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**DEVELOPMENT OF SCREENING AND  
THERAPEUTIC INTERVENTION STRATEGIES FOR  
CERVICAL CANCER IN RURAL REGIONS OF SUB-  
SAHARAN AFRICA**

**TSITSI GRACE CHITUKU**

A thesis submitted to the University of Huddersfield in partial fulfilment of the requirements for the degree of Doctor of Philosophy

The University of Huddersfield (in collaboration with – add any collaborating organisation otherwise delete this bracketed text)

May 2018

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## **Abstract**

Cervical cancer is the most common and lethal form of cancer in women in sub-Saharan African countries. Whilst screening for cervical cancer is available in the more affluent cities, it is currently not widely available in poor, rural areas and women typically present with advanced disease. The need for 'screen and treat' strategies in these regions is as obvious as it is imperative. The purpose of this study is twofold; (i) to conduct a pharmacoeconomic analysis of the benefits of early detection in Zimbabwe and (ii) to develop the preclinical rationale for the use of the enzyme activated prodrug EO9 as a locoregional therapy for cervical cancer.

We were able to show that it is feasible to provide cervical screening with VIA to a population of women in rural Zimbabwe by conducting two screening programmes where 469 women were screened for cervical cancer. The prevalence rate of VIA positive women was 1.5%. It would cost approximately US\$1.85 to screen each woman. Without screening, women would present with late stage disease and this is much more expensive to treat.

EO9 is a prodrug that is activated by oxidoreductases such as NAD(P)H:Quinone oxidoreductase 1 (NQO1) to DNA damaging species. A panel of cell lines were characterised with respect to NQO1 genotype and phenotype and chemosensitivity studies were conducted using the MTT assay. The results of this study demonstrate that a good correlation between NQO1 activity and response to EO9 exists and the role of NQO1 in the activation process was confirmed using the NQO1 inhibitor dicoumarol. Together with evidence of elevated NQO1 in CIN and SCC compared to normal cervix tissue, these results strongly support the use of EO9 as a locoregional therapy for pre-invasive cervical cancer.

In conclusion, this study has demonstrated that the simple use of VIAC screening in rural areas of Zimbabwe is feasible and popular amongst women. Early detection together with vaccination would significantly reduce the burden and suffering caused by this disease and the pharmacoeconomic benefits would be attractive to health care providers. Provided novel formulations for EO9 could be developed, there is a strong case for establishing clinical trials to test the safety and efficacy of EO9 as a locoregional therapy for cervical cancer.

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## **Dedications and Acknowledgements**

I would like to thank Professor Roger Phillips, Professor Zaheer Babar and Dr Patrick McHugh for their invaluable input and their guidance and direction over the course of this research. To Professor Phillips I would like to thank for his mentorship and constant encouragement even at times when it didn't seem possible and the odds were stacked against me. I am also grateful to Professor Rob Brown for his support in the early years of my research. During the course of my PhD study I have had the pleasure of working with colleagues from Zimbabwe. I would like to especially acknowledge Dr. Mawire, the Medical Superintendent of Chinhoyi Provincial Hospital and Dr. Wenceslas Nyamayaro the Provincial Medical Director for Mashonaland West Province. Their support for the work done in Zimbabwe made it all possible. It was my privilege to work with the dedicated and hardworking team of VIAC nurses at Karoi District Hospital led by Sister Rudo Chidavaenzi as well as the trained nurses including Mr. Mukwengwe who supported the team at both screening programmes. I would also want to acknowledge the army of young, passionate and inspirational volunteers who supported us in our screening programmes – too many to mention by name, but you guys are great. Chenai Muchena and Tino Madzima you deserve a special mention.

To colleagues and friends with whom we have shared this journey – Sam, Omar, Suleman and Scott, thank you for your support and for the memories in and out of the lab. I would like to acknowledge my friend and colleague Dr. Fadzai Kanyangara and all were with me at the start of this research and for one reason or the other have moved on, your input is appreciated. I acknowledge my church family who have supported me spiritually and materially – again too many to mention but you all know who you are. Finally, I am grateful for the constant and unwavering support of my family. To my mum who has been and is always a pillar of strength and source of advice. To Munyaradzi, the voice of reason in my life, and my sounding board. Tafadzwa, Isheanesu and Kuda. You all give me a reason to go on and try one more time. Thank you! Above all, I would like to thank God for His guidance and direction over the course of this research programme without Whom this would not have been possible and in Whom I have my being.

## List of abbreviations

<b>BSA</b>	Bovine serum albumin
<b>BMI</b>	Body mass index
<b>CIN</b>	carcinoma intraepithelial neoplasia
<b>CIS</b>	Carcinoma-in-situ
<b>CT</b>	Computerised tomography scanning
<b>DCPIP</b>	Dichlorophenolindophenol
<b>DNA</b>	Deoxyribonucleic acid
<b>DTD</b>	DT-diaphorase
<b>FDA</b>	United States Food and Drug Administration
<b>FIGO</b>	International Federation of Gynaecology and Obstetrics
<b>GAVI</b>	Global Alliance for Vaccines and Immunization
<b>GDP</b>	Gross Domestic Product
<b>GNI</b>	Gross National Income
<b>HCR</b>	Hypoxic cytotoxicity ratio
<b>HDI</b>	Human development Index
<b>HGSIL</b>	High grade squamous intraepithelial lesion

<b>HIV</b>	Human immunodeficiency virus
<b>HPV</b>	Human Papillomavirus
<b>IARC</b>	International Agency for Research on Cancer
<b>ICC</b>	Invasive cervical cancer
<b>ICER</b>	Incremental cost effectiveness ratio
<b>IHC</b>	Immunohistochemistry
<b>IHP</b>	International Health Partners
<b>LMIC</b>	Low-to middle-income countries
<b>LEEP</b>	Loop electrosurgical excision procedure
<b>LSIL</b>	Low grade squamous intraepithelial lesion
<b>MRI</b>	Magnetic resonance imaging
<b>MTT</b>	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<b>NAD</b>	Nicotinamide adenine dinucleotide
<b>NADH</b>	Nicotinamide adenine dinucleotide (reduced)
<b>NCD</b>	Non-communicable disease
<b>NQO1</b>	NAD(P)H:Quinone oxidoreductase 1
<b>PCR</b>	Polymerase chain reaction

<b>SCC</b>	Squamous cell carcinoma
<b>SCJ</b>	Squamo-columnar junction
<b>USS</b>	Ultrasound scan
<b>VIA(C)</b>	Visual inspection with acetic acid (and cervicography)

## MEDICAL GLOSSARY USED

<b>Brachytherapy</b>	A form of radiotherapy where a sealed radiation source is placed inside or next to the area requiring treatment.
<b>Dysplasia</b>	The presence of abnormal cells within a tissue which may signal a stage preceding the development of cancer.
<b>Koilocytosis</b>	The process whereby a squamous epithelial cell undergoes structural changes as a result of infection of the cell by human papillomavirus.
<b>Microinvasive</b>	Cancerous growth that has become invasive at a microscopic level.
<b>Paracolpos</b>	The connective tissue and membranous lining which envelopes the vaginal and inferior bladder blood and lymphatic vessels.
<b>Parametrium</b>	The fibrous tissue that separates the supravaginal portion of the cervix from the bladder. It helps to connect the uterus to other tissues in the pelvis.
<b>Pancytopenia</b>	A deficiency of all 3 types of blood cells (red blood cells, white blood cells and platelets).
<b>Pelvic exenteration</b>	A radical surgical treatment that removes all organs from a person's pelvic cavity leaving them with a permanent colostomy and urinary diversion.
<b>Trachelectomy</b>	Surgical removal of the uterine cervix leaving behind the uterine body.

# **Chapter 1 GENERAL INTRODUCTION**

## **1.1 Introduction to the cancer problem**

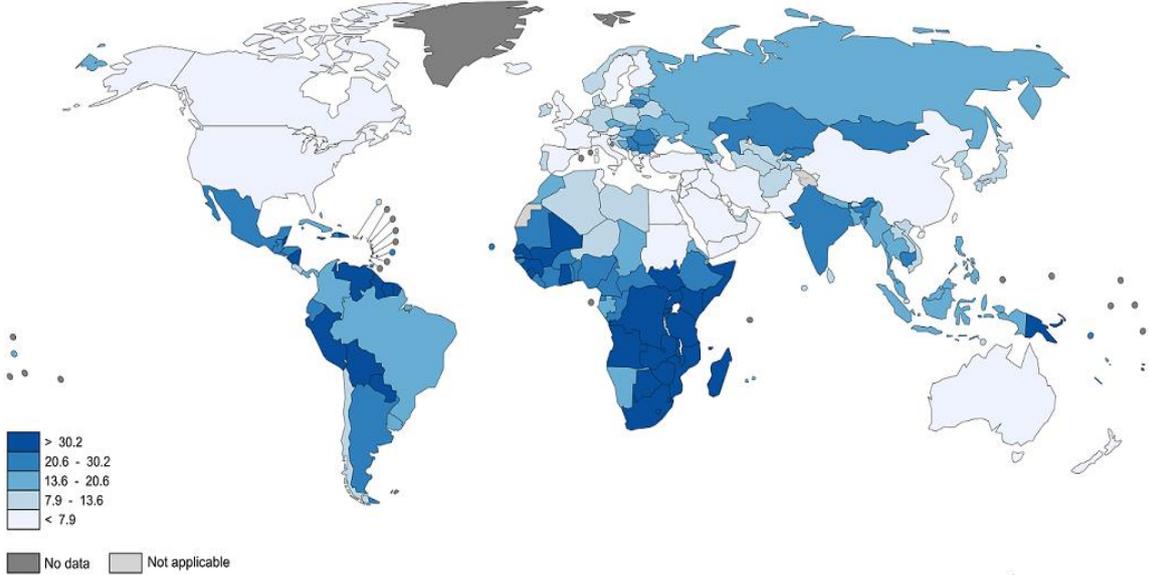
Current estimates predict that by 2030, the number of cancer cases will increase to 24.6 million, while the number of cancer deaths increase to 13 million annually (Bray et al., 2012). The low-income and middle-income countries (LMICs) will be particularly hard-hit as their health systems are the least prepared to manage this increase in cancer burden (Farmer et al., 2010). Cancer is already a major public health concern in the developing world, with 57% of the 14.1 million new cancer cases and 65% of the 8.2 million deaths that occurred worldwide in 2012 having occurred in the less developed world (Torre et al., 2015). As a matter of fact, it is estimated that in future, more than 2 in every 3 cancer deaths will occur in the less developed countries and yet Africa is the continent that is least prepared to deal with such a projected increase in cancer burden (Sylla & Wild, 2012).

There is therefore an urgent need to reduce this cancer burden. It is possible to reduce these unacceptable inequalities and thereby prevent unnecessary suffering caused by premature deaths by delivering cost-effective interventions for cancer screening, prevention, and treatment (Gelband et al., 2016). In the 'War on Cancer' early detection and improved intervention will play a significant role in reducing the burden of cancer. This thesis will highlight the problem of cervical cancer within the context of Zimbabwe, a low-income sub-Saharan country. This thesis will aim to (i) provide an economic rationale for introducing low-cost cervical screening to the majority of women who would otherwise have no access to it and (ii) provide the preclinical rationale for developing the quinone-based bioreductive drug EO9 as a locoregional therapy for cervical cancer.

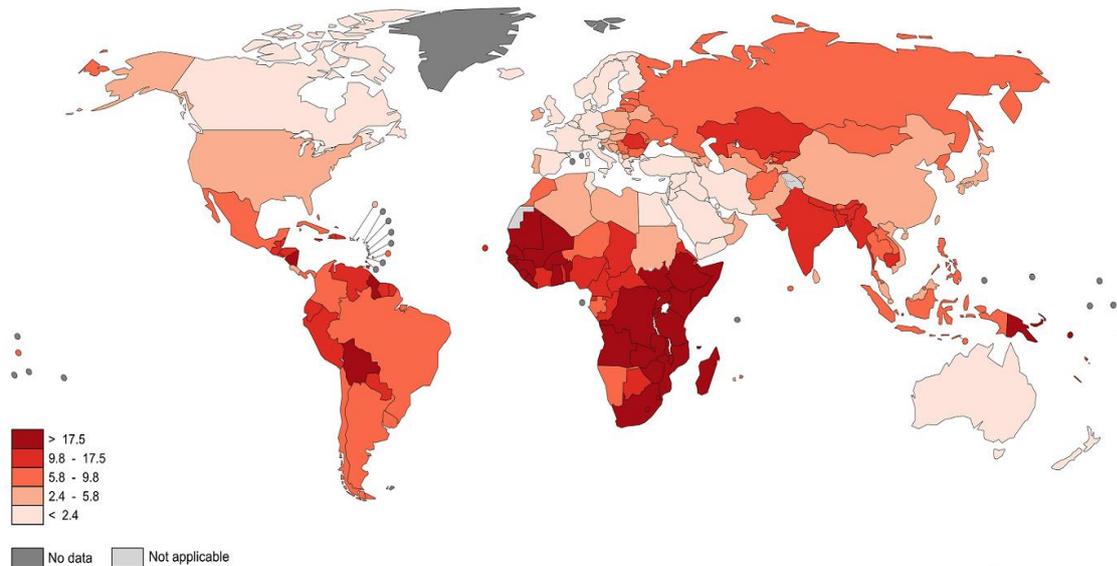
Before discussing the scientific background underpinning the rationale for the project, it would be pertinent to first review the incidence and impact cervical cancer has on populations around the world, and in Zimbabwe in particular.

## 1.2 Worldwide Incidence of Cervical Cancer

Cervical cancer kills more women than any other type of cancer in 55 countries of the world, and it is the most common form of cancer among women in 45 countries. These countries are primarily in Sub-Saharan Africa, Asia, and some Central and South America (World Health Organisation, 2014). Figures 1.1 and 1.2 shows the worldwide incidence and mortality of cervical cancer respectively.

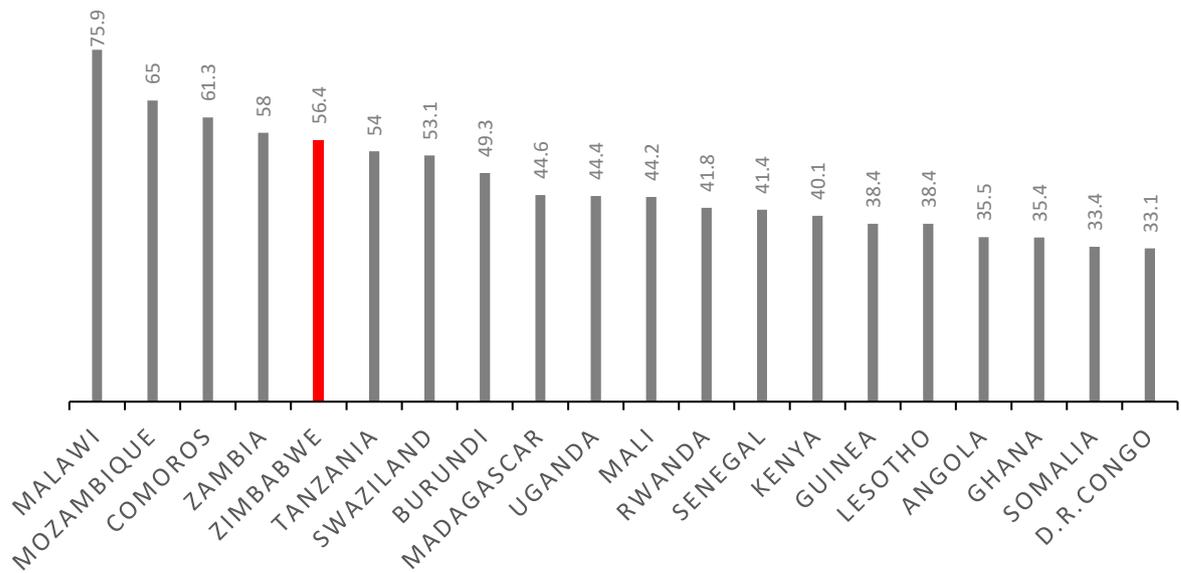


**Figure 1.1 Cervical cancer incidence in 2012. Estimated Age-standardized rates (ASR) (world) per 100 000 (IARC, 2012)**



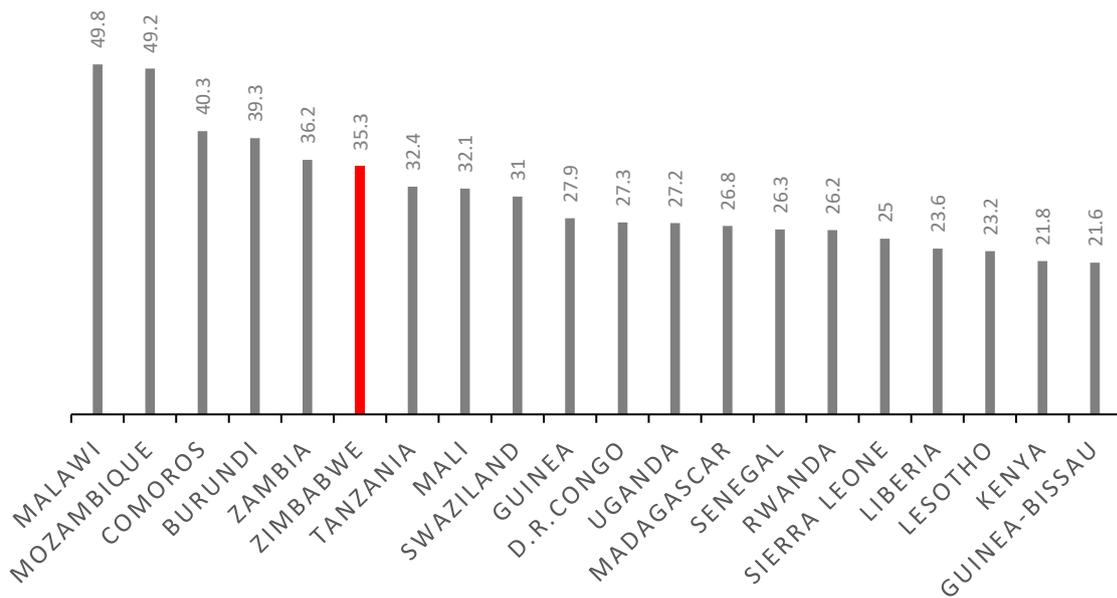
**Figure 1.2 Cervical cancer mortality in 2012. Estimated ASR (world) per 100 000 (IARC, 2012).**

In 2012, there were 528,000 new cases of cervical cancer worldwide with 85% of these cases occurring in the less developed countries of the world. Of the 266,000 women who died of cervical in the same year, almost 90% or a total of 231 000 women (Ferlay et al., 2013), lived and died in low to middle income countries, while 35,000 women, or just 10% of these women, lived and died in high-income countries (WHO, 2014). More specifically, in sub-Saharan Africa, 34.8 new cases of cervical cancer are diagnosed per 100,000 women annually, whilst 22.5 per 100,000 women die from the disease (IARC Press Release 223, 2013). In comparison, annual incidence rates in England are 9.27 per 100,000 women, with 2.72 per 100,000 women dying from the disease (NHS Information Centre Indicator Portal, 2015). The incidence and mortality figures for cervical cancer in Zimbabwe compared to other sub-Saharan countries is presented in figures 1.3.and 1.4. Being in the top 6 countries for incidence and mortality, cervical cancer in Zimbabwe is therefore a major problem.



**Figure 1.3 Incidence of cervical cancer in the top 20 countries of the world.**

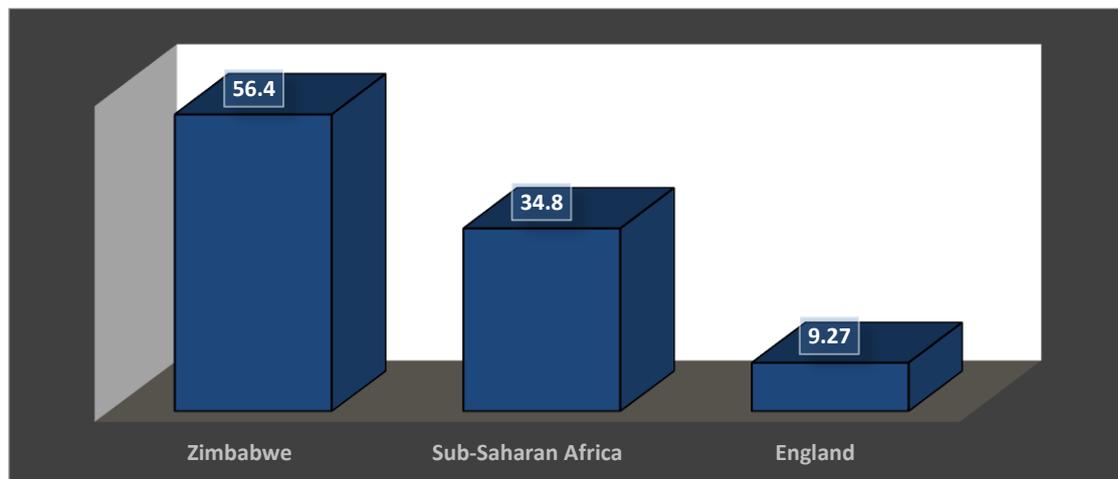
**Top 20 age standardised incidence rates for cervical cancer per 100,000 (World) (Afri.Dev.Info & Coalition, 2014).**



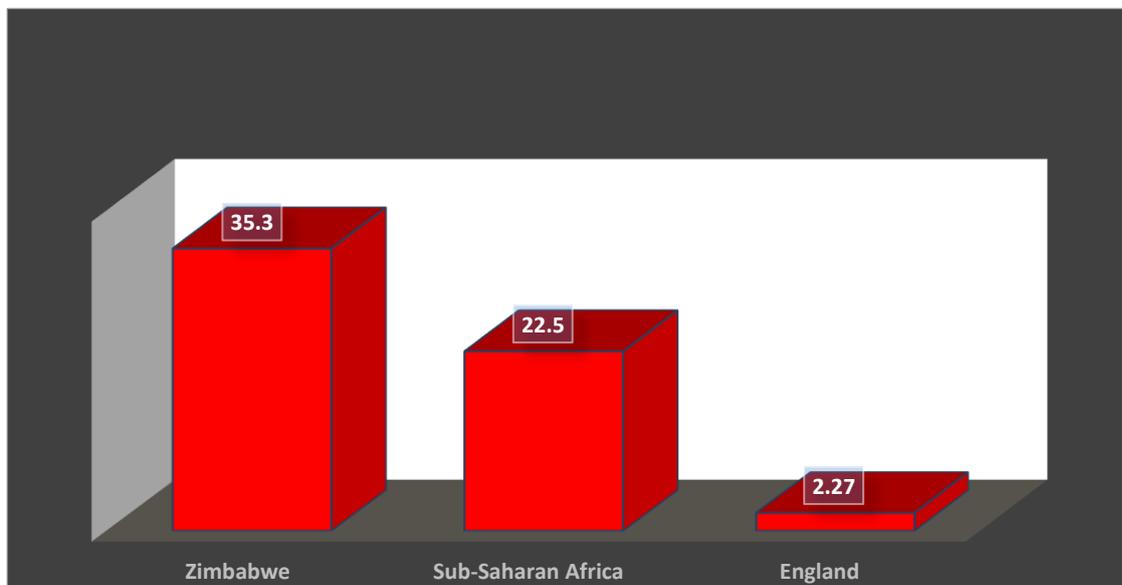
**Figure 1.4 Cervical cancer mortality in the top 20 countries of the world. Top 20 age standardised mortality rates of cervical cancer per 100,000 (World) (Afri.Dev.Info & Coalition, 2014).**

### 1.3 Incidence of cervical cancer in Zimbabwe.

In Zimbabwe, cervical cancer is the most common cancer and cancer death among women across all ages and the second most common cancer and cause of cancer death among women aged between 15 and 44 years old in Zimbabwe (ICO HPV Information Centre, 2017). Every year 2,270 women are diagnosed with cervical cancer and 1,451 die from the disease (ICO HPV Information Centre, 2017). It accounts for 33.9% of all cancer cases in Zimbabwe (ICO, 2014). Zimbabwe has the 5th highest incidence of cervical cancer in the world, with an age standardized incidence rate of 56.4/100,000 (figure 1.6) and an age standardized mortality rate of 35.3/100,000 (figure 1.7) (Afri.Dev.Info & Coalition, 2014).



**Figure 1.5 A comparison of age standardised incidence of cervical cancer between Zimbabwe, Sub-Saharan Africa and England. (IARC Press Release 223, 2013; NHS Information Centre Indicator Portal, 2015)**



**Figure 1.6 A comparison of age standardised mortality rates of cervical cancer between Zimbabwe, Sub-Saharan Africa and England. (IARC Press Release 223, 2013; NHS Information Centre Indicator Portal, 2015).**

Figures 1.5 and 1.6 highlight the scale of the problem of cervical cancer in Zimbabwe. Both incidence and mortality rates are higher in Zimbabwe than the rest of sub-Saharan Africa and significantly higher than the situation in the UK. It is clear that the challenge is significant but in addition to the suffering caused, there is an economic and societal element to consider too.

The Human Development Index (HDI) is a composite measure of the development of a country using its people and their capabilities as opposed to economic growth alone. three basic dimensions of human development (UNDP, 2013) (i) life expectancy at birth (ii) the average number of years of schooling achieved by adults aged 25 years and above and (iii) the gross national income per capita. When a comparison is made of the national incidence burden of cervical cancer by a country's HDI level, cervical cancer makes up to a third of all

cancers diagnosed (in both sexes) in many low HDI countries, whereas it comprises less than 10% of all cancers in very high HDI countries (Ginsburg et al., 2017). For this reason cervical cancer has been called “a case study in health equity” (Wittet & Tsu, 2008).

To summarise, cervical cancer is a major problem in Zimbabwe and other sub-Saharan countries. The need for strategies to reduce cervical cancer incidence, early detection of cervical cancer and application of novel treatments for this disease are as obvious as they are imperative.

## **1.4 Cervical Cancer and the Human Papilloma Virus**

Cervical cancer is caused by the presence of infection with one or more of the oncogenic human papilloma virus (HPV) genotypes for an ongoing period of longer than 2 years (Goodman, 2015; Muñoz & Bosch, 1996). About 90% of cervical cancer cases are squamous cell carcinoma while 10% are adenocarcinoma (NCI., 2011). HPV infection is the most common sexually transmitted infection (Dunne et al., 2007), and having other STIs including chlamydia, gonorrhoea and genital herpes increases the risk of HPV infection (Koutsky et al., 1993; Smith, 2002). The most important determinant of risk of HPV infection is age and most studies show that there is a sharp decrease in prevalence after the age of 30 which seems to be independent of sexual activity (Ho et al., 1998). The virus can also be transmitted by just skin to skin contact of the genital areas without sexual penetration (Burd, 2003).

There are over 120 HPV genotypes. The estimated average global prevalence of genital HPV infection is 12% and varies between countries (Bruni et al., 2010). HPV rates are high in sub-Saharan Africa, Latin America and India (Franceschi et al., 2006). HPV prevalence in women with normal cytology varies between 2 and 42% depending on age and population risk (Bruni

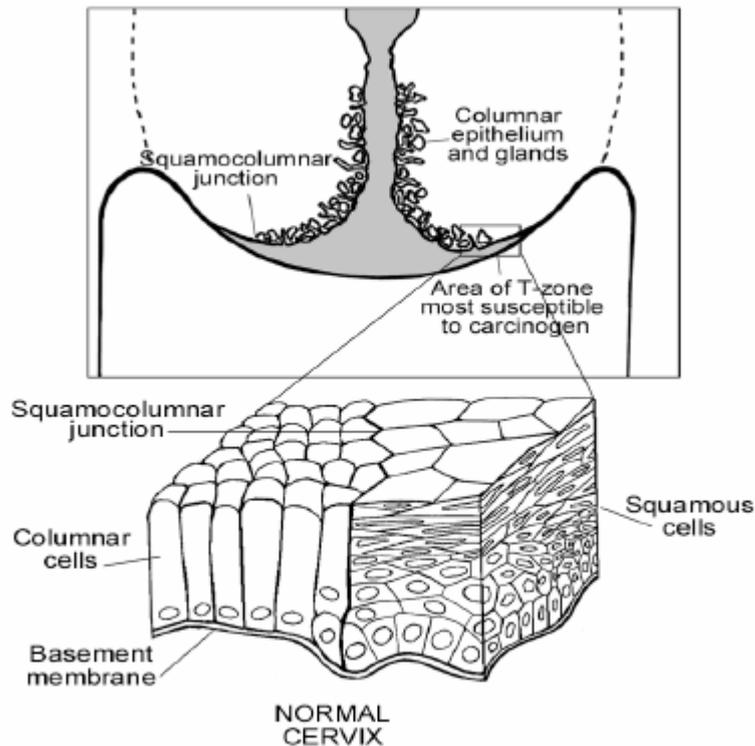
et al., 2010). In Zimbabwe, the prevalence of HPV among women with normal cervical cytology varied between 24.7 and 47.5% (Baay et al., 2004; Fukuchi et al., 2009; Nowak et al., 2011; Womack et al., 2000). In the USA, the lifetime cumulative risk of HPV infection is greater than 80% (Dunne et al., 2007).

The HPV genotypes that are carcinogenic to human beings are known as high risk HPV (hrHPV) types. These are HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66. HPV 16 and 18 cause 70% of all cervical cancer cases worldwide (Smith et al., 2007). Low risk (lrHPV) genotypes have a low oncogenic potential and these are HPV 6, 11, 42, 44, 51, 53 and 83 (zur Hausen, 2002). 90% of benign ano-genital warts are caused by HPV 6 and 11 (De Freitas et al., 2012).

The 5 most frequent HPV subtypes among Zimbabwean women with invasive cervical cancer are HPV16, HPV33, HPV18, HPV31 and HPV35 (ICO HPV Information Centre, 2017).

## **1.5 Anatomy of the cervix.**

The cervix is lined by two types of epithelium (figure 1.7). The stratified squamous epithelium which makes up the ectocervix and is a continuation of the vaginal covering and a single layer of columnar epithelium which is thinner and more fragile and covers the cervical canal. The two types of epithelium meet at the squamo-columnar junction (SCJ).



**Figure 1.7 Anatomy of the cervix (Blumenthal and MacIntosh, 2005).**

At birth, the original SCJ is found at the external cervical opening, which is known as the external OS. During puberty and under the influence of oestrogen, the cervix increases in size and the columnar epithelium from the endocervix as well as the original SCJ become visible on the ectocervix. When the columnar cells become exposed to the acidic environment of the vagina they undergo metaplasia resulting in a new SCJ formed by the metaplastic squamous epithelium which characterizes the cervix of a woman in her 30s. This area of variable size between the new SCJ and the old SCJ is called the transformation zone. It is these cells in the transformation zone that are particularly vulnerable to human papilloma virus (HPV) infection and most squamous cell carcinomas (SCC) develops here. The size of the transformation zone varies with age, hormonal status, history of birth trauma, pregnancy status and use of oral contraceptives. And consequently, these are risk factors for developing cervical cancer. As

the influence of oestrogen decreases at menopause, the cervix shrinks, and the columnar epithelium retreats back into the cervical canal.

In a cross-sectional study that examined 209 histopathology slides of cervical epithelium with normal, HPV or CIN diagnosis from a population-based study, the mean normal cervical epithelium thickness was 212.8 $\mu$ m. HPV- associated changes resulted in an increase in the mean cervical epithelium thickness to 297.3 $\mu$ m. In CIN, mean cervical epithelium thickness ranged from 245.3 $\mu$ m for CIN1 to 191.4 $\mu$ m for CIN2, and 218.5 $\mu$ m for CIN3 (Ghosh et al., 2016).

## **1.6 The Cervical and Vaginal Ecosystem**

The endocervical canal consists of a single layer of mucin-producing columnar epithelium (Moghissi et al., 1976). These cells produce mucus whose daily production varies in relation to the cyclical changes of the menstrual cycle from 600mg during mid-cycle to 20 - 60mg during the rest of the cycle (Sharif & Olufowobi, 2006). Cervical mucus contains mucins, plasma proteins, ions, enzymes, bactericidal proteins and water (Martyn et al., 2014). The pH of the cervical mucus is ~ 7.0. A study which looked at the pH values of 20 women found that the mean ectocervical pH was 6.91 ( $\pm$ 0.235) and the mean endocervical pH was 7.09 ( $\pm$ 0.161) (Correa et al., 2001). This study also showed that the longer the cervical mucus was exposed to atmospheric air during speculum examination, the more alkaline it became with ectocervical pH increasing to 7.16 ( $\pm$ 0.215) after 5 min, and to 7.27 ( $\pm$ 0.222) after 10 min. Similarly, the endocervical pH increased to 7.34 ( $\pm$ 0.159) after 5min and to 7.46 ( $\pm$ 0.164) after 10 min. The optimal pH for sperm viability is 7.2 to 8.5 (Haugen & Grotmol, 1998), therefore cervical mucus pH supports sperm viability (Eggert-Kruse et al., 1993).

The viscosity and water content of cervical mucus also varies during the menstrual cycle from around 93% during pre- and post- ovulatory periods to 98% during mid cycle (Pommerenke, 1946). The water content is determined by the influence of oestrogen and progesterone (Moghissi et al., 1960; K. S. Moghissi, 1972; K. S. Moghissi & Neuhaus, 1966) . During the mid-cycle under the influence of mainly oestrogen, the high water content makes the mucus thin and serous and more alkaline allowing it to function as a transport medium and producing a welcoming environment for spermatozoa. Outside of mid-cycle, under the influence of predominantly progesterone the mucus has a reduced water content and becomes thick and more acidic producing a hostile environment and functioning as a barrier for spermatozoa (Sharif & Olufowobi, 2006).

In contrast to the cervix, the vagina is maintained at an acidic pH of between 3.8 – 4.5 (Cohen, 1970; Kelly, 1990; Redondo-Lopez et al., 1990). This is because the epithelial cells of the vaginal wall are rich in glycogen, which is produced under the influence of oestrogen. Cell shedding and desquamation results in an accumulation of glycogen in the vaginal lumen. The anaerobic metabolism of vaginal glycogen by *Lactobacillus spp.* to pyruvic acid and lactic acid via glycogenolysis ensures that the vagina canal is maintained at an acidic pH. This acidic environment serves to protect the vagina from pathogenic microorganisms and the lower female genital tract from ascending infections (Carr et al., 1998).

## **1.7 The timeline and natural history of cervical cancer.**

Transient infections with hrHPV peak in prevalence among women during their teens and 20s after starting to engage in sexual activity. Most infections are cleared spontaneously by the

body within 2 years (Rodríguez et al., 2008). However lesions associated with HPV16 are less likely to undergo regression (Trimble et al., 2005). In a small percentage of women, infection with hrHPV persists resulting in a peak prevalence of precancerous conditions approximately 10 years later (Schiffman & Castle, 2005). Even fewer will progress to invasive cervical cancer (ICC), with an estimated number of no more than 2% of all women in low-resource countries expected to develop ICC during their lifetime. Peak prevalence of ICC is at ages 35 – 40 years (Schiffman & Castle, 2005).

According to recent data from the UK (adjusted for women who have had a hysterectomy), it is becoming apparent that there is a second peak in cervical cancer diagnoses after age 65 years (Sherman et al., 2015). In women aged over 65 years, HPV infection is more likely to be persistent and a positive HPV test more likely to be clinically significant (Datta et al., 2008). This observation that HPV prevalence in women peaks twice, in women under 30 and then in women in the mid 50's has led to the hypothesis that women with normal immunity can have latent HPV infection which is not detectable with an HPV test, which can later be reactivated at a later age (Del Pino et al., 2011).

## **1.8 Pathophysiology of cervical cancer**

Micro-abrasions in the cervical epithelium are a route through which HPV enters the basal cervical cells. Early HPV genes enhance the proliferative state of the host cell via the activity of oncogene proteins E6 and E7. In the upper layers of the epithelium the late genes L1 and L2 and E4 are expressed, and form virions in the nucleus which initiate new infections. Cervical intraepithelial neoplasm 1 (CIN1) or low grade intraepithelial lesions (LGIL) support productive viral replication. hrHPV infections progress to cervical intraepithelial neoplasm 2+ (CIN2+) or high grade intraepithelial lesions (HGIL). HPV DNA becomes integrated into the host's DNA, and this is the first step in the transformation of cells. E6 and E7 oncoproteins

promote the degradation of p53 and pRb respectively, and this is thought to be the main mechanism(s) by which HPV oncogenes induce genomic instability and cell transformation (Doorbar et al., 2015). HPV infection also induces oxidative stress in the form of reactive oxygen species (ROS) and this is believed to facilitate viral integration and oncogenic transformation of cells (Foppoli et al., 2015).

## **1.9 Risk factors for cervical cancer**

Early sexual activity (Schiffman & Brinton, 1995) and multiple sexual partners (Au, 2004; Finan et al., 2002) are independently associated with abnormal cytology and ICC. Also, HPV subtypes act synergistically, and women with multiple types of infection are more susceptible to developing ICC. Male circumcision is associated with a reduced risk of penile infection and consequently a reduced risk of ICC in their female partners (Castellsagué et al., 2002). Cofactors such as smoking and oral contraceptive use may play a role in the progression of ICC (Schiffman, 1992). Immunosuppression is another recognized risk factor, and there is a clear association between HIV infection, HPV infection and precancerous cervical changes (M. H. Schiffman & Brinton, 1995). HIV positive women are at an increased risk of developing invasive disease up to 10 years earlier than in HIV negative women (WHO, 2014). In fact, ICC is an AIDS-defining illness in patients with HIV, meaning that, in a woman infected with HIV, developing ICC is used worldwide, as a guideline for diagnosing that woman as having developed full-blown AIDS.

## **1.10 Pre-cancerous cervical lesions.**

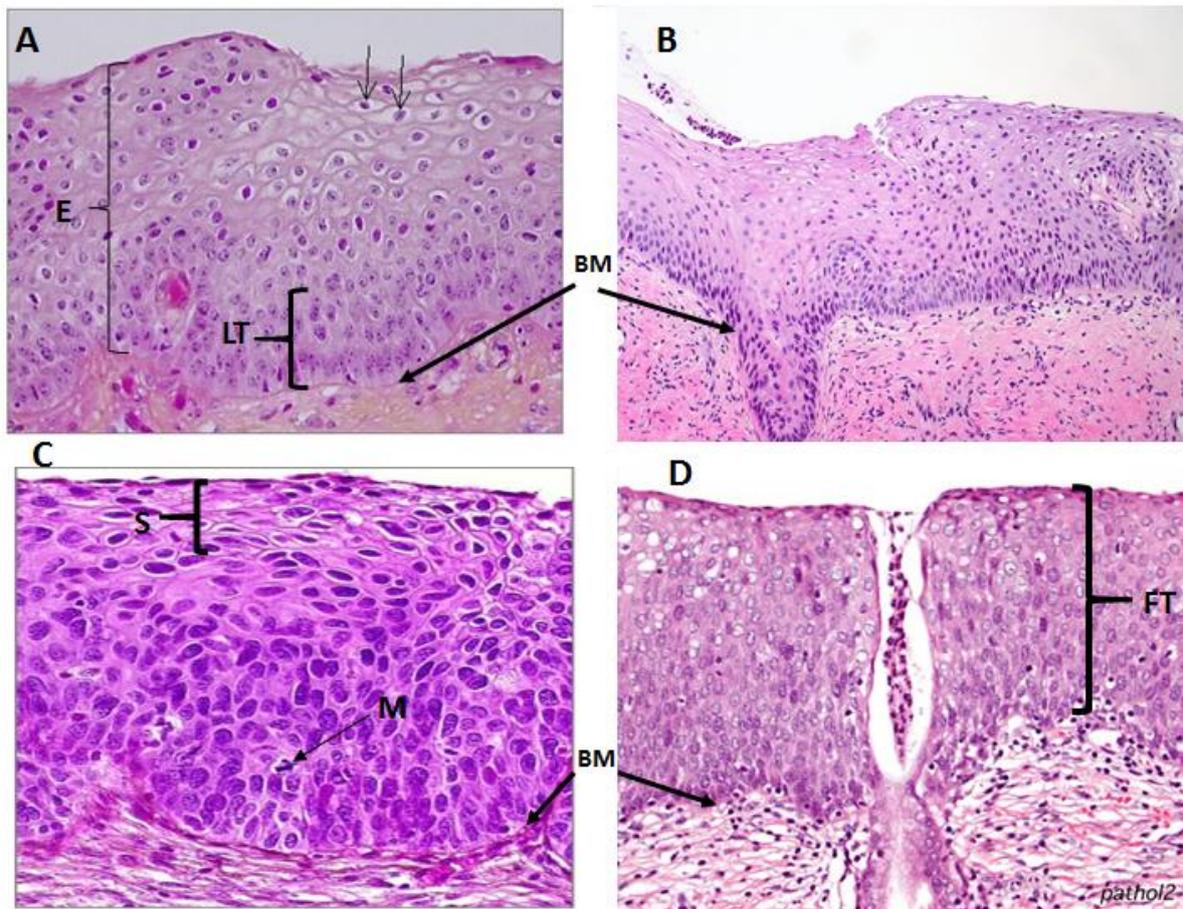
Precancerous changes in the cervix can be classified histologically as CIN 1, 2 or 3. Classification depends on differentiation, maturation and stratification of cells and nuclear

abnormalities. CIN can either regress, persist or progress to carcinoma *in situ* (CIS) and eventually to invasive carcinoma.

A more recent classification system, the Bethesda system (2001) reclassifies the histopathological diagnosis of CIN I as LSIL and groups together CIN 2 and 3, including CIS as HSIL (Solomon et al., 2002). Details are summarised in table 1.1 below:

Cytological classification (used for screening)		Histological classification (used for diagnosis)	
Pap	Bethesda system	CIN	WHO descriptive classifications
Class I	Normal	Normal	Normal
Class II	ASC-US ASC-H	Atypia	Atypia
Class II	LSIL	CIN 1 including flat condyloma	Koilocytosis
Class III	HSIL	CIN 2	Moderate dysplasia
Class III	HSIL	CIN 3	Severe dysplasia
Class IV	HSIL	CIN 3	Carcinoma in situ
Class V	Invasive carcinoma	Invasive carcinoma	Invasive carcinoma

**Table 1.1 The cytological and histological classification systems for precancerous and invasive cervical cancer.**



**Figure 1.8** The histological appearance of precancerous changes in the cervix classified as CIN 1 to 3 and carcinoma in situ (CIS). Panel A shows CIN1 which is characterized by good cell maturation with minimal nuclear abnormalities and few mitotic figures. Undifferentiated dysplastic cells are confined to the lower third (LT) of the epithelium (E). Koilocytes, which are benign changes brought about by HPV are represented by the two black arrows, and are seen in the full thickness epithelium. Panel B shows CIN 2 which is characterized by dysplastic cells in the lower half to two thirds of the epithelium with more marked nuclear abnormalities than in CIN1. Panel C shows CIN3 characterized by full thickness cellular dysplasia and nuclear abnormalities. Cellular differentiation is absent or present only in the superficial quarter of the epithelium (S) and there are numerous mitotic figures (M). Panel D shows CIS which is characterized by full thickness (FT) transformed cancerous cells which have not yet breached the basement membrane (BM).

## **1.11 Invasive cervical cancer (ICC).**

ICC may remain asymptomatic until the disease is advanced, especially in non-sexually active women. Early symptoms include vaginal discharge, irregular bleeding in women of reproductive age, post-menopausal spotting or bleeding and post-coital bleeding (World Health Organisation, 2014). Advanced symptoms which may indicate metastasis to other organs include weight loss, urinary frequency and urgency, lower abdominal pain, back pain/ache, peripheral leg oedema and shortness of breath (World Health Organisation, 2014).

## **1.12 Staging of cervical cancer**

Cervical cancer is staged clinically and pathologically. Clinical staging occurs after clinical examination and is most commonly classified using the Revised FIGO (International Federation of Gynaecology and Obstetricians) Staging System (2009) as follows:

Stage I: The disease is confined to the cervix (includes sub-stages IA1, IA2, IB1 and IB2).

Stage II: Cancer has spread outside the cervix into the upper vagina or to the tissue beside the cervix (parametrium), but not to the sidewall/s of the pelvis (includes sub-stages IIA1, IIA2 and IIB).

Stage III: Cancer has spread to the lower part of the vagina or all the way through the parametrium to the sidewall(s) of the pelvis (includes sub-stages IIIA and IIIB).

Stage IV: Cancer has spread to surrounding organs or distant tissue, such as the lungs and distant lymph nodes (includes sub-stages IVA and IVB) (World Health Organisation, 2014).

The treatment options and prognosis of ICC are dependent on the disease stage. The disease is staged by taking a full history and physical examination which includes a speculum vaginal

and rectal examination and pelvic examination under anaesthesia. Blood tests are taken for a full blood count, urea and electrolytes and liver function tests. Endocervical curettage and endoscopic examination using cystoscopy, hysteroscopy and proctoscopy is performed to ascertain tumour extension into the bladder, uterus or rectum respectively. An intravenous urogram is also performed which would indicate any involvement of the ureter/s and a chest x-ray is done to rule out lung metastases.

Further investigations which may be of benefit in planning treatment, but are not used in clinical staging include an abdominopelvic ultrasound, MRI scan of the abdomen and pelvis with intravenous and oral contrast, CT scan of the chest and laparoscopy. Pathological staging is done post-operatively and it is classified using the TNM classification system (Sobin et al., 2009) where:

T=tumour size

N=number of involved lymph nodes

M=any distant metastasis.

The outcome of pathological staging, however, does not change the clinical staging.

## **1.13 Management of cervical cancer**

Cervical cancer treatment options include surgery, radiotherapy and chemotherapy either alone or in combination. As with treatment of pre-cancerous lesions, patient wishes and reproductive needs are taken into consideration as far as possible. Other important considerations besides disease stage are the overall health of the woman, availability and whether or not it is recurrent disease.

Early stage disease can be treated equally by surgery or radiotherapy. The diagnosis of stage IA1 and IA2 microinvasive disease can only be made on a histological basis, and it depends on the depth of stromal invasion. Stage 1B1 and 1B2 lesions are gross with the former being less than 4cm and the latter more than 4cm in size. For women who want to preserve their fertility, stage IA1-1A2 can be treated by cone biopsy or trachelectomy. Trachelectomy is a procedure where the cervix and the upper part of the vagina is removed, leaving the rest of the womb intact. In women for whom fertility is not an issue, a simple hysterectomy is indicated. Stages IB and IIA are treated with either a radical hysterectomy with pelvic lymphadenectomy or radical radiotherapy with concomitant chemotherapy. Radical hysterectomy involves removal of the uterus, parametrium and paracolpos together with the upper one third of the vagina and a bilateral lymphadenectomy. The fallopian tubes and ovaries are not routinely removed. This can be discussed with the patient. In a carefully selected group of patients, pelvic exenteration can be offered as salvage surgery with the intention to be curative. This is carried out in the event of recurrent disease after initial treatment by surgery or chemo-radiotherapy, and consists of removal of all the reproductive organs including the bladder or the rectum or both, depending on where the disease is situated. In cases of advanced disease causing obstruction to organs such as the bowel or ureter, or fistula formation from radiotherapy, palliative surgery can be performed to relieve symptoms.

Primary radiotherapy is offered to women with stage IB2 disease or greater with curative intent. It is given daily for 5 weeks using either external beam therapy or brachytherapy. Radiotherapy with or without chemotherapy may also be given as adjuvant therapy in cases where cancer is found to have spread to the parametria or to other pelvic organs during primary surgery, if post-surgical histology shows any lymph node involvement of disease and if the histology specimen does not show adequate disease-free margins (Sedlis et al., 1999).

For patients who are not suitable for pelvic exenteration or where it is not a surgical option, radiotherapy can also be used as secondary therapy in women with recurrent disease in the pelvis, following primary surgery. Finally, radiotherapy can be used as palliative therapy in advanced disease to control symptoms such as pain, discharge or bleeding and to treat isolated metastases e.g. to the vertebrae or lymph nodes, without evidence of widespread metastases (World Health Organisation, 2014). The side effects of radiotherapy are local to the area exposed to radiation, which in the case of cervical cancer is the pelvic organs. These include menopause, dyspareunia, bladder and bowel problems and in rare cases, fistula formation. It is important to counsel women of childbearing age about these risks and the implications for future fertility.

Chemotherapy is rarely used alone as primary treatment for cervical cancer. However, there is good evidence (Peters et al., 2000; Rose et al., 1999; Whitney et al., 1999) that addition of platinum-based chemotherapy in patients treated with radical radiotherapy and in high risk patients with early stage disease treated post-operatively with radiotherapy improves both local control and survival. However, radiotherapy is a curative treatment and additional chemotherapy should not compromise radiotherapy treatment. There is a role for neo-adjuvant treatment with chemotherapy in women with large, bulky tumours in order to reduce tumour size, followed by radiotherapy because radiotherapy is more effective on less bulky tumours. Chemotherapy is also used in palliation of symptoms in women with advanced disease and widespread metastases to liver, lung and bone. Chemotherapy affects cancer cells together with other rapidly dividing cells in the bone marrow, digestive system, urinary system, skin and other organs lined by epithelia. Therefore, its side effects are widespread in the body and include pancytopenia which results in infections, bleeding and anaemia; kidney injury, nausea and vomiting and hair loss. The side effects of combination therapy may be additive.

## **1.14 Strategies to prevent cervical cancer**

Effective strategies to prevent cervical cancer exist. Primary prevention strategies include HPV vaccination which was launched in 2006, and secondary prevention strategies include cervical screening methods which detect oncogenic HPV infections and the precancerous changes caused by persistent HPV infection. Cervical cancer is one of the few cancers with a detectable, preclinical, asymptomatic stage which, if treated, can favourably influence the long-term course and prognosis of the disease. This pre-clinical stage allows time for identification and treatment of precancerous lesions before ICC develops. The role of cervical cancer screening is to identify these lesions and to treat them before they progress to ICC. The chance of surviving cervical cancer is directly related to the stage at which the disease is diagnosed. Women with cancer detected in the pre-invasive and even at the CIS stage, have a 5-year survival rate of essentially 100% (Fahs et al., 1996).

Screening with treatment of pre-cancerous lesions is among the few cancer-related so called “best buys” or “very cost effective strategies” according to WHO’s Global Action Plan for the Prevention and Control of Non-Communicable Diseases (2013–20) (WHO, 2016). However, it is access to these strategies which varies depending on where a woman lives, her socioeconomic status and her agency. The average cervical cancer screening coverage in developing countries has been shown to be 19% among women aged 25-64 years compared to 63% in developed countries (Gakidou et al., 2008). The global targets specified in the WHO Global Action Plan for the Prevention and Control of Non-communicable Diseases 2013-2020 (WHO, 2013a) and target 3.4 of the United Nations Sustainable Development Goals are to reduce premature mortality from NCDs. This can only be achieved if comprehensive cancer control is implemented in LMICs, which includes universal access to early diagnosis and accessible treatment for cancer.

## **1.15 Secondary prevention strategies – Methods of screening for cervical cancer.**

There are currently 3 different ways of screening for cervical cancer and these are discussed in detail below.

**Cytology/Papanicolou test.** The “pap” smear test or cytology, with colposcopy triage is the traditional cervical screening test, and is still the most common screening method worldwide (Sauvaget et al., 2011). It is attributed with reducing incidence and mortality of cervical cancer up to 80% in developed countries with organised wide coverage call-recall programmes (Peto et al, 2004; Kitchener et al, 2006). However, due to its low sensitivity to detect pre-cancer, the need for frequent screening, trained personnel, laboratory infrastructure and colposcopy, biopsy and histological follow-up at higher level facilities makes the pap test non-ideal in resource-poor LMICs. When cytology was compared with VIA and HPV test in a mathematical simulation model of carcinogenesis to project long-term health and economic impact of cervical cancer interventions in low resource settings, it was the least effective strategy due to low sensitivity and a high number of required clinic visits (Campos et al., 2015). The sensitivity of cytology varies between countries and laboratories depending on the medical infrastructure (Goodman, 2015).

There are two ways of performing cytology. The conventional way is the pap smear, where a healthcare provider uses a spatula or a small brush to scrape a small sample of cells from the outer layer of the cervix, after spreading the walls of the vagina apart with a speculum. The cells are then smeared onto a slide for examination under a microscope. The second way is the liquid based cytology (LBC), where a sample of cells is taken from the cervix using a small brush. The brush containing the cells is then placed into an ethanol or methanol based

medium for preservation. Once at the laboratory, the sample is processed into a single-cell thin layer on a slide. These slides are stained and can then either be examined under microscopy by skilled staff or subjected to partially automated imaging. Although there is no difference in sensitivity between conventional cytology and LBC, LBC was found to have a lower positive predictive value (Ronco et al., 2007). The automated LBC Thinprep® imaging system detected 1.29 more cases of HSIL per 1000 women screened than conventional cytology (Davey et al., 2007). Besides the platform for automation, the LBC sample has the added advantage of being able to be used for HPV testing too.

A systematic review and meta-analysis (Mustafa et al., 2016) compared the sensitivity and specificity of VIA, cytology and HPV testing . Sensitivity is also called the true positive rate or the probability of detection and measures the probability of the screening test to be able to detect the condition where it exists. Specificity is the true negative rate or the probability of the screening test to correctly identify a woman without the condition as having a negative test. The review compared two screening tests at a time on the same group of women. Cytology was found to less sensitive than HPV testing but more sensitive than VIA. Sensitivity pooled estimate was 0.70 compared to 0.94 for HPV and 0.84 compared to 0.77 for VIA. However, cytology was more specific than both HPV and VIA with a specificity pooled estimate of 0.95 compared to 0.90 for HPV and 0.88 compared to 0.82 for VIA (Mustafa et al., 2016).

A population based study (Katki et al., 2011) found that 30-60% of women found to have cervical cancer had had a normal pap smear 3-5 years before diagnosis. Potential sources of error in cytology include, not sampling the lesion, or the abnormal cells may not transfer from the smear applicator to the slide/vial or inadequate preservation of the cells. Cervical cytology is less sensitive at detecting pre-invasive and invasive glandular lesions than it is at detecting squamous cell lesions (Goodman, 2015).

**Visual inspection with acetic acid (VIA).** VIA is also known as direct visual inspection. It is done by trained personnel and involves staining the cervix with 3-5% solution of acetic acid using a cotton swab and then visualizing the cervix with the naked eye and then visualizing any changes on the cervix, but with no magnification. Abnormal tissue will stain white due to nuclear dense lesions, while normal tissue remains pink. Most precancerous lesions occur in the transformation zone, so VIA is most appropriate for use in women whose transformation zone is visible (typically in those younger than 50).

Acetic acid is cheap and readily available and VIA provides an immediate result in the clinic with no need for complex laboratory facilities to process any tissue. Treatment can therefore be given within the same setting if required. This reduces time, loss to follow-up and anxiety about future test results.

Cervicography is taking a digital photo of the cervix after VIA using an ordinary camera and lens. Once available on digital media the images can be viewed on a screen providing a visual record which can be discussed with colleagues and transmitted online. This has opened the way for innovations in cervical cancer screening in LMICs which will be discussed in more detail in a later chapter.

VIA has been validated as an alternative simple, cheap and valuable means of screening precancerous cervical lesions in resource-poor settings (Gaffikin et al., 1999). In a study of over 10,000 women in Zimbabwe, VIA was found to be more sensitive but less specific when compared with cytology, with a high negative predictive value. Sensitivity was 76.7% for VIA and 44.3% for cytology, with a specificity of 64.1% for VIA compared to 90.6% for cytology (Gaffikin et al., 1999). A systematic review and meta-analysis (Mustafa et al., 2016) found VIA to be less sensitive than both HPV and cytology with sensitivity pooled estimate of 0.69 compared to 0.95 for HPV and 0.77 compared to 0.84 compared to cytology. However, VIA

was more specific than HPV but less so than cytology with specificity pooled estimate of 0.87 compared to 0.84 for HPV and 0.82 compared to 0.88 for cytology (Mustafa et al., 2016). A drawback of VIA testing is that the test sensitivity is dependent upon the operator's training and experience which has to be intensive in order to reach acceptable levels, thereby limiting its potential for scalability (Campos et al., 2017).

**HPV testing.** The gold standard in HPV identification is via molecular detection methods, through DNA or RNA testing or detection of cellular markers in cells transformed to malignancy through HPV infection (Goodman, 2015). There are 148 HPV tests commercially available worldwide (Dillner, 2013). The two main methods by which commercial HPV kits test for HPV DNA are via signal amplified hybridization assays or PCR based target amplification assays (Zaravinos et al., 2009). HPV tests detect either hrHPV subtypes as a group, without specifying which ones, or they detect HPV16 and HPV18.

Specimens for HPV tests are obtained by scraping the ectocervix with either a cotton-wool swab, a cervical brush, or vaginal tampon and then placing the samples collected into HPV transport test medium. Before HPV testing, the material from the swab has to be reconstituted in a liquid medium (Goodman, 2015). An advantage of HPV testing is that the sample can either be collected by a healthcare provider, in which case a cervical sample is obtained, or by patient self-collection, in which case a vaginal sample is obtained. The latter reduces the time spent by women being screened, as well as the burden on health care workers, thereby potentially increasing the rate of screening uptake. Despite slightly reduced test sensitivity, HPV self-collection at home, is a cost effective alternative to provider-collection at the clinic (Campos et al., 2017).

Only four types of HPV tests are approved for primary cervical screening in Europe and the USA (Goodman, 2015). These are; the Hybrid Capture 2 Assay (Qiagen, Gaithersburg, MD,

USA), which is a nucleic acid hybridization assay that detects the amount of DNA of 13 hrHPV subtypes. In addition to the 13 subtypes detected by HC2, the Cervista HPV HR test (Hologic) also detects the presence of HPV 66. Another Cervista test (Cervista 16/18) detects the DNA of HPV 16 and HPV 18. The COBAS 4800 system (Roche Molecular Systems, Alameda, CA USA) is a PCR-based test which amplifies and detects the presence of HPV 16 and HPV 18 DNA and also gives a pooled result for another 12 high-risk subtypes. The Aptima mRNA test (Gen-Probe, San Diego, CA, USA) is an RNA-based test that identifies E6 and E7 protein RNA from 14 hrHPV subtypes (Goodman, 2015). The Cobas 4800 System is the only HPV test recommended for primary screening in the USA (FDA, 2014) because the ATHENA (Addressing THE Need for Advanced HPV diagnostics) trial showed that the Cobas 4800 System was superior to HC2 (Stoler et al., 2011).

A low-cost HPV DNA test, *careHPV* (Qiagen, Gaithersburg, MD, USA) was developed for use in low-resource regions. It has been clinically validated (Jeronimo et al., 2014; Qiao et al., 2008) and is now commercially available. It requires basic laboratory facilities, and detects 14 hrHPV subtypes. A result is available in just 2.5 hrs and each kit costs only US\$5.00. It has comparable sensitivity and specificity to the other higher cost HPV tests. In a study done in South Africa and Burkina Faso (Ngou et al., 2013) that compared cervical samples taken from the same women and tested with *careHPV* and HC2 there was 94.6% (95% CI, 89.7% - 97.7%) agreement between the two tests.

The HPV test is objective and reproducible and could result in cost savings by reducing the frequency of screening from the current 2-3 years that is required for cytology to every 5 years, because a negative HPV test virtually excludes any risk of having cervical disease for a period of at least 5 years (Castle et al., 2012). Additionally, HPV testing is less labour intensive, as one laboratory technician is able to process, in one working shift, the same

number of HPV samples, that would require the work of several cytotechnicians, in order to process the same number of cytology samples (Arbyn et al., 2010).

HPV testing is 30-40% more sensitive, but 3–5% less specific than cytology and leads to a 70% reduction in invasive cancer overall (Ronco et al, 2014). Although one screen with VIA remains cheaper compared to one screen with HPV testing, VIA and cytology screening must be done more frequently in order to achieve the same health benefit as is achieved with one HPV screen. As a result, health economists have shown repeatedly, that a screening programme using HPV testing would be more cost-effective than programmes that use either VIA or cytology testing (van Rosmalen, 2012; Campos NG, 2017). In addition to having a high negative predictive value, HPV testing has automated platforms which enable high throughput (Kitchener et al., 2009) and it is also less prone to human error in collection and interpretation of the specimen. However, the high cost of HPV testing which is caused by the logistical, and technological requirements for its successful implementation make it prohibitive for under-resourced health systems in LMICs (Denny, 2017).

The screening method(s) used in a country largely depend upon the resources available to that particular country and the perceived cost-effectiveness of the screening strategy. These strategies will be discussed separately for high income countries (HIC) and LMICs as they differ according to economic status of the region of the world the country lies in.

## **1.16 Cervical cancer prevention strategies in high income countries.**

**HPV vaccination:** The HPV vaccine has been licensed in the UK and USA since 2006, and vaccination in the UK started in September 2008 where it was targeted at 12 to 13-year old girls. By 2011, 28 countries had implemented HPV vaccination as part of their national immunization programme. There are 3 vaccines available – the bivalent Cervarix which protects against HPV16 and HPV18, the quadrivalent Gardasil which protects against HPV 6 and 11 in addition to 16 and 18, and the nonavalent Gardasil9 protects against an additional 5 HPV strains – 31, 33, 45, 52 and 58. Gardasil9 protects against the causative agents for 90% of all cervical cancers. Australia was the first country to introduce the quadrivalent vaccine which protects against HPV types that cause anogenital warts, in April 2007. The HPV vaccine has been shown to be effective in preventing genital warts, and is effective in protecting against oncogenic HPV types. These reductions in anogenital warts, HPV16/18 and HSIL have been well documented in young females and also in heterosexual males due to herd immunity effects (Drolet et al., 2015; Harrison et al., 2014; M. A. Smith et al., 2015; Tabrizi et al., 2014). Between 2004-6 and 2012, rates of CIN2/3 among women aged less than 20 years in Australia decreased by 53%, and the rate for women aged less 20-24 years was stable until 2010, then decreased by 21% in the following year (Australia Institute of Health and Welfare, 2014). So far the efficacy of the vaccine has been proven to last for up to at least 9 years and studies are still ongoing.

**Cervical cancer screening:** Cervical screening in the UK started in 1988 and is available to women aged 25-64 years old via a computerized call-recall system. Women aged between 25 and 49 are invited for screening every 3 years, and women aged 50 and over, every 5 years.

It has been attributed for detecting around 80% of cervical cancers in screened women (Peto et al, 2004) (Hoory et al., 2008).

There is an age-related decline in cervical screening uptake in the UK as women get older, because cervical cancer is thought to be a disease that predominantly affects younger woman (Sherman et al., 2015). This has been reinforced by high profile deaths of young women reported in the media. The death of television celebrity Jade Goody in March 2009, resulted in an additional 500 000 women, mainly below the age of 50 years, being screened between 2008 and 2009 (Lancucki et al., 2012). However, this increase in screening uptake was not sustained. The realization that there is a second peak in incidence of cervical cancer after age 65 years, has led to calls to extend the upper age limit for screening. Compounding this is fact that there are 25% or more of women who have not been regularly screened. These women are likely to be non-white and come from lower educational backgrounds (Moser et al., 2009). Extending screening to women aged 60-74 years would result in 90% prevention of cervical cancers.

Screening is primarily via cytology, and an abnormal cytology result requires biopsy and confirmation by histology. LBC has been used since 2008 (Denton, 2007). Cytological and histological diagnoses are in agreement in about 50% of cases. The NHS includes HPV triage in its screening programme. This means that if the initial cytology test shows borderline results or LSIL, the sample is further tested for HPV. If HPV is present the patient is referred for colposcopy, but if absent, the screen is negative, and she resumes the usual screening schedule.

The negative predictive value of HPV testing is high but it lacks specificity with sensitivity of about 90% (Zazove et al., 1998). Women who tested negative for hrHPV remained at low risk for developing precancerous lesions over a study period of 5 to 18 years (Castle et al., 2012).

The results of four randomized trials conducted in Sweden (Swedescreen), the Netherlands (POBASCAM), England (ARTISTIC), and Italy (NTCC) have shown that HPV-based screening provides 60-70% greater protection against invasive cervical carcinomas compared with cytology. The recommendation is for HPV-based screening to start from age 30 years and for the screening intervals to increase to at least 5 years (Ronco et al., 2014).

So far, the Netherlands and Australia are the only two countries that have transitioned to nationwide primary HPV testing screening. The Netherlands offers 10-yearly primary HPV screening with reflex cytology triage and cytology triage after six months for women aged 40 years and over Australia offers 5-yearly primary HPV screening with partial genotyping for HPV16/18 for women aged 25-69 years, with cytology triage for women who test positive for hrHPV types other than 16/18, and HPV exit testing at 70-74 years (Canfell et al., 2018). New Zealand will be introducing primary HPV screening in 2018, and Wales will be the first of the 4 devolved nations in the UK to introduce it in September 2018. This will be followed in England by the end of 2019, and in Scotland by January 2020.

## **1.17 Cervical cancer prevention strategies in low income countries.**

The technological advances that can prevent, detect and treat oncogenic HPV infections which are widely available in high income countries are not generally available in the low- and middle-income countries. This is because of limited access to high quality laboratory facilities and infrastructure, skilled personnel and health budgets to maintain them (Nkengasong et al., 2010).

**HPV vaccination:** Between 2006 and 2014, only 3% of females aged 10-20 years in LMICs had received even one dose of the HPV vaccine (Bruni L et al, 2016). The Vaccine Alliance (GAVI) has started the process of improving access to the vaccine. Since 2012, GAVI has approved HPV demonstration projects in 28 LMICs at a reduced vaccine price of \$4.50 per dose (Campos et al., 2017). Lesotho, Rwanda and Uganda have initiated national HPV vaccination programs.

Zimbabwe undertook a demonstration program in 2016 with their support which saw 10-year old girls in the two districts of Marondera and Beitbridge receive Cervarix. The demonstration programme has now been concluded and a national roll-out has now begun, as of August 2017. The government-funded vaccine has been made possible with the help of GAVI, and is available to all girls aged 10-14 years old for an initial two-year catch up period, after which the vaccine will be available to girls at age 10 years only (Hanson CM et al, 2015).

Whilst nationwide HPV vaccination is a welcome development in the fight against cervical cancer in Zimbabwe, the vaccine will not begin to reduce the burden of cervical cancer for 20 years. And for the rest of the women who are beyond the target age of vaccination, screening still remains the only form of protection. Even vaccinated women are still encouraged to go for screening since the vaccine does not protect against all HPV types that cause cancer. To this effect, screening remains an integral part of the fight against cervical cancer.

**Cervical cancer screening:** WHO recommends that LMICs with low coverage of screening and restrictions on resources, use a 'screen-and-treat' strategy, where the treatment decision of a precancerous lesion is based on a screening test and treatment is provided soon or ideally, immediately after a positive screening test in the same setting. The recommended age of screening is 30 years and older with priority to maximise population screening coverage rather than maximising the number of screening tests in an individual woman's lifetime (WHO, 2013;

WHO, 2014). The recommended screening test is the HPV test if resources allow, otherwise the visual inspection with acetic acid (VIA) test. The lifetime risk of cervical cancer was found to be reduced by 25-35% in women who are screened once in their lifetime at around age 35 years by VIA or HPV testing and by 40% in those screened twice (Goldie et al., 2005). Screening women three times, between 30 and 45 years reduced cancer risk by 50% (Campos et al., 2015). Cytology is not recommended unless an existing cytology programme meets quality indicators for training, high coverage, and follow-up (WHO, 2013b)

## **1.18 Cervical cancer screening in Zimbabwe**

The reasons as to why the incidence of cervical cancer is so high in developing countries are many. The most important one is the lack of availability of national screening programmes with a 'call and recall' system that can reach a high target population such as those found in developed countries. In Zimbabwe, 76% of women present at the later stages of the disease (Chinombe et al., 2014). Studies have shown however that even in the districts where cervical cancer screening is available, only a small percentage of women ranging from 4.3%-9% (Mangoma et al., 2006) (Mupepi et al., 2011) had accessed this service. Knowledge about cervical screening ranged from 2.9% among a cohort of HIV positive women of child bearing age (Gundani & Chipfuwa, 2013) to between 9.8% and 19% in rural women (Mangoma et al., 2006) (Mupepi et al., 2011). Lack of knowledge about disease progression and its consequences means that women view absence of symptoms and pain as an indicator of well-being and therefore focus on other more pressing issues whilst health is neglected (Mangoma et al., 2006). Other barriers to accessing screening included lack of funds to travel to testing centre, lack of trained staff to administer the tests and poor social marketing of the screening programme. Yet still, other reasons given included the embarrassment of being screened by

a male health professional and the discomfort of having to lay on your back with legs wide open during the procedure. Lack of understanding among the men in the women's lives and the social stigma associated with having a hysterectomy in the event that it was required also contributed (Mangoma et al., 2006).

The reproductive health beliefs of women in developing countries may also play a part in the increased rates of cervical cancer. A study done in two districts of Zimbabwe showed that 41-45% of women had used various forms of vaginal preparations including tree bark, herbs, leaves powders and soaps. These preparations, which are inserted per vaginally are used during pregnancy to dilate the vagina during labour (2.7% in the study). They are also used to dry and tighten the vagina in order to enhance sexual satisfaction and enjoyment for their partners (89.5% in the study) and women with fertility problems are given these herbs to enhance their fertility (5.9% in the study) (Mangoma et al., 2006).

## **1.19 Treatment of precancerous lesions.**

Effective cervical cancer screening involves identification and treatment of precancerous HPV-induced cervical lesions. The goal of treatment is to remove the lesion, which can be accomplished either by ablative methods such as cryotherapy, laser ablation and electrocautery or using excisional methods such as LEEP also known as large loop excision of the transformation zone (LLETZ) and cold knife cone biopsy (CKC). The method used primarily depends on the extent and severity of the lesion. Other factors which are taken into consideration include cost, availability, reproductive needs, and patient choice. Ablative methods destroy the precancerous cells in the transformation zone, by necrosis, using either ice-cold gas, laser or a heated wire electrode with no anaesthetic and no tissue is removed

so there is no histological specimen for evaluation. Excisional methods involve removal of the precancerous lesion together with a margin of healthy tissue, under either local or general anaesthetic. The tissue removed can be sent for histological evaluation. The most commonly used treatment methods are cryotherapy, LEEP and CKC and these are discussed further below.

Cryotherapy uses cold nitrous oxide or carbon dioxide gas to cool a metal disc (cryoprobe) to  $-50^{\circ}\text{C}$  (Gage & Baust, 1998), which is then applied to the pre-cancerous lesion on the cervix, thereby destroying the abnormal tissue. This is a quick procedure which lasts about 15 minutes and can be performed at all levels of the health system from the primary care all the way to the tertiary level by trained personnel including both nurses and doctors. It can also be done at the time of screening, with all these factors therefore making it an ideal procedure to be incorporated within a screen and treat strategy. There are, however, strict criteria in order for a patient to be eligible for cryotherapy. The whole of the lesion should be visible, the transformation zone should be visible and the lesion should cover less than 75% of the ectocervix (WHO, 2013b). Cryotherapy is effective, relatively low cost, low maintenance and safe, as major bleeding and infection are less common in cryotherapy compared with cold-knife conization and LEEP (Santesso et al., 2016). However, the evidence suggests that rate of recurrence of CIN2 to 3 is increased with cryotherapy treatment compared to LEEP and CKC (Santesso et al., 2016). Disadvantages of cryotherapy include the potentially high cost of obtaining and replenishing gas in LMICs and the cumbersome equipment which can be problematic in field settings.

LEEP is an excisional treatment method for precancerous lesions which is performed under local anaesthetic. Under direct vision, a hot wire loop electrode that cuts and simultaneously coagulates tissue is used to remove the entire transformation zone, including the abnormal tissue which is then followed by a ball electrode to complete the coagulation. The removed

tissue can be sent for histological evaluation, making it both a diagnostic and a therapeutic procedure. There is a higher rate of recurrence of CIN2 to 3 at 12 months, and an increase in major and minor bleeding with LEEP compared to CKC, however there is a decreased risk of major infection with LEEP compared with CKC (Santesso et al., 2016). LEEP is performed by an experienced clinician, generally an obstetrician/gynaecologist who can recognize and manage intra and post-operative complications, and in facility where surgical back-up is available if required (Maza et al., 2017). Ideally this should be done at least, at secondary care level i.e. a District Hospital. The use of LEEP as a first-line treatment in LMICs is limited due to its requirement to be performed by a trained clinician, as well as the need for electricity, local anaesthesia and back up theatre facilities (Maza et al., 2017).

CKC is performed in cases which are not amenable to cryotherapy or LEEP i.e. if the LEEP excision margins are inadequate; in cases of CIS or if there are glandular (adenocarcinoma) pre-cancerous changes. A scalpel is used to remove a cone-shaped piece of tissue which includes the endocervical canal and encompasses the lesion, under general anaesthesia. Using a scalpel ensures the removal of endocervical canal deeply, and avoids diathermy artefact caused by heat electrodes at the excision margins (Stern et al., 2012). Like LEEP, CKC is both a diagnostic and therapeutic procedure, as the removed tissue can be sent for histological evaluation. It is performed by a surgically trained health professional who is able to recognize and manage complications like major bleeding and damage to other organs requiring surgery. CKC has the lowest recurrence rate of CIN 2+ when compared with LEEP and cryotherapy (Santesso et al., 2016). There are also other complications such as major infection (including PID) and increased risk of premature labour (Santesso et al., 2016) which would need to be taken into consideration when treating women of childbearing age with CKC. It has no place in a screen and treat strategy because the potential complications of bleeding, and premature delivery outweigh potential benefits when compared to LEEP and cryotherapy (WHO, 2013b).

## **1.20 The Case for locoregional therapy for precancerous lesions.**

The treatments summarised above for pre-cancerous cervical lesions have been developed and been in use over the last five decades and are effective as they are responsible for reducing the incidence and mortality of cervical cancer in developed countries over the same period of time (Kitchener et al., 2006; Peto et al., 2004). However, since pre-cancerous lesions and consequently these treatments are typically in women of reproductive age locoregional strategies that address and circumvent some of the issues with current practice are required. The rationale for developing locoregional strategies is presented below.

While these surgical procedures remove the neoplasia, they do not target the cause of these lesions, which is the persistent HPV infection. Recurrence rate for CIN after treatment is between 5-26% (Gonzalez et al., 2001; Soutter et al., 2006). Although excisional therapy is effective for pre-cancerous lesions in immunocompetent patients, it seems to be effective only in slowing progression to cervical cancer in HIV positive women. The recurrence rates for women with HIV are much higher than for HIV negative women, even in the HAART era, with studies showing recurrence rates of up to 63% (Fruchter et al., 1996; Maiman et al., 1993; Paramsothy et al., 2004; Petry et al., 1994). Fruchter et al., reported that 90% of HIV-infected women with CD4 counts less than 200 cells/mm<sup>3</sup> developed recurrent dysplasia at 3 years (Fruchter et al., 1996). Antiretroviral therapy for HIV may delay the recurrence of HPV-related disease, however, persistent HPV DNA remains detected in patients on HAART, indicating that immune restoration on HAART is insufficient to clear HPV (Heard et al., 2004), furthermore, there has been no change in the incidence of invasive cervical cancer since the introduction of HAART (International Collaboration on HIV and Cancer, 2000). In addition,

treatment has failed to eradicate CIN in many HIV-infected women (Heard et al., 2005), with definitive management with hysterectomy being suggested for eradication of the disease.

A meta-analysis on perinatal mortality and adverse pregnancy outcomes showed that cold knife conization, laser conization and radical diathermy were associated with a significantly increased risk of peri-natal mortality, pre-term delivery and low birthweight (Arbyn et al., 2008; Kyrgiou et al., 2006a), premature rupture of membranes and caesarean section (Kyrgiou et al., 2006a). The literature is not consistent about effects of excisional therapy on fertility. A case-control study by Spracklen et al., (Spracklen et al., 2013) looked at the rates of subfertility, defined as prolonged time to pregnancy of more than 1 year, in women with a history of surgery for CIN, and found that they had more than a two-fold higher risk of prolonged time to pregnancy compared to untreated women. However a meta-analysis (Kyrgiou et al., 2014) found no evidence to support that treatment for CIN affects fertility outcomes and the chances to conceive, they did, however, find evidence that it increased the risk of a second trimester miscarriage possibly due to cervical incompetence after large excisions. Although CIN2 is commonly treated with local excision of the cervix recent data suggests that most CIN2 lesions regress spontaneously, particularly in young women. A recent systematic review and meta-analysis (Tainio et al., 2018) that looked at the clinical course, and risk associated with active surveillance of CIN2 found that over two years CIN2 will regress in 50% of women under surveillance, persist in 32% and progress to CIN3 or worse in 18%. For women under 30 years, the outcomes were more favourable at 60%, 23% and 11% respectively. Therefore, this means that the reclassification of CIN2 and 3 as HSIL has implications for many women, especially young women in their reproductive years with possibly spontaneously regressing lesions being classified as having high grade lesions warranting treatment and the attendant increased risks of reproductive morbidity (Kyrgiou et al., 2006b) (Santesso et al., 2016).

## **1.21 Locoregional therapy for cervical cancer (CIN).**

Based upon the discussion above, there is a case for developing locoregional therapies that can be applied directly to the surface of the cervix. The concept would focus treatment directly onto the preneoplastic lesion leading to local control of the disease and prevent recurrence. It would be particularly applicable in the case of HIV positive women for whom treatment is associated with high recurrence rates, and for whom treatment resistance is a problem. In a country like Zimbabwe where HIV prevalence rates are around 15% (UNAIDS, 2013), this would apply to a significant proportion of women. It would also reduce the need for yearly cervical screens following a diagnosis and treatment of high grade cervical dysplasia CIN2+. A treatment which negates the need to use excisional or ablative methods would be ideal as it would also reduce the incidence of obstetric complications that may follow, in women of reproductive age.

Locoregional therapies have been developed, but only a few drug-based locoregional therapies for HPV are approved for use in clinical practice. None of these therapies are licensed for the management of cervical dysplasia however. These therapies target either the molecular virology of HPV infection, the neoplasia, the immune response to the virus or indeed all three leading to viral clearance or lesion elimination (Stern et al., 2012). Strategies that target the virus aim to reduce the rate of recurrence by focusing on the cause of the lesions and thereby have an impact on the amount of latent and subclinical HPV DNA rather than just removing the neoplasia. Details of treatments currently used locoregionally are provided below.

**Cidofovir** is an acyclic monophosphate nucleotide analogue with broad-spectrum anti-DNA virus activity. It competitively inhibits the incorporation of deoxycytidine triphosphate into viral DNA by viral DNA polymerase, disrupting further chain elongation (Lea & Bryson, 1996). Cidofovir is primarily indicated for the treatment of AIDS-related cytomegalovirus (CMV) retinitis, and in the treatment of aciclovir-resistant mucocutaneous herpes simplex virus (HSV) infections in immunocompromised patients. A phase II double-blind prospective randomized placebo controlled trial of 46 patients treated for CIN2+ with either cidofovir formulated as a gel in a cervical cap, or placebo, 6 weeks before their planned conisation excisional procedure was carried out (Van Pachterbeke et al., 2009). Biopsy results from the excised cones showed that 60.8% of women in the cidofovir group were free from CIN compared to 20% in the placebo group.

**Lopimune** or **Kaletra** is an HIV-1 protease inhibitor which is a combination of lopinavir and ritonavir. It has shown some promising pre-clinical and early trial results for locoregional therapy in the treatment of CIN. In addition to its anti-HIV action, lopinavir has been shown to have anti-cancer activity (Batman, Hampson, & Hampson, 2011) by blocking the ability of HPV16 E6 protein to produce proteasomal degradation of p53 in cultured cervical carcinoma cells (Hampson et al., 2006), upregulation of the antiviral protein ribonuclease L in these cells and which has been subsequently confirmed in HPV 16 E6/E7 immortalized keratinocytes (Batman et al., 2011) and a high selective toxicity for E6/E7 expressing cells (Zehbe et al., 2011), but at a more than ten-fold higher concentration than is normally achieved in cervico-vaginal fluid by the doses used for oral HIV therapy (Kwara et al., 2008). In a single arm proof of concept trial done in Kenya (Hampson et al., 2016) 23 hrHPV positive women with HSIL were treated with a two-week course of Lopimune formulated as a self-applied twice daily vaginal pessary. Post-treatment cytology at 12 weeks which was confirmed by histology

showed 77.8% had achieved either complete resolution of dysplasia or a change from HSIL to LSIL, and HPV was no longer detected in 52.2% of women.

**5-Flourouracil** is an antimetabolite pyrimidine analogue which is licensed for the treatment of basal cell carcinoma and an off-label therapeutic option for VIN, VAIN and anogenital warts (Stanley, 2003; van de Nieuwenhof et al., 2008). A prospective non-blinded randomized trial of intravaginal 5% 5-FU formulated as a cream vs observation in women aged 18-29 years with CIN2 (Rahangdale et al., 2014), found that there was regression of CIN2 in 93% of women in the 5-FU group and 56% of women in the observation group. After 6 months, the women treated with 5-FU were twice as likely to be HPV negative, and to have negative cytology and cervical biopsies compared to the control group. It has also been previously shown to slow the time to, and rate of recurrence for HIV positive women previously treated with excisional methods for CIN2-3 (Maiman et al., 1999). However, its teratogenicity and the requirement for study participants to be on 2 forms of contraception may make this unacceptable to many women.

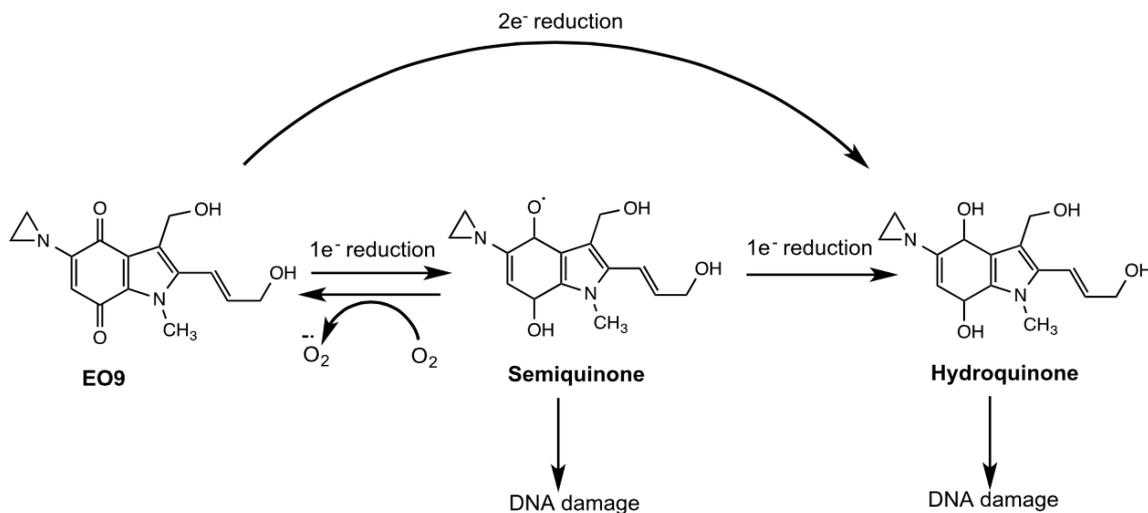
**Imiquimod** is an immune response modifier, which is licensed for the topical treatment of anogenital warts. It works through Toll-like receptor (TLR7) to stimulate the innate immunity through induction of cytokines and acquired immunity through interferons and interleukins. While imiquimod has proven efficacy against vulval intraepithelial neoplasia (Tristram et al., 2014), the literature is not consistent on its efficacy as a topical treatment for CIN (Grimm et al., 2012; Lin et al., 2012; Pachman et al., 2012).

**Artesunate** is a semisynthetic analogue of artemisinin that is used to treat malaria with possible strong antineoplastic activity. A phase I trial of intravaginal artesunate formulated as suppositories, is currently under way to evaluate its potential to treat precancerous cervical lesions (NCT02354534, Clinicaltrials.gov).

## 1.22 The potential for enzyme directed bioreductive therapy in cervical cancer.

As discussed above, there is precedence in the literature for locoregional therapies to treat cervical cancer either indirectly by targeting the HPV infection or directly by treating the cancer. The key for any chemotherapy based treatment of cancer is selectivity and this property is high on the 'wish list' for an effective locoregional therapy for cervical cancer. Here, the development of a rationale for the use of the enzyme activated bioreductive prodrug EO9 will be presented.

EO9 is an indolequinone based compound that is a prodrug. It requires enzymatic reduction to reactive intermediates that become toxic to cancer cells. The preclinical and clinical pharmacology of EO9 has been described in detail elsewhere (Phillips et al., 2017; Phillips et al., 2016; Phillips et al., 2013) and its mechanism of action is summarised in figure 1.10

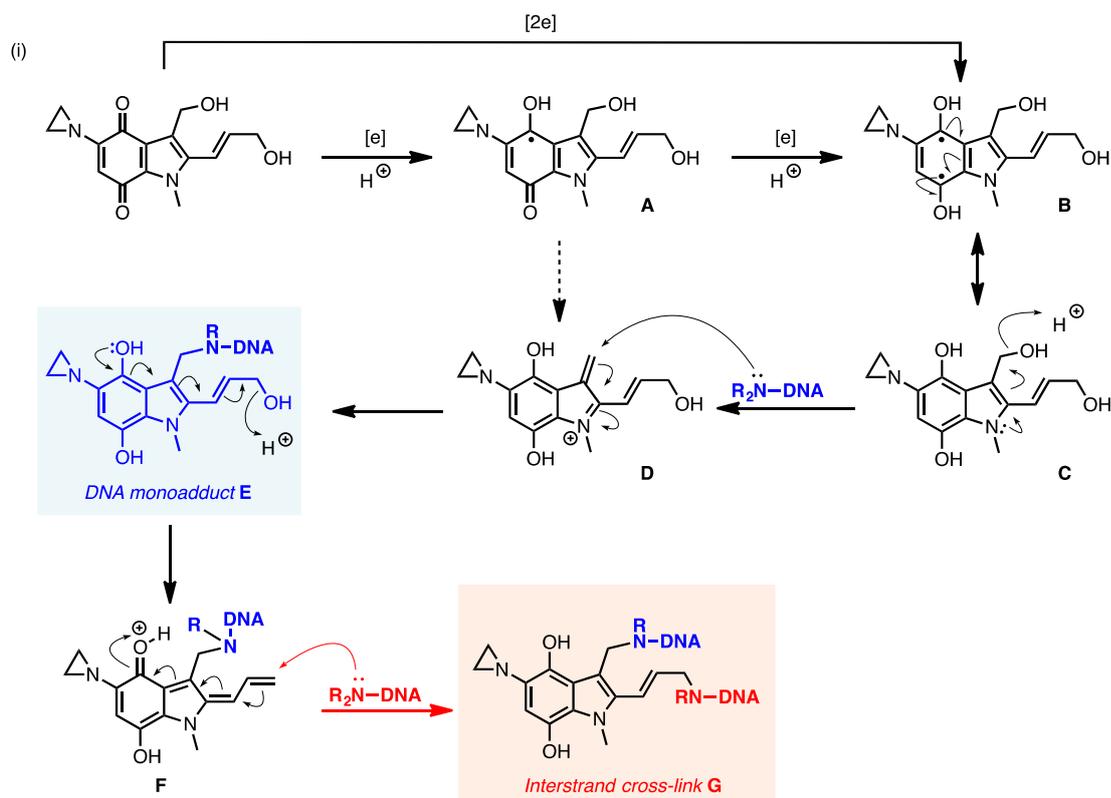


**Figure 1.9 Mechanism of action of EO9 (Phillips et al., 2017).**

EO9 has a complex mechanism of action and it has the ability to target hypoxic and aerobic cells depending upon its ability to undergo one or two electron reduction (Phillips et al., 2017). EO9 is reduced by either one or two electron oxidoreductases to generate the semiquinone (product of one electron reduction) or hydroquinone (product of two electron reduction). The parent compound is inactive but following enzymatic reduction, DNA damaging metabolites are generated (see figure 1.9).

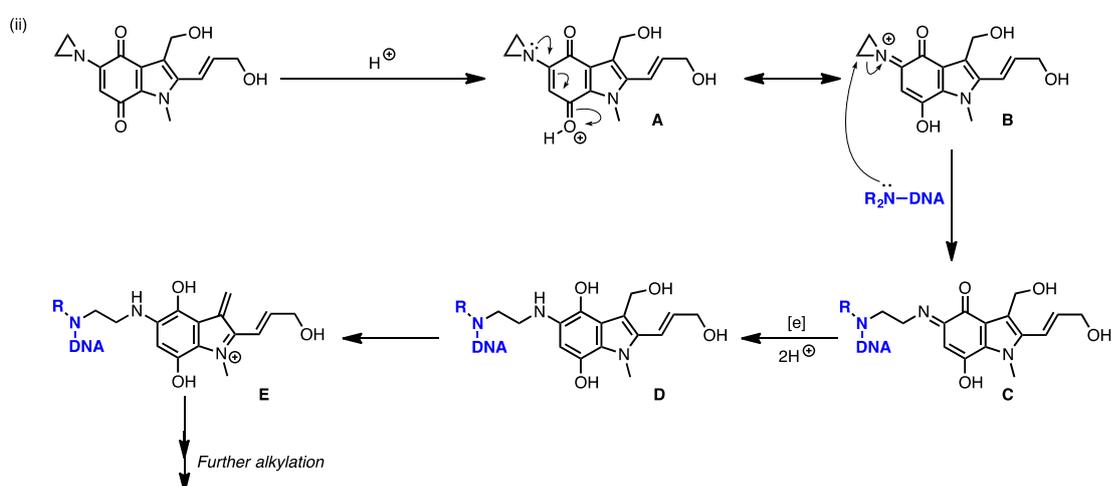
The key step in the activation of EO9 is enzymatic reduction and therefore selectivity will be determined by the enzymology of tumour cells. The pharmacodynamics of EO9 depend on the factors that influence the bioactivation process and the response of cells to the DNA damage that ensues. Bioreductive activation and the degree of cell kill of EO9 is dependent on the enzymology of the cells, specifically, the expression of one and two electron oxidoreductases such as cytochrome P450 reductase and NAD(P)H Quinone Oxidoreductase 1 (NQO1) (Phillips et al., 2013). Its activity is also influenced by the oxygen tension of the cells and the extracellular pH (Phillips et al., 2017).

Reduction of EO9 by purified NQO1 and cytochrome P450 reductase generates free radicals and DNA damaging species in cell-free assays (Bailey et al., 2001; Butler et al., 1996; Walton et al., 1991) (See figure 1.10 and 1.12). DNA damage causes interstrand cross-links, mono-adducts and strand breaks (Bailey et al., 1997; Bailey et al., 2001; Maliepaard et al., 1995; Phillips, 1996; Walton et al., 1991). There is some discrepancy in the literature with regards to DNA interstrand cross-links, with Bailey et al, 1997 and Maliepaard et al, 1995 reporting crosslinking which was not reported by Phillips, 1996. Conversely, anti-oxidants such as catalase and Tempol have been shown to reduce DNA damage and NQO1 activity (Phillips et al., 1999; Samuni et al., 2002).



**Figure 1.10 E09 mediated DNA alkylation via one- and two-electron reduction. Proton-coupled single-electron reduction of E09 generates stabilised radical **A**, which can be further reduced (via **B**) to key intermediate **C**; **C** can be alkylated via cation **D**, formed by acid-catalysed dehydration, to give DNA monoadduct **E**. **C** can undergo a second dehydration, giving diene **F**, which can be trapped in a second DNA alkylation event, giving interstrand cross-linked species **G** (Phillips et al., 2017).**

The activity of EO9 is increased under mildly acidic extracellular conditions of pH 6.0 without increasing substrate specificity (Choudry et al., 2001; Phillips & Ward, 2001; Phillips et al., 1992). However, it becomes less stable at this pH and breaks down into EO5A (Phillips et al., 1992) via proton-assisted aziridine ring opening and enhanced nucleophilic attack as shown in figure 1.11, this can lead to alkylation of DNA but in the presence of water only, the R-N-DNA group in blue is replaced by -OH to form EO5A.

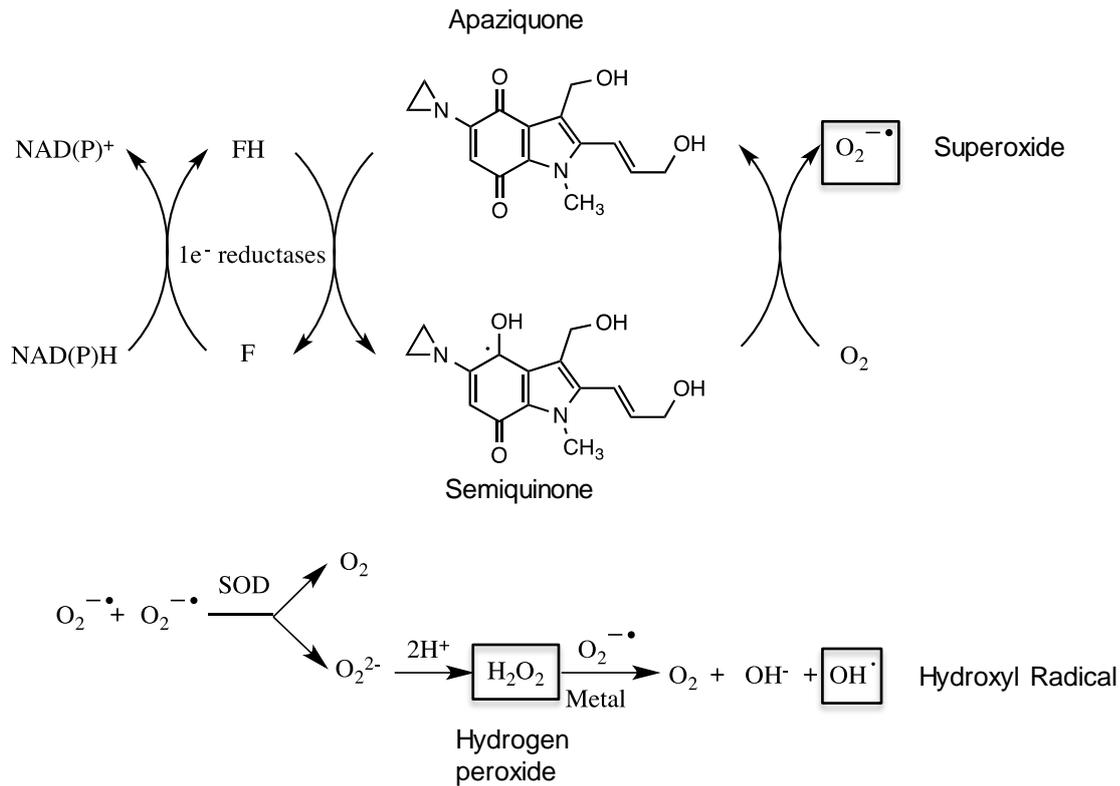


**Figure 1.11 EO9 mediated DNA alkylation via proton-accelerated mechanism.**

The lone pair of electrons on the aziridinyl group are conjugated into the quinone ring allowing for a proton-assisted addition DNA alkylation reaction. Thus, protonation leads to the formation of A, which is in resonance with B, nucleophilic attack of the reactive aziridinium group by DNA leads to ring opening of B forming the aza-quinone species C, which can undergo reduction to D. Acid-catalysed dehydration of D results in the methylene indolium cation E, which is an active alkylating agent (Phillips et al., 2017).

The oxygen tension of cells together with the expression of one electron and/or two electron reductases influences response to EO9. Typically, EO9 is predominantly reduced by NQO1, and studies have shown that cytotoxicity is inversely proportional to NQO1 activity (Fitzsimmons et al., 1996; Plumb et al., 1994; Robertson et al., 1994; Smitskamp-Wilms et al., 1996). In oxygen-poor tissues, increased EO9 cytotoxicity is seen in cells with little or no NQO1 activity (Plumb & Workman, 1994; Robertson et al., 1994) such as those with the NQO1\*2 polymorphic variant (Plumb & Workman, 1994; Traver et al., 1997). In this environment, one electron reductases take the predominant role of reducing EO9 and in this case, the reaction is oxygen-sensitive, with the semi-quinone becoming rapidly redox-cycled back to the parent compound, producing reactive oxygen species (see figure 1.12).

In a hypoxic cellular environment, the semi-quinone is not readily redox cycled back to the quinone, thus increasing its' half-life and therefore the opportunity for it to alkylate DNA directly or to be further reduced to the hydroquinone (Workman, 1994). In summary, *in vitro* studies have shown that the cytotoxicity of EO9 is dependent of NQO1 activity and in cells with high levels of NQO1, cell kill is oxygen independent. However, in cells with little or no NQO1 activity, EO9 selectively targets hypoxic cells. Predicting response to EO9 *in vivo* however, is more complex and tumour response could not be predicted based upon NQO1 activity (Collard et al., 1995).



**Figure 1.12 Reduction of E09 by one electron oxidoreductases. E09 is reduced by NAD(P)H dependent one electron oxidoreductases such as cytochrome P450 reductase to a semiquinone. In the presence of oxygen, the semiquinone undergoes redox cycling back to E09, generating superoxide anions in the process. The superoxide anions are converted to Hydrogen peroxide via superoxide dismutase (SOP) mediated reactions. Hydrogen peroxide is in turn, converted to hydroxyl radicals in the presence of trace metals, and these hydroxyl ions lead to subsequent damage to cellular macromolecules (Phillips et al., 2017).**

In summary, E09 has a complex mechanism of action and it has the ability to target hypoxic and aerobic cells depending upon its ability to undergo one or two electron reduction (Phillips et al., 2017). Brief details of the biochemical and physiological properties of NQO1 are presented below.

NQO1 is a multifunctional protein that (i) functions as an anti-oxidant enzyme by detoxifying potentially mutagenic and carcinogenic xenobiotics (Siegel et al., 2012), (ii) maintains  $\alpha$ -tocopherol and Coenzyme Q in their reduced state where they function as anti-oxidants (Dinkova-Kostova & Talalay, 2010) and (iii) functions as a 'gatekeeper' for 20S proteasomal degradation pathways preventing the degradation of biologically important proteins such as p53 (Asher et al., 2001; Asher et al., 2005). NQO1 is believed to stabilize p53 especially in response to oxidative stress, as well as partially inhibits p53 degradation mediated by E6 (Gad et al., 2002). In the context of the life cycle of hrHPV described above, loss of the antioxidant and p53 stabilization functionality of NQO1 could promote the oncogenic properties of these viruses.

The gene encoding for human NQO1 is polymorphic and several single nucleotide polymorphisms (SNPs) have been characterized. The most widely studied SNP is NQO1\*2 (rs1800566) that encodes for a proline to serine amino acid substitution in *NQO1* (Traver et al., 1997; Traver et al., 1992). *NQO1* mRNA is translated but the protein is rapidly degraded by ubiquitination and proteasomal degradation leading to complete loss of NQO1 activity (Siegel et al., 2001). Loss of the cytoprotective functions of NQO1 has significant implications and several studies have shown that the NQO1\*2 polymorphism is associated with an increased risk of several cancer types (Zhao et al., 2014) (Wang et al., 2013) (Li et al., 2014) (Gong et al., 2013) (Sun et al., 2014) (Liu et al., 2014) (Peng et al., 2014) (Lajin & Alachkar, 2013) including cervical SCC in Japanese subjects (Niwa et al., 2005).

In addition to its role in the detoxification of quinones and cytoprotective functions, NQO1 plays an important role in the bioreductive activation of prodrugs. The concept of enzyme directed bioreductive therapy depends on identifying compounds which are activated by specific reductases and identification of tumours which have elevated levels of enzyme activity (Workman & Walton, 1990). EO9 is a good substrate for NQO1 (Beall et al., 1994) and is

bioactivated to a species that is capable of inducing both single strands and interstrand crosslinks (Bailey et al., 1997; Maliepaard et al., 1995) in cell-free experiments. However, it can also be activated by other one or two electron transfer enzymes such as xanthine oxidase (Maliepaard et al., 1995) and NADPH cytochrome P450 reductase (Bailey et al., 1997). EO9 has activity under aerobic and anaerobic conditions (Collard et al., 1995; Hendriks et al., 1993; Phillips et al., 1992; Plumb et al., 1994; Roed et al., 1989).

In oxygen-rich tissues, high levels of NQO1 result in high cytotoxicity by EO9 (Collard et al., 1995; Fitzsimmons et al., 1996; Plumb & Workman, 1994; Plumb et al., 1994; Robertson et al., 1994; Robertson et al., 1992). In oxygen-poor tissues, high EO9 cytotoxicity is seen only in cells with little or no NQO1 activity (Plumb & Workman, 1994; Robertson et al., 1994) such as those with the NQO1\*2 polymorphic variant (Plumb & Workman, 1994; Traver et al., 1997). In oxygen-poor tissues EO9 is activated predominantly by one-electron reductases and the reaction is oxygen-sensitive (Phillips et al., 2017) In cell lines where NQO1 is high, the activity of EO9 is similar in both aerobic and hypoxic cells, suggesting that in NQO1-rich tissues the reduction of EO9 by NQO1 is independent of oxygen levels (Workman, 1994). In this environment EO9 is activated predominantly by two-electron reductases and the reaction is oxygen-insensitive (Phillips et al., 2017).

## **1.23 Regulation of NQO1**

*NQO1* is one of about 200 genes which are expressed by the activation of the transcription factor Nrf2 which collectively function to maintain a healthy intracellular redox balance, clear electrophilic xenobiotics, and degrade damaged and misfolded proteins (Kensler et al., 2007; Sykiotis & Bohmann, 2010). Nrf2 is normally kept under the regulation of Keap1 in the

cytoplasm. Oxidative stress causes abrogation of this regulation and results in Nrf2 being sequestered into the nucleus where it binds to the anti-oxidant response element, and activates transcription of protective genes.

Nrf2 and NQO1 are indeed found in increased levels in cervical carcinoma epithelia (Gaber et al., 2015; Ma et al., 2014) and the levels of the two proteins are correlated. Nrf2 is localised in the nucleus and cytoplasm of cervical epithelia cells. Levels of Nrf2 show an increase as cervical cells undergoing increasing degrees of dysplasia, with expression level increasing significantly from normal tissue to CIN, and being highest in invasive carcinoma (Gaber et al., 2015). NQO1 is mainly cytoplasmic and its levels also increase as the degree of cervical dysplasia increases. This increase in expression was also confirmed by qRT-PCR where levels of NQO1 mRNA were increased in SCC compared with normal fresh cervical epithelium (Ma et al., 2014a). There is no significant difference in NQO1 levels between CIN and SCC (Gaber et al., 2015; Ma et al., 2014a). The strongly positive rate of NQO1 protein is slightly higher in well-differentiated SCC than in CIN3, indicating that abnormal NQO1 expression may be an early event in the progression of cervical cancer. And also, the strongly positive rate of NQO1 expression was significantly higher in HPV-positive cervical SCC than in HPV negative cases (Gaber et al., 2015).

In addition, increased levels of NQO1 were positively correlated with poor differentiation (Gaber et al., 2015; Ma et al., 2014a), late clinical stage and the presence of lymph node metastases (Ma et al., 2014a). In a previous study it was shown that NQO1 overexpression induced tumour cell proliferation via the up-regulation of cyclins (Garate et al., 2010), and was accompanied by an increase in other anti-oxidant enzymes, such as HMOX-1 and GST, providing tumours with increased protection against cytotoxic agents, allowing for rapid cancer progression (Lau et al., 2008). Cervical SCC patients with high-level NQO1 expression had lower disease-free survival and 5-year overall survival indicating that NQO1 was a

potential predictor of poor prognosis, especially in patients with early stage cervical SCC (Ma et al., 2014b). Increased Nrf2 levels were significantly associated with cervical tumour grade and stage (Gaber et al., 2015).

## **1.24 EO9 as a locoregional therapy**

The clinical development of EO9 has been a difficult and turbulent journey. It was evaluated clinically in the 1990's where it was administered intravenously. Phase I and phase II studies were conducted but no clinical responses were recorded in phase II trials and EO9 was classified as inactive against human cancers (Dirix et al., 1996; Pavlidis et al., 1996). The reasons for the failure of EO9 in the clinic were attributed to poor delivery to tumours on account of the poor pharmacokinetic properties of EO9 in conjunction with its inability to rapidly penetrate through avascular tissue (Phillips et al., 1992). Based on this understanding together with evidence of elevated levels of NQO1 in superficial bladder cancer (Basu et al., 2004; Choudry et al., 2001), a further clinical trial against superficial bladder cancer was conducted. The rationale for this study was based upon the fact that intravesical administration of EO9 circumvents the drug delivery problem. In a bid to exploit EO9's poor pharmacokinetics in order to gain a therapeutic advantage it was postulated that if the drug was administered locoregionally, this would circumvent the problems of drug delivery, and the longer the drug could be retained at this site, the more improved drug penetration into the tumour would be. In addition, any drug that did manage to reach the systemic circulation would be rapidly cleared, reducing the risk of systemic toxicity (Phillips et al., 2013). This study demonstrated that EO9 had ablative activity against marker lesions at doses that were well tolerated locoregionally and systemically (Puri et al., 2006). The promising results in

phase I studies were replicated in phase II studies (van der Heijden et al., 2006) and EO9 is currently undergoing phase III clinical trials (Phillips et al., 2017).

EO9 is therefore clinically active against superficial bladder cancer when administered intravesically. The properties that make EO9 such a poor drug for systemic based therapies are paradoxically advantageous in a locoregional setting. Whilst studies in bladder cancer are advanced, the same concept can be applied to other clinical settings.

In view of the evidence discussed in the previous section demonstrating, that NQO1 is elevated in cervical cancers (CIN and SCC) compared to normal cervical epithelia together with the accessibility of the cervix via vaginal speculum examination, the application of EO9 to the treatment of cervical cancers via locoregional therapy is a potentially attractive option.

## **1.25 Rationale and aims of this study**

As described above, cervical cancer is a major problem in low to middle income countries and is particularly prevalent in sub-Saharan African countries including Zimbabwe. The management of the disease in these regions pose significant problems that are not encountered in more wealthy countries where there are established vaccination and screening strategies. There is therefore a need to explore approaches that can detect cervical cancer early and develop new treatment options for this disease.

## **AIMS and OBJECTIVES**

The AIMS of this thesis are:

1. To explore the feasibility of conducting a VIA based screening programme in rural regions of Zimbabwe.
2. To conduct a pharmacoeconomic analysis of the benefits that such a screening program could bring to the economy of Zimbabwe if implemented.
3. To develop the preclinical rationale for the use of the enzyme activated prodrug EO9 as a locoregional therapy for cervical cancer.

These aims will be achieved by pursuing the following OBJECTIVES:

- To conduct two cervical cancer screening programmes in rural Zimbabwe.
- To raise awareness and explore increasing uptake of the available cervical screening programme currently offered to the women of Karoi by the Zimbabwean government free of charge.
- To evaluate the burden of cervical cancer in Zimbabwe and the economic feasibility of providing once-in-a-lifetime cervical screening to all eligible women in rural Zimbabwe.
- To conduct laboratory based studies designed to generate evidence to support the development of EO9 as a loco-regional therapy for pre-invasive cervical cancer.

# **Chapter 2 EARLY DETECTION OF CERVICAL CANCER**

## **2.1 INTRODUCTION**

The likelihood of a woman developing cervical cancer and receiving timely access to affordable diagnosis and treatment of the disease is largely determined by which part of the world she lives in, her agency and socioeconomic status (Ginsburg et al., 2017). Early detection and prevention of cervical cancer remains the most effective strategy for control of the disease in low resource countries, because cervical cancer has a long pre-invasive phase which can be diagnosed and treated by screening and treatment of pre-cancerous cervical lesions. The incidence of cervical cancer is therefore directly related to a country's health system and its ability to provide its population with access to screening and treatment of pre-cancerous lesions (Goodman, 2015). Of the total global spending on cancer, only 5% is directed towards the majority of LMICs, where the need is greatest (Ginsburg et al., 2017). There were more than 700 million women in LMICs who were 20–49 years old in 2015 (United Nations, 2015) and will be at screening age in the next decade who have neither been vaccinated nor screened, and it is estimated that 19 million women will die from cervical cancer over the next 40 years if there is no increase in screening and preventive services in these countries (Tsu & Jerónimo, 2016).

The high cost of treatment is the main reason for inadequate delivery of cancer treatment in LMICs. In a study in Nigeria, 81% of cervical cancer cases referred for radiotherapy to the study centre did not receive the treatment because of financial issues (Datta et al., 2014). Usually, there is also inequality in affordability and access to care within a given country. Larger cities are more likely to have more advanced infrastructure for cancer care, as well as a higher proportion of people who could afford the care (Ministry of health and Child Care

Zimbabwe, 2014). Therefore, lower-income individuals in rural areas or smaller towns are less likely to afford or have access to cancer care in LMICs. Due to lack of widespread health facilities capable of providing cancer care in many LMICs, cancer patients and their caregivers may need to travel long distances to have access to cancer care. This can further increase non-medical costs of cancer treatment and be a major barrier for access to care (Ministry of health and Child Care Zimbabwe, 2014; Nyakabau, 2014). Although limited, the evidence available indicates that many cancer patients in LMICs who manage to receive treatments will have problems with some of their basic needs, such as purchasing food or paying for their utility bills (Zimstat, 2016). Many survivors may also need financial assistance from their relatives or friends (Nyakabau, 2014). This is despite the fact that many of those who afford cancer treatments in LMICs are of higher socioeconomic status.

### **2.1.1 The Economics of Cervical Cancer.**

Cancer places a burden on a nation's economy and its society, through increased spending on health, loss of labour and productivity, and reduced investment in human and physical capital. Whilst on an individual level, cancer has profound effects on women, their families, individual firms, and governments (Pakseresht et al., 2011). The economic cost of cervical cancer can be divided into direct and indirect costs. Direct costs are the value of resources used in the treatment of the disease. They can include medical costs such as medication, hospital stays, tests and procedures, or non-medical costs such as transportation to the healthcare provider, accommodation and hiring someone to look after children whilst a parent visits a healthcare provider. (Sam et al., 2009; Tarricone, 2006) Direct costs are an evaluation of the quantity of resources used to treat the disease. Indirect costs are the value of lost resources due to illness and death, which essentially places a monetary value on the value of

life. These include costs incurred through lost work days, decreased productivity, as well as the cost of unpaid assistance. Indirect costs address the magnitude of the negative economic consequence of the disease to society (Sam et al., 2009; Tarricone, 2006).

There are also intangible costs. Much of the work that is done by women does not involve monetary transactions, and will therefore not be reflected by aggregate changes in the economy of a country (Langer et al., 2015). However, a mother's death has profound effects on the children and families she leaves behind, because not only do women play central roles as caregivers within their families but they also contribute to the broader society through the roles they play in the provision of education, socialisation, and health and well-being of their children (Langer et al., 2015). Therefore, a mother's illness and untimely death will impact negatively on child survival and wellbeing, especially at key life stages such as adolescence. In addition, the slow, painful death from untreated and unpalliated cancer is a traumatic experience for patients and their families (Ginsburg et al, 2017). Therefore, in any bid to assess the economic burden associated with cervical cancer, the non-income-generating work done by women such as looking after children, tending to livestock, and housework, including preparing food and gathering water and firewood should ideally, also be included (Ginsburg et al., 2017).

### **2.1.2 The Economic Cost of Cervical Cancer in Low-Middle Income Countries compared to High Income Countries.**

In countries where there is organized screening the majority of the economic cost of managing cervical cancer relates to prevention, whilst in countries that lack organised screening, the bulk of the economic costs relate to management and treatment of the disease (Ginindza et

al., 2017). Low-to-middle-income countries are classified into 5 income groups based on World Bank defined income ranges (Campos NG et al; 2016 unpublished). To estimate the treatment cost the authors calculated the direct medical costs associated with the stage-specific FIGO treatment protocols, and to estimate the unit cost of each procedure they identified data from published and unpublished literature for various countries. Costs were then extrapolated to other countries. It was assumed that cancer treatment costs only applied to the proportion of women with access to cancer treatment in a given setting. See table 1 below:

<b>World Bank Income Tier</b>	<b>GNI Per Person Income Tier</b>	<b>Local cancer</b>	<b>Regional cancer</b>	<b>Distant cancer</b>
<b>Low income</b>	Low income (LI) (< \$1045)	628	887	601
<b>Lower middle income</b>	Lower-middle income 1 (LMI1) (\$1046 - \$2585)	1765	2494	1689
	Lower-middle income 2 (LMI2) (\$2586 - \$4125)	3780	5369	3636
<b>Upper middle income</b>	Upper-middle income 1 (UMI1) (\$4126 - \$8435)	8791	12421	8502
	Upper-middle income 2 (UMI2) (\$8436 - \$12745)	17642	24531	16564

**Table 2.1 The average stage-specific cervical cancer treatment cost by world bank income tier (2013 US\$).** Campos NG et al, 2016. Each of the 5 income tiers is colour-coded.

A literature search was performed to estimate cost of illness or burden of disease studies for cervical cancer. Eleven studies met the criteria for producing a final treatment cost expressed as the cost per patient case of cervical cancer which could be compared with other studies. The 11 studies were from Ethiopia, Uganda, Tanzania, Vietnam, Swaziland, Nicaragua, Morocco, Italy, United States and Canada as shown in the table below.

Country (Study Author).	World Bank Income Tier.	GNI (during year of study).	Cost of Cervical Cancer Treatment per patient (US\$ in year of study).
<b>Ethiopia</b> (Hailu & Mariam, 2013)	LI	<b>390</b> (2011)	<b>891.31</b>
<b>Uganda</b> (Campos et al., 2015)	LI	<b>670</b> (2015)	FIGO Stage: 1-2a- <b>888</b> 2b-4- <b>1 176</b>
<b>Tanzania</b> (Nelson et al., 2016)	LI	<b>900</b> (2016)	FIGO stage: 1- <b>2 988.84</b> 2- <b>2 999.28</b> 3- <b>1 744.39</b> 4- <b>1 741.47</b>
India (Campos et al., 2015)	LMI1	<b>1 600</b> (2015)	FIGO stage: 1-2a - <b>1 821</b> 2b-4- <b>2 652</b>
<b>Vietnam</b> (Van Minh, My, & Jit, 2017b)	LMI1	<b>1 860</b> (2014)	<b>368- 11 400</b>
<b>Nicaragua</b> (Campos et al., 2015)	LMI1	<b>2 010</b> (2010)	FIGO stage: 1-2a- <b>3 322</b> 2b-4- <b>4 268</b>
<b>Morocco</b> (Cheikh et al., 2016)	LMI2	<b>2 880</b> (2016)	<b>2 599</b>
<b>Swaziland</b> (Ginindza et al., 2017)	LMI2	<b>3 280</b> (2015)	FIGO stage: 1- <b>6 405</b> 2- <b>25 527</b> 3- <b>24 992</b> 4- <b>23 923</b>
<b>Italy</b> (Baio et al., 2012)	HI	<b>37 680</b> (2011)	<b>32 700</b>
<b>United States</b> (Chesson et al., 2012)	HI	<b>48 950</b> (2010)	<b>37 231</b>
<b>Canada</b> (Pendrith et al., 2016)	HI	<b>44 370</b> (2010)	Year 1- <b>38 560</b> Year 2- <b>14 194</b> Year 3- <b>11 100</b> Year 4- <b>8 309</b> Year 5- <b>5 393</b>

**Table 2.2 Studies evaluating the treatment cost of cervical cancer in various countries. The countries are grouped and colour-coded according to their world bank income tier. (LI- Lower Income 1, LMI1-Lower Middle Income 1, LMI2-Lower Middle Income 2, HI- High Income).**

The Low-to-Middle Income Countries were categorised according to their World Bank Income Tier and colour coded as in the table below. The income tier was classified according to the country's Gross National Income per person for the year that the study was carried out. The high income countries were left uncoloured. All costs were converted to US\$ in the year to allow comparison. The results are shown in the table below and discussed in the following sections.

### **2.1.3 Cost of Cervical Cancer Treatment in Low-to-Middle Income Countries.**

A prevalence-based cross-sectional study of 227 patients with invasive cervical cancer was conducted in Addis Ababa, Ethiopia which estimated the cost of cervical cancer treatment from the patient's perspective (Hailu & Mariam, 2013). Ethiopia is a Low-Income country. Data was collected using patient questionnaires and patient records in December 2011. Costs were in Ethiopian Birr which was converted to US dollar at a rate of 1US\$=14.5 Eth Birr and were calculated per patient, for the 12-month period which preceded the day the interview was held. Micro costing and bottom-up approach was used to estimate direct costs and indirect costs were calculated in terms of productivity losses. All costs were measured. The average outpatient cost was US\$486.91 per patient, whilst the average inpatient cost was US\$404.40 per patient giving an overall average cost of US\$891.31 as shown in the table above. Most study participants were diagnosed at FIGO stage 2 and 3 (Hailu & Mariam, 2013).

Tanzania, is another Low-Income country, which is similar to Zimbabwe in terms of access to cervical screening services. Like Zimbabwe, 75% of the population lives in the rural areas yet most of the screening centres are based in the urban areas. Consequently, only 1 in 20 women aged 30-50 years have received screening, and as a result most women present late with

advanced disease (Nelson et al., 2016). The cost of treatment for cervical cancer was derived from historical data and the per person cost was obtained by calculating the average of the cost of all patients treated from 2002-2011. The average cost of treating an early stage (1 and 2) patient was nearly \$3000 compared with about US\$1740 for late stage (3 and 4) cancer. Early stage patients receive curative radiotherapy and chemotherapy whereas late stage patients only receive palliative radiotherapy.

Vietnam is a Low-Middle-Income 1 country. A cost of illness study was conducted at 2 randomly selected central hospitals in Ho Chi Minh City and Hue in Vietnam (Van Minh et al., 2017). Data was collected from 40 healthcare workers in expert panel discussions which constructed scenarios that represent patient treatment situations. Each of these scenarios was then micro-costed by interviewing the healthcare workers in detail. Data was also collected from the patient records and financial bills of 38 randomly selected patients who were also interviewed together with their families to get detailed costing information. 10 different patient cancer treatment scenarios were identified costing between US\$368-11448 depending on the type of treatment (Van Minh et al., 2017).

A prevalence-based cost of illness study from a provider perspective was done to investigate the economic burden of HPV-related diseases in Swaziland (Ginindza et al., 2017). Swaziland is a Low-Middle-Income 2 country. The study used a top-down approach for the costs of healthcare performed at the hospital and a bottom-up approach for costs associated with primary care. They looked at direct medical costs related to cervical lesions, cervical cancer and genital warts diagnosed in 2015. The treatment for cervical cancer was estimated at US\$5 million in the study? Patients are transferred South Africa for staging and all treatment of cervical cancer FIGO stages 2-4, including radiotherapy, chemotherapy and surgery because these services are not available in Swaziland. Therefore, all costs for treatment were based on private hospital charges and included transport to and lodging in South Africa for duration

of treatment. Treatment of stage 1 cost US\$6 405, as it was assumed to be treated by hysterectomy alone which was done in Swaziland, stage 2 cost US\$ 25 527, stage 3 US\$24 992 and stage 4 US\$23 923. The difference in cost between stage 3 and 4 was because the dissemination of the disease in stage 4 required limited brachytherapy (Ginindza et al., 2017).

Morocco is also a Low-Middle-Income 2 country, like Swaziland. In a study done in Rabat, Morocco, 550 patients were treated for cervical cancer. The cost of illness was assessed using micro-costing of direct costs. The average cost was estimated at US\$2,599 per patient. The cost decreased with the stage of the disease due to the limited therapeutic choices for advanced cases (Cheikh et al., 2016). Radiotherapy accounted for 55% of total costs, followed by brachytherapy (27%) and surgery (7%). These three services and chemotherapy influenced the overall cost of care. Other services such as radiology, laboratory tests and consultations represented only 10% (Cheikh et al., 2016).

### **2.1.4 Cost of Cervical Cancer Treatment in High Income Countries.**

In the Italian study, investigators performed a systematic review of the literature available to identify the best secondary data available to produce lifetime costs of treating each of the 9 HPV-induced diseases including cervical cancer (Baio et al., 2012). The studies included reported incident cases by disease and prevalence of HPV, and direct cost data from the payer perspective. They used an incidence based approach, where a lifetime cost per case associated with each condition was applied to the estimated number of incident cases attributable to HPV 6, 11, 16 and 18 occurring in men and women over a 1- year period. The cost estimates for cervical cancer were obtained from a retrospective observational study conducted for 351 patients (Ferrandina et al., 2010). The mean total direct costs included

those generated by disease progression and recurrence and was estimated at €24 276 which equates to US\$32 700 (at 2011 values).

The study from Ontario, Canada looked at all the incident cases of ICC from 2007-2010 which were identified from the Ontario Cancer Registry and linked to administrative databases at their Institute for Clinical Evaluative Sciences (ICES) from which data on direct medical costs was obtained. Costs were estimated for the first 5 years after a diagnosis of ICC and a total of 779 cases were identified. Overall mean cost during the first year of diagnosis was US\$38 560 (converted from Canadian dollars at 2010 rates). Costs were highest in the first year after diagnosis since treatment is most aggressive during this period. After the first year costs dropped in each consecutive year following diagnosis. In the second year treatment costs dropped to US\$ 14 194 and then further to US\$11 100 in year 3, US\$8 309 in year 4 and US\$5 392 in year 5. Most of the costs were incurred from cancer clinics and hospital admissions (Pendrith et al., 2016).

The study from the US estimated the annual burden of ICC as the annual number of cases of ICC in the US multiplied by the estimated percentage of these cases multiplied by the cost-per-case estimates from literature (Kim & Goldie, 2008). The cost calculations reflected the lifetime costs of new cases that occur annually based on incidence estimates. All costs were in 2010 US\$. The treatment cost of cervical cancer was found to be US\$38 800 per case (Chesson et al., 2012).

## **2.1.5 Evidence-Base for Efficient and Equitable Cervical Cancer Screening Programmes in Low-to Middle-Income Countries.**

In countries where there is organized screening the majority of the economic cost of managing cervical cancer relates to prevention, whilst in countries that lack organised screening, the bulk of the economic costs relate to management and treatment of the disease (Ginindza et al., 2017).

In a 2003 study (Brown et al., 2006), the total annual estimated costs for cervical cancer screening and management in the UK was £185 million. The cost for screening and managing of CIN was £138.5 million, representing 67% of the total cost of managing HPV-related diseases. The cost of treating incident and prevalent cervical cancer cases was £46.8 million. In a Swedish study done in 2009 (Östensson et al., 2015), which calculated the cost of cervical cancer and genital warts from a societal perspective, found that the total estimated cost was €106.6 million, of which €81.4 million were direct medical costs. Prevention, management, and treatment of CIN cost €74 million; of which 76% of this sum was spent on screening and managing women with normal and inadequate cytology only. The cost of treating cervical cancer and palliative care was €23 million. A study from the USA (Chesson et al., 2012) found that in 2010, the annual direct medical cost of preventing and treating HPV-related diseases was US\$8 billion. Of this, US\$6.6 billion (82%) was used for cervical screening and prevention management, whereas only US\$1 billion (12%) was for treating cancer, of which US\$ 0.4 billion was for cervical cancer.

In study done in Swaziland (Ginindza et al., 2017), the annual direct cost of screening, managing and treating high-grade CIN and cervical cancer was approximately US\$12.6 million, of which screening represented 5% of the total cost at US\$900 000. The cost of treating high grade lesions was bulk of the cost at an estimated US\$7.6 million.

Developing an effective screening programme requires a fine balancing act between finding the most suitable screening test and delivery mechanism as well as the screening ages and interval that will lead to the greatest reduction in cancer burden while taking into account the available resources as well as logistical constraints within a particular setting (Campos et al., 2017). Various studies have compared various cervical screening methods and algorithms in low-resource settings in order to ascertain the method that provides the optimal balance between cost and effectiveness.

## 2.1.6 The Cost of Cervical Screening in Low-to-Middle Income Countries.

Country (Study Author)	World Bank Income Tier	GNI (during year of study)	Cost of VIA	Cost of Pap	Cost of HPV test	Cost of colposcopy	Cost of cryotherapy	Cost of LEEP
Uganda (Campos N et al, 2015)	LI	670 (2015)	2.90	12.25	8.78	32.90	13.49	139.54
Tanzania (Nelson S et al, 2016)	LI	900 (2016)	1.45	-	-	-	28.97	-
Zimbabwe (Mvundura M et al, 2014)	LI	810 (2012)	3.27 (two visit scenario)	-	-	-	37.00 (two visit scenario)	-
			8.04 (one visit scenario)				78.00 (one visit scenario)	
India (Campos N et al, 2015)	LMI1	1600 (2015)	3.55	15.15	9.24	30.06	38.13	N/A
Nicaragua (Campos N et al, 2015)	LMI1	2010 (2015)	9.61	13.71	15.61	39.38	33.04	133.64
Swaziland (Ginindza TG et al, 2017)	LMI2	3280 (2015)	53.00	22.00	-	58.00	96.00	2049.00
South Africa (Lince-Deroche N et al; 2015)	UMI1	7340 (2013)	3.67	8.17	54.34	67.71	-	-
23 Sub-Saharan countries (Mvundura M et al; 2014)	LMI	-	3.33 (two visit scenario)	-	-	-	37.58 (two visit scenario)	-
			7.31 (one visit scenario)				70.91 (one visit scenario)	

**Table 2.3 Studies evaluating the cost of cervical cancer screening in various countries. The countries are grouped according to their world-bank income tier. (LI- Lower Income 1, LMI1-Lower Middle Income 1, LMI2- Lower Middle Income 2, UMI1-Upper Middle Income 1).**

A study from Tanzania (Nelson et al., 2016) calculated the cost-effectiveness of cervical screening using VIA. They calculated the average cost of screening per patient to be US\$1.45 and the average cost of cryotherapy was US\$28.97 per patient. Cervical screening was found

to have an incremental cost effectiveness ratio (ICER) of US\$219 per life-year gained. Using the heuristic that an intervention with ICER of less than the GDP per capita is estimated to be “very cost-effective” and ICER less than 3 times GDP per capita is estimated to be “cost-effective” (World Health Organization, 2001), screening was therefore very cost-effective in increasing life-years. Additionally, screening was associated with averting 1.3 deaths each year due to earlier diagnosis, and in this case ICER was US\$4597 per life saved (Nelson et al., 2016).

A study by Mvundura and Tsu (Mvundura & Tsu, 2014a), looked at the cost of screening women aged 30-39 years from 23 Sub-Saharan countries, including Zimbabwe, once in a decade, using VIA. Costs were estimated using a computer-based screening and treatment programme. Screening was assumed to take place at primary level of the health system i.e. at health centres, while LEEP would be done only at a tertiary level facility such as a regional hospital. For cryotherapy two scenarios were considered. The first was a single visit approach where cryotherapy would be provided at all the centres which offer screening, and the second was a two visit approach where cryotherapy would only occur at the district level i.e. the level above the lowest level. The cost of the cryotherapy and LEEP equipment and supplies as well clinical staff and annual costs of equipment was included. The cost of treatment with cryotherapy and LEEP was combined into one figure by assuming that of the women requiring treatment 87.7% would require cryotherapy, while 12.3% would require LEEP. It was calculated that the two visit approach was cheaper with an average cost per woman screened and treated for precancerous lesions of US\$3.33 and US\$37.58 respectively, compared to a cost of US\$7.31 and US\$70.91 respectively for the single visit scenario. For Zimbabwe the cost of screening and treatment for precancerous lesions was estimated at US\$8.04 and US\$78 respectively for the single visit and US\$3.27 and US\$37 respectively for the two visit scenario.

Performing cryotherapy at all the screening sites resulted in 94% of all costs being allocated for cryotherapy equipment and 6% for LEEP equipment. However, where cryotherapy was performed only at district level, 58% of the costs were for cryotherapy equipment. When cryotherapy is provided at all the screening sites there is a trade-off between higher cost with increased accessibility of treatment services versus lower cost with reduced accessibility. The main factors affecting this trade-off are the distances the women would need to travel between local facilities and District referral facilities and whether they are motivated and suitably educated about the importance of completing their treatment through counselling at the time of screening; or by following up those who do not go for referral treatment and persuading them to do so (Mvundura & Tsu, 2014b).

A study by Campos et al (Campos et al., 2015) evaluated the optimal screening ages and intervals in order to maximize the health benefits for countries with limited screening opportunities, using a mathematical simulation model to compare the lifetime and economic costs associated with the 3 different screening methods of HPV DNA testing, VIA testing and cytology. Costs and screen test performance data were taken from the Screening Technologies to Advance Rapid Testing–Utility and Program Planning (START–UP) demonstration projects in India, Nicaragua, and Uganda (Jeronimo et al., 2014; Mvundura & Tsu, 2014a), because these 3 countries represented LMICs with varying epidemiologic profiles. For VIA they assumed women who had a positive screen and were eligible for cryotherapy were treated immediately, but a proportion either returned for treatment later or were lost to follow-up. HPV testing was done using either provider-collected (cervical sampling) or self-collected (vaginal sampling) on the first visit after which the women returned for their results on a second visit. If their result was positive, the majority received cryotherapy at the same visit. For cytology, there was an initial visit for screening, then a second visit to obtain results, if results were positive, there was a third visit for diagnostic

colposcopy and biopsy, and then a fourth visit for treatment, if required. Treatment protocols were based on current practice in each country. Screening tests were evaluated at various ages - once in a lifetime, twice in a lifetime and three times in a lifetime. The direct medical costs evaluated included staff time, clinical equipment and supplies, drugs and laboratory equipment and supplies. Costs were calculated in 2011 International dollars (I\$) and the cost effectiveness ratios were expressed using the ICER defined as the additional cost of a strategy divided by its additional health benefit, compared with the next costliest strategy after eliminating strategies that are dominated.

The study showed that HPV testing with cervical sampling was the most effective and cost-effective in all the 3 countries because of its superior test sensitivity, and the ability for it to be integrated into a screen and treat strategy requiring just two visits. VIA and cytology both had lower cancer risk reduction compared to *care*HPV testing, and the increased number of visits required with cytology led to increased loss to follow-up. The optimal screening ages associated with the greatest reduction in lifetime risk of cancer was between 30 and 45 years. This supports WHO guidelines which call for screening to take place between ages 30 and 49 years. However, due to differences in peaks of cancer incidence, once in a lifetime screening was most effective at age 40 or 45 years in India, while in Uganda and Nicaragua it was most effective at age 30 or 35 years. Overall, HPV testing women three times in a lifetime between 30 and 45 years was found to be very cost-effective and could reduce the risk of cancer by approximately 50% in all 3 countries.

A separate study by Campos et al (Campos et al., 2017) looked at data from 50 LMICs to compare the costs and lifetime health impact of 3 different interventions for cervical cancer. These interventions were, either vaccinating 10 year-old girls, screening women once-in-a-lifetime using VIA or HPV DNA or providing treatment for cervical cancer. The computer-based CERVIVAC model was used to create data on population, demographics and epidemiology and

costs were analysed from a payer perspective and all costs were in 2013 US\$. The current access to cancer treatment was estimated using published literature to project cervical cancer (Datta et al., 2014). Information on the effectiveness of screening and treatment was derived from microsimulation and the effectiveness of HPV16/18 vaccination was estimated using from the vaccine trials, as well as epidemiologic data on the numbers of cervical cancers caused by HPV16/18. The study showed that although vaccination averted more cases and deaths from cervical cancer than screening over a lifetime, the DALYs averted with screening were higher because screening led to earlier stage cancer detection which in turn resulted in fewer years lived with disability. The screening program was estimated to cost US\$8.88 per woman screened, and the net cost per DALY averted was US\$64. Having a screening program offset the cost of cancer treatment by approximately 38% of the screening program cost. This relatively small percentage is because treatment for cancer in LMICs is limited. The ICER for screening was below country per capita GDP for all countries and therefore making screening using VIA or HPV testing very cost effective (Campos et al., 2017).

Another study from South Africa estimated the costs and cost-effectiveness of conventional cytology, VIA and HPV testing in detecting CIN2+ in HIV+ women on antiretroviral treatment (Lince-Deroche et al., 2015). They looked at 1202 women aged 18-65 who presented at an HIV treatment clinic in Johannesburg and were screened using all of the 3 strategies. All positive, and 25% of negative VIA or cytology results were followed up by colposcopic biopsy to confirm result by histology. All HPV DNA results were obtained later, and were not used to determine whether colposcopic biopsy would be done. The study stopped at diagnosis and did not include treatment of pre-cancerous lesions. Costs were estimated for staff time, supplies and equipment required for each procedure through discussion with the clinic staff and were from a provider perspective. Unit costs for supplies and equipment were obtained from facility expenditure records and staff costs were obtained from public sector salary scales. They also

included the cost of the 4 or 6-year qualification for professional nurses who provided the screening. Costs which were fixed among the 3 screening methods such as infrastructure and utilities were not included in the analysis. Costs were collected in South African Rands and converted to 2013 US\$. As shown in the table 2.3, the average cost per procedure for VIA was US\$ 3.67, for cytology it was US\$ 8.17 and for HPV DNA testing it was US\$54.34. The cost of a colposcopic biopsy was US\$67.71 per procedure, and it is this cost that was mainly responsible for driving up the costs of cytology and HPV testing in this study. Overall, VIA was the most cost effective at US\$17.05 per true CIN2+ case detected, although it was least sensitive, followed by cytology at US\$130.63 per case of true CIN2+ detected and HPV DNA was the least cost-effective at US\$320.09 per case of true CIN2+ detected.

## **2.1.7 The Burden of Cervical Cancer in Zimbabwe**

Zimbabwe has experienced many economic and political problems in recent years. The national unemployment rate is estimated to be over 80% and that for youths over 90% (Masunungure & Koga, 2013). In 2015, 68% of the Zimbabwean population lived in the rural areas (IndexMundi). The country officially abandoned its currency in 2009 turning to the US dollar and South African rand.

WHO recommends that LMICs with low coverage of screening and restrictions on resources, use a 'screen-and-treat' strategy, where the treatment decision of a precancerous lesion is based on a screening test and treatment is provided soon or, ideally, immediately after a positive screening test in the same setting. The recommended age of screening is 30 years and older, with priority to maximise population screening coverage, rather than maximising the number of screening tests in an individual woman's lifetime (WHO, 2013; WHO, 2014).

The recommended screening test is the HPV test if resources allow, otherwise the visual inspection with acetic acid (VIA) test. The lifetime risk of cervical cancer was found to be reduced by 25-35% in women who are screened once in their lifetime at around age 35 years by VIA or HPV testing and by 40% in those screened twice (Goldie et al., 2005). Screening women three times, between 30 and 45 years reduced cancer risk by 50% (Campos et al., 2015).

## **2.1.8 Treatment Management of Cervical Cancer in Zimbabwe**

Cervical cancer is first detected when a patient attends for VIAC screening and is found to be screen positive with a suspicion of invasive cervical cancer. Or when they present for cytology screening and are found to have abnormal cells. The patient is then referred for colposcopy and a punch biopsy. If invasive cervical cancer is confirmed the patient is investigated with blood tests to check for renal and liver function as well as full blood count. This is followed by a chest x-ray to assess for lung metastasis and an abdominopelvic ultrasound for the purposes of assessing the extent of local spread and staging the disease. For patients who can afford it, this can be followed up by a CT or MRI scan to further evaluate the extent of disease spread. Management of cervical cancer is according to stage. In general, FIGO stage 1a is treated by radical hysterectomy without adjuvant chemo-radiation. Stages 1b -3 are managed by curative radiotherapy with adjuvant cisplatin-based chemotherapy and no surgery (Kuguyo et al., 2017). Stage 4 cervical cancer is managed with palliative radiotherapy and symptom control with powerful analgesics such as morphine for pain and blood transfusions for anaemia. Morphine is provided to all cancer patients free of charge by an NGO called Island Hospice. Island Hospice and Health and Hospice and Palliative Care Association of Zimbabwe (HOSPAZ) also provide hospice services at a low cost or free of charge for terminally ill

patients either in-hospital or at home (Kuguyo et al., 2017). But the rest of the treatment costs for cancer are met by the patient themselves.

## **2.1.9 Organisation of Cervical Cancer Treatment Services in Zimbabwe.**

The diagnostic and treatment infrastructure for cervical cancer in Zimbabwe is limited. Most facilities for the whole country, are available only at 2 central hospitals - Parirenyatwa in Harare or Mpilo in Bulawayo with a few private clinics/oncology centres also based in these 2 cities offering private treatment (Ministry of health and Child Care Zimbabwe, 2014; Nyakabau, 2014) . Screening for cervical cancer using VIA and treatment for CIN is provided free of charge to patients. However, the cost of treatment for invasive cancer is borne entirely by the patient and this can be accessed in the private or public sector. In order to access specialist cancer treatment, patients must spend large sums of money in travel and accommodation costs alone before factoring in the costs of the services and treatment itself.

Diagnostic and treatment services for cervical cancer in Zimbabwe are available as follows (Ministry of health and Child Care Zimbabwe, 2014): VIAC and cryotherapy and is done at District Hospital level. Colposcopy and biopsy, LEEP, cone biopsy, as well as investigations such as plain X-rays and blood pathology and are available at Provincial and Central hospital level and at a few mission hospitals. Histopathology services are available from private laboratories in Harare, so specimens are sent from the District General Hospitals to pathology centres in Harare and Bulawayo and normally have a turn-around time of at least 3 weeks. CT and MRI scanning is available only in Harare and Bulawayo. There are public facilities for CT scanning but these are inadequate and non-functional most of the time such that patients often have to resort to having it done privately. MRI scanning is available only in private

institutions. Radiotherapy and brachytherapy services are available only at Parirenyatwa and Mpilo Hospitals. Chemotherapy drugs are expensive and are not always available at the central hospitals so patients have to resort to buying the drugs themselves from private pharmacies. These inconsistencies in the availability of treatment have led a large number of the population who can afford it, to seek treatment from outside the country. The most popular treatment destinations are South Africa, India and Dubai.

## **2.1.10 BMA International Humanitarian Fund**

### **Sponsored Cervical Cancer Screening Saves Lives**

#### **Programmes in Zimbabwe.**

It is against this backdrop that between January 2015 and August 2016 we carried out two free cervical cancer screening programmes in rural Zimbabwe. The first screening programme was done in Karoi. The second programme was carried out in Magunje about 18 months later.

## **2.2 MATERIALS AND METHODS**

The British Medical Association (BMA) International Humanitarian Award (BMA, 2018) is an award that is given to teams which consist of at least one current NHS employer, that want to undertake humanitarian projects in low income countries. The projects are required to offer clear health benefits to the local population, to have a sustainable impact and to be in line with the UN Millennium Development Goals (UN, 2000) - now Sustainable Development Goals (UN, 2015). The award covers travel and accommodation but not equipment or drugs. A medical doctor colleague of mine and myself decided to apply for the International Humanitarian Award for 2014 in order to increase awareness of cervical and breast cancer and to carry out cervical cancer screening in marginalised communities of Zimbabwe. We

decided to start with my home area and the community in which I was raised, Karoi, since I was well acquainted with the area and the people. We were awarded the 2014 BMA International Humanitarian award, and started with our planning and preparations for the cervical screening programme.

**Karoi – Study Site 1.** Karoi town is a small town within a rural farming community of about 30 000 inhabitants, which is located in Hurungwe District, the 2nd largest district in the Mashonaland West Province of Zimbabwe. Karoi is approximately 200km north west of the capital city Harare, along the main road from Harare to Kariba. It is made up of 3 urban locations known as Karoi Town, and the high density areas of Chikangwe, and Chiedza (Goal, 2009). It is a farming area with fertile land. In 1945, Karoi was designated for World War 2 British white war veterans who obtained government assistance to embark on farming tobacco in the area. There is an estimated 12 000 people within the administrative rural farming area of 10km radius around the town (Goal, 2009). Many of the residents are informal traders, or subsistence farmers (Zimbabwe Democracy Institute, 2014). Karoi has a District General Hospital.

**Preparation Prior to Travel to Zimbabwe.** The planning team comprised of 4 doctors and 2 other non-medical professionals who offered a lot of technical knowledge and assisted in the initial set up of the project but were not able to be there on the screening days. Two of the doctors were based in Zimbabwe while the other two are based in the UK. The two non-medical members are based in the UK and South Africa. Communication between the members was carried out via WhatsApp group chat as this was the cheapest and quickest method of communicating information to all members. The Zimbabwe based doctors provided invaluable information on how the situation was and the protocols we had to follow to set up the project. We still had to contact some people via telephone and email. After coming up with a project proposal we sent this to the District Medical Officer at Karoi Hospital, who in

turn discussed it with the Provincial Medical Director. Once it was confirmed that we could go ahead and then set out to find donors for equipment as the BMA Award did not cover this. We were fortunate to receive help from the Calderdale and Huddersfield NHS Trust who linked us to Roche Diagnostics – UK, who donated blood glucose monitoring equipment and urine dipstix. We also received speculums from 2 GP surgeries in and from Williams Medical Supplies in Wales. We also acquired a substantial number of volunteers and helpers based in Zimbabwe, during the time of the project.

**Preparation in Zimbabwe.** The project was implemented as planned. Consultative forums were held with the Zimbabwe Ministry of Health through the local Karoi District Hospital. These forums were to help in explaining the purpose of the project. Meetings were held at Karoi Hospital, with the District Medical Officer (DMO), Dr. Chikutiro, Karoi Hospital Matron Sister Jimu, as well as the District Health Promotion Officer, Mr. Machacha. From the meetings it was agreed that we would work together to ensure the success of the project. Mobilizers attached to Health Promotion were tasked to mobilize communities about the programme. We printed flyers and posters that were used in mobilization. Trained VIAC nurses would be responsible for the cervical cancer screening and the hospital would provide the equipment for screening.

**Training the Mobilizers.** Training of the mobilizers was done on the 29th of December 2014. The purpose was to educate the volunteers on the rationale of the project and on what information to give to the community when they went out. There was a total of 10 mobilisers, who have experience in mobilizing the community for various Ministry of Health campaigns through drama and other ways. Owing to lack of funds we were not able to use drama or supply the mobilisers with T-shirts to advertise the project. A number of issues were raised by the mobilizers during the training which they felt were reasons why women were not accessing the cervical screening provided at Karoi Hospital. These were that communities are

ill-informed on the advantages of early testing – (one mobiliser expressed how his own mother died of cervical cancer, which he now felt could have been avoided had it been detected early). Women are ignorant about the disease including its signs and symptoms. There are also fears about the procedure itself and what it entails, and lack of funds to travel to the screening centre. Information on cervical screening and its availability at Karoi District Hospital was not available in smaller or rural communities in the district. Mobilization of the local population was carried out over 3 days by doing door-to door visits and leaving leaflets, placing posters in strategic areas frequented by women, and using a loudspeaker in the back of a car.

**Acquiring Appropriate Premises.** Initially we had planned to hold the screening at the hospital, however there was no room which was large enough to hold the anticipated numbers. We eventually settled on Chikangwe Hall, which was ideal as it was located right in the middle of the community we were targeting. This helped to offset transport costs for the majority of women. There was a maternity ward attached to the community clinic which is adjacent to the hall. It was still under construction but almost complete. The sister in charge at the clinic graciously offered us the use of the incomplete maternity ward. This was ideal as it contained separate rooms which allowed adequate privacy for a screening room, a room for weight and height measurements, and a separate “consultation room” where the doctors saw patients who had had all their investigations done. We were also able to use a room at the back near the toilets as a teaching/presentation room.

### **Cervical Cancer Screening at Karoi District Hospital.**

Karoi District Hospital started offering government-sponsored free cervical screening using VIA in 2014. Karoi District Hospital offers a screen and treat service. Cases that are VIA positive are treated by cryotherapy, if they are eligible. A patient is only eligible for cryotherapy if the whole of the lesion is visible, the transformation zone is visible and the

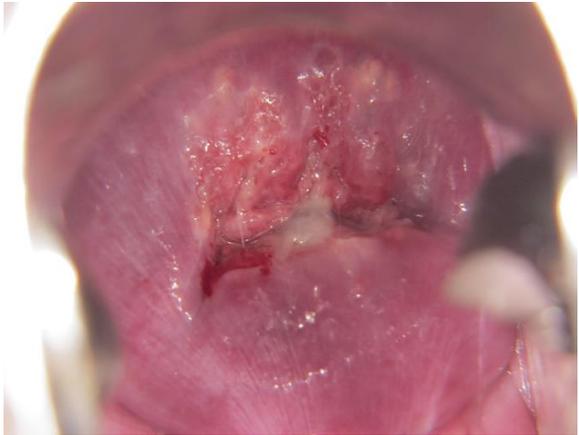
lesion covers less than 75% of the ectocervix (WHO, 2013b). As previously described in section 1.2.6.2, VIA involves staining the cervix with 3-5% acetic acid and viewing the colour change in abnormal tissue on the cervix which will stain white as shown in the diagram below:

**A**



Normal cervix remains pink.

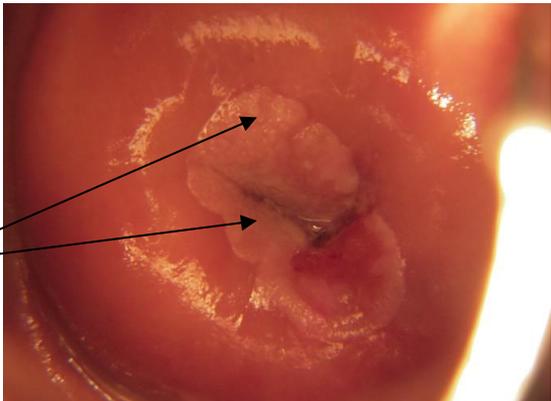
**B**



Cervix showing invasive cervical cancer.

**C**

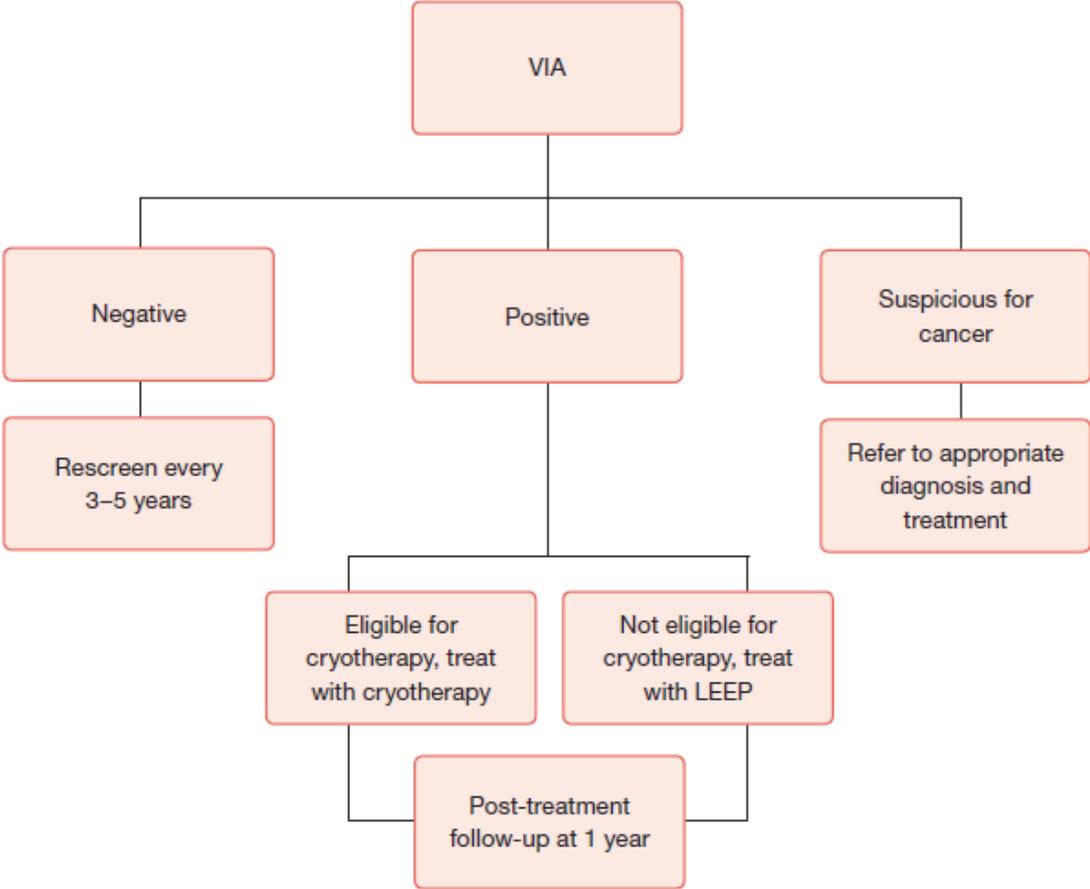
Aceto-white changes



Pre-cancerous lesions of the cervix stain white with 3-5% acetic acid.

**Figure 2.1 Results of cervical screening using VIA. A) normal cervix stays pink. B) cervix with invasive cervical cancer. C) cervix with pre-cancerous lesions stains white with 3-5% acetic acid.**

Even though the WHO recommends rescreening in 3 years for HIV positive women, and in 5 years for women who are HIV negative, the guidelines in Karoi state that HIV positive women are screened every year, while HIV negative women are screened every 2-3 years. The flowchart below recommended by WHO, documents the decision pathway used in the screen and treat strategy in Karoi:



**Figure 5.2 A flowchart of the screen and treat strategy offered at Karoi District Hospital (WHO, 2013b).**

**Screening Days 6-7 January 2015.** On the day, women arrived at the Hall, where they would be given the paperwork on which their results would be recorded. Various presentations

were given on Healthy living, breast cancer and breast awareness, cervical screening and the local cervical screening campaign, in the hall. The talks were repeated throughout the day with each new group of women who attended. This way we ensured that the women received health education prior to going for their cervical screening. From the hall, the women proceeded to the adjacent maternity suite to have their blood pressures measured and blood sugars taken, as well as weight, height and body mass index (BMI). After having their vital measurements done, they then proceeded to have their cervical cancer screening using the VIAC method.

VIAC screening was performed by 4 VIAC trained nurses from the hospital. The images taken of the cervix were to be later discussed with the VIAC trained local doctors at their weekly MDT meetings. The women with a VIAC positive result were offered a follow up appointment for cryotherapy or LEEP at Karoi Hospital the next day. For the obvious cancer lesion, they were referred to Chinhoyi Provincial Hospital for further management. The rest, who were VIAC negative, were given a date for a follow up appointment in either 2 or 3 years, if they were HIV negative. Women who are HIV positive were given a follow-up appointment in a year's time. All sexually active women, regardless of age, were eligible for screening.

After completing all tests and screening, the women proceeded to one of the doctors for a one to one consultation. During this time, the doctor reviewed the woman's individual results, and explained to her what they meant and what lifestyle adjustments they could make, if applicable. They also had an opportunity to ask the doctor questions and the importance of cervical screening was emphasized. The women were then finally asked to give feedback on screening programme, what they found most useful, what could have been done differently and whether they would recommend the service to a family member or a friend. The programme ran from 10.00am-5.00 pm on the first day when we screened 116 women, and from 8.30am to 4.30 pm on the second day when we screened 79 women.

**Challenges Faced and Overcome.** One of the biggest challenges we faced was financial. We relied mainly on contributions from well-wishers and voluntary service from the participants. However, there were other running costs such as providing food for the participants over the two days, paying the community mobilizers, giving small gratuities to the nurses and other helpers in line with the culture in Zimbabwe and incidental costs such as stationery, printing and photocopying, which we had to fund ourselves. This restricted the level of service that we were able to offer. For instance, we couldn't afford to get a drama group to mobilize the community, however, thankfully this proved unnecessary as we were not able to cope with the numbers of women who attended anyway. We are very thankful to those who donated and volunteered their time free of charge as they made this project possible. We managed to get accommodation for the doctors who volunteered at subsidized rates.

Co-ordinating the logistics of implementation of the project in Zimbabwe whilst we were in the United Kingdom also proved to be a challenge. We did not have full knowledge of what services were like that were available in Zimbabwe, and the costs that we would be looking at. It involved us making several calls to Zimbabwe and coordinating the involved parties and talking to different laboratories. A lot of correspondence was via e mail but we had to make telephone calls to Zimbabwe occasionally to ensure accurate, timely information. It was important to travel to Zimbabwe well in advance of the project date to mobilize people locally and to finalize arrangements which was very helpful. We had to get permission from local authorities, as standard practice whenever there is going to be a gathering of people in Zimbabwe. Also we needed permission to use the screening premises. Permission was obtained from the local town councillors.

The Karoi District Hospital matron was able to release the trained VIAC nurses who normally conduct screening at the hospital during the two days of the project. The screening service at

the hospital was moved to the premises where we were conducting the project. The four VIAC nurses were inundated with patients. The response of the women in the community to the call for screening was overwhelming. The nurses worked tirelessly but it was very obvious that we were short on manpower. This applied across the board for all the services we were offering during the two days. The doctors ended up going to give the talk on their designated subject, taking it in shifts. So while one doctor was giving a presentation, the others would be seeing patients and then take it in turns. They were also filling in the stations wherever there appeared to be delays, such as taking blood pressures. The biggest hold up on both days was at the cervical screening station. Firstly, because there were only 2 examining couches and two cameras, the VIAC nurses could only screen 2 patients at a time, even though there were 4 of them. Secondly, on the second day, we ran out of disposable speculums and this is what eventually brought the screening programme to its end. We would have benefited from deploying more VIAC trained personnel and having more equipment.

The other challenge was space. This was very limited due to the makeshift facilities we had which consisted of an incompletely built maternity suite. It was difficult to ensure privacy at the cervical screening station as it was in a single room housing two patients at a time. The nurses did their best by using screens. The consultation room was also a single room, which was being used by two doctors and two patients at any one time. There were times when the doctor had to discuss sensitive issues with the women and this proved to be challenging due to the presence of several women in the room.

The biggest challenge is the poverty. Some of the women simply could not afford to get to the local clinic and those who had been found to have cancer simply could not afford the treatment. It was a difficult position as some women came in desperate need of treatment for their extremely high blood pressure, or who were found to have evidence of infection following their per vaginal examination. All we could do was to signpost them to their local

clinic or health service provider. Sadly, it was obvious that for some of the women all this would end up being, is just another test, as they could not afford further treatment. And yet, they were the women with the greatest need.

**Sustainability of the Project.** As a result of the increased awareness of cervical cancer that was garnered in the community because of the project, the attendance at the VIAC clinic at Karoi District Hospital saw a sharp increase in the days and weeks following the project. Some of the women were women who had attended the screening but had not been able to have VIAC screening, due to one of the reasons described earlier. Others were family members and friends who had not been able to come to the hall on the two days due to other commitments, but had been told by those who had attended and therefore wanted to access the service. Each of the screened women was given a date to attend for their next screen, this will also go a long way towards ensuring sustainability of the awareness created through screening project.

**Magunje – Study Site 2.** Following on from the success of the first screening programme, we re-applied for, and were re-awarded the BMA International Humanitarian Award in 2015, to carry out another cervical screening programme. This time we decided on a village not far from Karoi, called Magunje.

Magunje lies in the northwest part of Zimbabwe. It is located in Hurungwe District which is in the Mashonaland West Province. It's the largest village and principal growth point in Hurungwe communal land. It has a clinic, three secondary schools and a Zimbabwe National Army base. The population is estimated at 13 000 (CSO, 2002). It is only 35km from Karoi, and public transport to Karoi is mainly via commuter omnibuses which charge US\$1 each way. Most of the population is made up of subsistence farmers in the surrounding communal lands and informal traders in the village. The reason for this choice of place for a cervical

screening programme is because the area is more rural and the population more impoverished compared to that of Karoi. There is no VIAC screening service at Magunje clinic, and the nearest one is at Karoi District Hospital. However, many of the women cannot afford the \$2 fare needed for a return trip by public transport in order to attend for screening in Karoi.

**Preparation in the UK - Procurement of Medication and Screening Equipment.** In order to address the problem of lack of medication, I approached International Health Partners (IHP) after seeing an advert in the weekly British Medical Journal. NIHP is a charity which supplies healthcare professionals with pre-packed mobile kits of essential medicines for use in medical relief work in low resource countries, and in war torn or disaster areas. Each pack consists of 2 x 23kg boxes of medication which would normally cost around £5000 in NHS prices. I procured 1 essential health pack from IHP at a cost of £250. I also approached Pelican Feminine Healthcare, London for help with disposable vaginal speculums. They supply Calderdale and Huddersfield NHS Trust with all their gynaecological treatment kits. They were able to donate 25 disposable vaginal speculums to us for free. I was limited only by the amount of luggage I was physically able to carry as they were willing to supply more, and since the equipment was being used for humanitarian purposes, the airline I was travelling to Zimbabwe on, South African Airways had agreed to let me carry extra luggage for free. Barnet Seventh-Day Adventist Church donated £800 towards the cost of the essential health packs. I contacted the Provincial Director Dr. Wenceslas Nyamayaro prior to leaving for Zimbabwe and was able to obtain permission and help on how to bring medication for use in the screening programme.

**Preparation in Zimbabwe.** Bringing medication into the country proved to be a challenge as import of medication is strictly regulated for the right reasons. Thankfully, I was able to collect the medication just in time for the screening programme, after it had been held at

airport customs for 2 weeks. However, with the help and support from the office of the Permanent Secretary of Health, it was released.

This time the screening programme was carried out over 5 days – from 1 – 5 August 2016. We had to transfer the entire screening unit from Karoi District Hospital to Magunje via ambulance. We were temporarily housed at the Magunje Youth Centre for the week, because that was the only place that could accommodate the 2 examining couches and in addition to all the screening equipment we had. We had the same VIAC nurses that we had the year before in Karoi. This time we had a team of 18 people comprising 4 VIAC-trained nurses from Karoi District Hospital, 4 qualified nurses from Magunje Hospital and 9 volunteers – 5 medical students, a physiotherapist, a fitness trainer, a sports TV presenter, and a graduate in Human Biology and 1 doctor. The duties of the volunteers included and were not limited to consultation for patients, group educational programs, patient registration & checking vitals, patient counselling and entertainment.

**Community Health Visitors and Mobilization of the Rural Community.** Magunje has community health visitors who are employed by the clinic to engage with the community when there are NGO-funded health programmes going, such as distribution of free mosquito nets, which was ongoing at the time of our visit. These health visitors are lay people who are known and respected in the community. They are essential because being a communal farming area, people's homesteads are spread far apart and it is harder for news of health initiatives to travel by word of mouth. They travel on bicycles going from home to home educating people and promoting various community initiatives. This time we decided to use these community health visitors to advertise the screening programme as they had knowledge of the area. However, after 2 days we had to recruit the mobilizers we had trained the year before as the turnout of women was low. After we recruiting the trained mobilizers from Karoi we had large numbers turning up on the last 2 days of screening.

**The Premises.** The Youth Centre consisted of 3 buildings which we had full access to. There was one examining room which was ordinarily used for this purpose. It contained a sink with running water which was essential to the screening. However, part of the challenges we faced was the local water supply was rationed, and so there were instances, especially in the first couple of days, where we would have no running water for several hours in the day. We were able to separate the examining couches with screens for privacy. This time there was only one functioning camera so the nurses would take turns in using it for each patient.

This time the one to one consultations were carried out by the trained nursing sisters from Magunje Clinic, in a separate building from the one housing the screening. Magunje Clinic does not have a regular resident doctor and so the nurses are able to prescribe medication. The medication from IHP meant that we could see other members of the public besides women, including men and children who required treatment.

Apart from cervical screening which was done by the trained VIAC nurses, the volunteers provided health education and awareness talks on cervical and breast cancer. These were very popular with the women, and the volunteers themselves also benefited from the knowledge of the more experienced nurses who live within the community. The volunteers also carried out measurements of blood pressure, height, weight, BMI and random blood sugar level, as was done in the previous screening programme in Karoi.

**Screening Days on 1-5 August 2016.** The first day was spent mainly travelling and setting up. We only managed to screen 7 women on the first day due to the low turnout. The turnout of women on the second day was disappointing, because news of the screening was slow to reach the community. However, the number slowly started to increase as the day progressed. We screened a total of 31 women and were limited by running out of clean speculums towards

the end of the day. By the third day news of the screening had reached the community, and turnout was high. We managed to screen 72 women. Time was now the limiting factor.

The Youth Centre we were based at, was also the local GUM clinic and was used for sexual health and contraception education and training, so they had speculums which we were able to use. In addition, they had a portable autoclave machine which proved to be invaluable because it allowed us to sterilise used batches of speculums, which could be re-used again later in the day. This increased our capacity of women we were able to screen per day. On the penultimate day we screened 106 women, which was our highest number for the day. The volunteers also put on a short play to educate the women about cervical cancer and as a way of entertaining them while they were waiting to be screened. The play was mainly focused on risk factors for cervical cancer and how to avoid them. On the fifth and final day we screened 62 women. In addition to the women we screened, many other women received the health education, BP, blood glucose and anthropometric measurements but no screening. Estimates are that up to 500 women in total attended the programme during the week excluding the men and children who attended for nurse consultation with various ailments that required medication.

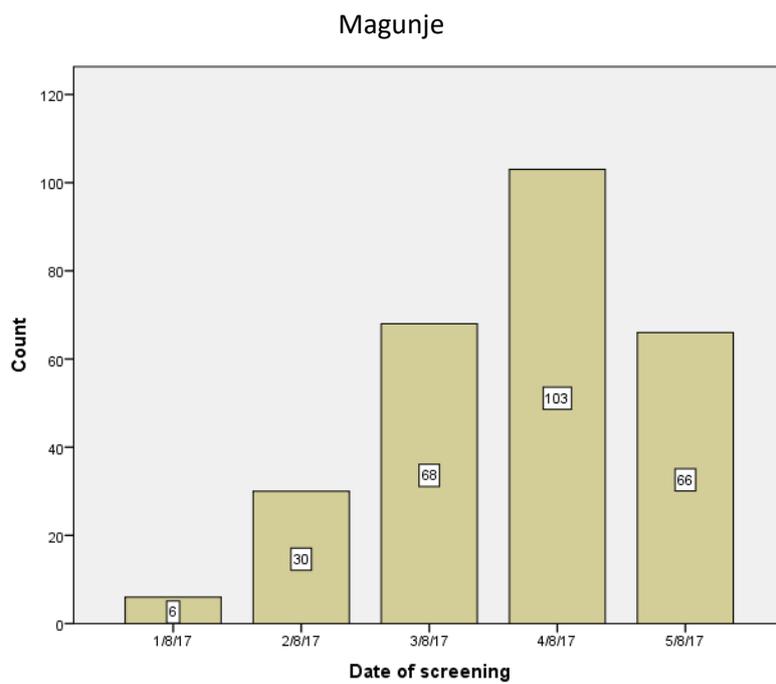
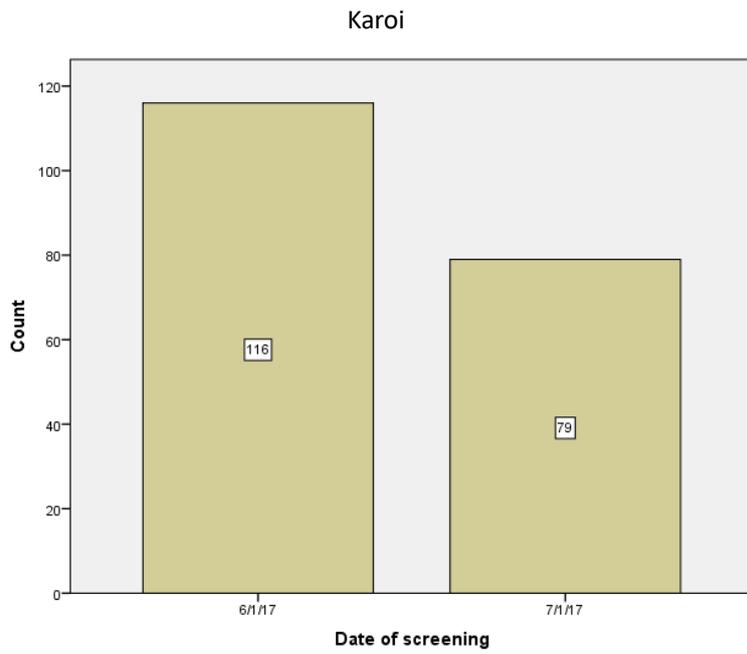
## **2.3 RESULTS**

In Karoi, the number of women who received cervical cancer screening over the two days between 6-7 January 2015, was 195 while the mean number of women screened per day was 98 (figure 15). The average age of women who came for screening was 36 years with their ages ranging from 18 to 77 years. The median age was 34 years. In Magunje, the total number of women who received cervical cancer screening over the five days between 1-5 August 2016, was 274 while the mean number of women screened per day was 55 (figure 15).

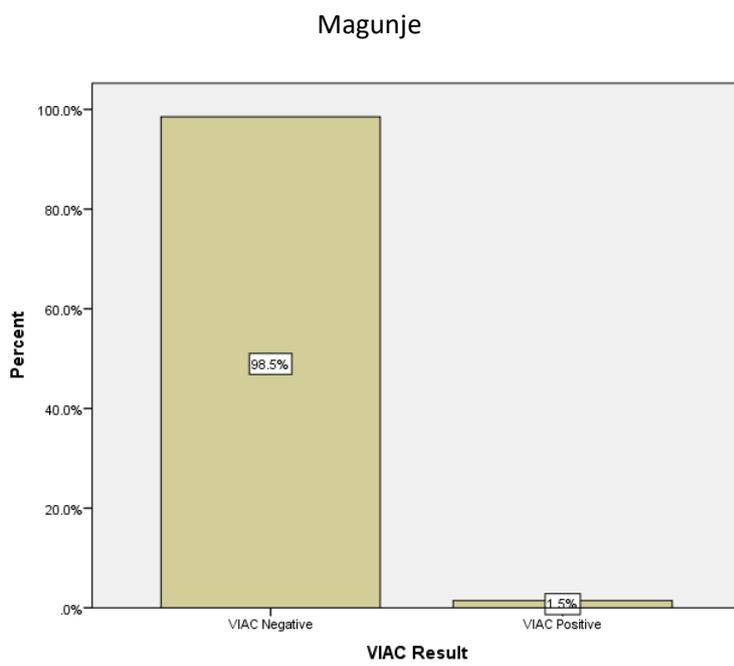
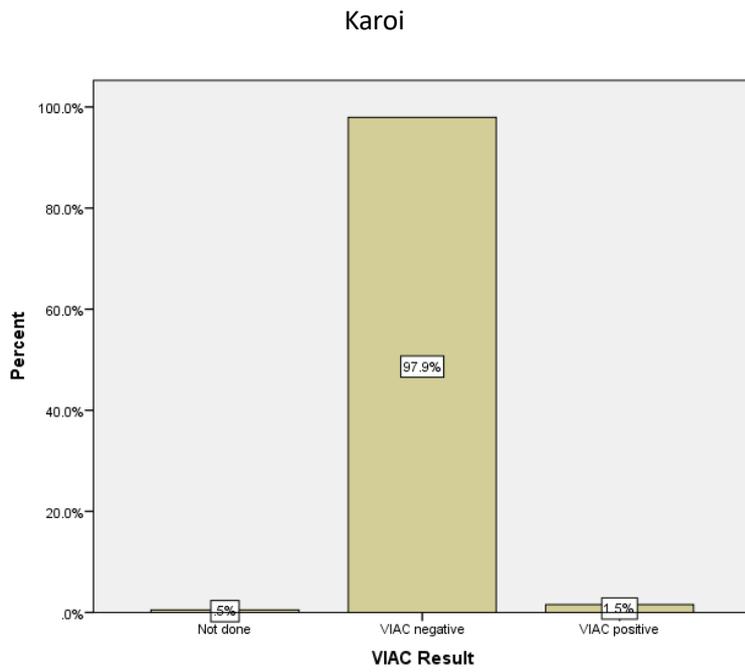
Indicators	Karoi	Magunje
Number of women screened	195	274
Average number of women screened per day	98	55
Mean Age	35.87 (95% CI 34.28- 37.46)	41.04 (95% CI 39.51-42.57)
Age Range	18-77 years	20-76 years
Median Age	34	39
Self-reported HIV+ prevalence	25.1% (49)	9.5% (26)
Percentage of women screened VIAC positive	1.5% (3) (All 3 referred for cryotherapy)	1.5% (4) (2 referred for cryotherapy. 1 referred for LEEP. 1 referred to gynaecologist)
Percentage of women treated for cervicitis/infection	0% (0)	4.4% (12)
History of previous cervical screening	2.6% (5)	-

**Table 2.3 Demographics of the women attending screening and results of the VIAC screening carried out in Karoi and Magunje.**

The average age of women who came for screening was 41 years with their ages ranging from 20 to 76 years. The median age was 39 years. The mode parity in Karoi was 2, while in Magunje it was 3 full-term births (figure 18). The prevalence of self-reported HIV positive women was 25.1% in Karoi, and 9.5% in Magunje (figure 17). The VIAC positive rates were the same at 1.5% in both Karoi and Magunje (figure 16). All of the 3 women who were found to be VIAC positive in Karoi were referred for cryotherapy which was done the day after the screening programme at the hospital. Of the 4 women who were VIAC positive in Magunje, 2 were referred to Karoi District Hospital for cryotherapy, 1 was referred to Chinhoyi Provincial Hospital for LEEP and 1 who was suspicious for invasive cervical cancer was referred to Chinhoyi Provincial Hospital for further investigations and biopsy. Other incidental findings on examination (figure 19) included cervicitis and vaginal discharge which are treatable with antibiotics. There was a similar prevalence rate of cervicitis in both sites – 0.4% in Karoi and 0.5% in Magunje (figure 19). None of the women in Karoi were treated and all 12 (4.4%) women with cervicitis and discharge in Magunje were treated with antibiotics (figure 20).

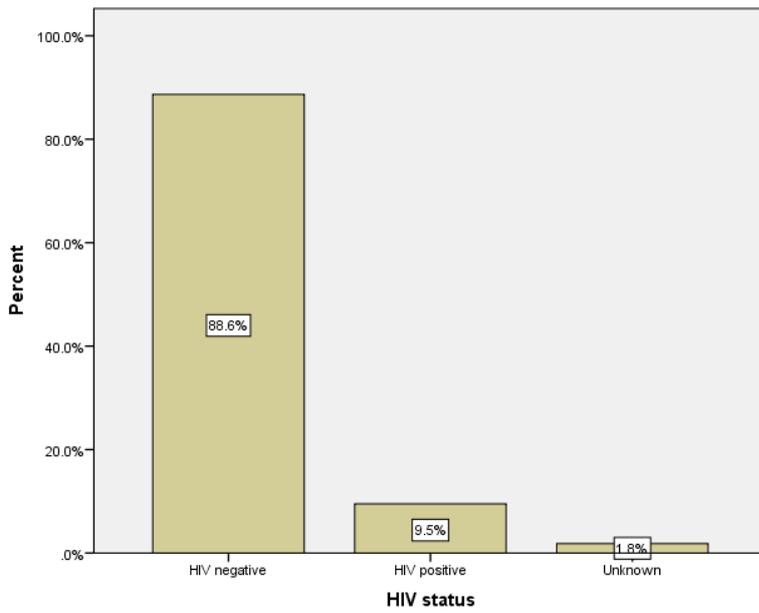


**Figure 2.3 Graphs showing the number of women attending for screening per day in Karoi (above) and Magunje (below).**

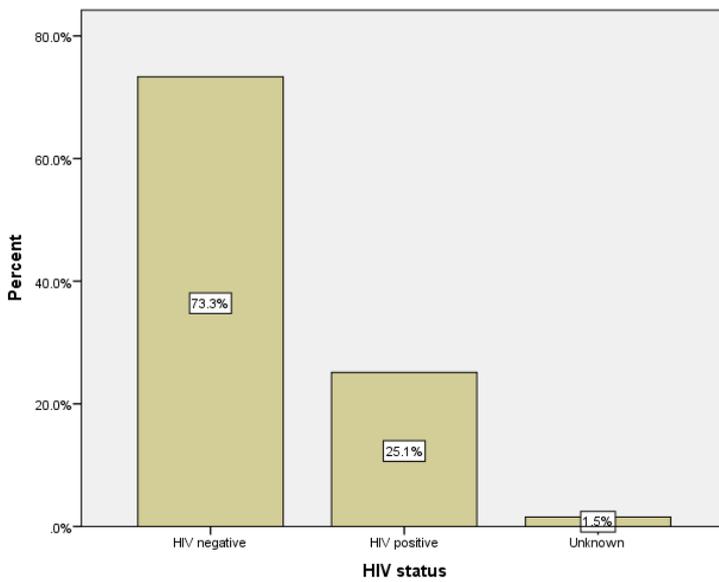


**Figure 2.4** Graphs showing VIAC results of women screened in Karoi (above) and Magunje (below).

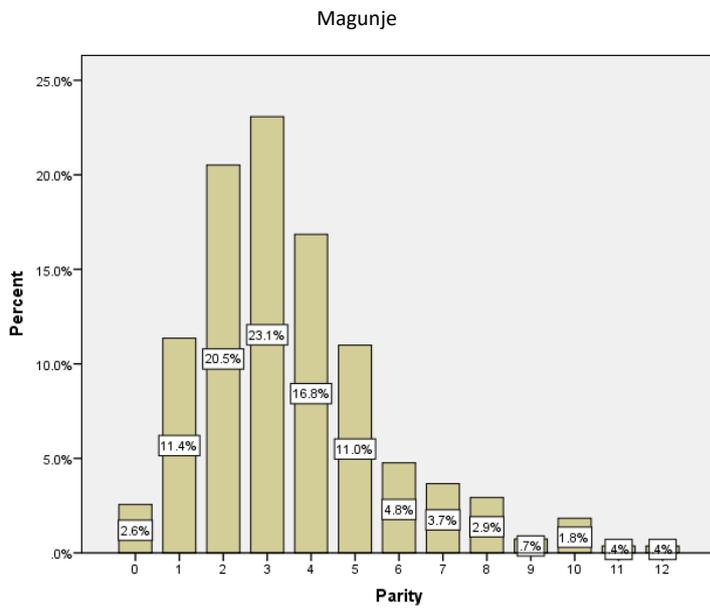
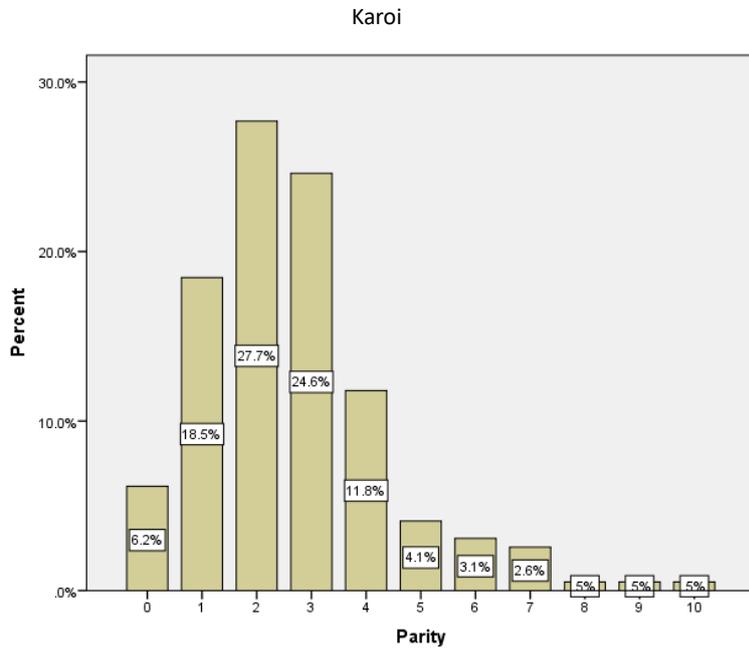
### Karoi



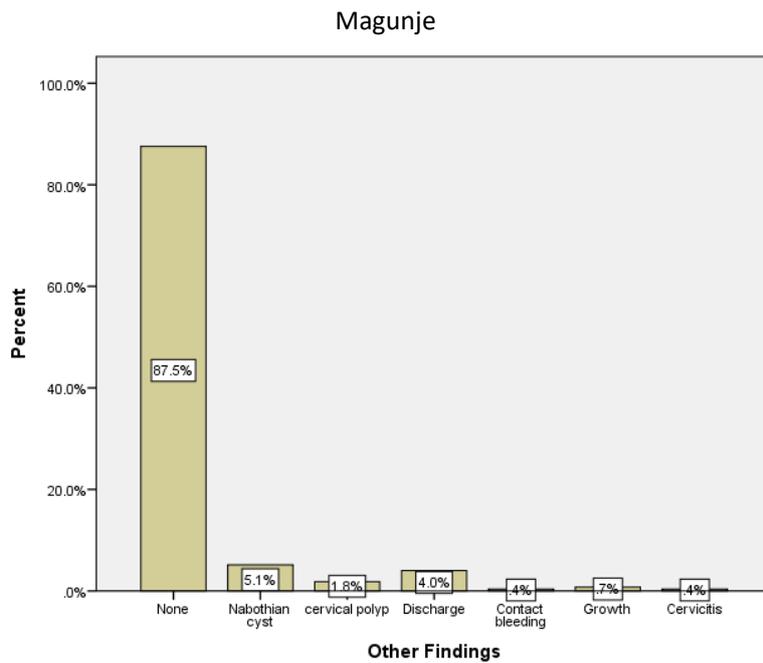
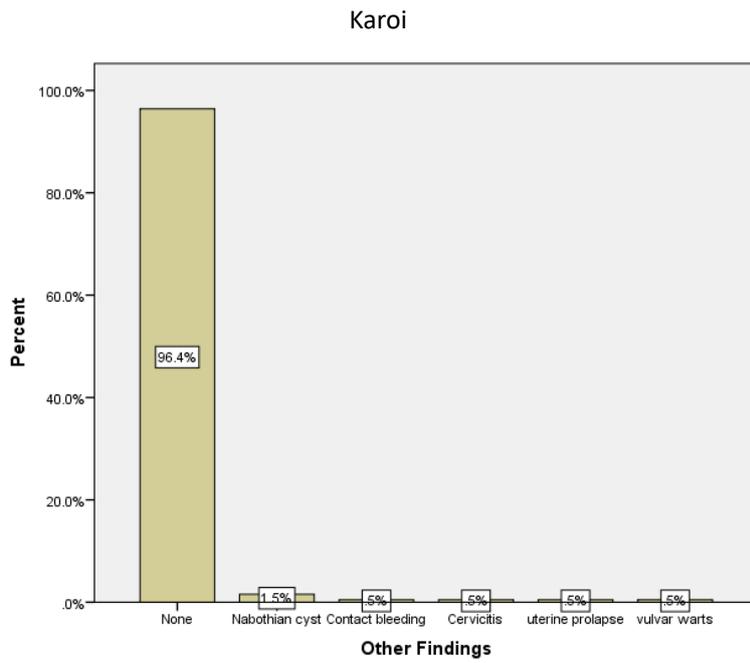
### Magunje



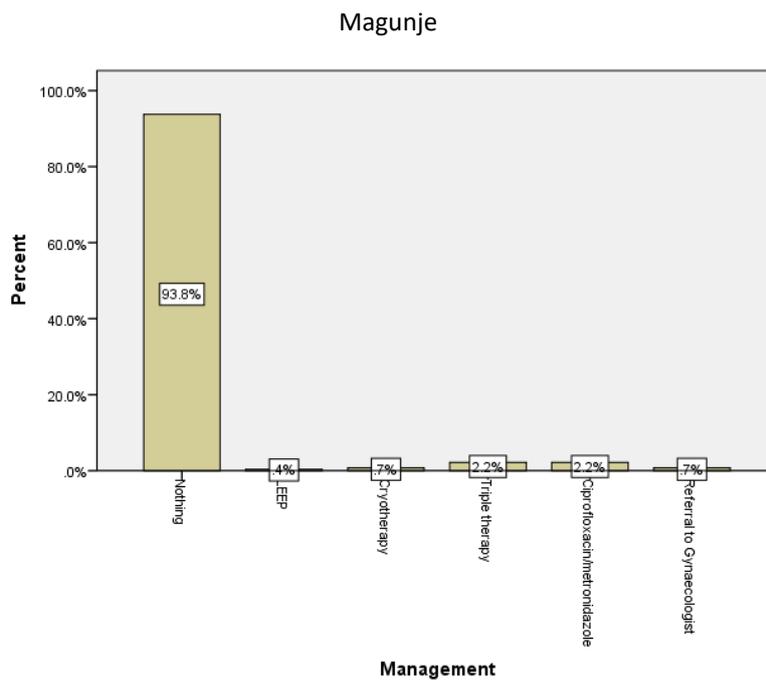
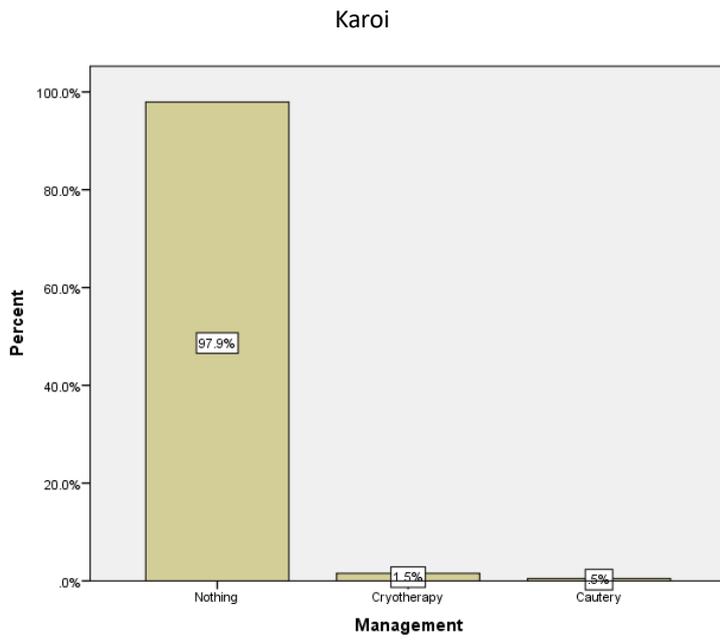
**Figure 2.5 Graphs of results showing percentage HIV status of women screened in Karoi (above) and Magunje (below).**



**Figure 2.6 Graphs of results showing parity frequency distribution of the women screened in Karoi (above) and Magunje (below).**



**Figure 2.7** Graphs showing other findings/diagnoses encountered during screening in Karoi (above) and Magunje (below).



**Figure 2.8** Graphs comparing the management options rendered to women in Karo (above) and Magunje (below).

### **2.3.1 Costs Associated with VIA Screening in Zimbabwe.**

The average VIAC screening cost per patient is US\$ 1.85. These costs include the staff salaries, and the cost of supplies from a provider perspective because screening is provided free of charge to the patient in Zimbabwe. **Table 2.5** itemizes the costs we used in calculating the cost of screening each woman during our screening programme. We therefore calculated that the approximate cost of screening all 469 women in our study was US\$867.65 (**Table 2.13**).

<b>Variable</b>	<b>Value</b>
Number of patients seen each day per VIAC nurse	15 patients
Time to perform VIA screening per patient	40 minutes
Daily salary for VIAC nurse	US\$22.7/day
Per patient cost of supplies for VIA	US\$0.34
<b>Total cost of screening per patient</b>	<b>US\$1.85</b>

**Table 2.5 The itemized cost of VIA screening per patient calculated from data collected during screening programmes in Karoi and Magunje.**

### **2.3.2 Costs Associated with Cervical Cancer Treatment Services in Zimbabwe**

The cost of screening which we calculated from our study was extrapolated to the female rural population of Zimbabwe aged 15 years and older. This was estimated at 3 536 205 (**Table 2.13**). The average cost of screening all rural women in Zimbabwe once in a lifetime would be approximately US\$6 577 341. Using the percentage of positive screens found in our study which is 1.5% and extrapolating it to the rural population of Zimbabwe, the number of women who would be found to have a positive VIAC screen would be approximately 53 043. Again using the proportions from our study, the majority of the screen positives would be amenable to cryotherapy which would be 38 898 women (1.1% of total population) while just 7 072 (0.2%) would need LEEP. 7 072 women (0.2%) would have been found to have lesions that are highly suspicious of invasive cancer and would need to be referred on for specialist investigations, and would therefore have likely subsequently required treatment for early stage cervical cancer at a cost of US\$7 114 432 to the patients in the public sector (**Table 2.7**), and US\$50 246 560 in the private sector (**Table 2.8**). If the screen positives had not attended for screening, they would have likely presented with late stage cervical cancer which extrapolates to 45 263 women (1.28% of the total population). This would have been at a cost of approximately US\$146 787 909 (**Table 2.9**) to the patients if they were treated in the public sector, and US\$980 170 265 (**Table 2.9**) if they were treated in the private sector.

Parameters	Cost US\$ (2018)	
	Private Sector	Public Sector
Hospital Admission	150/night	15/night
Total Abdominal Hysterectomy	5320	505
Radiotherapy	7468	500
F/up after radiotherapy	40	15
Chemotherapy (X6 sessions)	310/session	165/session
F/up during chemotherapy	40	15
F/up of treatment	40	15
MRI (available only in pvt sector)	1500	-
Staging CT scan	850	296
Bloods	50	50
Initial Dr. consultation	80	15
Colposcopy and biopsy	155	55
USS abdo/pelvis	220	40
LEEP	550	60
Cone biopsy	220	-
Blood transfusion	84	84

**Table 2.6 Average costs of investigations and treatment for cervical cancer in Zimbabwe in the private and public sector from a patient perspective (Courtesy of Parirenyatwa Hospital, March 2017).**

Parameters	Cost (US\$)	Stage 1a	Stage 1b-3	Stage 4
Hospital Admission	15/night	30	900 (x60 nights)	1350 (90 nights)
Total Abdominal hysterectomy	505	505	-	-
Radiotherapy	500	-	500	200
F/up after radiotherapy	15 +50 bloods		65	65
Chemotherapy X6 sessions	165/session (pvt)	-	990	-
F/up during chemotherapy	15	-	15	-
F/up of treatment	15	15	65	-
MRI	1500 (only in private sector)	-	-	-
Staging CT chest	296	296	296	296
Blood tests	50	50	50	50
Initial Dr. consultation	15	15	15	15
USS abdo/pelvis	40	40	40	40
Colposcopy and biopsy	55	55	55	55
Blood transfusion	84	-	252 (x3 transfusions)	1008 (x12 transfusions)
<b>Total Cost</b>		<b>1006</b>	<b>3243</b>	<b>3079</b>

**Table 2.7 The average cost of investigations and treatment for cervical cancer by FIGO stage per patient in the public sector from a patient perspective in Zimbabwe (Courtesy of Parirenyatwa Hospital, March 2017).**

Parameters	Cost (US\$)	Stage 1a	Stage 1b-3	Stage 4
Hospital Admission	150/night	300	9 000 (x 60 nights)	13 500 (x 90 nights)
Total Abdominal hysterectomy	5 320	5 320	-	-
Radiotherapy	7 468 (21 cycles)	-	7468	2987
F/up after radiotherapy	40 + 50 (blood test)		90	
Chemotherapy X6 sessions	310/session	-	1860	-
F/up during chemotherapy	40	40	40	
F/up of treatment	40 + 50 (blood test)	90	90	
MRI	1 500 (only in private sector)	1 500	1 500	1 500
Staging CT chest	850	850	850	850
Blood tests	50	50	50	50
Initial Dr. consultation	80	80	80	80
USS abdo/pelvis	220	220	220	220
Colposcopy and biopsy	155	155	155	155
Blood transfusion	84	-	252 (x3 transfusions)	1 008 (x12 transfusions)
<b>Total Cost</b>		<b>7 105</b>	<b>21 655</b>	<b>19 500</b>

**Table 2.8 The average cost of investigations and treatment of cervical cancer by FIGO stage per patient in the private sector from a patient perspective in Zimbabwe (Courtesy of Parirenytawa Hospital, March 2017).**

Parameters	Costs calculated from data collected during screening programmes in Karoi and Magunje.			Costs calculated from extrapolated rural Zimbabwe population data.	
	No. of women	Cost per woman screened/ treated (US\$)	Total Cost (US\$)	No. of women	Total Cost (US\$)
Female population 15 years and older in Zimbabwe*				5 132 373	
Percentage rural population in Zimbabwe*				68.9%	
Female rural population 15 years and older in Zimbabwe*				3 536 205	
Number of women screened	469	<b>1.85</b>	<b>867.65</b>	3 536 205	<b>6 577 341</b>
Number of women VIAC +	7 (1.5%)			53 043 (1.5%)	
Number of women treated with cryotherapy	5 (1.1%)			38 898 (1.1%)	
Number of women treated with LEEP	1 (0.2%)	<b>60</b>	<b>60</b>	7 072 (0.2%)	<b>424 320</b>
Number of women who would have been caught with early stage cervical cancer	1 (0.2%)			7 072 (0.2%)	<b>50 246 560 (private)</b> <b>7 114 432 (public)</b>
Number of women who would have progressed to late stage cancer without screening	6 (1.28%)	<b>1 006 (public)</b> <b>7 105 (private)</b>	<b>6 036 (public)</b> <b>42 630 (private)</b>	45 263 (1.28%)	<b>980 170 265 (private)</b> <b>146 787 909 (public)</b>

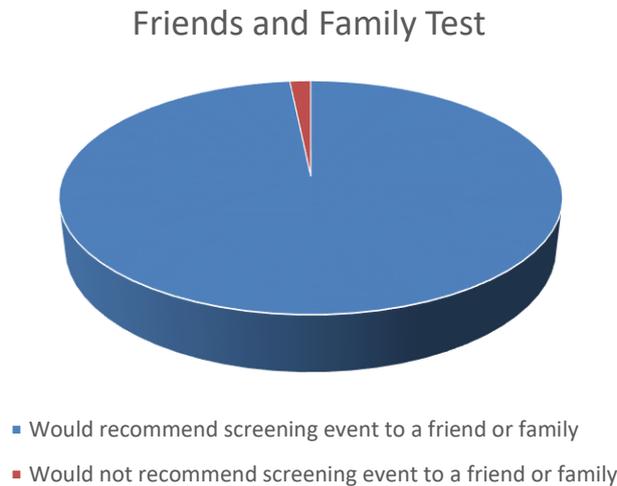
**Table 2.9 The average cost of VIA screening per woman as calculated from data collected from Karoi and Magunje screening programmes.** Also shown in the table is the average cost of VIA screening per woman if all the women aged 15 years and older in rural Zimbabwe were screened, and the total costs of not screening.

### 2.3 3 Feedback from Attendees

The feedback forms from Karoi screening programme revealed that of the 205 women whose forms we received back, 201 (98.1%) would recommend a friend or family member to attend cervical cancer screening (**Figure 2.9**). The most well received event on the day was the health education talk with 32% of all attendees saying it was the most useful and this was followed by B/P measurements (20%), BMI measurements (18%) and then cervical screening (15%) interestingly came 4<sup>th</sup> place (**Table 2.11**). Blood glucose measurements were also popular with 12% of attendees.

Overall Feedback	No. of Attendees (%)
Would recommend a friend or family member to attend a screening programme	201 (98.1%)
Would not recommend a friend or family member to attend a screening programme	4 (1.9%)

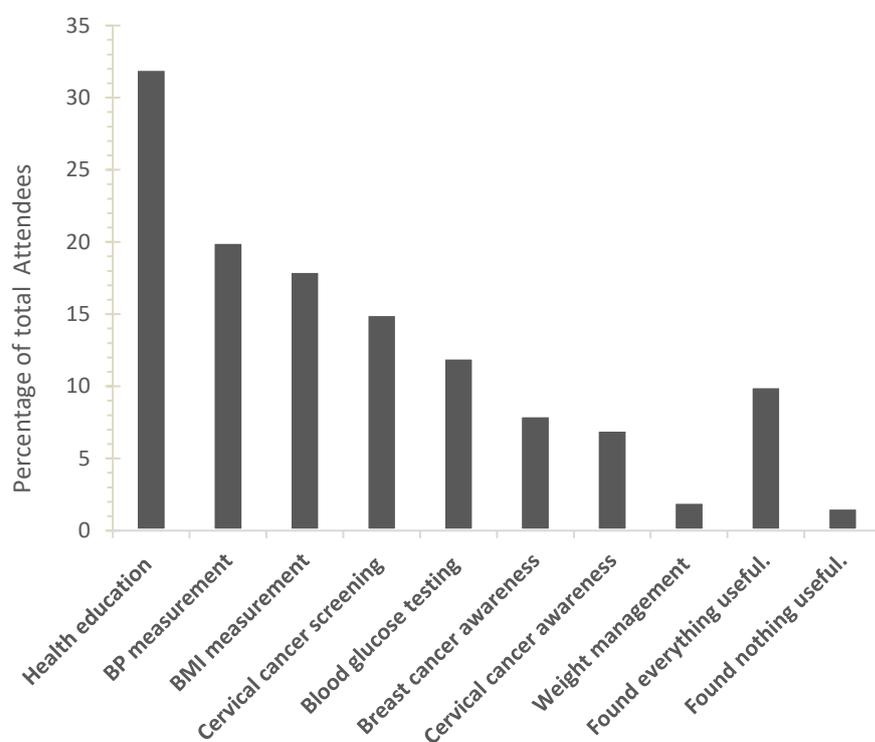
**Table 2.10 Results of the overall feedback from screening attendees.**



**Figure 2.9 A pie-chart showing the overall feedback result of the "Friends and "Family" test.**

Most Useful Event	% of total Attendees
Health Education	32
BP measurement	20
BMI measurement	18
Cervical cancer screening	15
Blood glucose testing	12
Breast cancer awareness	8
Cervical cancer awareness	7
Weight management	2
Found everything useful	10
Found nothing useful	1.6

**Table 2.11 Feedback results**



**Figure 2.10 Graph of the Feedback results in order of popularity.**

## 2.4 DISCUSSION

When evaluating cost-effectiveness for a screening programme, strategies which eliminate or reduce the need for follow-up visits and/or expensive colposcopy are most competitive. Concerns about VIA's sensitivity and specificity can be partly improved through training and regular quality assurance such as presentation of all images at multi-disciplinary team meetings where difficult cases can be discussed with more senior members of the healthcare team. Studies have shown that although comparable, and VIA recommendations specify that the service can be safely delivered by nurses, sensitivity and specificity outcomes for doctors are higher (Firnhaber et al., 2013).

Zimbabwe is a unique case in that it is a country with 4 separate economies working as one. There is the official currency which is the US dollar, but it is really being used as a commodity. There is also the country's own bond note which was supposed to be at a rate of 1:1 with the US dollar when it was introduced but it is not in reality. It is legal tender only in Zimbabwe and not anywhere else in the world, neither is it recognized as an official currency. There is also the "swipe" economy where consumers use their bank cards, and they have to pay an additional percentage which is variable depending on who is selling. Finally, there is Ecocash which is mobile money, and it has its own exchange rate too. Because of this unique situation, it is difficult to come up with a standard cost of goods or services because what a consumer pays depends on which "currency" they are using.

Our results for the cost of VIAC at US\$1.85 per patient screened, is in agreement with the results from other studies which evaluated the cost of VIAC in other Low Income 1 countries. In a study from Tanzania, they calculated an average cost of US\$1.45 per patient (Nelson et al., 2016), and in the study from Uganda it was US\$ 2.90 per patient (Campos et al., 2015).

A study based on economic modelling calculated a cost of US\$3.27 per patient in Zimbabwe (Mvundura & Tsu, 2014b).

There is a large divide in the cost of treatment in the private sector compared to the public sector. As shown in our study the private sector charges up to 7 times what the public sector charges to the patients, and these results are in agreement with those of Kuguyo et al, 2017 who found that private gynaecologists offer cytology testing at an approximate 7-fold of the cost offered in the public sector (Kuguyo et al., 2017). This puts cervical cancer screening out of reach of the majority of patients. Most of the patients who can afford such high prices, already seek medical attention outside the country, while those who cannot afford it, and have to use local services are the ones left to negotiate such exorbitant costs. Though the costs in the public sector are much lower, the prices are still out of reach for the average Zimbabwean (Nyakabau, 2014). Only 9.6% of Zimbabweans are on Medical Aid (Zimstat, 2016), however, even for those who have it, Medical Aid companies are also astute to the economic climate prevailing in the country and they can charge patients up to 50% of treatment costs upfront in order to cover shortfalls (Kuguyo et al., 2017).

In addition, the services available in the public sector are usually inadequate (Ministry of health and Child Care Zimbabwe, 2014). This problem is not limited to Zimbabwe only which has 2 radiotherapy units for a population of around 16 million people. The recommended ratio of units to individuals is 1 to 120,000–250,000 people. While high income countries average 1 unit to 130,000 people, that ratio increases to approximately 1 unit to 1.4 million people in LMIC, where out of 139 countries, only 4 are meeting their current radiotherapy needs (N. R. Datta et al., 2014).

There were limitations to our economic evaluation. We did not include the indirect costs such as loss of income due to time off work due to illness, or to caring for a loved one. We did not

include the cost of travel and accommodation to treatment centres, which is a large expense for the majority of patients who do not live in the two cities where cervical cancer treatment services are located. We also did not include fixed costs such as rent for buildings, water, electricity and the cost of acquiring equipment such as examining couches, cameras and laptop. We also did not account for the cost of training nurses for VIAC.

Karoi had a younger female screened population with an average age of 36 years compared to 39 years in Magunje, with mode parity of 2 and 3 respectively. In Magunje, 22.6% of women were aged 50 years and above, compared to 11.2% in Karoi. Younger women tend to move from the village to the more urban areas to look for work, or with their husbands or partners who move in search for employment.

The self-reported HIV prevalence of 25.1% in Karoi and 9.5% in Magunje is significantly different. Adult HIV prevalence in Zimbabwe is 15% (UNAIDS, 2013), but estimates are as high as 34% in urban areas (Averbach et al., 2010). The prevalence rate in Karoi is closer to the literature urban rate. The women in Karoi are more likely to have been tested and to be on anti-retroviral therapy, because they receive testing and free treatment for HIV/AIDS. In fact, during the time we were conducting screening, an HIV opportunistic infection clinic was running concurrently with our screening, at the premises we were based at. Karoi is also a more urban area compared to Magunje and as such HIV rates are likely to be higher in Karoi. HIV is a known risk factor for cervical cancer, it takes less time for HPV infection to progress to pre-cancer in HIV-positive women than in HIV negative women (Anorlu, 2008), HPV is more likely to persist in HIV + women and they have a higher rate of recurrence CIN (Paramsothy et al., 2004). Of the 7 women who were VIAC positive in both screening programmes, 2 women were HIV+, 1 had unknown status and 4 were HIV-. HIV infection is a known risk factor for persistent HPV infection and pre-cancerous lesions and recurrence of CIN2+ after treatment, so these results are surprising. Firstly, because the self-reported

prevalence of HIV in Karoi is more than double that of Magunje it was expected that the VIAC positive rate would similarly be higher in Karoi than in Magunje, however this was not the case. This could be attributed to the fact that HIV status was self-reported and not objectively tested. Also it is possible that in the case where the woman had not been tested for HIV she would more likely report herself as HIV negative in the absence of a formal diagnosis.

The prevalence of VIAC positive women screened during our project was the same in both areas at 1.5% which is much lower than the 20.2% and 39.8% prevalence rate found in a study done on over 10 000 women in Harare, Zimbabwe who were mostly in their late 20's or early 30's (Gaffikin et al., 1999). A separate study of 4641 women in Bulawayo, Zimbabwe found a VIAC positive rate of 10.8% (Fallala & Mash, 2015). The age group of the study compares well with the women in our project who had a mode age of 30 years so the difference is not likely attributed to age.

The studies quoted were done in the 2 major cities of Zimbabwe, while our study was done in a small rural town and a rural village. The sociodemographic factors of the women attending for screening in our programme are likely different to those of the women in the studies quoted, and this could account for the difference seen. There are likely differences in the educational levels, economic status and marital status and value systems. These factors were not taken into consideration in our study. Women from the cities are more likely to be more educated and therefore in employment, with a certain degree of economic independence compared to their rural counterparts. This brings with it an increased amount of social independence and the young women more likely to marry later compared to the less economically independent women in the rural areas who are forced to marry early as a way of gaining economic power, and therefore the women in the cities will likely have a higher number of sexual partners compared to the women from the rural areas. Historically people have moved from the rural areas to the city in a bid to seek for employment leaving behind

members of the extended family and going back home to the rural areas periodically. Therefore, the family unit in cities is not as strong as it is in the rural areas, and consequently, the family values are likely to be stronger in the rural areas compared to the cities. As a consequence, developmental milestones such as the age of sexual debut would be higher in the rural areas with less women having multiple partners. Furthermore, there is likely a larger cohort of sex workers within cities and it is possible that a proportion of the women in the studies from the cities could have been sampled from this cohort. The HIV prevalence in the cities is higher compared to rural areas, this will also have an impact on the VIA positive rates, with higher rates being expected to be seen in the cities as HIV is a risk factor for developing cervical cancer (Anorlu, 2008).

Studies have shown VIA to be a less specific screening test when compared to conventional cytology and HPV testing (Gaffikin et al., 1999; Mustafa et al., 2016) therefore it has a low positive predictive value, however, it was found to be more sensitive than cytology (Gaffikin et al., 1999). Sensitivity was 76.7% for VIA and 44.3% for pap smear, with a specificity of 64.1% for VIA compared to 90.6% for cytology (Gaffikin et al., 1999). The low specificity of the test could also account for the large differences in the results. As VIAC is operator dependent, a higher threshold for diagnosing a positive result could have played a role in our screening programme, in a bid to avoid overtreatment. However, 3 out of the 4 VIAC nurses who were providing the screening in Magunje were the same nurses who had done the screening in Karoi, even though there was a period of 1 year between the two screening programmes. This ensured a level of consistency which may have contributed to the similar result obtained at the two sites. Seven women in total were found to be VIAC positive, aged 24, 27, 31, 31, 32, 46 and 75 years with parity ranging from 1 to 12 full-term births. The small sample of VIAC positive women make it difficult to perform statistical analysis in order to establish correlation. Our sample size of 479 women is about 1/10<sup>th</sup> the size of the

Bulawayo study and about 1/20<sup>th</sup> the size of the Harare study, so the small sample size could have played a part in the difference seen in the VIAC positive prevalence rates between the studies quoted and our screening programme. The small sample size could have resulted in a type 2 error.

After the assessment in Karoi, some of the women were diagnosed with pelvic inflammatory disease, and cervicitis but could not afford the antibiotics which they needed, and many of the women on blood pressure medication were found to be hypertensive, indicating that their hypertension treatment was not optimized due to the lack of compliance brought about by lack of regular access to the medication. The medication we procured from IHP enabled us to treat all the women presenting with cervical or pelvic infection with antibiotics or antifungals, which was 12 (4.4%) women. There is a sense of helplessness when a patient is diagnosed with a treatable condition, but due to lack of access to appropriate medication, they remain untreated.

Only 5 (2.6%) of the women we screened in Karoi had received previous cervical cancer screening despite it being available free of charge, in Karoi. This is keeping with other studies which showed that uptake of screening is low even in areas with a screening programme in place (Mangoma et al., 2006; Mupepi et al., 2011). However, part of this could be due to the fact that screening has only been available in Karoi for the last year and uptake is still low due to lack of awareness.

Most women were very grateful for the screening project with 98.4% stating that they would recommend a family member or friend to come for the screening programme. Some were even saying that they were going home to tell neighbours, family and friends to come who were not aware. Many felt that 2 days was not long enough and we should have done at least a week, if not two weeks. Statements such as "I am glad I came because now I am

empowered as to the state of my health”, and “at least I am glad that the pain I have is not due to cervical cancer” were common. The women found health education to be the most useful as they were taught practical ways in which they could live healthier lifestyles which did not necessarily mean spending more money. The general health screening – blood pressure and BMI measurement and blood glucose were rated highly, because in Zimbabwe you have to pay to see a GP and some women had never had their blood pressure taken before. For diabetics, when they go to the clinic for blood glucose monitoring, they have to provide their own glucofix which costs US\$ 1.00 each. This is a prohibitive price to a substantial number of women who need it.

Many of the women expressed disappointment that we were not also doing HIV testing and counselling. HIV/AIDS is a disease which has affected the majority of people in Zimbabwe in some way. As a result, when it comes to matters of health is a top priority for most people who are either living with it or want to know if they have the virus. Breast cancer screening was another popular screening activity that the women would have liked us to provide. Some of the women confided in the doctors about issues of domestic violence and again we were limited in what we could offer them except to signpost them to social services. The issue of privacy – where two screening couches were in the same room, albeit with screens around each one, is another one which was brought up. This is something that next time when we plan a similar project we would be aware of and look to rectifying.

One of the most important things we realised when we first arrived at each screening programme site, was the need to quickly adapt to the cultural and social aspects of the community to promote the fluidity and expedient delivery of the screening program. At the beginning of the programme, the women were not as receptive as they were towards the end, because they were not accustomed to being served by such a young team of health professionals. We needed to build some professional trust with the community and ascertain

our roles in the program. As we organised group classes, teaching on cervical cancer, breast cancer and general health, this helped us to develop a certain level of confidence when addressing the patients. We also spent a considerable amount of time speaking to the locals while they waited for treatment to familiarise ourselves with them and to support them to speak freely around us. This managed to create a level of trust where the women felt confident to ask us for information concerning their wellbeing. From the feedback we got daily from the patients, we got to understand how they felt about our service delivery, thus, learn and make improvements each day.

Another important skill we learnt quickly was teamwork. All assigned duties and tasks were completed as the team was able to coordinate and designate duties within the group. This was particularly effective when we had to prepare patients for the VIAC screening by registering and checking their vitals; or covering each other for breaks. The team were also able to work together to organise a play on the risk factors of cervical cancer, which was performed for the women attending screening. The fact that we were from different fields also meant that we were able to bring different, creative ideas to the table throughout the week and establish a good working environment despite our differences.

#### ***2.4.1 Innovations in cervical cancer screening in LMICs***

Access to high quality laboratory diagnosis is a continuing challenge in LMICs (Nkengasong et al., 2010) caused by lack of essential infrastructure, laboratory supplies, basic equipment, skilled personnel, supply chain management and equipment maintenance. Dealing effectively with these challenges requires resources and time, which LMICs don't have, in the wake of the cancer epidemic they are facing. It is therefore imperative that innovations in early detection of cervical cancer are found, which move away from the traditional methods that

rely on established infrastructure and skilled personnel, which are in short supply in LMICs. Some of these innovations are discussed below.

**Downstaging cervical cancer.** Downstaging cancer is one way that countries in Africa can use, as an interim solution, to allow more effective cancer therapy while putting to better use the facilities that already exist. In a study done in 2 Kenyan villages in 2009 (Ngoma et al., 2015), village health aides with no prior medical training, underwent a 3-day course on symptoms and signs of cancer. They were trained to take focused histories and examinations and to take photos of suspected lesions. Educational material in printed and pictorial form was also provided to both villages.

In the interventional village, aides made proactive visits into the homes of the villagers and examined all consenting individuals. In the control village patients self-referred to the health aides at the local dispensary as per routine. Over a 3-year span, the incidence of cervical cancer diagnosed at early stages I and II rose from 9% to 14% to 33% in the control village, but in the interventional village it rose from 20% to 41% to 69% over the same period. This was primarily attribute to history taking because no pelvic examinations were done.

**HPV DNA testing.** A systematic review and meta-analysis of 15 studies from sub-Saharan Africa (Fokom-Domgue et al., 2015) which evaluated the performance of VIA, VILI (Visual Inspection with Lugol's Iodine) and HPV DNA testing as primary screening tests for CIN2+ found that there is considerable variation in VIA sensitivity and specificity despite there being no significant difference in the prevalence of CIN2+ between studies. This highlights the subjective nature of visual screening methods, while the effectiveness of HPV DNA testing has been demonstrated (Campos et al., 2015; Campos et al., et al., 2017; de Kok et al., 2012). WHO has recommended that HPV-DNA testing be integrated into cervical screening programmes in LMICs as part of a sequential test algorithm in a same day 'screen and treat'

strategy where HPV-DNA testing is followed by VIA (WHO, 2013b) (known as HPV testing with VIA triage). The emergence of HPV-DNA tests which can be offered at point of care like *careHPV* (Qiagen, Germantown, MD) and the Xpert® HPV test (GeneXpert; Cepheid, Sunnyvale, California) have made it possible to offer HPV testing in LMICs in this setting. However, recent research has suggested that VIA is not sufficiently accurate for inclusion in HPV based screening algorithms and offers no additional benefit compared to HPV-DNA testing alone (Toliman et al., 2018).

**Cervical 'Selfies'.** Integrating point-of-care diagnostics onto national or international laboratory networks can improve access to remote rural areas by effectively connecting a screen result to skilled personnel in a different part of the country or world. "Cervical selfies" is one way of doing so. Images at up to 25x magnification are taken using an Android mobile phone camera by smart medical device called the EVA (enhanced visual assessment) system. This mobile colposcope does the same job as a regular colposcope but comes at 10% of the size and 10% of the cost. It requires only basic training and is FDA cleared. It never touches the patient and the lens is 25-45cm away. It can document image and video findings, and collect patient information and remotely consult with experts during or after the examination. The information collected by the system is stored in a secure web storage. Currently this device is used in 23 states in the United States to conduct forensic examinations for sexual assault, and for diagnosis of oral cancer in India. The EVA system was designed and developed by an Israeli Biomedical start-up, has been used since May 2015, and costs around US\$1500. It has been piloted successfully in USA, Haiti, Guatemala, Botswana, Kenya, Nepal and Mexico and is now being used in 26 countries.

**Thermocoagulation.** Current screen-and-treat approaches rely on gas-based cryotherapy, which in turn relies on a consistent supply of gas, which is expensive to transport and not always available in low-resource settings. New non-gas ablative technologies that are smaller

and more portable than conventional cryotherapy equipment are currently undergoing testing. Thermocoagulation has been used successfully as part of a screen-and-treat program in Malawi (Campbell et al., 2016) and Cameroon (Viviano et al., 2017), and in addition it allows punch biopsies to be taken in the screening clinic. This treatment technology may improve management of screen-positive women by improving access to ablative therapy.

# **Chapter 3 TARGETING CERVICAL CANCER CELLS WITH QUINONE BASED BIOREDUCTIVE DRUGS.**

## **3.1 INTRODUCTION**

As described in the introductory chapter of this thesis, there is a case for developing loco-regional therapies for treating cervical cancer, particularly in the rural areas of low to middle income countries where access to or travel to medical facilities can be problematical. Whilst most currently available treatments for cancer are not applied loco-regionally, they could potentially be used in this setting. The design and development of therapies for loco-regional treatment however, will be distinctly different from traditional drug development pathways. The requirement for a pharmacokinetically stable compound is reduced in a loco-regional setting for example and paradoxically, poor pharmacokinetic properties could be advantageous in this setting. The bioreductive prodrug EO9 exemplifies this principle in that it failed to demonstrate activity in the clinic when administered intravenously but showed significant ablative activity against superficial bladder cancers when administered intravesically (Phillips et al., 2017; Phillips, 2016; Phillips et al, 2013).

EO9 is a quinone based bioreductive prodrug that requires enzymatic activation by oxidoreductases to generate toxic metabolites (Phillips et al., 2017; Phillips, 2016). Selectivity for tumour cells is determined by the enzymology of cancer cells, the presence or absence of oxygen and acidic extracellular pH. Numerous oxidoreductases have been shown to metabolise EO9 but the enzyme NAD(P)H:Quinone Oxidoreductase 1 (NQO1) plays a prominent role in the bioreductive activation process. NQO1, also known as DT-diaphorase (DTD) catalyses the two electron reduction of quinone based compounds and its physiological function is to protect cells from the damaging and potentially mutagenic effects of exogenous

quinone based compounds (Beyer et al., 1996). By converting the quinone directly to the hydroquinone, NQO1 circumvents the formation of the semiquinone intermediate (the product of one electron reduction) which can generate toxic free radicals via the redox cycling of the semiquinone in air (Cadenas, 1995). The gene encoding for NQO1 is polymorphic and individuals harbouring the C609T polymorphic variant (NQO1\*2) are completely devoid of NQO1 activity (Traver et al., 1997). The loss of the detoxifying function of NQO1 is of considerable interest as it may predispose individuals to diseases such as cancer (Lajin & Alachkar, 2013). NQO1 has a number of additional functions including the stabilisation of p53 and as described in the introduction, loss of NQO1 due to the polymorphic NQO1\*2 variant could predispose individuals to HPV generated cervical cancer. Details of these properties and functions are described in more detail in Chapter 1 and elsewhere in the literature (Asher et al., 2002; Asher et al., 2006; Tsvetkov et al., 2005).

From a drug development perspective, NQO1 can convert certain quinones into products that are more toxic than the parent compound. This is known as bioreductive activation and it is the chemistry of the groups attached to the quinone nucleus that determines whether a compound is more or less toxic following reduction (Cadenas, 1995). NQO1 levels have been characterised in a large number of cell lines and human tumours and in general, it is typically overexpressed in a number of cancers including non-small cell lung cancer (refs). In contrast, little is known about the expression of NQO1 in cervical cancer and no studies have evaluated whether or not EO9 has activity against cervical cancer cell lines. As cervical cancer is accessible in terms of loco-regional therapy, it is feasible that EO9 could eradicate cervical cancer cells by direct application to the surface of the cervix. As in the case of bladder cancer, any drug reaching the blood stream would be rapidly eliminated thereby reducing the risk of systemic side effects. The usual treatment of pre-invasive cervical cancer is surgical removal of cells from the cervix but this may not be feasible in rural areas of Zimbabwe for example.

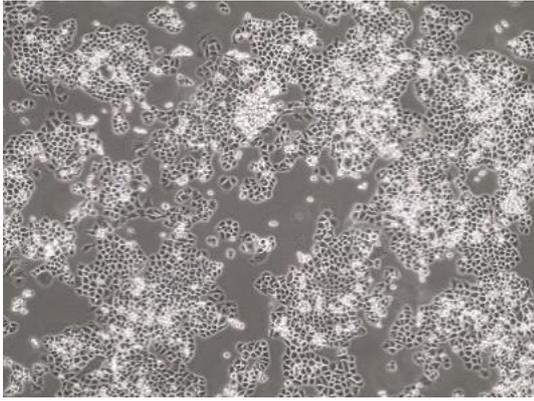
In this case, the application of drugs directly onto the cervix immediately following VIAC inspection could be a more cost effective and efficient way of dealing with this disease in this setting.

The purpose therefore of this chapter is to characterise the activity of NQO1 in a panel of cervical cancer cell lines and evaluate the preclinical activity of EO9 against these cell lines. In addition to EO9, a series of novel indolequinone derivatives were obtained from Dr Patrick Murphy (University of Bangor) and these were evaluated to determine if they are substrates for NQO1 and if they are selectively toxic to NQO1 rich cells *in vitro*. The primary objective of this chapter is to develop the preclinical rationale to support the development of EO9 (or novel quinone based derivatives) as loco-regional therapies for cervical cancer.

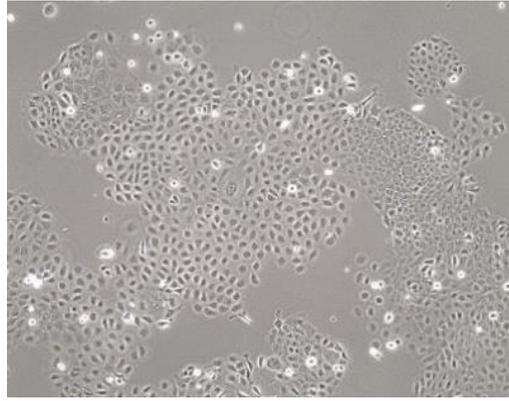
## **3.2 MATERIALS AND METHODS**

### **3.2.1 Cell lines**

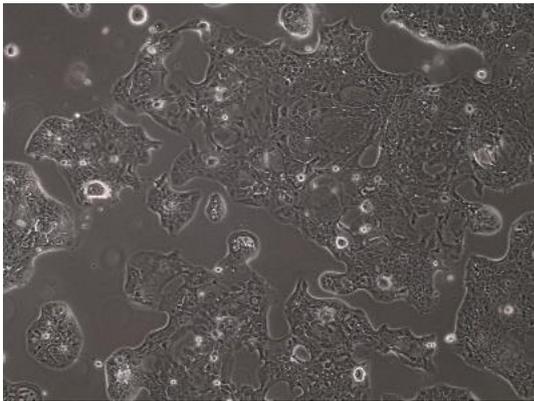
The panel of cervical cancer cell lines used in this study was obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) except SiHa which was a gift from Dr S. Allison (Huddersfield University). The cell lines and morphological appearance of the cell lines used are presented in figure 3.2.1 below.



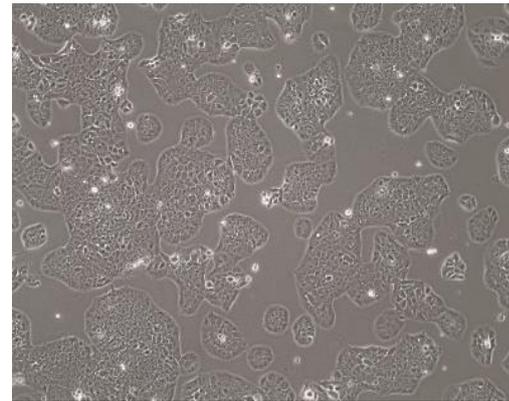
**C33A**



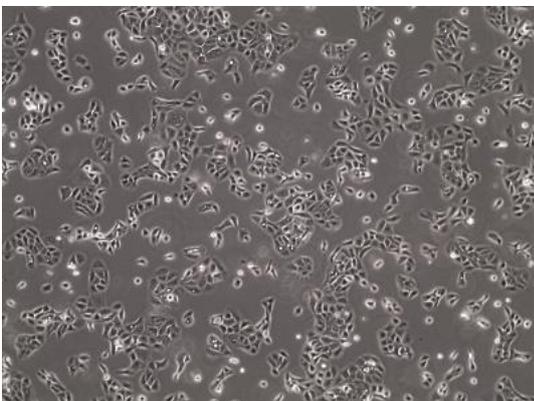
**CaSki**



**MS751**



**DoTc2**



**C4I**



**SiHa**

**Figure 3.2.1 The appearance of a panel of human cervical carcinoma cell lines under light microscopy (EVOS XL Core) at x20 magnification.**

### **3.2.2 Cell culture conditions**

Cell lines were cultured in media recommended by ATCC. C33A, MS751 and SiHa were grown in Eagles Modified Essential Medium (EMEM), DoTc2 was grown in Dulbecco's Modified Essential Medium (DMEM), CaSki was grown in RPMI and C41 were grown in Waymouth's medium. All media was supplemented with 10% foetal bovine serum (Sigma-Aldrich, Gillingham, Dorset, UK) and 2mM L-glutamine (Fisher, Loughborough, UK). Cells were routinely grown in vented cap T25 and T75 flasks (Sarstedt, Leicester, UK). Cell cultures were grown as monolayers and incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. All cell culture studies were carried out in a Class II biological safety cabinet (Triple Red, Buckinghamshire, UK).

### **3.2.3 Trypsinization of monolayer cultures.**

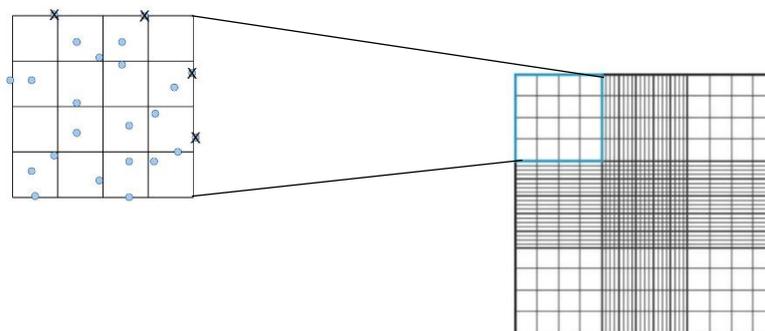
Trypsin EDTA solution at x10 concentration was obtained from Fisher (Loughborough, UK). It was stored in 5ml aliquots at -20° C and defrosted and diluted in Phosphate Buffered Solution (PBS) down to the working 1X concentration. PBS tablets were obtained from Sigma-Aldrich (Gillingham, Dorset), and were dissolved at 1 tablet to 200ml autoclaved, de-ionised water.

The media from flasks of cells at approximately 70% confluency was removed and cells were washed twice with 10ml of PBS after which, 1ml of trypsin was added to a T25 flask, and 2 ml to a T75 flask. Cell were incubated at 37° C for 5 minutes and examined under a microscope (EVOS) for complete separation into single cells. Cells were re-suspended in 5 ml of media and spun at 1500 rpm for 3 minutes. The supernatant was discarded and the cell pellet was finally suspended in 10ml of complete media. Cells were counted using a haemocytometer and  $1 \times 10^5$  cells were added to new T25 flasks containing 10ml of culture media for maintenance of stocks. The number of cells used varied depending on specific requirements

for experiments but these were the procedures used for the routine maintenance of cell cultures.

### 3.2.4 Cell counting

Cells were counted using Neubauer's modified haemocytometer as shown below. A coverslip was placed on top of the haemocytometer over the counting squares. 10µl of cell suspension was placed under the coverslip using capillary action in one chamber. A cell count was performed in the 4 large corner squares (blue border in the diagram below) using the EVOS XL Core cell imaging system. A total of 5 squares per grid were counted and two squares were used in total (10 squares counted). The average number of cells per grid was calculated and this was multiplied by  $10^4$  in order to get the number of cells per ml. Each large square of the haemocytometer represents a volume of  $10^{-4}\text{cm}^3$  which is the origin of the  $10^4$  multiplier described above.



**Figure 3.2.2 Neubauer's modified haemocytometer. The cells on 2 adjacent edges marked X are not counted. Each large square is  $1\text{mm}^2$ .**

The total number of cells per ml (N) is given by:

$$N (\text{cells/ml}) = n/10 \times 10^4$$

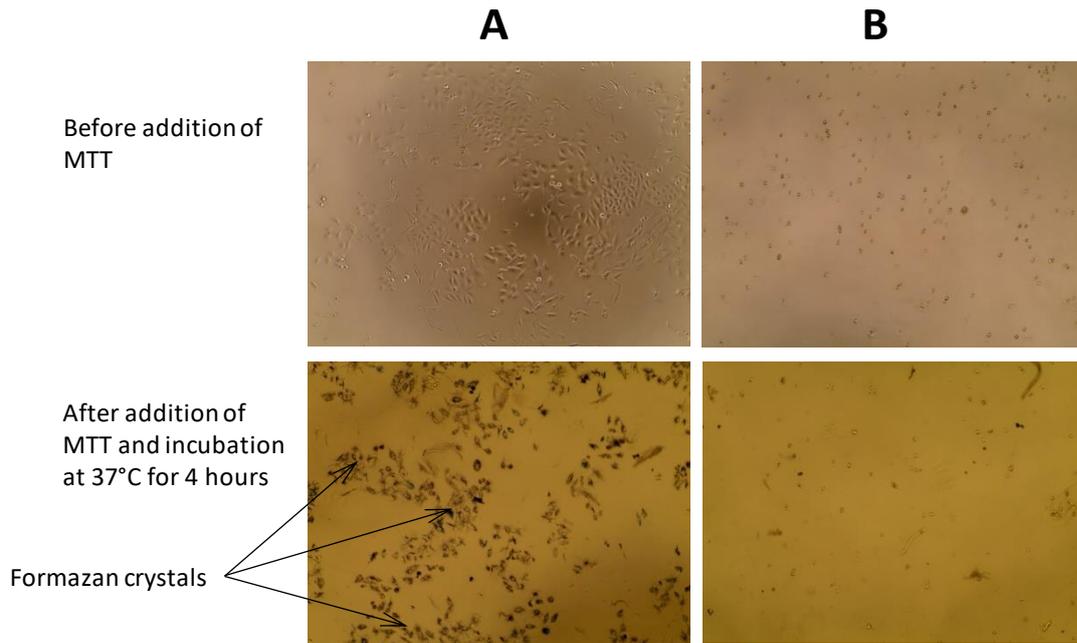
(where n= total number of cells counted and 10 is the number of grids counted).

### **3.2.5 Compounds**

EO9 was obtained from Spectrum Pharmaceuticals (California) and Tirapazamine was obtained from Sigma-Aldrich (Gillingham, Dorset, UK). Test Compounds were obtained from Dr P. Murphy (Bangor University). All compounds were dissolved in DMSO and stored at -20° C in small aliquots of 15µl each at a concentration of 100mM. The chemical structures of these compounds are shown in appendix 2. Purified human NQO1 enzyme and dicoumarol was obtained from Sigma-Aldrich (Gillingham, Dorset, UK)

### **3.2.6 MTT assay**

Chemosensitivity testing was done using the 3-(4,5, dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. This is a colorimetric assay which assesses cellular metabolic activity. Living cells use mitochondrial NAD(P)H-dependent cellular oxidoreductases to reduce the tetrazolium dye to its insoluble, purple formazan crystals. Dead cells do not contain these oxidoreductases and cannot catalyse this reaction. The MTT assay is therefore under defined conditions, able to reflect the number of viable cells present. The formazan crystals are dissolved in DMSO and the absorbance of the resulting solution correlates to cell viability. Images of control and dead cells before and after the addition of MTT are presented in figure 3.3 below. The images for controls show the presence of dark formazan crystals following exposure to MTT but prior to solubilisation in DMSO.



**Figure 3.2.3 The MTT assay. A 96-well plate containing A plenty of viable cells. B predominantly dead cells before and after addition of MTT. Formazan crystals are formed in the well with viable cells and are not formed in the well with dead cells.**

### **3.2.7 Validation of the MTT assay using SiHa cells.**

In order to ascertain the relationship between cell number and absorbance, a validation of the MTT assay was performed as follows. SiHa cells were grown in EMEM supplemented with 10% FBS and 1% glutamine until they reached 70% confluency. Following trypsinization and cell counting, a cell suspension containing  $6 \times 10^4$  cells/ml was prepared. To a clear, flat-bottomed 96-well-plate (Cyto-One, Starlab), 200  $\mu$ l of culture media was added to lane 1, rows A to H and this served as the blank. To lane 2, rows A to H, 200  $\mu$ l of cell suspension was added. To lane 3, rows A to H, 180  $\mu$ l of cell suspension plus 20  $\mu$ l of media was added.

This procedure was repeated across the plate where the volume of cell suspension added decreased by 20  $\mu\text{l}$  and the final volume was made up to 200  $\mu\text{l}$  using media in all cases. Once the cells had been plated out, MTT (20 $\mu\text{l}$ ) at a concentration of 5mg/ml was added to each well and the plate incubated at 37°C for 4 hours. Following the incubation period, the media/MTT solution was carefully discarded, and the resulting formazan crystals were dissolved in 150 $\mu\text{l}$  of DMSO per well. The mean absorbance for the solution in the wells was measured using a Tecan plate reader set at 540nm. The results were plotted as mean absorbance for each lane vs cell number.

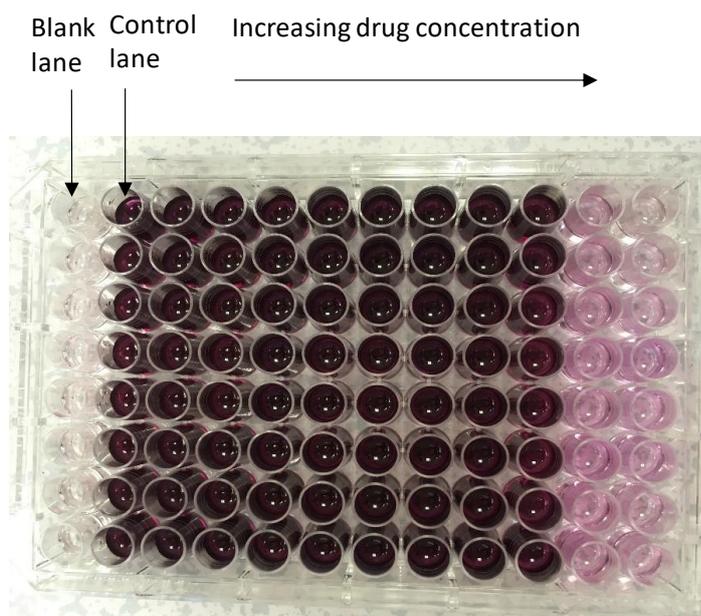
### **3.2.8 Seeding of the chemosensitivity plates.**

Stock cell cultures were grown to 70% confluency, harvested and diluted to form a working cell suspension concentration of  $1 \times 10^4$  cells  $\text{ml}^{-1}$ . To a 96 well plate, 200  $\mu\text{l}$  of complete medium only was added to lane 1 (rows A to H). To the remainder of the plate, 200 $\mu\text{l}$  of cell suspension was added to each well from lanes 2-12, giving a final concentration of 2000 cells per well. The plates were incubated for 24-48 hours depending on the cell line, at 37°C in a 5%  $\text{CO}_2$  enriched atmosphere to allow the cells to adhere to the wells.

### **3.2.9 Chemosensitivity assay (1-hour drug exposure).**

After cells had adhered, a series of 10 drug dilutions starting from either 10 $\mu\text{M}$  or 100 $\mu\text{M}$  maximum concentration (depending on the cell type) were prepared. The drug dilutions were poured into a 12 channel plastic reservoir. Media was then removed from all wells in the 96-well plate, and the cells were exposed to drug dilutions for 1 hour as follows: to lane 1, which had no cells, 200 $\mu\text{l}$  of media only was added, this served as the blank for the

spectrophotometer. To lane 2, again 200µl of media only was added, this was the control. To lane 3, 200µl of media containing the lowest concentration of drug to be tested was added, followed in the next lane by 200µl of media containing the second lowest concentration of drug until all lanes had been filled with successively higher drug concentrations until last lane, 12 which contained 200µl of media containing the highest drug concentration. Following drug exposure, all the drug containing media was removed and cells were washed twice with 200µl PBS per wash. Fresh media (200µl) was then added to all the wells and the plate incubated at 37°C for 96 hours. After this time, media was removed from all the wells and replaced with 200µl of fresh media before adding 20µl of MTT (5mg/ml). The plates were incubated for a further 4 hours during which time the enzymatic reduction of MTT in the mitochondria of live cells produced purple formazan crystals. After 4 hours, all the media was removed from the wells to dryness. DMSO (150µl) was added to all the wells and which formed a purple solution upon dissolution of the formazan crystals but stayed clear in wells not containing the crystals. The absorbance of the plates was measured at 540nm as described previously. The mean absorbance of the blank in lane 1, was subtracted from all the other lanes to get the true absorbance. The mean absorbance of control cultures was taken to be 100% cell survival. The mean absorbance of cell cultures was expressed as % cell survival. A dose response curve of % cell survival against drug concentration was plotted, and the concentration required to kill 50% of cells ( $IC_{50}$ ) was determined. All experiments were done in triplicate. A representative image of an MTT assay is presented in figure 3.2.4 below:



**Figure 3.2.4** The result of a chemosensitivity plate using the MTT assay. Lane 1 is the blank with no cells. The intensity of the formazan crystals in solution as measure by spectrophotometer correlates with the number of viable cells present per well.

### **3.2.10 Chemosensitivity assay (Timed Dose Response experiments).**

Stock cell cultures of SiHa and CaSki cell lines were grown to 70% confluency, harvested and diluted to form a working cell suspension concentration of  $1 \times 10^4$  cells  $\text{ml}^{-1}$  as described above. Chemosensitivity plates were prepared as described above and exposed to a series of drug dilutions. Each plate was exposed to drug for a time period of either 1hr, 2hr, 3hr, 4hr, 5hr, 6hr, 12hr or 24hr following which time the cells were washed twice with PBS and 200  $\mu\text{l}$  of media added to each well. Cells were incubated for a total of 96 hours (where  $t = 0$  is the start of the experiment) before the MTT assay was conducted as described above. For continuous drug exposure experiments, cells were exposed to drugs for 96 hours prior to the addition of MTT.

### **3.2.11 Response of cells to the NQO1 inhibitor dicoumarol.**

Dicoumarol (Sigma-Aldrich, UK) 20mg was first dissolved in 20 $\mu$ l of 2M NaOH and then made up to a total of 5.95ml with PBS to make a stock solution of 10mM dicoumarol. This was aliquoted and stored at -20°C until required for use. Stock cell cultures were grown to 70% confluency, harvested and diluted to form a working cell suspension concentration of  $1 \times 10^4$  cells ml<sup>-1</sup>, as described above and seeded into 96- well plates as described above. A stock solution of dicoumarol 10mM was diluted in media at a ratio of 1:40 to form a maximum concentration of 250 $\mu$ M, which was further serially diluted in 3x dilutions to form a total of 10 decreasing dicoumarol concentrations. The cells in the plates were then exposed to dicoumarol for 1, 3 or 6 hours, and the rest of the experiment proceeded as described above. Continuous drug exposure for 96 hours was also done. The purpose of this experiment was to determine a non-toxic dose of dicoumarol to use in combination with EO9.

### **3.2.12 Effect of dicoumarol on the response of SiHa cells to EO9.**

Stock cell cultures of SiHa were grown to 70% confluency, harvested and diluted to form a working cell suspension concentration of  $1 \times 10^4$  cells ml<sup>-1</sup>, as described above. Chemosensitivity plates were prepared as described in above and EO9 diluted with media containing a non-toxic concentration of dicoumarol as determined above. Full experimental details of concentrations used are presented in the results section. The response of cells

following exposure to EO9 (varying concentrations) and dicoumarol (fixed concentration) were determined using the MTT assay as described above.

### **3.2.13 Effect of drug/inhibitor administration schedule on chemosensitivity.**

The experiment above describes the concomitant administration of both EO9 and dicoumarol but it is possible to give dicoumarol before or after EO9. In this case, cells were exposed to dicoumarol (fixed concentration) either before or after exposure to EO9 (variable concentrations) using the experimental procedures described above.

### **3.2.14 Effect of dicoumarol on the response of a panel of cervical cancer cell lines to EO9.**

Once the initial studies using SiHa cells had been completed and procedures optimised, the experiment was expanded to include the full panel of cervical cancer cell lines. Stock cell cultures were grown to 70% confluency and seeded into 96- well plates as described above. A stock solution of dicoumarol at 10mM was prepared and diluted in media to 100µM. The dicoumarol at this concentration was used as a diluent to make up a total of 9 EO9 drug dilutions with a maximum concentration of 100µM. Plates were exposed to drug dilutions for 1 hour as follows: to lane 1, which had no cells, 200µl of media only was added, this served as the blank for the spectrophotometer. To lane 2, again 200µl of media only was added, this was the control. To lane 3, 200µl of the 100µM dicoumarol/media diluent alone with no EO9. To lane 4 was added 200µl of the lowest concentration of EO9/dicoumarol diluent to be tested

followed in the next lane by 200µl of media containing the second lowest concentration of EO9/dicoumarol. This was repeated until all lanes had been filled with successively higher EO9 concentrations until last lane, 12 which contained 200µl of EO9 100µM and dicoumarol (100 µM). For the CaSKi cell line, the maximum concentration of EO9 used was 500µM. Following drug exposure, all the EO9/dicoumarol was removed and cells were washed twice with 200µl PBS per wash. Fresh media (200µl) was then added to all the wells and the plate incubated at 37°C for 96 hours. The rest of the experiment was proceeded as for the chemosensitivity assay described above.

### **3.2.15 Influence of hypoxia on the activity of EO9 against cervical cancer cell lines.**

Experiments were performed on SiHa and C33A cells using EO9 and Tirapazamine. Tirapazamine is a 'gold standard' hypoxia activated prodrug and it was used here as a positive control to demonstrate that the experimental conditions used were appropriate for studying the effects of EO9 under hypoxic conditions. Stock SiHa and C33A cell cultures were grown until 70% confluency, harvested and diluted to form a working cell suspension concentration of  $1 \times 10^4$  cells ml<sup>-1</sup>, as described above. The cells were seeded into 96-well plates and incubated for 24 hours at 37°C in a normoxic environment of 5% CO<sub>2</sub> enriched atmosphere to allow the cells to adhere to the wells, as described in 1.1.9. After 24 hours the plates transferred to an H35 HypOxystation (Don Whitley Scientific, Shipley, UK) where they were incubated in chamber at hypoxic atmospheric conditions consisting of humidified gases at 5% carbon dioxide and 0.1% oxygen at 37°C. The cells were allowed to equilibrate to hypoxic conditions for a further 24 hours. After this time period, a series of drug dilutions was prepared

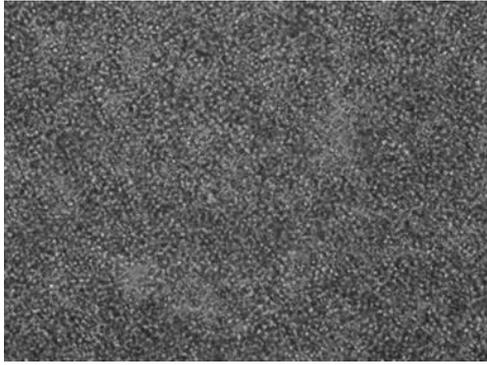
in the hypoxia chamber, using media which had been stored in the hypoxia chamber for at least 24 hours in order to equilibrate it to hypoxic conditions. Maintaining the plates under hypoxic conditions in the hypoxia chamber, the cells were treated with drug for 1 hour, as described in 1.1.10 and then the plates were incubated in the hypoxia chamber for 96 hours. After this period, the plates were removed from the hypoxic chamber and MTT was immediately added to each well, and the plates were incubated under normoxia at 37°C for a further 4 hours, following which the MTT assay was performed as described above. Each assay was performed in triplicate and IC<sub>50</sub> values were determined as described above. To measure the effect of hypoxia on the activity of EO9/Tirapazamine, the hypoxic cytotoxicity ratio (HCR) was calculated as described below:

$$\text{HCR} = \text{IC}_{50} \text{ of cells under aerobic conditions} / \text{IC}_{50} \text{ of cells under hypoxic conditions}$$

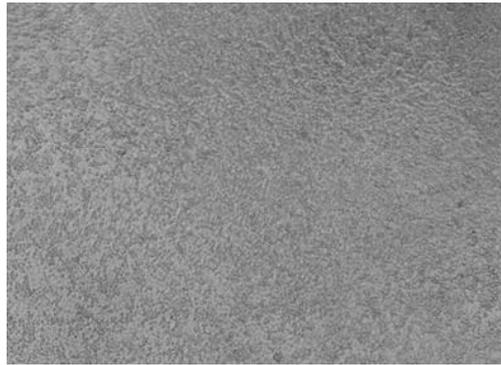
HCR values >1 indicate that the drug has preferential activity against hypoxic cells.

### **3.2.16 The activity of EO9 against confluent monolayer cultures.**

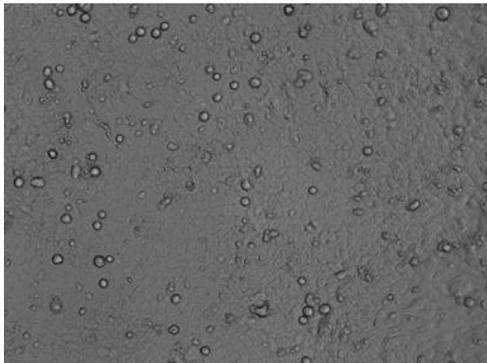
As cervical cancer cells clinically present themselves as a sheet or monolayer of cells, cells were grown to confluency to determine whether or not this altered the response of cells to EO9. Cells were plated into lanes 2-12 of a 96 well plate and incubated over 48 hours until the cells in each well had formed a monolayer. Representative images of these confluent monolayers is presented in figure 3.5. These cells were exposed to a range of EO9 concentrations for one hour as described above and the MTT assay was performed after 96 hours' recovery.



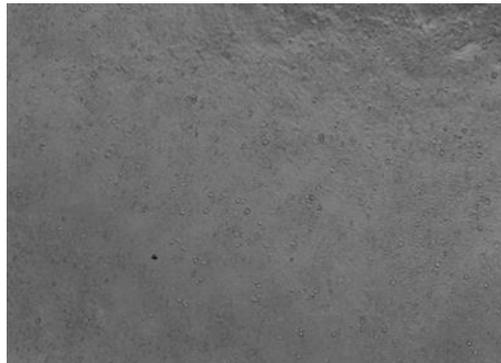
CaSki



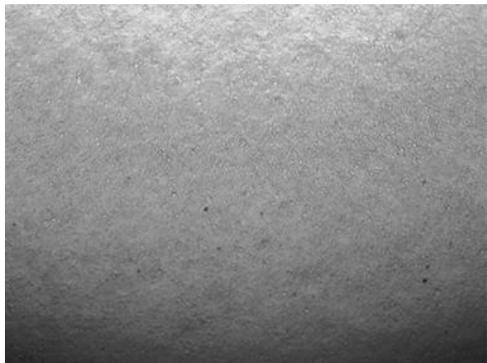
SiHa



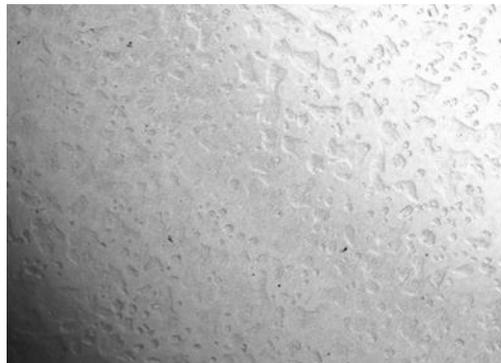
C4I



DoTc2



C33A



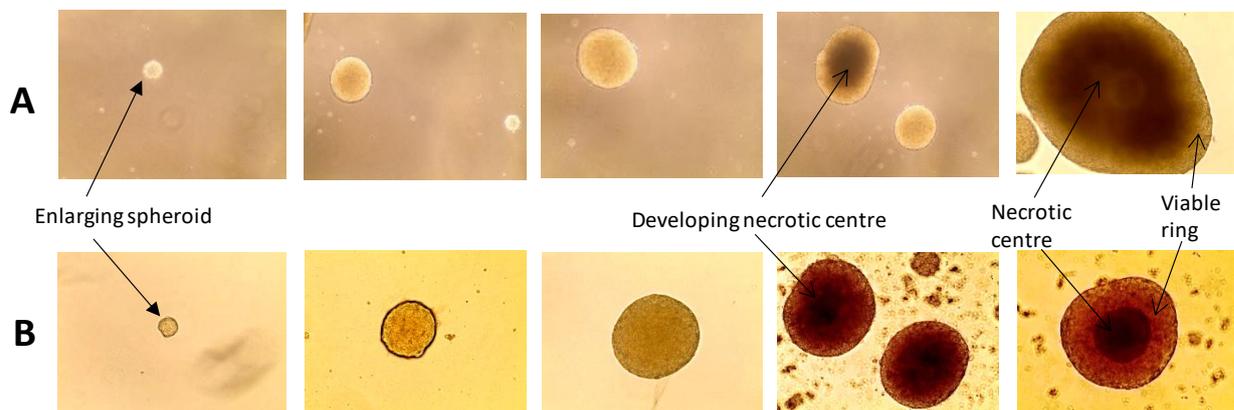
MS751

**Figure 3.2.5 The appearance of a monolayer sheet of cells at 100% confluency for a panel of human cervical carcinoma cells. Seen under light microscopy (EVOS XL Core) x40 magnification.**

### 3.2.17 Growth of DoTc2 and MS751 as Multicell Spheroids.

In a series of preliminary studies, the ability of the cervical cancer cell line panel to form multicell spheroids was determined. Of these cell lines, DoTc2 and MS751 showed signs of forming good, compact multicell spheroids and these were characterised further as described below.

Cell cultures at 70% confluency were harvested and prepared as single cell suspensions by trypsinization as previously described. T25 flasks (Sarstedt) were base coated with 1% agar and  $1 \times 10^5$  cells were seeded into the flask. The cells were left to grow in the flasks until spheroids were visible under the light microscope. As the size of the spheroids increased, a dark central region became visible which is indicative of the formation of a necrotic core. Representative images of spheroid morphology are presented in figure 3.2.6.



**Figure 3.2.6 Light microscopy photographs of an enlarging spheroid developing into a mature spheroid. Note the necrotic centre with a normoxic ring of viable cells. A The spheroids at x10 magnification B The spheroids at x40 magnification.**

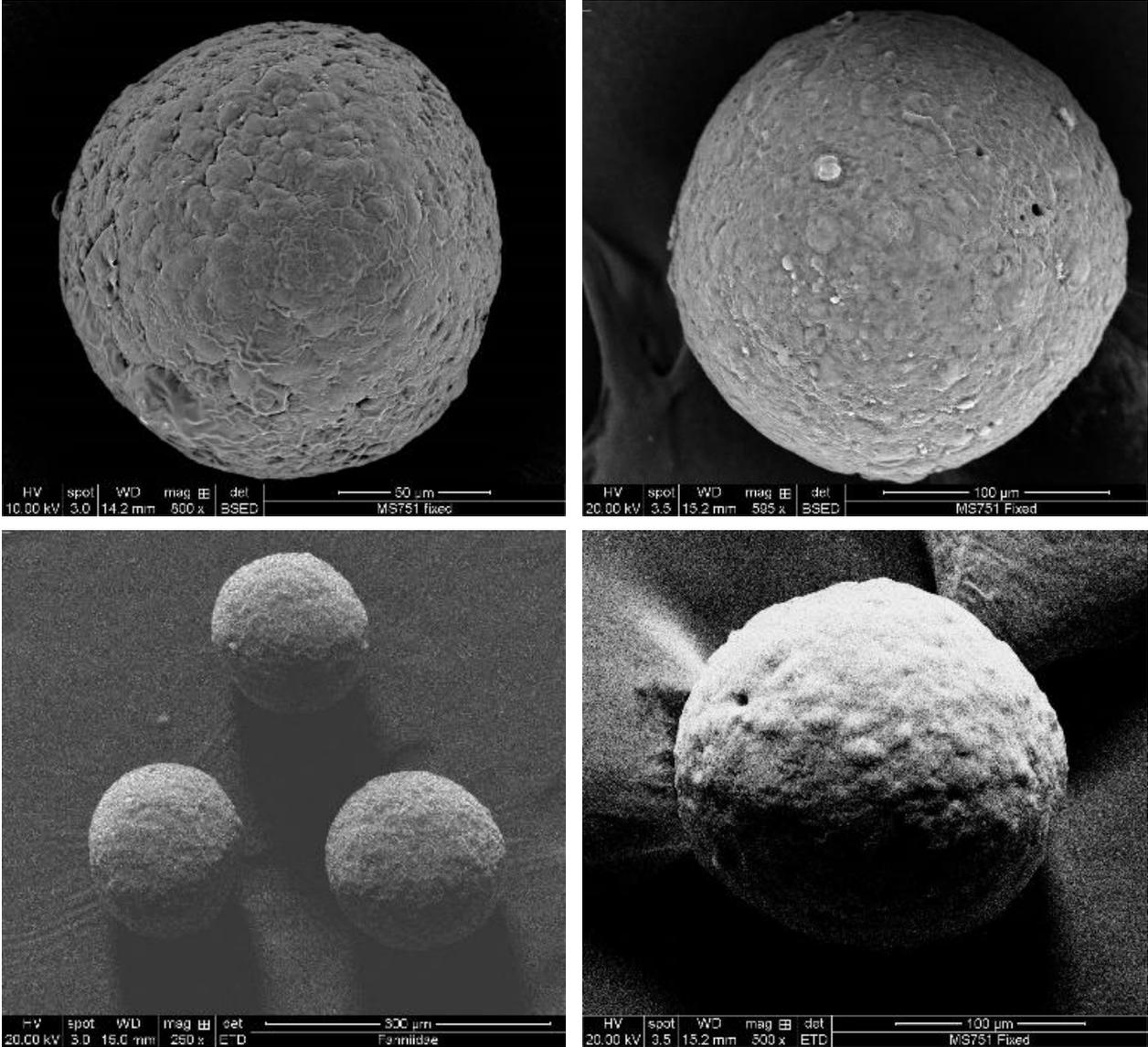
Differential sedimentation was used to separate out the spheroids into similar sizes. Spheroids were removed from the flasks and placed into a 25ml universal tube (Sigma/Fisher) and allowed to settle for 1 minute. The larger spheroids settled to the bottom of the tube and the smaller spheroids which remained in suspension were discarded. The remaining spheroids were re-suspended in 20ml of media and allowed to settle for 3 minutes. Spheroids of similar size and weight settled to the bottom of the tube whilst the smaller spheroids remained in suspension in the media. The media containing the smaller spheroids was discarded taking care not to decant the spheroids at the bottom of the tube. The spheroids which were remaining were of similar size and were re-suspended in media.

### **3.2.18 Fixation of the Spheroids**

Spheroids were collected from flasks as described in the section above. Approximately 3-5 spheroids were fixed in Karnovsky's fixative for 1hr at room temperature. (Karnovsky's fixative consists of 4% paraformaldehyde in phosphate buffered solution and 0.5% glutaraldehyde). After 1 hour the spheroids were rinsed twice with PBS and gradually dehydrated by suspension for 10 minutes at each concentration in an increasing concentration series of ethanol at 30%, 50%, 70%, 90% and then twice at 100%.

Scanning Electron Microscopy was carried out on fixed spheroid samples using a FEI Quanta FEG 250 Scanning Electron Microscope using the high vacuum mode. A sample specimen was fixed to a 50mm diameter aluminium stub plate using Agar Scientific adhesive conductive carbon sheet. The samples were coated using a Quorum Technology SC7920 Sputter Coater, coating in a thin layer of Gold/Palladium for a time period of 45 seconds, prior to examination.

The images were captured using the Back Scattered Electron detector to produce the Back Scattered Electron Image (BSEI).



**Figure 3.2.7 Scanning electron micrographs of MS751 spheroids after fixing with paraformaldehyde and glutaraldehyde.**

### **3.2.18 Response of MS751 and DoTc2 spheroids to E09.**

After resuspending similarly sized spheroids in 7ml of media, 1ml each of media and spheroids was pipetted into sterile 1.5ml Eppendorf tubes (Starlab) to make up a total of 7 microtubules. A series of drug dilutions starting from 10 $\mu$ M maximum concentration were made and added to spheroids. Media only was added to the spheroids in one of the microtubules to serve as a control. Spheroids were exposed to drug dilutions for 1 hour. After an hour, the drug/media solution was removed from all the microtubules, the spheroids were washed twice with PBS and 1ml of 10x concentrated trypsin was added to each microtube and incubated at 37°C for 10 minutes. The spheroids disaggregated to form single cell suspensions and cells were resuspended in medium following centrifugation. The cells were counted using a haemocytometer as previously described and each of the cell suspensions was diluted in media to form a working cell suspension concentration of 1 x 10<sup>4</sup> cells/ml. To a 96 well plate, 200  $\mu$ l of complete medium only was added to lane 1. To lane 2 was added 200 $\mu$ l of cell suspension from the control microtubule, giving a final concentration of 2000 cells per well. Each of the following lanes was filled with 200 $\mu$ l of cell suspension exposed to successively increasing drug concentration until a total of 8 lanes had been filled. The plates were incubated for 96 hours at 37°C in a 5% CO<sub>2</sub> enriched atmosphere and cell survival was determined using the MTT assay as described above.

### **3.2.19 Measurement of NQO1 activity in the panel of cervical cancer cell lines.**

Cell cultures at 70% confluency were trypsinized, suspended in 5ml of media and centrifuged at 1,500 rpm for 3 minutes. The supernatant was removed and cells were washed twice with PBS and re-suspended in 1ml of glucose (0.25M) solution. The cell solution was sonicated on ice using the sonicator at 75% intensity for 1 min using 10 sec bursts, with a break of 10s between cycles. The sonicated suspension of cells was then used to assay for NQO1 as described below.

NQO1 activity was determined by measuring the dicoumarol sensitive reduction of dichlorophenolindophenol (DCPIP) at 600 nm, using a Carys 60 UV-Vis Spectrophotometer (Agilent Technologies, Australia). All reagents were obtained from Sigma-Aldrich. Each reaction consisted of Tris-HCl buffer (25mM, pH 7.4) containing bovine serum albumin (0.7mg/ml), 40 $\mu$ l (200 $\mu$ M)  $\beta$ -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate ( $\beta$ -NADH) (Sigma Aldrich), DCPIP 40 $\mu$ l (80 $\mu$ M), 50 $\mu$ l of sonicated cell solution  $\pm$  dicoumarol 20 $\mu$ l (20 $\mu$ M), in a final reaction volume of 1ml. The reaction was measured over a period of 10-30s in order to establish the initial activity.

Enzyme activity was assessed in the presence and absence of dicoumarol which is a reversible inhibitor of NQO1. This was to ensure that any reduction occurring was due to NQO1 and not due to the presence of other reductases. The reactions were done in triplicate. The protein content of the samples was measured using the BCA assay as described in section 1.1.15.

The rate of DCPIP reduction was calculated using the linear part of the reaction curve at the start of the reaction. Specific activity was calculated using the Beer-Lambert Law with the following equation:

$$\text{Specific Activity} = \frac{\delta A/\text{min}}{(\Sigma) \times (P)}$$

where:

$\delta A/\text{min}$  = the rate of change of absorbance per min.

$\Sigma$  = molar extinction coefficient for DCPIP ( $21\text{mM}^{-1}\text{cm}^{-1}$ ).

P = amount of protein in the cuvette in mg.

### **3.2.20 Analysis of protein concentration using the bicinchoninic (BCA) assay.**

The amount of protein in each sample of cell lysate used was measured using the BCA assay. This assay combines the alkaline reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{+}$  (biuret reaction) with the colorimetric detection of  $\text{Cu}^{+}$  using bicinchoninic acid. The resulting purple colour reaction solution is formed by the chelation of two BCA molecules with one  $\text{Cu}^{+}$ . The colour formation results from the universal peptide backbone, the presence of cysteine/cysteine, tyrosine and tryptophan amino acid side chains and peptide bonds which are optimized at  $37^{\circ}\text{C}$ , thus making it more objective than the Bradford Assay. This complex strongly absorbs light at a wavelength of 562 nm. It is not a true end point assay as the final colour continues to develop.

The Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK) was used to quantify protein concentration in the cell lysates. A set of 8 bovine serum albumin (BSA) standards was freshly prepared using serial dilutions with distilled water, from a provided proprietary 2mg/ml concentration stock solution. Concentrations ranged from 0.2mg/ml to 2mg/ml, including a distilled water blank. BCA working reagent (WR) was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, reagent A: reagent B) forming a clear green solution. Each standard or protein sample (10µl) was pipetted into a well in a clear Cyto-One 96-well plate (Starlab, Milton Keynes, UK) in quadruplicate, and then 200µl of WR was added to each well. The plate was incubated at 37°C for 30 minutes and absorbance readings were taken at 562 nm on a Tecan plate reader. A linear standard curve was generated using the average absorbance vs BSA protein standard protein concentration. The test sample protein concentrations were determined using their absorbance values and extrapolating from the straight line generated by the standards, and the amount of protein in each cuvette was calculated. Specific activity was calculated as previously described above.

### **3.2.21 Characterisation of the NQO1 genotype in cervical cancer cell lines.**

As stated above, the gene for NQO1 is polymorphic and the presence of the C609T variant (termed NQO1\*2) is associated with loss of NQO1 activity compared to wild type NQO1 (termed NQO1\*1). Heterozygous cells (NQO1\*1/\*2) have intermediate levels of NQO1 activity. The specific genotype of the cervical cancer cell lines used in this study was determined as described below. In addition, the genotype of HT-29 (NQO1\*1) and BE (NQO1\*2) cells was also determined and as their genotype is known, they served as a positive control for the methods used.

DNA was extracted from cell lines using the ThermoScientific GeneJET Genomic DNA Purification kit (Thermo Fisher Scientific Inc., Loughborough, UK). Cultured cervical cancer cells were grown in T25 flasks, in their appropriate media supplemented with 10% foetal bovine serum, in a humidified incubator at 37°C until 70% confluency. The media was removed from the cells and the cells were rinsed twice with PBS. 1ml of trypsin (1X) was added to the cells and they were incubated at 37°C for 5 minutes. After the cells had dislodged, 2.5ml of media was added to inactivate the trypsin and the solution was centrifuged at 1,500 rpm for 5 minutes. The supernatant was discarded and the remaining cell pellet was re-suspended in 200µl of PBS.

200µl of lysis solution and 20µl of proteinase K was added to the re-suspended cells. The mixture was mixed thoroughly by vortexing and then incubated at 56°C in a water bath for 10minutes, with occasional vortexing until the cells were completely lysed. The mixture was removed from the waterbath and 20µl RNase A solution was added and mixed by vortexing. The mixture was left to stand for 10 minutes at room temperature. 400µl of 50% ethanol was added to the mixture and mixed by vortexing. The prepared lysate was then transferred to a GeneJET Genomic DNA Purification Column inserted in a collection tube. The column containing the lysate was centrifuged for 1 min at 6000 x g. The collection tube containing flow-through solution was discarded, and the purification column was placed into a new 2ml collection tube. 500µl wash buffer 1 was added to the column and it was centrifuged for 1 min at 8000 x g. The flow-through was discarded and the purification column was placed back into the collection tube. 500µl of wash buffer 2 was added to the purification column and it was centrifuged for 3 min at a maximum speed of 16000 x g. The collection tube containing flow-through solution was discarded and the purification column was transferred to a sterile, autoclaved Eppendorf tube.

200µl of elution buffer was added to the centre of the purification column membrane to elute the genomic DNA. This was left at room temperature for 2 minutes and then the column centrifuged for 1 min at 8000 x g. The purification column was discarded and DNA collected in the Eppendorf tube was aliquoted and kept for immediate use at 4°C and the remainder stored long-term at -20°C. DNA yield was measured using a Nanodrop 2000. DNA quality was assessed using gel electrophoresis as follows.

All reagents were purchased from Geneflow (Lichfield, UK). A 1.5% agarose gel with Midori Green dye was set up in a 12 well multiSUB mini gel tank and was loaded with a marker 10kbp DNA ladder and 9 samples of DNA each 100ng in weight. The gel was run at 100 volts of power for 35 minutes, and then read under ultraviolet light.

### **3.2.22 Genotyping of NQO1.**

Real time PCR Taqman assay was used to determine NQO1 genotype of the cell lines. The assay consists of two DNA sequence specific oligonucleotide Taqman probes and two primers – a forward and reverse primer which produce amplicons that span the target DNA using *Taq polymerase*. Differently labelled probes with fluorescent reporter dyes specific for the wild-type and the mutant allele are used, which are designed to bind to adjacent sequences on the target DNA. When intact, the probe does not fluoresce because of its close proximity to the quencher. Upon preferential hybridization of the probe of a specific allele to the target DNA sequence, the 5'- 3' exonuclease activity of *Taq polymerase* cleaves off the reporter dye and in turn allows it to fluoresce. The resulting fluorescence signal is proportional to the amount of amplified product in the sample.

All reagents were purchased from LifeTechnologies (Fisher Scientific, UK) unless otherwise stated. A pre-formulated PCR master-mix containing buffer, DNA polymerase and dNTPs, which contains 2 primer sets and 2 pairs of dye-labelled allele specific DNA probes to detect either NQO1\*1 or NQO1\*2 was used. The PCR reaction was set up in 8-strip PCR wells (with a total of 10µl in each well and a final concentration of 20ng genomic DNA, 4.5µl 2x Taqman® Master Mix, 0.5µl of 20x Pre-designed NQO1 (rs 1800566) Assay Working Stock and 2µl nuclease free water. In addition to the test DNA samples, there also included a no template control as a blank with no DNA and known homozygous wild-type sample HT-29 and known homozygous mutant sample BE as positive controls. The thermal cycling conditions were: an initial 95°C for 10 minutes for AmpliTaq Gold® enzyme activation, then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. After PCR the plate was read by the Biorad CFX96 thermocycler, and the results were analyzed using the Biorad CFX Manager 3.1 software (California, USA). Genotype is assigned by the multiplex four colour assay using an algorithm that analyses the final relative fluorescent units (RFU) and the data is presented as a scatter plot. DNA that is homozygous wild-type NQO1\*1 shows a different fluorescence signal to that which is homozygous mutant NQO1\*2. Heterozygous NQO1\*1\*2 generates a fluorescent signal with both probes for the target.

### **3.2.23 Substrate specificity studies using novel quinone based compounds.**

All chemical reagents were obtained from Sigma-Aldrich (Dorset, UK). The NQO1 substrate specificity of 12 test compounds including EO9 compound was measured using a Carey's 60 UV-Vis spectrophotometer (Agilent Technologies, Australia) by measuring the NADH dependent reduction of equine heart cytochrome c at 550nm. To make 100mM stock solutions the drugs which were obtained in solid form were weighed and measured (weights shown)

and dissolved in DMSO. The drugs which were in liquid form were diluted down in DMSO to 100mM stock solutions. Each reaction consisted of Tris-HCl buffer (50mM, pH 7.4) containing bovine serum albumin (0.7mg/ml), 20 $\mu$ l (2mM)  $\beta$ -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate ( $\beta$ -NADH), 20 $\mu$ l (70 $\mu$ M) cytochrome c (20 $\mu$ l), 10 $\mu$ l (10 $\mu$ M) purified human NQO1, 10 $\mu$ l (10mM) of the test substrate (drug), in a final reaction volume of 1ml. The reaction was measured over a period of 30s in order to establish the initial activity, and all reactions were done in triplicate. The rate of cytochrome c reduction was calculated using the linear part of the reaction curve at the start of the reaction. Specific activity (substrate specificity) was calculated using the Beer-Lambert Law as described above and the molar extinction co-efficient of cytochrome c is 21.1mM<sup>-1</sup> cm<sup>-1</sup>.

### **3.2.24 Enzyme Kinetics Studies**

Using the results obtained from the experiment described above, the test compounds which were substrates for NQO1 were selected for enzyme kinetic studies. Each test compound was serially diluted from a maximum concentration of 100mM to make a total of 9 concentrations. Each reaction was set up in cuvette, and consisted of Tris-HCl buffer (50mM, pH 7.4) containing bovine serum albumin (0.7mg/ml), 20 $\mu$ l (2mM)  $\beta$ -Nicotinamide adenine dinucleotide, reduced disodium salt hydrate ( $\beta$ -NADH), 20 $\mu$ l (70 $\mu$ M) cytochrome c and 10 $\mu$ l of each of the test compound to give a maximum initial concentration in the cuvette of 1000 $\mu$ M, in a final reaction volume of 1ml – Concentrations of reagents are needed here. This was repeated for each of the 9 decreasing drug concentrations. An initial blank of 10 $\mu$ l DMSO alone in place of the drug was used to blank the spectrophotometer and as the background absorbance in evaluating true absorbance. The reaction was measured over a period of 30s in order to establish the initial activity, and all reactions were done in triplicate.

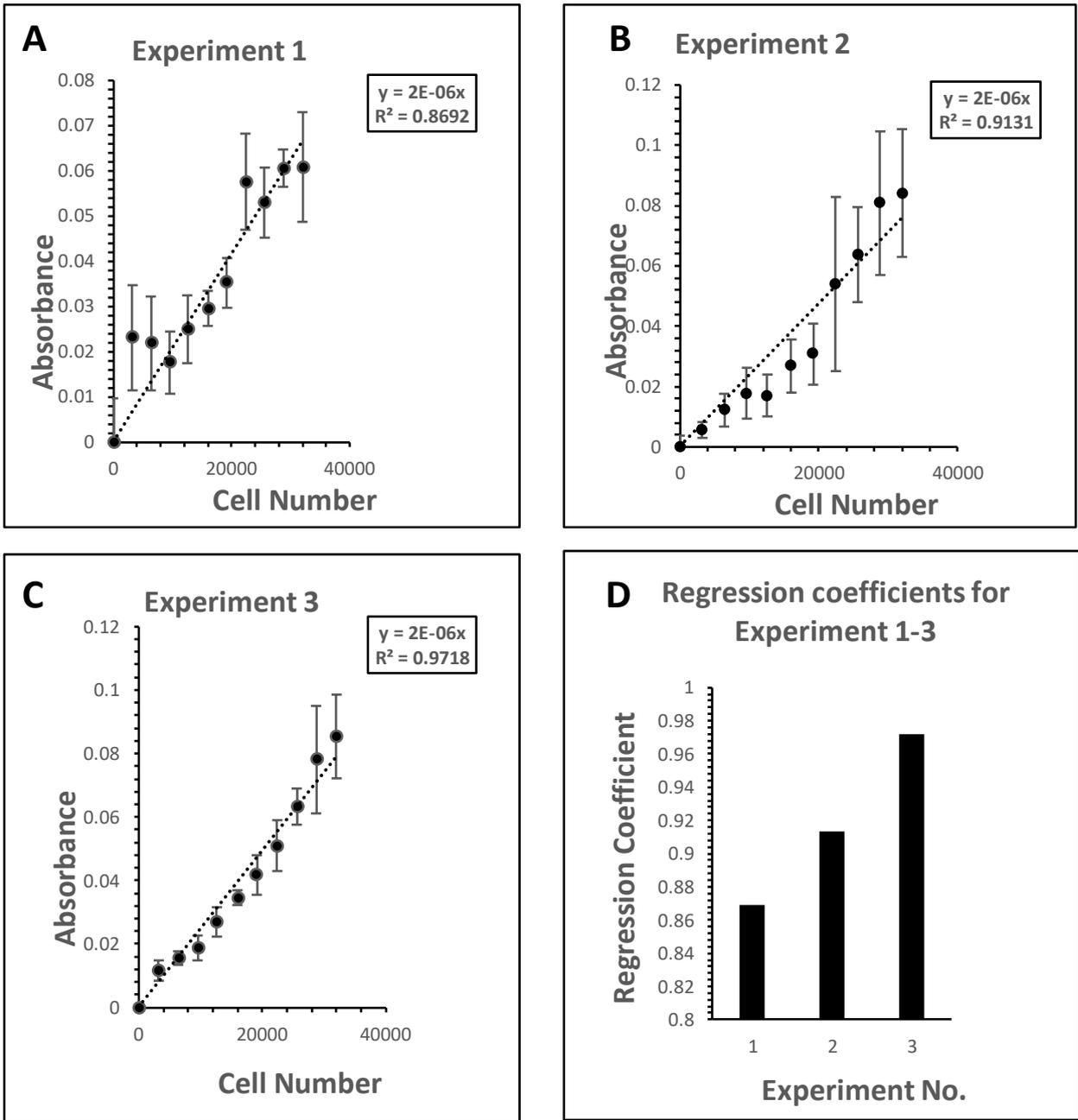
## **3.3 RESULTS**

### **3.3.1 Validation of the MTT assay**

The MTT assay was validated as a method of assessment of the cytotoxicity of a compound using SiHa cells initially. The robustness of the method is dependent on a linear relationship between the number of viable cells present and the amount of formazan produced. The absorbance of the solution of dissolved formazan crystals at 540nm provides a measurement of how much formazan is present. A known number of viable SiHa cells was added to each lane, and graphs of cell number vs absorbance were plotted. This experiment was carried out three times, and each time the relationship became more linear with the regression coefficients progressively increasing towards 1 (figure 3.3.1). This experiment demonstrates that the MTT assay is a valid assay for assessing chemosensitivity and it also serves as a training 'tool' for new workers to develop their cell culture and pipetting skills.

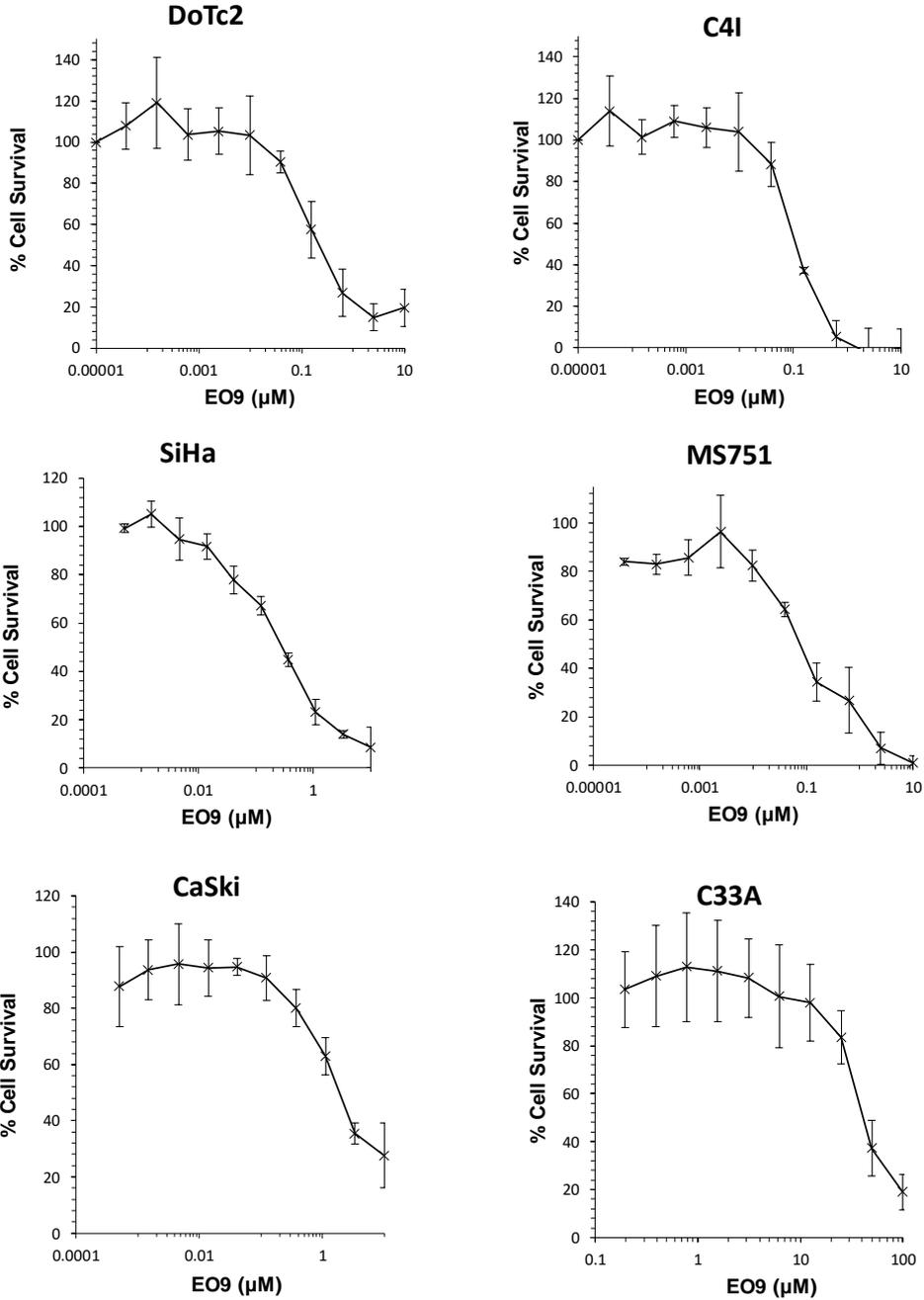
### **3.3.2 Response of a panel of human cervical cancer cell lines to EO9**

A panel of 6 cervical cancer cell lines were treated with EO9 for 1 hour as described above. The resulting graphs of percentage cell survival vs EO9 concentration were plotted as shown in 3.3.2. Each data point is represented by the mean and standard deviation of 3 independent experiments, for each cell line. The resulting IC<sub>50</sub> values were calculated and are presented in figure 3.3.3. DoTc2 was the most sensitive to EO9, with an IC<sub>50</sub> of 0.06 $\mu$ M ( $\pm$ 0.0048) closely followed by C4I with IC<sub>50</sub> of 0.07 $\mu$ M ( $\pm$ 0.0029). SiHa and MS751 had similar IC<sub>50</sub> at 0.22 $\mu$ M ( $\pm$ 0.07 and  $\pm$ 0.027 respectively), they were followed by CaSki with IC<sub>50</sub> of 1.6 $\mu$ M ( $\pm$ 0.22) and the least sensitive cell line to EO9 was C33A with IC<sub>50</sub> of 45.71 $\mu$ M ( $\pm$ 2.2).

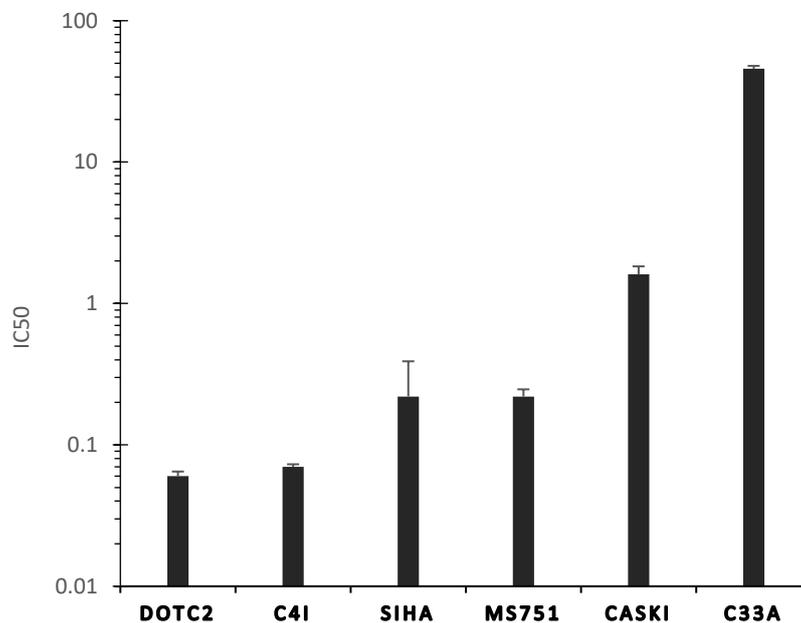


**Figure 3.3.1 MTT validation assay for human cervical cancer cell line SiHa. 3 experiments were carried out represented by the 3 graphs of true absorbance against cell number A-C. D shows a barchart of the regression coefficients of the graphs A-C, which tend more towards 1 as the experiment number progresses.**

In summary, the response of cell lines following 1hr exposure to EO9 was highly variable with IC50 values ranging from  $0.06 \pm 0.0048 \mu\text{M}$  to  $45.7 \pm 2.2 \mu\text{M}$  (a 761-fold range in sensitivity).



**Figure 3.3.2 The response of a panel of cervical cancer cell lines following one-hour exposure to E09. The results presented represent the mean  $\pm$  standard deviation for three independent experiments.**

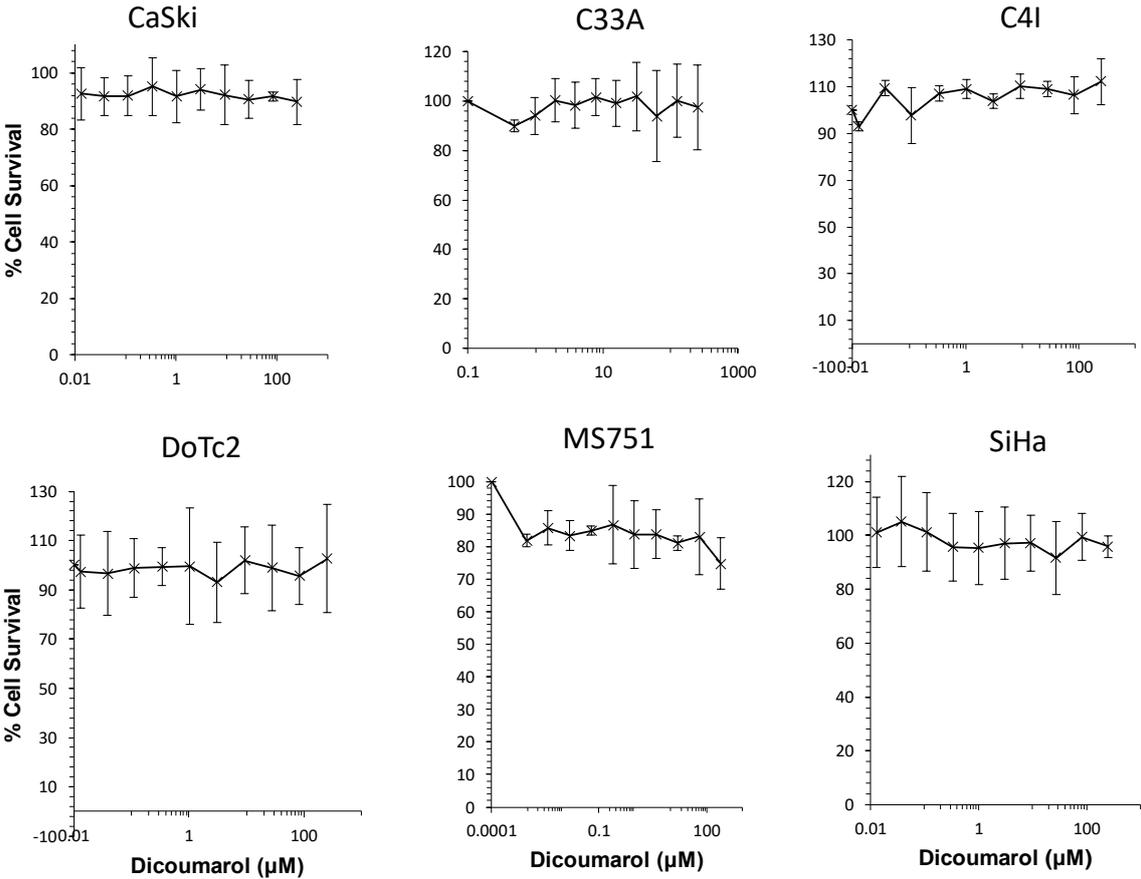


**Figure 3.3.3 Summary of IC<sub>50</sub> values (in  $\mu\text{M}$ ) following a one hour exposure to E09. Each value represents the mean  $\pm$  standard deviation for three independent experiments.**

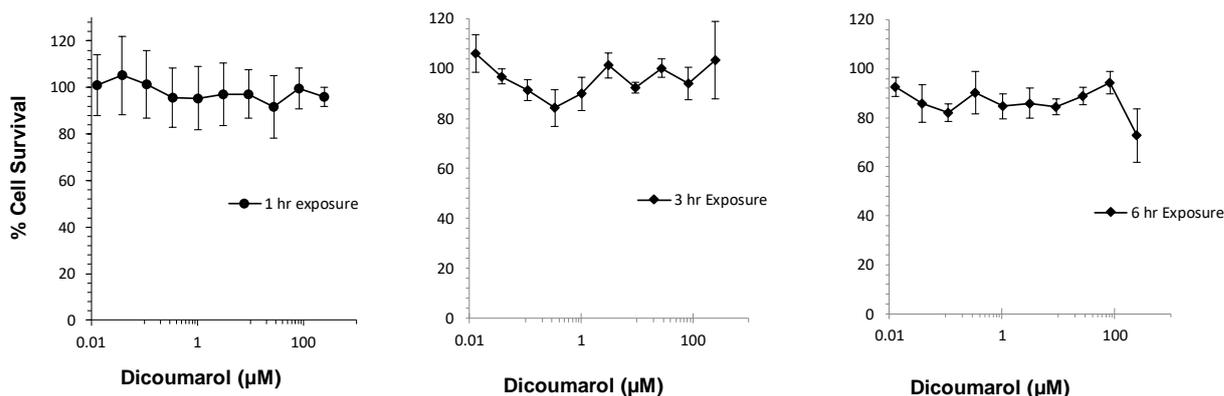
### **3.3.3 The effect of dicoumarol on the sensitivity of cell lines to E09.**

Dicoumarol is a known inhibitor of NQO1. In order to ascertain if indeed the cell death in our chemosensitivity assays could be attributed to the action of NQO1, dicoumarol was added to E09 and tested on a panel of cervical cancer cell lines as described above.

Dose response curves for the effect of 1 hr and 3 hr exposure of dicoumarol to a panel of 6 cervical cancer cell lines showed that the maximum dose of dicoumarol that was not toxic to cells was 100 $\mu$ M concentration. For 6hr drug exposure, 50 $\mu$ M concentration of dicoumarol was the maximum dose which was not toxic to the cells. So dicoumarol at a concentration of 100 $\mu$ M was used for 1hr and 3 hr drug exposures, and 50 $\mu$ M for 6 hr exposure.

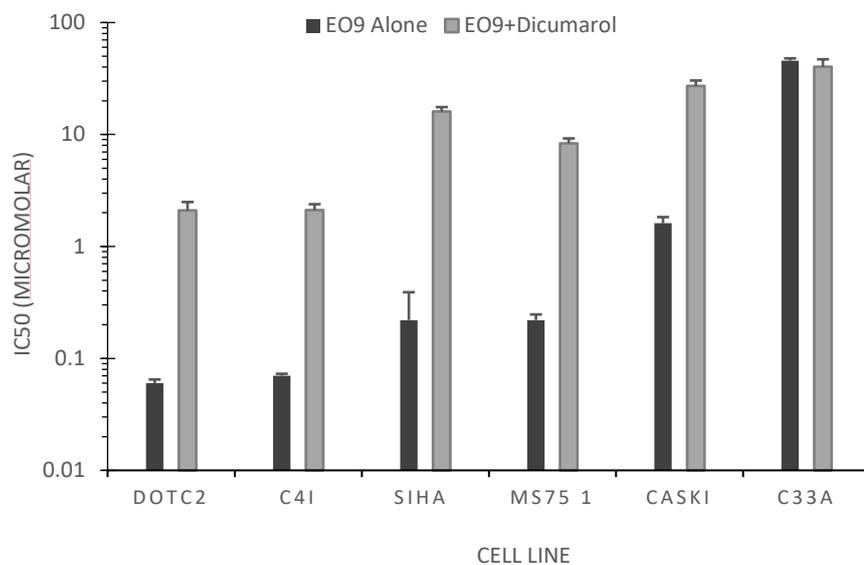


**Figure 3.3.4 Dicoumarol dose response curve for a panel of cervical cancer cell lines after 1 hr exposure. The results presented represent the mean  $\pm$  standard deviation for three independent experiments.**



**Figure 3.3.5 Dicoumarol dose response curves for SiHa at 1,3 and 6hr drug exposures. The results presented represent the mean  $\pm$  standard deviation for three independent experiments.**

The results are shown on the bar chart in figure 3.3.6. The  $IC_{50}$  of EO9 was increased in the presence of dicoumarol in all the cell lines except C33A. A calculation of the ratio of  $IC_{50}$  for EO9+Dicoumarol/EO9 alone provides a measure of the potency of the inhibitory effect of dicoumarol on EO9 for that particular cell line. The results showed that ratio was highest for SiHa at 73 (see table 3.3.1) and this decreased to 0.88 in the case of C33A cells. With the exception of C33A, the potency of EO9 decreased significantly when co-incubated with dicoumarol. This suggests that NQO1 plays a key role in the mechanism of action of EO9 in the majority of cell lines tested.



**Figure 3.3.6 Response of cells to EO9 in the presence and absence of dicoumarol. Each value represents the mean IC<sub>50</sub> value (μM) ± standard deviation for three independent experiments.**

Cell Line	IC <sub>50</sub> (μM)		Ratio (EO9+Dicumarol)/EO9
	EO9 Alone	EO9+Dicumarol	
SiHa	0.22 (±0.07)	16.04 (±1.54)	73
MS751	0.22 (±0.027)	8.36 (±0.87)	38
DoTc2	0.06 (±0.0048)	2.1 (±0.4)	35
C4I	0.07 (±0.0029)	2.11 (±0.27)	30
CaSki	1.61 (±0.22)	27.18 (±3.2)	17
C33A	45.71 (±2.2)	40.2 (±6.8)	0.88

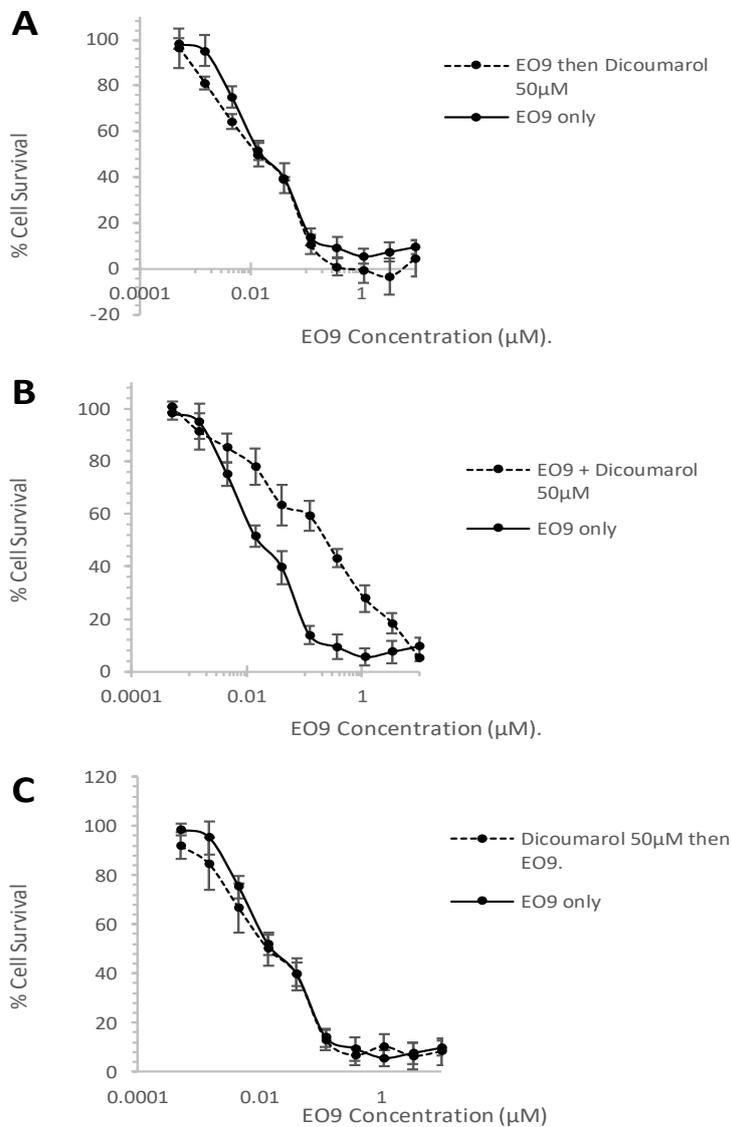
**Table 3.3.1: IC<sub>50</sub> values for a panel of cervical cancer cell lines exposed to EO9 alone or EO9 plus dicoumarol.** The ratio of IC<sub>50</sub> (EO9+Dicumarol/EO9 alone) is also shown.

### 3.3.4 The effect of scheduling of dicoumarol and EO9 treatment on chemosensitivity.

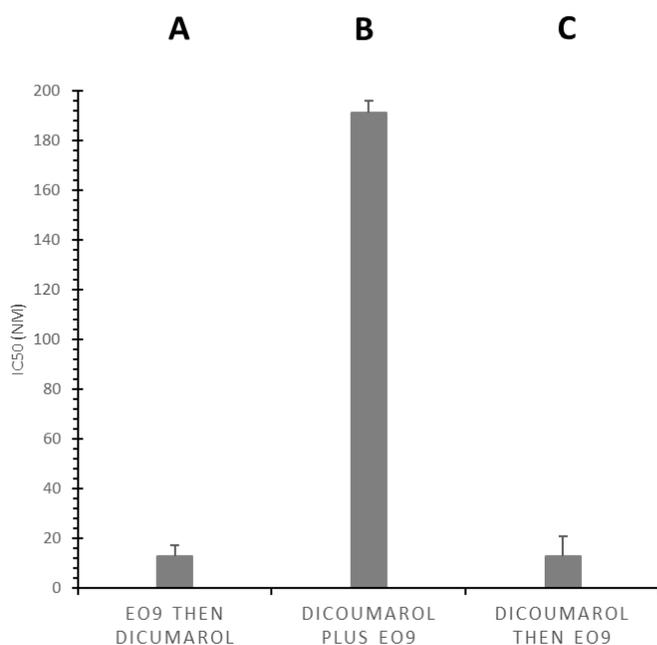
The effect of different scheduling of EO9 and dicoumarol on the response of SiHa cells was investigated in a series of experiments which looked at the effect of adding dicoumarol before or after EO9 on cell survival. In the first experiment (A) the cells were exposed to EO9 for 6 hours first, washed and then exposed to dicoumarol for 6 hours second. The result dose response curves in figure 3.3.7A shows that there is no difference in the dose response curves and IC<sub>50</sub> values. Administration of dicoumarol before EO9 therefore has no effect on cellular response to EO9. In the second experiment (B), the cells were exposed to EO9 and dicoumarol simultaneously for 6 hours. The resulting dose response curves in figure 3.3.7B show that dicoumarol has an inhibitory effect on EO9. These are effectively the same results as described above except the duration of drug exposure has changed. In the presence of dicoumarol, the dose response curve has shifted to the right and the IC<sub>50</sub> has increased 12-fold, from 0.015 ± 0.004 μM to 0.184 ± 0.005 μM). In the third experiment (C), the cells were first exposed to dicoumarol for 6 hours, washed and then exposed to EO9 for 6 hours secondly. Again there is no difference in the dose response curves for EO9 alone and for dicoumarol followed by EO9 in figure 3.3.7C. The IC<sub>50</sub> values are shown in table 3.3.2 and these results are presented graphically in figure 3.3.8. These results clearly demonstrate that inhibitory effects are seen only when EO9 and dicoumarol are administered simultaneously.

Time (hrs)	IC <sub>50</sub> (μM)		
	EO9 then Dicoumarol	Dicoumarol +EO9	Dicoumarol then EO9
1	0.115 (±0.046)	Not reached	0.164 (±0.065)
3	0.037 (±0.006)	Not reached	0.059 (±0.008)
6	0.015 (±0.004)	0.184 (±0.005)	0.013 (±0.008)

**Table 3.3.2 Response of SiHa cells following exposure to EO9 before, during or after dicoumarol. Each value represents the mean  $\pm$  s.d for N=3.**



**Figure 3.3.7** The dose response curve of the effect of dicoumarol on the IC50 value of EO9 compared with the effect of a 6-hour exposure of EO9 alone on SiHa cells. A) cells were exposed to EO9 for 6 hours, washed and immediately exposed to 50 $\mu$ M dicoumarol for a further 6 hours. B) cells were exposed to a mixture of EO9 and 50 $\mu$ M dicoumarol for 6 hours. C) cells were exposed to 50 $\mu$ M dicoumarol, washed and immediately exposed to EO9 for 6 hours.



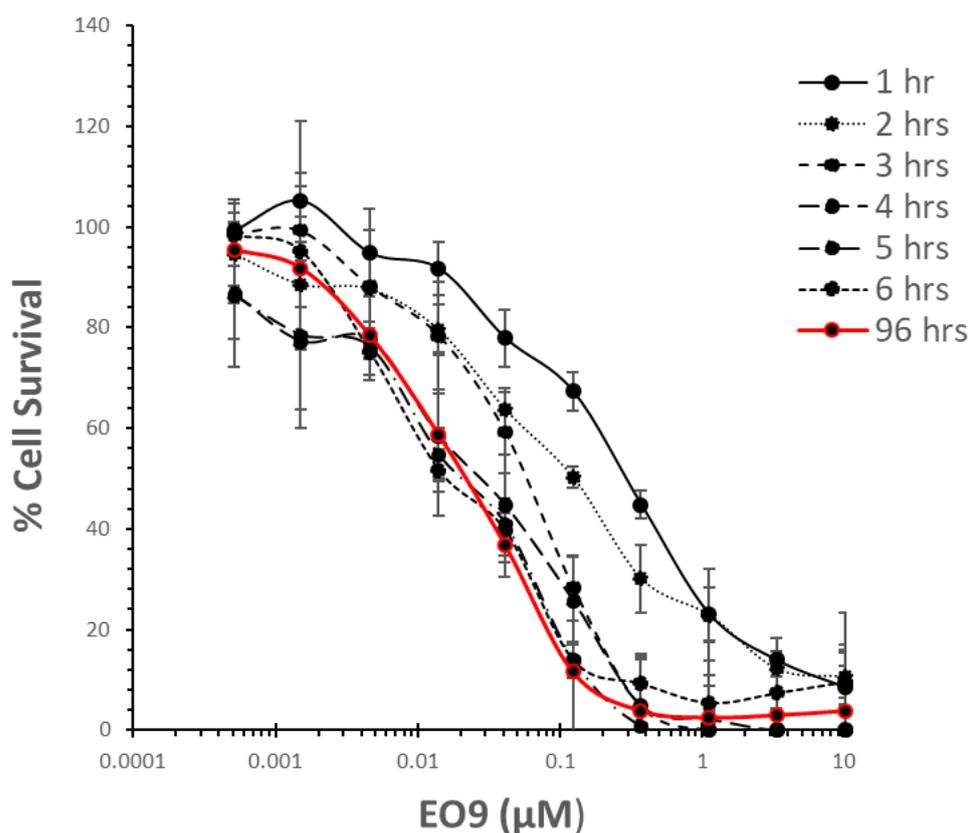
**Figure 3.3.8 The effect of dicoumarol on the potency of E09. The results presented represent IC50 values (nM) for different experimental procedures as indicated on the X axis. The duration of drug exposure was 6 hours and each value represents the mean  $\pm$  standard deviation for three independent experiments.**

### **3.3.5 Influence of duration of drug exposure on the response of SiHa and CaSKi cells to E09.**

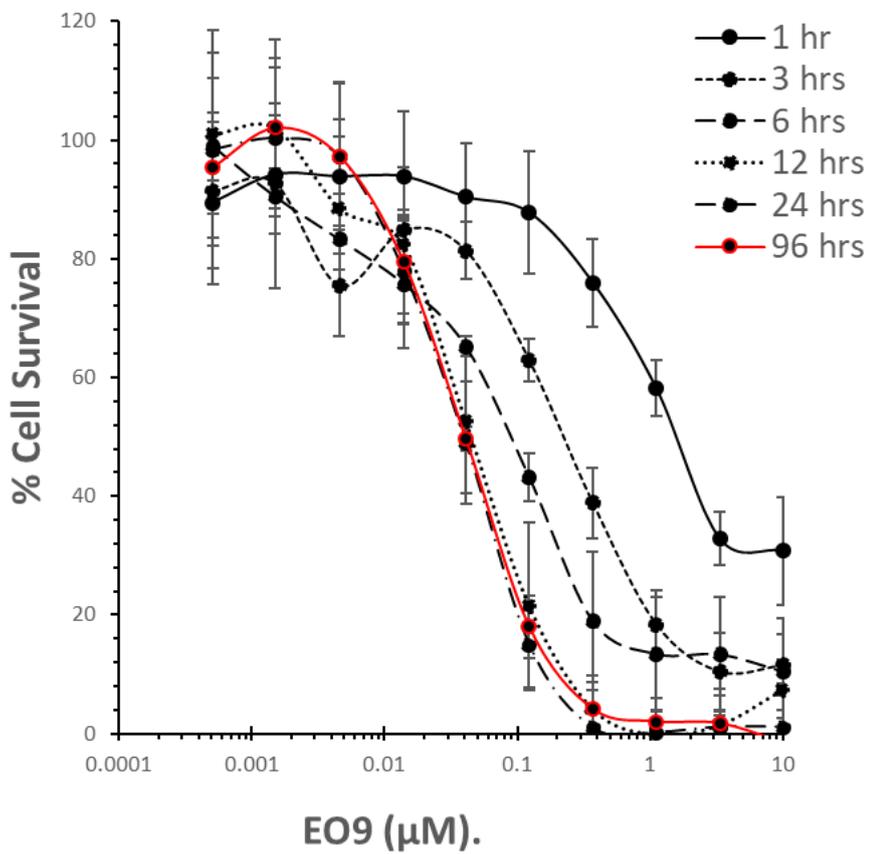
SiHa and CaSKi cells were exposed to E09 for varying lengths of time and timed dose response curves for each cell line were constructed as shown in figures 3.3.9 and 3.3.10. The results demonstrate that cell kill increases as the duration of drug exposure increases up to 4 hours in the case of SiHa cells and 6 hours in the case of CaSKi cells. Increasing the duration of drug exposure beyond these times does not significantly increase cell kill. This effect is seen

visually in figure 3.3.11 where the IC<sub>50</sub> values for SiHa cells exposed to EO9 are plotted against duration of drug exposure. IC<sub>50</sub> values decrease up to 4 to 5 hours of drug exposure but they then remain constant up to 96 hours. Similar results were obtained for CaSKi cells (figure 3.3.12).

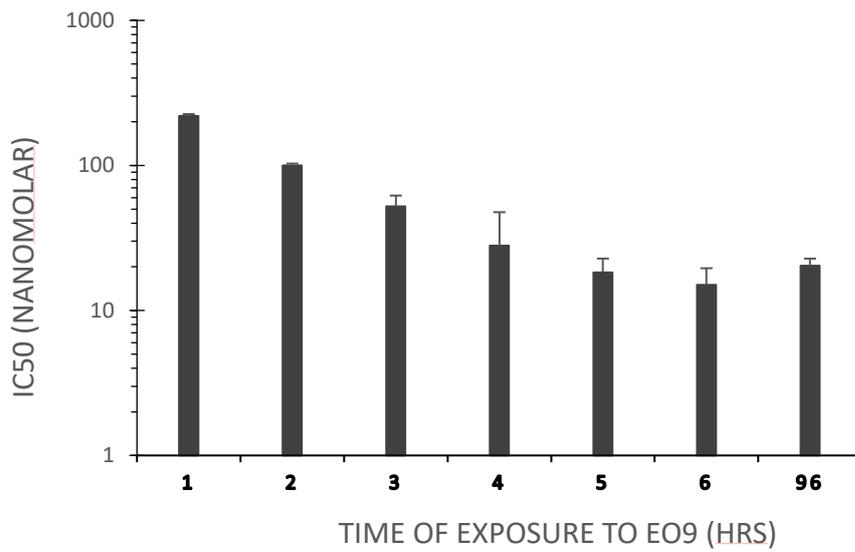
In summary, the results of this study demonstrate that the activity of EO9 decreases as the duration of drug exposure increases. Increasing the exposure time beyond 5 to 6 hours does not result in enhanced cytotoxic activity.



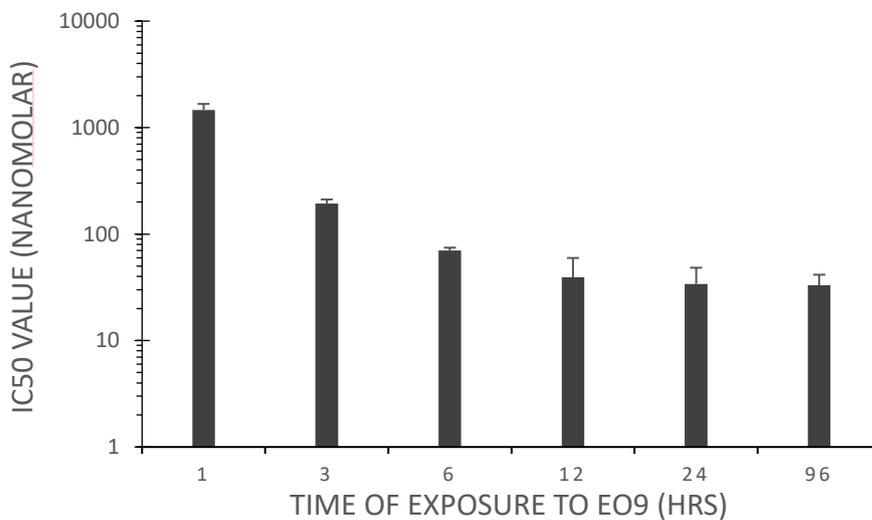
**Figure 3.3.9 Response of SiHa cells following exposure to EO9 for different durations. Each value represents the mean  $\pm$  standard deviation for three independent experiments.**



**Figure 3.3.10 Response of CaSki cells following exposure to EO9 for different durations. Each value represents the mean  $\pm$  standard deviation for three independent experiments.**



**Figure 3.3.11** The influence of duration of drug exposure on the IC<sub>50</sub> values obtained for SiHa cells. The results represent the mean IC<sub>50</sub> values ± standard deviation for three independent experiments.



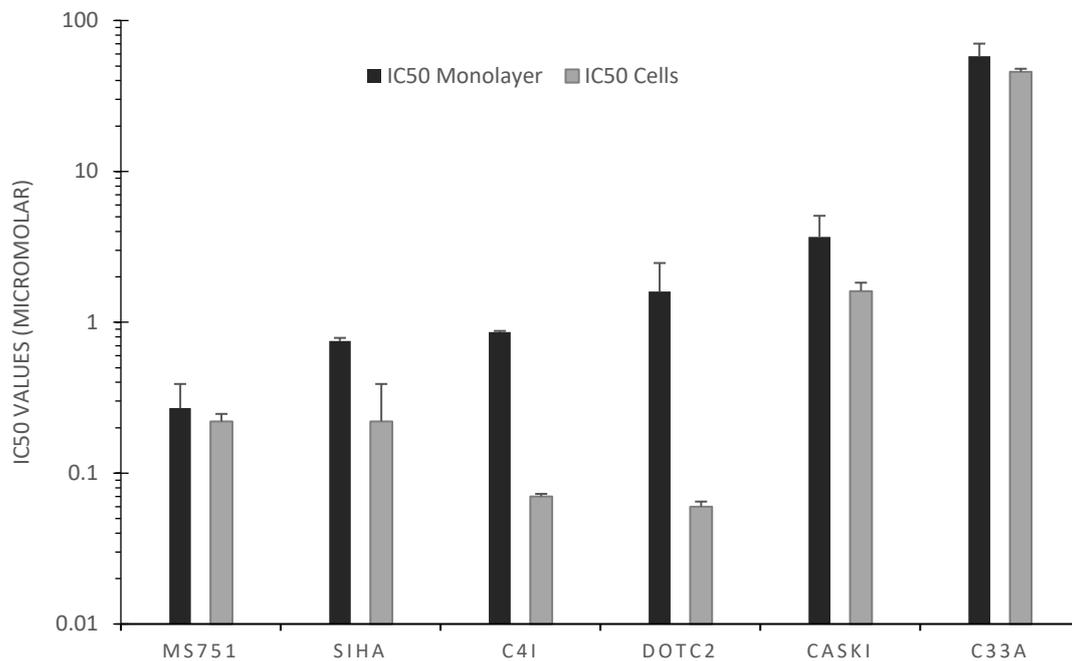
**Figure 3.3.12.** The influence of duration of drug exposure on the IC<sub>50</sub> values obtained for CaSki cells. The results represent the mean IC<sub>50</sub> values ± standard deviation for three independent experiments.

### 3.3.6 Response of monolayer cultures to EO9.

The activity of EO9 was tested on monolayer cultures as opposed to single cells to more closely mimic cells growing in the cervix. The results are shown in figures 3.3.13 and summarised in table 3.3.3. EO9 activity was greatest against the continuous monolayer of MS751 with IC<sub>50</sub> of 0.27µM this was similar to its activity on the single cells with an IC<sub>50</sub> of 0.22µM shown in table 3.3.3. The activity of EO9 decreases and IC<sub>50</sub> conversely increases in continuous monolayers in the order SiHa 0.75µM (±0.038), C4I 0.86µM (±0.016), DoTc2 1.6µM (±0.87), CaSki 1.87µM (±0.14) and C33A 57.9µM (±12.4) as shown in figure 3.3.13 and table 3.3.3. The IC<sub>50</sub> of continuous monolayers was most increased in comparison to the single cells in DoTc2 and C4I and these cell lines consequently had the highest IC<sub>50</sub> monolayer/IC<sub>50</sub> cells ratios of 26.7 and 12.3 respectively (table 3.3.3). The IC<sub>50</sub> values for CaSki, MS751 and C33A continuous monolayers was similar to that of the single cells and their subsequent ratios were lowest at 1.16, 1.23 and 1.27 respectively (table 3.3.3).

Cell Line	IC <sub>50</sub> (µM)		Ratio (IC <sub>50</sub> Monolayer/IC <sub>50</sub> Cells)
	Continuous monolayer	Cells	
MS751	0.27 (±0.12)	0.22 (±0.027)	1.23
SiHa	0.75 (±0.038)	0.22 (±0.07)	3.40
C4I	0.86 (±0.016)	0.07 (±0.016)	12.3
DoTc2	1.6 (±0.87)	0.06 (±0.87)	26.7
CaSki	1.87 (±0.14)	1.61 (±0.22)	1.16
C33A	57.9 (±12.4)	45.7 (±2.2)	1.27

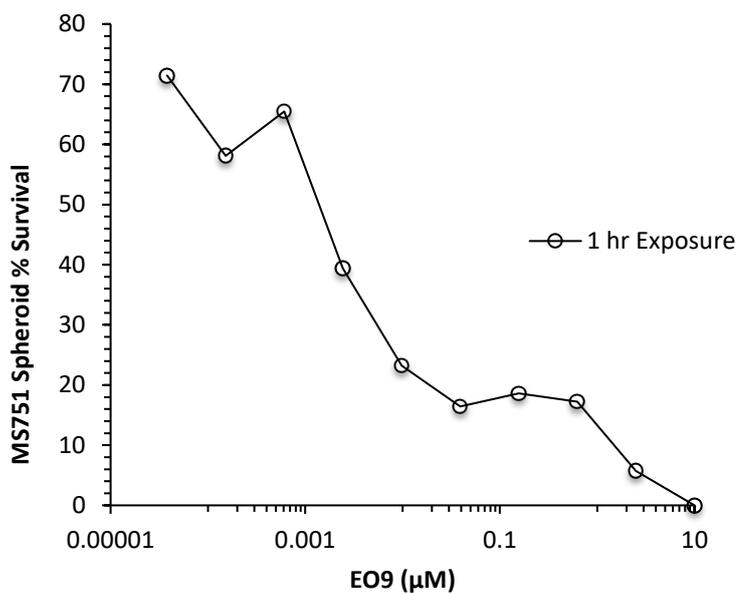
**Table 3.3.3 Comparison of IC<sub>50</sub> values obtained when single cells or monolayer cultures were exposed to EO9.**



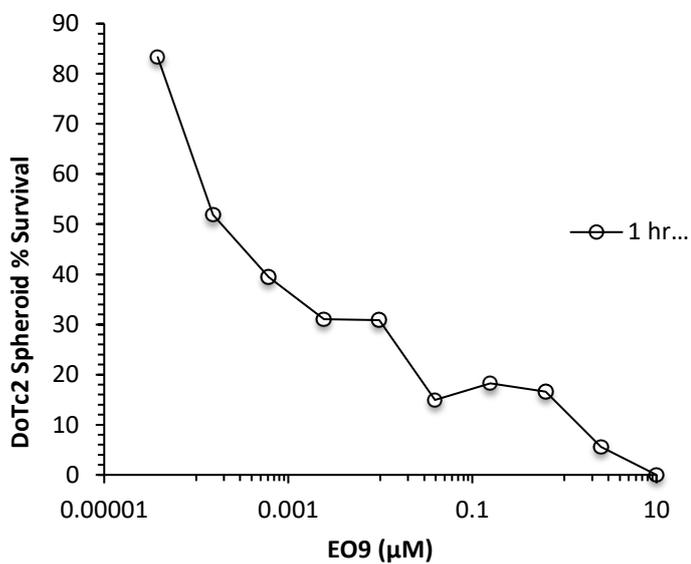
**Figure 3.3.13 Response of single cells or monolayer cultures to EO9. A barchart of IC50 values for monolayer cultures and single cell cultures.**

### **3.3.7 Response of Muticell Spheroids to EO9.**

The activity of EO9 was tested on MS751 and DoTc2 multicell spheroids. Cervical carcinoma cells were able to form 3-D spheroids which were more complex compared to the cell monolayers. The spheroids showed increased sensitivity to EO9 compared to single cell or monolayer cell cultures. These are preliminary results as we ran out of time and were only able to do one set of experiment for each cell line.



**Figure 3.3.14 Response of MS751 spheroids to EO9. (Each datapoint is represented by N=1).**



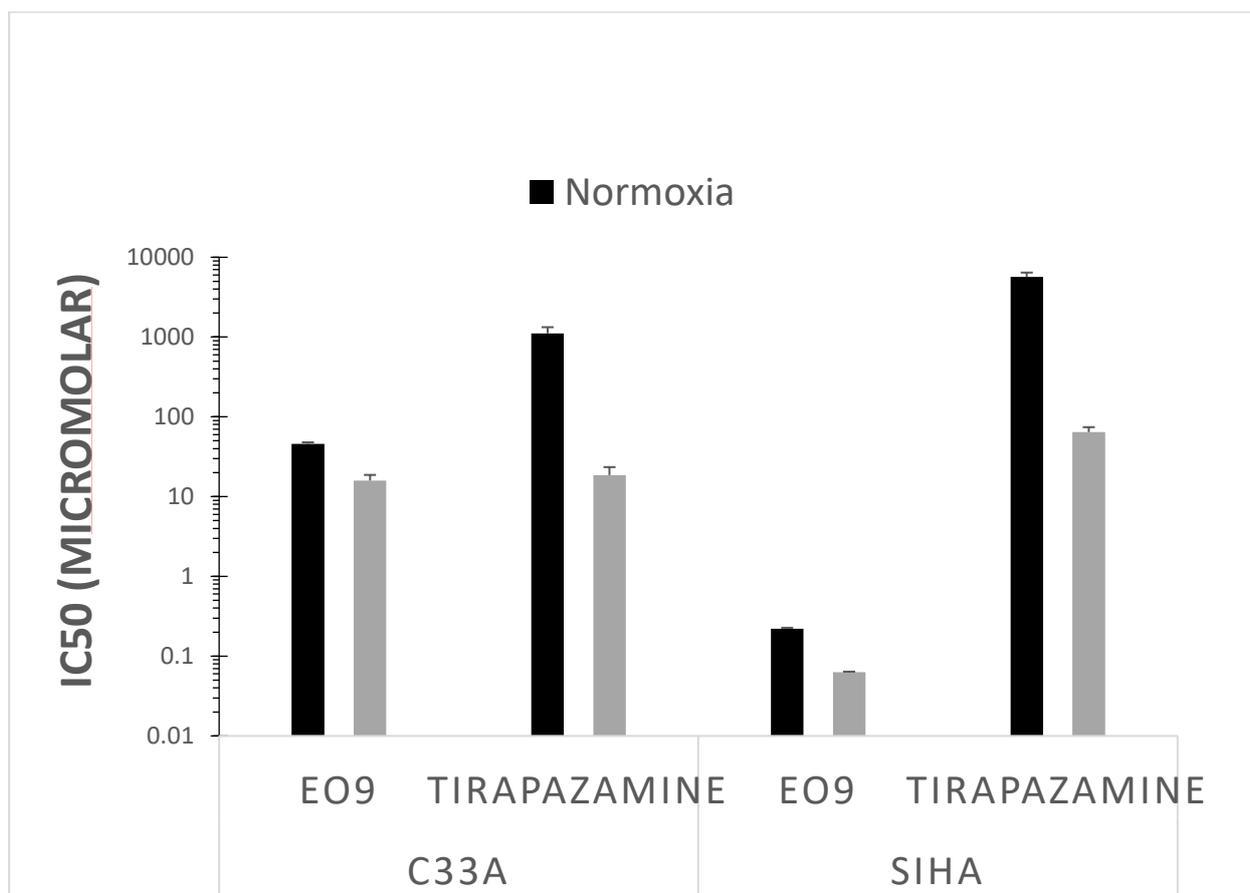
**Figure 3.3.15 Response of DoTc2 spheroids to EO9. (Each datapoint represents N=1).**

### 3.3.8 Influence of hypoxia on the activity of EO9.

The influence of hypoxia on EO9 was tested on SiHa and C33A cells was performed as described in the methods section. As a positive control, Tirapazamine was used as it is a known hypoxia-activated prodrug (Ahmadi et al., 2014). The IC<sub>50</sub> results were compared with those obtained in normoxic conditions and the results are presented in table 3.3.4 and figure 3.3.16. In both cell lines, hypoxia potentiates the activity of EO9 and Tirapazamine although the effect is greater for Tirapazamine than for EO9.

Cell Line	Drug	IC50 (μM)		Hypoxia Cytotoxicity ratio (HCR)
		Normoxia	Hypoxia	
C33A	EO9	45.7 (±2.2)	15.9 (±2.8)	2.87
	Tirapazamine	1112.3 (±217)	18.6 (±4.8)	59.8
SiHa	EO9	0.22 (±0.007)	0.063 (±0.00093)	3.49
	Tirapazamine	5700.3 (±731)	64.4 (±9.83)	88.5

**Table 3.3.4. The influence of hypoxia on the activity of EO9 and Tirapazamine. Each value represents the mean ± standard deviation for three independent experiments.**

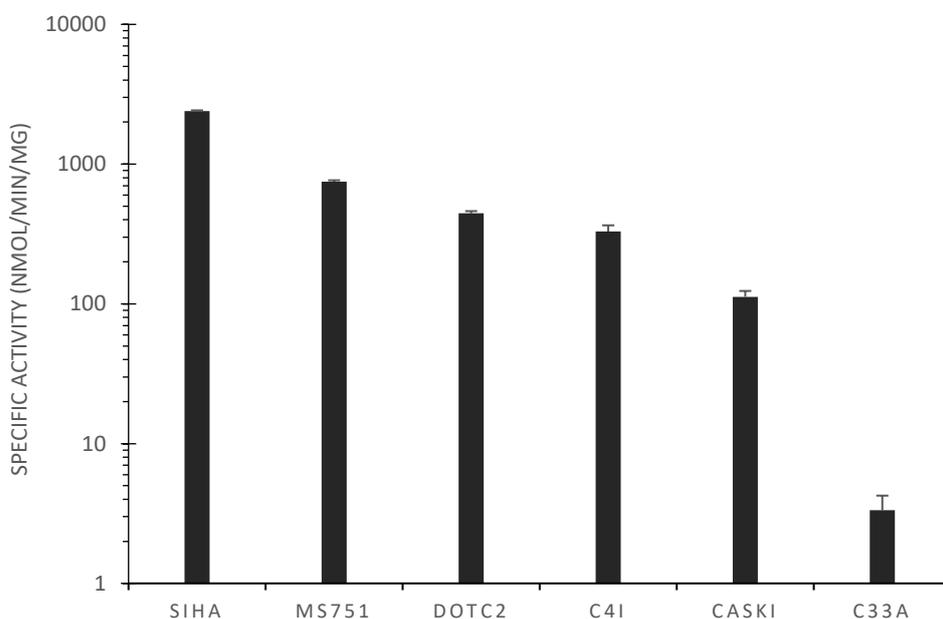


**Figure 3.3.16 Response of C33A and SiHa cells to Tirapazamine and EO9 under normoxic and hypoxic (0.1% oxygen). Results presented and the mean IC<sub>50</sub> values ± standard deviation for three independent experiments.**

The hypoxia cytotoxicity ratio (HCR) was calculated for each drug and cell line tested. This is a measure of the potentiation of a drug working under hypoxic conditions as compared with the same drug working under normoxic conditions. It is calculated by dividing the IC<sub>50</sub> of the drug in normoxia by the IC<sub>50</sub> of the drug in hypoxia. The higher the HCR of a drug, the more potent it becomes when it is tested under hypoxic conditions. The HCR of EO9 in C33A is 2.87 compared to 3.49 in SiHa, and for Tirapazamine it is 59.8 in C33A compared to 88.5 in SiHa. These results are shown in table 3.3.4. In both cases there was a greater potentiation of drug action in SiHa compared to C33A cells.

### 3.3.9 NQO1 activity in a panel of cervical cancer cell lines.

The level of NQO1 activity in a panel of cervical cancer cells was measured as described in the methodology section and the results are presented in figure 3.3.17 and summarised in table 3.3.5. A large range of NQO1 activity was observed in the cell panel tested. NQO1 activity was highest in SiHa cells, with an activity of  $2396 \pm 29$  nmol/min/mg, followed by MS751 with NQO1 activity of  $748 \pm 18$  nmol/min/mg. DoTc2 is next with activity of  $444 \pm 17$  nmol/min/mg followed by C4I with activity  $329 \pm 36$  nmol/min/mg. CaSki has the second lowest activity at  $113 \pm 11$  nmol/min/mg and finally C33A has the least NQO1 activity of  $3.34 \pm 0.9$  nmol/min/mg. It should be noted that the activity of NQO1 in C33A cells is close to the limit of detection of the enzyme assay.

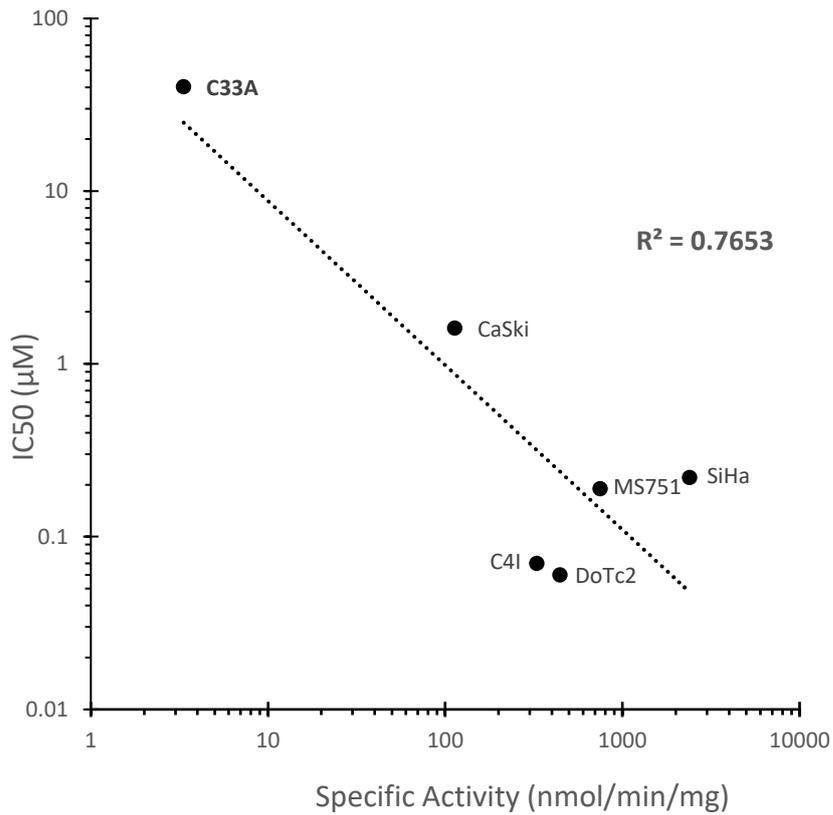


**Figure 3.3.17 Activity of NQO1 in a panel of cervical cancer cell lines. Each value represents the mean  $\pm$  standard deviation for three independent experiments.**

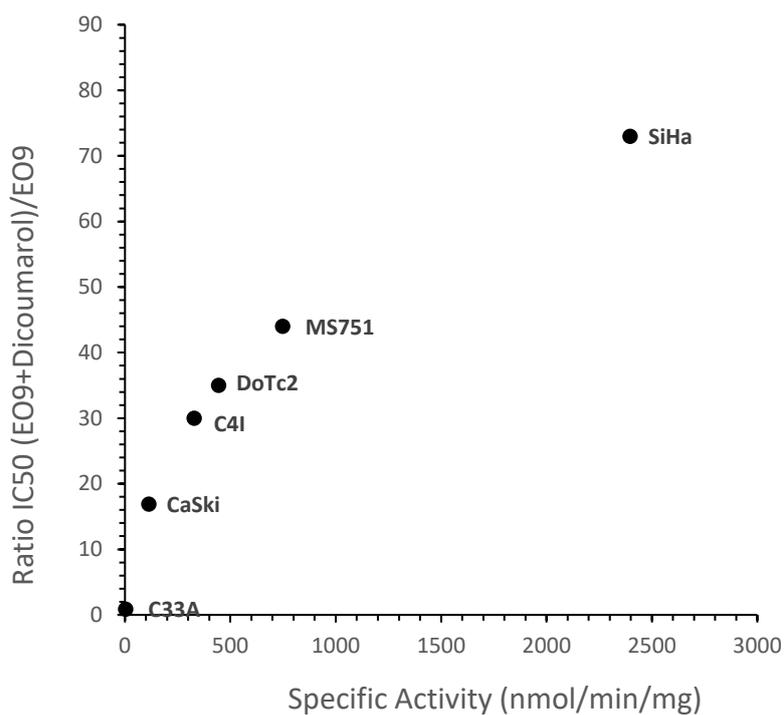
### **3.3.10 Relationship between NQO1 activity and response to EO9 in a panel of cervical cancer cell lines.**

A graph of IC<sub>50</sub> values vs NQO1 activity for the panel of cervical cancer cell lines was plotted and the results are shown in figure 3.3.18. It can be seen from the general trend that the cells with the lowest NQO1 activity are most resistant to EO9. In particular, C33A which has the least NQO1 activity is the most resistant to EO9. This is contrasted with MS751 which has the second highest NQO1 activity and is subsequently sensitive to EO9. In general, there is a correlation between NQO1 activity and response to EO9 but it is not absolute and there are variations. Generally, if cells have NQO1 activities above 100 nmol/min/mg, they are typically sensitive to EO9 and this could serve as a potential 'threshold' value for NQO1 in terms of defining which cell will respond well to EO9.

Earlier in this chapter, the ratio of IC<sub>50</sub> values in the presence and absence of dicoumarol was presented (figure 3.3.6 and table 3.3.1) and here, the relationship between the ratio of EO9+Dicoumarol to EO9 was plotted against NQO1 activity (figure 3.3.19 and table 3.3.5). This demonstrates that the effect of dicoumarol on the activity of EO9 is related to NQO1 activity although the curved nature of the relationship suggest that additional factors play a role in determining response.



**Figure 3.3.18 The relationship between NQO1 activity and the response of cervical cancer cell lines to EO9. The line of best fit was constructed using linear regression analysis and the linear regression coefficient is presented on the graph.**



**Figure 3.3.19 Relationship between the response of cells to EO9 in the presence and absence of dicoumarol and NQO1 activity.**

Cell Line	IC50 EO9	NQO1 Activity (nmol/min/mg)	Ratio (EO9+Dicoumarol/EO9)
SiHa	0.22 ( $\pm 0.07$ )	2396 ( $\pm 29$ )	73
MS751	0.22 ( $\pm 0.027$ )	748 ( $\pm 18$ )	38
DoTc2	0.06 ( $\pm 0.0048$ )	444 ( $\pm 17$ )	35
C4I	0.07 ( $\pm 0.0029$ )	329 ( $\pm 36$ )	30
CaSki	1.61 ( $\pm 0.22$ )	113 ( $\pm 11$ )	16
C33A	45.71 ( $\pm 2.2$ )	3.34 ( $\pm 0.9$ )	0.88

**Table 3.3.5. Summary of the relationship between NQO1 activity, response to EO9 and the effect of dicoumarol on the activity of EO9 in a panel of cervical cancer cell lines.**

### 3.3.11 Genotyping of cell lines for polymorphic variants of NQO1.

The polymorphic variants of NQO1 present in a panel of cervical cancer cell lines were determined. Firstly, the DNA from each cell line was extracted as described in the methods section and the results of the DNA yield and quality as determined by Nanodrop reading and A260/A280 ratio is shown in table 3.3.6 below:

Lane	Sample	DNA conc (ng/ $\mu$ L)	Total DNA Yield ( $\mu$ g)	A260/A280 ratio
M	Mass Ladder			
S1	C33A	79.2	15.8	1.82
S2	C4I	34.3	6.9	1.83
S3	MS751	40.1	8.0	1.82
S4	BE	53.2	10	1.90
S4	SiHa	123	24.6	1.82
S5	CaSki	54.7	10.9	1.82
S6	DoTc2	80.9	16.2	1.80
S7	HT29	71	10	1.89
M	Mass Ladder			

**Table 3.3.6. Quantity and quality of DNA extracted from cancer cell lines. The order of presentation of the data (lane) refers to the order used to load the gels in figure 3.3.20 where 'mass ladder' refers to the molecular weight markers used in the gel electrophoresis experiments.**

Good yields of DNA were achieved for all cells and all the A260/A280 ratios were above 1.80 signifying good quality DNA.

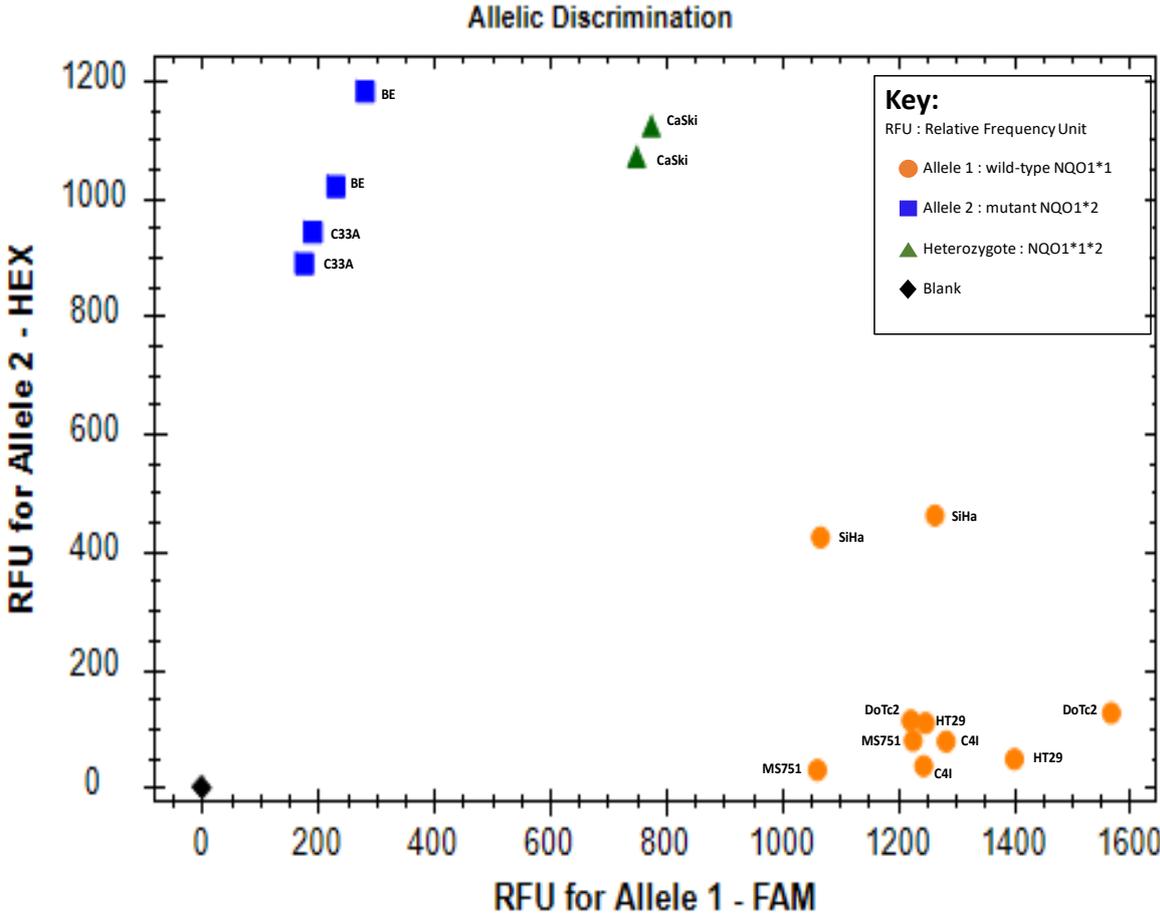
The DNA quality was further determined by gel electrophoresis as described in the methods section. Good quality (little smearing), high intensity and high molecular weight bands were seen under UV light for all cell lines (figure 3.3.20).



**Figure 3.3.20 Analysis of DNA extracted from a panel of cell lines. L6 and L11 were blank and L1 and L12 were molecular weight markers. L2 to L5 contained DNA extracted from C33A, C4I, MS751 and BE cells respectively. L7 to L12 contained DNA extracted from SiHa, CaSki, DoTc2 and HT-29 cells respectively. Samples were separated on a 1% agarose gel and stained with midori green dye.**

Real time PCR using the Taqman assay was used for NQO1 SNP analysis and allelic discrimination. HT29 and BE were used as positive controls because their NQO1 polymorphic status has been previously established. HT29 is NQO1 homozygous wild type (NQO1\*1) while BE is NQO1 homozygous recessive (NQO1\*2). These known genotypes were used to ascertain the validity of the test and the results are presented in figure 3.3.21. The allelic discrimination

showed SiHa, MS751, DoTc2 and C4I to be NQO1 homozygous wild type (NQO1\*1) while C33A is homozygous recessive (NQO1\*2). CaSki was shown to be NQO1 heterozygous (NQO1\*1\*2). According to Traver et al, 1997, whilst cells that are heterozygous for NQO1\*2 have reduced NQO1 functional activity compared to the homozygous wild type, cells lines that are homozygous for NQO1\*2 have between 2-4% of activity of the wild type protein (Traver et al., 1997). The results of this study show that indeed SiHa, MS751 DoTc2 and C4I which are homozygous wild type have high NQO1 activity (table 3.3.7). CaSki which is heterozygous



**Figure 3.3.21 Allelic discrimination for NQO1 in a panel of cell lines. Each assay was duplicated and the results of both assays are presented on this figure (two cell line names presented on the figure). HT-29 and BE were used as positive controls and these were classified as allele 1 (NQO1\*1) and allele 2 (NQO1\*2) respectively.**

had intermediate NQO1 activity whereas C33A which is homozygous recessive has no appreciable NQO1 activity (table 3.3.7).

Cell line	NQO1 activity (nmol/min/mg)	NQO1 genotype
SiHa	2,396 ± 29	NQO1*1
MS751	748 ± 18	NQO1*1
DoTc2	444 ± 17	NQO1*1
C4I	329 ± 36	NQO1*1
CaSki	113 ± 11	NQO1*1/*2
C33A	3.34 ± 0.9	NQO1*2

**Table 3.3.7. Relationship between NQO1 genotype and NQO1 phenotype (activity).**

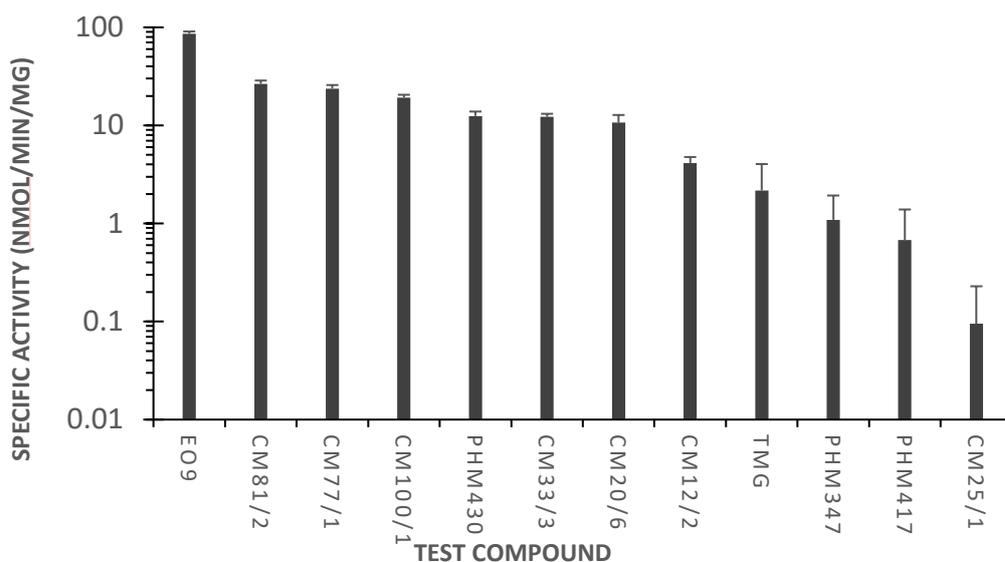
### **3.3.12 Evaluation of novel quinone based bioreductive drugs.**

The ability of novel compounds (structures shown in appendix 1) to serve as substrates for purified human NQO1 was determined and compared to EO9. The results are presented in figure 3.3.22.

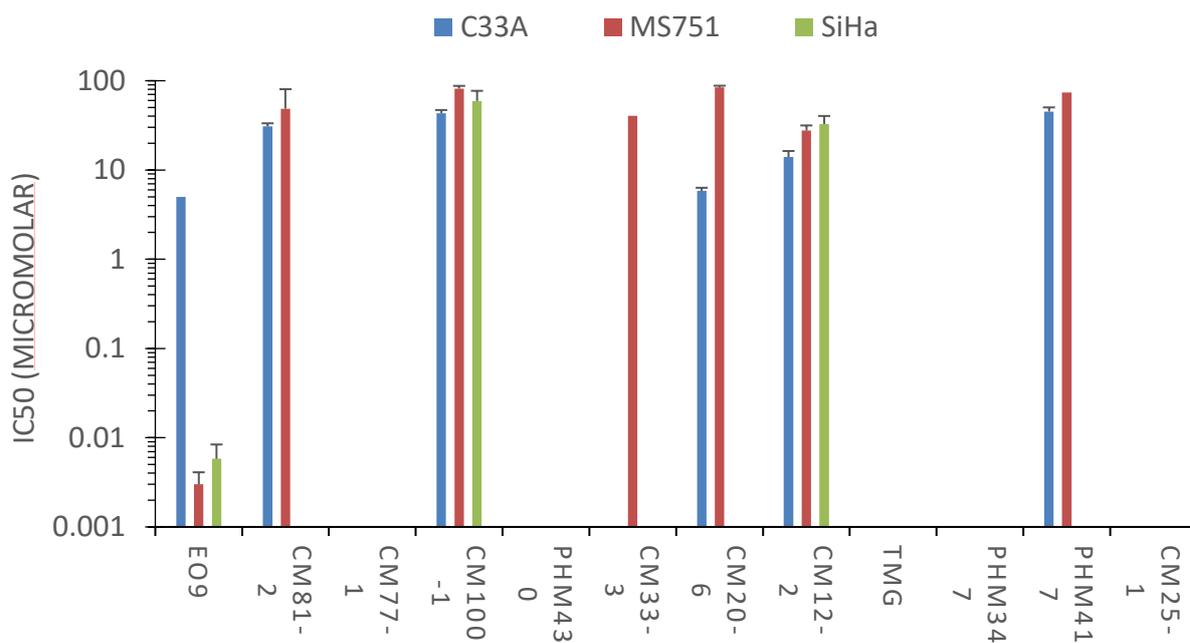
Several compounds were substrates for NQO1 but none were better substrates than EO9. To determine whether or not these compounds were selectively toxic to NQO1 rich cells, they were evaluated against C33A (NQO1 deficient) and the NQO1 rich SiHa and MS751 cell lines.

The results of these studies are presented in figure 3.3.23 and summarised in table 3.3.8. Whilst EO9 was much more effective against NQO1 rich cells, none of the novel compounds evaluated were selectively toxic to NQO1 rich cells. In several cases (e.g. CM20-6), the IC<sub>50</sub> values were much higher in NQO1 rich cells suggesting that NQO1 is functioning as a detoxification enzyme in these cases (table 3.3.8).

Physiologically, NQO1 functions as a detoxification enzyme by reducing quinones to hydroquinones in a single step that bypasses semi-quinone radicals produced by one-electron reduction, thereby eliminating toxic reactive oxygen species. The route taken by the reduced hydroquinone depends on the functional groups attached to the parent quinone (Cadenas, 1995). The functional groups can either form redox stable hydroquinones, which are subsequently conjugated to non-toxic glucuronides and easily excreted, or they form hydroquinones which can rearrange themselves to form toxic DNA alkylating agents as in the case of EO9. They can also form redox labile hydroquinones which can auto-oxidise in air to either stable hydroquinones which follow the route of conjugation or to toxic reactive oxygen species (Cadenas, 1995). This would suggest that in the cases of CM81-2, CM20-6, CM12-2 and PHM417 where cytotoxicity was reduced in NQO1-rich cell lines compared to the NQO1-deficient cell line, the functional groups attached to the quinone nuclei resulted in formation of stable and non-toxic hydroquinones.



**Figure 3.3.22 Substrate specificity for purified human NQO1. All compounds were tested at a concentration of 100 $\mu$ M and each value represents the mean  $\pm$  standard deviation for three independent experiments.**



**Figure 3.3.23 Response of NQO1 rich (MS751 and SiHa) and NQO1 deficient (C33A) cells to EO9 and a series of novel quinone based compounds. Where no bars are presented on the graph, the IC50 values are >100 $\mu$ M. Each value represents the mean IC50  $\pm$  standard deviation for three independent experiments.**

Compound	IC <sub>50</sub> ( $\mu$ M)			Substrate Specificity (nmol/min/mg)
	C33A cells	MS751 cells	SiHa cells	(100 $\mu$ M)
EO9	5.01 ( $\pm$ )	0.003 ( $\pm$ 0.0011)	0.0058 ( $\pm$ 0.0026)	86.0 ( $\pm$ 4.76)
CM81-2	30.81 ( $\pm$ 2.53)	48.64 ( $\pm$ 31.74)	IC <sub>50</sub> > 100	26.6 ( $\pm$ 2.10)
CM77-1	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	23.7 ( $\pm$ 2.11)
CM100-1	43.26 (3.7)	81.61( $\pm$ 6.00)	59.25 ( $\pm$ 17.84)	19.2 ( $\pm$ 1.34)
PHM430	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	12.5 ( $\pm$ 1.38)
CM33-3	N/A	40.5	N/A	12.2 ( $\pm$ 0.92)
CM20-6	5.83 (0.49)	84.05 ( $\pm$ 3.89)	IC <sub>50</sub> > 100	10.7 ( $\pm$ 2.10)
CM12-2	13.95 ( $\pm$ 2.37)	27.71 ( $\pm$ 3.86)	32.69 ( $\pm$ 7.62)	4.1 ( $\pm$ 0.62)
TMG	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	2.2 ( $\pm$ 1.86)
PHM347	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	1.1 ( $\pm$ 0.84)
PHM417	45.21 ( $\pm$ 5.06)	73.78	IC <sub>50</sub> > 100	0.7 ( $\pm$ 0.71)
CM25-1	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	IC <sub>50</sub> > 100	0.1 ( $\pm$ 0.13)

**Table 3.3.8 Summary of the response of cells to EO9 and novel test compounds and its relationship to NQO1 substrate specificity. N/A refers to compound CM33-3 which was not available for chemosensitivity studies. All other values represent the mean  $\pm$  standard deviation for three independent experiments.**

### 3.4 DISCUSSION

In this chapter, preclinical evidence has been generated to evaluate the use of EO9 against cervical cancer. This work has predominantly focused on initial studies in cell lines and further work is required to develop this concept further as will be discussed later. Initially, the focus of this discussion will be on the evaluation of EO9 against a panel of cervical cancer cell lines.

The pharmacological properties of EO9 have been extensively evaluated in a range of different cell lines (Hendriks et al., 1993; Phillips et al., 2013) but no studies have evaluated the use of EO9 against cervical cancers. There has been extensive studies looking at the NQO1\*2 polymorphism and susceptibility to HPV mediated cervical cancers (Hu et al., 2010) and studies have been conducted looking at the characterisation of NQO1 in squamous cell carcinoma (SSC) of the cervix. Importantly, immunohistochemical analysis of NQO1 protein expression in SSC cervix have demonstrated that NQO1 protein expression was significantly higher in cervical SCC and CINs than normal cervical epithelia (Ma et al., 2014a). Furthermore, high levels of NQO1 was associated with poor prognosis and NQO1 expression was identified as an independent hazard factor for disease free survival for SCC patients (Ma et al., 2014a). Whilst this study has not evaluated the expression of NQO1 in cervical cancer tissue, there is evidence in the literature that CINs and SCC cervix have elevated levels of NQO1 and this suggests that the biochemical target for EO9 is present in cervical cancer tissues.

Hypoxia is another factor that influences the pharmacology of EO9 and hypoxia has been shown to be one of the factors leading to the failure of stage III cervical cancer to radiotherapy (Markowska et al., 2007). Similarly, immunohistochemical studies looking at endogenous hypoxia markers (HIF1, c-Met, carbonic anhydrase IX and glucose transporter 1) have

demonstrated that higher expression was detected in cervical cancers but expression levels in CIN and normal cervix were much lower (Kim et al., 2013). It is unlikely therefore that hypoxia in CIN is common and therefore hypoxia is unlikely to be an important consideration for the treatment of CIN using EO9.

Turning more towards this study, a broad range of NQO1 activity in cervical cancer cells was obtained. Values ranged from  $2,396 \pm 29$  nmol/min/mg in SiHa cells to  $3.34 \pm 0.9$  nmol/min/mg in C33A cells. Genotyping of these cell lines demonstrated that C33A cells are homozygous for the C609T variant (NQO1\*2) and this is a novel finding. The lack of NQO1 activity in C33A cells is consistent with other studies demonstrating that NQO1\*2 variants have low or no NQO1 activity (Traver et al., 1997). The NQO1\*2 variant produces protein but this is rapidly ubiquitinated and degraded in the proteasome (Siegel et al., 2001) and is a valuable experimental tool for the evaluation of NQO1 activated prodrugs. The wide range of NQO1 activity therefore provides a good opportunity to assess the activity of EO9 in vitro. The response of cells to EO9 demonstrates that there is a good correlation between NQO1 activity and IC<sub>50</sub> values (**figure 3.3.2**). For NQO1 activities above 100 nmol/min/mg, there was generally a very good response to EO9 and this could serve as a threshold value for predicting sensitivity to EO9.

The relationship between NQO1 and sensitivity to EO9 has been demonstrated previously for a number of cells lines (Collard et al., 1995; Fitzsimmons et al., 1996; Plumb et al., 1994; Plumb & Workman, 1994a; Robertson et al., 1994, 1992; Smitskamp-Wilms et al., 1994) and the results of this study are consistent with these findings. This relationship occurs under aerobic conditions but under hypoxia, the potency of EO9 is generally enhanced only in cells with low levels of NQO1. In particular, HCR values are very high in cell lines that harbour the NQO1\*2 variant (Plumb & Workman, 1994; Plumb et al., 1994; Robertson et al., 1994). In contrast to these studies, our findings did not show significant hypoxia sensitisation of EO9 in

C33A, despite the fact this cell line harbours the NQO1\*2 polymorphic variant. The reasons why low HCR values were obtained is not known but as stated previously, hypoxia is unlikely to play a major role in CIN and therefore the focus needs to be on the properties of EO9 under aerobic conditions.

Using the NQO1 inhibitor dicoumarol, the potency of EO9 is significantly reduced when EO9 and dicoumarol are added together (**figure 3.3.6**). These results suggest that NQO1 plays a significant role in the mechanism of action of EO9. Interestingly, the relationship between NQO1 activity and the ratio of IC<sub>50</sub> values with and without dicoumarol (**figure 3.3.19**) is much stronger than the relationship between IC<sub>50</sub> values and NQO1 (**figure 3.3.18**). This analysis hasn't been conducted previously and it further confirms the role that NQO1 plays in the activation of EO9.

This study also explored the influence of duration of drug exposure on the activity of EO9. In **figure 3.3.9**, it is clear that the activity of EO9 does not increase significantly after 4 to 6 hours of treatment and this is consistent with the fact that EO9 is unstable *in vitro* (Phillips et al., 1992). It breaks down to the aziridine ring opened compound EO5A and this has little or no cytotoxic activity *in vitro*. The breakdown of EO9 is pH dependent and it becomes very unstable when pH becomes acidic. Paradoxically, EO9 becomes much more active under acidic conditions (Phillips et al., 1992) and this is attributed to proton assisted aziridine ring opening (Phillips et al., 2017). In the context of using EO9 in a loco-regional setting against cervical cancer, this property could be valuable as the drug could be applied to the cervix and left in situ in the knowledge that the drug will decay to a non-toxic product within a few hours. In a rural setting where medical facilities are not advanced, this could have significant practical advantages.

The final section of this chapter explored the activity of a series of novel quinone based compounds. The purpose was to determine whether or not new substrates for NQO1 could be identified and whether these were selectively toxic towards NQO1 rich cells. The results demonstrate that whilst several of these test compounds were substrates for purified human NQO1, EO9 was by far and away a better substrate (**figure 3.3.22**). In addition, evaluation of these compounds against NQO1 rich and deficient cells clearly demonstrated that they were not selectively toxic to NQO1 rich cells and in many cases, they were actually less active against NQO1 rich cells than NQO1 deficient cells. This suggests that NQO1 was functioning as a detoxification enzyme in these cases. The novel compounds were therefore not NQO1 activated and are therefore of limited interest.

In conclusion, the results of this chapter have demonstrated that EO9 can selectively target NQO1 rich cervical cancer cell lines *in vitro*. EO9 was potent and for cells with very high levels of NQO1, IC<sub>50</sub> values were in the nM range and activity was confined to the first 6 hours of drug exposure. The use of the NQO1 inhibitor dicoumarol confirms that NQO1 plays a significant role in the activation of EO9 under aerobic conditions. Finally, the evidence obtained from the literature confirms that NQO1 levels are elevated in CIN and SCC of the cervix suggesting that these cancers have the right biochemical machinery to activate EO9. Taking all the evidence from this study and the literature together, there is a strong case for developing EO9 as a loco-regional therapy for cervical cancer that could have particularly significant applications to treating this disease in low to middle income countries in sub-Saharan Africa and elsewhere in the world.

## **Chapter 4 GENERAL DISCUSSION**

Early detection with appropriate treatment intervention for pre-invasive cervical cancer has proved to be a game-changer in the incidence and mortality rates of cervical cancer over the last 50 years in the developed world. Unfortunately, these gains have not been realised in the developing world, which has incidence and mortality rates currently approaching up to 90% of the world's burden. Alarming, this is projected to rise even further. There has since been a realisation that the cytology screening method which is credited for the decrease in incidence of cervical cancer in the developed world has proved inappropriate for practical application in developing countries due to economic, infrastructure and skilled personnel constraints.

The VIA screening method developed in 2000 has proved to be the valuable tool for cervical cancer screening that the developing countries so desperately need. The attractiveness of this screening method lies in its ability to be incorporated into a 'screen and treat' strategy that bypasses the need for a protracted wait for screen results and potential loss to follow-up which is endemic in this part of the world. This is in addition to its low cost, ability to be performed by trained nurses and lack of need for laboratory facilities or diagnostic equipment. Despite its attractiveness, VIA has been slow in being rolled out in the developing countries and especially in the rural areas where it is needed most.

The current study demonstrated that VIA is an effective screening method which can be just as feasible to implement within a rural Zimbabwean setting as it has already been shown to be within a Zimbabwean city setting (Fallala & Mash, 2015). We were able to screen a total of 469 women in Karoi and rural area of Magunje in Zimbabwe and found a consistent prevalence of 1.5% of women in both study sites. These women who were VIA positive

received treatment for precancerous cervical lesions or were referred to specialist gynaecology services for further investigation and management for cervical cancer. These women would have otherwise been unlikely to have been screened due to lack of opportunity to travel to a screening site in Karoi due to lack of money, time or knowledge of the importance of being screened.

The National Cancer Prevention and Control Strategy for Zimbabwe 2014-2018 sought to reduce cancer morbidity and mortality through the implementation of evidence-based and cost-effective prevention and control interventions achieved through 6 goal areas (Ministry of health and Child Care Zimbabwe, 2014). Goal 3 addressed early detection of cancer including cervical cancer, and it sought to reduce late presentation of cervical cancer by providing VIA screening at all levels of the health delivery system by 2018 and by increasing the proportion of people utilising the service (Ministry of health and Child Care Zimbabwe, 2014). Although this has not yet been achieved in Zimbabwe, our study has shown that this goal is possible to achieve for cervical cancer. We have shown that it is possible to deliver VIA screening to women within a poor, rural setting, and that this is cost-effective and will result in identifying women with precancerous and early cervical cancer lesions which are amenable to curative treatments, instead of having them present at a later stage with advanced disseminated disease.

The average cost of VIA screening per patient based on our study was US\$1.85 which was comparable with that from other cost of screening studies from Tanzania and Uganda which are both in the same world bank income tier as Zimbabwe (Campos et al., 2015; Nelson et al., 2016). Comparing the costs of screening and treatment, calculated using the data from our study and extrapolating costs to the rural population of Zimbabwe, we found that screening the entire female population aged 15 years and older in the rural population of Zimbabwe once in a lifetime would cost approximately US\$6,577,341. This would result in

cost saving of US\$146,787,909 to patients who would have otherwise presented with late stage disease if they are treated in the public sector, and US\$980,170,265 if they are treated in the private sector. Although these costs represent the 'out-of-pocket' cost to the patients, they don't include the indirect costs to the patient such as transport and accommodation costs or labour and productivity loss. They also do not take into account the intangible costs to the patients and their families brought about by the illness and probable eventual death of a loved one.

The feedback we received from the screening programme is evidence that the screening was well-received and resulted in word of mouth dissemination of the importance of cervical cancer screening. From our feedback results, 98.4% of attendees at the Karoi screening programme said they would recommend a friend or family member to attend a similar screening programme. If a community of women can be educated about the importance of cervical cancer screening, they can in turn impart that knowledge to their daughters and granddaughters, aunts, sisters and significant female relations.

The second aim of this thesis was to generate evidence to support the development of EO9 as a locoregional therapy for pre-invasive cervical cancer. Our study has also shown that EO9 can selectively target NQO1 rich cervical cancer cell lines *in vitro*, and literature confirms that NQO1 levels are elevated in CIN and SCC of the cervix. This suggests that the biochemical machinery that is required to activate EO9 is present in pre-cancerous cervical lesions and in cervical SCC. EO9 has undergone successful Phase I and II trials for locoregional therapy in bladder cancer and is currently undergoing Phase III trials (Phillips et al., 2017), thereby establishing proof of concept in a locoregional setting and that the drug is safe, non-toxic and it works in bladder cancer.

The pharmacokinetics of EO9 can potentially be exploited for therapeutic advantage as a locoregional therapy in a 'screen and treat' strategy for pre-cancerous cervical lesions. Experiments on the influence of duration of drug exposure on SiHa and CaSki cell lines have shown that after 4-6 hours the activity of EO9 decreases as the drug becomes unstable and breaks down into an inactive compound EO5A (R M Phillips et al., 1992). This is ideal in a situation where the drug is applied to the cervix. As previously discussed, the cervical and vaginal ecosystem is complex due to the balance between the conflicting need for protection against infection and allowing entry of sperm to the upper genital tract. As such, there is a pH gradient from the acidic vaginal canal at pH 3.8-4.5 to the alkaline cervical mucus at pH ~7.0. EO9 is known to be more active at pH 6.0 but paradoxically also more unstable. The pH at the ectocervix is not optimal for EO9 activity. In order for EO9 to be administered in an environment where it can exert its optimal activity, the challenge is to be able to formulate the drug immediately prior to administration because while EO9 works optimally at pH 6.0 it is also unstable at that pH and is rapidly metabolised into an inactive compound EO5A (R M Phillips et al., 1992). The solubility of EO9 in 40% tert-butanol in water is at least 9.5 mg/ml whereas in water it is approximately 0.2 mg/ml (Evans et al., 2014). This formulation could be in the form of lyophilized drug which is mixed with a reconstitution solution buffered at pH 6.0 at the time of application to the cervix. Previously discussed formulations of loco-regional drug therapy for the cervix have been gels, creams or a vaginal pessary. An ideal situation would be to provide the reconstituted drug in a formulation contained within a biodegradable device such as a cervical cap similar to a contraceptive cap that can be attached to the cervix and left in-situ for 4-6 hours while the drug is active before biodegrading. This device would also give the maximum chance for the drug to work locally across the full thickness of the cervical epithelium which is approximately 200-300µm thick (Ghosh et al., 2016) as EO9 was previously shown to only penetrate a few microns from a blood vessel within its pharmacokinetic lifespan (Phillips et al., 1998). Furthermore, the cervical cap would enable

drug release to be in a unidirectional flow onto the ectocervix, and not backwards into the vaginal canal. This is because if the drug remains within the acidic vaginal canal one of two scenarios may arise. It may become rapidly inactivated at the low vaginal pH, before it has had a chance to act upon the cervical lesions, or it may become activated within the vaginal canal and exert its pharmacological action there instead of the cervix where it is desired. The effect of EO9 of the vagina has not been ascertained, however, the safety profile of EO9 has been established in cancer patients following iv administration where dose-limiting toxicity was reversible proteinuria due to damage to glomeruli (Aamdal et al., 2000; McLeod et al., 1996; Schellens et al., 1994) with no myelosuppression observed (Schellens et al., 1994). The drug concentration that ends up in the systemic circulation from the cervix is likely to be negligible and any EO9 that does find its way into the systemic circulation should undergo rapid clearance, as previous studies have shown that it has a short half-life ranging from 0.8 to 19 min (Schellens et al., 1994). Thus the possibility of systemic toxicity is low and no prolonged adverse effects should occur. Importantly, immunohistochemical analysis of NQO1 protein expression in SSC cervix have demonstrated that NQO1 protein expression was significantly higher in cervical SCC and CINs than normal cervical epithelia suggests that the biochemical target for EO9 is present in cervical cancer tissues and selective toxicity to SCC and CIN should occur (Gaber et al., 2015; Ma et al., 2014).

Within the context of a 'screen and treat' strategy, the cervical cap (or similar device) loaded with EO9 could be applied by the VIAC nurse straight after viewing the aceto-white changes associated with a positive VIAC screen and pre-cancerous lesions. The cervix will be mildly acidic due to the acetic acid used in the screening process at this point, and this may act to potentiate drug action. The cervical cap could be removed again by the VIAC nurse after 1 to 2 hours, which may prove to be problematic in terms of time and requirements for an observation area for the screen positives who are being treated. A situation where the cap is

removed after 24 hours would prevent this from being a strictly screen and treat strategy with the attendant risk of increased likelihood of loss to follow-up. Alternatively, the cervical cap may be formulated in such a material that it disintegrates after the drug has been inactivated, which would be the ideal situation.

Future work will focus on translational research. Activity of EO9 on NQO1-rich cervical cancer cell lines *in vitro* has been established. EO9 is active on single cells and on a continuous monolayer of cells. Activity has also been shown on 3-D spheroid models of MS751 and DoTc2. To progress this work further, the activity of EO9 on animal models of cervical cancer should be investigated. Experimental cervical cancer has been reported only in mice (IARC, 1993). *In utero* exposure of diethylstilboestrol in dalb/c mice produces female offspring with CIN with reversible neoplastic lesions which can be used as *in vivo* models (Bustamam et al., 2008). Although literature has already established the increased expression of NQO1 in CIN and cervical cancer, a confirmatory immunohistochemical study to confirm the enzymology of CIN could also be carried out.

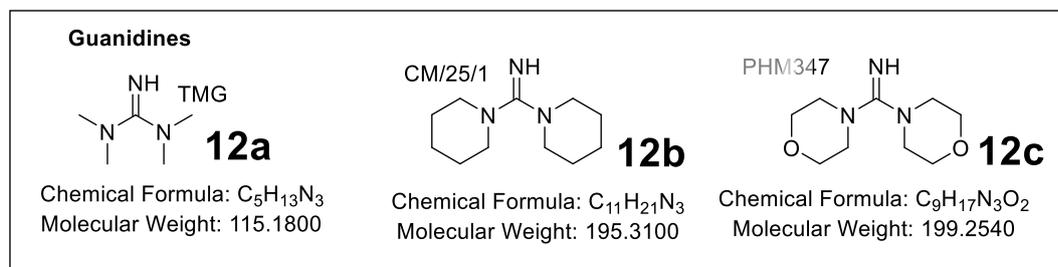
## CONCLUSION

Cervical cancer is a major problem in low to middle income countries and is particularly prevalent in sub-Saharan African countries including Zimbabwe and is set to become an even bigger problem if there is no effective intervention. The effective strategies that the high income countries have in place for prevention and early detection of cervical cancer have been slow to be implemented in developing countries largely because of economic constraints. And it is also because of these economic constraints that treatment for cervical cancer in developing countries is only accessible to the minority. It is therefore imperative that urgent solutions that are sustainable and that will work within the economic environment of low to middle income countries are found. We have shown that makes better economic sense and that it will result in lives saved, to provide nationwide once-in-a-lifetime low cost cervical screening using VIA to women aged 15 years or older in the rural areas of Zimbabwe by bringing it to where they are. These women who make up the larger proportion of women have not routinely been able to access available screening services which are mainly located in the towns and cities of the country. Our study has also evaluated the potential of developing a new locoregional therapy for precancerous cervical lesions, which can be used in a 'screen and treat' strategy in conjunction with VIA screening. The promising results from our laboratory studies and the literature together, have shown that there is a strong case for developing EO9 as a loco-regional therapy for cervical cancer that could have particularly significant applications to treating this disease in low to middle income countries in sub-Saharan Africa.

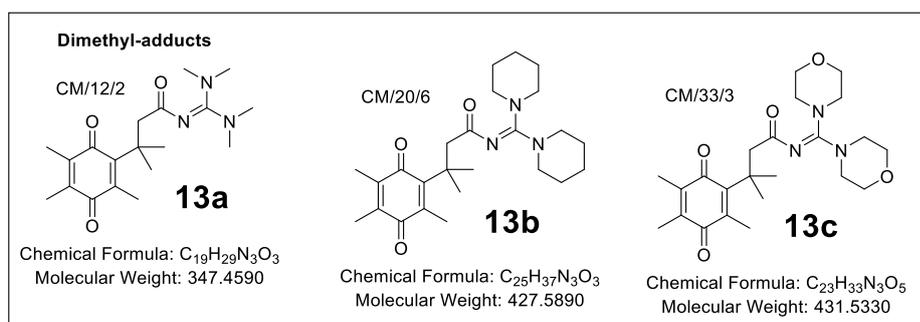
# Appendices

## Appendix 1

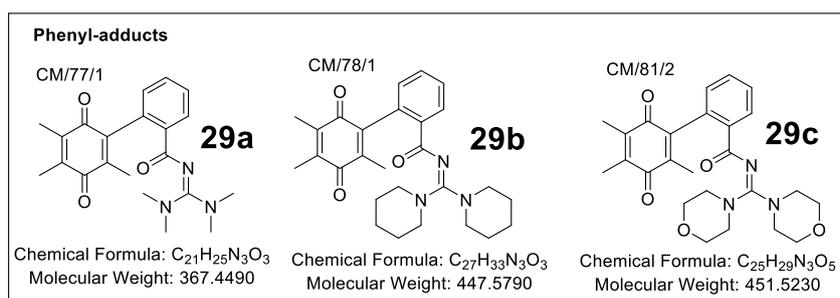
Guanidines :12a-c. 12a) TMG (liquid) 12b) CM/25/1 (liquid) 12c) PHM347



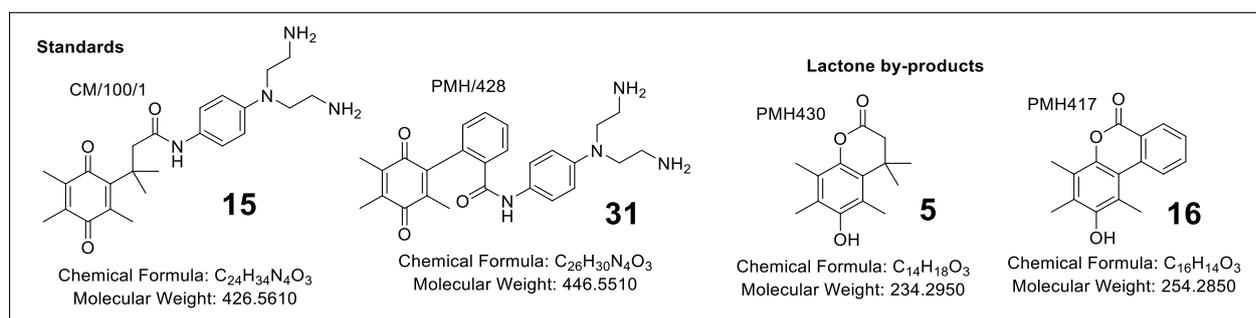
Dimethyl lock pro-drugs: 13a-c. 13a) CM/12/2 13b) CM/20/6 13c) CM/33/3



Phenyl lock prodrugs: 29a-c. 29a) CM/77/1 29b) CM/78/1 29c) CM/81/2



Standards: 15) CM/100/1 31) PMH/428 5) PMH/430 16) PMH417



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