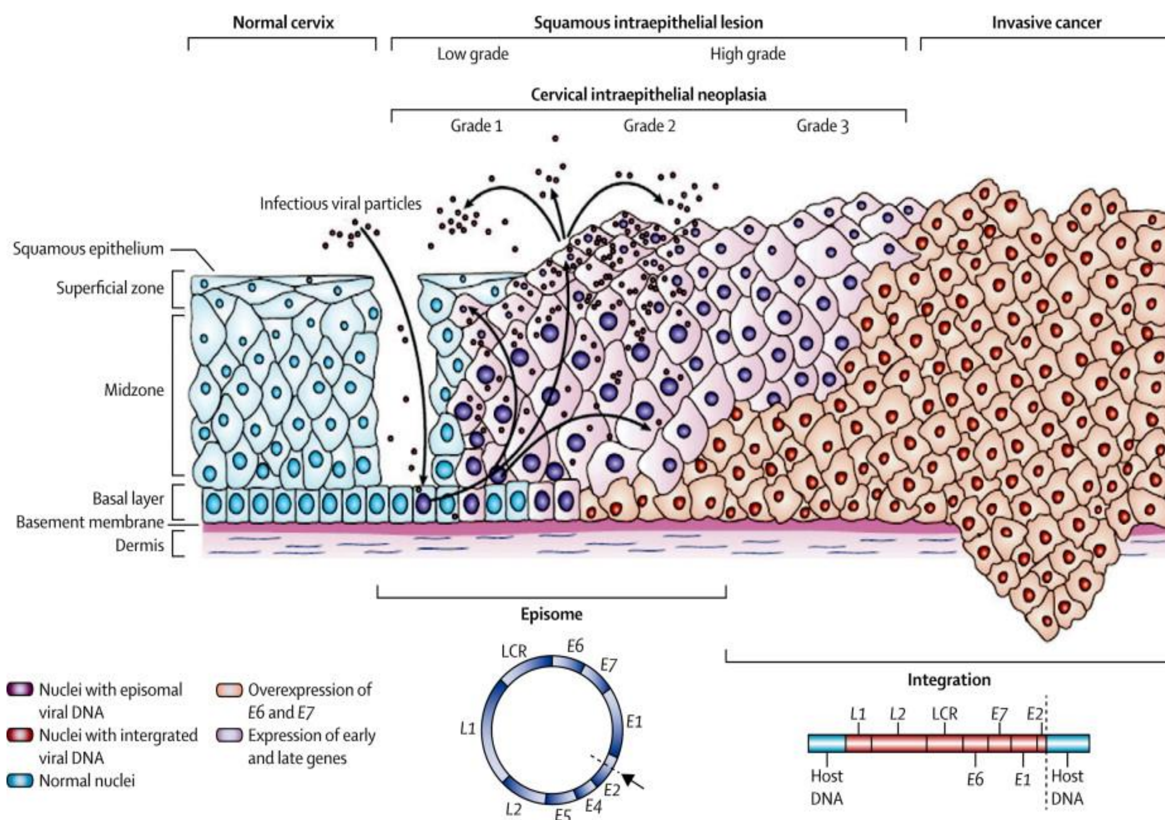

Figure 1.9: Representation of CIN development to Invasive SCC. (Figure 1 in (Crosbie *et al.*, 2013)

Figure 1.10 shows the basic interactions of E6 and E7 on the host's genome. However, as with many things, the interplay between HPV and the host is highly complex, Figure 1.11 gives a brief summary of this interplay. This eventually culminates in the production of a cervical lesion and progression from CIN 1 towards CIN 3 and invasive cervical cancer. This knowledge of the association between the specific hrHPV types and cervical cancer and pre-cancer has become exceedingly important in the role of cervical cancer detection. Over 90% of cervical cancers are positive for HPV (Walboomers *et al.*, 1999) which has led to the option of HPV testing as a valid form of screening for cervical cancers. This has been backed up over time by a series of randomised control trials (Naucler *et al.*, 2007, 2009; Ronco *et al.*, 2008, 2010; H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; D. C. Rijkaart *et al.*, 2012; C Kitchener *et al.*, 2014; Maaike G. Dijkstra *et al.*, 2014) supporting the use of HPV testing incorporated into cervical screening practices. HPV testing has allowed screening to have a more automated, objective and sensitive test with overall cost reductions for the screening service (HIQA, 2017). HPV testing has been utilised in serveral ways over the years. HPV testing has been utilised as a Test of Cure (TOC), for the management and triage of low grade cytological abnormalities and recently as the primary screening test in lieu of cytology.

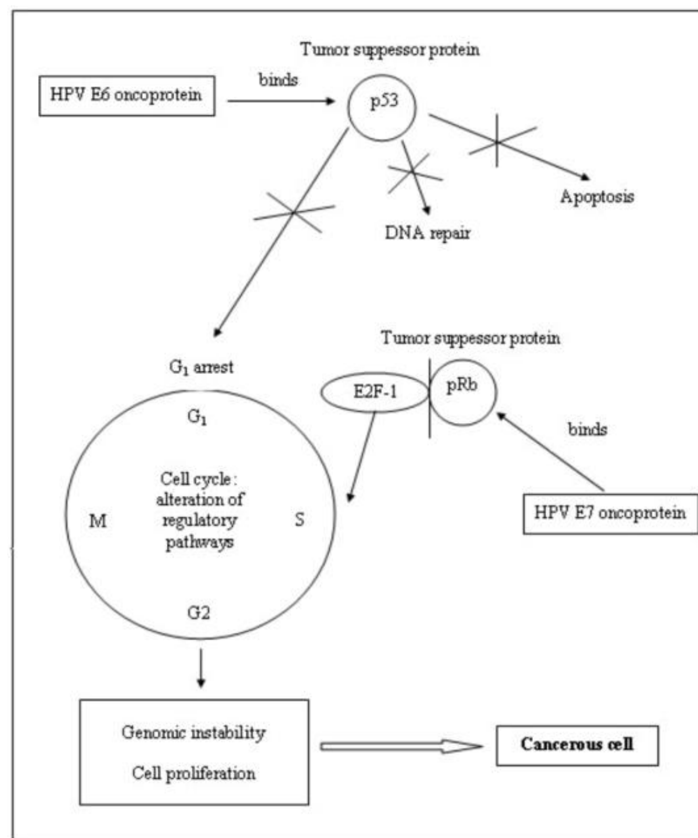

Figure 1.10: E6 and E7 basic cellular Interactions (Gómez and Santos, 2007)

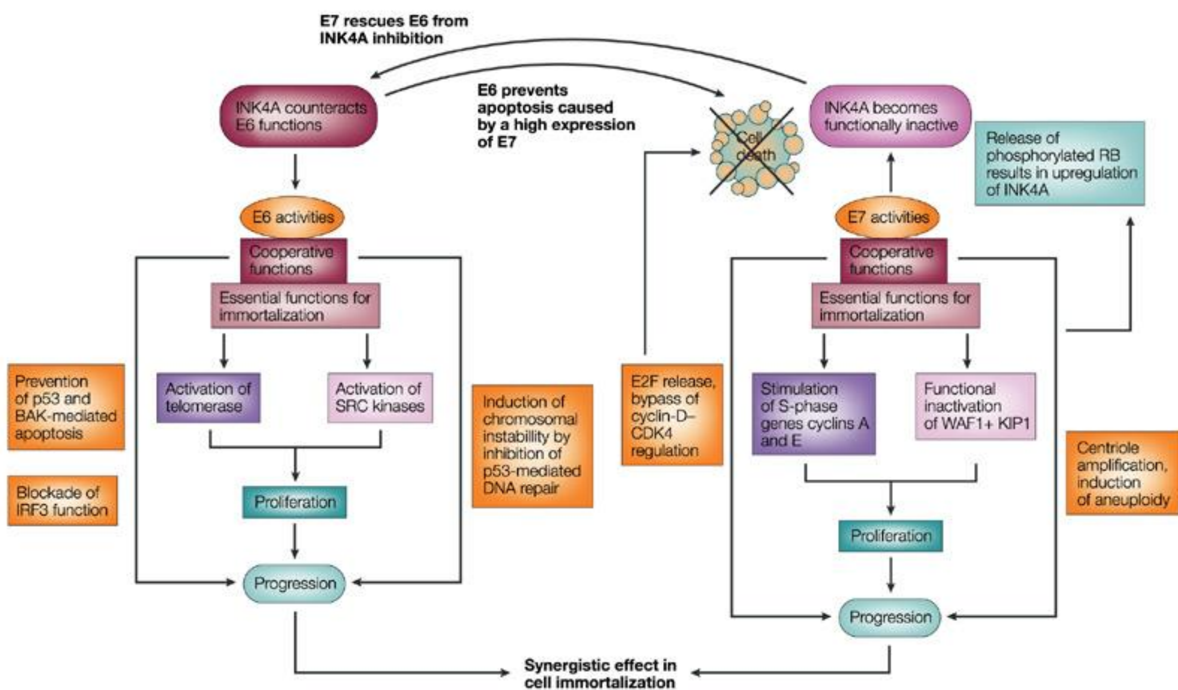

Figure 1.11: Other functions of E6 include activation of telomerase and SRC kinases, inhibition of p53 and BAK. E7 inhibits RB and releases E2F leading to the upregulation of INK4A. E7 also inactivates INK4A. E6 and E7 lead to cell immortalization and malignant transformation: E7 relieves E6 from INK4a inhibition allowing E6 to prevent apoptosis induced by high E2F levels. (Zur Hausen, 2002)

1.4. Cervical Cancer Screening

There are typically two ways in which a woman is initially suspected to have an underlying cervical malignancy: either they present symptomatically with symptoms such as bleeding between periods or heavier menstrual bleeding, pelvic pain, vaginal discharge and post-menopausal bleeding (HSE, 2018) or they can present during routine cervical screening. The ideal outcome in this case is for women to present earlier in the disease course, during screening, as symptomatic presentation is generally associated with more advanced lesions.

Cytology is performed on a sample of cervical cells taken by either a broom, brush or spatula and commonly suspended in the LBC vial. This sample from the cervix should include cells from the ectocervix, transformation zone and endocervical canal. When processed the end result is a monolayer of adhered cervical cells that is subsequently stained using the PAP stain. This is a trichrome stain which uses a nuclear stain, haematoxylin, alongside two counter stains, OG-6 and EA-50. OG-6 stains the keratin and EA-50 stains the cytoplasm of squamous epithelium as well as the nucleoli and red blood cells present. Once stained the slide can be viewed by a Cytopathologist who looks for the presence of dysplastic and malignant cells. The general feature of a dysplastic or malignant cell is its nucleus which is generally increased in size from its benign version. The ratio of the nucleus to cytoplasm is an important factor with the nuclear cytoplasmic ratio (N:C), being an initial indicator of dyskaryosis. Following the classification of an abnormal smear result only histological classification can confirm the final diagnosis. Terminology varies based on the region for describing the dyskaryotic cells found in cytology. Ireland and Europe in general follow the Bethesda system of classification which also loosely correlates with the probable histological outcome (Soloman, 1989; Solomon *et al.*, 2002; Nayar and Wilbur, 2015, 2017). These abnormalities are classified as either equivocal results such as ASC-US (Atypical Squamous Cells of Undetermined Significance) and ASC-H (Atypical Squamous Cells but cannot exclude high grade) or a Squamous Intraepithelial Lesions (SIL) being either low grade or high grade, LSIL or HSIL (Figure 1.12). Abnormalities originating in glandular cells are termed AGC (atypical glandular cells) or adenocarcinoma in situ (AIS). Table 1.2 shows the Bethesda classifications with histological correlation along with a visualisation of varying grades of cytological abnormalities shown in Figure 1.12.

Although a PAP test has a reasonable specificity (~76%) it lacks sensitivity (~57%) with a Negative Predictive Value of approximately 92% and Positive predictive value of approximately 26% and demonstrates limitations with reproducibility with the overall sensitivity of the test varying country to country and lab to lab (Cuzick *et al.*, 2006; Barut *et*

al., 2015). This variation can be attributed to a combination of operator variation both within the same laboratory and between labs nationally and globally which is mainly due to the relative subjectivity of cytology. Due to human error, there is always the issue of false positive and false negative results occurring. However, thanks to quality assurance protocols this can be kept to a reasonable level in accredited laboratories.

Table 1.2: Cytology Terminology in order of severity with best fit Histology correlation.

Cytology Classification (for Screening)		**Histology Classification (For Diagnosis)**
Cytology Grade	**Interpretation**	**CIN**
Negative	No Abnormal cells detected	Normal
ASCUS	Atypical Squamous Cells of Undetermined Significance	Atypia (Squamous or HPV related)
LSIL	Low Grade Squamous Intraepithelial Lesion (SIL)	CIN 1
ASC-H	Atypical Squamous Cells of Undetermined Significance. High grade abnormalities cannot be ruled out.	CIN 1, CIN 2, CIN 3
HSIL	High Grade SIL	CIN 2, CIN 3
AGC	Atypical Glandular Cells. The glandular cell type is; endocervical, endometrial or glandular cells	
?SCC	Query Squamous Cell Carcinoma	CIN 3, Invasive Cancer
AIS	Endocervical Carcinoma in Situ	Adenocarcinoma, Invasive Cancer

Cytology Terminology table based of CervicalCheck's Current practice (CervicalCheck, 2015). Histology classification based off Bethesda system (Soloman, 1989; Solomon *et al.*, 2002; Nayar and Wilbur, 2015, 2017)*. Cytology to histology grade correlation is meant as an approximate reference guide.*

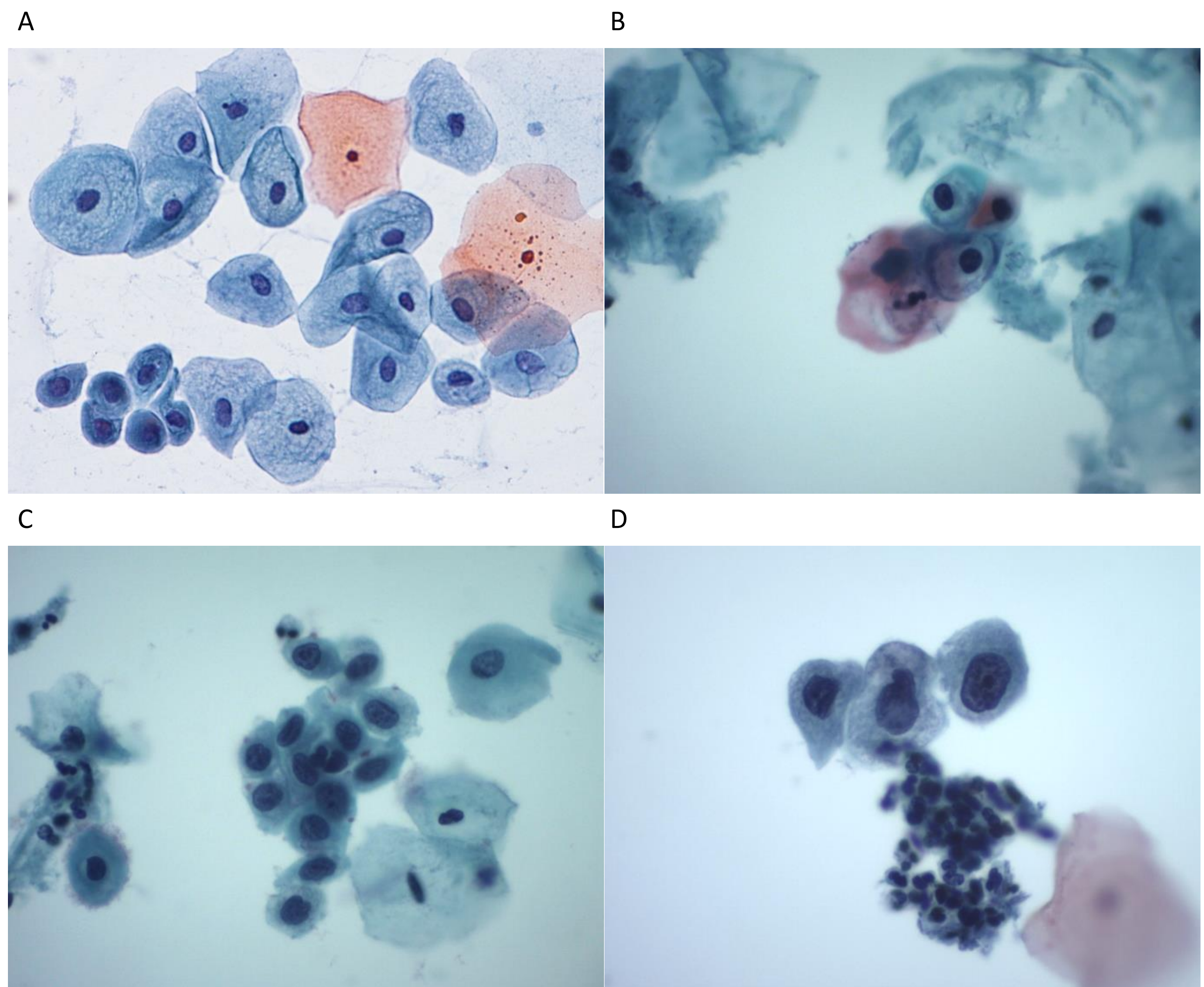

Figure 1.12: ThinPrep PAP smears across increasing cytological severity. (60x magnification)

(A) Normal: Superficial cells with orangeophilic cytoplasm (orange) and pyknotic nuclei, Intermediate and parabasal cells with cyanophilic cytoplasm (blue) and vesicular nuclei. Note the fine chromatin pattern and normal N:C ratio.

(B) LSIL cytology: Squamous cells in the centre showing slightly enlarged nuclei with hyperchromasia and slightly increased N:C ratio. Note the koilocytic appearance of the cells.

(C) HSIL cytology: Three squamous cells in the centre showing enlarged, hyperchromatic nuclei, raised N:C ratio, irregular nuclear border and coarse chromatin pattern.

(D) Queried SCC on cytology: Cyanophilic squamous cells showing enlarged hyperchromatic nuclei with coarse chromatin pattern, irregular nuclear border, raised N:C ratio.

Images obtained with permission from the Cytopathology laboratory in the Coombe Women’s and Infants University Hospital. Images reviewed by Padmaja Naik, Senior Cytologist.

1.4.1. CervicalCheck The National Cervical Screening Programme

In Ireland since 2008, we have the National Cervical Screening Programme, CervicalCheck. Each year over 1 million women between the ages of 25 to 60 years are invited attend for cervical screening. CervicalCheck operates a call/recall-based screening programme for eligible women with either a three-year screening interval for woman aged 25-44 or a five-year screening interval for women aged 45-60.

Initially in 2008, CervicalCheck offered Liquid Based Cytology (LBC) testing for all women however, as technologies and understanding improved, HPV testing was implemented as a “Test of Cure” for CervicalCheck colposcopy clinics which utilised a combined cytology and HPV test approach. In May 2015, HPV triage was introduced for the triage of low-grade cytological abnormalities (ASC-US/LSIL).

1.4.1.1. Triage of Low-Grade Cytology

Studies showed that triaging low grade cytological abnormalities with HPV reduced the rates of repeat smears by up to 74% and performed better in women over 35 compared to the under 35 population which had a higher level of transient HPV infections (Moss *et al.*, 2006; Ronco *et al.*, 2007). As other key developments came to light such as the high NPV of HPV testing and the long-term protective nature of a HPV negative result, the use of HPV testing as a cytology triage test came to be utilised in many cervical screening programmes. In 2015, CervicalCheck implemented HPV triage of low-grade (ASC-US/LSIL) abnormalities. Women with low grade cytology abnormalities and a negative HPV test return to the routine screening intervals and women with low grade abnormalities and a positive HPV test are referred to colposcopy. From the first year of introduction into the programme, CervicalCheck reported the success in HPV triage within their first year with the detection of above average levels of high grade abnormalities attributed to the use of HPV triage (CervicalCheck, 2016). The current national screening procedure is shown in Figure 1.13.

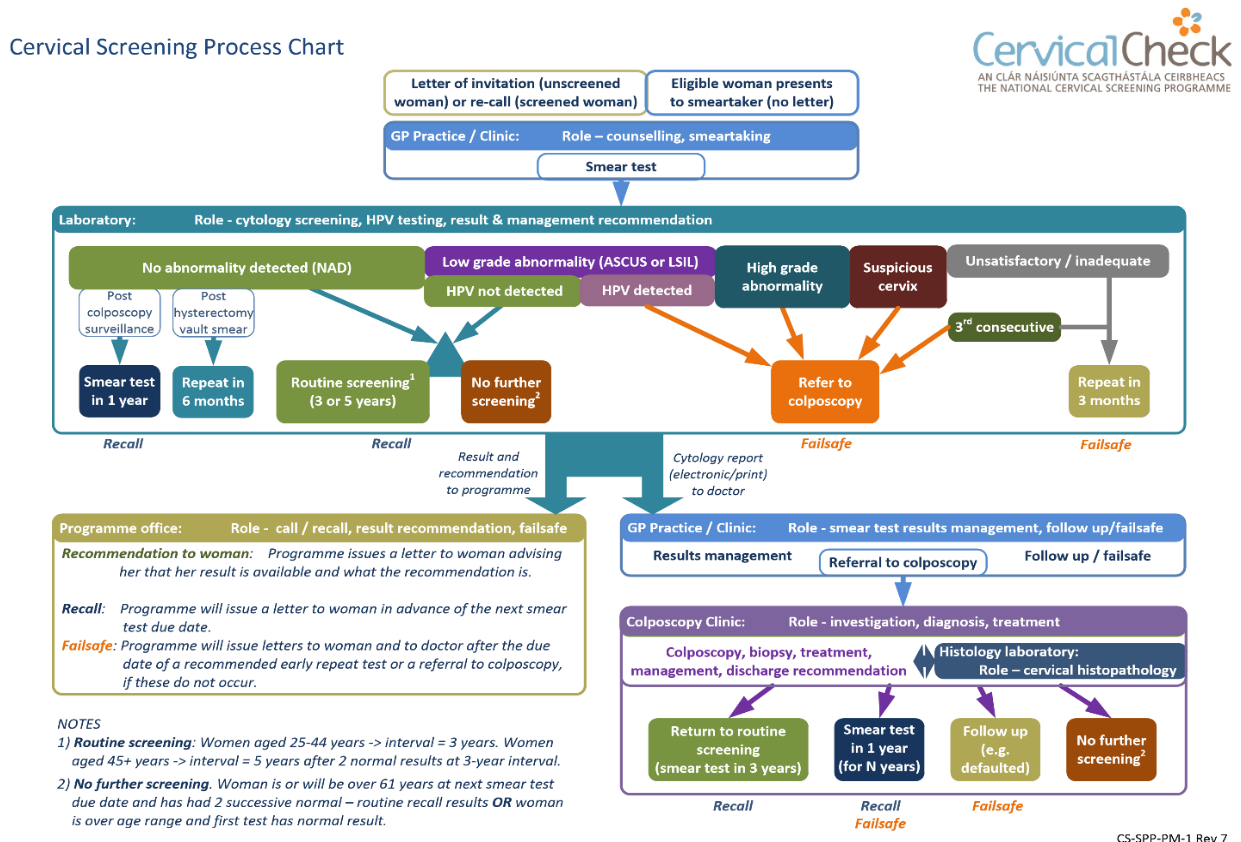

Figure 1.13: Current CervicalCheck Screening Protocol for women attending for routine smears (CervicalCheck, 2017)

1.4.1.2. Test of Cure

LLETZ (Large Loop Excision of the Transformation Zone) (72.5%) and ablation (26.3%) are the two most common forms of treatment for CIN (CervicalCheck, 2016). LLETZ is a procedure where the region of effected tissue is cut away using an electrified loop while the woman is under a local anaesthetic. Though LLETZ has a high success rate with 85% to 95% of CIN or cGIN being cleared after the initial treatment there is a 5-15% chance of a recurrent CIN or cGIN forming post LLETZ (Onuki *et al.*, 2016). This means that women require follow up after treatment to ensure that re-occurrence, if it happens, is dealt with in its earlier stage as the LLETZ procedure carries its own risks such as bleeding as well as increasing the risk of pre-term birth and pre-term membrane ruptures during pregnancy (Kyrgiou *et al.*, 2006). Minimising the number of treatments therefore is a desired goal. The previous standard procedure was annual cytology over a ten-year period which began 6 months post LLETZ. With the understanding that a negative HPV result indicated a very low risk of CIN 2+, HPV was introduced in 2012 as a test of cure, 6 months post treatment women are now co-tested with HPV and cytology. If one of the hrHPV types is detected with an LSIL+ cytology the women is referred to colposcopy and managed as required. If the woman was HPV negative with either an NAD or ASC-US cytology, she is discharged with a 12 month follow up screening test. If HPV negative at the repeat test with either an NAD (No Abnormality Detected), ASC-US or LSIL cytology, she is discharged back into the routine screening system. Any other result warrants further investigation by colposcopy (CervicalCheck - The National Screening Service Programme, 2015). This was a policy change where previously all women would have been followed up annually for 10 years post CIN 2+ treatment (HIQA, 2017).

1.5. HPV Primary Screening

HPV is set to supersede cytology as the primary screening test for cervical cancer in most counties. In May 2017, the Health Information and Quality Authority (HIQA) Ireland published a Health technology assessment of human papillomavirus testing as the primary screening method for prevention of cervical cancer. The primary aim and the scope of the assessment was to examine the impact of switching to a HPV based primary screening test. The report recommended “A change to primary HPV screening followed by liquid-based cytology (LBC) triage at five-yearly intervals for all eligible women aged 25 to 60 years would improve the efficiency of the CervicalCheck programme… would lead to a net cost saving of up to €35 million over the first eight years of its implementation (2018 to 2025)” (HIQA, 2017).

Over the last decade, there have been several randomised control trials (RCT) assessing the use of HPV primary screening including the NTCC Trial (New Technologies in Cervical Cancer Trial) (Ronco *et al.*, 2008, 2010), POBASCAM (Population-based Screening Study Amsterdam) (Dorien C. Rijkaart *et al.*, 2012; Maaike G. Dijkstra *et al.*, 2014), the ARTISTIC Trial in the UK (A Randomised Trial in Screening to Improve Cytology (H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; C Kitchener *et al.*, 2014), and the Public Health Trial Finland, Swedescreen (Naucler *et al.*, 2007, 2009). There have also been a host of observational studies, general papers as well as national health technology papers and Cochrane systematic reviews on the use of a HPV primary screening programme (Moss, 2005; Arbyn *et al.*, 2007; Naucler *et al.*, 2007; Vink *et al.*, 2015; Doorbar *et al.*, 2015; Altobelli *et al.*, 2016; HIQA, 2017; Koliopoulos *et al.*, 2017; Chrysostomou *et al.*, 2018; Clarke *et al.*, 2018; Cuschieri *et al.*, 2018).

From this mass of data there are several clear, repeatable and pertinent results that are consistently shown by these studies. Firstly, HPV testing has a far superior sensitivity for detection of CIN 2+ than cytology. HPV testing had a sensitivity of 95.4% (95% CI: 88.6-98.7) vesus cytology 71.3% (95% CI: 60.6-80.5) from the Sweedescreen RCT (Naucler *et al.*, 2007). HPV testing also has a far superior negative predictive value (NPV) where a single HPV test has an NPV in the range of 99.7% (95% CI:99.6-99.9) (Dillner *et al.*, 2008) and offers a much higher assurance over a 6 year period that a CIN 3+ lesion will not occur. Women who tested negative on cytology but were positive for HPV had a cumulative incidence rate of 10% for CIN 3+ compared to just 3% for those who had a negative HPV test (Dillner *et al.*, 2008). This brings an unparalleled level of assurance into a HPV primary screening programe for those who test negative for HPV. The specificity and positive predictive value (PPV) of HPV primary screening however have consistantly scored lower than cytology with the specificity of HPV DNA tests being 89.9% (95% CI: 89.7-90.00%) compared to cytology which can be as high as 98.6% (95% CI: 98.3-98.9) depending on the center (Cuzick *et al.*, 2006; Naucler *et al.*, 2007; Koliopoulos *et al.*, 2017). The large multicentre based ATHENA trial in the US, (Cox *et al.*, 2013) demonstrated that cytology alone (n=35,546) had a sensitivity of 57.7% and specificity relative to ASC-US triage of 84% compared to HPV testing alone (n=34,254) which had a sensitivity of 89.9% and specificity relative to ASC-US of 71.0%. Overall, the conclusion was that HPV testing can lead to earlier detection of clinically relevant CIN 2+, which, following treatment reduces the subsequent levels of CIN 3+ for those women. HPV testing improves the protection against CIN 3+ compared to cytology (Dorien C. Rijkaart *et al.*, 2012). From the ARTISTIC trials, HPV primary screening was significantly more protective than cytology over a 6 year period (H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; C Kitchener *et al.*, 2014). On the back of these RCTs and many other studies and input from the scientific community

governments and screening programmes are beginning to adopt HPV as the primary screening test for cervical cancer (C Kitchener *et al.*, 2014; HIQA, 2017; Cuschieri *et al.*, 2018).

Much of the initial HPV studies were conducted using the Hybrid Capture 2 (HC2) test, which is considered the gold standard and benchmark HPV test since its introduction. Its importance is such that in 2009 the Meijer crtiteria (Meijer *et al.*, 2009) were set out, in which all other HPV tests must meet a defined set of standards based of its clinical performance. New HPV tests have to meet these criteria such as "the candidate test should have a sensitivity for ≥CIN 2 not less than 90% of the clinical sensitivity of the HC2 in women of at least 30 years." A pooled sensitivity of 89.9% (95% CI: 88.6-91.1) and pooled specificity of 89.9% (95% CI: 89.7-90.00%) has been reported for the HC2 DNA test based on analysis of data from over 138,000 women across 25 different studies (Koliopoulos *et al.*, 2017). Many other competitor HPV tests from the original Hybrid Capture 2 now exist such as the CareHPV test, GP5+/GP6+ bio PCR-EIA, Cervista HPV HR, Cervista HPV 16/18, Cobas HPV test, Xpert HPV, Abbot RealTime hr HPV assay, PapilloCheck and BD Onclarity as well as mRNA based tests such as the Aptima HPV Assay, PreTect HPV-Proofer and Avantage HPV E6 test. This thesis focuses on two of the main HPV tests currently on the market, the Cobas HPV test (DNA based) and Aptima HPV test (mRNA based). Both the Cobas HPV test (Heideman *et al.*, 2011; Stoler *et al.*, 2011; Lloveras *et al.*, 2013) as well as the Aptima HPV test (Heideman *et al.*, 2013; Cook *et al.*, 2017) have met the Meijer criteria (Meijer *et al.*, 2009).

Both these tests have the same end goal, the detection of HPV, but both have starkly different methodologies and targets to do so. The Cobas HPV test targets DNA from the L1 region of the HPV's episome using more classical PCR techniques to detect HPV 16, 18 and the 12 hr HPV's (31/33/35/39/45/51/52/56/58/59/66/68). The Aptima HPV mRNA test however targets the E6 and E7 mRNA in the same 14 hrHPV types as the Cobas test with a second reflex test that allows for detection of HPV 16 and the combined HPV 18/45. The Aptima however does not use the classical PCR technique. It is a three-step process with target capture by oligomers attached to magnetic microparticles. This is followed by transcription mediated amplification (TMA) where the mRNA is transcribed by creating a complementary DNA strand on the mRNA which is followed by cleavage to produce mRNA transcripts which then repeat to form dsDNA which in turn creates more mRNA allowing for a highly sensitive assay. Lastly DNA hybridisation probes are used to detect the product.

The kit specified clinical performance of the Cobas HPV DNA test was a sensitivity of 88.2% (95% CI:84.8-90.9), specificity of 57.8% (95% CI:56.6-58.9) with a PPV of 10.9 (95%

CI:10.4-11.3) and NPV of 98.8 (95% CI: 98.5-99.1). This was in comparison to cytology with a sensitivity of 51.5% (95% CI:46.8-56.2), specificity of 73.4 (95% CI:72.4-74.4) with a PPV of 10.2 (95% CI:9.3-11.1) and NPV of 96.3 (95% CI:95.9-96.6) all for detection of CIN 2+ (Roche Molecular Systems Inc., 2017). This was comparable to the trends and results from the previous RCT studies. From a systematic review published by Cochrane with a sample size of 11,666 women it found that the Cobas HPV DNA test had a sensitivity ranging from (88-100%) and specificity ranging from 50-91% for detection of CIN 2+ (Koliopoulos *et al.*, 2017). To date, the Cobas HPV DNA test has had up to a four year longitudinal follow up period and from the end of the ATHENA trial paper and showed that the 4 year cumulative index rate of CIN 3+ in those women who tested HPV negative at baseline was 0.3% (95% CI 0.1-0.7). The RCT also reported that the sensitivity of the HPV arm of the study was 76.1% (95% CI: 70.3-81.8) with a specificity of 93.5% (95% CI: 93.3-93.8) compared to the control cytology arm with a sensitivity of 47.8% (95% CI: 41.6-54.1) and specificity of 97.1% (95% CI: 96.9-97.2) (Wright *et al.*, 2015).

The kit specified clinical performances for the Aptima HPV mRNA tests state a sensitivity of 90.8% (95% CI:84.9-94.5) and a specificity of 55.7% (95% CI:51.8-59.6) along with a PPV of 32.1% and NPV of 96.3% for detection of CIN 2+. An unnammed comparator HPV DNA test was used for comparison with it having a sensitivity of 95.0% (95% CI:90.1-97.6), specificity of 47.4% (95% CI:43.5-51.4) and a PPV and NPV of 24.9% and 97.6% respectively. Overall, the Aptima HPV mRNA test states it had comparable sensitivity but a significantly higher specificity for detection of CIN 2+ that the use of hrHPV DNA detection alone. From the same independent Cochrane systematic review the Aptima HPV mRNA test was also assessed which included a sample size of 15,895 women. It found that the mRNA test had a sensitivity ranging from 83 to 100% and specificity ranging from 90-97% for detection of CIN 2+. Compared to the Cobas HPV DNA test the Aptima HPV mRNA test has longitudinal data on two consecutive screening rounds across a six year interval from the German AHPV Screening Trial (GAST). It showed that the absolute risk of developing CIN 3+ over the six year period in women who tested negative for HPV on the Aptima tests was 3.1 (95% CI:1.7-5.7) per 1000. This was compared to an absolute risk of 9.3 (95% CI:2.9-30.2) per 1000 for those women who tested negative by cytology. They also compared the Aptima HPV mRNA test against the HC2 test with a relative sensitivity of 91.5% for detection of CIN 3+ and the NPV for the Aptima HPV test was 99.8% (95% CI: 99.5-99.9) (Iftner *et al.*, 2018). Another trial focusing on the Aptima test was the FOCAL trial which had comparable results with the rate of detection of CIN 3+ being higher in the HPV arm (7.5 per 1000) compared to the cytology control arm (4.6 per 1000). A retrospective analysis of Swedish biobanked specimens seven years prior to CIN 3+ both the Cobas and Aptima HPV tests showed a similar concordance. The longitudinal sensitivities for both tests

were assessed and found comparable based of the 80 cases of CIN 3+ that developed after >5 years of follow up. Forslund et al. concluded that HPV mRNA testing could be used safely for cervical screening (Forslund *et al.*, 2018).

The last key point made in these studies is the necessity of triaging those women who test positive for HPV. Due to the lower specificity for CIN 2+ it would be expected that many women who test positive for HPV will have a transient HPV infection with no underlying lesion. To process all HPV positive women based on this single test alone would overburden colposcopy clinics as well as subject women to a more invasive confirmatory procedure with the associated risks over diagnosing non-relevant lesions (Ronco *et al.*, 2010). Many recommend cytology become the initial triage test (Naucler *et al.*, 2007, 2009; Cuschieri *et al.*, 2018). Both the Cobas 4800 HPV DNA test and the Aptima HPV mRNA test have shown repeatedly that they have the ability to better detect CIN 2+ and CIN 3+ lesions compared to the current cytological screening practices. They both however still lack the necessary specificity to function as a standalone test for cervical cancer even with the improvement in specificity that mRNA testing provides. Just as in the original RCTs, triage tests will be required in a HPV primary screening setting. This can be through cytology triage, use of p16^{INK4a}/ki-67 dual staining, methylation markers, HPV genotyping for HPV 16/18 or even other novel biomarkers (Figure 1.14).

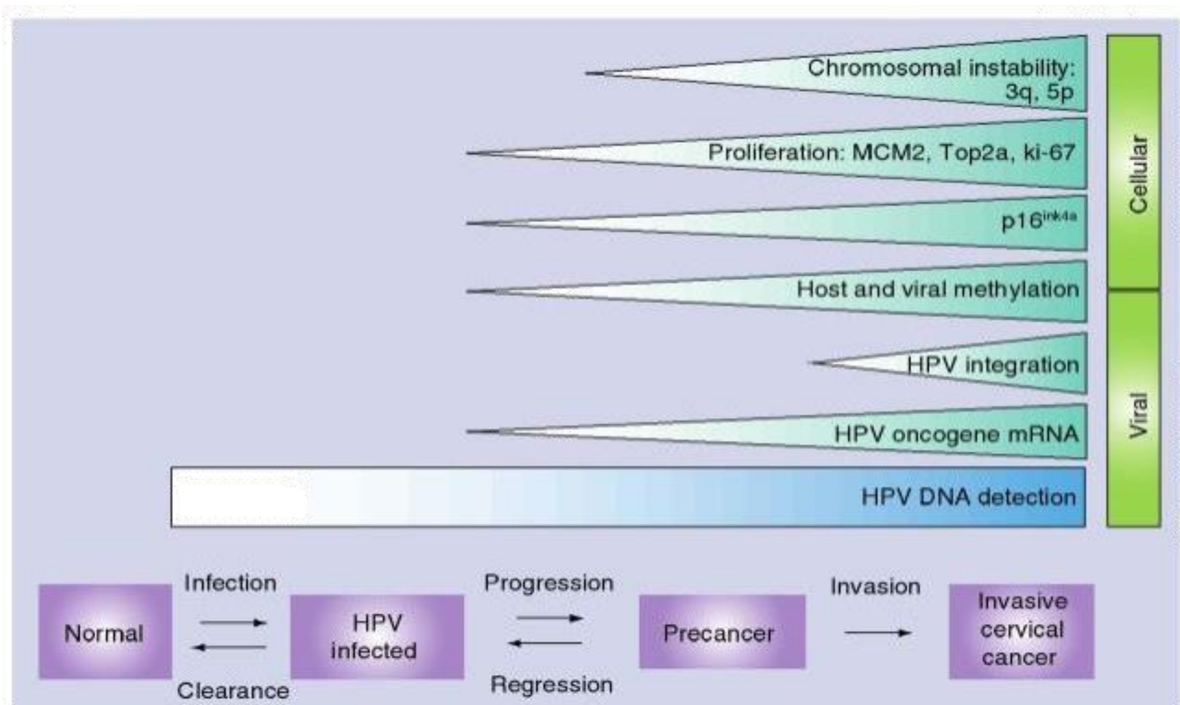

Figure 1.14: Categorised biomarkers for cervical cancer. Amended from Schiffman & Wentzensen 2013

1.5.1. Biomarkers for the Triage of HPV Positive Women.

The current consensus is the requirement of triage test(s) for those women who test positive for HPV. This will be required to refine primary screening and the management of those women at true risk for disease whilst returning those at low risk back to routine screening or a less intensive management programme. Currently there are several options for triaging HPV positive women from utilising the currently established cytology programme or dual staining with p16^{INK4a}/ki-67. There are also other options now available, thanks to the better understood nature of HPV infection and cervical cancer we have a better understanding of not only the phenotypical changes that occur in the cell but also the genetic and molecular alterations that occur (Figure 1.15). Potential biomarkers that follow a more molecular approach have the ability to be a non-subjective test and in theory, indicate potential precancers before the phenotypical changes can be viewed by the conventional cytological approach. Such an epigenetic modification event that has shown promise in this area is that of methylation.

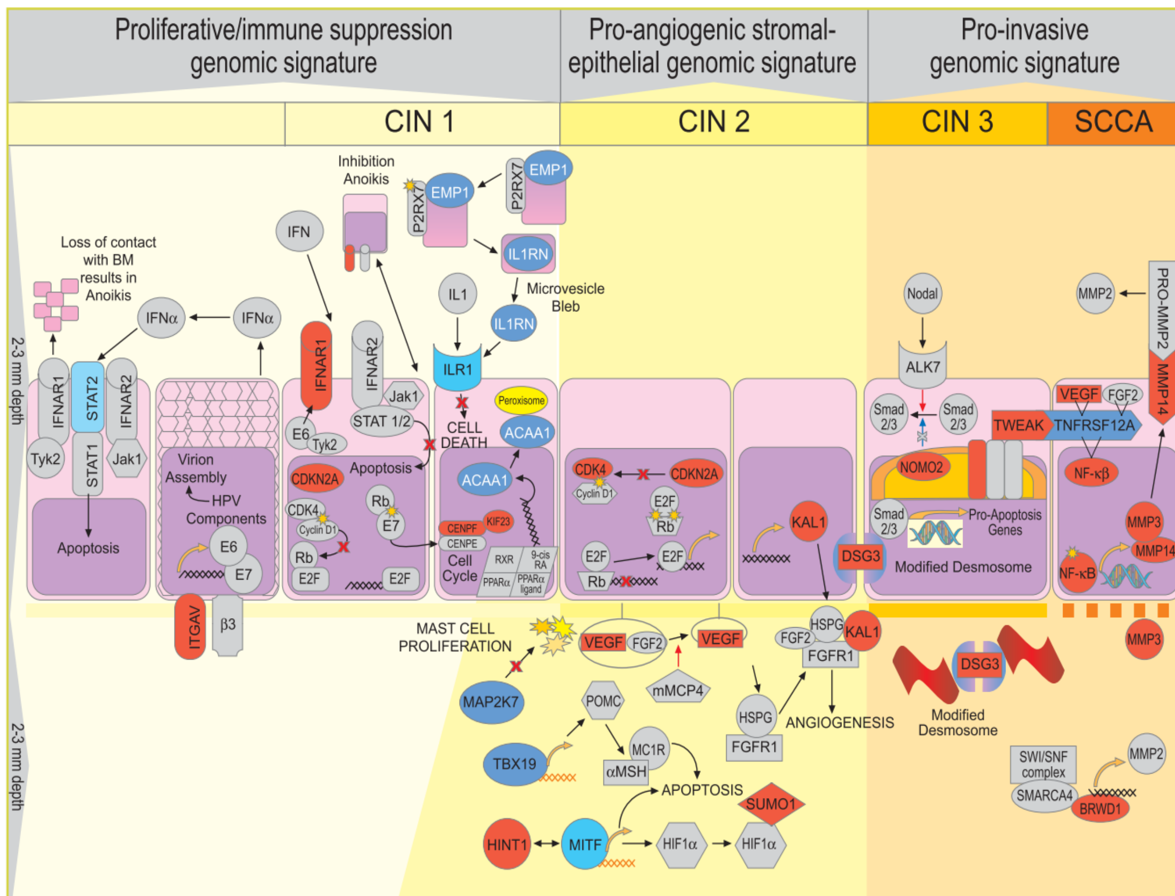

Figure 1.15: Over the course of oncogenic progression there are both phenotypical as well as gene changes occurring. During the transition to CIN 1 there is an increase in gene activities associated with cell proliferation as well as immune suppression. During the transition stages of CIN 1 to CIN 2 there is an upregulation of genes involved in angiogenesis as well as altering the stromal-epithelial environment. In the transition towards CIN 3 and onwards there is the upregulation of genes involved in invasion. Phenotypically these changes can be seen by histology in their CIN classification or by cytology following the Bethesda classifications (Centre for Cancer Research, 2006)

1.5.1.1. Cytology Triage

The most likely triage candidate in any HPV primary screening programme is using its already established cytology screening programme. In theory, it makes the transition period smoother and allows only one shift in practice to occur rather than introducing a new triage test as well as a new primary screening test. To date there have been 3 RTC trials that have looked at cytology triage, namely the ARTISTIC, POBASCAM and Swedescreen (Naucler *et al.*, 2007; H. C. Kitchener *et al.*, 2009; Dorien C. Rijkaart *et al.*, 2012). The general procedure in these trials was to triage HPV women with cytology, those with an ASC-US+ were referred to colposcopy and those that were normal by cytology received a follow up HPV test anywhere between 6 to 18 months later. In the follow up, another HPV test is performed and if the women still had an HPV positive result she would be referred to colposcopy for assessment. The overall outcome was a reduction in CIN 3+ detection in the

second round of screening which indicates that most were discovered in the first screening round with HPV screening. Overall the biopsy rates were similar in the cytology control arm and the HPV with cytology triage arm. Though promising, some issues are still present in cytology triage. Firstly, it is still a subjective morphological assessment of the cervical cells and variation between sites is expected as well as a wide degree of performance variation (Bergeron *et al.*, 2015). This was shown in a Norwegian study where agreement between cytologists was only moderate (kappa: 0.45-0.58) and using ASC-H+ as a cut off the sensitivity varied from 68.8% to 93.8% with the specificity ranging from 70.6% to 95.6%. What was also seen was that those pathologists who showed the highest sensitivity for detecting CIN 2+ were also the least specific (Sørbye *et al.*, 2017). In the role of triage, where HPV provides a high level of sensitivity, what cytology will need to prove is the specificity while maintaining a reasonable and consistent level of sensitivity. A more practical limitation that should be considered is the requirement of a highly skilled and specialised workforce of cytologists required to maintain the service. In high income countries this is less of a challenge, but the likelihood is that HPV primary screening will reduce the overall workload and over time the majority of significant lesions will reduce as has been seen in the RTC's. Long-term, maintaining a cytology staff in this environment may be challenging and attention must be given to alternative options should this become the case.

1.5.1.2. p16INK4a/Ki-67 Dual Staining

p16^{INK4a}/Ki-67 dual staining allows for the simultaneous detection of both the p16^{INK4a} protein as well as the ki-67 protein through immunohistochemical staining of a cervical smear. In the normal cell expression of one or the other proteins is not considered a significant finding, however the dual staining of both proteins is indicative of cellular dysregulation, specifically cell cycle dysregulation. Co-expression of both these markers is considered to be highly indicative of CIN. p16^{INK4a} has been used as a surrogate histological marker for HPV infections. HPV E7 interacts with the retinoblastoma protein (Rb). Normally p16^{INK4a} blocks both CDK 4 and CDK 6 from phosphorylating Rb and thus halting the release of E2F and arrests the cell cycle in G1. In a HPV infection E7 binds Rb releasing E2F and allowing cell cycle progression. This results in a feedback look of p16^{INK4a} overexpression as it attempts to self-correct the progression of the cell cycle (Romagosa *et al.*, 2011). This has been classically used in histology as a surrogate marker for HPV. However, in cervical screening there are no histological waypoints to use as cells are in suspension, so a secondary marker is required to utilise p16^{INK4} in LBC. Ki-67 is a nuclear proliferation marker and the co-expression of p16^{INK4a} which is involved with arresting the cell is indicative of a highly dysregulated cell with a high likelihood of CIN progression. In the ATHENA trial researchers

found that p16^{INK4a}/ki-67 dual staining was significantly more sensitive that cytology (74.9% vs 51.9%) for triaging HPV positive women. The specificity was also comparable (74.1% vs 75.0%). Using HPV 16/18 genotyping with triage for the other hrHPV types showed p16^{INK4a}/Ki-67 had a much greater sensitivity for CIN 3+ (86.8%) compared to cytology (78.2%) whilst still requiring similar levels of colposcopy referral (Wright *et al.*, 2017). p16^{INK4a}/Ki-67 dual staining is showing significant value for triaging HPV positive women, ideally this process is less subjective that cytology but still requires some level of competence. To date, the CINtec PLUS is the only patented kit for p16^{INK4a}/Ki-67 dual staining and there are some issues over the feasibility of introducing the kit in larger scale centralised laboratories though incorporation into automated stainers is a simple and relatively easy solution that will likely be brought forward in the future (Ikenberg *et al.*, 2013).

1.5.1.3. Methylation Markers

In recent years, attention has been drawn to methylation as a potential triage marker for HPV positive women. The knowledge of abnormal methylation patterns is not a new concept with evidence being shown as far back as 1994 that abnormal methylation is present in cancer. The limitations of technology at that time however hindered much progress to make it of use as a test for cancer (Susan *et al.*, 1994). As technology has progressed since then, methylation has taken a more prominent role in our understanding of cancer such as with the methylation of the TSLC1 gene in small cell lung cancer (Kuramochi *et al.*, 2001). TSLC1 would later be re-named as CAD M1 and be shown to be upregulated in cervical cancer cases along with a host of other potential host gene promoter regions. The HPV episome itself has been shown to have increased levels of methylation in cervical carcinogenesis especially in the L1, L2 and E2 gene regions with the logical conclusion that E2 methylation further promotes the upregulation of the two oncogenic E6 and E7 proteins (Lorincz, 2014). A potential draw back with HPV episomal methylation could be the variability over time with the episome itself leading to false negative results however pairing both host and viral methylation markers may be of some use.

In this thesis, host methylation markers will be assessed for their use as a HPV primary screening triage tool. To best understand the potential of methylation testing the process of methylation itself must be well understood.

1.5.2. DNA Methylation

The basis of DNA methylation stems from the methylation of CpG promoter regions. These CpG regions are defined as longer regions of the gene with a higher ratio of cytosine and guanine pairs that can be 50 to 1000bp's long. If the CpG promoter region of a gene is methylated, there is inhibition of that particular gene or in the case of an unmethylated CpG promoter it is free to actively transcribe that genes product(s). Methylation can be thought of in terms of an on or off switch for gene expression as shown in Figure 1.16.

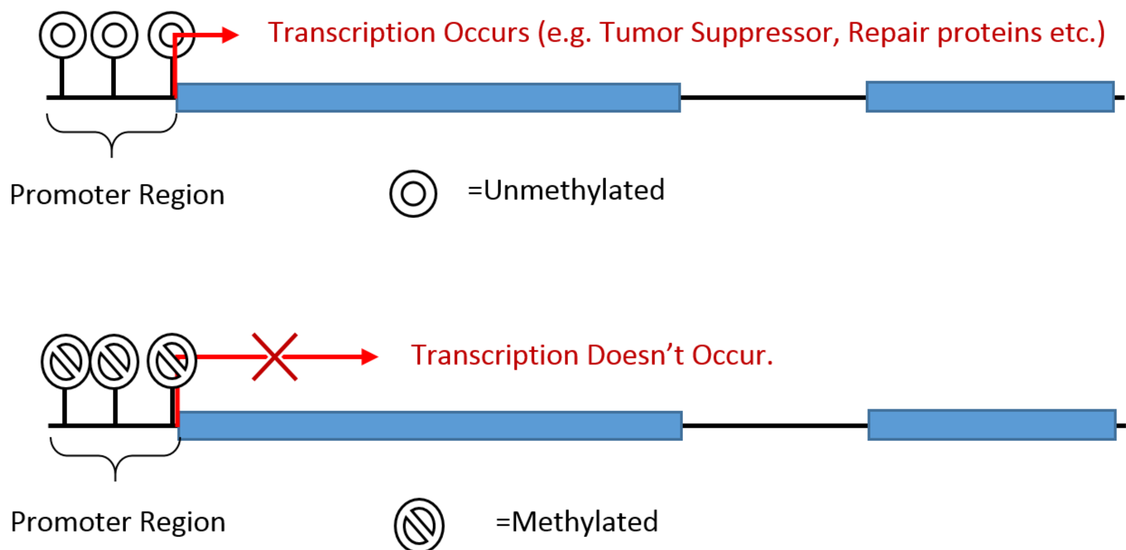

Figure 1.16: Unmethylated vs Methylated Promoter Region

Under normal cellular conditions there is a normal methylation pattern typical to each cell. Depending on the requirements of the specific cell, there are regions of unnecessary gene sequences that are methylated and may have been methylated since embryonic development, and there are those genes that are unmethylated and free to actively transcribe as required. In cancer, this methylation profile is known to change regardless of whether it is a virally driven cancer or a more typical self-derived cancer. Altered methylation patters have been observed in different tumour suppression genes in several different cancer types from ovarian, breast, cervical, endometrial, lung, stomach, liver, colon, brain and skin (Vadakedath and Kandi, 2016). In many cancers, there are altered pattern of methylation present in the cell and that many tumour suppressor sites or novel regions are methylated compared to normal controls (Vadakedath and Kandi, 2016). This indicates that at some point in the oncogenic process there was unwanted methylation of these sites of which some of this altered methylation pattern gave an advantage to the oncogenic process while the impact of methylation on particular sites is not always clear, DNA methylation offers potential as a biomarker for cancer.

In cervical cancer and pre-cancer, there is altered methylation in HPV infected cells. The mechanism behind the alteration is most likely due to the release of DNA Methyltransferase Enzyme 1 (DNMT 1) gene from inhibition as seen in Figure 1.17.
As p53 and Rb are inhibited via E6 and E7 and degraded through the ubiquitination and proteasomal destruction pathway there is the release of the inhibitors of the DNMT gene. This leads to an uncontrolled upregulation of DNMT. This increased level of DNMT increases global methylation in the host cell and it thus acquires an abnormal methylation profile.

DNMT's act via a reversible catalytic reaction where cytosine is converted to 5-methylcytosine. S-Adenosylmethionine (AdoMet) is the donor of the methyl group (Moison, Guieysse-Peugeot and Arimondo, 2014). DNMT's in mammals come in two functional

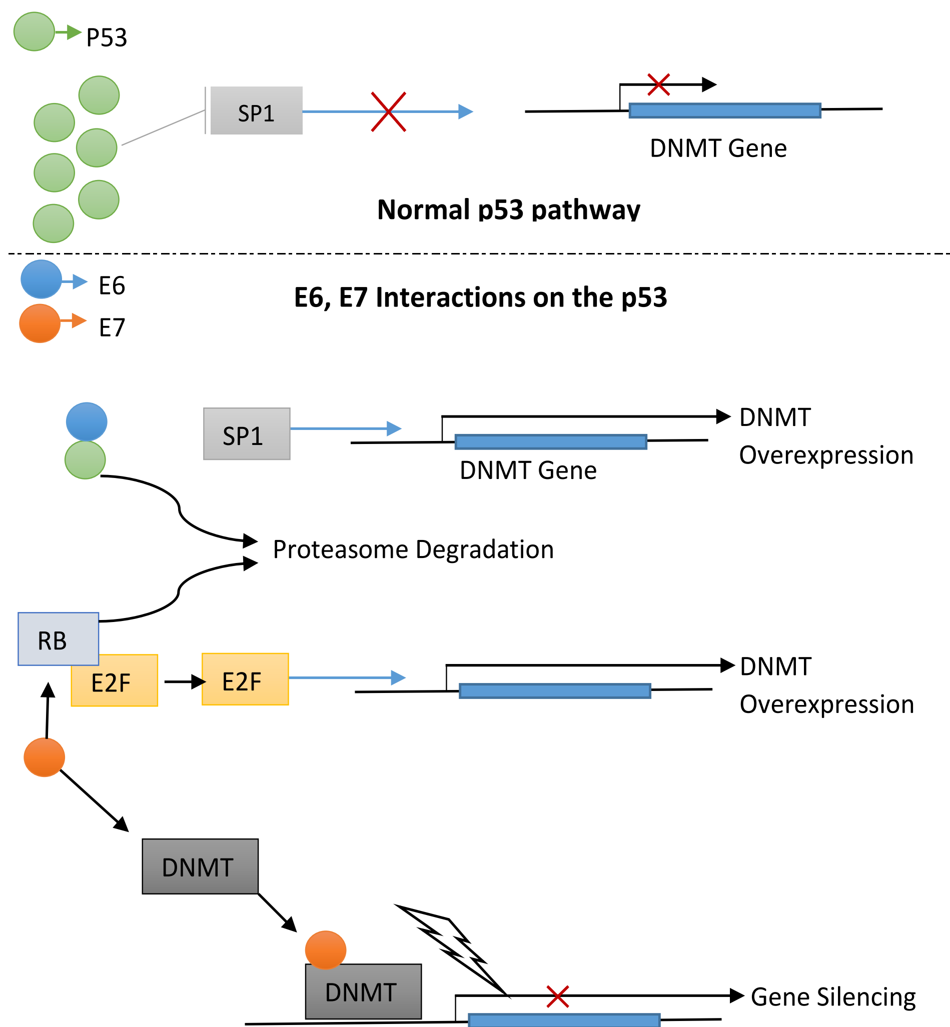

Figure 1.17: p53 Inactivation of the DNMT 1 gene is supressed by E6. Rb inhibition of E2F (promotes G1 to S phase in the cell cycle) is supressed by E7. Upregulation of DNMT enzyme and methylation of genes occurs (Amended from (Jiménez-Wences, Peralta-Zaragoza and Fernández-Tilapa, 2014)

groups, de novo and maintenance DNMT's and to date three active DNMT's have been identified in mammals. De novo DNMT's are present in the embryonic stages of development and stage the pattern of 'normal' methylation in mammals. Maintenance DNMT's act to maintain methylation patterns in the cell as well as correct hemimethylated DNA (DNA that has one strand methylated due to a misstep in replication machinery or a *de novo* methylation event) as shown in Figure 1.18.

The three active DNMT's are:

- DNMT 1: most abundant and key maintenance DNMT. Active in *de novo* methylation.
- DNMT3A: structurally similar to DNMT 1. Active in *de novo* methylation.
- DNMT3B: structurally similar to DNMT 1. Active in *de novo* methylation.

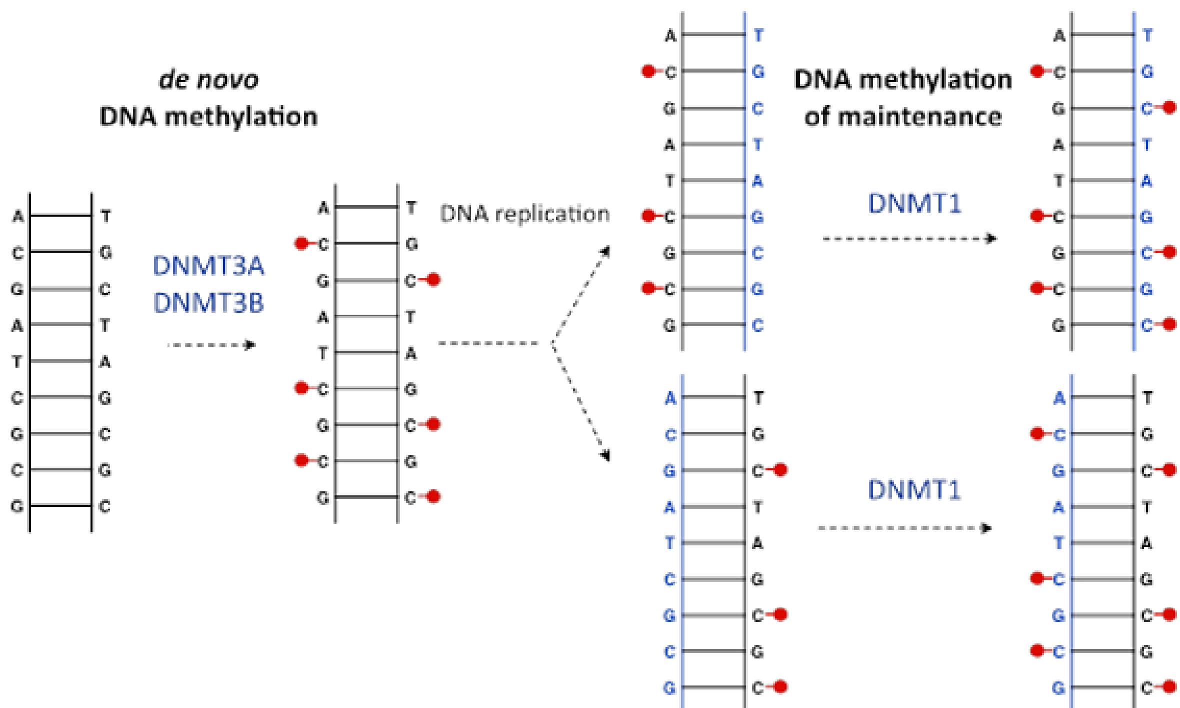

Figure 1.18: Red sticks indicate methylated cytosine residues. Hemimethylated DNA is converted by DNMT 1 as shown on the Right. DNMT3A and DNMT3B are shown on the Left (Moison, Guieysse-Peugeot and Arimondo, 2014)

There is also a fourth DNMT3L: however this does not possess any enzymatic abilities but can be found in a complex with the other DNMT3's and may function as a co-activator and improve the methyltransferase activity by up to 1.3 to 4 times (Castillo-Aguilera *et al.*, 2017). Due to E6 and E7 activity in cervical cancer DNMT 1 is the most likely cause for the upregulated methylation activity and altered methylation profiles (Yang, Coukos and Zhang, 2007; Valeri *et al.*, 2009; Jiménez-Wences, Peralta-Zaragoza and Fernández-Tilapa, 2014).

DNMT's catalytic conversion of cytosine is reversible the current consensus is that TET (Ten-eleven translocation (TET) family) is the main component of the demethylation pathway (Figure 1.19). Depending on the individual cell, there is both methylation and demethylation occurring at varying rates. As the level of HPV E6 and E7 increases, it can overwhelm the demethylation pathways and there is an increase in the global methylation levels of the cell. In fact TET genes are often found to be mutated in other various cancers (Rasmussen and Helin, 2016). It is hypothesised in some groups that monitoring methylation could be used in a similar fashion as HPV was for the test of cure post LLETZ treatment. If the tissue mass is successfully cleared, with the methylation status decreasing from the baseline, may correlate with regression. A stable or rising methylation status may then correlate to incomplete clearance or progression though this has yet to be shown.

During cervical carcinogenesis many sites seem to be commonly methylated though the reason behind the apparent specific targeting of these genes is unclear and remains an

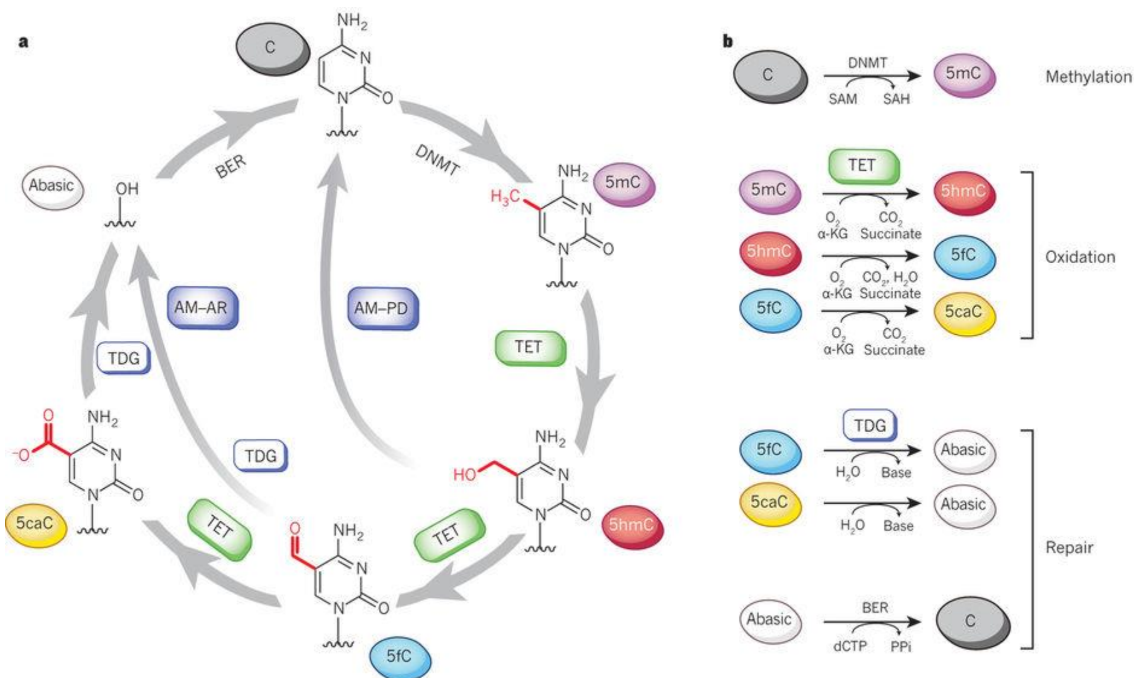

Figure 1.19: Methylation and Demethylation cycle of the Cytosine group (Kohli and Zhang, 2013)

area of active research. New research may be shedding light into the specific/targeted methylation process. There are certain DNMT complexes that show a mix of either specific or nonspecific binding to DNA, most of this seems to be involved in the normal regulatory process of the cell though there are some repeated patterns of methylation in cancers which still suggests that there may be specific methylation occurring. This could be due to complexes forming or potentially DNMT having an affinity for specific regions such (A/G/T)(T/G/A)(T/A/C)**CG**(T/G/A)(C/A/T)(A/T/C), (T/A/C)(A/T)(T/G/A)**CG**(T/G/C)G(G/C/A), and (A/C)(C/G/A)(A/G)**CG**T(C/G)(A/G) (Hervouet *et al.*, 2018). There is some evidence to suggest that viral components can also influence DNMT activity such as E1a in adenovirus and HPV's E7. DNMT1/E7 complexes seem to increase inheritance DNA methylation in the cell. This may be occurring as DNMT1/E7 may be promoting DNA binding and AdoMet recruitment allowing for more efficient methylation. The question of specificity and whether the DNMT1/E7 complex has other complexes involved is still unanswered (Burgers *et al.*, 2007; Hervouet *et al.*, 2018).

1.5.2.1. Methylation and Cervical Cancer

Understanding that HPV infection leads to the eventual and consistent upregulation of methylation in the host cell has allowed researchers to proceed with whole genome wide methylation analysis (Wang *et al.*, 2016; García *et al.*, 2017; Verlaat *et al.*, 2017). This showed that in CIN 3 lesions there were higher frequencies of methylation in the promoter regions for genes involved in transcription (27%) and cell structure (26%) followed by genes involved in neuronal activity, signal transduction, cell cycle and metabolism (14%, 12% 8% and 8%, respectively) (García *et al.*, 2017). From this and previous studies, researches have proffered potential individual candidate genes for detection of cervical cancer. In the EUROGIN roadmap for 2017 a list of the current prominent markers was published which included: C13ORF18, JAM3, ANKRD18CP, FAM19A4, EPB41L3, HPV16/18/31/33, CADM1, MAL, miR-124, PAX1, SOX17, TERT along with others. Of these markers, two of the most well researched and highest potential methylation markers to date in HPV positive women has been the combination of CAD M1 and MAL. In combination these markers have been shown to achieve a sensitivity of 84% and specificity of 52% for detection of CIN 2+ (Hesselink *et al.*, 2011). Other marker combinations such as FAM19A4 and hsa-mir-124-2 in combination with HPV 16/18 genotyping has been shown to greatly increase the sensitivity for detection of CIN 2+ (De Strooper *et al.*, 2016). The use of viral methylation markers has also been used in combination with EPB41L3 in a population of hrHPV positive women with a reported sensitivity of 74% and specificity of 65% (Lorincz *et al.*, 2016). Methylation markers have also been investigated as nested studies in recent RCTs such as the PROHTECT trial. This compared the combination of MAL and hsa-mir-124-2 to cytology

triage of HPV positive self-sampled women. On comparison, the methylation test was non-inferior to cytology (Verhoef, Bosgraaf, *et al.*, 2014). While these markers did not show a superior clinical performance there are still a host of advantages to using methylation in lieu of cytology. There is the logistical advantage of being able to test both the primary and triage test on the same sample or even on a self-sample in what is likely to become a fully automated process. Added to this the non-subjective nature of molecular testing can allow a greater degree of consistency, reproducibility and objectivity to the triage test.

1.5.2.2. Methylation Markers [CAD M1-M18, MAL M1 and hsa-mir-124-2] for Detection of Cervical Cancer and Pre-Cancer

For this study, three markers were chosen to represent the more well documented and researched markers at the time the study commenced. This was an important consideration at the time as the chosen markers would, for the first time, be tested in a HPV Primary Screening population and also at a larger scale than previously published. Selecting the most researched and promising methylation markers in lieu of more novel markers would allow this methylation panel to have a solid basis in which to exist. To that end the markers chosen were CAD M1 M18, MAL M1 and hsa-mir-124-2 which showed the most promise for triaging HPV positive women and comprised the majority of literature on methylation testing at the time. Methylation biomarkers are generally poorly understood or defined. As these markers are generally pulled from whole genome methylation analysis studies functional data can be lacking. The currently known functions for each of the methylation markers are listed below.

CAD M1 M18 (Cell Adhesion Molecule (1/18)) was originally associated as a tumour suppressor in lung cancer and was referred to originally as TSLC1. Some functions have been elucidated over time but due to its frequent relabelling (Also known as; BL2; ST17; IGSF4; NECL2; RA175; TSLC1; IGSF4A; Necl-2; SYNCAM; sgIGSF; sTSLC-1; synCAM1) (Gene ID: 23705) consensus on the functional aspects are limited. CAD M1 M18 encodes a member of the immunoglobulin superfamily (Kuramochi *et al.*, 2001). The protein is an immunoglobulin-like cell surface protein involved in intracellular adhesion through homophilic and heterophilic trans-interactions (Masuda *et al.*, 2002). Loss of CADM1 function is associated with reduced cell adhesion and suppression of tumourigenicity, invasion, and anchorage-independent growth. Moreover, CADM1 overexpression has been shown to inhibit proliferation and induce apoptosis in epithelial cells (Kuramochi *et al.*, 2001; Ito, Shimada and Hashimoto, 2003). CAD M1 is frequently down regulated in many cancers i.e. lung, prostate, liver, pancreas, and breast, etc (Kuramochi *et al.*, 2001; Murakami,

2005). Reduced CAD M1 expression leads to the disruption of cell-cell adhesion in the epithelial cells and can lead to tumour cell invasion and metastasis (Murakami, 2005).

The MAL (myelin lymphocyte proteolipid) gene encodes a highly hydrophobic integral membrane protein belonging to the MAL family of proteolipids (NCBI, 2018). MAL has been shown to be localized to the endoplasmic reticulum (ER) of T-cells and is a candidate linker protein for T-cell signal transduction (NCBI 2018). MAL is also localized in compact myelin of cells in the nervous system and may be involved in myelin biogenesis and/or function (NCBI 2018). MAL is involved in the formation, stabilization and maintenance of the glycosphingolipid-enriched membrane microdomains (NCBI 2018). When down-regulated, this gene has been associated with a variety of human epithelial malignancies (NCBI 2018). Mal may have an important role in vesicular trafficking cycling between the Golgi complex and the apical plasma membrane (GeneCards, 2018).

hsa-mir-124-2 is a micro RNA (miRNA), a small non-coding RNA strand. miRNA is a known component in epigenetic regulation of gene expression. In viral infection, to help achieve a suitable environment, the infecting virus has been shown to either modify or downregulate miRNA's (Skalsky and Cullen, 2010). The mature hsa-mir-124 sequence is processed from 3 separate premature sequences, located at chromosomes; 8p23.1 (miR-124-1), 8q12.3 (miR-124-2), and 20q13.33 (miR-124-3) all of which contain CpG islands in their promoter regions and thus are viable methylation targets. hsa-mir-124-2 and hsa-mir-124-3 promoters were commonly hypermethylated in Androgen Receptor negative Prostatic Cancer (PCa) cells. Overexpression of hsa-mir-124 inhibited the proliferation rates and invasiveness of PCa cells in vitro, and suppressed xenograft tumour growth in vivo (Chu *et al.*, 2015). hsa-mir-124 may inhibit lung cancer cell migration and invasion through suppressing epithelial-mesenchymal transition (EMT) and inducing apoptosis of the lung cancer cells. hsa-mir-124 has been shown to suppress tumour proliferation and aggression by directly targeting oncogenic CD164 signalling pathway in non-small cell lung cancer (Lin *et al.*, 2016).

Following on from genome methylation analysis studies, CAD M1 M18 via bisulphite sequencing of a 93 bp promoter region was investigated. Methylation of CADM1 was demonstrated in most cervical SCCs and a subset of high-grade CIN lesions (Overmeer *et al.*, 2008). From this, many subsequent papers determined the viability of CAD M1 M18 methylation as a plausible biomarker for cervical cancer. Many of these papers also included MAL M1 and hsa-mir-124-2 (Buffart *et al.*, 2008; Overmeer *et al.*, 2008, 2011; Hesselink *et al.*, 2011; Snellenberg *et al.*, 2012; Bierkens *et al.*, 2013; De Strooper, Hesselink, *et al.*, 2014; De Strooper, van Zummeren, *et al.*, 2014; Verhoef *et al.*, 2015).

These are summarised in Table 1.3 and contributed to the primary papers available for these markers at the start of this study. hsa-mir-124-2 in relation to cervical cancer is currently the least understood function-wise. It is known that miRNA loci are significantly associated with insertion sites of HPV in cervical cancers and that HPV- encoded genes were shown to influence the miRNA expression of its host cell, generally through down regulation (Jiménez-Wences, Peralta-Zaragoza and Fernández-Tilapa, 2014). Increased methylation levels of hsa-miR-124-1 and hsa-miR-124-2 in cervical tissue specimens are significantly correlated with lower hsa-miR-124 expression levels (Wilting *et al.*, 2010). In HPV-immortalised keratinocytes increased methylation levels are related to reduced hsa-miR-124 expression and higher mRNA expression of IGFBP7, a potential hsa-miR-124 target gene (Wilting *et al.*, 2010). hsa-mir-124-2 represses the migration and invasion ability of cervical cells, and it supresses vasculogenic mimicry (VM) in cancer cells. VM is an important step in cancer metastasis (Wilting *et al.*, 2010).

Table 1.3: Current literature on CAD M1, MAL M1, hsa-mir-124 and other markers

Study	Country	No. of Women	Sample Type	Methylation Marker*	Outcome
Overmeer et al. 2008	Netherlands	n=171	Cervical Tissue and scrapes	CADM1 via immunostaining	Dense methylation increased from 5% in normal samples to 30% in CIN 3 and 83% in SCC ($p<0.00005$).
Overmeer et al. 2011	Netherlands	n=261	FFPE cervical specimens	CADM1/MAL	HR-HPV+CAD/MAL markers outperformed HR-HPV+LBC in the detection of CIN 1, CIN 2, CIN 3 and Cancerous specimens.
Hesselink et al. 2011	Netherlands	n=275	Cervical scrapes	CADM1-M12, CADM1-M18, MAL M1, MAL M2	Combination of CADM1-M18 and MAL M1 was the best combination (cross validated partial AUC=0.719). Validation panel CIN 3+ sensitivity 100%-60.5%, specificity ranging 22.7% to 83.3%.
Bierkens et al. 2013	Netherlands	n=388	Cervical Scrapes	CADM1/MAL	Statistically significant difference in baseline scrapes vs CIN2+ scrapes ($p=0.001$). Statistically significant difference in CIN 2 to CIN 3 scrapes ($p=0.031$).
De Strooper, Hesselink, et al., 2014	Netherlands	n=268	Cervical Scrapes	CADM1/MAL/mir-124-2	Tri-Marker panel; 100% Detection of cervical cancers. (at least one marker positive) and 76% of endometrial cancers. 68% CIN 3, 37.5% CIN 2 positive for 1/3 methylation markers.

De Strooper, van Zummeren, et al., 2014	Netherlands	n=250	Cervical Scrapes	CADM1/MAL	Cytology with bi-marker sensitivity (84.5%), Specificity (69.9%), PPV (48%), NPV (93.2%) for CIN2. Cytology with bi-marker sensitivity (86.8%), Specificity (64.8%), PPV (32.4%), NPV (96.2%) for CIN 3+. Recommended as a triage test for HR-HPV types.
Verhoef et al. 2015	Netherlands	n=364 from PROHTECT-3 Study	Cervical Scrape	CADM1/MAL	Cytology with bi-marker increased CIN3+ sensitivity (88.7%) specificity (53.6%). Reduced risk of missed high grade lesions.
*Keyword search of CADM1 methylation markers via PubMed. *All methylation markers are detected via qMSP unless otherwise stated*					

Several studies have demonstrated the utility either singly or in combination of the expression patterns of the methylation biomarkers CAD M1 M18, MAL M1, hsa-mir-124-2 for use in cervical screening (Table 1.3). Detection of a combination of all three biomarkers in cervical scrapes from women consistently detects CIN 3 (De Strooper, van Zummeren, *et al.*, 2014). Furthermore, in a HR HPV DNA positive groups of HIV positive women the specificity, sensitivity of the combined tri-marker test for CIN2+ was comparable with cytology [atypical squamous cells of undetermined significance or worse] (89% and 95%, respectively) (De Vuyst *et al.*, 2015). The use of methylation biomarkers for triage of HPV positive women from primary screening has been explored in self-samples taken from a cohort of 355 hrHPV-positive self-collected specimens from women in routine screening in the Netherlands. At clinical specificities of 50 and 70%, the MAL- m1/ miR-124-2 methylation markers showed sensitivity for detection of CIN3 which ranged from 77.0% to 87.8% and from 64.9% to 71.6%, respectively (Hesselink *et al.*, 2014). Other studies have shown that combined CAD M1 M18/MAL panels have a CIN3+ sensitivity of 86.8% compared with 65.8% for sole cytology triage testing. Corresponding CIN3+ specificity was 64.8% for combined triage and 78.6% for sole cytology triage testing. For CIN2+, the sensitivity of combined triage testing was 84.5% compared with 65.5% for sole cytology triage, with corresponding specificities of 69.9% and 83.5%, respectively (De Strooper, Hesselink, *et al.*, 2014). These studies do however have several limitations, many studies have had relatively small sample sizes from 50 to 500 women. This coupled with the higher prevalence of CIN 2+ in these studies likely skews the overall clinical performance of these tests. Many studies also utilised cervical scrapes or self-collected sampled and not cervical brush samples used in the Irish cervical screening programme or in the case of triage data utilise retrospective study samples. There is also no current evidence in the effectiveness of these markers in a HPV primary screening population. Lastly there is no standardised approach to methylation testing. There is no consensus of what molecular approach to take from PCR based or pyrosequencing yet to be decided. There is also no consensus on the appropriate approach to defining clinically relevant cut offs for methylation with some studies approaching each methylation marker in an individual manor or utilising combined scoring approaches with positivity definitions varying from group to group.

This highlights two aspects, firstly the potential of CAD M1 M18, MAL M1 and hsa-mir-124-2 for use in triage of HPV positive women. All three markers have shown consistently positive results in regard to the detection of CIN 2+ and compared to other markers present prior to 2016 they also contributed the majority or research in this field making them prime candidates for this study. Secondly, though these are the most researched methylation markers in this space they still lack key data points such as sample size, reflective of

disease prevalence and use in a primary screening population on top of methodological questions such as the right technique, classification and scoring methods.

This thesis will further expand this this area by examining for the first time the methylation profile of these three methylation biomarkers for triage of HPV DNA positive women from primary screening population. It will be one of the largest studies undertaken on these methylation markers and is part of a larger trial underway within CERVIVA which is also examining other triage approaches for managing HPV positive women. This thesis will also examine the utility of the methylation biomarker panel [CAD M1 M18, MAL and hsa-mir-124-2] in ThinPrep liquid-based cytology specimens. As well as a novel approach in defining clinically relevant methylation and novel scoring methods.

1.6. CERVIVA

This study stems from previous work carried out under CERVIVA, the Irish cervical screening consortium. CERVIVA is a multi-investigator, multi-institutional consortium which was established in 2005 by Prof. John O'Leary and Prof. Cara Martin. This collaborative research effort between various academic institutions, hospitals and industry partners is pursuing a number of research projects which will aid the cervical screening programme in Ireland and provide information and guidance on its delivery. CERVIVA has focused on the development of biochip technologies, virtual slide technologies and quality assurance systems, the impact of vaccination, social acceptance of HPV testing, education and outreach programmes, HPV in HIV infected women, the development of automated screening technologies, HPV primary screening and HPV primary screening triage tests. Phase 3 (CERVIVA CARG) is funded by the Health Research Board Ireland (CARG 29/2012). Reagents and consumables for the Aptima HPV E6/E7 mRNA test were supplied by Hologic®. A portion of the reagents and consumables for the Cobas 4800 HPV DNA test were supplied by Roche. This study was performed partnership with CervicalCheck, the National Cervical Screening Programme in Ireland. CERVIVA CARG will focus on the areas of HPV primary screening, molecular biomarkers and HPV screening in vaccinated cohorts.

1.7. Current Challenges

Cervical cancer is a preventable disease in the vast majority of cases, if detected early enough. At the beginning of this PhD project, cytology was the primary test for detection of cervical cancer and precancer with a handful of countries moving to implementation of HPV primary screening. Over the course of the three years since beginning this project, Ireland has tendered for the introduction of a HPV primary screening in line with EU counterparts. From a population perspective this is an excellent prospect. The limitations of cytology from its sensitivity and poor reproducibility are well documented. HPV primary screening will undoubtably provide a greater level of detection of high grade pre-cancerous lesions allowing for the timelier treatment of many women. There still remains the challenge of both what HPV detection platforms to use in this new programme. The biggest challenge facing HPV primary screening is how to adequately triage HPV positive women. Though the HiQA HTA has recommended cytology to be the initial triage test, the longevity of maintaining an adequate cytology service may become an issue over time and still suffers from the lack of sensitivity (HIQA, 2017; Cuschieri *et al.*, 2018). Cytology is also incapable of referring HPV positive cytology negative women back to routine screening leading to subsequent shorter interval testing to ensure no relevant cases are missed (Stanczuk *et al.*, 2017; Wentzensen *et al.*, 2017; Cuschieri *et al.*, 2018). The options for molecularly triaging HPV positive women show great potential from the current literature yet lack reproduction in other research groups and in general have too small a sample size and lack of definition for what classifies a positive methylation test. To date there has been minimal research into HPV based screening in an Irish population with CERVIVA being the primary research consortium in this space. There has also been no research into methylation testing for cervical cancer in an Irish population either. To allow for the best possible start of the HPV primary screening programme in 2020 this study aims to provide this valuable data which has been lacking to date. Though cytology will likely be the triage test used in the initial programme there is a high probability that methylation testing will be a potential alternative and hopefully address issues cytology triage suffers from. To that end it is important that longitudinal data exists for these markers to truly understand their utility in this area. This longitudinal aspect is incorporated into this project but won't pay dividends for several years but remains a vital part of this observational study.

Thus, it is the aim of this project to assess a three-marker methylation panel for the triage of HPV positive women. This will be done in a two-part manner, firstly, the aim is to validate and determine an ideal methylation testing approach that will best classify CIN 2+ lesions. Secondly, it is the aim of this project to assess the optimal approach on the HPV positive triaged population in this study and determine if it further stratify HPV positive women.

1.8. Hypothesis

HPV primary screening is more sensitive for detection of CIN 2+ than standard cytology primary screening. Different HPV assays perform differently in different populations. This thesis will assess the performance of two different HPV assays for use in primary screening. I hypothesise that HPV DNA and HPV E6/E7 mRNA assays are comparable for use in a primary screening context. However, HPV primary screening will be less sensitive and refer more women to colposcopy without adequate triage. I hypothesis that a panel of three methylation biomarker [CAD M1-M18, MAL M1 and hsa-mir-124-2] used either singly or in combination will be able to stratify HPV positive women with an adequate sensitivity and specificity for detection of CIN 2+. Methylation biomarker panels have the potential to reduce the risk and number of women referred to colposcopy in a HPV primary screening programme.

1.9. Aims

The aims of this project were to;

i. to determine the baseline prevalence of HPV DNA and HPV E6/E7 mRNA in the population and evaluate and compare HPV DNA and HPV E6/E7 mRNA tests for detection of CIN 2+ in a HPV primary screening population.

ii. to establish a validated and clinically relevant threshold for positive methylation for detection of CIN 2+.

iii. to assess the utility of the methylation panel for triage of HPV positive women in a "real world" primary screening population and to determine its clinical performance, associated risks and evaluate its referral rates in comparison to cytology with and without HPV 16/18 genotyping.

iv. to assess the clinical performance, associated risks and referral rates of a custom methylation panel for triage of HPV positive women in a "real world " primary screening population in comparison to cytology with and without HPV 16/18 genotyping.

1.10. References

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-Chapter 2-

Materials and Methods

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2.1. Study Population

This chapter contains a detailed description and accompanying background information on all the Materials and Methods used in this thesis. These methods will be referred to again in subsequent chapters briefly.

2.1.1. Patient Recruitment

Women attending for their routine cervical smear offered by CervicalCheck were recruited from 33 different primary care centres in Ireland. These centres were chosen to reflect the normal screening population as well as sending their smears to the Coombe Women & Infants University Hospital for cytological review. Prior to a woman having her smear taken she is provided with a Study Information Leaflet and a consent form. All women attending for their routine smear test were eligible to participate in the study. Exclusion criteria include women with a previous history of CIN2+, an abnormal smear within the past 3 years or women who had undergone hysterectomy where the cervix has been removed.

2.1.2. Study Design

The HPV primary screening pilot study is an observational cohort study conducted on women attending primary care for their routine CervicalCheck smears. The study is examining the utility of different HPV tests for primary screening and different molecular triage tests for management of HPV positive cases (Figure 2.1). A total of13,446 women were enrolled into this study. Of this, 1277 of these were invalid for this study, 91 gave incomplete consent, 111 did not give full consent, 301 were duplicate annual smears and not part of the routine screening programme and 764 were additional annual smears. This left the study with 12,169 valid fully consenting women enrolled into this study. Once routine screening was performed by the Cytopathology laboratory in the Coombe Women and Infants University Hospital (CWIUH) the sample was given a unique ID (CARG Number, CARG xxxxx) and pseudo-anonymised for research use. The Cytology laboratory maintains and holds the database of all women enrolled including their key identifiers and linker to their assigned CARG Study ID. Once the samples had been coded, they were labelled with the appropriate CARG ID sticker and other identifiers on the vial were removed or obscured before transferring the sample into the molecular pathology laboratory for research testing. The Cytology laboratory at the CWIUH acts as the gatekeeper to the coded data. Once

transferred to the molecular research lab all samples were processed for HPV DNA and mRNA testing as described in Section 2.2. HPV DNA positive cases were further tested for a panel of biomarkers including: methylation markers (CAD M1-M18, MAL M1 and hsa-mir-124-2), p16/ki-67 dual staining, cytology, HPV mRNA partial genotyping and DNA HPV 16/18/HR genotyping (Figure 2.1).

This PhD thesis work is focused on the HPV testing, both DNA and mRNA as well as the analysis of methylation markers in the context of triage for HPV positive results.
All participants provided written informed consent access their CervicalCheck follow-up data in relation to subsequent smear tests or treatments the women may have had. This data was used to determine allowed the clinical performance of the study tests. This data is provided coded from CervicalCheck. The individual study results were not disclosed to the women, GP or smear taker and participating in the study had no impact on their standard of care.

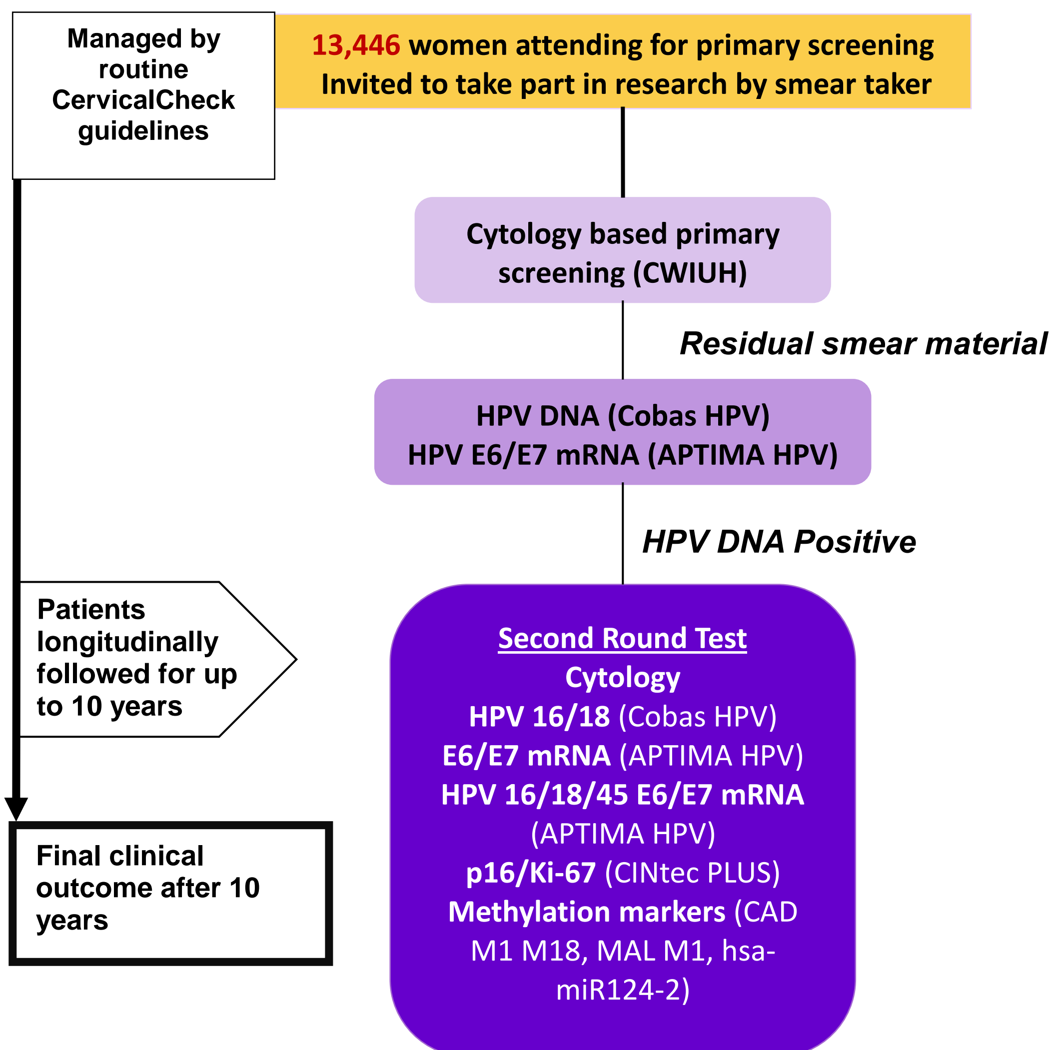

Figure 2.1: HPV Primary Screening Pilot: Study Design

2.1.3. Ethical Approval

Ethical approval was granted by the Research Ethics Council at the Irish College of General Practitioners (ICGP). All women provided written informed consent to be included in the study.

2.2. HPV Testing

2.2.1. The Cobas 4800 System and HPV Test

The Cobas 4800 HPV Test [Roche] is a qualitative *in-vitro* test for the detection of HPV DNA. This system is a full flow system coupling both the Cobas x480 for sample preparation and the Cobas z480 for amplification and detection of PCR product.

The Cobas 4800 HPV Test targets DNA from the L1 region. This region is amplified via PCR to detect HPV 16, 18 and 12 hr HPV's (31/33/35/39/45/51/52/56/58/59/66/68) in a single analysis. This testing method requires a dead volume of 3mls from a primary ThinPrep vial or 1000µl from secondary tubes with a consumption of 400µl of sample per run. β-globin acts as the Internal Cellular Control for this assay with built in pass/fail limits (Roche Molecular Systems Inc., 2017). The instrument software specifications states that results are positive at clinically relevant infection levels. The Limit of Detection (LOD) for this assay was defined as "the level of HPV DNA in the sample that has a positive test result at least 95% of the time". The LOD for plasmids of HPV 16 and HPV 18 was 600 copies/ml. The LOD for HPV infected cell lines were as follows: SiHa (HPV 16), 100 cells/ml; HeLa (HPV 18), 40 cells/ml. The Cobas 4800 HPV Test is run as per manufacturer's guidelines and kit inserts (Roche Molecular Systems Inc., 2016, 2017). This involved two major process DNA extraction, purification and PCR plate set up on the Cobas x480 and real-time PCR amplification on the Cobas z480. The Cobas 4800 System is shown in Figure 2.2.

Figure 2.2: The Cobas 4800 system. Left to Right; The Cobas x480 unit and the Cobas z480 unit (Roche Molecular Systems Inc., 2009b)

2.2.1.1. Cobas x480 Unit and the HPV DNA Test

The Cobas z480, heater shaker unit, Cobas x480 and Cobas 4800 software were turned on. Periodic maintenance was performed on the Cobas x480 instrument, daily and weekly maintenance was mandatory and prompted by the system software, the ability to perform a kit run was locked out pending completion of the required maintenance. To perform maintenance the “Run Daily/Weekly Maintenance” icon was clicked under maintenance status in the Cobas x480 tab.

All reagents were removed from the fridge and allowed to come to room temperature. In total 96 samples were processed per run including a positive and negative kit control that was provided with the assay. An on-screen wizard guides you through the loading of the instrument. ThinPrep vials were vortexed and the lid was removed before loading into carrier racks twelve at a time. The sample barcode was read as the sample is loaded on to the instrument. The controls and reagents were loaded in separate racks to samples, once equilibrated to room temperature. Reagents including; Magnetic Glass Particles (MGP), Elution Buffer (EB), System Wash Buffer (WB), Proteinase K (PK), SDS, System lysis Buffer (LYS), HPV Master Mix (HPV MMX), HPV Mg/Mn Solution, HPV Positive Control (HPV 39 for High Risk, HPV 16, HPV 18 plasmids), HPV Negative Control (Tris Buffer). Only the MGP reagent required vortexing to bring all magnetic glass particles into suspension.

The Cobas x480 workflow overview was as follows; The Deepwell Plate (DWP) was moved to the Processing Location onboard the Cobas x480. 100µl of SDS and PK was dispensed

into each well that will contain a sample. 400µl of NC and PC were added to wells A1/B1 and underwent an aspirate/dispense (A/D) mixing step. 400µl of specimen was transferred into the DWP and underwent an A/D mixing step. The DWP was transferred to the Heater/Shaker onboard unit. 110µl of MGP and 350µl of LYS was added. Lysis and binding occurred in the DWP. Nucleic acids bind to the silica surface of the MGP in the presence of chaotropic salts, high detergent concentrations and low Ph. The DWP was transferred to the Magnetic Separator. On an activated Magnetic Separator which bind the MGP: Nucleic Acids to the bottom of the DWP, the lysate is removed. The bound MGP: Nucleic Acids undergo a wash step. The DWP is transferred to the Processing Location as shown in Figure 2.3. 950µl of WB was added to each well. The DWP was transferred to the Magnetic Separator. The WB was then removed. This step was repeated for a total of two wash cycles. Once the wash step was complete the DWP was transferred to the processing location. 150µl of EB was added to the DWP and Mixed 3x. The DWP was transferred to the Heater/Shaker unit. Elution occurs at this point as the nucleic acids unbind from the MGP. The DWP was transferred to the processing location and allowed cool for 15 minutes.

The HPV MMX and HPV Mg/Mn Solution were combined onboard to form Working MMX. 25µl of this Working MMX was dispensed into the Microwell Plate. After 15 minutes of cool down the DWP is transferred to the Magnetic Separator. The Eluate was added to the Microwell Plate and was moved to the Start position. Racks were unloaded, and the Microwell Plate was sealed using Sealing Film. This process takes approximately 160 minutes to complete. Figure 2.3 shows the Cobas x480 deck overview.

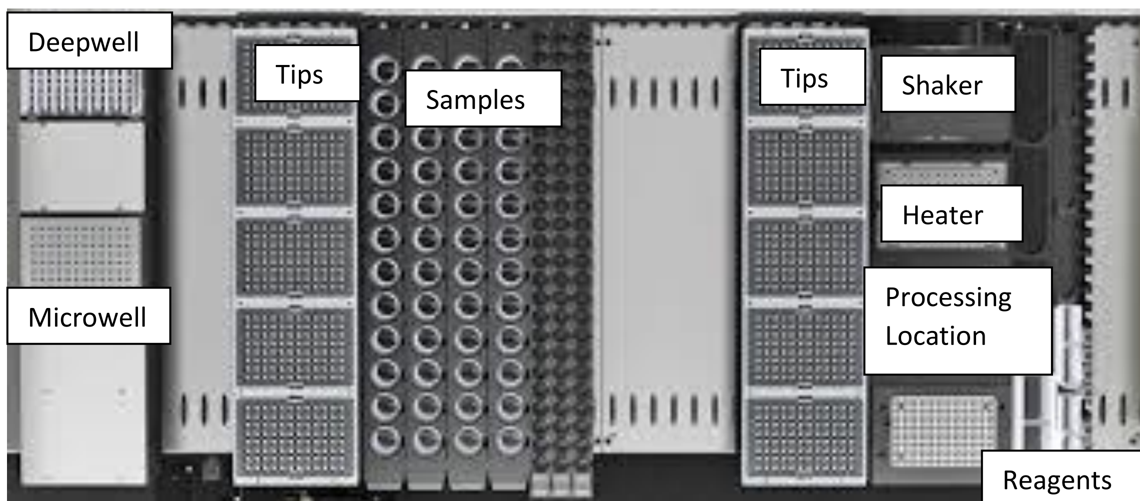

Figure 2.3: The Cobas x480 deck overview (Roche Molecular Systems Inc., 2009b)

2.2.1.2. Cobas z480 Unit and the HPV DNA Test

PCR amplification was achieved using primer sequences of approximately 200 base pair length within the L1 region of HPV. EagleZ05 DNA Polymerase (a chemically modified version of Thermus species Z05 DNA polymerase) was used for a Hot-start amplification. The heat activates EagleZ05 DNA Polymerase and denatures the viral and genomic DNA. During cooling, primer binding occurred and during the rise in temperature EagleZ05 DNA Polymerase extended the primers forming a duplicate strand. This process is repeated in the usual PCR fashion. Amplification only occurs for the HPV specific primers and the genomic β-globin control (Figure 2.4). Detection is based on four different fluorescent dyes, HPV 16, HPV 18, HR HPV (31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68) and β-globin amplicons are labelled by their own fluorescent dye allowing each to be measured independently as shown in Figure 2.5.
Channel 1 (FAM) – "other" high risk HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68
Channel 2 (HEX) - HPV 16 only
Channel 3 (JA270) - HPV 18 only
Channel 4 (Cy5.5) - β -globin Target

For the classification of a positive result, Roche Molecular Systems have shown that the LOD is sensitive enough to detect clinically relevant levels of infection and that any well showing a valid β-globin and amplification of either HPV 16, HPV 18 or HR HPV is deemed as a positive result.

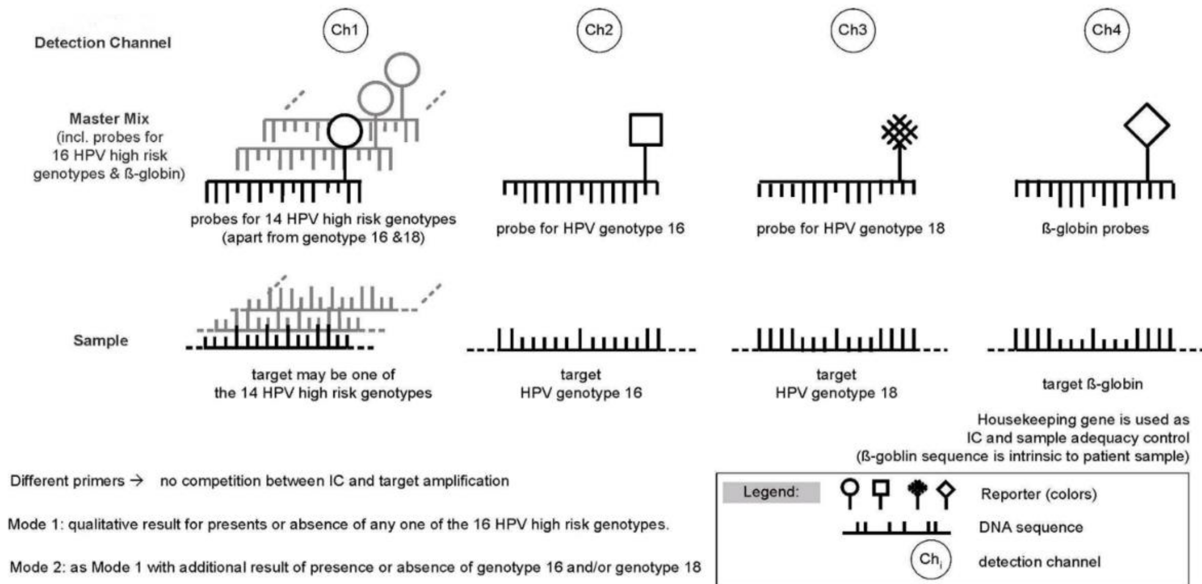

Figure 2.4: Cobas 4800 HPV DNA Test, Amplification and Detection (Roche Molecular Systems Inc., 2009a).

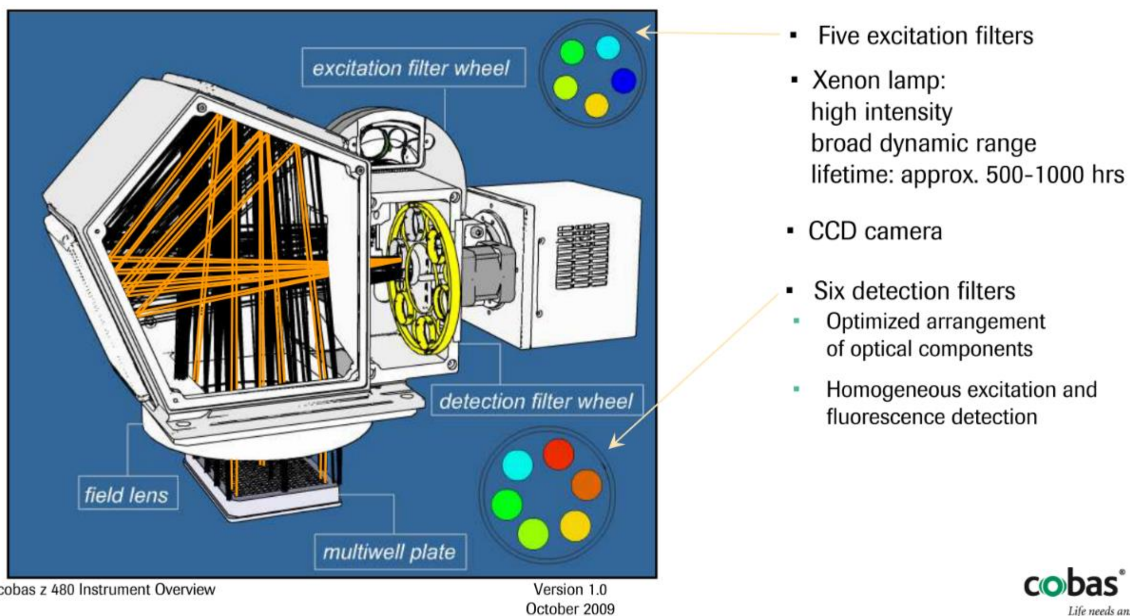

Figure 2.5: Cobas z480 Optical System (Roche Molecular Systems Inc., 2009c)

2.2.2. The Panther System and Aptima HPV mRNA Assay

The Aptima HPV Assay by Hologic® was run on the Panther system for the qualitative detection of E6 and E7 mRNA in 14 HPV types (16/18/31/33/35/39/45/51/52/56/58/59/66/68) via an amplification nucleic acid probe test. The Aptima HPV mRNA test does not distinguish between the 14 hr HPV types. However, as part of this study a second Aptima genotyping test was used to distinguish HPV 16 and a combined HPV 18/45 in samples that were positive for the initial Aptima HPV mRNA test. The Aptima HPV 16 18/45 Genotyping test was performed in part by an MSc project student Katherine Murphy.

The APTIMA HPV Assay is a three-step process, which takes place in a single tube on the fully automated panther system. The on-board process includes: target capture, target amplification by Transcription-Mediated Amplification (TMA) and detection of the amplification products (amplicon) by the Hybridization Protection Assay (HPA).

1) Target Capture

Target mRNA was isolated from the specimen using capture oligomers that were linked to magnetic microparticles. The capture oligomers contain complementary sequences to the specific regions of the HPV mRNA target as well as a string of deoxyadenosine residues. During the hybridization step, these sequence-specific regions of the capture oligomers bind to the specific regions of the HPV mRNA target molecule. This oligomer target complex was then brought out of solution by decreasing the temperature of the reaction to room temperature. This temperature reduction allows the hybridisation to occur between the deoxyadenosine region on the capture oligomer and the poly-deoxythymidine molecules that were covalently attached to the magnetic particles. The magnetic particles, with the captured HPV mRNA target molecules bound to capture oligomers, were pulled to the side of the reaction tube using magnets and the supernatant was aspirated. The particles were then washed to remove any residual solution that may contain amplification inhibitors.

2) Target Amplification

After target capture the HPV mRNA was amplified using TMA which utilizes two enzymes, Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase and T7 RNA polymerase. A specific mRNA primer binds to the mRNA strand where the reverse transcriptase enzyme can create a complementary DNA strand (cDNA). An RNase H enzyme cleaved the RNA component from the cDNA leaving a single stranded DNA (ssDNA) strand. A second specific DNA primer binds to the DNA strand and the reverse transcriptase replicated a

Double Stranded DNA (dsDNA) template. From this RNA polymerase can create hundreds to thousands of mRNA amplicons. These new RNA amplicons can then repeat the cycle of creating cDNA followed by ssDNA to dsDNA and produce its own mRNA amplicons allowing a very small number of captured mRNA amplicons in the sample to be rapidly replicated to the billion-fold in a short space of time and in a single reaction tube. (Figure 2.6)

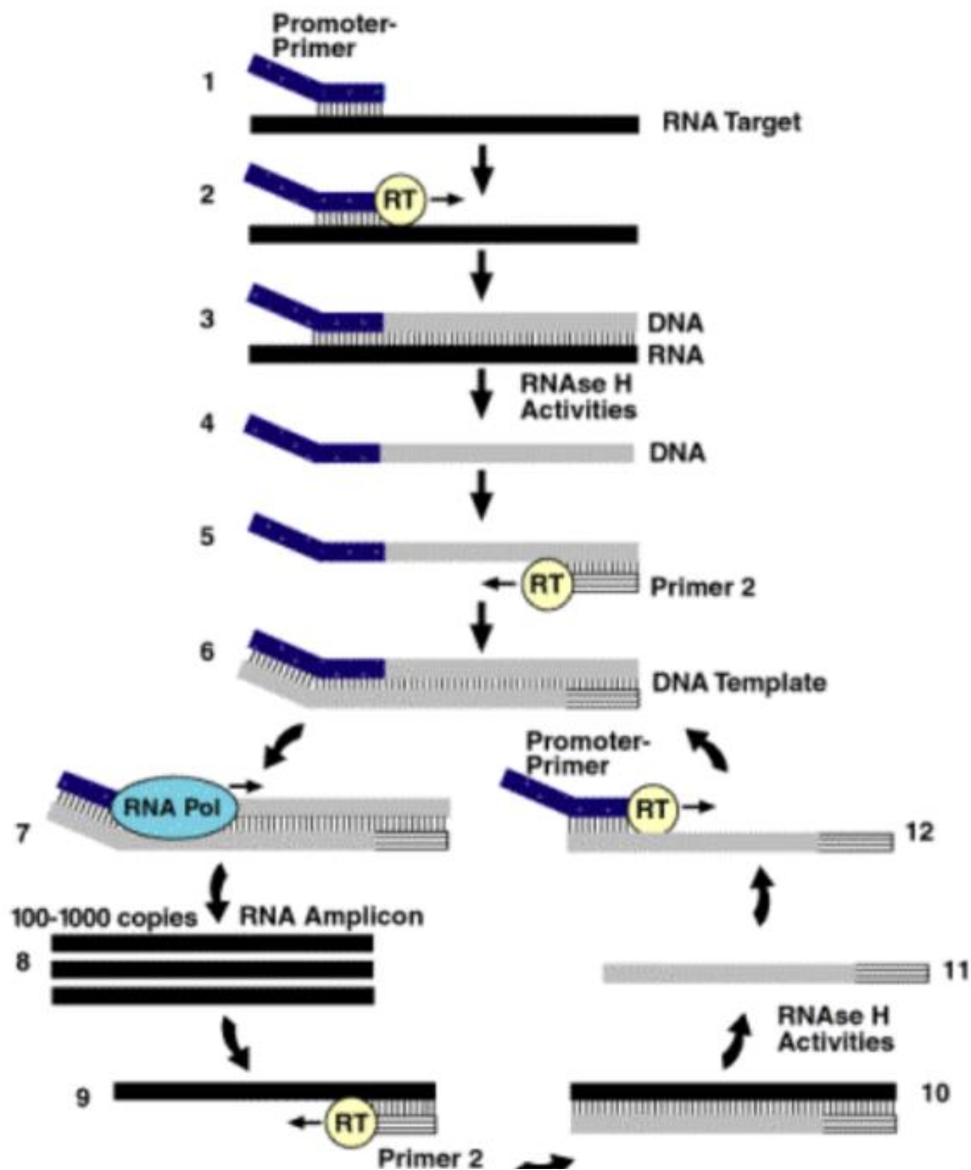

Figure 2.6: Transcription Mediated Amplification (TMA) Process (CHIHWARO, 2015)

3) Target Detection

Detection of the amplicon was achieved by Hybridization Protection Assay (HPA). By using single-stranded DNA probes with a chemiluminescent label the labelled nucleic acid probes hybridize specifically to the target amplicon (Figure 2.7). The Selection Reagent allows differentiation between the hybridised and unhybridized probes through inactivation of the chemiluminescent label on the unhybridized probes. During detection the light emitted from the labelled RNA: DNA is measured as a photon signal through a luminometer and were reported as Relative Light Units (RLU) but the final assay result was interpreted based on the signal to cut-off value (S/CO). During the reagent preparation step an Internal Control (Single stranded amplicon) was added to target Capture Reagent (TCR). During the assay this allowed the Internal Control to be added to each individual reaction. The Internal Control allowed the target capture, amplification and detection steps of the assay to be monitored. The Internal Control could be detected through differential kinetics of light emission from the probes with different labels. The Internal Control utilised a rapid emission probe (flasher) while the amplicon specific probes used a relatively slower emission probe (glower). The on-board system used this Dual Kinetic Assay (DKA) method to differentiate the signals from the 'flasher' and 'glower' probes. The Internal Control did not control for sample quality, it was a method control only, care must be taken when preparing the sample tube that cellular material was transferred from the primary ThinPrep vial into the specimen transfer tube. (Hologic®, 2015) .

There were several steps in determining the positivity of a sample all done automatically onboard the Panther. IC Cut Off = 0.5 x [mean IC RLU of the valid Negative Calibrator

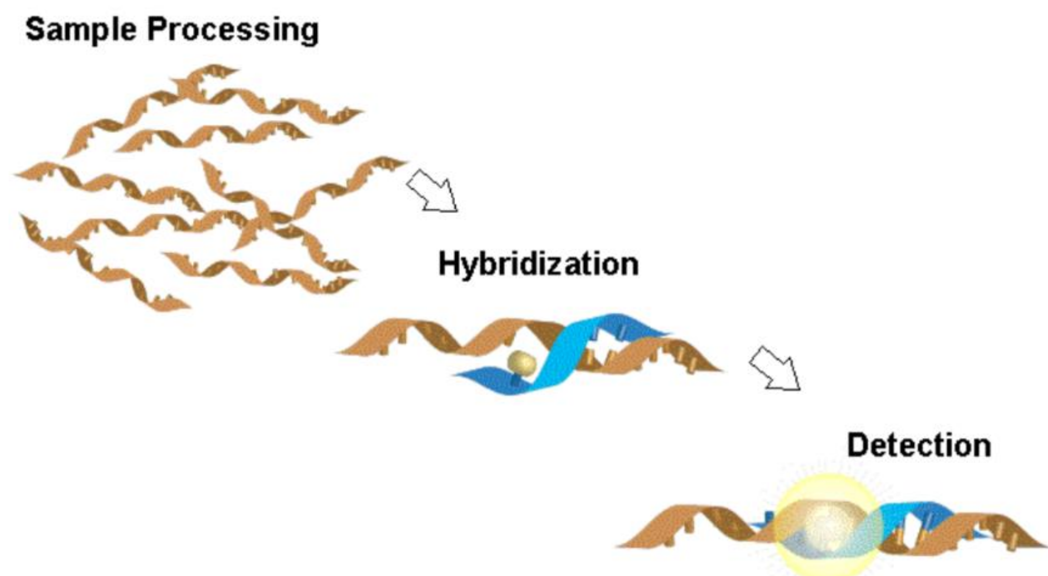

Figure 2.7: DNA probe hybridisation diagram

replicates]. The Analyte Cut-off= [mean analyte RLU of the valid Negative Calibrator replicates] + [0.09 x mean analyte RLU of the valid Positive Calibrator replicates]. The Analyte Signal Cut Off (S/CO) = [test sample analyte RLU] / [analyte cut off]. For a positive result three criteria had to be met, Analyte S/CO ≥ 0.50, IC ≤ 2,000,000 RLU (i.e. over the IC Cut Off) and Analyte ≤ 13,000,000 RLU.

Approximate analytical sensitivity following probit regression analysis had a 'predicted 95% detection limit less than 100 copies/reaction for HPV 16 (74 copies/reaction), 18(52 copies/reaction), 31, 33, 35, 39, 45, 56, 58, 59, 66, and 68 whilst HPV 54 and 52 had limits between 100/300 copies/reaction (Hologic®, 2015).

2.2.2.1. mRNA Testing with the Aptima HPV Assay and Aptima HPV 16 18/45 Genotyping Kit

Kit Reagents Included; HPV Amplification Reagent (A), HPV Enzyme Reagent (E), HPV Probe Reagent (P), HPC Internal Control Reagent (IC), HPV Amplification Reconstitution Solution (AR), HPV Enzyme Reconstitution Solution (ER), HPV Probe Reconstitution Solution (PR), HPV Selection Reagent (S), Target Capture Reagent (TCR), HPV Positive Calibrator (PCAL, 1000 copies of HPV 16 per ml), HPV negative Calibrator (NCAL), HPV Positive Control (PC), HPV Negative Control (NC). Onboard Reagents Included; Aptima Auto Detect Kit (Auto Detect 1 and 2), Aptima Assay Fluids (Aptima Wash Solution, Aptima buffer for Deactivation Fluid, Aptima Oil Reagent), 5% Sodium Hypochlorite Solution, Specimen Transfer Tubes.

1ml of vortexed sample was transferred to the specimen transport tube ensuring that there was some cellular material being transferred into the tube, this tube contained a solution that lyses the cells and releases the mRNA whilst it protects it from degradation during storage. The specimen transport tube and sample are stable at 2°C-30°C for up to 30 days. It was recommended that after sample has been added to the specimen transport tubes that a minimum of 15 minutes was allowed prior to loading the specimen. Prior to running any assay, the operator logged on with their unique log on and the maintenance status on the instrument was assessed. Weekly tasks include the changeover of the sample shield and system reboot and were performed if necessary. There was a programmed monthly maintenance and fluids such as wash buffers, cleaning solutions, auto detect kits and plastics such as Multi-Tube Units (MTU's), pipette tips and waste were monitored continuously and prompted when a change was required. A system prime was required

before a new assay run begins and was valid for 6 hours after initiated to ensure all components were primed with the necessary on-board reagents.

The reconstitution of the HPV Assay and HPV 16 18/45 Assay followed the same procedure. All work surfaces were decontaminated with 2.5%-3.5% sodium hypochlorite (allowing the solution to remain in contact with the surface for 1 minute then followed by a rinse with water and drying with clean tissue). A clean tissue or pad was placed on the work surface. The Aptima HPV lyophilised reagents and Aptima HPV Calibrators and Controls were removed from the fridge and allowed reach room temperature. Once the kits had reached room temperature, each reconstitution solution was matched with its lyophilised reagent (these were colour coded green, purple and orange). The Master Lot sheet was checked to ensure the correct reagents were paired and the Lot numbers match. Taking the first lyophilised reagent the sealed cap was removed. The reconstitution collar was firmly fastened to the lyophilised reagent bottle touching only the central notched segment of the collar (Figure 2.8 , Step 1). The collar was carefully inserted into the corresponding reconstitution bottles opening (Figure 2.8, Step 2). Slowly the assembled bottle and vial was inverted allowing the solution to drain into the glass bottle (Figure 2.8, Step 3). The solution was gently swirled to mix the contents and to try avoid creating bubbles or a foamy solution (Figure 2.8, Step 4) and allowed to sit until fully reconstituted (this time varied but took around 15 minutes on average). (Figure 2.8, Step 5). The reconstitution collar and glass vial were removed and disposed of and the reconstituted solution was recapped with the same cap that was removed earlier (Figure 2.8, Step 6-8). These steps were used for all three lyophilised reagents.

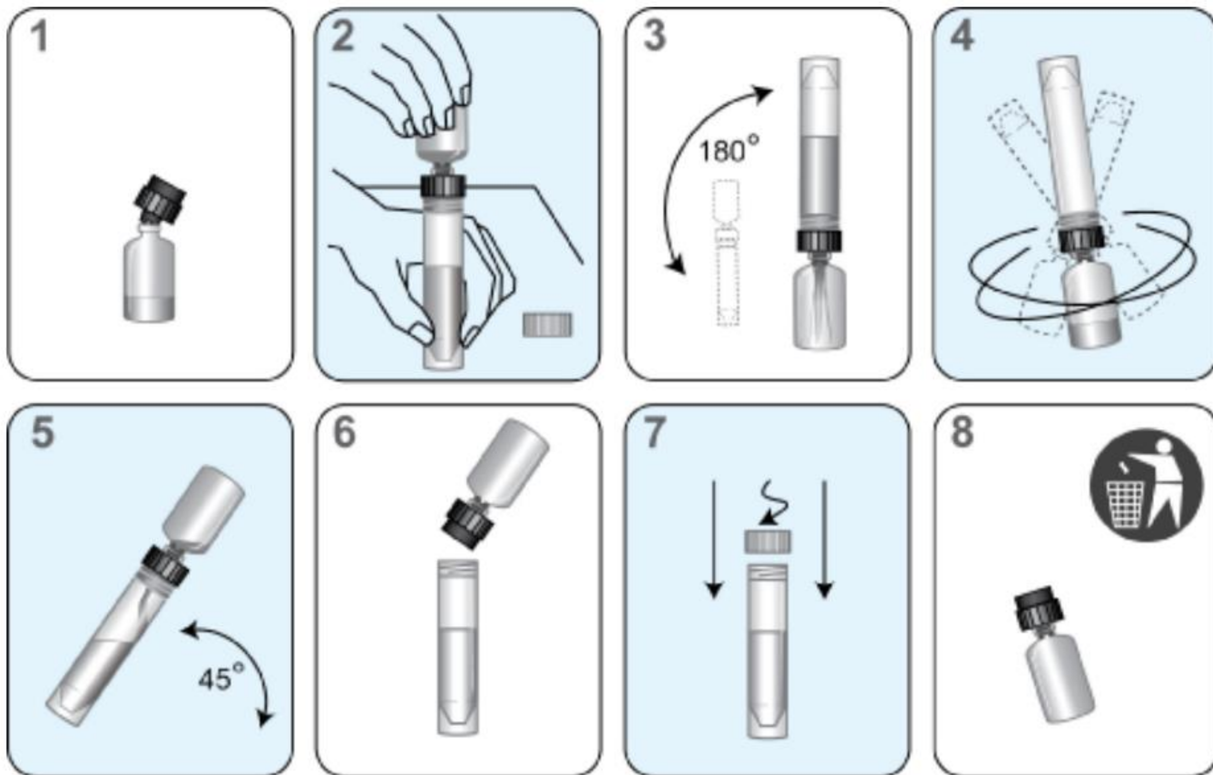

Figure 2.8: Lyophilised Reagents Reconstitution process (Hologic®, 2015)

The working TCR (wTCR) was created by pairing the TCR and IC bottles (coded black in colour) and checking to ensure the Master Lot sheet matched the two vials. the entire contents of the IC poured, carefully into the TCR bottle (a small amount of liquid may remain in the IC, this is acceptable). The TCR was resealed with the cap and gently swirled and mixed while attempt to avoid creating a foam. The wTCR was left to rest for 15 minutes alongside the other reconstituting reagents. Occasionally a precipitant was noticed after this time. This was dissolved by warming the wTCR to between 42°C and 60°C for up to 90 minutes. The wTCR must equilibrate back to room temperature when this step was required. The Selection Reagent required no additives. The assay regents were then loaded on to the Panther System as prompted.

Before samples were loaded, the samples, controls and calibrators were brought up to room temperature. Each rack held 10 samples and the Panther System accommodated 8 racks at a time. Once everything had come to room temperature, the Calibrators followed by the Negative and Positive controls were placed into the first positions in the first rack followed by the samples ensuring the barcode was facing outwards. When loading the samples, it was noted if there was any bubbles between the space of the liquid and the cap, if noticed the sample was centrifuged for 5 minutes at 540 RCF to eliminate the bubbles. Samples were then be loaded on to the Panther System for processing. Once sampled, the racks

were removed, and new samples loaded on for continuous analysis. Once the assay was complete, the results were viewed, and any Invalid vials were removed and recapped with a penetrable cap for re-testing and all positive samples were removed, capped with a non-penetrable plastic cap and stored upright in the -20°C freezer for 24hours and then stored in bags in the same freezer for later HPV 16, 18/45 Genotype testing.

The HPV 16, 18/45 genotyping test followed the same preparation and loading steps as the previously described. The only variation is that the Panther System was set to ‘GT HPV’

2.3. Methylation Analysis

2.3.1. Cell Culture

In this thesis, SiHa cells (ATCC: HTB-35, reported to contain 1-2 copies of integrated HPV 16per cell) (SiHa (ATCC® HTB-35™), 2018) were used to perform test validation and optimisation of methylation specific PCR. SiHa cells were also used as positive controls for methylation analysis. Cell culture was performed in part by BSc project student Rita Ladapo. Stocks of SiHa cells stored in liquid nitrogen were thawed to 37ºC. SiHa cells were cultured in Buffered Minimum Essential Medium (MEM) (Sigma-Aldrich Cat No. M4655-500ml). In 500mls of MEM media 50ml of Foetal Bovine Serum (FBS) (Sigma-Aldrich Cat No. 12103C), 10mls of Penicillin/streptomycin (5000 units of Penicillin, 5mg/mL of Streptomycin in 100ml.) (Sigma-Aldrich) and 4ml of L-Glutamine (200nm stock concentration) (Bio-Whittaker) was added to form the Buffered MEM.

SiHa cells were initially cultured in T25 cell culture flasks. 1ml of thawed SiHa cells were reconstituted with 10mls of Buffered MEM media. Flasks were incubated at 37°C and checked daily for confluency. When confluency reached 80%+ the cells were split. The media in theT25 flask was aspirated gently and removed. 4ml of TrypLE (1X) (Gibco) was added over the cells and left to incubate at 37°C for 5 minutes. The flask was viewed under an inverted microscope (Zeiss, Axiovert 35 Inverted Phase Contrast Microscope) to confirm that the cells detached from the flask surface. 10mls of Buffered MEM was added to the flask to halt the Trypsinisation process. Cells were brought up to T175 for subsequent culture.

Cell pellets were collected from each passage by centrifugation of cell suspension at 3000 rpm for 5 minutes at 4°C. The supernatant was removed and 5mls of PBS (1X, 7.4PH, Ambion™) was added to wash the cells. The cells were spun again at 3000rpm for 5 minutes and the supernatant was removed, and the tubes were left upside down in the fume hood for 20 minutes to remove all supernatant. Cell pellets were stored at -80°C for later use.

2.3.2. DNA Extraction from ThinPrep Samples

DNA extraction from cell lines and cervical cytology samples was performed using the Qiagen DNeasy blood and tissue kit in accordance with the manufacturer's protocol for DNA Purification from Blood or Body Fluids (Spin Protocol) (Qiagen, 2016). All reagents were reconstituted as required and outlined in the product protocol (Qiagen, 2016). Pre-extraction sample washing steps were performed as follows; 2ml of cervical sample was aliquoted into labelled eppendorf tubes and centrifuged at 14,000 rpm for 5 minutes. The supernatant was removed and 800µl of 1X PBS (10X, 7.4PH, Ambion™) was added, vortexed and centrifuged at 14,000 rpm for 5 minutes. All supernatant was removed, and the pellet was resuspended in 200 µl of PBS for DNA extraction.

20 µl of Proteinase K was pipetted into the sample tube containing the cell pellet. 200µl of AL Buffer was aliquoted into the sample tube and the sample was mixed thoroughly by vortexing. The sample was incubated at 56°c for 10 minutes. The sample was briefly centrifuged to remove any droplets from the tubes lid. 200 µl of ethanol (96-100% required) was added (≥99.5% Ethyl alcohol, Pure. Honeywell Riedel-de Haën™) and mixed thoroughly by vortexing. The sample was briefly centrifuged to remove any drops in the lid. The sample was pipetted into a labelled DNeasy Mini spin column placed in a 2ml collection tube. The mini spin column was centrifuged for 1 minute at 8000rpm/6000 x g. The spin column was removed, and the upper spin column was placed in a new 2ml collection tube and the old tube with the flow through was discarded. 500 µl of Buffer AW1 was added. The spin column was centrifuged for 1 minute at 8000rpm/6000 x g. The spin column was removed, and the upper spin column was placed in a new 2ml collection tube and the old tube with the flow through was discarded. 500 µl of Buffer AW2 was added. The spin column was centrifuged for 3 minutes at 14,000rpm/20,000 x g to remove all wash buffer from the membrane. The spin column was removed from the centrifuge and the upper spin column was placed in a new epindorff tube with corresponding label ID's. 50µl of Buffer AE was pipetted into the centre of the spin column membrane and incubate for a minimum of 1 minute at room temperature (15-25°c) and up to 5/10 minutes at room temperature to increase the yield in smaller cell pellets. The average incubation time was 5 minutes. Centrifuge for 1 minute at 8000rpm/6000 x g. The spin column was removed, and the upper spin column was discarded, and the extracted DNA was stored at -20°C for later use.

2.3.3. Estimation of DNA yield

DNA concentrations were determined using the Nanodrop 2000c (Thermo Fisher Scientific, 2009). 1µl of sample was pipetted onto the sensor on the lower pedestal of the Nanodrop 2000c instrument.

A fibre optic cable (the receiving fibre) is embedded within the lower pedestal whilst a second fibre optic cable (the source fibre) is then brought into contact with the liquid sample causing the liquid to bridge the gap between the ends of the two fibres. A pulsed xenon flash lamp provides the light source and a spectrometer utilizing a linear CCD array analyses the light passing through the sample.

To run the analyser the Nanodrop programme was set to determine Nucleic Acids on the home screen. Once its initial validation step was complete a blank sample was run to calibrate the Nanodrop to the sample medium. 1 µl of Qiagen elution Buffer AE was used to blank the devise. 1 µl of mixed sample was pipetted onto the lower pedestal. The arm of the nanodrop was lowered and the measure button was pressed on the accompanying computer. Once the measurement was made, the arm was raised and using a dry, lint free wipe or tissue the top and bottom pedestal were cleaned of the DNA sample. 1ul of deionised water was added to the lower pedestal, the nanodrop arm was lowered and raised and the water was wiped off both the bottom and top of the pedestal. The Nanodrop was now ready for the next sample (Figure 2.9). Each sample was measured twice to ensure the accuracy of the reading. The Nanodrop has a limit of detection of 2 ng/µL dsDNA and an upper limit of 15,000 ng/µL dsDNA. To aid in the bisulfite conversion samples found to exceed 500ng/ul of DNA were diluted to approximately 100-400ng/µl with AE Buffer and remeasured. This is to ensure the volume of DNA used for the conversion isn't less than 1µl and allow more accurate pipetting and more accurate conversion and later qMSP results. DNA purity has been suggested to fall between an A 260/280 ratio of 1.6-2.3 for the following bisulfite conversion.

Figure 2.9: NanoDrop 2000c Clean down procedure post DNA measurement (Thermo Fisher Scientific, 2009)

2.3.4. Bisulfite Conversion of DNA

Bisulfite conversion of DNA was performed using the Zymo EZ DNA Methylation-Gold™ Kit (D5006) in accordance with manufacturer's protocol (Zymo Research, 2017).

The principle of the Bisulfite Conversion method relies on protection of the methylated cytosine residue on DNA from conversion to a uracil residue. DNA is treated with bisulfite and in its presence any unmethylated cytosine residues are converted into uracil residues. Methylated cytosine residues are protected from this process and remain unchanged. Bisulfite converted DNA templates are now single stranded and due to the now non-complimentary nature of each strand that contains uracil remain that way up to the PCR stage. Of note, when PCR (qMSP) occurs with bisulfite converted DNA, uracil will pair with adenine in the first PCR cycle, following this the PCR will pair it with thymine. This results in the end PCR product having thymine in place of uracil (originally unmethylated cytosine). The Zymo EZ DNA Methylation-Gold™ Kit (D5006) has the capability to convert DNA concentrations of between 500pg to 2µg but the optimal conversion DNA input amount is between 200-500ng. For the downstream amplification of the converted DNA, 500ng of input DNA was used in the conversion and eluted in 10µl of M-Elution Buffer. This allowed the required 50ng's per PCR well from 1µl of elute.

500ng of DNA was made up to a final volume of 20µl in a PCR tube. 130 µl of CT Conversion Reagent was added to 20 µl of the DNA sample for a final volume of 150ul's. In cases where there was insufficient DNA to have 500ng of DNA in 20µl a separate conversion reagent stock was made such that the water component of the stock was reduced by 10µl for every 10µl above 20µl of sample added. For samples that require >20 µl to achieve the required 500ng concentration a separate CT conversion reagent was prepared as follows, for every 10 µl increase in DNA sample there was a 100 µl decrease in dH20 added to the CT conversion reagent. DNA samples mixed with CT conversion reagent were placed in a thermocycler (GeneAmp PCR System 9600, Perkin Elmer) at 98°C for 10 minutes, 64°C for 2.5 hours and an optional 4°C storage for up to 20 hours. 600 µl of M-Binding Buffer was added to labelled Zymo-Spin™ IC Column that was placed in the provided Collection Tube. The converted DNA samples from the thermocycler were pipetted into the Zymo-Spin™ IC Column. The cap of the spin column was closed and mix by inverting the column several times (3/5 times). The spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds. The flow through collected in the tube of the spin column was discarded and the collection tube was retained. 100 µl of M-Wash Buffer was added to the spin column. The spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds. 200 µl of M-Desulphonation buffer was added to the column and let stand at room temperature

(20-30ºc) for 15-20 minutes. After incubation the spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds. 200 µl of M-Wash buffer was added to the column. The spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds and another 200 µl of M-Wash buffer was added to the column. The spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds. The 30 second centrifuge at full speed was repeated to ensure all M-Wash Buffer is removed. The upper column of the spin column was placed into a labelled 1.5ml microcentrifuge tube. 10 µl of M-Elution Buffer was added to the column matrix. The eppendorf: spin column was centrifuged at full speed (14,000rpm/20,000 x g) for 30 seconds to elute the DNA. The DNA was ready for immediate use or could be stored at -20ºc for later use.

2.3.5. Methylation Specific PCR (qMSP)

Real time singleplex qMSP was performed for detection of methylated DNA.
Primer and Probe sequences for CAD M1, MAL M1 and hsa-mir-124-2 have been published previously are listed in Table 2.1.

A methylation specific primer and probe for β-Actin (ACTB) was employed as an endogenous control (Overmeer *et al.*, 2011). A Non-Methylation Specific β-Actin (NcACTB) (TaqMan™ DNA Template Reagents, Life-Technologies Ref;401970) was employed as a methylation control to show if successful bisulfite conversion had occurred.
Primer design was previously optimised for specific methylation regions (Wilting *et al.*, 2010; Overmeer *et al.*, 2011).

All TaqMan probes were labelled with 5' FAM and 3' TAMRA
Each qMSP was run in duplicate and included controls for each target
Controls:

- SiHa DNA Converted Control (Positive Control).
- Known Repeat Negative ThinPrep Sample, Converted DNA (Repeat Cytology Negative Pooled samples) (Negative Control).
- SiHa DNA Unconverted (Bisulfite Conversion Control).

Table 2.1: Methylation Primer and Probe Sequence					
Target Gene	**Forward Primer 5'-3'**	**Reverse Primer 5'-3'**	**Probe 5'-3'**	**Amplicon Size**	**Annealing Temperature**
CAD M1-M18	ATTTTATTAG TTGTTGGTT CGGGT	CTCGACA ACACTACT CGCC	ACCTACCT CAAACTAA CGACGTT AACTACCT CCGA	90	60 °C
MAL M1	GCGTAGTAT TAAGTAGAG AGGTTCG	AATAAAAA ATAAAACC GACCGC	ACTAAACC GACGCTA ATTCGAC GACGCT	107	60 °C
hsa-miRNA-124-2	GGGTAATTA ATTTGGATTT ACGTCGTTA T	CGTAAAAA TATAAACG ATACGTAT ACCTACG T	TTTACAAC ACACGCC TAAA	138	60 °C
ß-Actin (IC)	TGGTGATGG AGGAGGTTT AGTAAGT	AACCAATA AAACCTAC TCCTCCCT TAA	ACCACCA CCCAACA CACAATAA CAAACAC A	133	60 °C
(Wilting et al., 2010; Overmeer et al., 2011)					

All qMSP's were carried out in 12ul reaction volumes as per Table 2.2. 1µl of DNA was used in each reaction. This was equivalent to approximately 50ng's of DNA per reaction well from the starting 500ng's/10µl's. All methylation primers and probes were sourced from Bio-Sciences Limited using their custom primer and probe ordering system (Cat. 450025 and 4304970). All primers were received lyophilised at a concentration of 10000 pmol (10nmol), all probes were at a stock concentration of 100pmol/µl. The stocks of

primer were maintained at 417nM which equated to 0.5µl per reaction. For the probe the stocks were maintained at 208nM which equated to 0.25µl per reaction.

Table 2.2: Master Mix Components	
qMSP reaction Components	**Volume(µl)**
Forward Primer(417nM)	0.5
Reverse Primer(417nM)	0.5
Probe(208nM)	0.25
QuantiTect Probe PCR Kit Master Mix(1X)	6
Template DNA (50ng)	1
DNase Free water	3.75
Reaction Total volume	12
Master Mix for 1 qMSP well.	

The NcACTB was at a stock concentration of 3µM (3000nM) for the Forward and Reverse primers and 2µM (2000nM) for the Probe. These were pre-optimised primer and probe sequences and using the C1V1=C2V2 equation. This gave a volume of 1.2µl for both the primers and the probes per PCR reaction.

Thermocycling was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, 2010) under the following cycle conditions; 95°C for 15 minute followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

2.3.6. qMSP Analysis

All controls were monitored on Jenkins control charts.

Post qMSP the following criteria had to be met prior to analysis (Bierkens *et al.*, 2013).

- Controls valid.
- ACTB Ct<30.
- Target Ct<40.

Baseline and threshold limits were set and recorded for each run as per Applied Biosystems (Applied Biosystems, 2010) instructions.

Post-conversion semi-quantitative analysis was conducted using the formula 2^[ct(B-actin)-ct(Target)]x100 to calculate a ratio for each sample (Hesselink *et al.*, 2011).

2.4. Data and Statistical Analysis

For the study, a HPV prevalence of 15% was assumed based on a previous HPV population prevalence study performed during phase 1 of CERVIVA studies. To achieve the necessary sensitivity and specificity for the HPV assays a sample size calculation with a precision of 0.05 determined that a target of 13,000 women would yield approximately 2,000 HPV DNA positive cases that would be available for second-round molecular tests. Sample size calculations for the methylation triage panel were calculated based on the data obtained for sensitivity and specificity from the initial validation panel lower 95% CI discussed in Chapter 4 as well as powered based on previously publish data. Sample size calculations were estimated based on a sensitivity of 72% and specificity of 84%with the expected prevalence of “positivity” (35-40%) and a precision of 0.05. These calculations determined a sample size of 886 HPV positive woman would be required.

Throughout this thesis several statistical packages have been used to analyse and store data. Depending on the outputs required individual or multiple programmes and statistical packages have be used to ensure the accuracy of the results.

- CARG Consent Form Storage; Microsoft Access (Primary Database) and Microsoft Excel (Secondary back-up Database).
- CARG Study Results (HPV DNA, HPV mRNA, Methylation, Age, smear date, Cytology, Colposcopy, Histology); Microsoft Excel.
- Statistical Analysis: Minitab Statistical Software version 17, IBM SPSS Statistics 25, MedCalc Version 18, GraphPad Prism 7, QuickCalcs and Microsoft Excel. The statistical programme used for each test will be given in the methods section of each chapter.

The disease endpoint for this observational cohort study is the detection of CIN 2+, taken as histological diagnosis of CIN 2 or greater, and CIN 3+ taken as CIN 3 or greater. Normal cytology and Normal/CIN 1 histology are considered as no disease (<CIN 2). For all statistical analysis a p-value was considered statistically significant when $p < 0.05$.

2.4.1. Primary Screening Test

Statistics were calculated on Minitab Statistical Software version 17. Comparisons between HPV DNA and mRNA were performed on cross-sectional cytology data and available histology data. Kappa correlation (k) was used determine agreement between the two tests. Binary Logistic Regression was used to calculate Odds Ratio (OR) and 95% Confidence Intervals (CI's) to determine the association of age and cytology grade with risk of testing HPV DNA or mRNA positive. Each platforms performance was assessed though the calculation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) with the associated 95% CI for the detection of both CIN 2+ and CIN 3+. The McNemar's test was used to compare HPV DNA and mRNA platforms regarding their positivity rates and sensitivity and specificity for CIN 2+.

2.4.2. Methylation Validation Panel

Statistical analysis of the methylation data was calculated using Minitab Statistical Software version 17, IBM SPSS Statistics 25 and MedCalc Version 18. Following qMSP for each sample a Methylation Score was calculated for each individual methylation marker; $2^{(Ct\ of\ \beta-actin)-Ct\ (Target\ gene)}\ x\ 100$ (Hesselink et al., 2011).

A Total Methylation Score was derived from this calculation by taking the value derived from the 2^[ct(B-actin)-ct(Target)]x100 equation for each marker and summing the values together. CAD M1-M18 + MAL M1 + hsa-mir-124-2 = Total Methylation Score. No weighting was applied to any individual marker. A Paired Methylation Score was also calculated using two of the methylation markers methylation scores. The methylation nomenclature and descriptions are outlined in Table 2.3.

Table 2.3: Description of nomenclature for the different Methylation Scores	
Methylation Score Grouping	**Description**
Individual Methylation Markers (Methylation Score)	A Methylation Score for each individual marker was derived from: 2^[ct(B-actin)-ct(Target)]x100.
Total Methylation Score	A Total Methylation Score was calculated by taking the value derived from the 2^[ct(B-actin)-ct(Target)]x100 equation for each marker and summing the values together: CAD M1-M18 + MAL M1 + hsa-mir-124-2 = Total Methylation Score.
Paired Methylation Score Nomenclature: Paired Methylation Score (xxx : xxx)	A Paired Methylation Score was derived by taking the value derived from the 2^[ct(B-actin)-ct(Target)]x100 equation for two markers and summing the values together: CAD M1-M18 + MAL M1= Paired Methylation Score (CAD:MAL) or MAL M1 + hsa-mir-124-2 = Paired Methylation Score (MAL :miR)

The Wilcoxon signed rank test was used to determine if a statistical difference exists between each of the Methylation Scores outlined in Table 2.3. The Kruskal Wallis test was used to determine if there was a statistical difference between the Methylation Scores and their respective histology grades. This was performed on the IBM SPSS Statistics 25 package.

For the determination of cut off values for a positive methylation test Receiver Operating Characteristic (ROC) Curves were used. These were calculated for detection of CIN 2+. For this study the two groups were classified as Disease or No Disease. Disease being CIN 2+ and No Disease being classified as either Normal (Negative HPV and normal Cytology) and CIN 1. Statistical tests associated with ROC analysis included the Area Under the Curve and its 95% CI as well as a z-statistic was calculated to determine the statistical difference between the two groups. The Youden Index J was calculated to capture the performance of this dichotomous test. Youden's index J is the likelihood of a positive result in subjects with disease [CIN2+] versus those with no disease [<CIN2]. It has a value between 0-1 and in the perfect test the Youden's J index=1. It is also mapped to the vertical axis of the ROC's diagonal chance/no discrimination line to the ROC curve for a single point, this point is the Associated criterion and has a corresponding sensitivity

and 1-specificity around this point as shown in Figure 2.10. These points were classified as cut offs for each methylation marker and the combined Total and Paired Methylation Scores.

The clinical performance of each potential cut off value was also assessed during ROC analysis with the sensitivity, specificity, PPV and NPV being calculated for each cut off value. A heat map of the validation panel for the positive and negative results from the proposed cut off points was created using the online heat mapper tool: http://www.heatmapper.ca/

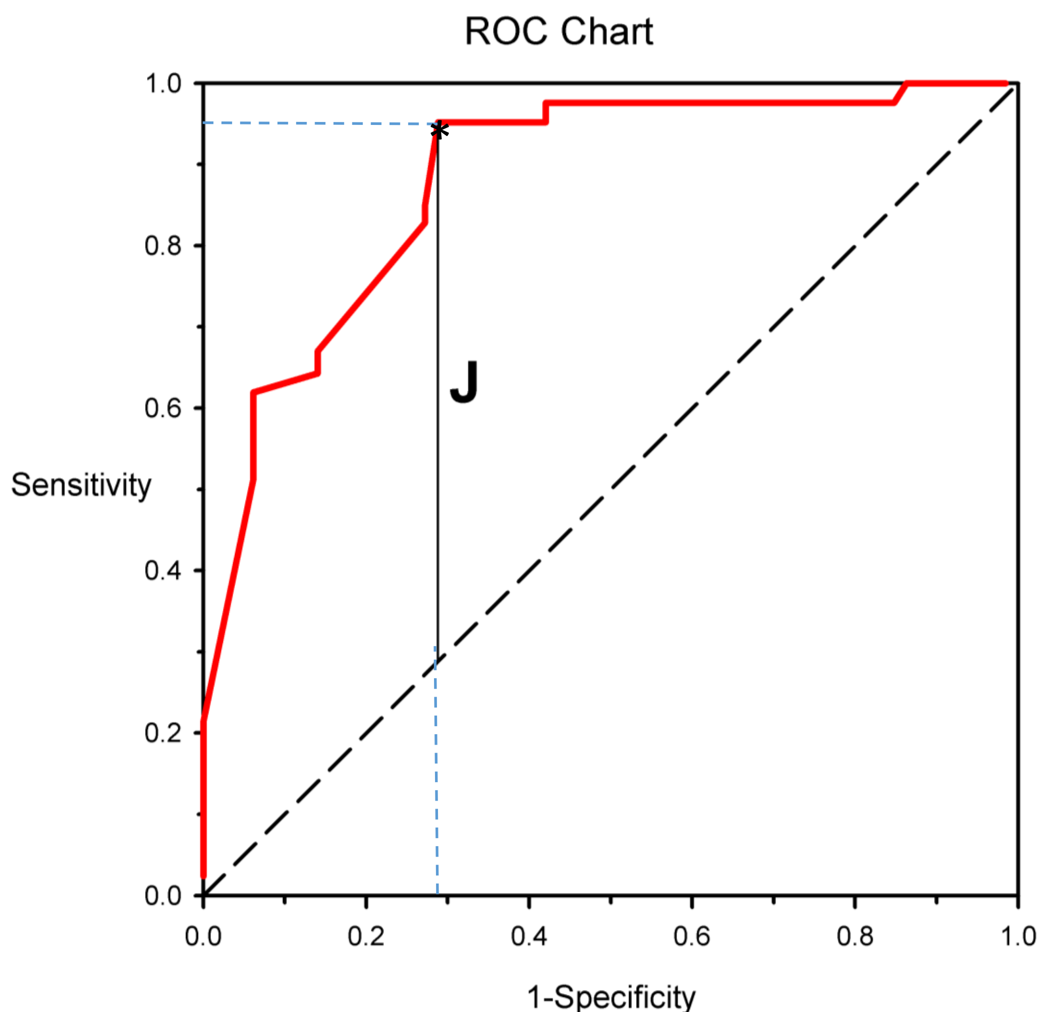

Figure 2.10: An example of a ROC curve. Red Line: ROC curve, Dashed line, Chance/no discrimination line; Vertical line, (J) maximum value of Youden's index for the ROC curve; Blue dashed line, Estimated sensitivity and 1-specificity of the associated criterion (*) (Kognos, 2019)

2.4.3. Methylation Triaged Population

The Total Methylation Score was used to determine positive methylation tests in this triage population based off the validation work from Chapter 4. Binary Logistic Regression was used to calculate Odds Ratio (OR) and 95% Confidence Intervals (CI's) to determine the association of age, cytology and HPV genotyping and the risk a positive result for the Total Methylation Score. An associated Wald's p-value was used to show statistical significance in relation to age, cytology and HPV genotype. 5 triage strategies were assessed, cytology triage alone, HPV 16/18 genotyping with cytology triage of the other hrHPV positive women, Methylation testing alone using the Total Methylation Score, HPV 16/18 genotyping with methylation triage of the other hrHPV positive women and lastly cytology triage with methylation testing of NAD cytology. Each triage strategy performance was assessed though the calculation of sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) with the associated 95% CI for the detection of CIN 2+. The HPV positive triage population that was tested for methylation was compared against alternate triage strategies including HPV genotyping and cytology. The number of women referred to colposcopy was calculated and compared using Chi Square test. This was termed the Triage Referral Rate: this was subdivided into women <30 and >30 years of age. To show the risk stratification of a negative or positive triage test result the PPV and 1-NPV were used to classify women following their triage test. A risk classification graph (adapted Wentzensen et al. 2016) was generated for the potential triage strategies. The risk of having a CIN 2+ follow a positive triage result (PPV) was shown against risk of CIN 2+ following a negative triage result (1-NPV). Risk classification is based on the current EU guidelines: women with a risk >20% for CIN 2+ warrants a colposcopy referral. Those with a risk <2% for CIN 2+ can return to routine recall (Castle et al., 2008; Arbyn et al., 2011, 2017). Women with a risk classification between 2-20% would remain under surveillance.

2.5. References

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-Chapter 3-

A Comparison of HPV DNA and HPV mRNA Testing in a HPV Primary Screening Population

3.1. Introduction

It is well established that long term and persistent infection with hrHPV is the primary aetiological agent for the development of cervical cancer and pre-cancers (Bosch *et al.*, 2002). Since this development, and due to the fact that HPV cannot be cultured, several different molecular based HPV tests and detection platforms have been developed that can detect HPV DNA or HPV mRNA from the main hrHPV types. Several of these have been clinically validated for use as triage tests for low grade cytological abnormalities, as a test of cure for women following ablative or excisional surgery to remove high grade cervical lesions and more recently for use as a primary screening test for cervical cancer and pre-cancers.

Over the past decade, HPV testing has become the preferred method for triage of low-grade cytological abnormalities and is now being implemented as the primary cervical screening method. Several countries including, the Netherlands, Turkey and Australia, have already introduced population-based HPV primary screening for women aged 30-64 years and others are in the process of implementation, including; Denmark, Finland, France, Germany, Italy, Sweden, the United Kingdom, New Zealand and Norway all with varied screening protocols including different screening ages, ranging from 23 to 69 years, and screening intervals from 3 to 5 years (Figure 3.1) (Chrysostomou *et al.*, 2018). CervicalCheck, the National Cervical Screening Programme in Ireland are expected to move to HPV based primary screening by Q1 2020, following recommendations from a Health Technology Assessment carried out by HiQA the Health and Quality Authority Ireland (HIQA, 2017), which recommended a switch to a HPV-based primary screening programme with cytology triage at 5-year intervals for women aged 25 to 60 and with unvaccinated women being recommended to have a smear test at 28 (HIQA, 2017).

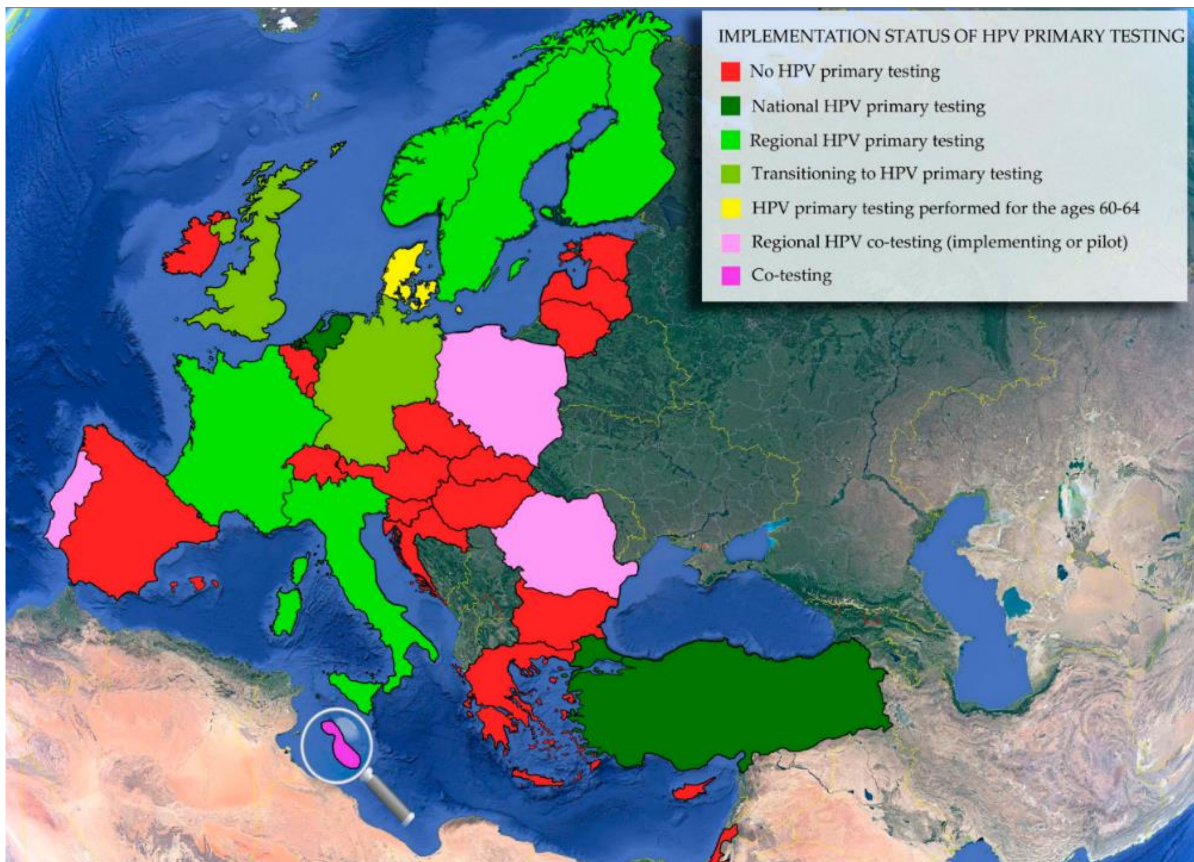

Figure 3.1: HPV Primary Screening implementation across Europe. The magnifying glass serves to enlarge Malta. Image from Figure 3 (Chrysostomou et al., 2018)

The evidence supporting the use of HPV-based primary screening comes from several large scale randomised control trials (RCTs) that have been conducted over the last 10 years, including the NTCC Trial (New Technologies in Cervical Cancer Trial) (Ronco *et al.*, 2008, 2010), the ARTISTIC Trial in the UK (A Randomised Trial in Screening to Improve Cytology (H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; C Kitchener *et al.*, 2014), POBASCAM (Population-based Screening Study Amsterdam) (Dorien C. Rijkaart *et al.*, 2012; Maaike G. Dijkstra *et al.*, 2014) and the Public Health Trial Finland, Swedescreen (Naucler *et al.*, 2007, 2009). These four RCTs showed that HPV primary screening can provide 60-70% greater protection against invasive cervical carcinomas compared to cytology based screening and support a HPV primary screening model for over an 30's population with a 5 year screening interval. (Ronco *et al.*, 2014). These RCTS also showed that HPV primary screening had a superior sensitivty compared to cytology as well as a higher NPV which supported more extended screening intervals for those women who are negative for HPV.

The next section outlines the main findings from these individual studies:

- NTCC: Detection of invasive cervical cancer was similar between cytology and HPV (9 cases vs 7 cases) tested in the first round of screening with cytology detecting more invasive cancers during the second round screening (7 cases vs 0 cases). However the detection rate of CIN 2+ was greater in the HPV screened cohort during the first screening round (107 cases vs 55 cases (HPV vs cytology)) and 11 vs 15 cases in the second screening round. The final conclusion of the study was that HPV screening is more effective than cytology in the prevention of invasive cervical cancer through the earlier detection and treatment of high-grade pre-cancerous lesions. However without appropriate triage protocols will over refer women to colposcopy (Ronco *et al.*, 2010).

- ARTISTIC: The ARTISTIC trial, amonst other findings, showed that HPV negative women in their intital screening test had a significantly higher level of protection from CIN 2+ than cytology negative women. The cumulative incidence rate of CIN 2+ for women with a HPV negative test was 0.87% (95% CI: 0.70% to 1.06%) after three screening rounds (6 years) compared to a negative cytology result at the studies entry, 1.41% (95% CI 1.19% to 1.65%). Women with a negative HPV test at entry had a similar protection from CIN 2+ after a 6 year period compared to women who had normal cytology at baseline after 3 years. The ARTISTIC trial also showed the importance of genotyping for HPV 16 as HPV 16 positive women had a cumulative incidence rate for CIN 2+ of 43.6% over 3 screening rounds compared to 20.1% for women positive for other hrHPV types. HPV primary screening was significantly more protective than cytology over a 6 year period with potential for extending screening intervals for HPV negative women (H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; C Kitchener *et al.*, 2014).

- POBASCAM: In the POBASCAM study the population was divided either into a cytology arm or HPV and cytology co-testing arm. At baseline in both cytology and HPV of the trial, detection of CIN 3+ did not differ significantly between the two groups but was significantly more common in women with a normal cytology result compared to HPV and cytology co-testing (34 of 19 286 vs 12 of 19 373; Relative Risk; 2·85, 95% CI: 1·47–5·49; p=0·001). There was significantly more CIN 2+ cases detected at baseline in the intervention arm of the study (p=0.015). During the follow up screening round using HPV and cytology co-testing significantly fewer CIN 3+ lesions were detected in the intervention arm (88 of 19 579 vs 122 of 19 731; Relative Risk 0·73, 95% CI: 0·55–0·96; p=0·023). The cumulative detection of CIN 3+ and CIN 2+ did not differe significantly between the two study arms. Overall the conclusion was that HPV DNA

testing leads to the earlier detection of clinically relevant CIN 2+, which, following treatment reduces the subsequent levels of CIN 3+ for those women. HPV testing improves the protection against CIN 3+ compared to cytology (Dorien C. Rijkaart *et al.*, 2012).

- Swedescreen: The intervention arm (HPV testing with cytology co-testing) detected 51% more CIN 2+ cases than that of the control (cytology only) arm while in the follow up screening round the intervention arm had 42% less cases of CIN 2+ indicating the protective benefits of HPV screening for the protection against CIN 2+ (Naucler *et al.*, 2007). Subsequent analysis of the data showed that cytology has a sensitivity of 71.3% (n=62/87, 95% CI: 60.6-80.5) compared to a sensitivity of 95.4% (n=83/87, 95% CI: 88.6-98.7) for HPV alone and a specificity for cytology of 98.6% (95% CI: 98.3-98.9) vs HPV only of 94.2% (95% CI:93.5-94.7). The PPV for cytology was 42.5% (95% CI:34.3-50.9) compared to 19.2% (95% CI:15.6-23.2) for HPV alone. It was concluded that primary HPV DNA screening with cytology triage and repeat follow up HPV testing for those who had a normal cytology on triage was a good approach.

Several different approaches to HPV testing exist, while several of the randoimised control trials described above use the traditional PCR based approach using the GP5/GP6 primers sets, or the hybid capture 2 (HC2) test. Several newer technologies have emerged in recent years including the Cobas HPV DNA test, Aptima HPV mRNA test, Cervista HPV HR, Xpert HPV, Abbot RealTime hr HPV assay, PapilloCheck and BD Onclarityas and AVantage HPV E6 to name but a few.

HC2 was one if the first clinically valdiated commercial HPV tests and heretofore has been regarded as the gold standard HPV assay for use in cervical screening and used as the primary competitor assay for validation of new HPV tests. This is based on the Meijer criteria as outlined in Chapter 1, Section 1.5 (Meijer et al., 2009). A pooled sensitivity of 89.9% (95% CI: 88.6-91.1) and pooled specificity of 89.9% (95% CI: 89.7-90.00%) has been reported for the HC2 DNA test based on analysis of data from over 138,000 women across 25 different studies (Koliopoulos *et al.*, 2017). Comparing this to other studies such as the large multicentre based ATHENA trial in the US, (Cox *et al.*, 2013) demonstrated that cytology alone (n=35,546) had a sensitivity of 57.7% and specificity relative to ASC-US triage of 84% compared to HPV testing alone (n=34,254) which had a sensitivity of 89.9% and specificity relative to ASC-US of 71.0%.

Despite the improved sensitivity and high negative predictive value of HPV testing, the the specificity and PPV is consistently reported as lower than cytology. However, HPV testing

has a higher NPV than cytology, reducing the rate of false negatives (Naucler *et al.*, 2007, 2009; Ronco *et al.*, 2008, 2010; H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; Dorien C. Rijkaart *et al.*, 2012; C Kitchener *et al.*, 2014; M. G. Dijkstra *et al.*, 2014). Due to the limited specificity of HPV testing, this could potentially lead to unnecessary referrals to colposcopy which necessitates the need for an appropriate triage test(s) to be in place in a HPV screening setting to further stratify HPV positive women.

The majority of studies on HPV based primary screening have focused on detection of HPV DNA and has been shown to be effective, as HPV DNA testing does not discriminate between transient and transforming HPV infections (Burd, 2016). The majority of HPV infections are transient infections, which will clear without developing a clinically significant lesion. Those that do not clear, can lead to transforming infections resulting in replication, propagation and cellular abnormalities, with the over expression of viral oncogenes E6 and E7 being a hallmark of the transforming HPV infections. This logic has lead to the development of many competitor HPV tests from the original Hybrid Capture 2 test, to other HPV DNA tests such as the Cobas HPV DNA test as well as mRNA based tests such as the Aptima HPV E6/E7 mRNA test.

This thesis focuses on two of the main valdiated HPV tests currently on the market, the Cobas HPV test (DNA based) and the Aptima HPV test (mRNA based). Both these assays are CE-IVD markers and have received FDA approval for use in cervical screening (Arbyn *et al.*, 2015). Both the Cobas HPV test (Heideman *et al.*, 2011; Stoler *et al.*, 2011; Lloveras *et al.*, 2013) as well as the Aptima HPV test (Heideman *et al.*, 2013; Cook *et al.*, 2017) meet the Meijer criteria (Meijer *et al.*, 2009).

A Cochrane review published in August by Koliopoulos et al. 2017 looked at over 19,000 records culminating in 40 accepted studies for meta-analysis comparing cytology to HPV testing (including HPV DNA dn HPV mRNA assays) for cervical cancer screening in the general population, this included data from the ARTISTIC, ATHENA, POBASCAM, Swedescreen and the VUSA-Screen study. The study concluded that the HPV assays higher sensitivity outperformed cytology even at its lowest positivity threshold of ASC-US and HPV testing will be less likely to miss CIN 2+ and detect progressive rather than regressive lesions compared to cytology. However, the specificity was higher in the cytology cohort (LSIL threshold) compared to HPV DNA tests but had an equivalent specificity in HPV mRNA tests (Aptima). This was mainly due to the high rate of non-relevant productive HPV infections in the population which HPV testing alone cannot differentiate (Koliopoulos *et al.*, 2017). From this Cochrane study the Cobas test had two accepted studies (n=11,666) with a sensitivity ranging (88-100%) and specificity ranging (50-91%) for the detection of

CIN 2. The Aptima test had three accepted studies for CIN 2+ (n=15,895) with a sensitivity ranging (83-100%) and specificity ranging (90-97%) for CIN 2+.

Since the original RCTs which utilised either the HC2 or the GP5+/GP6+ assays there have been a number of observational or interventional studies assessing both the Cobas and Aptima assays. To date, the Cobas has had a 4 year follow up period while the Aptima currently has data up to 6 years longitudinal data from different study cohorts. Recently the Cobas and Aptima tests have had data published around the retrospective detection of HPV in samples up to seven years before severe dysplasia (Forslund *et al.*, 2018).

The ATHENA trial was an interventional clinical trial evaluating the Cobas HPV test to routine cytological screening. The Cobas HPV test from the ATHENA's end of study paper shows that the 3 year cumulative index rate of CIN 3+ in cytology negative women was 0.8% (95% CI: 0.5-1.1) and 0.3 (95% CI: 0.1-0.7) for those women who had a negative HPV test on entry into the study. The sensitivity of cytology for CIN 3+ was 47.8% (95% CI: 41.6-54.1) compared to 76.1% (95% CI: 70.3-81.8) for HPV and a specificity of 97.1% (95% CI: 96.9-97.2) versus 93.5% (95% CI: 93.3-93.8) for HPV (Wright *et al.*, 2015).

The GAST trial was an observational trial for the evaluation of the Aptima HPV mRNA assay based in Germany. Women were tested for HPV mRNA with the Aptima assay , HC2 and cytology. While the APTIMA HPV Assay is a newer technology and therefore has less data available, the longituional data recently published from the GAST trial demonstrates that the absolute risk of developing a hrHPV positive CIN 3+ over six years in women who tested negative for HPV at baseline was 2.2 (95% CI:1.0-4.9) and 3.1 (95% CI: 1.7-5.7) per 1,000 women screened by HC2 and APTIMA HPV assays respectively. This compares to an absolute risk of 9.3 (95% CI: 2.9-30.2) for women with a normal cytology at baseline. The relative sensitivity of the Aptima HPV test compared to the Hybrid Capture 2 was 91.5% for the detection of CIN 3+ and the NPV for the Aptima HPV test was 99.8% (95% CI: 99.5-99.9) (Iftner *et al.*, 2018).

The FOCAL trial which is a randomised trial comparing HC2 testing to cytology, reports that the HC2 assay had a higher detection rate for CIN 3+ in the HPV arm (7.5/1000, 95% CI: 6.2-839) compared to the cytology control arm (4.6/1000 95% CI: 3.4-6.2). The FOCAL trial also compared several other HPV tests to the HC2. When the Cobas HPV DNA test was compared to the HC2 with follow up LBC 24 months later, the study reported that the two assays had a very high agreement (96.1%, kapp=0.75). They also reported that the use of cytology for the triage of HPV positive women was appropriate (Cook *et al.*, 2015). When compared to the Aptima test the agreement was also high (96.5%, kapp=0.76) but noted

that the Aptima assay with HPV 16/18/45 genotyping and cytology triage would lead to a reduced colposcopy referal with similar detection rates for CIN 2+ compared to the use of the HC2 test (Cook *et al.*, 2017). Both reported that CIN 3+ was significantly corrolated with HPV 16.

The data published around HPV detection in samples up to seven years before severe dysplasia (Forslund *et al.*, 2018) compared both the Cobas and Aptima tests based on biobanked samples from the Swedish population. Though this study had a relatively small study population in comparison to prior studies (n=1,172) with CIN 3+ occuring between 4 months to seven years after the initial screening test both the Cobas and Aptima tests showed similar concordance. For women both <30 and >30 years the sensitivity of the Aptima test was non-inferior compared to the Cobas (p=0.0217 and p=0.0123 for relative sensitivity of >90%) as well as an overall kappa value of 0.611). Longitudinal sensitivities for both tests were also comparable based of the 80 cases of CIN 3+ that developed after >5 years of follow up. Forslund concluded that HPV mRNA testing could be used safely for cervical screening.

HPV primary screening has been shown exhaustively to provide a higher level of sensitivity and NPV than the current cytology based screening with a lower specificity and PPV for the detection of CIN 2+. For this reason, primary HPV testing alone is not recommended in any of the screening trials referenced, HPV primary screening is recommended in combination with cytology as a triage test based of many of the RCT trials. The combination of HPV primary screening and cytology triage increases the overall specificity and negates the issues around false positivity rates while keeping the high sensitivity. Thus keeping the false negative rates lower than that of our current cytological screening and improving the overall safety of the cervical screening programme. While many studies have shown that both HPV assays perform well there is little prespective population data on head to head comparisons. In relation to Ireland there has been no HPV primary screening studies performed. With HPV primary screening set to begin in 2020 it is nessessary to provide data like this that would be needed in the cervical screening programme.

To this end, this study will evaluates the Aptima HPV mRNA assay and the Cobas HPV assays for their use in a primary screening population and will undertake a comparative assessment of both technologies for use in this context. This will be one of the first direct comparisons between the APTIMA HPV test and Cobas HPV test in a primary screening population.

3.2. Aims

The aims of this chapter are:

i. to assess the baseline population prevalence of HPV (DNA, mRNA) in women attending for routine CervicalCheck smear test;
ii. to compare the performance of HPV DNA and mRNA for detecting CIN2+ in a primary screening population;
iii. to assess the specific HPV genotype prevalence in those HPV positive cases and compare the genotyping capabilities of both the HPV DNA and mRNA assays.

3.3. Methods

3.3.1. Study Population

CERVIVA, in partnership with CervicalCheck, are currently undertaking a HPV primary screening study, which is evaluating and comparing different strategies for the triage of women with a HPV positive primary screening test. This study, funded by the HRB, is an observational cohort study which has enrolled >13000 women aged 25-60y attending primary care for their routine CervicalCheck smear test. Women attending for their routine CervicalCheck smear tests at 33 different primary care centres across Leinster were invited to participate in the study and gave written informed consent to participate. Residual smear samples following routine cytological diagnosis were retained and tested for HPV DNA (Cobas HPV DNA test (Roche)) and HPV mRNA (The Aptima HPV test (Hologic)). The women are being followed longitudinally through CervicalCheck for up to 10 years through several screening rounds (Chapter 2, Section 2.1.2 Figure 2.1). To be included in this study, women must be attending for their routine cervical smear. Exclusion criteria include women with a previous history of CIN2+, an abnormal smear within the past 3 years or women who had undergone hysterectomy where the cervix has been removed.

3.3.2. Cobas 4800 HPV Test

Cervical cytology samples collected in PreservCyt were tested using the Cobas HPV Assay and the Cobas 4800 instrumentation in accordance with the manufacturer's instruction and as detailed in Chapter 2 Section 2.2.1. Briefly, the Cobas 4800 HPV test is a qualitative *in-vitro* test for the automated real time multiplex PCR detection of HPV DNA. The Cobas 4800 HPV test detects HPV 16, HPV 18 and 12 other hrHPV genotypes (31,33,35,39,45,51,52,56,58,59,66 and 68) as well as a separate β-globin control for each sample.

3.3.3. Aptima HPV Assay

Cervical cytology samples collected in PreservCyt were tested using the Aptima HPV assay. 1ml of sample was aliquoted into a specimen transport tube containing a lysis solution prior to testing by the Aptima HPV assay on the Panther system.

The Aptima HPV test is a qualitative *in vitro* test for the detection of E6 and E7 mRNA in 14 HPV types (16/18/31/33/35/39/45/51/52/56/58/59/66/68) via an amplification nucleic acid probe test. There was no discrimination between the 14 high-risk types, results are reported as either Positive or Negative.

3.3.4. Aptima HPV 16 18/45 Genotyping Kit

All HPV mRNA positive samples were tested by the Aptima HPV 16 18/45 genotyping kit on the Panther system. During the study the majority of HPV genotyping tests were conducted on the original HPV mRNA positive sample that were stored after initial HPV mRNA testing at -20°C over the course of the study. As per the kit insert, samples were stored in the specimen transport tube stored at -20°C to -70°C and are stable for up to 24 months. The Aptima HPV 16, 18/45 test detects either HPV 16 or the combination of HPV 18/45.

3.3.5. Statistical Analysis

Overall prevalence of HPV DNA and mRNA was reported as percentages with associated 95% confidence intervals (95% CI). Binary Logistic Regression was used to calculate the Odds Ratio (OR) and 95% Confidence Intervals (CI) to determine the risk of testing positive for either HPV DNA or HPV mRNA in relation to age and cytology grade. Assay comparisons were based on matched data from women with both a valid HPV DNA and HPV mRNA result. Kappa correlation (k) was used to determine the level of agreement between the two tests. The agreement level based of this statistic are as follows: 0-0.2 (None), 0.21-0.39 (Minimal), 0.40-0.59 (Weak), 0.60-0.79 (Moderate), 0.80-0.90 (Strong), 0.90+ (Almost Perfect).

Clinical performance for the Cobas HPV DNA and Aptima HPV mRNA tests were assessed by calculating sensitivity, specificity, PPV and NPV for detection of high-grade disease. The primary disease endpoint was histologically confirmed CIN 2+ diagnosed at first colposcopy visit. High grade disease was classified as CIN 2 or greater [CIN 2+]. Histological confirmed CIN1/Normal and women with NAD on cytology were categorised as no high-grade disease (<CIN 2). Due to limited numbers, CIN 3+ was considered a secondary disease endpoint. CIN3 or greater was groups as CIN 3+, and less than CIN 3, including NAD cytology, grouped as <CIN 3. An associated McNemar's statistic was compare differences in the detection of the respective disease endpoints for each testing platform. Minitab Statistical Software version 17, IBM SPSS Statistics 25 and Microsoft excel 2017 where used for statistical analysis.

3.4. Results

3.4.1. Study Population

13,446 women consented and were enrolled into the study. Of these 13,446 women a total of 1433 were excluded from the study for various reasons including incomplete consent forms (n=194), not giving full consent for the study (n=111) duplicate consents (n=358), women presenting for non-routine repeat annual smears from past CervicalCheck post treatment follow up protocols (n=756). This resulted in 12,013 eligible women for inclusion in the study of which cytology data has been retrieved [to date] for 11,074 women.

3.4.2. Study Population Demographics

The age distribution of the study population is presented in Table 3.1 and is representative overall of the cervical screening population (CervicalCheck 2016, Annual report). The median age of the study population is 38 years, mean age 39 and range 22 -76 years. Overall, 19.4% of the women are under 30 years old and 80.6% of the women are over 30 years old (Table 3.1)

Table 3.1: Baseline Age demographics for the Study population with the CervicalCheck 2015-2016 Report age demographics

Age	Study Population (n=12013)	Study Population %	CervicalCheck % (2015-2016 Report*)
<25	8	0.07	0.20
25-29	2319	19.30	16.40
30-34	2161	17.99	17.80
35-39	2158	17.96	18.90
40-44	1972	16.42	17.20
45-49	1318	10.97	12.20
50-54	891	7.42	7.20
55-59	822	6.84	5.60
60	134	1.12	0.90
61+	230	1.91	3.50

**CervicalCheck report for end of year cytology figures does include a small fraction of cytology taken during colposcopy visit but it is largely representative of the primary screening population.*

3.4.3. Baseline Cytology and Follow-Up Histology Results in the Study Population

The baseline cytology results for the first 11,074 women enrolled in the study were obtained from CervicalCheck. In total, 8.61% have an abnormal ASC-US or greater (ASCUS+) cytology with LSIL being the most common at 5.17% (Table 3.2).This is broadly representative of the abnormal cytology rate observed by CervicalCheck (CervicalCheck 2016, Annual report). We did note a higher proportion of LSIL cytology and lower proportion of ASCUS in the study population (Table 3.2) but this is within the recommended limits

(CervicalCheck, 2013) and is likely due to the cytology for the specific study cohort being performed in one laboratory. In total, 599 histology results have been retrieved from CervicalCheck [to date] with CIN 1 being the most commonly found lesion (45.08%) and CIN 3+ accounting for 29.05% of the cases. Currently the prevalence of CIN 2+ in our primary screening population is 2.37%.

Table 3.2: Cytology and Histology Results in the study population

Baseline Cytology Breakdown			
	n	Study Population %	CervicalCheck (2015-2016 Report) %
NAD	9878	89.20	90.80
ASC-US	172	1.55	3.70
LSIL	572	5.17	3.80
ASC-H	7	0.06	0.40
HSIL	191	1.72	1.20
Other (AGC, AIS, SCC)	12	0.11	0.07
UNSAT	242	2.19	1.60
Total cytology	11,074		
Baseline Histology Breakdown			
	n	%	CervicalCheck (2015-2016 Report) %
Normal	66	11.02	12.1
CIN 1	270	45.08	34.5
CIN 2	89	14.86	22.1
CIN 3*	174	29.05	23.1
Total histology	599		

CIN 3 includes histology of AIS (n=2) and SCC (n=9)*

3.4.4. Overall Prevalence of HPV in the study population

3.4.4.1. HPV DNA and mRNA Prevalence in the population

Of the 12,013 women enrolled into the study, HPV DNA testing has been performed to date on the first on 9,577 consecutively enrolled women who were valid for inclusion in the study, while HPV mRNA testing was performed on all 12,013 women of which 11,990 had valid results. The 23 missing mRNA tests were invalid for testing due to insufficient sample remaining. Figure 3.2 depicts the overall HPV positivity with HPV DNA and HPV mRNA

tests. Overall, 15.98% (1530/9577) of study population were positive for HPV DNA. The Cobas HPV test simultaneously provides partial genotyping data for HPV16/18. Of the overall population, 3.95% (378/9577) were HPV 16 positive and 1.09% (104/9577) were HPV 18 positive with the remaining 10.94% (1048/9577) having a hrHPV infection for one or more of the other 12 HR HPV types. In the HPV positive cohort of women HPV 16 made up 24.72% (378/1530) of cases, HPV 18 was present in 6.80% (104/1530) of cases and hrHPV accounted for 68.48% (1048/1530) of HPV positive cases.

Overall, 13.22% (1585/11,990) of the study population were positive by the Aptima HPV mRNA test. Subsequent partial genotyping data indicated that 2.35% (282/11,990) of the HPV mRNA positive cases were positive for HPV 16, 1.41% (169/11,990) were positive for HPV 18/45 positive with 0.26% (31/11,990) having a dual positive HPV 16,18/45 positive result and the remaining 9.10% (1134/11,990) had a hr mRNA positive result. In the HPV positive cohort of women HPV 16 made up 17.79% (282/1585) of cases, HPV 18/45 was present in 10.66% (169/1585) of cases and hrHPV accounted for 71.54% (1134/1585) of HPV positive cases.

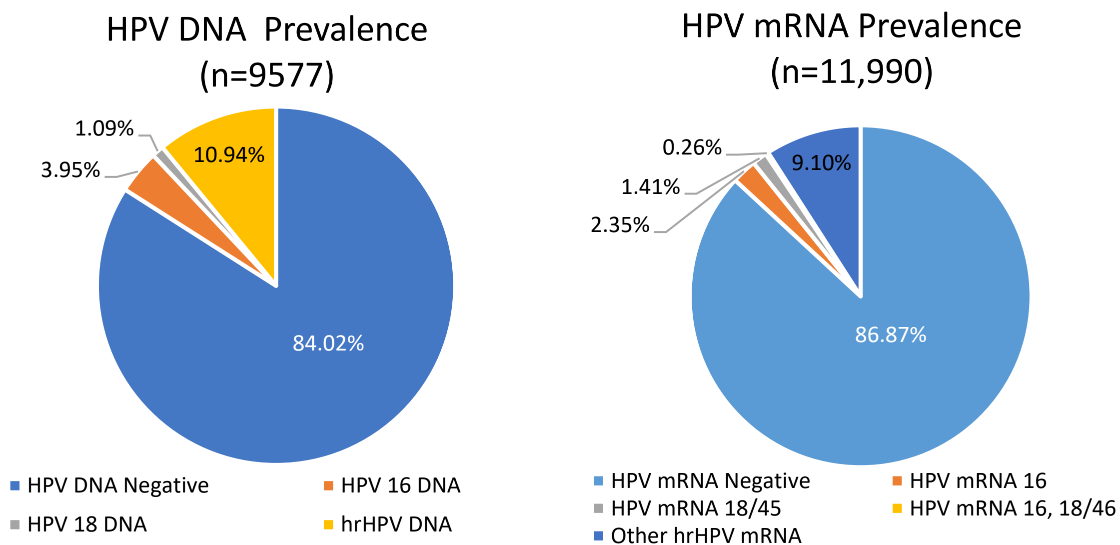

Figure 3.2: HPV DNA and mRNA prevalence of the study population

3.4.4.2. HPV Positivity in Relation to the Age of the HPV Primary Screening Population

The overall prevalence of HPV DNA and mRNA is higher in the younger women (<30 years) than that of the older age groups (40+ years), with the over 40 years age group having nearly 50% less HPV positive results than the 30-39 year age group alone for both HPV DNA and HPV mRNA (Table 3.3). The data shows that the odds of having a HPV positive DNA and mRNA test decreases with increasing age (Table 3.3). This trend is also seen in the HPV DNA positive population with 40.22% (615/1529) of HPV positive women being <30 years old. 36.43% (558/1529) are between 30-39 years, 15.63% (239/1529) between 40-19 years and 7.72% (118/1529) being over 50 years old. This trend is true in the mRNA positive women also with 43.84% of <30-year olds, 36.84% of 30-39 years, 12.80% of 40-49 years and 6.43% of 50+ being positive for HPV mRNA.

Table 3.3: HPV DNA and mRNA prevalence in relation to age. Associated percentages and odds ratio.

	% HPV DNA Positive	**OR (95% CI)**	**% HPV mRNA Positive**	**OR (95% CI)**
<30	34.40% (615/1788)	-	29.92% (696/2326)	-
30-39	16.25% (558/3433)	0.3675 (0.32-0.41)	13.54% (584/4312)	0.3633 (0.3220-0.40)
40-49	8.95% (239/2670)	0.1939 (0.16-0.19)	6.19% (203/3279)	0.1604 (0.13-0.18)
50+	7.00% (118/1686)	0.1634 (0.13-0.19)	4.92% (102/2073)	0.1370 (0.11-0.16)

3.4.4.3. HPV Positivity in Relation to the Cytology

The overall prevalence of HPV DNA and HPV mRNA when stratified by cytology increases as the severity of the cytological grade increases. Of the 9577 HPV DNA tests performed 9502 had a corresponding cytology result and of the 11,990 HPV mRNA tests performed 11,042 had a corresponding cytology also. Overall 11.24% and 8.28% of samples with a normal cytology (NAD) had a positive HPV DNA or HPV mRNA result respectively with the prevalence of HPV DNA and mRNA increasing with higher grades of disease (Table 3.4). The data shows that the odds of having a HPV positive DNA or mRNA test increases with the severity of the cytological findings.

In the HPV DNA and HPV mRNA positive population, the breakdown of cytology is shown in Figure 3.3. NAD is the most commonly reported cytology result in the HPV positive cohort at 63.4% and55.62% for HPV DNA and mRNA respectively. LSIL is the second most commonly reported cytology finding at 22.23% and 25.60%.

Table 3.4: Breakdown of the Percentage Cytology stratified by HPV result

Cytology Grade	% HPV DNA Positive	OR (95% CI)	% HPV mRNA Positive	OR (95% CI)
NAD	11.24% (964/8547)	-	8.28% (817/9862)	-
ASC-US	43.70% (59/135)	6.1284 (4.33-8.66)	38.60% (66/171)	6.9589 (5.07-9.54)
LSIL	71.52% (339/474)	19.8232 (16.06-24.46)	65.73% (376/572)	21.2382 (17.61-25.60)
ASC-H	66.67% (2/3)	15.7884 (1.43-174.28)	57.14% (4/7)	14.7613 (3.29-66.06)
HSIL*	89.19% (132/148)	63.6179 (38.26-105.77)	93.68% (178/190)	138.0184 (81.17-234.67)
UNSAT	9.74% (19/195)	0.8522 (0.52-1.37)	7.92% (19/240)	0.9518 (0.59-1.52)

HSIL includes AGC, SCC and AIS*

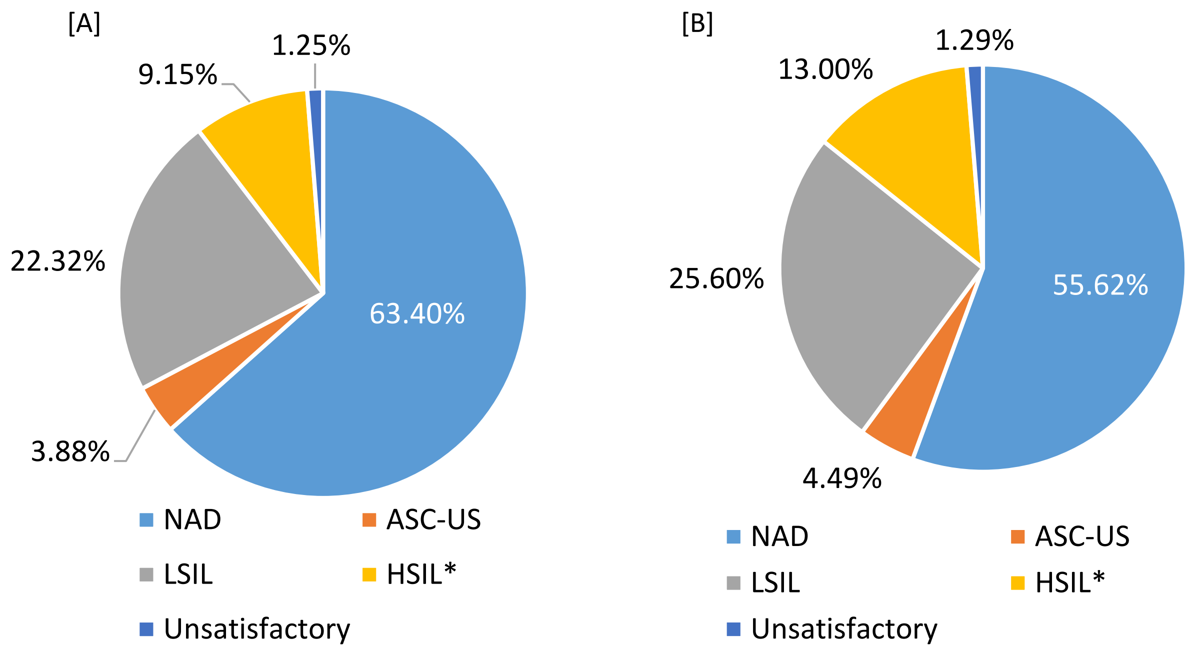

Figure 3.3: Distribution of Cytology in the HPV DNA [A] and mRNA [B] positive population (n=1519). HSIL* includes ASH-H and query AIS, SCC and AGC.

3.4.5. Comparison of the performance characteristics of HPV DNA and HPV mRNA assays in a primary screening cohort

Of the 9577 HPV DNA tests and 11,990 HPV mRNA tests performed, there were 9559 samples that had paired HPV DNA and HPV mRNA results. Of these results, there was a HPV DNA prevalence of 15.99% (1529/9559) and HPV mRNA prevalence of 13.35% (1276/9559) with a kappa value of 0.704 between the two platforms, (McNemar $p<0.001$). Of the 9559 HPV DNA and HPV mRNA results, there were 9517 with a valid cytology result. Within this, the HPV DNA positivity was 15.96% (1519/9517) and HPV mRNA positivity was 13.32% (1268/9517) with a kappa value of 0.707, (McNemar $p<0.001$).

3.4.5.1. Comparison of HPV Positivity in Relation to the Age for the Cobas and Aptima assays.

As expected, the prevalence of HPV DNA and mRNA decreased with increasing age (Table 3.5), and the prevalence of HPV DNA was significantly higher than HPV mRNA across all age groups ($p<0.001$) In particular women <30's had high prevalence rate of for HPV DNA 34.40% (615/1788) and 30.54% (546/1788), for HPV mRNA.

Table 3.5: HPV DNA and mRNA in relation to age. Associated percentages and statistics.

	% HPV DNA Positive	% HPV mRNA Positive	McNemar	Kappa
<30	34.40% (615/1788)	30.54% (546/1788)	<0.001	0.761
30-39	16.25% (557/3428)	13.65% (468/3428)	<0.001	0.701
40-49	8.89% (239/2661)	6.73% (179/2661)	<0.001	0.584
50+	7.02% (118/1682)	4.93% (83/1682)	<0.001	0.529
All Ages	15.99% (1529/9559)	13.35% (1276/9559)	<0.001	0.704

3.4.5.2. Comparison of HPV Positivity in Relation to the Cytology in a primary screening cohort

As expected, there prevalence of HPV DNA and mRNA increased with the increasing grade of disease (Figure 3.4). The prevalence of HPV DNA was significantly higher than HPV mRNA in those women with LSIL (p=0.002) or no abnormality detected on cytology (p<0.001). A kappa statistic was calculated for each cytological group between HPV DNA and HPV mRNA with NAD having a kappa of 0.609, ASC-US having a kappa of 0.807, LSIL having a kappa of 0.728, HSIL* with a kappa of 0.657 and Unsatisfactory having a kappa of 0.636.

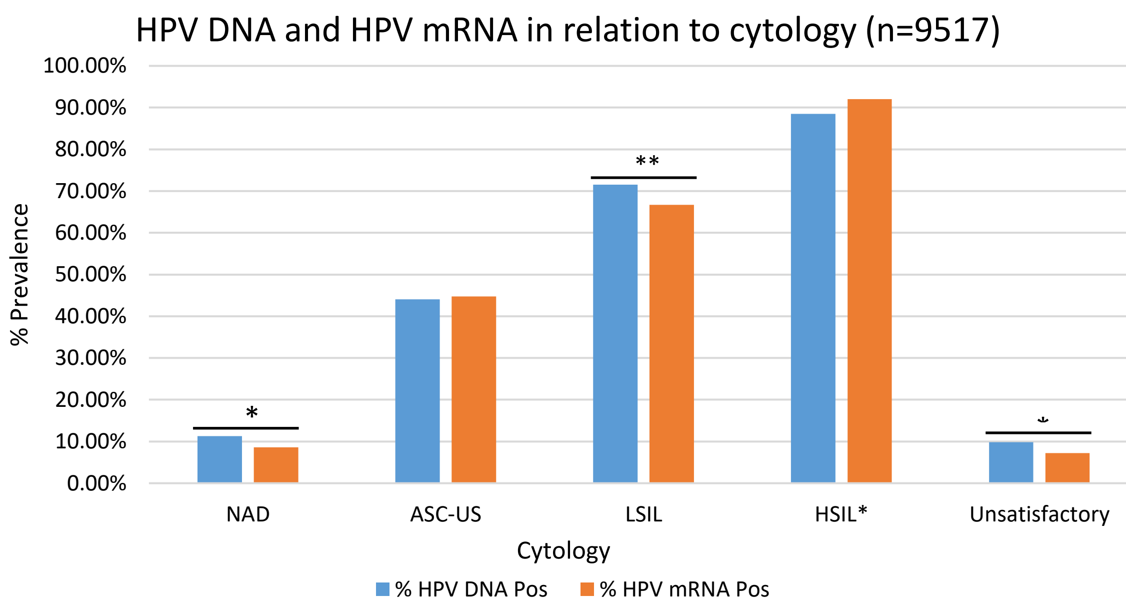

Figure 3.4: HPV DNA and mRNA in relation to their cytology.
HSIL* includes ASC-H, SCC, AIS, AGC.
*p-value <0.001, **p-value=0.002, p-values are derived from the McNemar's test

3.4.5.3. Sensitivity, Specificity and Predictive values of HPV DNA and HPV mRNA test for the detection of CIN 2+ and CIN 3+

The sensitivity, specificity, PPV and NPV for the HPV DNA test and HPV mRNA test for the detection of both CIN 2+ and CIN 3+ are shown in Table 3.6. In total there were 263 confirmed CIN 2+ cases and 174 confirmed CIN 3+ cases with corresponding HPV DNA and HPV mRNA tests.

Between the Cobas HPV DNA test and the Aptima HPV mRNA test there were no statistical differences shown between the two platforms for sensitivity of either CIN 2+ (p=0.550) or CIN 3+ (p=0.461). Specificity was higher for HPV mRNA but was not statistically different to HPV DNA (p=0.062). The PPV of the HPV mRNA test was higher for both the detection of CIN 2+ and CIN 3+ (19.34%, 12.66%) compared to the HPV DNA test (14.87%, 9.75%). The NPV of both tests DNA and mRNA was comparable at 99.90% (CIN 2+) and 99.93% vs 99.92% (CIN 3+).

Table 3.6: Clinical performance of the Cobas 4800 HPV test and Aptima HPV test in the detection of CIN 2+ and CIN 3+ (n=9517).

	CIN 2+		**CIN 3+**	
	HPV DNA Test	**HPV mRNA Test**	**HPV DNA Test**	**HPV mRNA Test**
Sensitivity (95% CI)	96.26% (95.78-96.74)	96.55% (96.15-96.96)	96.43% (95.86-97.00)	95.93% (95.35-96.51)
Specificity (95%CI)	86.63% (86.38-86.87)	89.65% (89.47-89.83)	85.94% (85.69-86.19)	88.89% (88.70-89.08)
PPV (95%CI)	14.87% (14.21-15.54)	19.34% (18.49-20.19)	9.75% (9.28-10.21)	12.66% (12.06-13.26)
NPV (95%CI)	99.90% (99.89-99.90)	99.90% (99.90-99.90)	99.93% (99.93-99.94)	99.92% (99.92-99.92)

Cross sectional data not all histologically verified; true and false negatives include cytology NAD

Table 3.7 shows the clinical performance of both HPV DNA and mRNA assays in an under 30-year-old population. There was no statistical difference between the Cobas HPV DNA test and the Aptima HPV mRNA for the detection of either CIN 2+ (p=0.829) or CIN 3+ (p=0.878). Specificity was statistically higher for HPV mRNA than HPV DNA (p=0.032). The PPV for both CIN 2+ and CIN 3+ is higher for the Aptima mRNA test compared to the Cobas HPV DNA test (18.85% vs 22.82%; 12.75% vs 14.80%). The NPV for both HPV tests for both disease endpoints were comparable (99.82% vs 99.86%; 99.82% vs 99.86%).

Table 3.7: Clinical performance of the Cobas 4800 HPV test and Aptima HPV test in the detection of CIN 2+ and CIN 3+ in an under 30-year-old population (n=1656)

	CIN 2+		**CIN 3+**	
	HPV DNA Test	**HPV mRNA Test**	**HPV DNA Test**	**HPV mRNA Test**
Sensitivity (95% CI)	98.13% (97.78-98.48)	98.46% (98.20-98.72)	97.26% (96.65-97.87)	97.65% (97.16-98.14)
Specificity (95%CI)	70.82% (69.79-71.85)	76.42% (75.59-77.24)	69.30% (68.25-70.35)	74.59% (73.73-75.44)
PPV (95%CI)	18.85% (17.58-20.12)	22.82% (21.36-24.27)	12.75% (11.82-13.67)	14.80% (13.75-15.84)
NPV (95%CI)	99.82% (99.81-99.83)	99.86% (99.85-99.87)	99.82% (99.81-99.83)	99.86% (99.85-99.87)

Cross sectional data not all histologically verified; true and false negatives include cytology NAD

There was no statistical difference between the Cobas HPV DNA test and the Aptima HPV mRNA for the detection of either CIN 2+ (p=0.677) or CIN 3+ (p=0.580). The clinical performances are shown in Table 3.8 and both tests have a comparable NPV (99.91%; 99.95% vs 99.94%) for the detection of both CIN 2+ and CIN 3+. The specificity was marginally higher for HPV mRNA compared to HPV DNA (p=0.159). The PPV of the HPV DNA test was lower than the HPV mRNA for the detection of both CIN 2 + and CIN 3+ (12.20% vs 16.71%; 7.73% vs 11.05%).

Table 3.8: Clinical performance of the Cobas 4800 HPV test and Aptima HPV test in the detection of CIN 2+ and CIN 3+ in an over 30-year-old population (n=7797)

	CIN 2+		**CIN 3+**	
	HPV DNA Test	**HPV mRNA Test**	**HPV DNA Test**	**HPV mRNA Test**
Sensitivity (95% CI)	94.39% (93.39-95.40)	94.66% (93.79-95.52)	95.52% (94.50-96.55)	94.25% (93.11-95.39)
Specificity (95%CI)	90.00% (89.79-90.20)	92.57% (92.42-92.72)	89.54 (89.33-89.76)	92.10% (91.95-92.26)
PPV (95%CI)	12.20% (11.47-12.93)	16.71% (15.71-17.71)	7.73% (7.24-8.22)	11.05% (10.34-11.76)
NPV (95%CI)	99.91% (99.91-99.91)	99.91% (99.91-99.91)	99.95% (99.95-99.96)	99.94% (99.93-99.94)

Cross sectional data not all histologically verified; true and false negatives include cytology NAD

3.4.6. Comparison of HPV DNA and HPV mRNA in Relation to their HPV Genotype

Of the 9577 HPV DNA tests and 11,990 HPV mRNA tests performed, there were 9517 samples that had paired HPV DNA and HPV mRNA and genotyping results. Of these results the prevalence of HPV 16/18 detected by Cobas HPV assay was 5.04% (482/9517) compared to a prevalence of 4.28% (409/9517) for HPV 16/18/45 detected using the APTIMA genotyping assay. Both tests were deemed statistically significant from each other with a McNemar's value of $p>0.001$ and a kappa value of 0.354.

3.4.6.1. HPV 16/18 DNA and HPV 16/18/45 mRNA in Relation to Age

The prevalence of HPV 16/18 and HPV 16/18/45 mRNA decreased as the age increased in the population (Figure 3.5). There is a statistical difference between the two tests for each age grouping: <30's ($p=0.008$), 30-39 ($p=0.001$), 40-49 ($p<0.001$) and 50+ ($p=0.003$). However, the overall agreement between each HPV test and HPV genotype is quite strong with a corresponding HPV 16/18 or 16/18/45 genotype in the <30's 94.30% of the time. 30-39 had an agreement of 95.25%, 40-49 had an agreement of 98.54% and woman over 50 had an agreement of 98.58%.

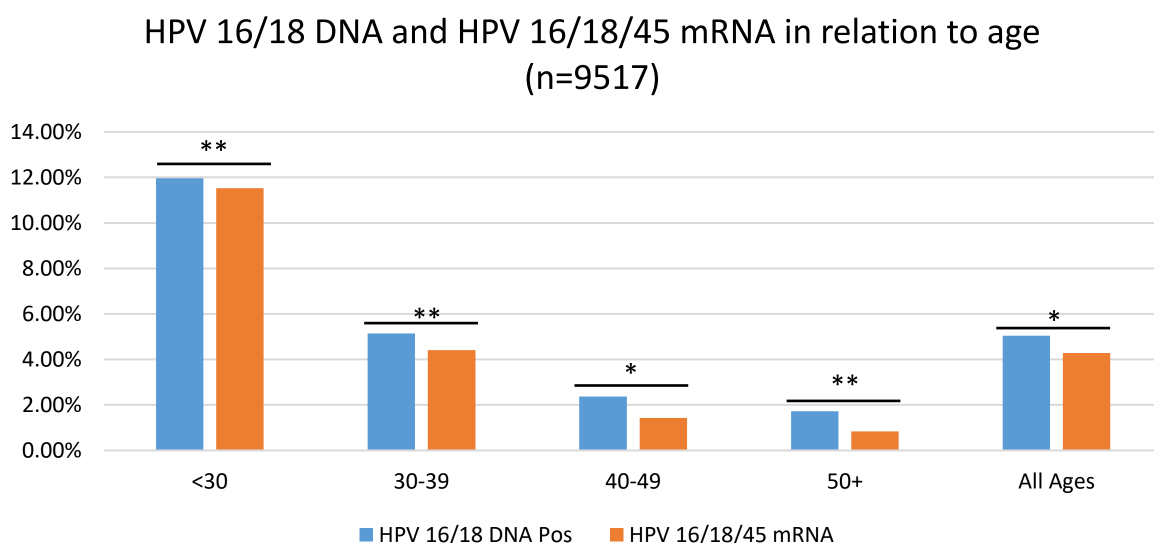

Figure 3.5: HPV 16/18 DNA and HPV 16/18/45 mRNA in relation to age.
*p-value <0.001, **p-value <0.05, p-values are derived from the McNemar's test

3.4.6.2. HPV 16/18 DNA and HPV 16/18/45 mRNA in Relation to Cytology

The prevalence of HPV 16/18 DNA and HPV 16/18/45 mRNA increased as the cytological grade increased (Figure 3.6) with HSIL cytology being positive for HPV 16/18 DNA in 56.95% of the population and HPV 16/18/45 mRNA in 68.21%. This is a marked increase from the 21.10% and 21.31% of the LSIL cytology cohort. There was only a statistically significant difference between the two tests for NAD cytology (p<0.001). HPV 16/18 DNA and HPV 16/18/45 mRNA were in agreement in 98.12% of NAD cytology results. 90.43% for ASC-US, 83.40% for LSIL and 92.05% for women with HSIL* cytology

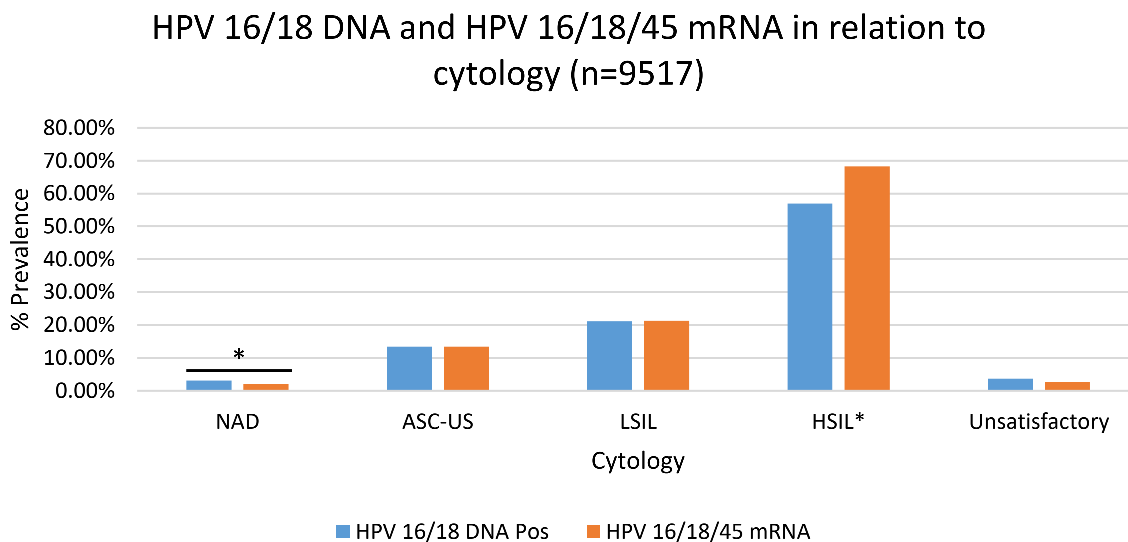

Figure 3.6: HPV 16 DNA and HPV 16 mRNA in relation to their cytology.
HSIL* includes ASC-H, SCC, AIS, AGC.
*p-value <0.001, p-values are derived from the McNemar's test

3.4.6.3. Other hrHPV DNA and other hrHPV mRNA genotypes in Relation to Age

The remaining HPV DNA and HPV mRNA tests make up the other hrHPV genotype category. Of these results, there was a hrHPV DNA prevalence of 10.95% (1047/9559) and a hrHPV mRNA prevalence of 9.21% (880/9559) with a kappa value of 0.617 and both tests were statistically different (p<0.001). The prevalence of hrHPV DNA and mRNA decreased with the increase in age (Figure 3.7) and there is statistical difference between both tests across each age group (p<0.001). Agreement across each age group was good with 90.45% of <30, 93.41% of 30-39, 94.78% of 40-49 and 95.33% of 50+ women agreeing with their hrHPV DNA or mRNA result.

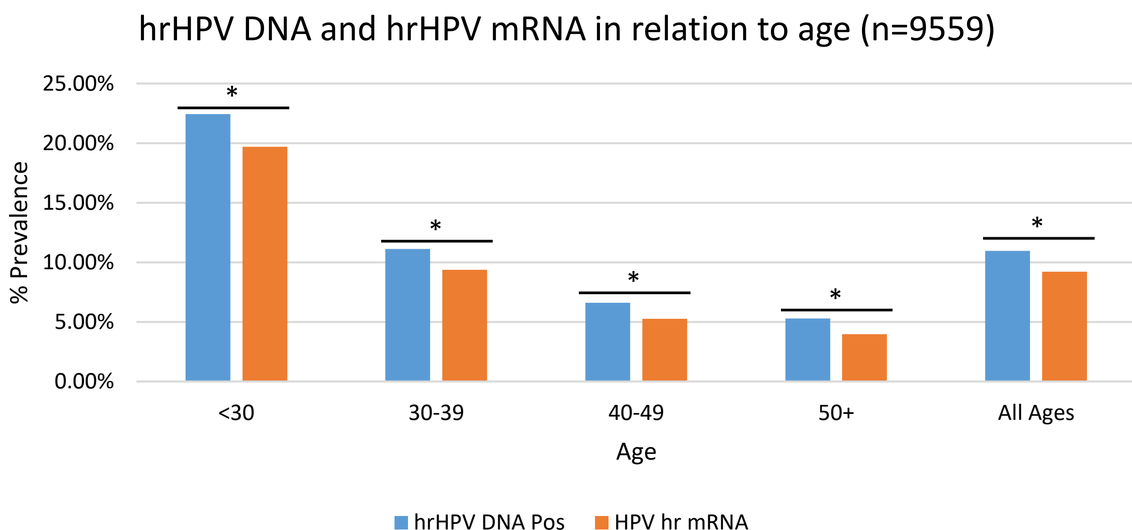

Figure 3.7: hrHPV DNA and hrHPV mRNA (not including HPV 45) in relation to their age. *p-value <0.001

3.4.6.4. Other hrHPV DNA and hrHPV mRNA in Relation to Cytology

The prevalence of other hrHPV DNA and mRNA genotypes increased from NAD to LSIL followed by a decreased prevalence in HSIL (Figure 3.8). There was a statistical difference between NAD and LSIL cytology and the two HPV tests DNA and mRNA (p <0.001, p=0.044). There was good agreement in hrHPV DNA and mRNA in respect to their cytology result. 98.12% of women with an NAD cytology had a matching HPV result, 90.43% for ASC-US, 83.40% of LSIL's and 91.76% of HISL's.

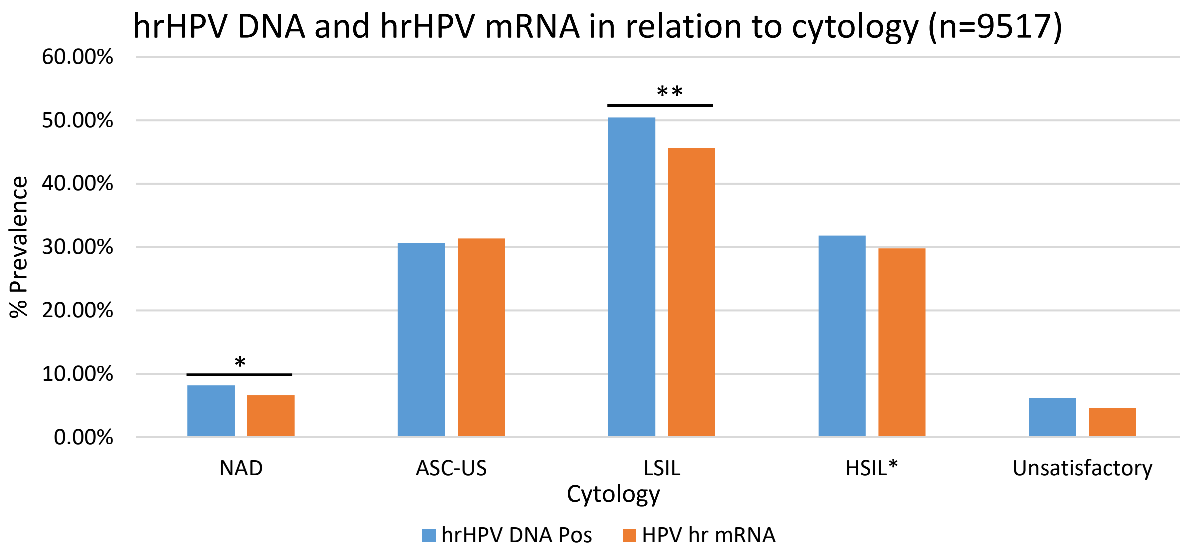

Figure 3.8: hrHPV DNA and hrHPV (not including HPV 45) mRNA in relation to their cytology.
HSIL* includes ASC-H, SCC, AIS, AGC.
*p-value <0.001, **p-value <0.05, p-values are derived from the McNemar's test

3.4.7. HPV DNA Genotyping in relation to Age and Cytology

In a HPV positive primary screening population the age trend is similar to what has previously been shown. As the age of the population increases the prevalence of both HPV 16/18 and hrHPV declines. The <30-year-old population show the highest prevalence of both HPV 16/18 (14.00%, 214/1529) and hrHPV (26.23%, 401/1529). Overall 31.52% (482/1529) of women had a HPV16/18 positive test Figure 3.9.

Of those women who had a positive HPV DNA test the majority had a corresponding NAD cytology result. 17.31% (263/1519) of women had a NAD cytology with a positive HPV 16/18 DNA result and 46.08% (700/1519) of women who also had an NAD cytology has a hrHPV result. For both ASC-US and LSIL a hrHPV result was more prevalent than HPV 16/18 (ASC-US: 1.18% vs 2.70%, LSIL: 6.58% vs 15.73%). For those women who had a HSIL* result HPV 16/18 was more prevalent than hrHPV (5.99% vs 3.16%) (Figure 3.10).

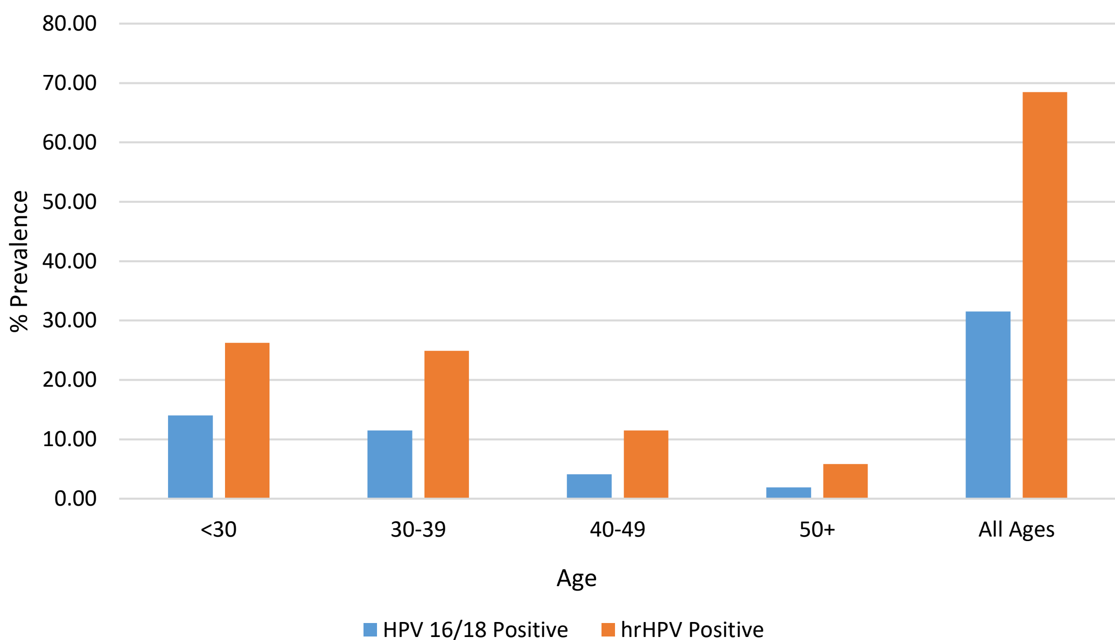

Figure 3.9: HPV Positive Genotyping in relation to Age (n=1529)

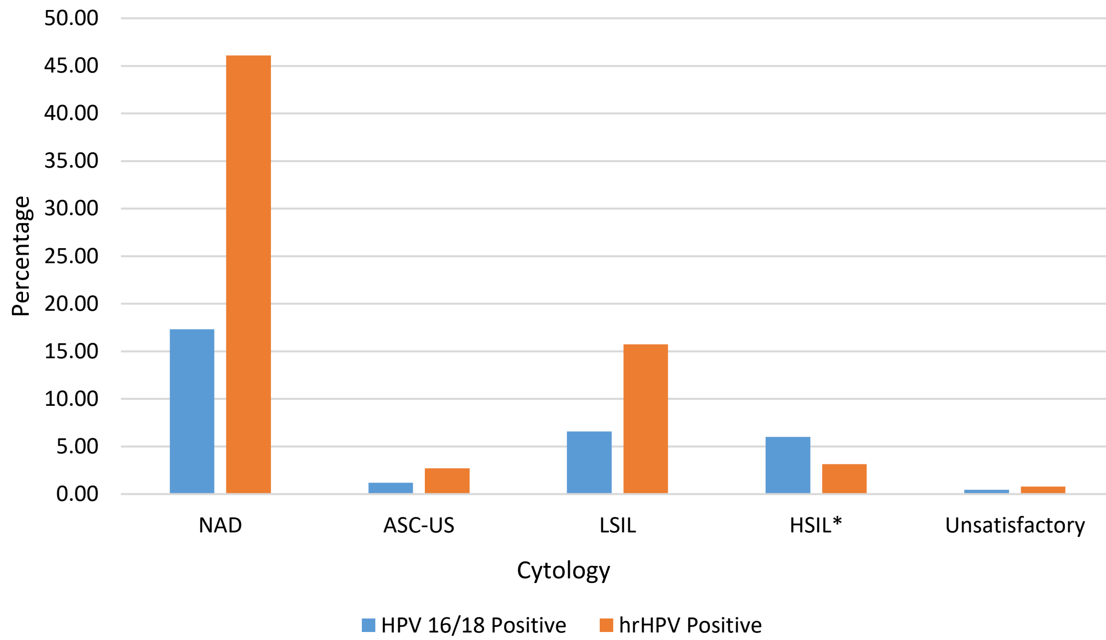

Figure 3.10: HPV Positive Genotyping in relation to Cytology (n=1519).
HSIL* includes ASH-H and query AIS, SCC and AGC.

3.5. Discussion

The aim of this chapter was to assess the baseline prevalence of HPV using the Cobas HPV DNA test and the Aptima HPV mRNA test with partial genotyping in women attending for their routine CervicalCheck smear test and to compare the performance of both HPV DNA and mRNA for detecting CIN 2+ in a primary screening population.

Overall, the Cobas HPV DNA test consistently had a higher positivity rate compared to the Aptima HPV mRNA test, with an overall prevalence of 15.98% for HPV DNA compared to 13.22% for HPV mRNA, across all age groups. This rate would be higher than most European countries with rates ranging from 21.4% in Eastern Europe to 8.8% to 10.0% in West, South and Northern Europe (Bosch *et al.*, 2013). The Caribbean had the highest reported prevalence at 35.4% and Western Asia had the lowest at 1.7%. The broad range can be due to the variance in high risk activities in each country such as unprotected sexual intercourse but also could be due to the lower rate of HPV testing or less sensitive testing used in the reporting of HPV prevalence. When stratified by age, the highest prevalence of HPV DNA and mRNA is observed in the <30's with the prevalence decreasing with age as expected. Several other studies have shown similar results (Leinonen et al. 2013; C Kitchener et al. 2014).

Consistent with literature the prevalence of HPV increases with the cytological grade with 89.19% and 93.68% of HSIL* samples having a positive HPV DNA and mRNA result. In total 11.24% and 8.28% of cytology NAD samples were positive for both HPV DNA and mRNA. Though this group has the lowest prevalence of HPV, of all the cytology grades it accounts for the highest number of HPV positive women. This will be an important factor to consider later if cytology were to be utilised in the triage of HPV positive women as the options for how to manage HPV positive, cytology negative women is still uncertain with retesting in 12 to 24 months being the current favoured option (Rijkaart et al. 2010; HIQA 2017).

Comparison between HPV DNA and HPV E6/E7 mRNA.

Assessing the agreement between the two HPV tests is not a straightforward matter, due to the inherent differences in the platforms target. By virtue of the biological process, HPV DNA can be detected when an infection is present, whereas mRNA is detected only when viral oncogenes E6 and E7 are transcribed. In addition, to the difference in DNA and mRNA assays, both target differing regions of the HPV genome with the Cobas 4800 HPV test detecting the L1 region while the Aptima assay detecting the oncogenic E6 and E7 mRNA.

A second compounding factor is the difference in chemistries between the two platforms with the Cobas HPV Assay using a more conventional real time PCR detection method and the Aptima utilising its three-step capture, TMA amplification and detection steps. Overall, while there is a statistically significant difference between the two tests ($p>0.001$) for detecting HPV, there is a good kappa agreement between the two platforms (0.704) indicating that both platforms are in general agreement over the HPV test results. When stratified by age in the <30 and 30-39-year-old cohorts both assays again show good agreement (kappa 0.761, 0.701), while the agreement is only moderate in women over 40 years. The reason for the drop-in agreement in the older age cohorts is unknown but the disagreement between HPV assays is a known factor in HPV testing. Positive agreement was also found to be lower in women aged 30-65 compared to 23-29 years in the HORIZON dataset (Rebolj *et al.*, 2014). Also with this there is the possibility of a reactivated HPV infection rather than a newly acquired infection in these older women (Brown and Weaver, 2013). The more important factor for these two assays is the agreement between relevant CIN 2+ lesions. Agreement between cytology grades showed moderate to good agreement only having a statistical difference in the NAD, LSIL and Unsatisfactory cytology groups.

Overall for the broad classification of a positive or negative HPV test there is a good agreement between the two platforms however there is still a significant difference between them in terms of their positive results. Depending how these results are stratified will affect the overall agreement but importantly there is good agreement in the HSIL* cytology cohort with no statistical difference for the high-grade cytology. There was however both a statistical difference ($p<0.001$) as well as low kappa value for NAD cytology (0.609) between HPV DNA and mRNA which may be an important consideration at a later point if cytology will be utilised as a triage test for HPV positive women. This decrease in agreement may be due in part to the mRNA target of E6 and E7. In cytology normal women with more transient infections would not overexpress these transcripts in comparison to HISL samples that had high mRNA positivity. This is the potential reason for the increased specificity seen in mRNA testing. Agreement has been assessed briefly before in the Horizon study on 5,064 women with a kappa of 0.62 (95% CI: 0.59-0.64) (Rebolj *et al.*, 2014). This was also shown again more recently with a kappa of 0.611 (95% CI: 0.547-0.675) in 1,172 cervical samples (Forslund *et al.*, 2018).

In total, 5.04% of study population were positive for HPV 16/18 genotype detected by the Cobas HPV Assay, and 4.28% having a HPV 16/18/45 mRNA genotype. Regardless if the genotyping is stratified by age, cytology or neither both assays showed a strong level of agreement between each other. If HPV 16 DNA and mRNA are compared directly there is a strong percentage agreement between the two platforms indicating the majority of the

disagreement shown in the McNemar statistic is likely due to the inclusion of the additional type HPV 45 in the mRNA genotyping test.

Clinical Performance of HPV Testing.

Based on the Cochrane review by Koliopoulos et al. 2017 the Cobas HPV Assay has a sensitivity range of [88-100%] and specificity range of [50-91%] for the detection of CIN 2+ and [92-100%] and [57-90%] for the detection of CIN 3+. The Aptima has a sensitivity range of [83-100%] and specificity range of [90-97%] for CIN 2+ and [78-100%] and [89-96%] for CIN 3+. In this study, we found that both HPV tests had comparable sensitivity and NPV. The Cobas HPV DNA Assay having a sensitivity of 96.26% (95% CI; 95.78-96.74%) and specificity of 86.63% (95% CI; 86.38-86.87%) for the detection of CIN 2+ and a sensitivity of 96.43% (95% CI; 95.86-97.00%) and specificity of 85.94% (95% CI; 85.69-86.19%) for CIN 3+. The Aptima assay mirrored this with a sensitivity of 96.55%, (95% CI; 96.15-96.96%) but a higher specificity of 89.65% (95% CI; 89.47-89.83%) for CIN 2+ and a sensitivity of 95.93% (95% CI; 95.35-96.51%) and higher specificity of 88.89% (95% CI; 88.70-89.08%). Both the sensitivity and specificity are comparable for the Cobas and Aptima tests to previous studies as well as the initial validation studies. The PPV of the Aptima HPV test was marginally higher than that of the Cobas HPV test for the detection of both CIN 2+ and CIN 3+ (14.87% vs 19.34%) (7.75% vs 12.66%). As expected, both tests had very high NPV's and were both identical at 99% for both.

The current sensitivity and specificities for both the Cobas HPV assay and Aptima platform show its ability to detect CIN 2+ precancerous lesions. The caveat is that the data is based on cross sectional data from a cytological screening programme and the clinical performance of both tests is likely to be underestimated in this first round of assessments. The long-term follow-up of these women may uncover underlying lesions that were not initially picked up through routine cytology in this first round and though a very small proportion of CIN 2+ lesions were HPV negative in this instance there may be a larger proportion of HPV positive cytology NAD women present in this study that are currently undetected.

Association of Age in HPV Primary Screening

Across Europe, HPV screening age brackets vary with countries like the Netherlands opting to begin screening at 30 years compared to countries like Italy who would begin at 25 years. The main impact of the younger age groups is the expected decrease on the specificity of both tests and a higher rate of false positives in this age bracket (Chrysostomou et al. 2018).

From our study population we assessed the clinical performances of under and over 30's population. The under 30's category of women are generally considered to have a higher level of non-relevant/transient HPV infections which if detected for could increase the burden on follow-up colposcopy services. Ireland however will not be screening only women over 30 years but from 25 to 60 year (HIQA, 2017). These women will be managed appropriately through triage testing in the cervical screening programme. HPV mRNA testing in this light, however, may be able to provide a higher level of specificity for this younger age group.

For women under 30 years, the sensitivity of both tests remains in the upper 98% range and the NPV remains at ~99% we see a marginally higher PPV compared to the baseline performances. However, the specificity for detecting both CIN 2+ and CIN 3+ is less in the under 30's with a specificity for the Cobas HPV Assay of 70.82% compared to 86.63% for the detection of CIN 2+ and for the Aptima, 76.42% compared to 89.65% for the detection of CIN 2+ also. This occurs for CIN 3+ also (69.30% vs 85.94% for the Cobas HPV assay, 74.59% vs 88.89% for the Aptima). Arbyn et al. 2012 noted that the use of HPV primary screening for women over the age of 30 years was linked to higher sensitivity and specificity for the detection of CIN 2+. This holds true in our population in regard to specificity with the over 30's seeing comparable results for the Aptima test with a slight increase in specificity to 92.57%. However, we see a decrease of around 2% in the sensitivity of the Cobas HPV Assay for both CIN 2+ and CIN 3+. Regardless of the starting age of the HPV primary screening programme both tests show a high level of sensitivity for the detection of either CIN 2+ or CIN 3+ in both <30 and >30's.

There is one point of consideration between these two tests that should be addressed at this point relating to the use of an internal control. The Cobas HPV Assay contains a β-globin internal control to ensure that cellular material was present in this automated process prior to testing. The Aptima platform however contains an internal control that monitors the process rather than the specimen leaving the possibility that a reported negative result may be negative due to the lack or no cellular material present in the sample. A letter from Dr Thomas Schelhorn, the Manager for Scientific Affairs in Hologic, notes that "A cellularity control based on a human housekeeping gene, gives a positive signal for all kinds of human cell material in the sample (skin cells, mucosal cells etc.). Therefore, a positive control signal does not prove cellular adequacy [that the specimen contains cervical cells] and does not prove that there are sufficient cells for quality reflex cytology. This still can lead to a false negative result, as an insufficient sample cannot be excluded". Though true in regard to adequacy of cells for cytological triage, with the advent of molecular testing such as methylation markers this may be a cause for concern. The ATHENA trials invalid rate of

0.21% to 0.61% for HPV testing and subsequent no invalid HPV tests for CIN 2+ specimens may mean a cellular control was not needed but in a future technology that may test million upon millions of cervical samples a small percentage can lead to larger real world number women developing an cervical lesion between screening and in screening that may recommend extended intervals, this is an important consideration. However, as shown in here the sensitivity of both tests even in the absence of an internal control are comparable for detection of CIN 2+ and CIN 3+ and that the Aptima test is not missing disease cases. Interestingly there was a difference in positivity between HPV DNA and HPV mRNA in women with unsatisfactory cytology (Figure 3.4). Whether this was due to a true decrease in mRNA positivity or a decrease in the adequate cellular material is unknown. This may however be an important consideration to make in future work.

Conclusions

As has been noted in nearly all papers concerning HPV primary screening, is the issue around increased rates of positive HPV tests compared to abnormal cytology due to the lower specificity. In our cohort, ~15% of women would require colposcopy assessment due to the HPV positive result without a triage test in place. Triaging these HPV positive women is a necessary and recognised step in any HPV primary screening programme and it is the aim of the further chapters to assess and investigate alternative strategies for HPV triage beyond just cytological triage which still contains many of its limitations even in a triage role and leaves a population of HPV positive cytology NAD women in a holding pattern within the screening system. Ideally, a triage test should allow for a single screening event and minimise the need for subsequent follow-up of women.

It is a well-established that no screening test will achieve 100% in its clinical performance. Where a 'gold standard' test may approach that mark its methods are either too invasive via a biopsy, too unpleasant for the general public which directly impacts attendance. A screening test on the other hand must balance these lines to being generally acceptable test as well as having adequate sensitivities and specificities with a good NPV between screening intervals.

Findings from this chapter show that regardless of the HPV test both have good sensitivities and NPV's. The biggest challenge is the high burden of HPV infections in the general public will lead to a higher burden on colposcopy clinics and it has been long known that the introduction of a HPV primary screening practice, regardless of the platform used for testing, will require adequate triage of those HPV positive cases to classify those women who are

at a true risk of cervical cancer/pre-cancer from those with a transient or regressive infection. This will be discussed and investigated in further chapters.

In summary, the Cobas HPV DNA test and Aptima HPV mRNA test both maintain a high level of clinical performance that is concurrent with previously reported levels from both the Cochrane meta-analysis and previous literature. Though there is a level of disagreement between the platforms both show strong agreement in the detection of both CIN2 + and CIN 3+ lesions. Both assays have been shown here and in many previous papers to perform well in a HPV primary screening population. In this study, the Aptima platform shows a general trend of higher specificity and PPV values as well as higher rates of positivity in clinically relevant lesions. It also seems the Aptima test has a lower positivity level in non-relevant NAD cytology findings that will be clarified in the longitudinal aspect of this study. This, if true, over the long term could reduce the unnecessary colposcopy follow up or extend screening intervals for women with an initial negative HPV test which would extend to a cost savings from both extended intervals and in reducing the number of triage tests required due to the lower positivity rate. Regardless of the platform both require follow on triage to reduce unnecessary follow up of HPV positive test results which the following chapters will address.

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-Chapter 4-

Classification of Cervical Precancer Based on the Semi Quantitative Analysis of DNA Methylation

4.1. Introduction

HPV based primary screening is due to be implemented in Ireland in 2020. As evidenced from several RCTs, HPV based primary screening is more sensitive than cytology, but lacks specificity. The key challenge is to balance sensitivity and specificity to avoid over treatment and over referral to colposcopy. Therefore, appropriate triage tools are required. Currently cytology is the preferential triage test with extensive longitudinal evidence supporting its use (Naucler *et al.*, 2007; H. Kitchener *et al.*, 2009; Dorien C. Rijkaart *et al.*, 2012). However, cytology still suffers from its limitations such as a lower specificity as well as variability between laboratories. A high proportion of HPV positive women will have a normal cytology which may require further surveillance as these women have a risk of disease too high to allow routine screening. This along with the investment of skilled cytologists, quality assurance protocols, as well as the requirement to maintain a cytology service in a period when cytologist recruitment and retention may be a critical factor makes cytology an uncertain long term strategy for triage of HPV positive women (Cuschieri et al. 2018). This chapter will focus on an alternative triage option, namely the use of host specific methylation markers [CAD M1-M18, MAL M1 and hsa-mir-124-2] for the triage of HPV positive women in a HPV primary screening programme. This will be the first instance of these markers in used in a HPV primary screening population to date.

The field of methylation and specifically HPV driven methylation is an area of active research. Methylation is a normal control mechanism in the body. During the process of methylation, facilitated by the DNMT enzymes, a methyl group can be added to the DNA molecule at either a cytosine or adenine residue. Cytosine methylation is the most common form of methylation in mammalian cells and when located on a gene promoter's CpG island, rich in cytosine residues, it acts in a repressive manner silencing gene transcription. The process of DNA methylation is vital for normal development and gene regulation (Rose and Klose, 2014). In HPV infections however, this process is hijacked by the two HPV oncogenic proteins, E6 and E7. Under normal cell cycle conditions, p53 inhibits the SP1 protein from transcriptionally activating the DNMT gene and Rb inhibits transcription factor E2F. When a HPV infection occurs, initially, there is no major disruption of this balance. However, over the course of a prolonged infection or if E6 and E7 are released from their transcriptional control though the loss of E2 via viral methylation or integration events. The levels of E6 and E7 increase in the host cell and can lead to an increased degradation of p53 via E6 as well as loss of the inhibitory effect of the Rb protein binding E2F through E7. This results in the over expression of DNMT allowing an increased level of methylation to occur in the host

cell, increasing the oncogenic potential of the cell (Burgers *et al.*, 2007; Lin and Wang, 2014; Castillo-Aguilera *et al.*, 2017). This has led to the understanding that HPV infections can lead to an altered methylation genome in the cell from which specific allelic targets could be used to classify women at risk of cervical cancer (Chen *et al.*, 2014; Wang *et al.*, 2016; Verlaat *et al.*, 2017).

There are two approaches to methylation testing for cervical cancer or pre-cancer, host DNA methylation and/or HPV episomal methylation. HPV specific methylation has been looked at in a few studies in the L1, L2 and E2 genes of different HPV types and has shown a strong association with cervical carcinoma (Brentnall *et al.*, 2015; Lorincz, 2016; Lorincz *et al.*, 2016). Though potentially useful, with the advent of HPV vaccination, how these viral methylation markers will fare long term remains to be seen. Viral transformation may also be an issue over time, with slight episomal alterations resulting in potential false negative results compared to a generally more stable eukaryotic DNA. A multitude of studies have shown that host hypermethylation occurs in cervical pre-cancer, increasing with the increase of disease severity (Wilting *et al.*, 2010; Hesselink *et al.*, 2011; Lin and Wang, 2014; Lorincz, 2014, 2016; Steenbergen *et al.*, 2014; Schmitz *et al.*, 2017; Cook *et al.*, 2018; Cuschieri *et al.*, 2018; Sen, Ganguly and Ganguly, 2018; Verlaat *et al.*, 2018). Potential methylation markers such as C13ORF18, JAM3, ANKRD18CP, FAM19A4, hsa-mir-124-2, EPB41L3, CAD M1-M18, MAL M1, PAX 1, PCDHA4, PCDHA13, DLX1, ITGA4, RXFP3, SOX17, ZNF671, JAM 3, and TERT and several others have been proposed as potential triage markers for HPV positive women. Of all these potential methylation markers at the time, CAD M1-M18, MAL M1 and hsa-mir-124-2 have the most evidence for their effectiveness at detecting CIN 2+. While there is substantial evidence for the effectiveness of these markers in triage or self-sampling population there is, however, no data in the HPV primary screening population for these methylation markers. For these reasons, they are ideal candidates to bring forward to test in this HPV primary screening population and provide new data in a much larger study cohort that will match a ”real world” HPV positive triage population.

Different combinations of methylation biomarkers have shown promise in a triage setting, for example, the combination of MAL M1 and hsa-mir-124-2 showed a sensitivity for detection of CIN 3+ which ranged from 77.0 to 87.8% and from 64.9 to 71.6%, (Hesselink *et al.*, 2014). While other studies have shown that the combined CAD M1 M18 and MAL M1 biomarker panel has a sensitivity and specificity of 86.8% and 64.8% for detecting CIN 3+ compared to 65.8% and 78.6% for cytology triage (De Strooper, Hesselink, *et al.*, 2014). The application of methylation biomarkers has also been explored in self-samples taken from a cohort of 355 hrHPV positive specimens from women in routine screening in the Netherlands. At a set clinical specificity of 70%, the combination of MAL M1 and hsa-mir-124-2 showed a sensitivity of between 64.9% to 71.6% for detection of CIN 3+ (Hesselink *et al.*, 2014). In the PROHTECT-3 self-sampling study, methylation patterns for MAL M1 and hsa-mir-124-2 were examined as a triage tool and compared to cytology. 509 women were enrolled into the cytology triage arm with 515 being tested for both methylation markers. Methylation testing has been used in a hrHPV DNA positive group of HIV positive women. The specificity, sensitivity of the combined tri-marker test (CAD M1-M18, MAL M1 and hsa-mir-124-2) for CIN2+ was comparable with cytology [ASC-US+] (De Vuyst *et al.*, 2015). Overall, the study concluded that the use of these two methylation markers was non-inferior to cytology but had a significantly reduced time to CIN 2+ diagnosis, 94 days versus 158 days (p=0·00084) (Verhoef, Bosgraaf, *et al.*, 2014). Table 4.1 shows a systematic review conducted by Lorincz *et al* 2016 outlining the sensitivity and specificity of CAD M1, MAL M1 and hsa-mir-124-2.

Table 4.1: A review of the performance of CAD M1, MAL M1 and hsa-mir-124-2 for the detection of CIN 2+

Genes*	Study Design	Test	Sensitivity %	Specificity %	PPV %	AUC	Endpoint
FAM19A4 MIR124	Screening	qMSP	69	76	54	NA	CIN 3+ (self-collected samples
CADM1 MAL MIR124	Screening	qMSP	89	50	52	0.8	CIN 2+ (HIV positive women)
CADM1 MAL	Screening	qMSP	62	78	49		CIN 2+
			68	76	35		CIN 3+
MAL MIR124	Screening	qMSP	72	49	29	NA	CIN 2+
			73	47	19		CIN 3+ (self-collected samples)
MAL MIR124	Screening	qMSP	82	60	NA	0.76	CIN 3+ (self-collected samples)
CADM1 MAL MIR124	Screening and gynae. clinic	qMSP	48	81	NA	NA	CIN 2 and CIN 3
CADM1 MAL	Screening	qMSP	84	52	25	0.72	CIN 2+

Table adapted from (Lorincz, 2016). Colposcopy studies redacted.
**Combination tests; any single marker classified as a positive test unless specified otherwise*
qMSP; Methylation Specific PCR

What these and many other studies have shown clearly is the need for a panel of markers as no single marker is adequately sensitive and specific enough currently. The apparent nature of transforming HPV infections seems to indicate a semi-consistent methylation profile with some gene promoters often being methylated in cervical pre-cancers and cancer but not in all. For this reason, a panel of markers, for which individually they may be insufficient, together, provide a higher sensitivity, specificity, PPV and NPV for cervical cancers and pre-cancers. Knowing this, many studies now select a multitude of markers

usually with the criteria that a single positive marker denotes a high enough risk level to warrant colposcopy review.

The ability to test for epigenetic changes is possible through the development of a method for sodium bisulfite modification of DNA. This allows the conversion of unmethylated cytosine to uracil which is subsequently amplified as thymine during PCR whilst methylated cytosine is unaffected (Susan *et al.*, 1994). The majority of DNA methylation analysis is based around this methodology. The two most commonly seen techniques for methylation testing in a triage capacity are Pyrosequencing and qMSP, both considered a relatively sensitive, quantitative approach to methylation testing Figure 4.1. qMSP is considered a rapid and very sensitive method to detect specific methylated candidate genes with the capability of functioning well on DNA samples of both limited quantity and quality. Pyrosequencing (PSQ) however allows for the easier quantification of the methylated gene in question. Consensus on the best approach and techniques is still a matter of debate, though each has its place in methylation analysis.

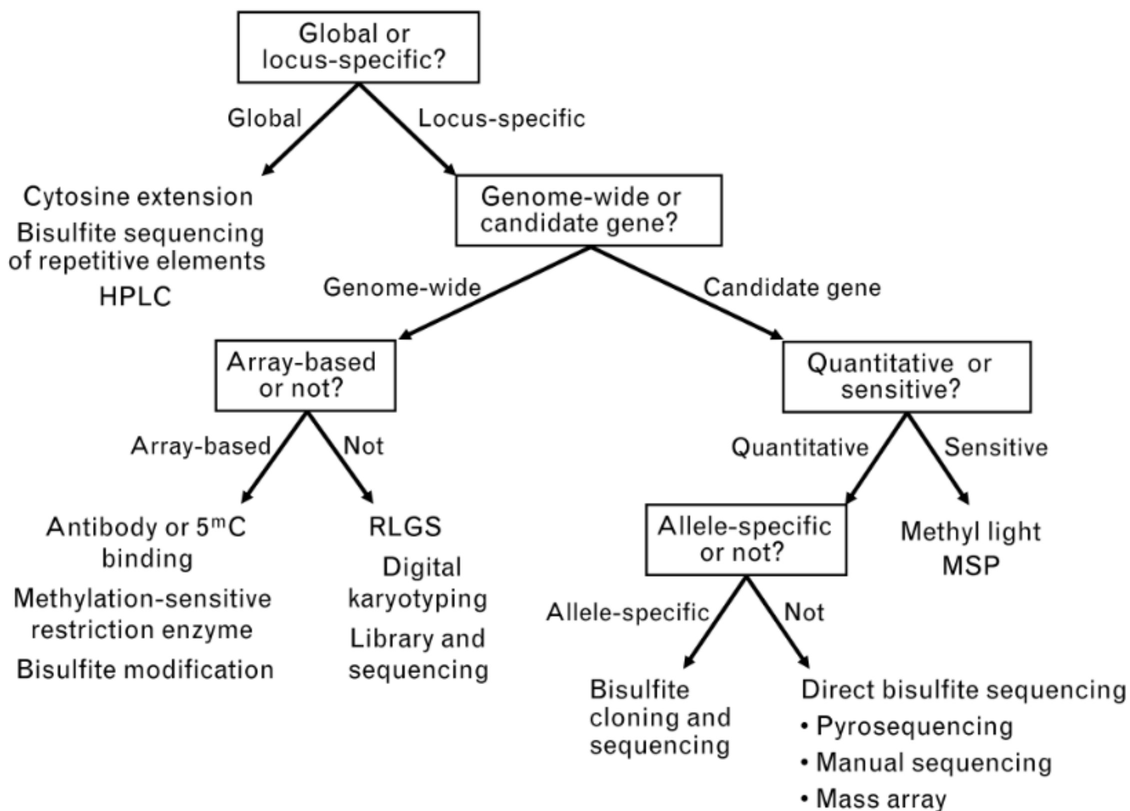


Figure 4.1: Decision tree for the selection of an appropriate DNA methylation analysis method (Shen and Waterland, 2007)

The use of methylation testing has not yet been perfected for clinical testing and there is a lack of an agreed, standardised approach for testing and analysis. For any methylation marker across different studies, there is a broad range of reported clinical performances. This is due in part to the methodology in defining positivity in these cohorts. There are several common approaches to this. Firstly to define a positive methylation result some researchers utilise the ΔΔct methodology or derivations of it from their qMSP data to compute rational values (Hesselink *et al.*, 2011, 2014; Verhoef, Bosgraaf, *et al.*, 2014; Verhoef, Heideman, *et al.*, 2014). One such derivation is 2^[ct(B-actin)-ct(Target)]x100 shown by Hesselink *et al.,* 2011 and in other later papers from the Netherlands. This can be a powerful approach for qMSP as it allows individualised values relating to each specific sample and not reliant on standard curves. This is of benefit due to the degree of variability in bisulfite conversion which makes the standard curve approach an unsatisfactory method. Others may use logistic regression analysis on pyrosequencing data or qMSP methylation data to determine rational values (Brentnall *et al.*, 2015; Lorincz *et al.*, 2016; Cook *et al.*, 2018). One such approach utilised by Lorincz *et al.,* 2016 was the S5 classifier determined through logistic regression and PSQ which showed promise in combining methylation markers (weighted scoring method). In this thesis a novel summation score will be utilised from the data to determine a single methylation value for which a single cut-off point can be calculated. Each methylation marker in this novel scoring method will be unweighted compared to the S5 score which was weighted. This decision was made due to the inherent variability in host methylation and CIN and weighting one host methylation marker can inhibit the other two in the case where the positively weighted marker is negative. The weighted markers individual sensitivity or specificity can also heavily impact the summation score. In regard to cut off points for methylation Receiver Operating Characteristic (ROC) curves, regression analysis or setting defined specificity and/or sensitivity thresholds have been used to determine what constitutes a positive methylation test in the past. Between this and the variability in target sequences there is an inherent amount of variability in studies in addition to different sample cohorts. Most data highlights the potential of methylation markers for detection of cervical precancer and cancers. Others may use their own classification formulas and methods such as the S5 classifier score (Cook et al., 2018).

A core issue throughout the current literature is the variability in the clinical performances of methylation markers. This is not due to the inadequacy of methylation testing but rather inter-laboratory variation, lack of standardised procedures, kits, biological variance, researcher variance and methodology for setting thresholds. These are all, fortunately, short term issues that will eventually be addressed through technological advancements in the area such as automation of the qMSP or PSQ process and as validated kits come to market.

This thesis will further expand the knowledge base of these three methylation markers as well as using novel, semi quantitative, scoring methods to optimise the value of the methylation markers for the triage of HPV positive women in a HPV primary screening population which has not been shown previously. We hypothesis that the combination of these three markers will broadly cover the abnormal methylation profile of women with a transforming HPV infection at high risk of CIN 2+. Currently there is no Irish data on the use of methylation markers within a real-world population-based screening programme. To this end this chapter will address a potential approach for the standardisation of methylation analysis as well as standardising the three selected methylation markers with clinically relevant cut off points for the determination of CIN 2+.

4.2. Aims

The aims of this chapter are:

i. to establish a novel assessment method for the methylation status of Thinprep specimens for a panel of triage markers; CAD M1, MAL M1 and hsa-mir-124-2
ii. to validate the methylation panel for detection of CIN 2+
iii. to establish a clinically relevant cut-off for the biomarker panel for detection of CIN 2+.

4.3. Materials and Methods

4.3.1. Study Population

CERVIVA, in partnership with CervicalCheck, are currently undertaking a HPV primary screening study, which is evaluating and comparing different strategies for the triage of women with a HPV positive primary screening test as described in Chapter 2, Section 2.1.2 (Figure 2.1). For establishing the methylation biomarker assays and associated cut offs, a validation panel was formed using the ThinPrep sample from the overall study population with confirmed histology follow-up. The panel comprised of 50 CIN 1, 34 CIN 2 and 50 CIN 3 ThinPrep samples as well as 50 Negative ThinPrep samples; all HPV DNA and mRNA negative with two previous NAD cytology results prior to entry into the study. The CIN 1 to CIN 3 samples were chosen sequentially from the initial pool of follow up data received from CervicalCheck while the cytology NAD and HPV negative samples were selected through random number generation. For clarity the validation panel comprises of the residual ThinPrep sample obtained at enrolment with the associated cytological outcomes and not the subsequent histological tissues.

4.3.2. Cell Culture

SiHa cells (ATCC: HTB-35, reported to contain 1-2 copies of integrated HPV 16 per cell) (SiHa (ATCC® HTB-35TM), 2018) were used to perform the initial test validation and optimisation. SiHa cell pellets were cultured and stored following the methods outlined in Chapter 2, Section2.3.1. Prior to use the SiHa pellet was allowed to come to room temperature.

4.3.3. DNA Extraction

DNA extraction was performed using the Qiagen DNeasy blood and tissue kit in accordance with manufacturer's protocol on: DNA Purification from Blood or Body Fluids (Spin Protocol) (Qiagen, 2016). The extractions were carried out on 2ml's of PBS washed ThinPrep sample and all reagents were reconstituted as required and outlined in the protocol and as described in Chapter 2, Section 2.3.2 and 2.3.3. DNA was eluted in 50µl of AE buffer to maximise the final concentration. DNA concentrations were determined using the nanodrop 2000c (Thermo Fisher Scientific, 2009). To aid in the bisulfite conversion method, samples found to exceed 500ng/µl of DNA was diluted to approximately 300-400ng/µl with AE Buffer.

This was to ensure the volume of DNA used for the conversion was not less than 1µl and allow more accurate pipetting and more accurate conversion and later qMSP results.

4.3.4. Bisulfite Conversion

The bisulfite conversion of DNA was performed using the Zymo EZ DNA Methylation-Gold™ Kit (D5006) in accordance with manufacturer protocol (Zymo Research, 2017). All reagents were reconstituted as required as outlined in Chapter 2, Section 2.3.4. As per the manufacturer's recommendations, sample DNA concentrations can range from 500pg to 2µg with an "ideal" range being between 200-500ng of DNA. To determine the optimal amount of input DNA a range of 300ng – 600ng of SiHa DNA was converted and tested by qMSP. Bisulfite conversions were performed on the GeneAmp PCR System 9600, Perkin Elmer thermocycler at 98ºC for 10 minutes, 64ºC for 2.5 hours and an optional 4ºC storage for up to 20 hours. This was followed by several wash steps with a final elution volume of 10µl of bisulfite converted DNA. With each bisulfite conversion batch of samples, the following controls were used: SiHa positive control, a pooled known HPV and methylation negative control and a SiHa non-converted control.

4.3.5. Methylation Specific PCR (qMSP)

Real time singleplex qMSP was performed for detection of DNA methylation. Primer and probe sequences and PCR conditions for CAD M1-M18, MAL M1 and hsa-miR124-2 are listed in Table 4.2 from pre-published papers (Wilting *et al.*, 2010; Overmeer *et al.*, 2011).

A methylation specific primer and probe for β-Actin (ACTB) was employed as an endogenous control (Overmeer *et al.*, 2011). A non-methylation specific β-Actin (NcACTB) (TaqMan™ DNA Template Reagents, Life-Technologies) was employed as a methylation control to show successful bisulfite conversion had occurred. All primers and probes were labelled with 5' FAM and 3' TAMRA. Each qMSP was run in duplicate and included controls for each target (SiHa DNA (positive control), pooled negative ThinPrep samples (negative control) and unconverted SiHa DNA (bisulfite conversion control)). The master mix components are outlined in Chapter 2, Section 2.3.5 and 2.3.6.

Thermocycling was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, 2010) under the following cycle conditions; 95°C for 15 minute followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

Table 4.2: Methylation Primer and Probe Sequence					
Target Gene	Forward Primer 5' to 3'	Reverse Primer 5' to 3'	Probe	Amplicon Size	Annealing Temperature
CAD M1-M18	ATTTTATTAGTTGTTGGTTCGGGT	CTCGACAACACTACTCGCC	ACCTACCTCAAACTAACGACGTTAACTACCTCCGA	90	60 °C
MAL M1	GCGTAGTATTAAGTAGAGAGGTTCG	AATAAAAAATAAAACCGACCGC	ACTAAACCGACGCTAATTCGACGACGCT	107	60 °C
hsa-miRNA-124-2	GGGTAATTAATTTGGATTTACGTCGTTAT	CGTAAAAATATAAACGATACGTATACCTACGT	TTTACAACACACGCCTAAA	138	60 °C
ß-Actin (IC)	TGGTGATGGAGGAGGTTTAGTAAGT	AACCAATAAAACCTACTCCTCCCTTAA	ACCACCACCCAACACACAATAACAAACACA	133	60 °C
(Wilting et al., 2010; Overmeer et al., 2011)					

4.3.5.1. Determination of the Methylation Status in Women with no Cervical Abnormalities.

To demonstrate that methylation biomarkers panel are not elevated in women with no cervical abnormalities, a pool of 20 HPV Negative, Cytology Normal (2 previous screening rounds) were tested for all three methylation markers. This panel formed the pooled negative control.

4.3.5.2. Determination of the Limits of Detection for the qMSP with CAD-M1 M18, MAL-M1 and hsa-mir-124-2

To determine the limits of detection (LOD) of the methylation marker panel two approaches were used.

1. To determine the minimum number of methylation positive cells that could be detected by qMSP, in a background of normal cells. Known quantities of SiHa cells were spiked into the pooled negative control sample. Cell counts of $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, $1x10^1$, $1x10^0$ cells were resuspended in 1500µl of the pooled negative control sample and mixed by vortexing. Cells were centrifuged, the supernatant removed and resuspended in 200µls of PBS as per the DNA extraction protocol prior to extraction, bisulfite conversion and qMSP.

2. Serially diluted SiHa DNA post bisulfite conversion was used to determine the LOD of the qMSP to estimate how much methylated DNA is required post bisulfite conversion for a positive qMSP result. 500ng of SiHa DNA underwent bisulfite conversion. After bisulfite conversion the 500ng stocks were serially diluted from 500ng to 0.5pg and tested via qMSP.

4.3.6. Statistical analysis

The disease endpoint for this observational cohort study is detection of CIN 2+ or greater. Following qMSP for each sample, a Methylation Score was calculated for each of the methylation markers using the formula; $2^{(Ct\ of\ \beta-actin)-Ct\ (Target\ gene)}\ x\ 100$ (Hesselink *et al.*, 2011). From each individual Methylation Score a Total Methylation Score was also derived. This was achieved by taking the value from the $2^{(Ct\ of\ \beta-actin)-Ct\ (Target\ gene)}\ x\ 100$ equation for each marker and summing the values together; $CAD\ M1\ M18\ +\ MAL\ M1\ +\ hsa\ mir\ 124\ 2\ =\ Total\ Methylation\ Score.$ No weighting was applied to any individual marker. The Paired Methylation Score was also calculated using only two methylation markers. Table 4.3 shows a description of each marker combination used in this Chapter.

Table 4.3: Description of nomenclature for the different Methylation Scores

Methylation Score Grouping	**Description**
Methylation Score and Individual Methylation Markers	A Methylation Score for each individual marker is derived from: 2^[ct(B-actin)-ct(Target)]x100. When each marker is assessed as an individual value the criteria for a positive methylation test is any one individual methylation marker positive result.
Total Methylation Score	A Total Methylation Score was calculated by taking the value derived from the 2^[ct(B-actin)-ct(Target)]x100 equation for each marker and summing the values together: CAD M1-M18 + MAL M1 + hsa-mir-124-2 = Total Methylation Score.
Paired Methylation Score **Nomenclature: Paired Methylation Score (xxx : xxx)**	A Paired Methylation Score was also derived by taking the value derived from the 2^[ct(B-actin)-ct(Target)]x100 equation for two markers and summing the values together: CAD M1-M18 + MAL M1= Paired Methylation Score (CAD:MAL) or MAL M1 + hsa-mir-124-2 = Paired Methylation Score (MAL :miR)

The Methylation Scores were assessed across the different histology grades using the Kruskal Wallis test on the IBM SPSS Statistics 25 package. The Wilcoxon signed rank test was performed on the IBM SPSS Statistics 25 package to determine the statistical differences between the individual methylation markers, Total Methylation Score and Paired Methylation Score with or without stratification by histology.

ROC analysis was performed on both the MedCalc Version 18 statistical platform and IBM SPSS Statistics 25 software. ROC analysis is a performance measurement between two dichotomous groups. It is an indicator of how much a test is capable of distinguishing itself from a baseline or control group. ROC analysis can allow for the selection of optimal cut offs associated to the sensitivity or specificity of the control and test groups. For determination of the optimal cut-off point for detection of CIN 2+, the Youden Index J statistical output was used. The Youden Index J is a single statistic that captures the performance of a dichotomous diagnostic test. The index is defined for all points on a ROC curve, and the maximum value of the index may be used as the criterion for selecting the optimum cut-off point. Figure 4.2 is a graphical representation of the AUC and potential Youden index J values. The Likelihood Ratio (LR) was also computed from ROC analysis. A positive LR (>1.0) informs an increased probability of disease given a positive test result

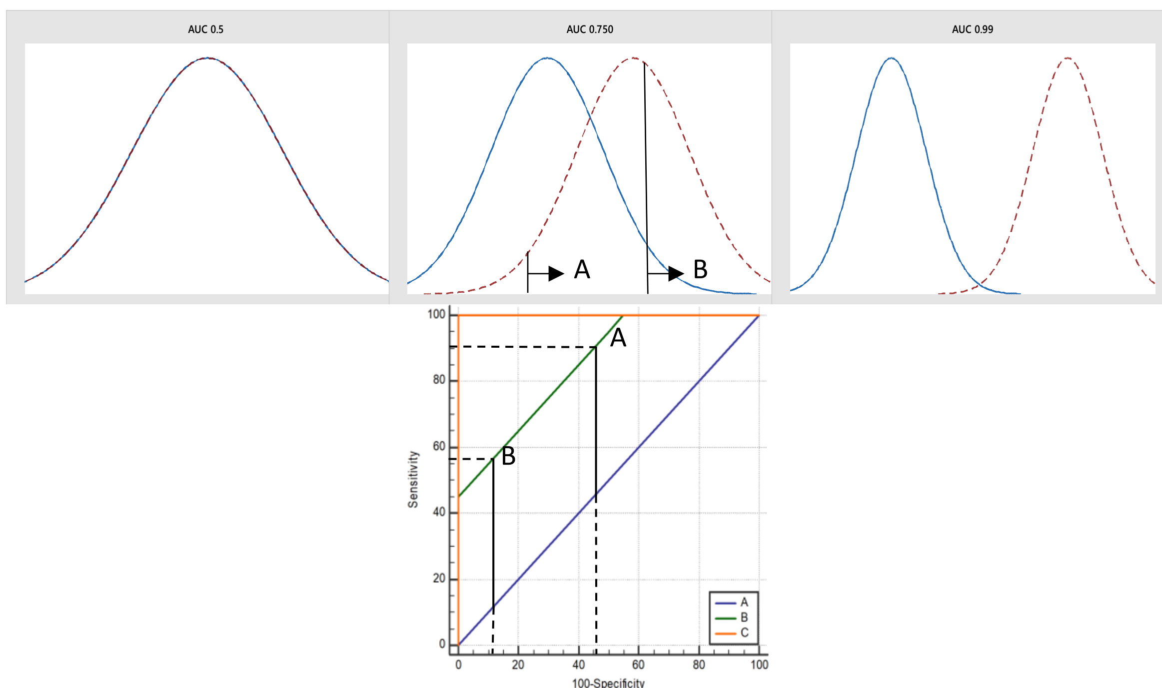


Figure 4.2: ROC curves and the Area under the Curve
Hypothetical example of the AUC and the separation of the Negative Group (blue) from the Positive Group (blue). Left to Right; an AUC of 0.5 with no separation of the two groups (identical) AUC of 0.750 with approximately 50% separation of the two groups, AUC 0.99+ near perfect separation of the two groups. Line A and B are examples of potential areas to set a cut-off point. A is optimised for Sensitivity with the loss of Specificity compared to B which is optimised for Specificity with the loss of Sensitivity.

and a negative LR informs the decreased probability of disease given a negative test (<1.0) (McGee, 2002). Pairwise comparisons of each individual ROC curve were also assessed using the DeLong et al. (1988) methodology. This is a calculation for the standard error of the AUC and calculating the difference between two AUCs with $p<0.05$ indicating a statistical difference between the two AUC's. The clinical performance for the individual and combination of methylation markers was assessed by calculating sensitivity, specificity, PPV and NPV for detection of CIN 2+. A heat map of the positive and negative results from the proposed cut off points was created using the online heat mapper tool; http://www.heatmapper.ca/

4.4. Results

4.4.1. Optimisation of the Methylation Panel

4.4.1.1. Optimal DNA concentration for Bisulfite Conversion

An optimal DNA input concentration between 200-500ng's of DNA is recommended for bisulfite conversion using the Zymo EZ DNA Methylation-Gold™ Kit (D5006). To validate this, a range of input DNA concentrations was assessed between 300ngs to 600ngs (Figure 4.3), to establish the optimum concentration of DNA to add to the bisulfite conversion assay to yield maximum concentration of converted DNA. All methylation biomarkers were detected using all four concentrations of input DNA. A DNA concentration of 500ng for all subsequent bisulfite conversions was chosen firstly, to ensure there was adequate converted DNA for three biomarkers to be amplified in duplicate. Secondly, to allow more consistency between subsequent qMSP's, by using 1µl of converted DNA instead of 0.83µl if the input concentration was 600ng.

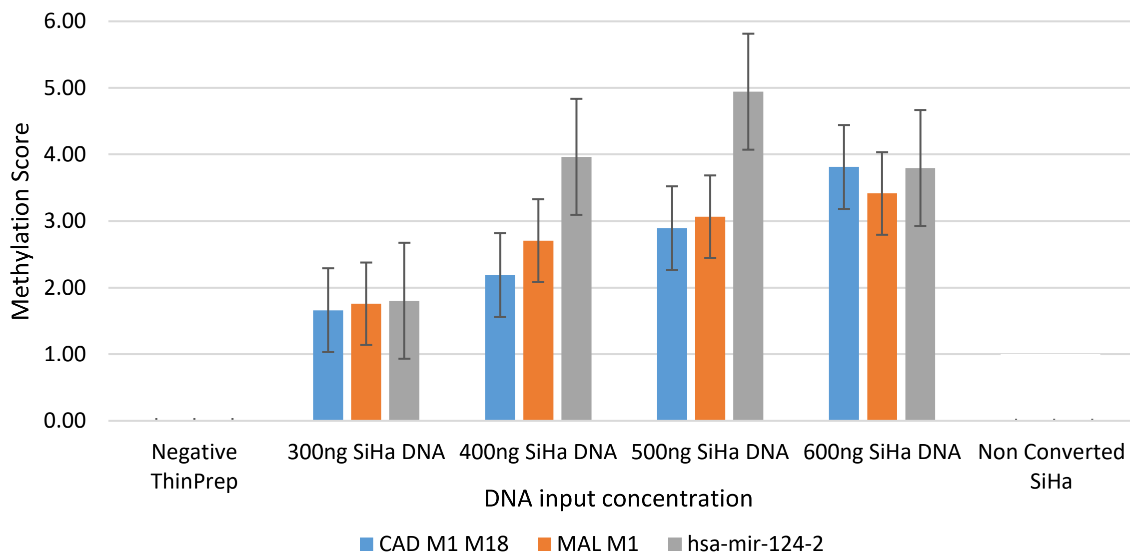


Figure 4.3: Optimal DNA input concentration for bisulfite conversion

4.4.1.2. Determination of the Methylation Status in Women with no Cervical Abnormalities.

To demonstrate that HPV negative women exhibit negligible methylation levels, a group of 15 women from the study population were selected with a negative HPV test and no abnormality on cytology. Figure 4.4 shows the methylation score for each of these women labelled 1 to 15. CAD M1 methylation is not detected in any of the 15 samples, MAL M1 methylation was detected in 5 of the samples and hsa-mir-124-2 methylation was detected in 14 of the samples. However, the methylation levels for these markers are low. In comparison values for the SiHa control were 2.19, 2.73 and 3.43 respectively.

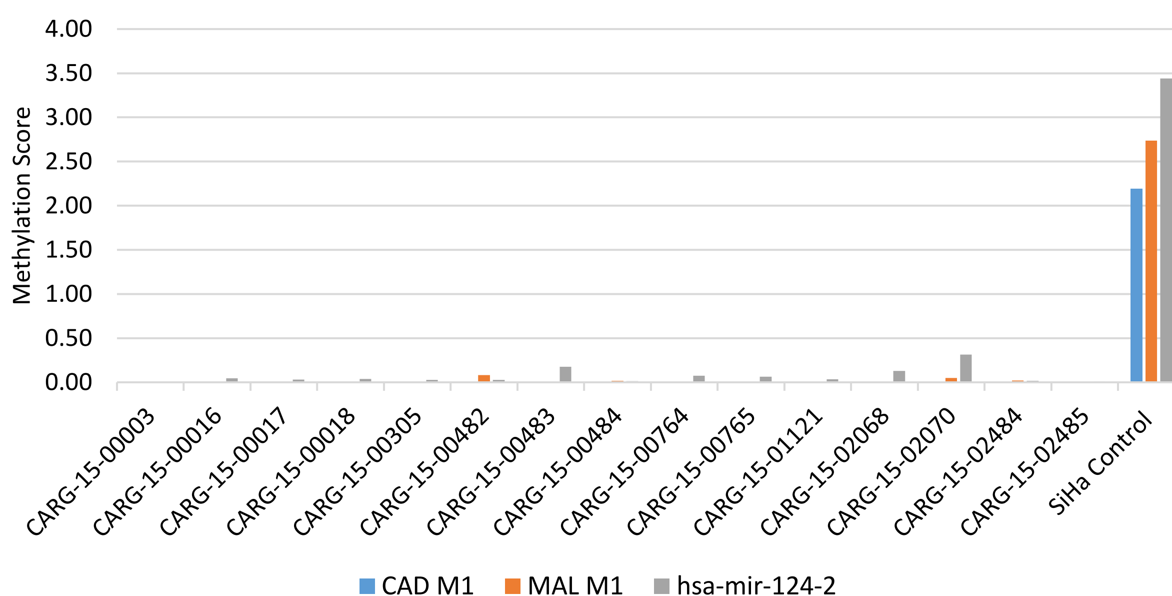
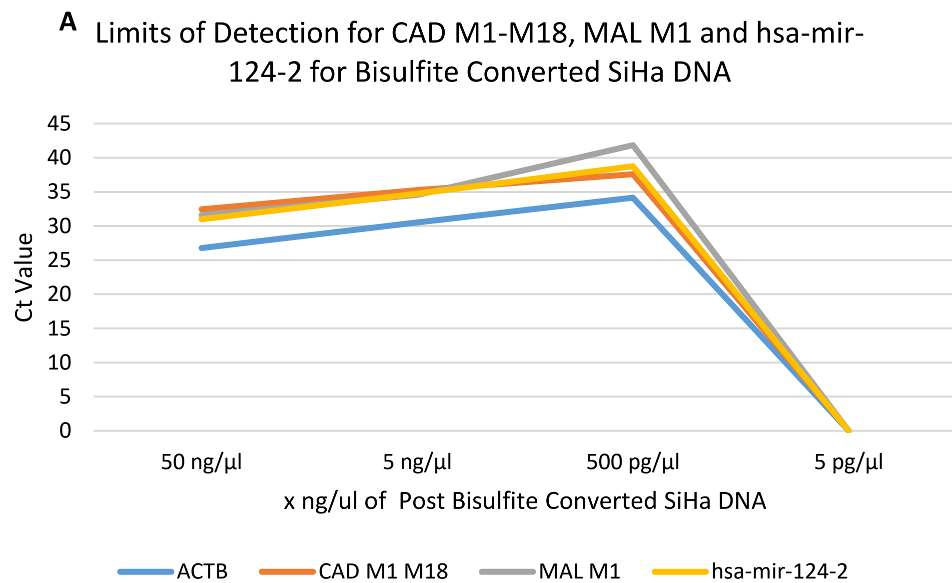


Figure 4.4: Methylation status of HPV negative women with no cytological abnormalities

4.4.1.3. Determination of the Limits of Detection for the qMSP with CAD-M1, MAL-M1 and hsa-mir-124-2

Bisulfite converted DNA was serially diluted to determine the limit of detection for the qMSP. SiHa DNA was serially diluted post bisulfite conversion from 500ng/µl to 0.5pg/µl. Figure 4.5 [A] shows the results of the qMSP. All three methylation markers and the internal control could be detected as low as 500pg/µl. To examine the limit of detection in clinical samples, SiHa cells were spiked in to the pooled HPV negative cytology normal, methylation marker negative control to simulate the pre-cancerous cells present in a cervical smear. Figure 4.5 [B] shows the results of the qMSP represented by the Ct-value in this instance. Both CAD M1-M18 and hsa-mir-124-2 had detectable products up to $1x10^1$ spiked SiHa cells. For MAL M1 there was detectable product up to $1x10^2$ spiked SiHa cells.



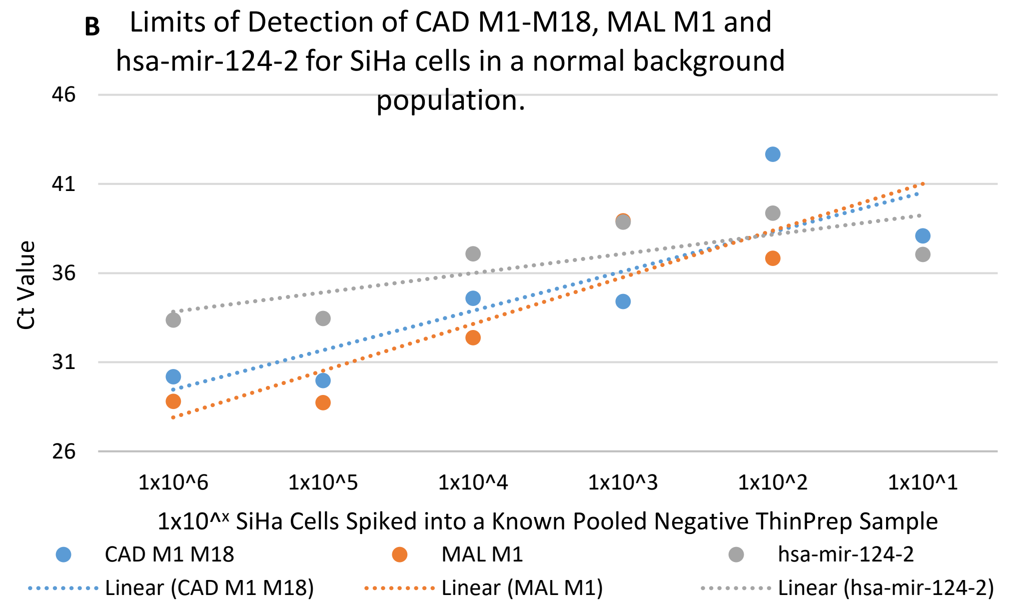


Figure 4.5: Limits of detection for the methylation panel. [A] the LOD for detecting methylation positive, bisulfite converted DNA. [B] the LOD using known concentrations of SiHa cells spiked into a background of normal cells

4.4.2. A Validation panel for the use of determining appropriate Cut Offs for CIN 2+

4.4.2.1. Validation Panel Composition

The validation panel comprised of cervical smear samples taken from women during their routine primary screening test for whom histology follow-up was available. The samples were classified based on their histology results. Table 4.4 shows the distribution of the cytology grade in relation to follow up histology result.

Table 4.4: The cytology grade in relation to histology for the Methylation Validation Panel

	n	NAD	ASC-US	LSIL	HSIL*
Negative**	50	100.00% (n=50/50)	0.00% (n=0/50)	0.00% (n=0/50)	0.00% (n=0/50)
CIN 1	50	0.00% (n=0/50)	16.00% (n=8/50)	84.00% (n=42/50)	0.00% (n=0/50)
CIN 2	34	0.00% (n=0/34)	17.65% (n=6/34)	64.71% (n=22/34)	17.65% (n=6/34)
CIN 3	50	0.00% (n=0/50)	2.00% (n=1/50)	32.00% (n=16/50)	66.00% (n=33/50)

HSIL includes ?SCC (n=1), AGC (n=1) and ASC-H (n=2). One ASC-H finding related a CIN 2 on histology. The remaining related to a CIN 3 finding on histology.*

*** ThinPrep samples, which were HPV DNA and mRNA negative that have had two previous NAD cytology results.*

4.4.2.2. The Methylation Profile of the Validation Panel

For each methylation marker the average methylation scores across each histological grade is shown in Figure 4.6. Generally, the average methylation score for each of the three independent markers increases with increasing grade of disease. There is a significant difference between each disease grade and CAD M1-M18, for example between CIN 1 and CIN 2 (p=0.003), for all other combination the significance range was p=<0.05 - <0.001. For MAL M1 and hsa-mir-124-2 there was a significance range was p=<0.05 - <0.001 for all combinations with the exception of Negative and CIN 1 in which there was no significant difference between these two grades for MAL M1 and hsa-mir-124-2 (p=0.111 and p=0.063 respectively).

Each methylation marker was also assessed against each other to determine if there was a statistical difference across different grades of disease. For example, there was a statistical difference between CAD M1-M18 and MAL M1 in the Negative category (p=0.008) but not in CIN 1, CIN 2 or CIN 3 (p=0.614, 0.412, 0.960). There was a statistical difference between CAD M1-M18 and hsa-mir-124-2 as we as between MAL M1 and hsa-mir-124-2 across each histological grade (p<0.05).

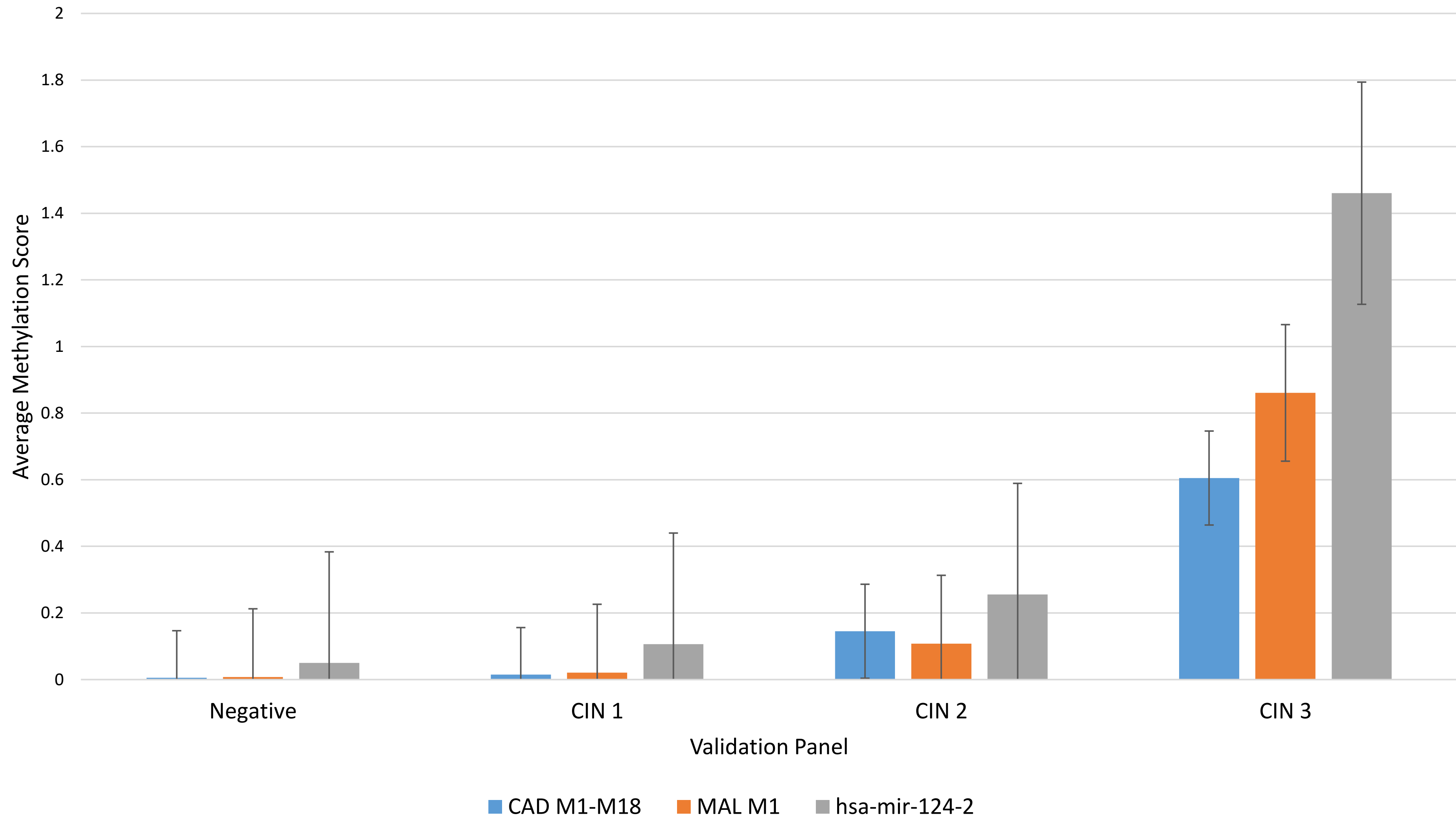


Figure 4.6: The average methylation scores of the validation panel stratified by histology. Negative (n=50), CIN 1(n=50), CIN 2(n=34), CIN 3 (n=50)

4.4.2.3. The Total Methylation Score of the Validation Panel

For each sample the three individual methylation markers scores were summed together to calculate the Total Methylation Score as described in Table 4.3and Table 2.3. A boxplot of the Total Methylation Score for each histological grade is shown in Figure 4.7. There is a statistically significant difference between each disease grade and the Total Methylation Score (significance range p=<0.05 - <0.001) with the Total Methylation Score increasing with disease severity. When the individual methylation markers (CAD M1-M18, MAL M1 and hsa-mir-124-2) and the Total Methylation Score are compared against each other across the different histology grades, the Total Methylation Score had a significantly higher methylation score compared to the individual markers ($p<0.001$ for all combinations).

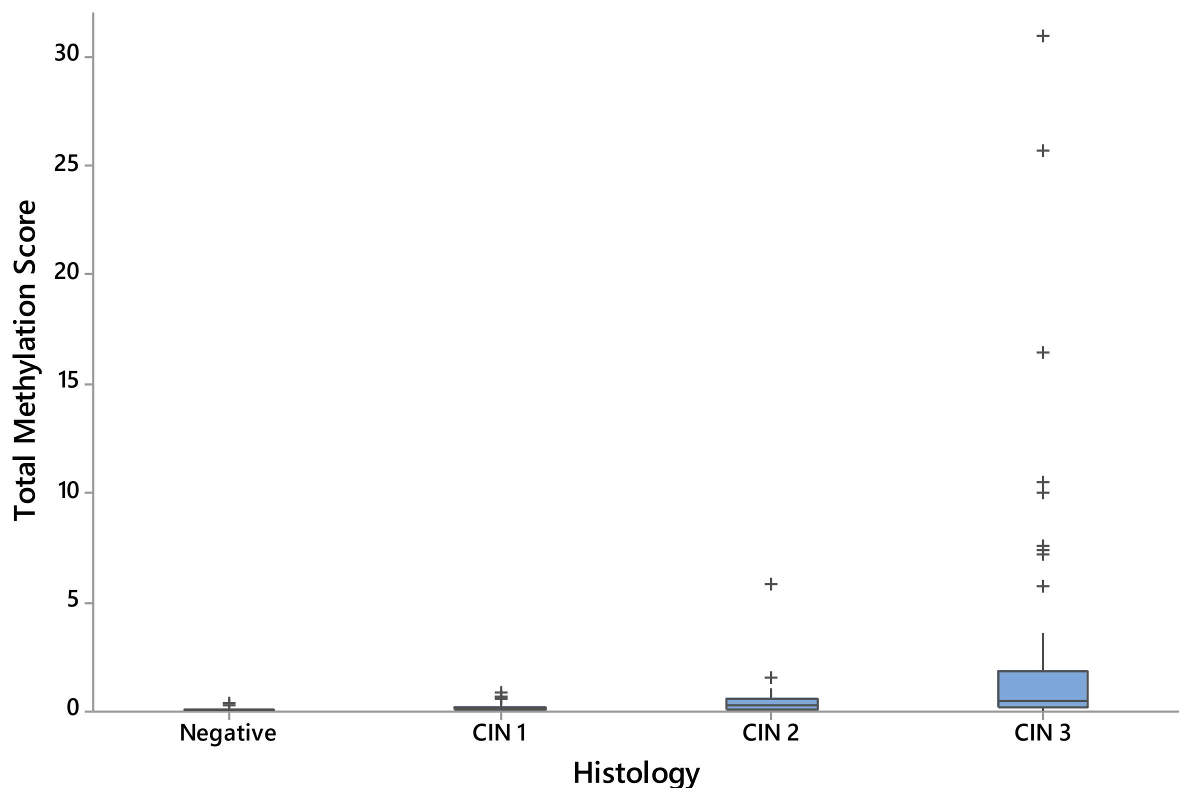


Figure 4.7: Boxplot of the Total Methylation Score stratified by histology
+ denotes outliers on the boxplot

4.4.2.4. Paired Methylation Score

As the Total Methylation Score is a summation of all three individual methylation markers it is possible to calculate a Paired Methylation Score for combinations of any two individual methylation marker. A boxplot of the Paired Methylation Scores is shown in Figure 4.8. As expected, the Total Methylation Score utilising all three methylation markers has a significantly higher methylation score compared to each of the Paired Methylation Scores (significance range $p=<0.05$ - <0.001). When the Paired Methylation Scores were compared against each other the only non-significant results were between the Paired Methylation Score for CAD M1: hsa-mir-124-2 and MAL M1: hsa-mir-124-2 across the grades CIN 1, CIN 2 and CIN 3 ($p=0.059$, 0.546, 0.466 respectively) a trend similar to the Total Methylation Score.

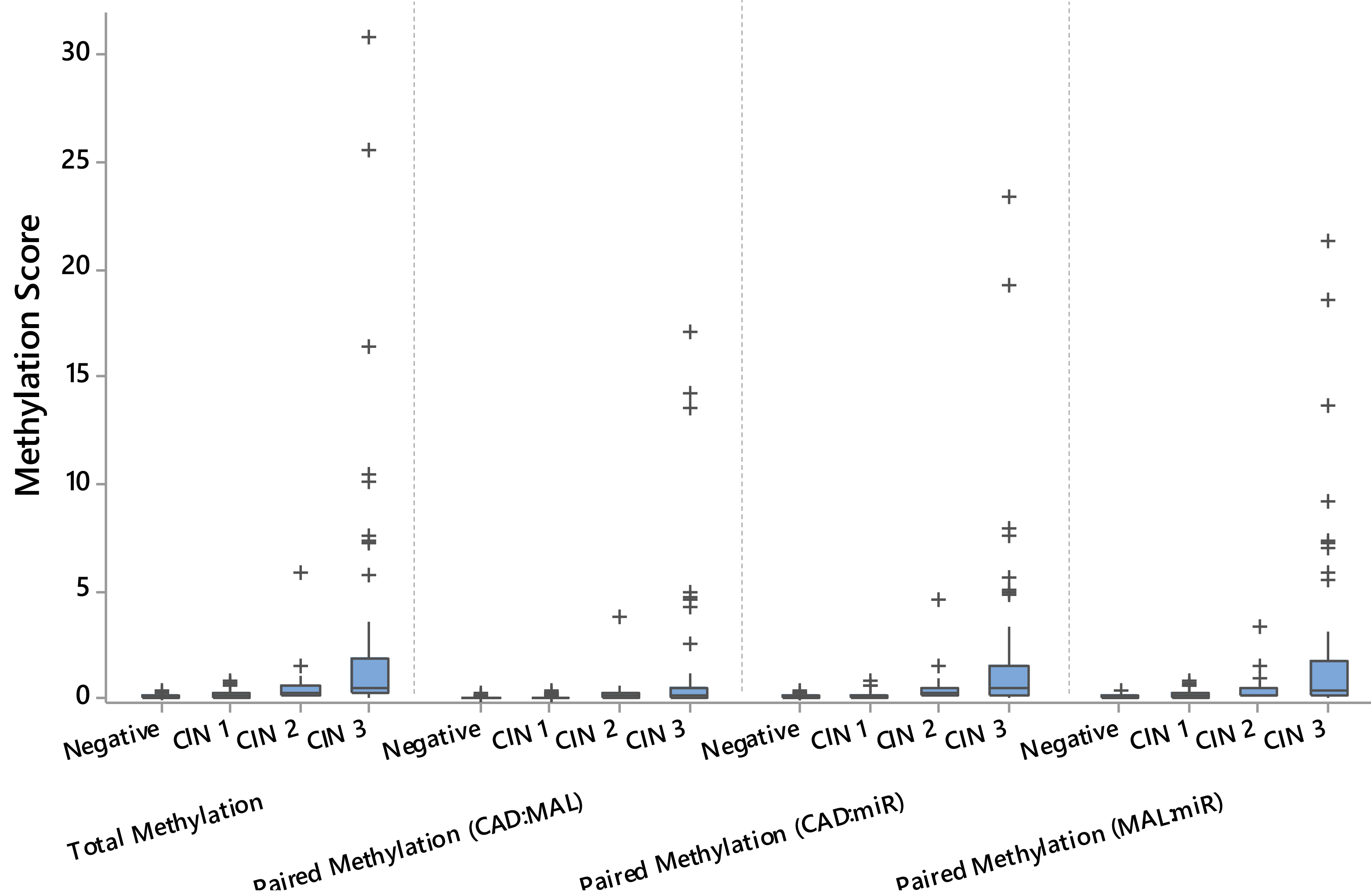


Figure 4.8: Paired Total Methylation Scores. Left to right: Total Methylation Score for all three markers, Paired Methylation Score for the combination of CAD M1-M18 + MAL M1, Paired Methylation Score of CAD M1-M18 + hsa-mir-124-2, Paired Methylation Score of MAL M1 + hsa-mir-124-2. + denotes outliers in the boxplot

4.4.3. Determination of Methylation Biomarker Panel Cut-Off Value

Assessment of the methylation biomarker panel methylation status in the validation cohort has demonstrated increased methylation of all three markers as the severity of the lesion increases This data suggest that the combination of these three markers has the potential to stratify HPV positive women into at risk groups with those exhibiting a high methylation level at a higher risk of having some form of cervical lesion. In order to use the Validation Panel for determining those at a high risk of a pre-cancerous lesion, the optimal cut-off points for the marker combinations was established using the receiver operating characteristic (ROC) curves.

4.4.3.1. ROC curve for Individual and Total Methylation Scores. <CIN 2: CIN 2+

ROC curves were generated with the outcomes of CIN 2+ as the disease endpoint and Negative, CIN 1 as the non-diseased outcome. Two Youden J values were used to set two independent cut-offs for the Total Methylation Score, one being the Youden index J value from the ROC output, the second manually selected from the other Youden J values. This was based off the desire to have a cut-off value at the optimal sensitivity as well as a cut-off value at the optimal specificity to provide flexibility when recommending a cut off value. Table 4.5 and Figure 4.9 presents the data from the ROC analysis.

The highest AUC value of 0.826 (95% CI: 0.764-0.878) was generated by the Total Methylation Score (Table 4.5). When pairwise comparisons of the AUC between each marker, with CIN 2+ as the outcome, was assessed CAD M1-M18, MAL M1 and hsa-mir-124-2 show no statistically significant differences between each other (p>0.05). There was a statistical difference between Total Methylation and each of the other methylation markers AUC's (p=0.0017, <0.0001, 0.0004). For each individual methylation marker, the Youden index J value was calculated to serve as a cut off value for the detection of CIN 2+. The estimated sensitivities around these cut offs, for each individual marker, is relatively low ranging from 53.57% to 66.67%. The Total Methylation Score has both the highest and second highest sensitivities at 78.57% (95% CI: 68.3%-86.8%) and 72.62% (95% CI: 61.8-81.8). All show a reasonable specificity ranging from 78.00% to 90.00%. Figure 4.9 shows the combined ROC curve for each of the markers with the central diagonal line representing the region where a non-discriminatory test would lie. All four methylation approaches show that they are discriminatory from the baseline population of Negative and CIN 1. The Total Methylation Score is shown to be the most distinct classifier in this grouping.

The positive (+) and negative (-) Likelihood ratio (LR) were also calculated. CAD M1-M18 showed the highest LR+ (5.84, 95% CI (2.9-10.2)) which equates to an increase of over 30% in the probability of disease given a value that exceeds the cut-off value of >0.012. However, it is the Total Methylation Score that has the lowest LR- value if either cut off is implemented with a LR- of 0.27 (0.2-0.4) which equates to an approximate 25% decrease in the probability of disease if the given methylation score was <0.13838.

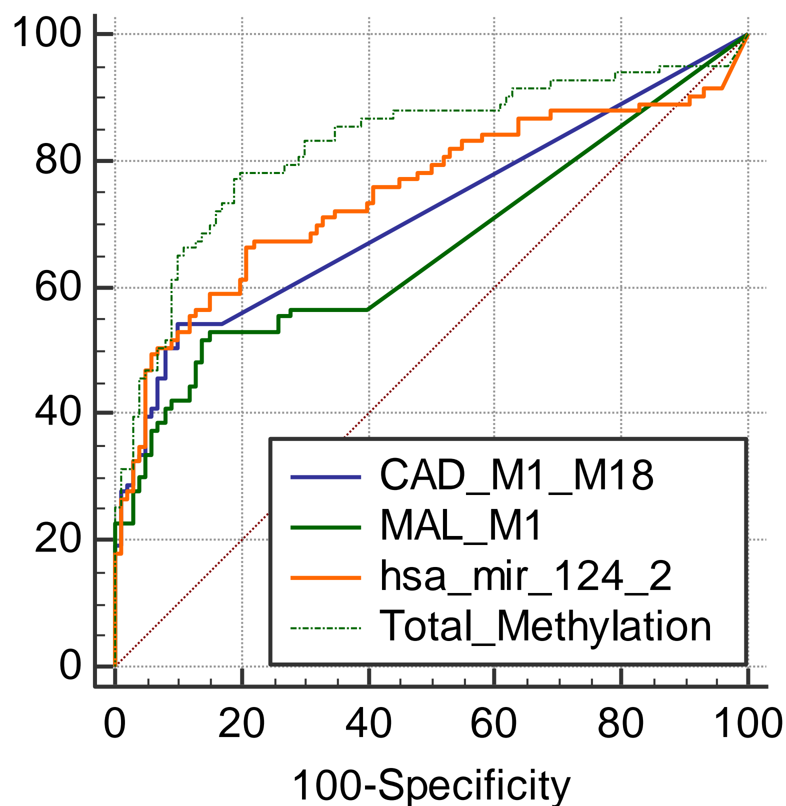


Figure 4.9: Combined ROC curves for CAD M1-M18, MAL M1, hsa-mir-124-2 and Total Methylation for the detection of CIN 2+

Table 4.5: ROC derived outputs for the detection of CIN 2+ for the individual methylation markers and the Total Methylation Score

	Outcome 1, Non-disease: Negative, CIN 1 (n=100) Outcome 2, Disease: CIN 2+ (n=84)								
	AUC (95% CI)	p-value	Youden index J (cut-off value)	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)	PPV (95% CI)	NPV (95% CI)
CAD M1-M18	0.718 (0.647-0.781)	<0.0001	>0.012	54.76% (43.5-65.7)	90.00% (82.4-95.1)	5.84 (2.9-10.2)	0.50 (0.4-0.6)	82.14 (78.30-85.98)	70.31 (66.70-73.93)
MAL M1	0.665 (0.592-0.733)	<0.0001	>0.02606	53.57% (42.4-64.5)	85.00% (76.5-91.4)	3.57 (2.2-5.9)	0.55 (0.4-0.7)	75.00 (70.26-79.74)	68.55 (64.75-72.34)
hsa-mir-124-2	0.739 (0.669-0.801)	<0.0001	>0.09766	66.67% (55.5-76.6)	78.00% (68.6-85.7)	3.03 (2.0-4.5)	0.43 (0.3-0.6)	72.15 (67.72-76.58)	74.29 (70.63-77.94)
Total Methylation Score	0.826 (0.764-0.878)	<0.0001	>0.13838*	78.57% (68.3-86.8)	80.00% (70.8-87.3)	3.93 (2.6-5.9)	0.27 (0.2-0.4)	76.74 (72.97-80.52)	81.63 (78.66-84.60)
			>0.17584**	72.62% (61.8-81.8)	84.00% (75.3-90.6)	4.54 (2.8-7.2)	0.33 (0.2-0.5)	79.22 (75.54-82.90)	78.50 (75.31-81.70)

LR= Likelihood Ratio (positive or negative)

**Youden index J specified value, **Manually selected Youden index J value*

Sensitivity, specificity, LR (+/-), PPV and NPV are calculated for the detection of CIN 2+

The heatmap shown in Figure 4.10 depicts each methylation marker and its status as either positive (green) or negative (red) when the cut-offs derived from the ROC analysis (Table 4.5) for each marker is applied (e.g. MAL M1 cut-off of ≥0.02606 denotes a positive result shown in green). From the heatmap CAD M1-M18 has a low positivity rate in the Negative group compared to the other markers. As the lesion grade increases, there is an increase in the positivity for all markers with dual or triple positive results becoming more frequent. The Total Methylation Score also has a low level of positivity in the Negative and CIN 1 grades. The cut-off point is sufficiently high enough samples do not pass the threshold for a positive result even when one of the other methylation markers has passed the threshold for positivity in this category. For example, in the Negative cohort at seven positions [6,12,18,20,28,29 and 40] and in the CIN 1 cohort at another 7 positions [51,60,63,67,72,82 and 89] at least one individual methylation marker was positive but the combination of all three was insufficient to surpass the lowest threshold of >0.13838. This occurs in the higher-grade lesions also but not to the same extent with only 4 position [106,114,117 and 128] in the CIN 2 region and 2 [170 and 172] in the CIN 3 region being positive for a single methylation marker and not for the Total Methylation Score.

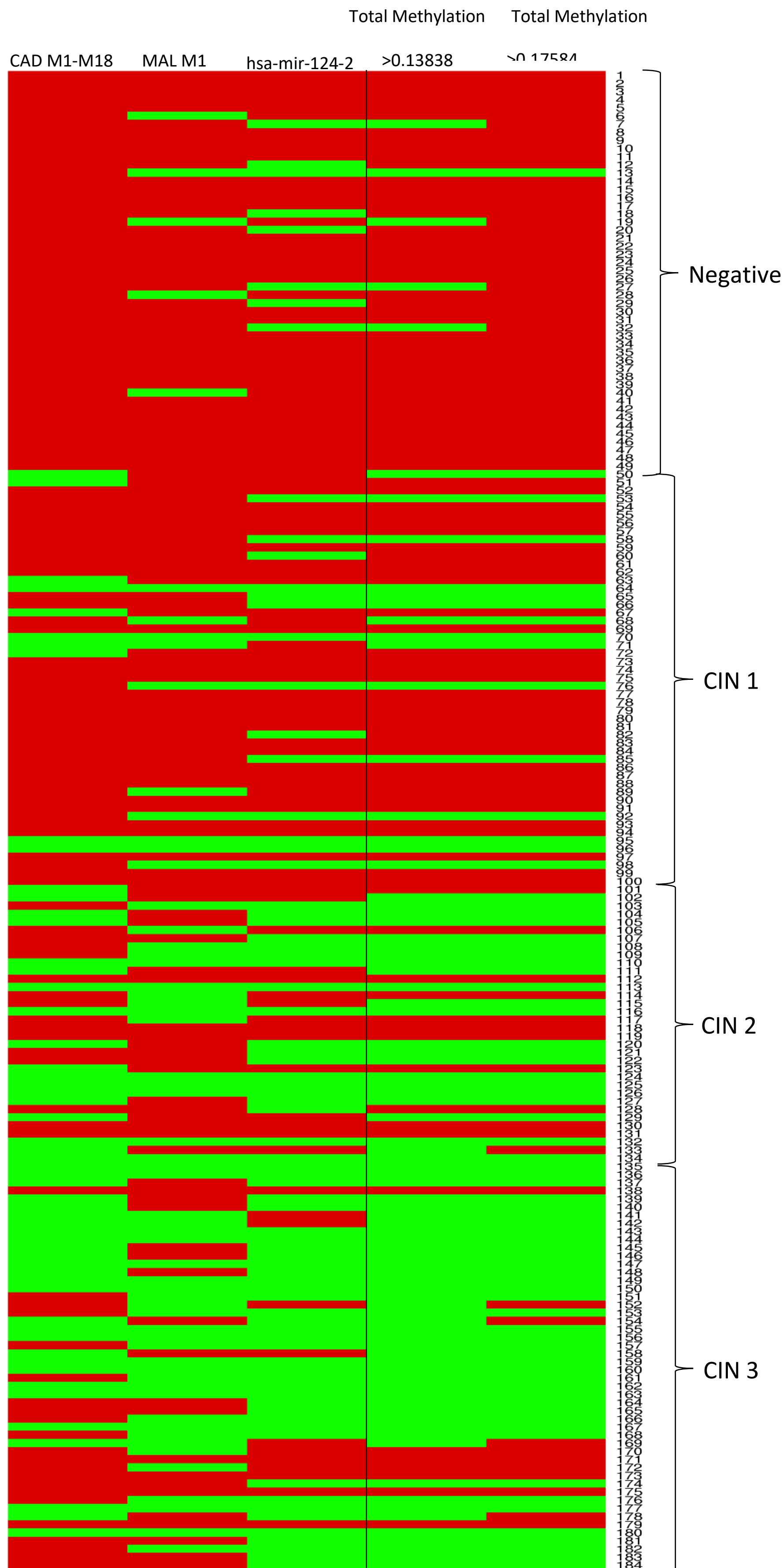


Figure 4.10: Heatmap of the validation panel across their respective histological grade stratified as positive (green) or negative (red) for the respective methylation marker. Left to right: CAD M1-M18, MAL M1, hsa-mir-124-2, Total Methylation Score (cut off ≥0.13838), Total Methylation Score (cut off ≥0.17584).

4.4.3.2. ROC curve for Paired Methylation Scores. <CIN 2: CIN 2+

The use of Paired Methylation Scores as outlined from Figure 4.8 were also assessed using ROC analysis with the Youden index J was utilised as a cut-off value. Overall the Total Methylation Score shows the best-balanced sensitivity and specificity when compared to the three Paired Methylation Scores (Table 4.6). Paired Methylation Scores for (CAD M1: hsa-mir-124-2) and (MAL M1: hsa-mir-124-2) have higher specificities but lack an adequate sensitivity compared to the Total Methylation Score. The Paired Methylation Score for (CAD M1: MAL M1) however had the best overall performance of the three paired scores with a reasonable sensitivity and specificity compared to the other two Paired Methylation Scores. When pairwise comparisons between each Methylation Score were assessed there was no statistical difference in the AUC's with the exception of Total Methylation Score versus Paired Methylation Score (MAL M1: hsa-mir-124-2) (p=0.0216).

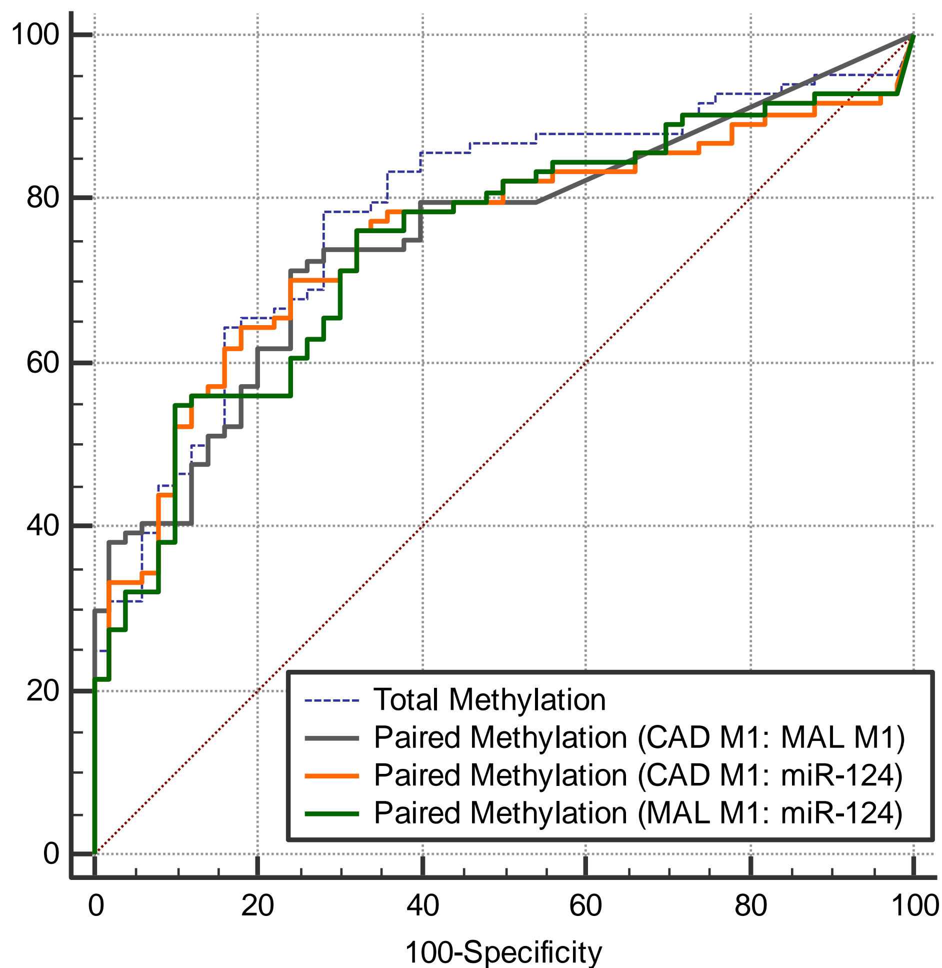


Figure 4.11: Combined ROC curves for defection of CIN 2+ using alternative Total Methylation Scores

Table 4.6: ROC curve derived outputs for the detection of CIN 2+ for Paired Methylation Scores

	Outcome 1, Non-disease: Negative, CIN 1 (n=100), Outcome 2, Disease: CIN 2+ (n=84)								
	AUC (95% CI)	p-value	Youden index J (cut-off)	Sensitivity (95% CI)	Specificity (95% CI)	LR+ (95% CI)	LR- (95% CI)	PPV (95% CI)	NPV (95% CI)
Paired Methylation Score (CAD M1: MAL M1)	0.755 (0.673 to 0.825)	<0.0001	>0.02449	71.43% (60.5-80.8)	76.00% (61.8-86.9)	2.98 (1.8-5.0)	0.38 (0.3-0.5)	76.19 (72.31-80.07)	80.00 (76.86-83.14)
Paired Methylation Score (CAD M1: hsa-mir-124-2)	0.753 (0.671 to 0.823)	<0.0001	>0.1984	64.29% (53.1-74.4)	82.00% (68.6-91.4)	3.57 (1.9-6.6)	0.44 (0.3-0.6)	70.53 (66.35-74.71)	80.90 (77.69-84.11)
Paired Methylation Score (MAL M1: hsa-mir-124-2)	0.745 (0.663 to 0.817)	<0.0001	>0.24864	54.76% (43.5-65.7)	90.00% (78.2-96.7)	5.48 (2.3 -12.9)	0.50 (0.4-0.6)	69.79 (65.57-74.01)	80.68 (77.43-83.94)
Total Methylation Score	0.826 (0.764-0.878)	<0.0001	>0.13838*	78.57% (68.3-86.8)	80.00% (70.8-87.3)	3.93 (2.6-5.9)	0.27 (0.2-0.4)	76.74 (72.97-80.52)	81.63 (78.66-84.60)
			>0.17584**	72.62% (61.8-81.8)	84.00% (75.3-90.6)	4.54 (2.8-7.2)	0.33 (0.2-0.5)	79.22 (75.54-82.90)	78.50 (75.31-81.70)

*LR= Likelihood Ratio (positive or negative).*Youden index J specified value, **Manually selected Youden index J value. Sensitivity, specificity, LR (+/-), PPV and NPV are calculated for the detection of CIN 2+*

4.4.3.3. Clinical Performance of the Individual Methylation Markers

Separate from using a single classifier such as the Total Methylation Score or one of the Paired Methylation Scores each methylation marker can be used on an individual basis grouped in a panel of methylation markers. In this instance the criteria for an overall positive result is a minimum of one of the methylation markers achieving a result over the defined positive cut off set in by the ROC curve in Section 4.4.4.1. Using these cut offs for each of the individual methylation markers the sensitivity, specificity, PPV and NPV were calculated for each possible panel combination of CAD M1-M18, MAL M1 and hsa-mir-124-2 as shown in Table 4.7. The Total Methylation Score has also been included for comparison.

The use of all three individual markers (All Markers) gives the highest sensitivity for CIN 2+ (88.10%) but the lowest specificity at 66.00% compared to using a combination of any two markers. A panel using CAD M1-M18 and MAL M1 gave the highest specificity for CIN 2+ at 80.00% but the lowest sensitivities at 76.19%. Each combination of individual methylation markers also had a reasonable high NPV for both CIN2 + (80.00% to 86.84%) and CIN 3 (87.91% to 92.96%). In comparison the Total Methylation Score with a cut off set at >0.13838 showed the best combined approach for sensitivity and specificity for the detection of CIN 2+. The difference in sensitivities between a panel of All Markers and the Total Methylation Score decreased for detection of CIN 3 with a difference of only 4% while maintaining a high level of specificity. Across all the potential markers the NPV is comparable and the Total Methylation Score shows a higher PPV especially compared to the use of a panel of All Markers.

Table 4.7: Clinical performance of an individual methylation marker panel for the detection of CIN 2 + and CIN 3

	Clinical performance for the detection of CIN 2+ (n=84)			
	Sensitivity (95% CI)	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**
Total Methylation	78.57% (68.3-86.8)	80.00% (70.8-87.3)	76.74 (72.97-80.52)	81.63 (78.66-84.60)
All Markers	88.10% (85.85-90.34)	66.00% (61.60-70.40)	68.52% (64.45-72.59)	86.84% (84.27-89.41)
CAD M1-M18 and MAL M1	76.19% (72.31-80.07)	80.00% (76.86-83.14)	76.19% (72.31-80.07)	80.00% (76.86-83.14)
CAD M1-M18 and hsa-mir-124-2	79.76% (76.31-83.21)	72.00% (68.05-75.95)	70.53% (66.35-74.71)	80.90% (77.69-84.11)
MAL M1 and hsa-124-2	79.76% (76.31-83.21)	71.00% (66.96-75.04)	69.79% (65.57-74.01)	80.68% (77.43-83.94)

Criteria for a positive methylation test when in combination is any single methylation marker positive result

4.4.3.4. Comparison of Methylation Strategies for the detection of CIN 2+

Eleven potential strategies, utilising the primary three methylation markers (CAD M1-M18, MAL M1, hsa-mir-124-2) for detection of CIN 2+ have been assessed in this chapter. The trade-off of each methylation strategy is shown in Figure 4.12 which plots the sensitivity against the specificity of each methylation strategy. Each strategy is coded (a to k) as shown in Table 4.8 along with their sensitivity and specificity. Using a Methylation Panel of the individual methylation markers shows the highest ranges of sensitivity with the trade-off of the lowest sensitivity of the eleven strategies (h, k, j). A Methylation Panel using CAD M1-M18 and MAL M1 (i) showed the best approach out of these four strategies. Using CAD M1-M18 (a) or MAL M1 (b) individually allows for a highly specific methylation test but lacks overall sensitivity for CIN 2+. However, using hsa-mir-124-2 (c) individually shows an increased sensitivity but a lower specificity in comparison. Both Paired Methylation Score strategies (e, f, g) had a range of clinical performances with the Paired Methylation Score (MAL M1: hsa-mir-124-2) (g) having a comparable performance to CAD M1-M18 alone (a). Both the Paired Methylation Score (CAD M1-M18: MAL M1) (e) and Paired Methylation Score (CAD M1-M18: hsa-mir-124-2) (f) show a trade-off of sensitivity and specificity associated with the use of either MAL M1 or hsa-mir-124-2 in its calculation. Lastly the two Total Methylation Scores (d) (d*) shows a good combination of both sensitivity and specificity. Total Methylation Score (d*) using the higher cut-off of >0.17584 has a higher specificity to sensitivity profile than most other methylation strategies. The Total Methylation Score (d) using the lower threshold of >0.13838 shows the best balance of both sensitivity while remaining reasonable specific for CIN 2+. Of the Paired Methylation Scores the combination of CAD M1 and MAL M1 performed the best (i) and hsa-mir-124-2 (c) was the best individually functioning methylation marker.

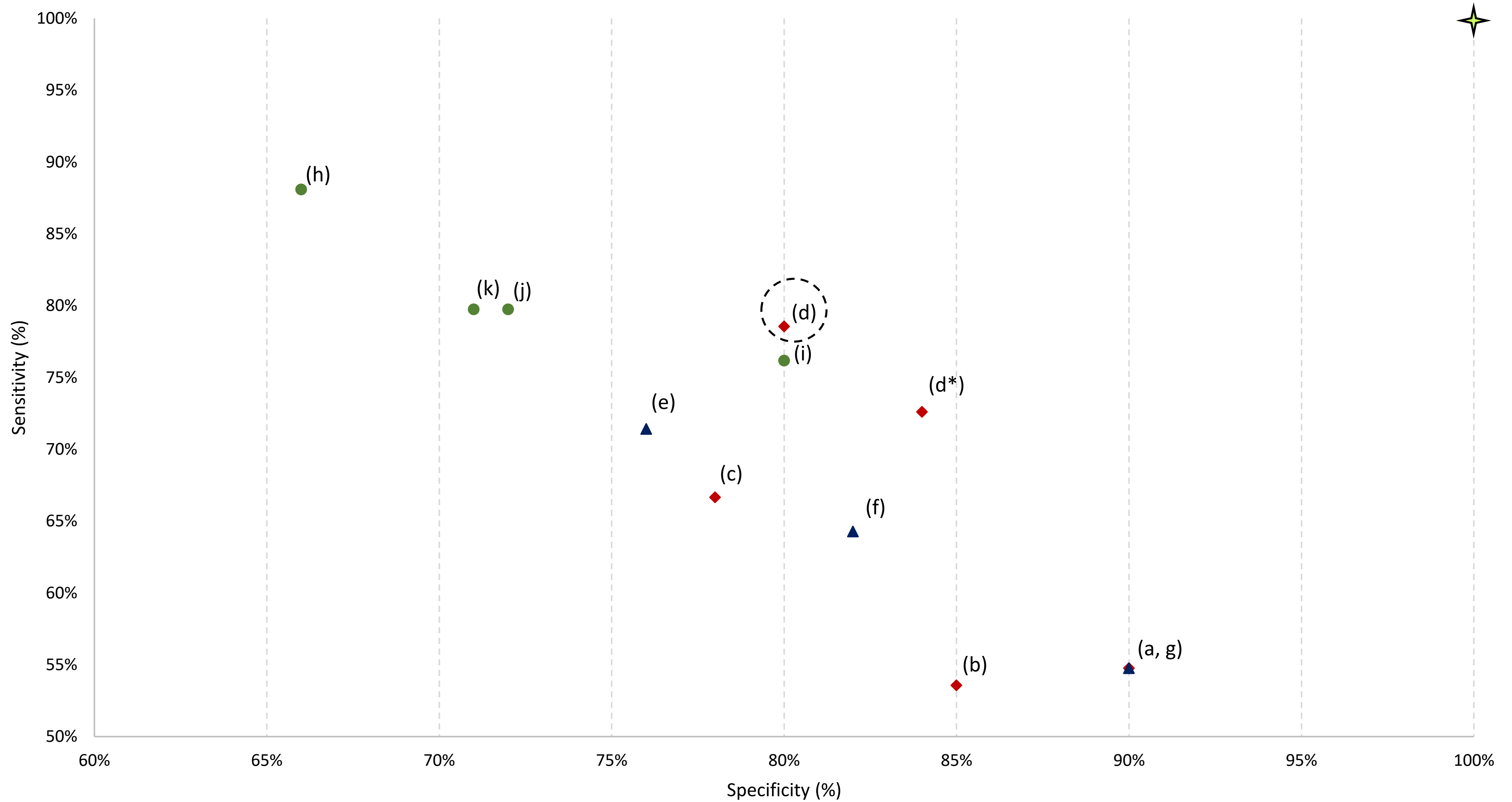


Figure 4.12: Scatterplot of sensitivity by specificity for the detection of CIN 2+ for each potential methylation strategy. Each strategy is coded (a-k) as shown in Table 4.8. The star represents a perfect test with 100% sensitivity and specificity. The best candidate marker is circled by a dotted line.

Table 4.8: Sensitivity and specificity of each methylation strategy for the detection of CIN 2+

	Symbol		Sensitivity % (95% CI)	Specificity % (95% CI)
Individual Methylation Markers	◆	CAD M1-M18 [a]	54.76% (65.70-43.50)	90.00% (95.10-82.40)
		MAL M1 [b]	53.57% (64.50-42.40)	85.00% (91.40-76.50)
		hsa-mir-124-2 [c]	66.67% (76.60-55.50)	78.00% (85.70-68.60)
		Total Methylation (>0.13838) [d]	78.57% (86.80-68.30)	80.00% (87.30-70.80)
		Total Methylation (>0.17584) [d*]	72.62% (61.80-81.80)	84.00% (75.30-90.60)
Paired Methylation Score (x:x)	▲	(CAD M1: MAL M1) [e]	71.43% (80.80-60.50)	76.00% (86.90-61.80)
		(CAD M1: hsa-mir-124-2) [f]	64.29% (74.40-53.10)	82.00% (91.40-68.60)
		(MAL M1: hsa-mir-124-2) [g]	54.76% (65.70-43.50)	90.00% (96.70-78.20)
Methylation Panel *(Criteria for a positive methylation test when in combination is any single methylation marker positive result)*	●	All Markers [h]	88.10% (90.34-85.85)	66.00% (70.40-61.60)
		CAD M1-M18 and MAL M1 [i]	76.19% (80.07-72.31)	80.00% (83.14-76.86)
		CAD M1-M18 and hsa-mir-124-2 [j]	79.76% (83.21-76.31)	72.00% (75.95-68.05)
		MAL M1 and hsa-124-2 [k]	79.76% (83.21-76.31)	71.00% (75.04-66.96)

4.5. Discussion

The aim of this chapter was to determine and appropriate method and validate a set of clinically relevant cut off values for detection of CIN 2+ using a set of three methylation markers [CAD M1-M18, MAL M1, hsa-mir-124-2]. This set of methylation markers could potentially be used as a triage test for HPV positive women in a HPV primary screening population. To date, there has been a large body of evidence supporting the use of methylation markers as a triage test in the case of HPV primary screening. Several methylation markers have become prominent in the literature but there is no single adequate marker and it is the general consensus that a panel of methylation markers will be required for any methylation test.

In this chapter, CAD M1-M18, MAL M1 and hsa-mir-124-2 were optimised on a validation panel of cervical smears with histologically confirmed disease. Cut off values were established defining clinically relevant methylation levels for detection of CIN 2+ using ROC curves. This was achieved by using a derivation of the 2^-ΔΔ Ct method which was 2^(Ct(β-Actin)-Ct(Target)x100) (Hesselink *et al.*, 2011) to calculate individual methylation scores for the methylation markers. Another approach also adopted including the combining of all three individual methylation scores calculating the overall Total Methylation Score as well the combination of only two of the three methylation scores giving us another three different Paired Methylation Scores (Table 4.2, Table 2.3) with ROC curve derived cut-off values. This allowed for a novel and simplified approach for methylation analysis compared to the current methods. Commonly the literature, will classify a methylation test as positive if at least one individual methylation marker surpasses the threshold for positivity. This can lead to a very sensitive test the more methylation markers are incorporated into the panel but would lose specificity and would lead to over referral. A single scoring method such as the Total or Paired Methylation Score(s) would however provide a better-balanced option for triage as cut off points are determined around the one score rather than three individual markers. The benefits of this approach have been shown previously with classifiers such as S5 published by Lorincz *et al.,* 2016.

Initial assessment of the methylation markers on a cohort of HPV negative cytology normal women (n=15) showed that there was a low level of methylation detected by qMSP. Establishing the low level of methylation in what is considered a normal cervical smear highlighted the need to define a clinically relevant cut off point to correctly identify those women who have a true risk of cervical cancer or pre-cancer.

Determination of Clinically Relevant Cut-Offs for Detection of CIN 2+.

To define what a clinically relevant level of methylation is for each of the three methylation markers a validation panel was established from the CERVIVA primary screening study population which included cervical smears from women with histologically confirmed disease. Assessment of the methylation biomarker panel scores in this validation cohort, clearly demonstrated that the pattern of methylation increased with increasing grade of disease with a statistical difference between each grade of CIN for each of the methylation markers ($p<0.05$) (Figure 4.6). This trend was also true when the Total and Paired Methylation scores were calculated and shows that the methylation markers have the ability to distinguish CIN 2+ from CIN1 and histologically normal samples. To fully utilise this data, ROC analysis was employed to set positive cut off thresholds for each of the individual markers as well as the Total and Paired Methylation Scores for detection of CIN 2+. This culminated in eleven unique strategies for evaluation.

Performance of the Individual Methylation Markers.

The initial three individual cut off points for CAD M1-M18, MAL M1 and hsa-mir-124-2 showed low levels of sensitivity (53.57% to 66.67%) with reasonable specificity (78.00% to 90.00%) which was expected when using these markers in isolation (Table 4.5). No individual methylation marker in the panel has an adequate sensitivity to detect CIN 2+ but they are specific for CIN 2+. This is an aspect of methylation markers that is well known, and it is the contribution of multiple markers in a panel that improves the sensitivity and specificity for detection of CIN 2+. When all three individual markers are used with the criteria that a positive result is when at least one individual methylation marker surpasses the threshold for positivity, the true value of the methylation panel can be seen. When all three markers are used, there is a sensitivity of 88.10% and specificity of 66.00% with a PPV of 68.52%. Previous studies utilising these three markers in this fashion have reported sensitivities of 48% to 89%, specificities of 48% to 50% as well as a PPV of 50% (De Strooper, van Zummeren, *et al.*, 2014; De Vuyst *et al.*, 2015). In this validation set the methylation panel show an overall improved performance with comparable sensitivities and improved specificities and PPV of detection of CIN 2+. As this is a validation panel the performance of the specificity can be inflated due to the disproportion of disease versus normal samples. This is an important limitation to be aware of in validation panels, however, it is necessary to carefully form a panel that allows for the controlled setting of cut-off values.

When the methylation panel is limited to two methylation markers, sensitivity is reduced (76.16-79.76%) compared to the three-marker panel (88.10%). However, specificity is higher (71.80% vs 66.00%). Compared to previous studies summarized by Lorincz, 2016

(Table 4.1), using a two marker panel, comparable outcomes in regards to their clinical performance can be seen. For example, the combination of CAD M1-M18 and MAL in this study has a sensitivity of 76.19% and specificity of 80.00%, compared to two similar studies with a sensitivity of 62% and 84% and specificity of 78% and 52% respectively (Hesselink *et al.*, 2011; De Strooper, Hesselink, *et al.*, 2014). The combination of MAL and hsa-mir-124-2 in this study has a sensitivity of 79.76% and specificity of 71.00%, which is comparable to previously published studies with a sensitivity of 72% to 80% and specificity of 49% to 60%, respectively (Hesselink *et al.*, 2014; Verhoef, Heideman, *et al.*, 2014).

Performance of the Total and Paired Methylation Scores.

One of the novel aspects of this study was the introduction of the Total Methylation Score which was the summation of the individual methylation scores. The use of two cut off values for the Total Methylation Score was based on the desire to have a value at the optimal sensitivity to specificity as well as one at the optimal specificity to sensitivity. At the threshold of >0.13838 the Total Methylation Score has a sensitivity of 78.57% and specificity of 80.00%. Using a higher threshold of >0.17548 however increases the specificity to 84.00% with an associated decrease in sensitivity to 72.62%. These are comparable to the previous studies using these three markers. However, it shows the highest combined sensitivity and specificity of any methylation panel to date. Previous studies have shown either a reasonable sensitivity or specificity but not both. The caveat of setting any cut off point is the trade-off between sensitivity and specificity, to achieve a higher sensitivity the specificity inherently decreases and vice versa. This was well shown in De Vuyst et al. 2015, with a sensitivity of 100% the specificity was only 10% and to achieve >90% specificity the sensitivity was only 51%. This method of combining the individual scores to a single value and defining a relevant cut off around it has shown the best-balanced approach in regards the clinical performance of the test. Of the two thresholds however, a Total Methylation Score using the lower threshold of >0.13838 is the preferred option. Though there is a loss in specificity there needs to be a reasonable level of sensitivity if it is to be used in a triage capacity. A second approach to scoring methylation was the Paired Methylation Scores which showed comparable clinical performances to previous studies but overall the use of the Total Methylation Score was superior in regard to the sensitivities and specificities as well as showing statistically higher methylation scores compared to the Paired Methylation Scores ($p <0.05$-<0.001). However, of the three the combination of CAD M1 and MAL M1 provided the best performances of the three. Compared to all the potential methods of classifying a positive methylation test the Total Methylation Score shows the best-balanced approach for the triage of HPV positive women (Figure 4.12 and Table 4.8). This approach could, ideally, allow for a single triage test to be used in this population compared to a very

specific but non-sensitive test which would necessitate repeat screening at a later point or another subsequent triage test to ensure all relevant cases of CIN 2+ are detected. It would also allow an acceptable referral rate compared to highly sensitive but non-specific tests which would catch the majority of CIN 2+ cases with the caveat of a very high colposcopy referral rate as well as the referral of women with no disease.

The heatmap from Figure 4.10. best shows how the Total Methylation Score interacts in the validation panel. Where one individual methylation marker may just surpass the threshold for positivity, it is not sufficient in combination to surpass the thresholds set by the Total Methylation Score. This is seen quite clearly in the Negative and CIN 1 groups on the heatmap where several individual markers are considered positive but fails to reach the threshold of positivity for the Total Methylation Score. This reflects the increased specificity of the Total Methylation Score in comparison to the individual methylation markers and that the combination of all three to calculate the Total Methylation Score maintains both the sensitivity and specificity of the overall test. Though the sensitivity for detection of CIN2+ is reduced compared to using the individual markers in a methylation panel (88.10% vs 78.57%), for the detection of CIN 3 the impact on sensitivity is less (90.00% vs 86.00%) while keeping the higher level of specificity (66.00% vs 80.00%).

Conclusions.

As early as 2004, it was shown that methylation could be detected in cervical samples up to 7 years prior to diagnosis using the TSLC1 gene that would be later reclassified as CAD M1 (Steenbergen *et al.*, 2004). Since then, in a multitude of studies across a variety of genes it has been shown that the DNA methylation is strongly associated with cervical cancer and can be used in a triage capacity. There has been little work done however on the question of progression, regression and methylation. There are a host of advantages to using methylation panels as a biomarker in the triage of HPV positive women. Abnormal methylation is directly linked to the activity of both E6 and E7 viral oncoproteins with the increasing levels of cellular methylation linked to an increasing lesion severity (Moore, Le and Fan, 2013; Jiménez-Wences, Peralta-Zaragoza and Fernández-Tilapa, 2014; Lorincz, 2016). Methylation testing has been shown to be non-inferior to cytology in a triage setting (De Vuyst *et al.*, 2015) with the benefit of it not being a subjective test open to varied interpretation. Due to the relatively straight forward procedure once in place it has a quicker overall turnaround time versus cytology (Verhoef, Bosgraaf, *et al.*, 2014). Considering the use of methylation testing in cervical cancer screening is relatively novel, it is likely that methylation testing will further improve over time as more novel methylation markers are discovered.

HPV primary screening represents the biggest change in cervical screening since the introduction of cytology in the 1950's and it necessitates the use of a triage test. Though HPV primary screening will detect more cases of cervical pre-cancer and cancer earlier it lacks the overall specificity to be used on its own. From this thesis, we have already shown that 15.98% of women are positive for HPV DNA compared to 8.6% who were positive for cytology. This would represent a 7.38% increase in the number of cases referred to colposcopy on a transient, non-relevant HPV infection. In many screening programmes, Ireland being no exception (HIQA, 2017), cytology will likely become the initial triage test due to its pre-establishment and familiarity however it will still carry its own limitations in sensitivity, subjectivity and necessitate further recall of cytology NAD women. Chapter 5 will assess the use of methylation testing as the triage test in a HPV primary screening programme.

In conclusion, the findings from this set of validation work demonstrates that the use of CAD M1-M18, MAL M1 and hsa-mir-124-2 offer comparable sensitivity and specificities for the detection of CIN 2+, in a validation population, compared to previous studies. However, the use of the Total Methylation Score offers an improvement on the overall clinical performance of the methylation test compared to previous studies by being both sensitive and specific for the detection of pre-cancer and cancer. In Chapter 5 those women who have tested HPV positive will be tested against the methylation panel with a larger population size and more realistic proportion of CIN 2+ present in the population. The Total Methylation Score, Paired Methylation Score (CAD M1-M18: MAL M1) and the individual methylation marker hsa-mir-124-2 will be evaluated in this HPV triage population to determine its clinical utility.

4.6. References

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-Chapter 5-

Molecular Triage Strategies for Managing HPV Positive Women

5.1. Introduction

HPV testing is considered a "test of risk" rather than a "test of disease" (O'Leary *et al.*, 2018). This potentially could lead to an increased rate of referrals for women with transient, non-progressive HPV infections. This warrants the use of triage tests to take this population and stratify them to those with sufficient risk. Triage tests, for example, HPV genotyping, cytology or methylation markers, would further stratify HPV positive women act as a 'test of disease'. A positive triage test would imply that the women is at a higher risk of a cervical cancer or precancer and requires further follow up. A negative triage test can either imply a low enough risk to return to routine screening intervals or a reduced risk that would still require surveillance at a shorter interval of between 6 to 12 months. Each triage test performs differently in this regard the best triage system may not be a single triage test but potentially two sequential triage tests such as HPV 16/18 genotyping with cytology triage for those positive for the other hrHPV types. This is a risk-based system of screening which allows sequential testing with each new test adding to the previous risk until certain thresholds are met which warrants colposcopy or treatment. An idealized version of this is shown in Figure 5.1 (Wentzensen *et al.*, 2016).

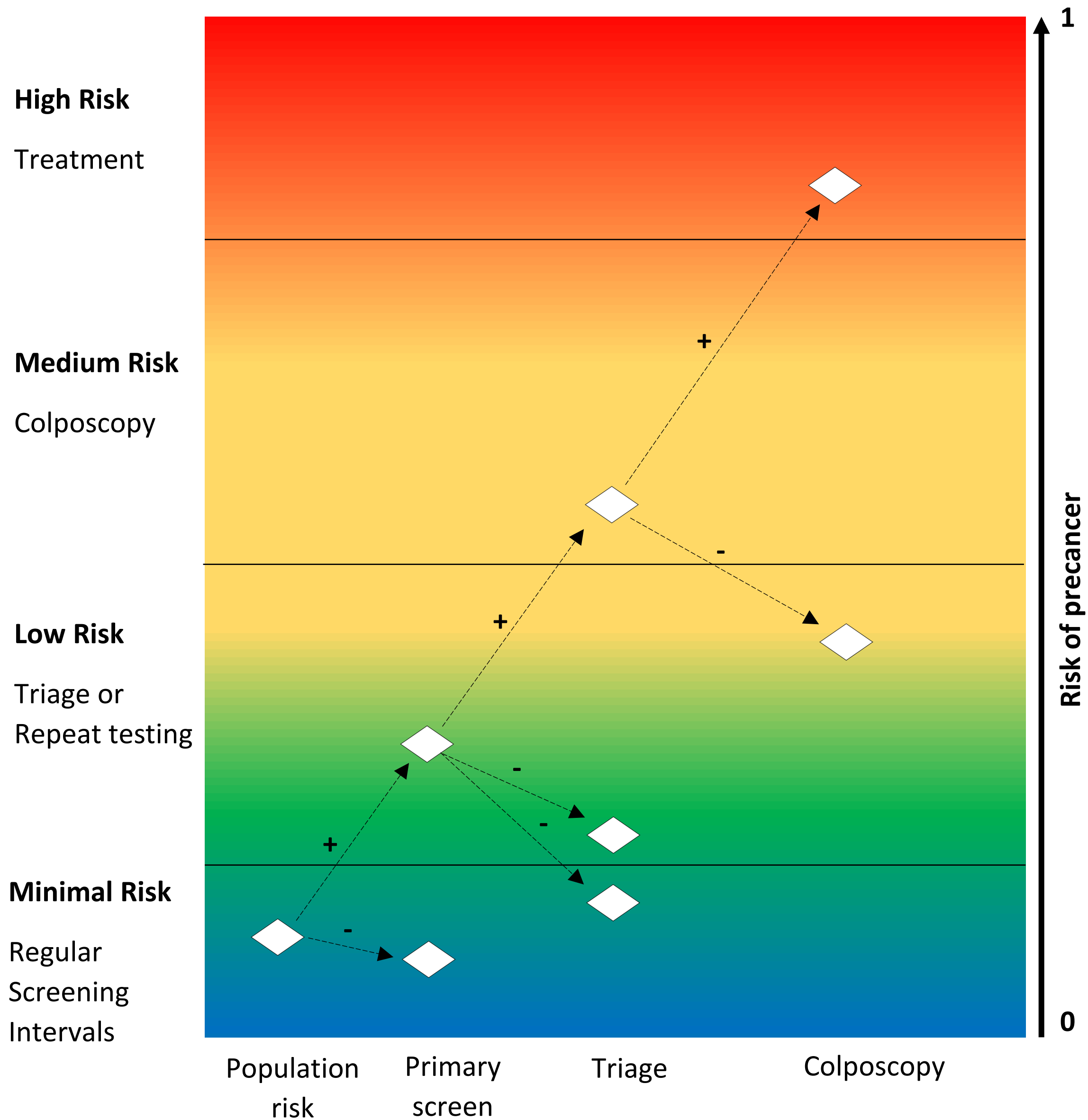

Figure 5.1: Risk based management in cervical screening. Different primary screening and triage tests may group women in different risk strata. Those categorised as Minimal Risk (Blue) are considered safe to be in the routine screening system. Those at Low Risk (Green/Yellow) are considered to be at a higher risk and cannot return to routine screening but are not considered at a high enough risk to warrant colposcopy. Medium to High Risk is considered sufficient risk to warrant colposcopy and treatment. A positive screening/triage/colposcopy result should impart an increased level of risk for the women to move them up in the risk categories. A negative test should impart a decrease in the woman's risk. Ideally a negative triage test should allow the woman to return to the minimal risk category though this may not always be possible. (Amended from Wentzensen et al. 2016)

An important factor to consider is that there are no universal thresholds for what denotes low, medium or high risk for these screening strategies. They depend on many factors such as the benefits and harms associated with treatment of the cancer or pre-cancer as well as the benefits and harms associated with confirmatory test such as colposcopy. Risk thresholds will also vary across differing diseases and healthcare settings such as national screening programmes or opportunistic screening. For example, in the USA risk is standardised (Katki *et al.*, 2013). If the risk of a positive result from new screening or triage test is similar to the risk of CIN 3+ following a LSIL cytology result (5-year associated risk: 5.2%), then the women should be referred to colposcopy. This has the benefit of being less variable across differing populations but is also a very low bar in which to refer to colposcopy (Katki *et al.*, 2013). This compares with Europe where the guideline is that a positive test (screening with/without triage) result indicates a >10% risk of CIN 3+ then the women should be referred to colposcopy or if the test indicated a >20% risk for CIN 2+ (Arbyn *et al.*, 2011). There are also risk guidelines for a negative triage test. If a screening or triage test is negative and imparts a risk of <1% for CIN 3+ or <2% for CIN 2+ then it is considered safe for the woman to return to the routine recall. If not then the possibility of retesting over a shorter interval should be considered (Arbyn *et al.*, 2017).

There have been an extensive number of RCTs investigating triage strategies for HPV primary screening programmes from the NTCC, ATHENA, ARTISTIC and POBASCAM. The most commonly seen triage strategies included triage with cytology, HPV 16/18 genotyping and p16INK4a/Ki-67 dual staining either individually or in combination with each other. These triage options can be broadly classified into four strategies:

Strategy 1: HPV primary screening with cytology triage.
Strategy 2: HPV primary screening with HPV 16/18 triage.
Strategy 3: HPV primary screening with HPV 16/18 and cytology triage.
Strategy 4: HPV primary screening with p16INK4a/Ki-67 triage.

Strategy 1, was the most common approach with ATHENA, ARTISTIC, NTCC and the Public health trial Finland publishing data. The clinical performance of cytology as a triage test for the detection of CIN 2+ ranged from 46.5% to 97.6% and 65.6% to 97.6% for sensitivity and specificity respectively. Overall, colposcopy referral ranges from 2.8% to 12.1% with between 25.9% to 38.7% of women triaged were referred to colposcopy (Ronco, Giorgi-Rossi, *et al.*, 2006; Ronco, Segnan, *et al.*, 2006; Castle *et al.*, 2011; Leinonen *et al.*, 2013; C Kitchener *et al.*, 2014; Bergeron *et al.*, 2015; Wright *et al.*, 2017).

Strategy 2, using HPV 16/18 partial genotyping, as expected showed a high level of specificity for CIN 2+ but as hrHPV positive women were not referred to colposcopy it suffered from a low sensitivity. Sensitivity ranged from 51.8% to 56.4% (95% CI: 46.8-60.3) in the ATHENA and ARTISTC studies respectively (Castle *et al.*, 2011; C Kitchener *et al.*, 2014). Adopting this triage strategy of referring HPV 16/18 positive women only to colposcopy, resulted in 4.5-12.3% of the screening population being referred to colposcopy. This would reflect 27.6-28.8% of the HPV positive triage population from these studies being referred.

Strategy 3, involved HPV 16/18 genotyping with cytology triage. This approach showed a higher level of sensitivity and more consistent specificity compared to Strategy 1. The ATHENA assessed this in two studies. This approach had a sensitivity range of 66.7% to 74.5% and specificity of 82.5% to 82.7% (Castle *et al.*, 2011; Wright *et al.*, 2017). This resulted in 20.1% and 20.2% of the screening population being referred to colposcopy or 44.8% and 43.3% of the triaged women to be referred to colposcopy.

Strategy 4, is the same approach as Strategy 1 but using the p16INK4a/Ki-67 as the triage test. This approach was assessed in the ATHENA trial but was also broken into three categories, (1) triage with p16INK4a/Ki-67 alone, (2) co-testing with HPV 16/18 genotyping with referral if both tests were positive or (3) co-testing with HPV 16/18 genotyping with referral if either were positive. (1) had a sensitivity of 63.1% and specificity of 89.6%. (2) had a sensitivity of 35.5% and specificity of 96.6%. (3) had a sensitivity of 74.3% and specificity of 82.5%. The referral rate for all women screened was 12.1%, 5.2% and 20.6% respectively and the referral rate for the triaged women was 28.4%, 11.1% and 44.1% respectively (Wright *et al.*, 2017). The use of a double positive triage test such as p16INK4a/Ki-67 and HPV 16/18 positive reduces the number of women referred to colposcopy and allows for a highly specific screening programme with the loss of the overall sensitivity. Compared to the first and last option of p16INK4a/Ki-67 testing alone or in combination with HPV 16/18 genotyping these approaches allow the tests to remain sensitive and specific. This does however affect the end referral rates with the addition of HPV 16/18 genotyping increasing the referral rate by 15.7%.

These four strategies are currently the most commonly accepted potential triage options. Strategy 1 utilising cytology is the most commonly accepted approach in most screening practices in part due to the long-term evidence and clinical performances where cytology standards are maintained at a high level. In contrast other triage options from p16INK4a/Ki-67, HPV 16/18 genotyping and methylation testing have shown insufficient sensitivities and/or a lack of longitudinal follow up and small study populations with respect to

methylation testing. There is a requirement for more larger scale studies in HPV primary screening populations on these triage approaches to evaluate their performances as well as their longitudinal performances. The longitudinal results for cytology triage are also the most well documented with the 4 year sensitivity and specificity being 66.0% (95% CI: 59.6-71.9) and 81.4% (95% CI: 78.0-81.1) from the POBASCAM trial (Maaike G. Dijkstra *et al.*, 2014). If co-testing with HPV genotyping, the 4 year longitudinal sensitivity increases to 90.3% (95% CI: 85.7-93.5) while specificity is reduced 57.6 (95% CI: 53.3-61.7) for the detection of CIN 2+ (Maaike G. Dijkstra *et al.*, 2014).

An important point to consider is the risk associated with a negative triage test. Based on the guidelines that a negative triage test should imply a <2% risk of CIN 2+ to allow a women to return to normal recall (Arbyn *et al.*, 2017) any potential triage tests should attempt to meet that criteria alone or in combination with another triage test to avoid the recall of the women over a shorter interval. The PaVDaG (Papillomavirus Dumfries and Galloway) trial that was conducted on a Scottish population assessed the pre and post-test risk of several triage strategies including cytology (ASC-US+), HPV 16/18 genotyping, p16INK4a/Ki-67 dual staining. They found that no individual triage test, when negative, had a low enough risk to allow women to return to routine screening (Stanczuk *et al.*, 2017). However, a combination of a negative p16INK4a/Ki-67 dual staining test and negative HPV 16/18 genotyping test brought the risk to <2% which would allow for a return to routine recall (Stanczuk *et al.*, 2017).

In terms of triage utilising methylation markers from RCT trials, there has been several retrospective case control/observational sub-studies from the FOCAL, POBASCAM and PROHTECT-3 trials. The FOCAL trial (n=257) utilised the S5 classifier (weighted methylation testing of EPB41L3 and HPV16L1, HPV16L2, HPV18L2, HPV31L1 and HPV33L2 genes) and the PROHTECT (n=364) utilised CAD M1-M18 and MAL M1 retrospectively on stored HPV positive samples. Both studies showed that methylation increased in relation to disease severity. Both also showed the methylation markers were comparable to cytology for the detection of CIN 3+. Colposcopy referral rates between cytology and methylation testing were also comparable for both studies with 3.0% and 4.3% of screened women being referred from the FOCAL trial (Cook *et al.*, 2018) and in the PROHTECT-3 trial 34.9%, 35.7% of triaged women would be referred (Verhoef *et al.*, 2015). The S5 classifier had a PPV of 33.1% for detection of CIN 2+ showing that a positive methylation test reaches both the USA and European thresholds for colposcopy referral (Arbyn *et al.*, 2011; Cook *et al.*, 2018). In another retrospective analysis from the PROHTECT-3 trial on a population of self-sampled women the combination of MAL M1, hsa-mir-124-2 and HPV 16/18 genotyping was assessed (n=515). It showed again that

methylation testing was non-inferior to cytology (Verhoef, Bosgraaf, *et al.*, 2014). Lastly, a retrospective analysis from the POBASCAM trial was assessed to determine the longitudinal outcome of methylation testing with FAM19A4 and hsa-mir-124-2 a HPV positive cohort that had a 14 year follow up period (n=1,040). At baseline 36.1% of women tested positive on the methylation test which showed 24 cancers from the follow up compared to cytology which was positive in 30.6% of women with 14 cancers on follow up. Kaplan-Meier estimates of the 14-year cumulative incidence rates for cervical cancer was 1.7% for those women who tested negative on the methylation test at baseline compared to 2.4% for those who were cytology negative at baseline. This showed that women who tested negative by these two markers had a much lower risk of cervical cancer in this HPV positive population (De Strooper *et al.*, 2018). These studies do however have their limitations. Both the FOCAL and PROHTECT trails had low population numbers and the PROHTECT trial was in self-sampled women. Common throughout each study too was the use of retrospective material with the 1,040 samples from the POBASCAM trial dating from 1999 to 2002. Though each study is of value they contain important limitations when used to draw conclusions of methylation triage in a HPV primary screening population in the present day. These limitations are addressed in this study such as the small sample size, retrospective nature of the studies and the lack of primary screening population data.

Methylation markers have been shown repeatedly to be non-inferior to cytology however, there is a need for further studies with longitudinal follow up assessing methylation tests alongside other triage options to correctly assess their long-term safety. To date there is no current Irish data on the use of methylation markers in a HPV primary screening programme. This chapter aims to show the utility of methylation testing and show that methylation markers can provide comparable to improved performances for the triage of HPV positive women. This study also has the benefits of longitudinal follow up that will go beyond this project. Though this will not be addressed at this time, the predictive power of the methylation test can be determined in subsequent follow ups.

5.2. Aims

The overall objective of this study was to examine, for the first time, the expression patterns of these three methylation markers for use in the context of a primary HPV screening programme.

The specific aims of this chapter are

i. to assess the Total Methylation Score, Paired Methylation Score and hsa-mir-124-2 in a large cohort of HPV positive cervical screening population;
ii. to assess the performance of the methylation panel as a triage test for HPV positive women;
iii. to compare the methylation triage test to other alternative triage strategies and assess the post-test risk of each triage strategy.

Materials and Methods

5.2.1. Study Population

CERVIVA, in partnership with CervicalCheck, are undertaking a HPV primary screening study, which is evaluating and comparing different strategies for the triage of women with a HPV positive primary screening test as shown in Chapter 2, Section 2.1.2 (Figure 2.1). In this chapter women who tested positive for HPV DNA on the Cobas HPV DNA test were assessed for their methylation status by CAD M1-M18, MAL M1 and hsa-mir-124-2 methylation markers (n=993). Each woman's methylation status was scored and classified using the Total Methylation Score (Cut Off 1: >0.13838), Paired Methylation Score(CAD:MAL) and hsa-mir-124-2 and classified as either positive or negative for methylation as outlined in Chapter 4.

5.2.2. Methylation Analysis

Real time singleplex qMSP was performed for detection of DNA methylation. Primer and Probe sequences and PCR conditions for CAD M1-M18, MAL M1 and hsa-miR124-2 and β-Actin have been previously described in Chapter 2, Section 2.3.5 and 2.3.6. All primers and probes are labelled with 5' FAM and 3' TAMRA. Each qMSP is run in duplicate and include controls for each target (SiHa DNA (positive control), pooled negative ThinPrep samples (negative control) and SiHa DNA that is Unconverted (bisulfite conversion control)). Thermocycling was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, 2010) under the following cycle conditions; 95°C for 15 minute followed by 45 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

5.2.3. Statistical Analysis

The disease endpoint for this observational cohort study is the detection of CIN 2+; normal cytology and CIN 1 histology are considered as no disease. For this study, the entire HPV DNA positive population have not yet been assessed for methylation. The methylation status was assessed using the Total Methylation Score, the Paired Methylation Score (CAD:MAL) as well as hsa-mir-124-2. Sample size calculations were calculated based on the initial validation panels lower 95% CI. To achieve the necessary sensitivity and

specificity of 72% and 84% respectively, with a precision of 0.05 a target of 886 HPV positive samples were required.

Binary logistic regression was used to calculate the Odds Ratio (OR) and 95% Confidence Intervals (CI) to determine the risk of testing positive for one of the triage tests in relation to age, cytology and HPV genotype. An associated Wald's p-value was calculated to determine if there was a statistical difference for each triage test in relation to their age, cytology or HPV genotype. McNemar's test was used to compare each triage strategy as well as both methylation tests in regard to their positivity rates. Kappa correlation (k) was used to determine the level of agreement between the two tests. The agreement level based of this statistic are as follows: 0-0.2 (None), 0.21-0.39 (Minimal), 0.40-0.59 (Weak), 0.60-0.79 (Moderate), 0.80-0.90 (Strong), 0.90+ (Almost Perfect). For all other statistics a p-value <0.05 was deemed statistically significant.

As this study is an observational study, all women enrolled are managed by their routine cytology screening result. As a result, the clinical performances cannot accurately be calculated for cytology or cytology containing triage options as women with a NAD on cytology are returned to routine recall and do not have histology follow up available. The clinical performances of the other triage options assessed by calculating sensitivity, specificity, PPV and NPV for detection of high-grade disease. The primary disease endpoint was histologically confirmed CIN 2+ diagnosed at first colposcopy visit. Histological confirmed CIN1/Normal and women with NAD on cytology were categorised as no high-grade disease. Those without this follow-up were not included in the calculations.

Each triage test was classified into Triage Strategies and the percentage referred to colposcopy was calculated. Those who tested negative for the triage test(s) were classified as "Not Referred to Colposcopy". This indicates either a shorter recall period or a return to routine recall depending on risk stratification. Risk for a positive or negative triage test was based on the triage tests PPV for a positive test result and 1-NPV in the case of a negative triage test. The cut off for acceptable risk limits are based off the current EU guidelines which states a post-test risk of >20% for CIN 2+ warrants a colposcopy referral while if a negative test imparts a risk of <2% for CIN 2+ then the women can return to routine recall (Castle *et al.*, 2008; Arbyn *et al.*, 2011, 2017). Those women who have a risk classification between 2-20% are considered to be at too low a risk for referral to colposcopy but also to high a risk to return to routine screening intervals. This group would be followed up with a repeat screening test at around 12 months.

5.3. Results

5.3.1. Population Characteristics for Methylation

In total, 993 HPV positive samples have been tested using the methylation panel with hsa-mir-124-2, the Total Methylation Score and the Paired Methylation Score (CAD:MAL) determining positivity. This population is representative of the study's overall HPV triage population with respect to the distribution of age and cytology. A boxplot of hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score (CAD:MAL) across each cytological grade is shown in Table 5.2. There is a statistically significant increase in the expression of hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score for those with a HSIL ($p<0.001$) compared to NAD, ASC-US and LSIL. The Total Methylation Score also had a statistically higher methylation score compared to hsa-mir-124-2 and the Paired Methylation Score in LSIL and HSIL categories ($p <0.005$). The Paired Methylation Score had a significantly lower methylation score compared to either hsa-mir-124-2 or the Total methylation Score ($p<0.001$).

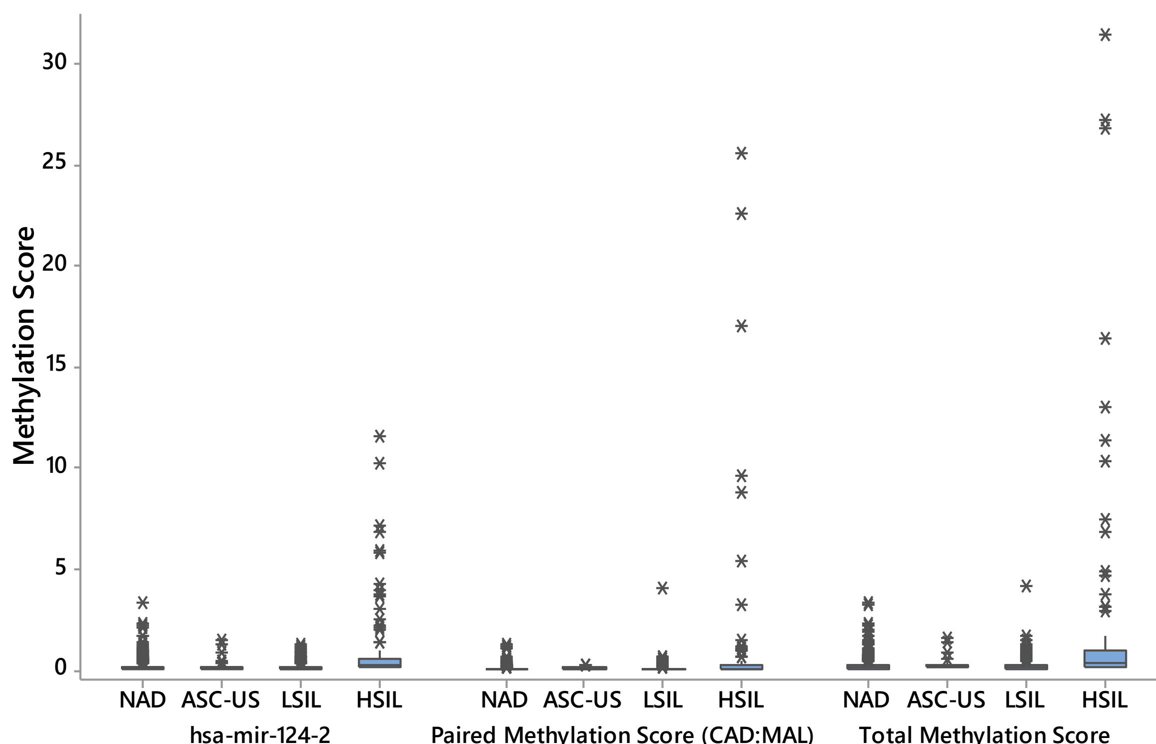

Figure 5.2: Boxplot of hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score (CAD:MAL) stratified by cytology
+ denotes outliers on the boxplot.

5.3.2. HPV Positive Population Stratification

Overall, 41.59% (413/993), 39.58% (393/993) and 37.76% (375/993) of the HPV positive women were classified positive by either hsa-mir-124-2, the Total Methylation Score (>0.13838) or the Paired Methylation Score. If triaged using cytology, 38.27% (380/993) had an ASC-US+ finding while if triaged by HPV 16/18 alone 30.72% (305/993) of women were positive. When hsa-mir-124-2, Total Methylation Score, Paired Methylation Score were stratified by age there is a significant trend of increasing positivity for each of the methylation triage approaches with increasing age most notably in hsa-mir-124-2 and the Total Methylation Score (significance range p= 0.035 to <0.001) (Table 5.1). This is in contrast to cytology triage which has a significant decreasing trend of positivity with increasing age (significance range p= 0.005 to <0.001)). The Total Methylation Score, hsa-mir-124-2 stratified by cytology showed that there was no significant difference between NAD and ASC-US/LSIL but women with a HSIL* finding had increased odds of having a positive methylation test (OR:3.73, p<0.001, OR:2.47, p<0.001) (Table 5.2). However, the Paired Methylation Score was significant for ASC-US, LSIL and HSIL compared to NAD cytology (significance range p=0.016-<0.001). Lastly, when the three methylation approaches were stratified against HPV genotypes there was no significant difference in methylation positivity (Table 5.3). This was also true when cytology (ASC-US+) was stratified by HPV genotype

Table 5.1: Rates of Positivity for hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score across age with the associated Odds Ratio and Wald's p-value.

Age	% hsa-mir-124-2 Positive	OR (95% CI)	p-value	% Total Methylation Positive	OR (95% CI)	p-value	% Paired Methylation Positive	OR (95% CI)	p-value
<30	35.16% (135/384)	-	-	31.77% (122/384)	-	-	30.73% (118/384)	-	-
30-39	42.59% (161/378)	1.3685 (1.02-1.83)	0.035	40.21% (152/378)	1.4401 (1.06-1.95)	0.020	39.95% (151/378)	1.4995 (1.11-2.02)	0.008
40-49	45.70% (69/151)	1.5520 (1.05-2.27)	0.024	47.02% (71/151)	2.0469 (1.38-3.04)	<0.001	45.03% (68/151)	1.8468 (1.25-2.71)	0.002
50+	60.00% (48/80)	2.7667 (1.68-4.53)	<0.001	60.00% (48/80)	3.4969 (2.10-5.79)	<0.001	47.50% (38/80)	2.0395 (1.25-3.32)	0.004

Table 5.2: Rate of Positivity for hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score across cytology with the associated Odds Ratio and Wald's p-value.

Cytology	% hsa-mir-124-2 Positive	OR (95% CI)	p-value	% Total Methylation Positive	OR (95% CI)	p-value	% Paired Methylation Positive	OR (95% CI)	p-value
NAD	40.95% (9251/613)	-	-	37.19% (228/613)	-	-	31.00% (190/613)	-	-
ASC-US	37.50% (15/40)	0.8653 (0.44-1.67)	0.668	35.00% (14/40)	0.9505 (0.48-1.88)	0.884	62.50% (25/40)	3.7105 (1.91-7.19)	<0.001
LSIL	35.51% (87/245)	0.7941 (0.58-1.07)	0.141	35.92% (88/245)	1.0596 (0.77-1.45)	0.721	39.59% (97/245)	1.4591 (1.07-1.98)	0.016
HSIL*	63.16% (60/95)	2.4724 (1.58-3.86)	<0.001	66.32% (63/95)	3.7343 (2.32-5.99)	<0.001	66.32% (63/95)	4.3831 (2.77-6.93)	<0.001

HSIL included any ASC-H, AGC, SCC and AIS cytology*

Table 5.3: Rate of Positivity for hsa-mir-124-2, the Total Methylation Score and Paired Methylation Score across HPV genotyping with the associated Odds Ratio and Wald's p-value.

HPV Genotype	% hsa-mir-124-2 Positive	OR (95% CI)	p-value	% Total Methylation Positive	OR (95% CI)	p-value	% Paired Methylation Positive	OR (95% CI)	p-value
hrHPV	40.26% (277/688)	-	-	38.66% (266/688)	-	-	35.90% (247/688)	-	-
HPV 16	43.19% (101/230)	1.1617 (0.85-1.57)	0.330	41.74% (96/230)	1.0168 (0.73-1.40)	0.920	41.74% (96/230)	1.2791 (0.94-1.73)	0.114
HPV 18	46.67% (35/75)	1.2983 (0.80-2.09)	0.285	41.33% (31/75)	0.9352 (0.56-1.54)	0.794	42.67% (32/75)	1.3287 (0.81-2.15)	0.249
HPV 16/18	44.59% (136/305)	1.1940 (0.90-1.56)	0.202	41.64% (127/305)	1.1319 (0.86-1.48)	0.376	41.97% (128/305)	1.2912 (0.98-1.70)	0.069

Abnormal cytology= ASC-US+

HPV 16/18 includes all samples that are HPV 16 or HPV 18 positive or both

The rate of positivity for the Total Methylation Score, hsa-mir-124-2 and the Paired Methylation Score across histology is shown in Table 5.4. There was a significant increase in the number of positive results for all three tests for detection of CIN 3+ ($p<0.001$). For CIN 1-3 there was no statistical difference between each of the methylation tests (significance range: p=0.829-0.115).

In women with normal histology, the Paired Methylation Score was significantly less positive compared to both the Total Methylation Score and hsa-mir-124-2 (p=0.012, 0.001). The Total Methylation Score was also significantly less positive than hsa-mir-124-2 in this normal group also (p=0.026). The Total Methylation Score and hsa-mir-124-2 showed a moderate to strong agreement across histological grades (kappa range: 0.732-0.829). However, both the Total Methylation Score and hsa-mir-124-2 did not have a strong agreement with the Paired Methylation Score (kappa range: 0.500-0.115). In this cohort, there was 1 case of AIS, and 5 SCC cases were all detected by both the Total Methylation Score and hsa-mir-124-2 with 1 SCC case being negative for the Paired Methylation Score.

Table 5.4: Rate of Positivity for hsa-mir-124-2 Total Methylation and Paired Methylation Score positivity stratified by histology with the associated Odds Ratio and Wald's p-value (n=876)

	% hsa-mir-124-2 Positive	OR (95% CI)	p-value	% Total Methylation Positive	OR (95% CI)	p-value	% Paired Methylation Positive	OR (95% CI)	p-value
Normal	40.92% (259/633)	-	-	37.60% (238/633)	-	-	32.07% (203/633)	-	-
CIN 1	34.07% (46/135)	0.7483 (0.50-1.10)	0.145	31.85% (43/135)	0.7777 (0.52-1.15)	0.213	38.52% (52/135)	1.3302 (0.90-1.95)	0.146
CIN 2	42.42% (14/33)	1.0669 (0.52-2.16)	0.858	45.45% (15/33)	1.3866 (0.68-2.80)	0.363	60.61% (20/33)	3.2664 (1.59-6.69)	0.001
CIN 3*	56.00% (42/75)	2.5740 (1.56-4.23)	<0.001	53.33% (40/75)	2.6392 (1.61-4.31)	<0.001	52.00% (39/75)	3.0135 (1.84-4.91)	<0.001

HSIL included any ASC-H, AGC, SCC and AIS cytology*

CIN 3+ includes SCC (n=6)

Normal includes histologically confirmed Normal and cytology NAD

5.3.3. Clinical Performances of Potential Triage Approaches

The clinical performances for each triage strategy are shown in Table 5.5. This is calculated on a sub population (876/993) that had available histological follow up as well as women with a normal cytology result which were grouped as No Disease. As the study is an observational study all women enrolled are managed their cytology screening result. As a result, an accurate clinical performance cannot be calculated for cytology as women with a NAD on cytology are returned to routine recall and do not have histology follow up available (TS 1 and TS 2). If HPV 16/18 genotyping were to be the sole triage test it would have a sensitivity of 55.56% with a specificity of 73.05%. Methylation testing alone (TS 3) had a reasonable sensitivity and specificity for CIN 2+. However, the Paired Methylation Score had a statistically higher sensitivity compared to both hsa-mir-124-2 and the Total Methylation Score (p=0.045, 0.033) with the Paired Methylation Score also having a higher specificity (p=0.033, 0.020). Utilising HPV 16/18 partial genotyping with methylation testing on women with another hrHPV type (TS 4) showed comparable performances for all three methylation tests with a significant increase in sensitivity for CIN 2+ detection as well as an increase in the NPV. However, the specificity also decreased. The Paired Methylation Score had a marginally higher specificity compared to the other two methylation tests. Both the PPV and NPV for the Paired Methylation Test was higher than that of the Total Methylation Score and hsa-mir-124-2.

The clinical performance of methylation testing is altered when stratified by women <30 (n=329) and >45 years (n=120). For women <30 the sensitivity of the Total Methylation Score decreases to 50.98% with an increase of specificity to 70.61%. This also occurs for the Paired Methylation Score with a sensitivity and specificity of 49.02% and 72.76% for women <30. The sensitivity of hsa-mir-124-2 isn’t affected at 56.86% however the specificity increases to 66.67% for the detection of CIN 2+. For women >45 the inverse occurs for the Total Methylation Score with a sensitivity and specificity of 75.00% and 49.11% and for the Paired Methylation Score a sensitivity and specificity of 75.00% and 59.82%. The specificity for hsa-mir-124-2 increased slightly to 62.50% however specificity for CIN 2+ decreased to 49.11%. For women <30 years the PPV of both the Total Methylation Score Paired Methylation Score and hsa-mir-124-2 increased to 24.07%, 24.75% and 23.77% respectively with an associated decrease in the NPV to 88.74%, 92.10% and 89.37% respectively. However, for women >45 the NPV increased to 96.49%, 97.10% and 94.83% respectively. There was also a decrease in the PPV in women >45 years to 9.52%, 11.76% and 8.06 respectively.

Table 5.5: Clinical performance for the detection of CIN 2+ in a HPV positive triage cohort (n=876)

Triage Algorithm	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
Cytology alone (TS 1) *	98.15% (97.81-98.49)	79.56% (78.41-80.71)	40.30% (37.40-43.21)	99.67% (99.65-99.70)
HPV 16/18 Genotyping with Cytology (TS 2) *	100.00% (100.00-100.00)	57.16% (55.43-58.89)	24.71% (22.97-26.46)	100.00% (100.00-100.00)
Methylation Testing alone [a, b, c] (TS 3)	57.41% [a] (52.80-62.02)	60.29% [a] (58.59-61.98)	16.89% [a] (15.46-18.33)	90.96% [a] (90.25-91.68)
	56.48% [b] (51.85-61.12)	63.41% [b] (61.77-65.05)	17.84% [b] (16.28-19.39)	91.20% [b] (90.52-91.88)
	59.26% [c] (54.71-63.81)	66.80% [c] (65.23-68.37)	20.06% [c] (18.30-21.82)	92.10% [c] (91.50-92.70)
HPV 16/18 Genotyping with Methylation Testing [a, b, c] (TS 4)	78.70% [a] (75.54-81.86)	44.27% [a] (42.53-46.02)	16.57% [a] (15.37-17.77)	93.66% [a] (93.05-94.27)
	80.56% [b] (77.60-83.51)	46.48% [b] (44.72-48.24)	17.47% [b] (16.20-18.74)	94.44% [b] (93.92-94.97)
	80.56% [c] (77.60-83.51)	48.83% [c] (47.06-50.60)	18.13% [c] (16.80-19.45)	94.70% [c] (94.20-95.19)

- denotes data points that could not be computed. * Women are managed by cytology, this does not reflect an accurate clinical performance hsa-mir-124-2 [a], Total Methylation Score [b], Paired Methylation Score (CAD:MAL) [c].

5.3.4. Potential Triage Options for HPV Positive Women

There are several potential triage pathways that can be used in this HPV positive triage cohort, this includes the use of HPV genotyping, cytology and methylation testing in an individual basis or in combination with each other. Each triage pathway is shown below and denoted as Triage Strategy 1 to 4. Figure 5.3 shows Triage Strategy 1 which is triage of HPV positive women by cytology alone. Figure 5.4 shows the combined triage of HPV genotyping with reflex cytology on hrHPV positive women (Triage Strategy 2). Figure 5.5 shows triage by methylation alone (Triage Strategy 3). Figure 5.6 shows triage using HPV genotyping with methylation testing on hrHPV positive women (Triage Strategy 4). Triage Strategies 3 and 4 utilise the Total Methylation Score, Paired Methylation Score and hsa-mir-124-2. The prevalence of CIN 2+ in this cohort was 10.80% this represents a prevalence of 1.12% in the total population.

Each strategies end point is either referral to colposcopy or not referred to colposcopy. Management of women who are not referred to colposcopy may include either a return to routine recall or a repeat screening test within 12 to 48 months. This will be addressed in the following section. In our study cohort if HPV primary screening was the sole screening test it would refer 15.98% (1530/9577) of women to colposcopy. In comparison if cytology was used as the primary screening test alone it would refer 8.61% (n=954/11074) of women. From the 4 presented triage strategies Triage Strategy 3, Paired Methylation Score has the lowest overall referral rate at 37.76% followed by cytology alone at 38.27%. Triage Strategy 3 for both the Total Methylation Score and hsa-mir-124-2 have reasonable referral rates of 39.58% and 41.59% respectively. In the perspective of the entire HPV primary screening population this represents 3.92%, 3.97%, 4.10% and 4.31% of the overall population being referred to colposcopy by these approaches. Compared to the current screening approach of cytology triaged by HPV which would refer 5.61% (608/11074) of women. Cytology alone (Triage Strategy 1) detected 106 of the 108 cases of CIN 2+. The two cases that were cytology NAD were likely detected during the cervical smear. The use of HPV 16/18 genotyping with cytology triage (Triage Strategy 2) however detected the 2 cases of CIN 2+ that was not previously detected by cytology alone. None of the methylation Triage Strategies detected 100% of CIN 2+ cases. Methylation Testing alone detected 61, 64 and 62 of the 108 cases of CIN 2+ using the Total Methylation Score, Paired Methylation Score and hsa-mir-124-2 methylation marker respectively. The addition of HPV 16/18 genotyping to methylation testing increased to overall detection of cases of CIN 2+ to 80.56% for the

Total and Paired Methylation Score and 78.70% for hsa-mir-124-2. This however increased the referral rate of the triage population by around 17%.

For Triage Strategies 1 and 2 under 30 had a higher referral rate than those over 30 years of age by up to 10% (Table 5.6). Triage Strategies 3 and 4 however had a lower rate of referral in the under 30's (Table 5.6). This reflects the trend seen in Table 5.1 where methylation testing increases with age. When each Triage Strategy is compared to each other we find that Triage Strategy 1 was statistically different from Triage Strategy 2 and 4 ($p<0.001$) but not Triage Strategy 3 ($p=0.669$-0.469) in relation to referrals. Triage Strategy 2 was statistically different from Triage Strategy 3 ($p<0.001$) but not Triage Strategy 4 ($p=0.129$). Triage Strategy 3 was statistically different from Triage Strategy 4 and 5 ($p<0.001$). This shows that there was no statistical difference between cytology alone and methylation alone for triaging HPV positive women for the overall referral rates. As well as this there was no difference between the two strategies that incorporated HPV genotyping with either cytology or methylation testing on the hrHPV genotype.

When Triage Strategy 3 was assessed in women <30 the referral rate decreases to 31.59%, 30.55% and 35.25% for Total Methylation Score, Paired Methylation and hsa-mir-124-2. Of the cases of CIN 2+ (n=58) the Total Methylation Score detected 50.98% (n=26), Paired Methylation Score detected 49.02% (n=25) and hsa-mir-124-2 detected 56.86%(n=29). For women >45 years the referral rates increased to 52.21%, 44.12% and 51.47% respectively. Of the cases of CIN 2+ (n=8) these three approaches detected 75.00% (n=6), 62.50% (n=5) and 75.00% (n=6) respectively.

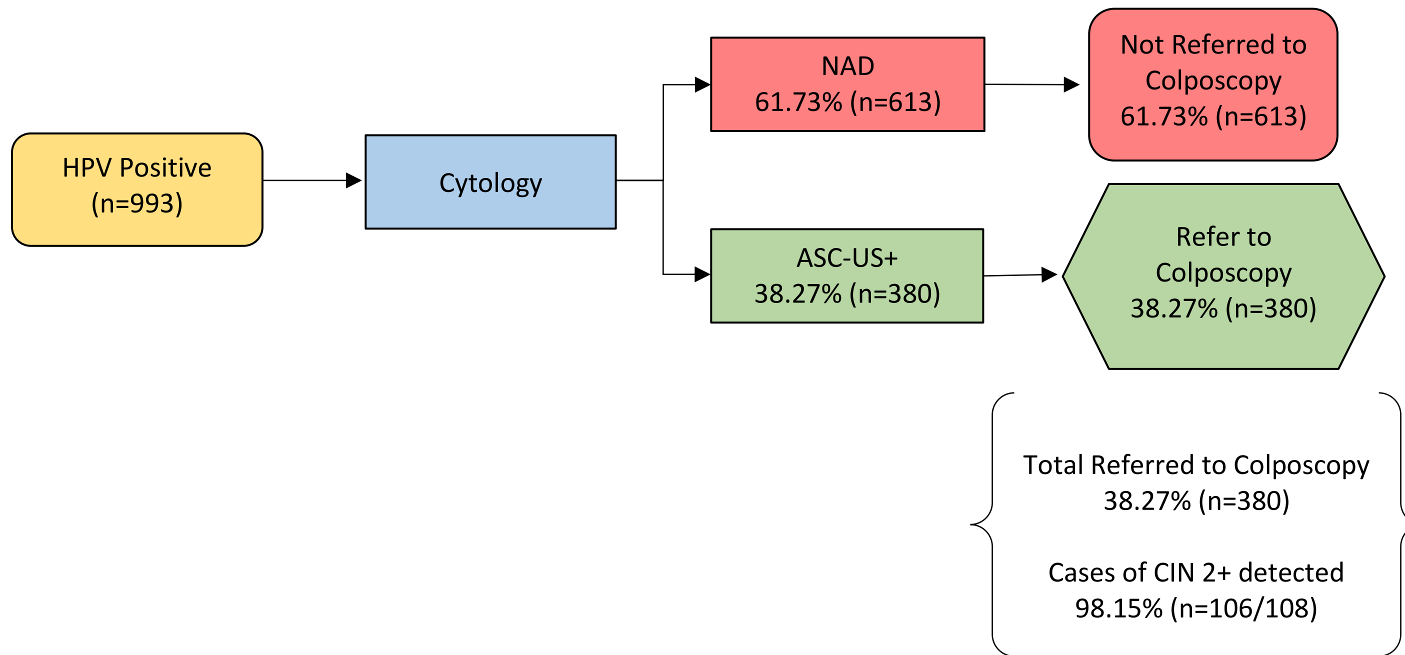

Figure 5.3: Triage of HPV positive women by cytology alone. Positive cytology is considered ASC-US+ (Triage Strategy 1). 2 cases of CIN 2+ were diagnosed during the cervical smear and reported on at that time.

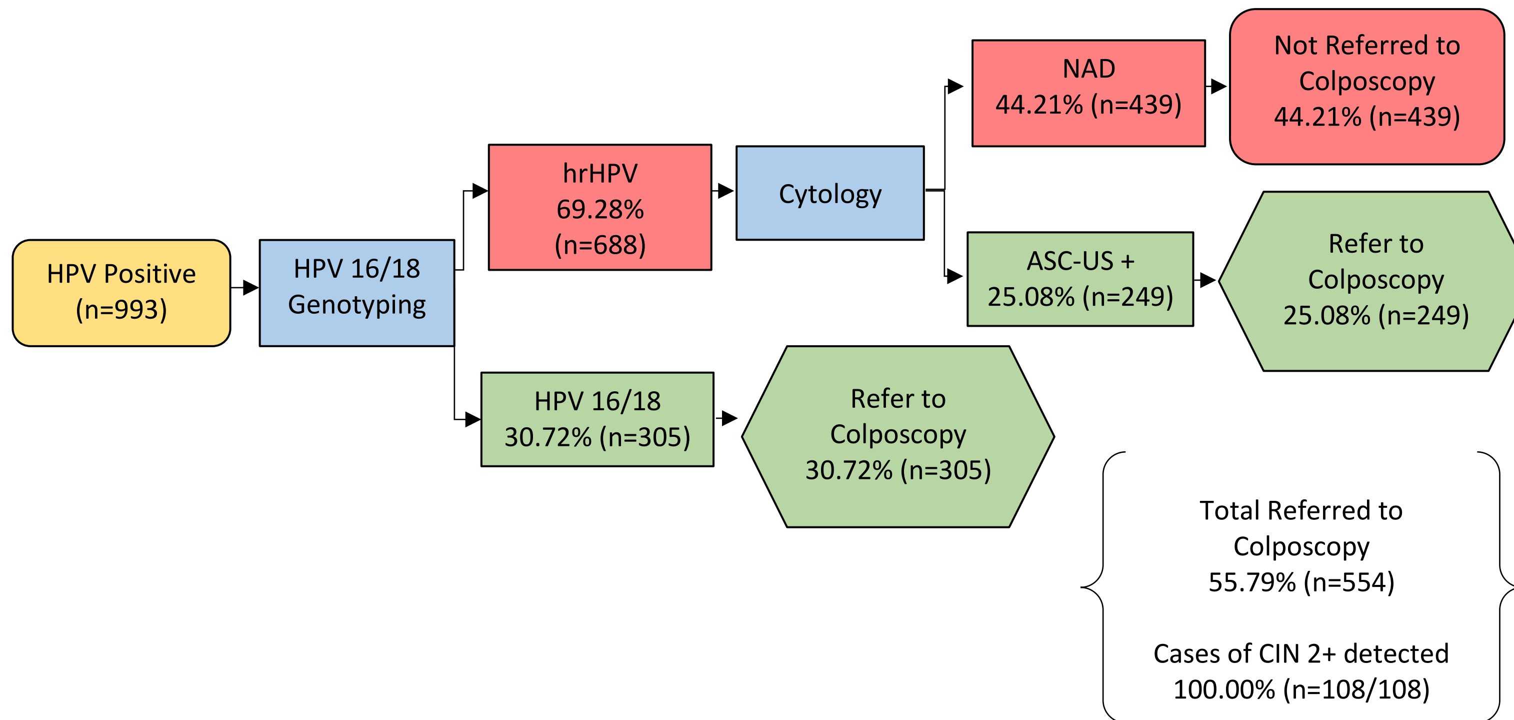

Figure 5.4: Triage of HPV positive women by HPV genotyping with reflex cytology. Positive cytology is considered ASC-US+. (Triage Strategy 2)

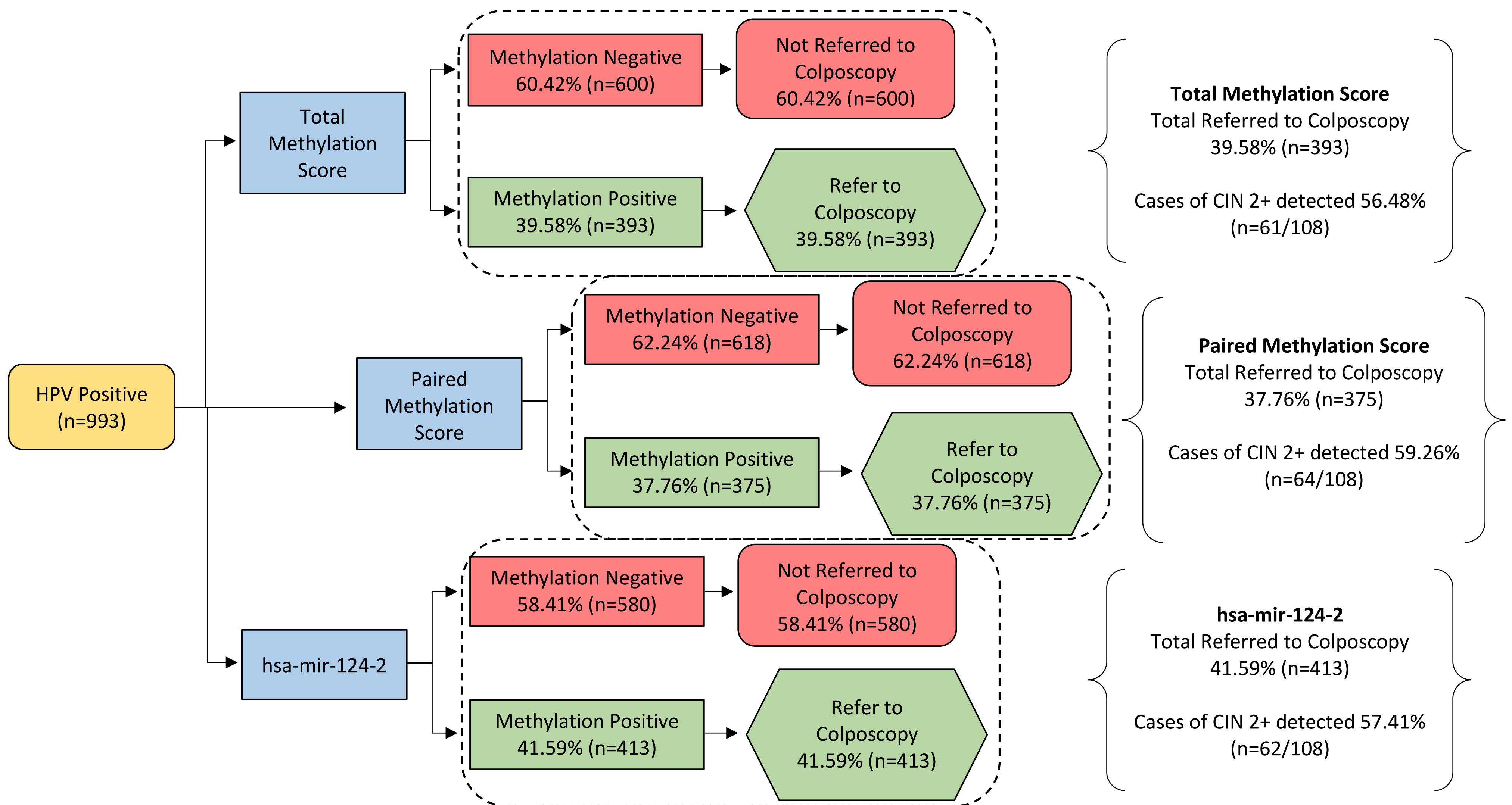

Figure 5.5: Triage of HPV positive women by methylation testing with either the Total Methylation Score, Paired Methylation Score or hsa-mir-124-2 (Triage Strategy 3). Each methylation test is shown independently of each other here, separated by a dotted box.

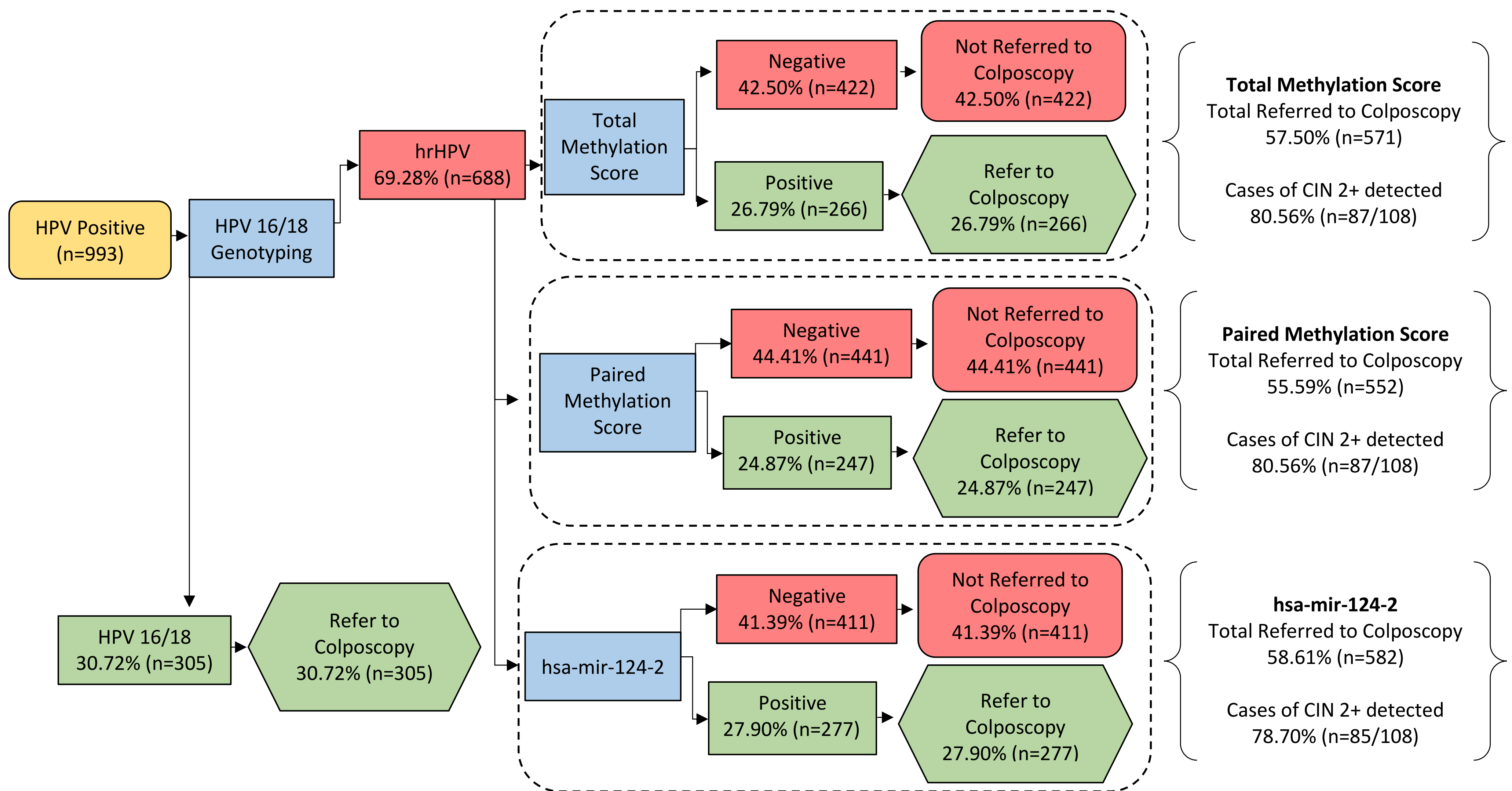

Figure 5.6: Triage of HPV positive women by HPV genotyping with reflex methylation testing of hrHPV women with either the Total Methylation Score, Paired Methylation Score or hsa-mir-124-2 (Triage Strategy 4). Each methylation test is shown independently of each other here, separated by a dotted box.

Table 5.6: Referral Rate for each triage strategy for the entire HPV primary screening cohort, the triage cohort and the triage cohort stratified by age.

	Primary Screening Population Referral Rate	**HPV Positive Triage Referral Rate**	**Triage Referral Rate <30's**	**Triage Referral Rate >30's**
Cytology alone (TS 1)	3.98%	38.27%	47.14%	32.68%
HPV 16/18 Genotyping with Cytology (TS 2)	5.78%	55.79%	61.98%	51.98%
Methylation Testing alone [a, b, c] (TS 3)	4.31% [a]	41.59% [a]	35.16% [a]	45.65% [a]
	4.10% [b]	39.58% [b]	31.77% [b]	44.50% [b]
	3.91% [c]	37.76% [c]	30.73% [c]	42.20% [c]
HPV 16/18 Genotyping with Methylation Testing [a, b, c] (TS 4)	6.07% [a]	58.61% [a]	53.65% [a]	61.74% [a]
	5.96% [b]	57.50% [b]	53.13% [b]	60.26% [b]
	5.76% [c]	55.59% [c]	51.82% [c]	57.96% [c]

hsa-mir-124-2 [a], Total Methylation Score [b], Paired Methylation Score [c].

5.3.5. Risk of CIN 2+ Following Triage

The current European guidelines for risk states that a woman must have a >20% risk for CIN 2+ to warrant colposcopy referral or a <2% risk of CIN 2+ to allow the safe return to routine screening. Figure 5.7 shows the risk stratification of each of the Triage Strategies. As this population is managed by cytology it is over estimated in this analysis. Cytology only (Triage Strategy 1) achieves a positive triage test risk of 40.30% with a negative triage test risk of 0.33%. The use of HPV 16/18 genotyping with cytology has a lowered the positive triage test risk to 24.71% but improved the negative triage test risk (0.00%). For Triage Strategy 3 the risk following a positive methylation test for either the Total Methylation Score or hsa-mir-124-2 is comparable (17.84%, 16.89%) as well as with a negative triage test (8.08%, 9.04%). The Paired Methylation Score however had a positive triage test risk of 20.06% (95% CI:18.30-21.89) indicating a positive Paired Methylation test is sufficient to refer those women to colposcopy. The use of HPV 16/18 genotyping in combination with methylation testing (Triage Strategy 4) does not affect the positive triage test risk for the Total methylation Score or hsa-mir-124-2. It does however lower the PPV of the Paired Methylation Score to 18.13%. HPV 16/18 genotyping does decrease the risk from a negative triage test to 5.56%, 6.34% and 5.30%.

Shown is also the Paired Methylation Score for women <30 and >45. In women <30 years the positive triage test risk increased to 25.00% but the negative triage test risk also increased to 11.35%. In women >45 years the risk from a positive triage test decreased to 11.76%. However, the risk from a negative triage test decreased to 2.89% (95% CI: 2.34-3.56)

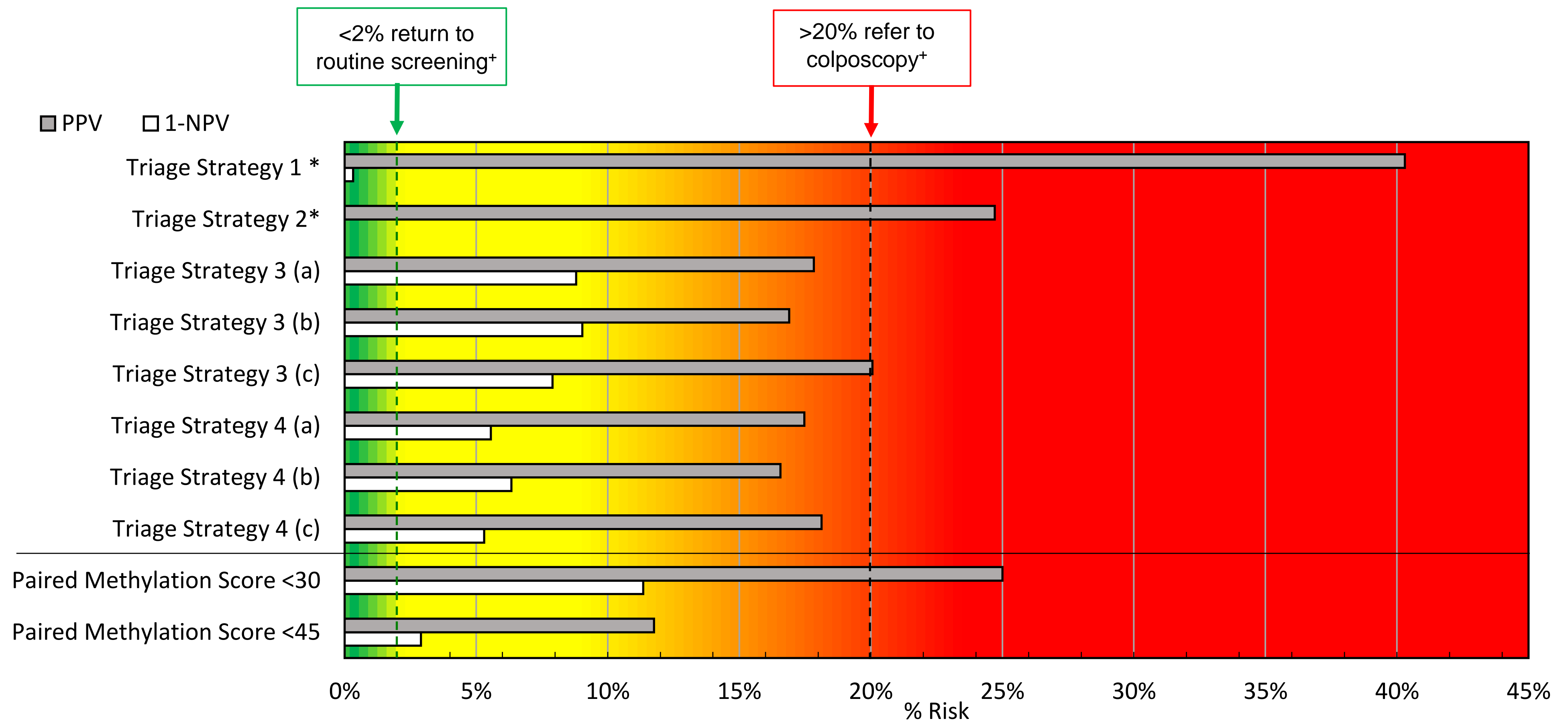

Figure 5.7: Risk stratification of each triage strategy the for safe referral to colposcopy or to the routine screening population following a positive (grey) or negative (white) triage test. Risk for the Paired Methylation Score for women <30 and >45 is also shown.
*Includes cytology managed triage pathways, +Risk stratification adapted from Castle et al., 2008; Arbyn et al., 2011, 2017, (a) Total Methylation Score, (b) hsa-mir-124-2 methylation marker, (c) Paired Methylation Score.

5.4. Discussion

The aim of this chapter was to assess the methylation panel validated in Chapter 4. For the first time these three methylation markers were examined for the triage of HPV positive women from a "real world" primary screening population. Three of these approaches were assessed in the general HPV positive triage population, the Total Methylation Score, the Paired methylation Score (CAD:MAL) and hsa-mir-124-2. It also aimed to assess the methylation panel in regard to its clinical performances, referral rates and how it compares overall to cytology triage with and without prior HPV 16/18 partial genotyping.

From the initial assessment of the Total Methylation Score, Paired Methylation Score and hsa-mir-124-2 in this HPV positive triage population there was the expected increase in methylation associated with the increased severity of the cytological findings. This trend is consistent with previous literature for the vast majority of methylation markers (Steenbergen et al., 2004; Wilting et al., 2010; Overmeer et al., 2011; Bierkens et al., 2013; De Strooper, van Zummeren, et al., 2014; Verhoef et al., 2015; Lorincz, 2016; Cook et al., 2018). Interestingly when the three methylation tests were stratified by age and compared against cytology we see that the rate of positive methylation tests increases with age in comparison to abnormal cytology which has a decreasing trend with age, consistent with current literature (Monsonego et al., 2015). This would be consistent with the hypothesis that DNA methylation is also an age related alteration (Xu and Taylor, 2014). Previous studies have postulated that those who have increased levels of site-specific methylation associated with age could be at a higher risk for malignant transformation. We see this in our population, with significant increases in methylation related to increased age ($p<0.05$). Interestingly there was no associated difference between the HPV genotypes 16, HPV 18 or the remaining 12 hrHPV types and the rate of positive methylation tests in the overall population. This may suggest that although HPV 16/18 are the two leading HPV genotypes for the development of cervical cancer that, at the population level, methylation for these markers progresses at a similar rate regardless of HPV type. This may be an important consideration in the context of a HPV vaccinated cohort.

For each of the three methylation tests, there was no statistical difference between them in regard to their CIN grades. The Paired Methylation Score was more specific compared to the Total Methylation Score and hsa-mir-124-2 detecting significantly fewer of the normal cohort. The Paired Methylation Score was also significantly more positive in the CIN 1 to CIN 3 cases compared to normal cohorts unlike the other two methylation tests which were

only significantly different for CIN 3+. The Paired Methylation Score did however miss 1 of the 5 SCC cases.

Producing every possible triage strategy from the methylation data in Chapter 4, cytology and HPV genotyping would not have been feasible in this thesis. Four strategies were selected to be focused on utilising cytology and the three methylation biomarkers with or without prior HPV genotyping. As women were managed by cytology the sensitivity was quite high in this triage pathway but the specificity does fall into the range previously shown in the ATHENA, ARTISTIC, NTCC and the Public health trial Finland data (Ronco, Giorgi-Rossi, *et al.*, 2006; Ronco, Segnan, *et al.*, 2006; Castle *et al.*, 2011; Leinonen *et al.*, 2013; C Kitchener *et al.*, 2014; Bergeron *et al.*, 2015; Wright *et al.*, 2017). Utilising HPV 16/18 genotyping prior to cytology increased the sensitivity slightly however the specificity decreased due to the increase in false positive results. If HPV 16/18 genotyping were to be the sole triage test however the sensitivity would remain at 55% with a specificity of 73%.

Performance of Methylation Testing in a HPV Positive Triage Population.

Strategy 3 utilising methylation testing alone showed the Paired Methylation Score had the highest sensitivity and specificity compared to the other two methylation tests. Overall, this was a decrease in the clinical performance compared to the validation panel in Chapter 4. This range of sensitivities and specificities is however comparable to previously reported sensitivities and specificities ranging from 48% to 89% and 47% to 81% (Lorincz, 2016). There has been no published clinical performance, data to the best of my knowledge, on the use of hsa-mir-124-2 alone with the exception of its initial discovery paper (Wilting et al., 2010). This methylation marker has been commonly used in combination with MAL M1 (Hesselink et al., 2014), CAD M1-M18 and MAL M1 (De Strooper, Hesselink, *et al.*, 2014) or FAM19A4 (De Strooper et al., 2016). In this cohort, hsa-mir-124-2's clinical performance for a single methylation marker is extremely positive. For example the tri-marker panel of CAD M1-M18, MAL M1 and hsa-mir-124-2 only achieved a sensitivity of 48% for the detection of CIN 2+ (De Strooper, van Zummeren, *et al.*, 2014). When each of these methylation approaches are stratified by women <30 which would represent the majority of women who would test positive for HPV the specificity of the methylation approached decreases slightly with an associated increase in the specificity. The PPV in these women also increased to >20% indicating that in this population a positive methylation tests could provide the adequate risk to refer a woman to colposcopy. For women >45 years (managed under a 5-year interval in the current cytology screening programme) the sensitivity of the three methylation approaches increases with a decrease in the specificity. This had the benefit of decreasing the NPV for all three methylation approaches. The use of HPV 16/18

genotyping prior to methylation testing in Triage Strategy 4 significantly increases the sensitivity of the triage test overall. For both the Total Methylation Score and the Paired Methylation Score the sensitivity increased to 80.56% for detection of CIN 2+.

In regard to each strategy that utilised a single triage test, these showed a lower referral rate than triage strategies incorporating a two-step serial triage approach with HPV genotyping. This results in the associated loss in specificity for this serial approach compared to a single triage test. However, compared to previous trials such as the FOCAL and PROHTECT-3 the overall referral rates are comparable. In this study cohort cytology, testing alone would refer 3.98% of the total screened population or 38.27% of the triaged population to colposcopy. The Paired Methylation Score had the lowest referral rate of any triage test with 3.91% or 37.76% respectively. For the Total Methylation Score this was comparable with referral rates of 4.10% and 39.58% respectively. hsa-mir-124-2 had a marginally higher referral rate of 4.31% and 41.59%.These were comparable to the two retrospective studies in which the FOCAL trial would refer 3.0% of cytology triaged women and 4.3% of methylation tested women to colposcopy (Cook *et al.*, 2018). In the PROHTECT-3 trial 34.9% of women triaged by cytology were referred by colposcopy, 35.7% by methylation testing. Methylation triage testing alone therefor is comparable to cytology for its overall referral rates in this population.

Post-Test Risk of Methylation Triage Testing.

It is a known issue with cytological triage that women who test NAD on cytology do not have a low enough risk of CIN 2+ to safely return to the routine recall (Stanczuk *et al.*, 2017; Wentzensen *et al.*, 2017; Cuschieri *et al.*, 2018). In fact many triage tests do not meet this criteria currently (Stanczuk *et al.*, 2017). This can lead to those women being recalled over shorter period of 12 months for a repeat HPV screening test. This shorter interval is required to ensure the safety of the women before being discharged back into the routine recall population. A major issue with this process is that HPV infections can persist up to 18 months, if not longer in women and thus require two or more annual screening checks before they return to routine screening (Trottier *et al.*, 2008; Ramanakumar *et al.*, 2016). Ideally, methylation testing of these HPV positive, NAD women which make up the majority of this triage population (61.73%) would allow for the safe return of up to <60% of this population while referring around 35% of them either to colposcopy or a 12 month follow up. Though this thesis cannot vouch for the safety of this approach the longitudinal aspect of this study will hopefully address this. A secondary potential aspect of methylation testing could also be its use in returning a portion of women back to routine screening by utilising a highly specific cut-off point.

The last important factor for any triage test is the positive and negative risks associated with the test. The Paired Methylation Score was the only methylation test to exceed the 20% required risk. However, in women <30 all three methylation approaches achieved a positive triage test risk of >20%. Neither the Total Methylation Score or hsa-mir-124-2 alone or in combination with HPV 16/18 was sufficient to reach the 20% threshold in this cohort. Previous studies have shown methylation markers to have a high enough risk to warrant colposcopy referrals however many of these generally have had smaller sample sizes ranging from 171 to 388 with skewed disease prevalence (Overmeer *et al.*, 2008, 2011; Hesselink *et al.*, 2011; Bierkens *et al.*, 2013; De Strooper, Hesselink, *et al.*, 2014; De Strooper, van Zummeren, *et al.*, 2014; Verhoef *et al.*, 2015). Larger studies have looked at combinations of these markers such as the PROHTECT-3 (n=515) (MAL M1, hsa-mir-124-2 and HPV 16/18 genotyping) but have been retrospective studies. This subpopulation from the HPV positive cohort matches both age, cytology and HPV 16/18/hr genotype distributions as is found in the overall HPV positive population. From this, we can expect the cases of CIN 2+ as well as <CIN 2 to be representative of an Irish HPV primary screening cohort. This does not seem to be the case in many of these other papers, which may suffer from a skewness towards CIN 2+ cases and a slightly inflated PPV. Risk of CIN 2+ with a negative test however is too high to warrant a return to routine screening for any of the methylation triage test approaches. In women >45 years the Paired Methylation Score did show promise with a negative triage test risk of 2.89%, the lowest value from any approach investigated but still insufficient for the safe return of these women to routine screening. This is a known problem with the currently available triage test as many do not currently meet this criteria (Stanczuk et al., 2017). Potentially, retesting HPV positive methylation negative women in 12 to 48 months would identify women with persistent HPV infection and also detect most of the remaining CIN2+ cases. (Lorincz 2016). This could be a future potential option for triage testing and an approach likely to be taken with cytological triage.

Conclusions.

Though HPV primary screening will undoubtably be a superior test than cytology, the increased rate of referrals to colposcopy would be greatly increase the burden on colposcopy clinics. At this point in time, there is no ideal triage test available to allow a HPV positive woman to return to a routine recall system. The likelihood is women who are HPV positive with a negative triage test will have to return in 12 months to ensure no cervical abnormality is missed and from this thesis this would still hold true for methylation triage currently. Many researchers believe methylation panels to be the ideal triage test for these

women, but it is still an evolving field of research and the optimal methylation markers and approach to testing has yet to be determined. The novel summation approach to create a Total Methylation Score does show promise in this field. As has been shown however, the combination of the individual methylation markers is important and where the Total Methylation Score performed the best in Chapter 4 both hsa-mir-124-2 and especially the Paired Methylation Score incorporating CAD M1-M18 and MAL M1 showed a superior ability to detect CIN 2+ in this cohort which reflects a true HPV triage population. It is an important factor to remember that much of the work in methylation testing for cervical cancer is based on small validation cohorts and the need for more larger scale studies to show if the performance of these markers' scales appropriately as has been attempted in this thesis. This is also the first study to examine these markers outside the confines of triage or self-sampling populations and assess them in a "real world" setting of a HPV primary screening population. The combination of HPV 16/18 genotyping and methylation testing does however show great potential in triage. Though this would increase the referral rates in the initial screening rounds, we would expect that over time the referral rates would decrease as many early pre-cancerous lesions would be detected as was seen with cervical screening with the introduction of CervicalCheck in 2008 (National Cancer Registry Ireland, 2018b, 2018a).

In conclusion, the findings support the incorporation of methylation testing as a triage test for HPV positive women. Of the three methylation approaches alone the Paired Methylation Score shows the best sensitivity, specificity and PPV of the three. With the incorporation of HPV16/18 genotyping either the Total Methylation Score or Paired Methylation Score could be used effectively. Though hsa-mir-124-2 performs remarkable well as a single methylation biomarker. However, it lacks the overall sensitivity and specificity that the other two summation scores have. It would still be advisable to incorporate multiple methylation markers into any triage panel to allow for the broad range of abnormal methylation to be detected. Triage tests to date have shown the inability to reach a <2% risk of CIN 2+. In this study however, methylation testing if properly applied to an older cohort may provide a sufficiently reduced risk from a negative methylation test. The overall referral rates for both cytology and each methylation approach with or without HPV 16/18 testing is comparable.

5.5. References

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-Chapter 6-

Discussion

In 2017, HiQA recommended a change to primary HPV screening followed by cytology triage at five-yearly intervals for all women aged 25 to 60 years with unvaccinated women aged 28 also requiring a HPV screening test (HIQA, 2017). This was based on the knowledge that HPV testing was very specific for high-grade precancerous disease in comparison to the current cytology-based screening. However, due to the high prevalence of transient HPV infection HPV testing suffers from a low specificity for disease. This forms the primary challenge for HPV primary screening; how to adequately triage HPV positive women. CERVIVA are undertaking the HPV primary screening pilot programme in partnership with CervicalCheck, the National Cervical Cancer Screening Programme, with the aim to address these issues. This thesis has two primary aims, firstly, to assess the HPV prevalence of HPV DNA and mRNA and compare two HPV testing approaches. Secondly, to assess a panel of methylation biomarkers [CAD M1-M18, MAL M1 and hsa-mir-124-2] to triage the population of HPV positive women and correctly identify those with high grade, cervical pre-cancer.

There are many HPV testing platforms available on the market utilising different testing methods and targets. For those platforms that are validated based on the Meijer criteria (Meijer *et al.*, 2009), studies have shown HPV testing for DNA and mRNA to be relativly comparible (Koliopoulos *et al.*, 2017). There have, however, been few head to head comparisons performed in prosepctive studies. In this study, two potential HPV testing platforms utilising two unique testing approaches, HPV DNA and HPV E6/E7 mRNA were assessed using the Cobas HPV DNA test and Aptima HPV E6/E7 mRNA test. Data on an Irish screening population is vitally important now to inform the best practice of the screening programme going forward.

Performance and Comparison of HPV DNA and HPV E6/E7 mRNA Testing.

A portion of the work presented in this thesis was on the performance and comparison of two potential HPV platforms in a screening population (Chapter 3). 12,013 eligible, fully consenting women, attending for their routine cervical smear with the prevalence of HPV DNA being 15.98% and the prevalence of HPV mRNA being 13.22%.

In the context of a new cervical screening programme, what HPV test is implemented can be very important and having several laboratories utilising different tests with different targets and different detection limits could possibly lead to unnecessary intra-laboratory variation, something with which cytological based screening programmes suffer with globally. Chapter 3 aimed to assess and compare both the Cobas 4800 HPV DNA test and the Aptima HPV E6/E7 mRNA test. The clinical performance of both tests is comparable in

this cohort, with the sensitivity for both at around 96% and specificity at around 87-89% for detection of CIN 2+. HPV mRNA however is more specific at 76.42% compared to 70.82% for HPV DNA testing in women <30 (p=0.032). It is known that women <30 years generally have higher rates of transient HPV infection and in this study cohort, the majority of HPV infections occurred in women <30 years (34.40%, 30.54% for HPV DNA and mRNA respectively), double the rate of women between 30-39. With the current HiQA recommendation of 5 year screening intervals for women aged 25 to 60 (HIQA, 2017), this would constitute a large proportion of the HPV positive women. Unvaccinated women would also be recommended to be screened at 28, again, increasing the number of women <30 years requiring triage testing. Though the sensitivity of both HPV tests increased in women <30, the specificity for CIN 2+ decreased. With the high prevalence of transient HPV infections in women the difference in positivity of over 2.5% between HPV DNA and HPV mRNA testing would have long term benefits to the number of women requiring triage testing.

The NPV of both the Cobas and Aptima tests, regardless of age, remain very high at >99%, indicating an extremely low risk of missing relevant disease when a woman tests negative for either test. This would give an excellent level of assurance to women compared to cytological screening in which a negative cytology test has a much lower NPV (H. C. Kitchener *et al.*, 2009; H. Kitchener *et al.*, 2009; C Kitchener *et al.*, 2014). Though cytology detects the majority of CIN 2+ cases it still does not detect a small portion of CIN 2+ (Cuzick et al., 2006; Barut et al., 2015); to that end, women are screened over a shorter interval compared to HPV primary screening.

Both the HPV DNA and HPV mRNA tests have comparable sensitivities and NPV. However, HPV mRNA testing is more specific than HPV DNA testing. In any HPV primary screening programme however, both tests would provide the necessary level of sensitivity and reassurance around negative HPV results. My data suggests that the mRNA test may be more appropriate in women<30 years of age due to its increased specificity. This is relevant to the Irish cervical screening programme given that the decision has been made to screen women aged 25 to 65 years of age. Regardless of HPV mRNA's increased specificity in women <30, both testing approaches would still require an adequate triage test to reduce a referral rate of 13-15%. The choice of triage test therefore would dictate the final performance of the screening programme as well as the proportion of the population that would be referred on to colposcopy, back to routine recall or into a shorter recall group.

Triage Tests for HPV Positive Women.

Cytology is the most likely candidate for the triage of HPV positive women for the foreseeable future (HIQA, 2017). Logistically, this makes sense. Firstly, Ireland has well-trained cytologists as well as the necessary infrastructure and quality control systems in place. Secondly, a staged transition across to HPV primary screening utilising cytology triage would likely reduce any issues that may arise if the programme directly switched to HPV primary screening with a different triage test. Studies have shown that knowledge of a women's HPV status can lead to an increase in reporting of abnormalities resulting in increased sensitivity (Bergeron *et al.*, 2015). However, with HPV knowledge, there may also be a loss in the specificity of cytology (Richardson *et al.*, 2015). A Norwegian study also showed that those cytologists who had a high sensitivity for cervical pre-cancer were those with the lowest specificity overall (Sørbye *et al.*, 2017). The inherent subjective nature of cytology would also still exist when used in a triage capacity. As noted in the EUROGIN Roadmap 2017, over the long term, recruitment, training and retention of cytology staff may prove a challenge with HPV screening reducing the overall cytology work-load (Cuschieri *et al.*, 2018). Cytology is generally considered an appropriate interim for the triage of HPV positive women, but a more robust triage test will be required in the near future.

To that end, this thesis assessed a panel of the three most prominent and promising methylation markers at the time: CAD M1-M18, MAL M1 and hsa-mir-124-2. At the time of this study these three methylation markers constituted the majority of literature in the area of cervical cancer. Novel methylation markers are important in this field, however, there is a pressing requirement to compile data on the existing methylation markers and define relevant levels of methylation. This area of research lacks verification from other research groups and the longitudinal data that would be required to validate the safety of methylation testing in a screening programme. This is an aspect that can take a great deal of time and requires study. This thesis set out to address several issues. Firstly, to define what a clinically relevant level of methylation was in the context of the Irish population and to define relevant cut-off values for each of the methylation markers. Previous studies have used the criteria that the value of the 99% confidence interval of the methylation levels obtained in normal cervical controls would act as the cut-off for a positive methylation test. These normal specimens were generally normal cervix from hysterectomy specimen with no HPV infection present (Wilting *et al.*, 2010; Overmeer *et al.*, 2011). In other cases the thresholds for positivity were set my maximising the sensitivity for CIN 3+ at set specificity levels such as 70% (Hesselink *et al.*, 2011; Verhoef *et al.*, 2015). Using the 99% CI of women with a normal cervix or fixing the specificity can lead to underspecific or undersensitive thresholds. This been shown before with the combination of these three markers in the past having a

sensitivity of 48% and specificity of 81% (De Strooper, van Zummeren, *et al.*, 2014). This project aimed for an alternative approach and utilised a panel of normal, CIN 1, CIN 2 and CIN 3 samples to define clinically relevant methylation levels. This would hopefully improve the overall specificity of methylation testing as in the case of women with no HPV infection when methylation levels are exceedingly low (Chapter 4, Figure 4.4). This would hopefully allow methylation to be a single use triage test and not require the recall of women who test negative for methylation like the current cytology triage option.

A validation panel was used to determine clinically relevant cut off points in Chapter 4. Of all the combinations, the novel summation scores; Total Methylation Score, Paired Methylation Score (CAD:MAL) and the individual methylation marker hsa-mir-124-2 showed the best combination of sensitivity and specificity for detection of CIN 2+ with comparable performances to previous studies (Hesselink *et al.*, 2011, 2014; De Strooper, Hesselink, *et al.*, 2014; De Strooper, van Zummeren, *et al.*, 2014; Verhoef, Heideman, *et al.*, 2014; De Vuyst *et al.*, 2015; Lorincz, 2016). Of the three, the Total Methylation Score had the highest sensitivity and specificity. Going forward, it was decided that one of each of the scoring methods would be brought forward to stratify the HPV positive population. This included the Total Methylation Score, Paired Methylation Score and hsa-mir-124-2 as the top three potential methylation triage approaches. As outlined in Chapter 4 these three approaches would hopefully allow for a single triage test to be required in the HPV positive population. There are however two alternate options this thesis did not pursue. A highly sensitive but non-specific triage test such as a methylation panel utilising all three markers or a highly specific but non-sensitive test such as CAD M1-M18 alone or the Paired Methylation Score (MAL M1: hsa-mir-124-2) (Chapter 4 Figure 4.13, Table 4.8).

The rule of thumb is that high sensitivity helps to rule out disease and high specificity helps rule in disease (Parikh *et al.*, 2008). In the case of a highly sensitive test which lacks specificity the majority of disease would be detected. However, this approach would inevitably increase, not only colposcopy referrals, but also the rate of false positives. This could lead to over-referral to colposcopy and its associated risks, but it may allow women who test negative the security to be referred back to routine screening due to its lower inclusion criteria. Considering the natural progression of cervical cancer however a highly specific test with a lower sensitivity may be more applicable. In this instance women would benefit from a high specificity and confidence around a positive methylation test, but a negative methylation test would not rule out disease. This population would require more screening in the future under a shorter timeframe to ensure all relevant CIN 2+ is detected. The 'perfect' approach to triage is heavily driven by the context of the screening programme and the disease in question. As Solomon (2003) stated "an efficient triage test should

reduce overtreatment, patient anxiety, inconvenience and overall management costs, usually by reducing the number of diagnostic procedures performed, all without sacrificing sensitivity for detection of disease." Where previous studies have geared their methylation panels to fit either paradigm it was the aim of this thesis to determine an approach that could provide both specificity and sensitivity. In the context of the Irish screening population this is important as the public is more aware of the limitations of screening and the tolerance for repeat screening and recall is currently an unknown factor.

Chapter 5 assessed these three methylation strategies in a cohort of 996 HPV positive women, which was representative of the overall HPV positive triage population. This, compared to previous studies, would demonstrate the utility of methylation testing in HPV primary screening in a more realistic population and determine whether the positive results from previous studies scaled appropriately with a more reflective disease prevalence. Age does seem to be a contributing factor for these three methylation markers with the proportion of positive methylation results increasing with age; the inverse of HPV primary screening. It has been previously shown that age is related to increasing methylation and that cells have a lower threshold for a malignant transformation with this increase in methylation (Xu and Taylor, 2014; Jung and Pfeifer, 2015). The NPV for detection of CIN 2+ increased for all three methylation approaches in women >45 years, compared to women <30 years. This may suggest that methylation testing in older women could provide a high enough NPV to allow a return to the routine screening population. This is also useful in women <30 years, who will likely make up the majority of women who will test HPV positive and require triage by methylation. In women <30 years, there is an increase in the sensitivity of each methylation approach. In the case of the Total Methylation Score, the sensitivity increased from 56.48% to 75.00% in women <30. If utilised appropriately in these cases, methylation testing may be a very powerful tool. The clinical performance of these methylation approaches is also comparable to the performance of cytology in triage setting which ranged from 46.5% to 97.6% and 65.6% to 97.6% for sensitivity and specificity respectively. (Ronco, Giorgi-Rossi, et al., 2006; Ronco, Segnan, et al., 2006; Castle et al., 2011; Leinonen et al., 2013; C Kitchener et al., 2014; Bergeron et al., 2015; Wright et al., 2017). Unexpectedly, the HPV genotype does not seem to affect a person's odds of having a positive methylation test at a population level. This may be due to the more realistic prevalence of CIN 2+ in this population. However, this may be an important consideration in HPV 16/18 vaccinated cohorts in the future. Across each of the methylation strategies, there was no statistical difference regarding their positivity and CIN grade. The Paired Methylation Score however had the lowest rate of positivity in women with no apparent cervical abnormality. Of the three, the Paired Methylation Score had the highest sensitivity and specificity of the three. The incorporation of HPV 16/18 genotyping increased the

sensitivity for CIN 2+ to 80.56% with an associated decrease in specificity for each methylation marker.

In this population, cytology would have referred 38.27% of the HPV positive women. For all three methylation approaches, this was comparable with the Paired Methylation Score having the lowest referral rate of 37.76%. This would be considered comparable to previous cytology referral ranges of 25.9% to 38.7% (Ronco, Giorgi-Rossi, *et al.*, 2006; Ronco, Segnan, *et al.*, 2006; Castle *et al.*, 2011; Leinonen *et al.*, 2013; C Kitchener *et al.*, 2014; Bergeron *et al.*, 2015; Wright *et al.*, 2017) as well as referral rates for other methylation tests (Verhoef *et al.*, 2015; De Strooper *et al.*, 2018). The combination of HPV genotyping with either cytology or one of the methylation approaches increased the rate of referral to between 51.82% to 61.98% with the Paired Methylation Score referring the least number of women and cytology referring the most. Direct comparison between methylation testing and cytology could not be made in this study however methylation and cytology have been shown to be comparable in previous studies (Cook *et al.*, 2018).

These three methylation approaches have demonstrated comparable performances to previous studies, which have shown methylation biomarker to be comparable to cytology-based triage performances (Verhoef *et al.*, 2015; De Strooper *et al.*, 2018). Using the novel summation scoring approach such as the Total Methylation Score or Paired Methylation Score seems to provide a more balanced level of sensitivity and specificity to methylation testing that had not been seen previously. Subsequent literature searches showed a similar approach with the S5 classifier (Brentnall *et al.*, 2015; Cook *et al.*, 2018), indicating a combination approach to methylation testing is more powerful than taking individual panels of makers forward. This could provide the ideal balance for clinical performance and with the inclusion of new methylation markers could provide even more power to methylation triage.

The current European guidelines for risk states that a woman must have a >20% risk for CIN 2+ to warrant colposcopy referral or a <2% risk of CIN 2+ to allow the safe return to routine screening (Arbyn *et al.*, 2011). These risk limits are based off the PPV and 1-NPV respectively. The PPV is considered the risk that a person testing positive for a test is also positive for disease, while the NPV is the probability of a person having a negative test truly does not have disease. In this study cohort, no individual methylation approach was able to achieve a negative post-test risk of <2%. However, the combination of HPV 16/18 genotyping was able to reduce the negative post-test risk by up to 3%. Only one methylation approach achieved a positive post-test risk of >20%, the Paired Methylation Score. However, for women <30 years, the PPV for all three methylation approaches alone were

>20%. HPV 16/18 genotyping reduced the PPV for each test by between 0.5% to 2%. Triage tests failing to achieve this <2% risk threshold is a known issue with HPV primary screening (Stanczuk *et al.*, 2017). Cytology too has been shown not to meet this standard with women who test normal on cytology in HPV triage having a risk of >2% (Stanczuk *et al.*, 2017; Wentzensen *et al.*, 2017; Cuschieri *et al.*, 2018). To date very few triage tests have shown the ability to safely refer women back into routine screening. These women will likely require either a secondary triage test or return for a second screening test within 12 to 18 months, to ensure an interval lesion has not occurred. At the outset it was hoped methylation testing may negate the need for repeat testing. However, from this research, recalling women who test negative would be required. The risk denoted by a positive HPV test is still too high to allow the safe return to routine screening, but as stated previously methylation testing for women >45 years may be able to safely refer women back to routine screening at some point.

This is one of the first prospective studies of methylation markers with a sample size of >900 women as well as the first study assessing these markers in a HPV primary screening programme. Many previous studies have had between 40 to 500 women enrolled into their respective studies (Wilting *et al.*, 2010; Hesselink *et al.*, 2011; Lin and Wang, 2014; Lorincz, 2014, 2016; Steenbergen *et al.*, 2014; Schmitz *et al.*, 2017; Cook *et al.*, 2018; Cuschieri *et al.*, 2018; Sen, Ganguly and Ganguly, 2018; Verlaat *et al.*, 2018). The PPV of different combinations of the three methylation biomarkers in these studies have ranged from 29% to 54% for the detection of CIN 2+ (Lorincz, 2016). In the validation panel from this study, the PPV has was >70% for the majority of methylation combinations. This is due to the fact that both the PPV and NPV are not intrinsic to the test. Both also depend on the prevalence of the disease (Altman and Bland, 1994). A common aspect of previous studies for methylation markers and other triage tests is the prevalence of CIN 2+ used to determine the performance of the methylation biomarkers. Just like in the validation panel in Chapter 4, the higher prevalence of CIN 2+ leads to an increase PPV. This also effects the NPV, which is generally lower in these cases. In our cohort of HPV positive women, the distribution of CIN 2+ is more typical to how a true HPV triage population would be. From this, we can see, that the PPV of methylation testing may not be as powerful as has previously been shown, however as the population is managed by cytology there may be some underlying disease that is unknown to the study at present. Regardless of this, methylation testing, as showed by the Paired Methylation Score, can provide the necessary risk as outlined by the EU guidelines to refer women to colposcopy. It is however an important consideration to make when basing decisions around imbalanced prevalence data.

Methylation testing does have limitations. From this study, it was shown that the PPV may not be as high as previously suggested. However, the Paired Methylation Score did achieve a PPV >20%. This indicates that though PPV may be low the correct combination of methylation markers with appropriate thresholds is capable of exceeding the required 20% limit. This is an important point to consider for all triage tests and how risk is calculated and managed. More technical limitations revolve primarily around qMSP, which remains a time and labour-intensive approach with multiple steps involved, to take the sample through to a useable result. It is also a relatively new approach, that has been taken in cervical screening and consensus on the correct methodologies has yet to be made along with other aspects, such as quality control or even automation. The few commercial kits that exist still require manual labour and can introduce variables. Many of these limitations are likely short-term, namely the lack of automation for qMSP or other methylation detection methods, cost, standardisation and quality control. In today's technology driven world, these limitations could be dealt with relatively rapidly. Much as with the first genome sequenced and the subsequent plummeting of costs associated with technological advances, methylation analysis will likely one day be very cost effective with automated platforms being able to provide a high throughput and high capacity option for screening programmes along with potential cost savings. It would be well within the realm of possibility that cervical screening could be a track-based system with automatic reflex testing of HPV positive results. One possible longer-term issue that will need to be addressed going forward however, is the introduction and training of clinicians for interpreting this new testing platform and result output, as it may take clinicians longer to become acquainted and comfortable with the newer methylation tests (Lorincz, 2016). However, this is less of an issue in national screening programmes but will likely still be an issue in more opportunistic screening environments. Regardless of the hurdles taking a molecular based primary screening test with a follow-up molecular based triage in place of cytological triage will help condense the workflow process as well and create an overall leaner and more efficient workflow for a robust National Screening Service.

6.1. Conclusion

HPV primary screening has been shown in this study to conform to the high sensitivity that has been shown in previous studies. For the Irish population, both the Cobas 4800 HPV DNA test and the Aptima HPV E6/E7 mRNA test are comparable for detection of CIN 2+. However, the prevalence of HPV mRNA is less than that of HPV DNA, without compromising the sensitivity of the mRNA test while providing a minor increase in the overall specificity of the test. For the new Irish HPV primary screening programme set to begin in 2020, the use of either test would be applicable to the needs of the screening programme with the primary difference being the number of women requiring triage. Methylation testing has been shown in two parts in this thesis. Firstly, it was validated to established cut off values to determine what constitutes a positive or negative methylation result. This led to the to the development of the Total Methylation Score and the Paired Methylation Score (CAD:MAL). These clinically relevant cut-off points provided a high sensitivity for CIN 2+ while remaining relatively specific. In a HPV positive triage population, each of these three methylation approaches in combination with HPV 16/18 genotyping show a high degree of sensitivity for CIN 2+ with a reasonable specificity. The referral rates of cytology triage or methylation triage with or without the use of HPV 16/18 genotyping are also comparable.

6.2. Future Direction

This is a longitudinal, observational cohort study and women who consented into this study will be followed up over a 10-year period, allowing follow-up for between two or three subsequent screening rounds. This will allow the study to determine the final endpoint for these women with knowledge of their baseline HPV result and triage test result. A short-term goal following this project could also be the examination of other computational approaches to determine relevant methylation. This can include other combination approaches aside from the Total Methylation Scores to re-defining cut off points. Following this optimisation work on Multiplex qMSP would be advantageous to allow a more stream-lined work flow process.

Methylation testing has another potential use in a HPV primary screening programme that the longitudinal data may reflect on but would also require its own controlled studies. Methylation is a hallmark for transforming and progressive HPV infections. Therefore, in theory those women who have negative methylation tests are at low risk of progression with a higher likelihood of regression, versus those with a positive methylation test who would be at a higher risk of progression. This longitudinal study will hopefully elucidate this matter.

Women who were HPV positive with an NAD cytology result who had a positive methylation test, should be at a higher risk of progression than if they tested negative for methylation. This could allow methylation testing to not only triage women but also be a predictor of disease in women with no cytological/phenotypical indicators of disease. It may also allow clinicians to determine who may require treatment and who could be monitored and allow the lesion to regress naturally. The outcomes for these women will be followed up and the methylation panel will continue to be investigated to identify if it shows a predictive ability for disease progression and the requirement of treatment. The cohort of women with a CIN 1 lesion who will not be immediately treated and follow a "wait and see approach", will also be followed up.

How these methylation markers and HPV primary screening in general will operate in a HPV vaccinated cohort is also a question currently being researched. The potential elimination of HPV 16/18 infections may affect the overall performance of HPV testing as most CIN 2+ results in studies have been in HPV 16/18 rich populations. How these tests will operate with the other hrHPV types is still unknown. In Ireland with varied vaccination rates, we would expect to see a mixed population for another 50 years which may change how these two groups are screened. Previous vaccination coverage in Ireland ranged at 80% to 88% between 2009 and 2014. However with the rise in anti-vaccination groups, this declined to around 51% in 2017 (HPSC, 2018). There is however a reported increase in vaccination again thanks to education efforts. The current study has investigated an unvaccinated population, however, CERVIVA are undertaking the CERVIVA-VAX study which will investigate the utility of a range of triage approaches, included in this study, in the catch-up cohort of vaccinated women.

One last aspect of potential future work for this study is the addition of other methylation markers or triage tests such as p16/ki-67 dual staining to this study. Current research from the UK and the Netherlands have shown that EPB41L3 and FAM19A4 show great potential as methylation markers and the inclusion of those into this panel may provide increased performances. This will allow a broad range of paired triage test data to be assessed in the same population over time and provide a good deal of insight into how each triage test may work in the HPV primary screening programme. The option also exists to peruse alternative methods such as pyrosequencing. Overall, methylation testing still suffers from its relative novelty and determining a consensus on the best methodologies and approaches to classifications are yet to be determined but will be necessary to progress methylation testing from a research test to an accredited laboratory test.

6.3. References

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