THE ASSOCIATION BETWEEN PLACENTAL HUMAN PAPILLOMAVIRUS DETECTION AND PRE-ECLAMPSIA IN ADULT WOMEN GIVING BIRTH IN TWO ACADEMIC HOSPITALS IN JOHANNESBURG

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand in partial fulfilment of the requirements for the degree of

Master of Medicine in the branch of Obstetrics & Gynaecology

Pretoria, 2017

I, Pieter Francois Retief, declare that this research report is my own work. It is being submitted in partial fulfilment of the requirements for the degree of Master of Medicine in Obstetrics and Gynaecology at the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at this or any other university.

Signed

On this 14th day of November, 2017.

I dedicate this research report
to Anchen, Mairin and Trudie,
the women of my life.

“As a matter of fact,
we know practically nothing of the causation of eclampsia.

A theory has only to be set up by one investigator
to be knocked down by another,
and since there are a large number of theories advanced,
we must give both sets of workers credit for the immense amount of labor and time consumed in building up these theories
and in knocking them down.”

Joseph Bolivar DeLee
Read before the Chicago Gynecological Society,
December 16, 1904

PRESENTATIONS ARISING FROM THIS STUDY

1. Poster presentation, Faculty of Health Sciences Research Day, University of the Witwatersrand, 1 September 2016.
2. Oral presentation, South African society of Obstetricians and Gynaecologists (SASOG) Southern Gauteng Continued Medical Education event, 29 October 2016.
3. Poster presentation, Royal College of Obstetricians and Gynaecologists (RCOG) World Congress, Cape Town, 20-22 March 2017.

ABSTRACT

Background and objectives

Evidence supporting an association between HPV infection and pre-eclampsia has recently been published. Pre-eclampsia is a common, serious complication of pregnancy of complex aetiology that to date has not been fully described. Human papillomavirus (HPV) is a ubiquitous, DNA-virus with tropism for human mucosal commonly found in the female genital tract. Association between placental HPV infection and preterm labour and pregnancy loss has previously been described. This study tested the hypothesis that an association exists between HPV in the placenta or the cervix and clinical pre-eclampsia, or levels of its associated biomarkers, soluble fms-like tyrosine kinase (sFLT1) and placental growth factor (PlGF).

Methods

Women with pre-eclampsia were matched to healthy controls. All subjects were delivered by caesarean section, and cervical and placental samples were collected at the time of delivery. These samples were tested for HPV using a polymerase chain reaction (PCR) assay. Serum levels of soluble fms-like tyrosine kinase (sFLT1) and placental growth factor (PlGF) at the time of delivery were tested. Placental and cervical HPV was compared to the outcomes of clinical pre-eclampsia and serum sFLT1 and PlGF levels.

Results and conclusion

While clinically apparent disease was associated with increased levels of sFLT1 and decreased levels of PlGF, HPV was not detected in any of the placental specimens using the PCR assay. As a result, no association was found between placental HPV detection and clinically apparent pre-eclampsia or deranged serum levels of sFLT1 or PlGF. HPV was very common in cervical samples and showed a non-significant trend towards negative association with clinical pre-eclampsia and sFLT1, and a positive association with PlGF. This may be an effect of cervical HPV infection on the vascular endothelial growth factor (VEGF) signalling system that may explain its association with miscarriage.

ACKNOWLEDGEMENTS

The author would like to acknowledge the help and assistance of the following individuals and institutions:

Ms Merle Laubscher, Ms Sandra Rowe, Ms Stephanie van Niekerk and Ms Claire Pow-Chung of Roche Diagnostics.
Dr Etienne Müller of the NICD and Dr Taryn Pillay of the NHLS for laboratory support.
Dr Noelyn Hung of the University of Otago for her time and generous advice.
Prof GJ Hofmeyr and Sr Catherine Parker of the CAP trial for logistical support.
The Wits Health Sciences Research Office for assistance with statistical analysis.
Dr Haroun Rhemtula and Dr Marlene Bothma who supervised the project.
My head of department, Prof Yasmin Adam for the opportunity and encouragement.

The study was financially aided by an Individual Grant from the Faculty Research Committee of the Faculty of Health Sciences, University of the Witwatersrand

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LIST OF ABBREVIATIONS

VEGF	Vascular endothelial growth factor
sFLT1	Soluble fms-like tyrosine kinase 1
PlGF	Placental growth factor
HPV	Human papillomavirus
DNA	Deoxyribonucleic acid
ACOG	American College of Obstetricians and Gynaecologist
SOMANZ	Society of Obstetric Medicine of Australia and New Zealand
NICE	National Institute for Clinical Excellence
BMI	Body mass index
$HBA1_c$	Haemoglobin $A1_c$
PAPP-A	Pregnancy-associated plasma protein A
CSF-1	Colony-stimulating factor 1
KDR	Kinase Domain Receptor
FLT1	Fms-like tyrosine kinase 1
FLT4	Fms-like tyrosine kinase 4
sEng	Soluble endoglin
hrHPV	High-risk human papillomavirus
lrHPV	Low-risk human papillomavirus
PCR	Polymerase chain reaction
mmHg	Millimetres of Mercury
g	Gram
kg	Kilogram
mmol	Millimol
pg	Picogram
ml	Millilitre
CI	Confidence interval

1. INTRODUCTION

Pre-eclampsia is a serious complication of pregnancy of complex aetiology that to date is not completely understood. The search for factors that may play a role in the development of the disease is ongoing. An imbalance of pro- and anti-angiogenic factors of the vascular endothelial growth factor (VEGF) signalling protein family and their receptors are thought to play a central role in the disease process (1). Soluble fms-like tyrosine kinase-1 (sFLT1), a circulating splice variant of the VEGF-receptor-1, and placental growth factor (PlGF), a member of the VEGF family largely derived from the placenta, are the most widely used biomarkers in clinical studies of pre-eclampsia (2).

Human papillomavirus (HPV) is a ubiquitous, small, double-stranded DNA-virus that displays tropism for human mucosal and cutaneous epithelial tissues. It causes verrucomatous lesions in the skin and condylomatous lesions of the mucosa. Infection may also be latent with no clinical manifestations visible. In infected cells it integrates into the host DNA and interferes with mechanisms that inhibit cell division. It is well-described as a carcinogenic factor in cervical and oropharyngeal mucosa (3,4).

The common prevalence of HPV in the genital tract raises the possibility that it may have a role in adverse pregnancy outcomes. An association between detection of HPV in products of conception in preterm labour (5) and pregnancy loss (6) has been reported in observational studies. Evidence for a role for cervical or placental HPV in pre-eclampsia is conflicting, but recent studies have reported an association between presumed cervical (7) as well as demonstrated placental HPV infection and pre-eclampsia (8).

This experimental research study tests the hypothesis that placental or cervical HPV is associated with clinical pre-eclampsia, or with changes in the serum levels of sFLT1 and PlGF.

2. LITERATURE REVIEW

2.1 Pre-eclampsia

2.1.1 Introduction

Pre-eclampsia, the clinical syndrome of hypertension, proteinuria and organ system dysfunction is one of the biggest challenges of obstetric care. It remains one of the leading killers of mothers in both developed and developing countries, and causes severe morbidity (9). The proven cure is delivery, but this often results in prematurity at birth, so that in its resolution pre-eclampsia causes neonatal morbidity. There are few other instances in obstetrics where the well-being of the mother and the fetus can be so diametrically opposed.

Pre-eclampsia remains incompletely understood. It was first named “Krankheit der Theorien” or the “disease of theories” by the German obstetrician Paul Zweifel (1848-1927) in his textbook *Lehrbuch der Geburtshülfe* (10). This description has been repeatedly re-examined by different writers (11–14) but remains apt. Despite intense scientific scrutiny, the pathophysiology of the disease defies easy description and definition.

Much of the existing knowledge on pre-eclampsia is drawn from observational cohort studies. Many of the known risk factors for pre-eclampsia have been discovered in this way. For most of the risk factors so identified, odds ratios achieving statistical significance may be expressed. However, not all of them have clinical utility. Few screening risk factors have proven robust enough to form the basis for effective preventative treatment (15), and the search for effective screening tools continues.

Pre-eclampsia may manifest with a variety of clinical presentations. This reflects the endothelial lesion that is the hallmark of the disease, and virtually any tissue, organ or system may be affected. The observation that the presence of placental tissue is the mandatory precondition for the development of pre-eclampsia implicates pathophysiology in the placenta as the source of the disease (16).

Many observations and theories of the nature of the placental pathology have been proposed, starting with the publication in 1958 of the observation that remodelling of the myometrial spiral arteries is incomplete by Dixon and Robertson working in Jamaica (17). However, this widely-described pathological finding in the placenta is not invariably present in pre-eclampsia, and the mechanism by which it may precipitate pre-eclampsia is likewise subject to much theorizing. Indeed, ultimately whether this finding is the cause or the consequence of the disease is not settled.

In recent decades measurable serum biomarkers of pre-eclampsia have been identified. Prominent among these are anti-angiogenic factors (sFLT1) and soluble endoglin (sEng) (18). While these show promise in determining the prognosis of the disease (19) and may come to play a role in early screening, how, and indeed whether they are involved in the pathophysiology of the disease is still not completely understood.

2.1.2 Historical aspects of pre-eclampsia

Pre-eclampsia is defined by the International Society for the Study of Hypertension in Pregnancy (ISSHP) as new-onset hypertension after 20 weeks' gestation, with either proteinuria, or evidence of organ system dysfunction (20). There are several other internationally recognized sets of diagnostic criteria published by major professional bodies such as the American College of Obstetrics and Gynecology (ACOG) in the United States (21), the Society for Obstetric Medicine of Australia and New Zealand (SOMANZ) in Oceania (22) and the National Institute for Clinical Excellence and Health (NICE) in the United Kingdom (23).

While there are differences in approach and diagnostic criteria, they all contain certain key diagnostic elements. These are hypertension with onset in the second half of pregnancy, accompanied by proteinuria or other organ system dysfunction (20–23). Each of these represents in its own way a major breakthrough in the study of pre-eclampsia.

At least some of the manifestations of pre-eclampsia as a disease of pregnancy had been observed for millennia. A serendipitous recognizable description of pre-eclampsia as 'drowsiness and headache accompanied by heaviness and convulsions' already appears in the *Prognosis,* one of the ancient texts from the school of Cos, thought to predate Hippocrates (24). Some authors also claim that recognizable descriptions of eclampsia exist in ancient Chinese and Egyptian writings (25).

During the middle ages in Europe, childbirth was the realm of lay midwives. Further record of eclampsia was only made after trained physicians became increasingly involved in labour and delivery in the seventeenth and eighteenth centuries, especially in France. Francois Mariceau (1637-1709) discussed convulsions in pregnancy and labour in his treatise *Traité des maladies femmes grosses et accoucheés* (1688), but did not distinguish eclamptic convulsions from epilepsy (26).

Eclampsia was first formally distinguished from epilepsy by Francois Boissier de Sauvages (1706-1767). He proposed a classification system for convulsions, distinguishing chronic "epilepsy" from acute "eclampsia", which included "eclampsia parturiemtum" as acute convulsions during labour (26).

The description of the syndrome remained limited to the symptoms of headache, oedema and convulsions – essentially a description of eclampsia and its prodrome, until two key discoveries during the nineteenth century laid the foundation of the current definition of pre-eclampsia. These were the discovery of the association of proteinuria and hypertension with the classical symptoms of oedema, headache and convulsions. These two observations can be rapidly and cheaply made, and remain the cornerstone of pre-eclampsia screening in most of the world more than a century later.

In France the pathologist François Rayer (1793-1867) found protein in the urine of three pregnant women with oedema in 1840. This was followed in November 1843 by the simultaneous publication by John Charles Lever (1811-1858) and James Young Simpson (1811-1870) of the observation of albumin in the urine of eclamptic patients (27).

The association of hypertension with proteinuria in pregnancy was first described by Charles Vinay (1845-1908) in 1894 in Paris. Henri Vaquez (1860-1936) and Pierre Nobécourt (1871-1943) first extended this association to eclampsia in a paper in 1897 (26). Again it took time for hypertension and proteinuria in pregnancy to be distinguished from chronic renal disease with proteinuria, or Bright's disease, as it was also known. Once established, blood pressure measurement subsequently became the screening observation of choice for predicting eclampsia.

Once these diagnostic criteria of proteinuric hypertension in pregnancy were known, the study of pre-eclampsia proceeded on a firmer footing during the twentieth century, and large observational studies could be carried out to determine the epidemiology of what came to be termed pre-eclampsia (26).

2.1.3 Epidemiology and demographic risk factors for pre-eclampsia

The true incidence of pre-eclampsia in a population is seldom known. Incidence rates are often estimated from sometimes very biased samples. The incidence has been quoted as 3-5% (28). In addition, pre-eclampsia adds disproportionally to obstetric morbidity. Women with pre-eclampsia are significantly more likely to suffer severe outcomes, near-misses or death (9). Hypertension, including pre-eclampsia, was responsible for 640 maternal deaths (14.77% of the total) in South Africa for the 2011-2013 triennium (29).

Making comparisons between populations is problematic as the diagnostic criteria used may differ. The availability of screening and antenatal care between countries may also vary. There may also be selection bias present if the sample reported was drawn from women who delivered in hospital in countries with high rates of home birth or low rates of antenatal care access. With that in mind, while the reported incidence varies among populations, it does so within a range of 1.4 to 4.6 per cent with few outliers outside this range.

A large review of eight similar-sized population databases from New South Wales, Western Australia, Sweden, Norway, Scotland, Massachusetts, Denmark and Alberta found rates of pre-eclampsia that varied from 1.4% in Alberta to 4.0% in Norway (30). Secondary sources give some impression of a similar situation in developing countries. Secondary analysis of the World Health Organization Multi-country Survey on Maternal and Newborn Health showed reported incidence of pre-eclampsia in 27 countries in Africa, South America and Asia. The incidence ranges from 0.46% in Niger to 4.6% in Brazil, with Mongolia (6.71%) and Nicaragua (7.67%) notable outliers (31).

Prospective cohort studies, which should in theory provide the most accurate reflection of the incidence, are congruent with the above ranges. A prospective cohort study from Switzerland took a sample of 1300 women who presented for booking between 11-14 weeks and followed them for the duration of the pregnancy. The sample had an incidence of pre-eclampsia of 2.31% with a 95% confidence interval of 1.62-3.28% (32).

In South Africa, studies examining possible risk factors for pre-eclampsia give an idea of the incidence of pre-eclampsia. Frank *et al* studied a random sample of pregnant women in Soweto for the effects of HIV (Human immunodeficiency virus) seroprevalence on the incidence of pre-eclampsia-eclampsia and found an overall incidence for hypertension of 17.3% and for pre-eclampsia of 5.7% (33).

Pre-eclampsia is more common in older women. In studies that dichotomise age above and below 35 years, the association between higher age and pre-eclampsia is straightforward (34). Similarly, when women are sorted into age brackets of five years, there is a linear relationship between increasing age and pre-eclampsia incidence (35).

Pre-eclampsia is more common in first pregnancy. This was first published by Francois Mariceau who observed that “primigravidas are at far greater risk of convulsions than multiparas.”(24) This medieval observation has stood the test of time. Observation from the Swedish Medical Birth Register over 19 years covering 763 795 births found a 4.1% risk of pre-eclampsia in first pregnancies and a 1.7%

risk in higher-order pregnancies overall (36). The protective effect of previous pregnancy however is lost, and indeed reversed to statistically significant degree, if the outcome of the previous pregnancy was a preterm delivery or second trimester loss (37).

One of the strongest risk factors for pre-eclampsia is a previous pregnancy complicated by pre-eclampsia. In the analysis of the Swedish Medical Birth Register the incidence for pre-eclampsia in subsequent pregnancies in women who previously had pre-eclampsia increased from an overall risk of 4.11% in first pregnancies to 14.69% in subsequent pregnancies. This implies that an inherent susceptibility to the disease exists in some women. (35)

The effects of change of partner on the risk of pre-eclampsia are controversial. Increased length of exposure to partner sperm appears to have a protective effect (38,39). In addition, pregnancies conceived using donor sperm (implying minimal or no pre-conception sperm exposure) have an increased risk of pre-eclampsia (40,41). On the other hand, large registry based studies showed that after statistical correction for inter-pregnancy interval, this effect disappears and even reverses (42). However, the validity of this statistical approach has also been questioned (43).

The heritability of pre-eclampsia is complex and is likely to be multi-factorial. Although the reported numbers are small (two out of four), concordance for pre-eclampsia in monozygotic twins have been reported (44). In addition, the presence of a second genome in the placenta makes the analysis of heritability factors in pre-eclampsia even more complex (45).

Pre-eclamptic women are more likely to have a sister with a history of pre-eclampsia (46). Similarly, they are more likely to be the daughters of women with pre-eclampsia. While this bears out some degree of heritability, the risk remains small, and inheritance remains far from predicting even a majority of pre-eclampsia (47).

2.1.4 Modifiable risk factors for pre-eclampsia

In addition to the epidemiological features of affected women, there are some independent modifiable or variable risk and protective factors for pre-eclampsia. They can be roughly grouped for purposes of discussion. These groups are prior illness, diet, habits and environment and pregnancy-related factors.

2.1.4.1 Prior illness

The prior illnesses that bear increased risk for pre-eclampsia are a diverse group. These include diabetes mellitus, thrombophilia, auto-immune disorders, chronic hypertension and chronic renal disease (48). It is of note that they are all pro-inflammatory diseases that carry increased risk for cardiovascular disease in general.

Chronic hypertension and renal disease alone or in combination are associated with an increase in risk for pre-eclampsia (49). The difficulties in diagnosis that arises from the common diagnostic criteria has thus led to a separate group in the classification of hypertensive disorders of pregnancy: chronic hypertension with superimposed pre-eclampsia (20). Hopefully once reliable diagnostic biomarkers for pre-eclampsia are in wide use, this category may be done away with (2). Regardless of diagnostic difficulties, in the setting of hypertension existing prior to pregnancy, the risk of pre-eclampsia is increased (50).

Pre-existing diabetes at the time of booking increases risk more than three-fold (51). The increase in risk is greater for type 1 than for type 2 diabetes (52). Gestational diabetes similarly holds increased risk (53). The risk is independent of body mass index (BMI) and an increase in BMI has an additive effect to the risk (52). In women with pre-gestational insulin-dependent diabetes mellitus, tighter control of hyperglycaemia as reflected by $HBA1_c$ level is associated with smaller increase in risk (54).

Inherited thrombophilia shows a weak association with risk for pre-eclampsia (55). A much larger risk is present with anti-phospholipid syndrome, the most important acquired thrombophilia in pregnancy (56). Similarly auto-immune disease, primarily systemic lupus erythematosus, is associated with increased risk (57).

2.1.4.2 Diet, habits and environment

A number of maternal environmental factors modify risk of developing pre-eclampsia. An increase in BMI has a linear relationship with risk for pre-eclampsia (52). Dietary deficiency of calcium and residence at altitude are associated with increased risk (58). Tobacco smoking during the third trimester is associated with a decreased risk (59).

Increased BMI has a linear relationship with pre-eclampsia risk. This relationship holds true in multiple large reported series (51,53,60). This linear relationship has been shown independent of other disease associated with obesity such as diabetes and hypertension (52).

Women living at altitudes higher than 3000m have an increased risk of pre-eclampsia compared to women living at lower altitudes (61). The evidence for this comes from studies in Latin America, where women living at high altitudes in the Andes were compared to women near sea-level. Whether a linear relationship between altitude or partial pressure of oxygen and the incidence of pre-eclampsia exists, remains to be determined.

The observation that a deficiency of dietary calcium is associated with pre-eclampsia was first made in the 1980s (62) and has been the subject of intense investigation. For women with a deficiency, calcium supplementation can indeed decrease the incidence (58). Whether this is due to its effects on blood pressure in later pregnancy or whether it has an intrinsic role in the prevention of the initiation of the disease process has not been determined and is the subject of a large ongoing randomized controlled trial (63).

The smoking of tobacco products during the third trimester of pregnancy has a protective effect against pre-eclampsia (59). The effect is not seen for women who smoke in earlier pregnancy, and holds true for those who start smoking in the third trimester. Non-combustion use of tobacco products do not show the same effects (59).

2.1.4.3 Pregnancy-related factors

The nature of the conceptus affects the risk of pre-eclampsia. Multiple gestation (64) and molar pregnancy (16) are associated with increased risk. Pregnancies conceived by artificial reproductive techniques have varying increases in risk (65).

Women with twin gestation have a more than two-fold increase in risk for pre-eclampsia (66). The increased risk is common to both the early severe and later-onset pre-eclampsia (64). The zygosity of the twins in some studies do not have an effect on the rates of pre-eclampsia (67). Other investigators however found differences when stratifying by severity of disease, but again overall no significant difference in rates (68).

Current diagnosis and management of molar pregnancy means that most molar pregnancies are terminated during the first trimester. However, in historical reports, molar pregnancies were associated with a significant increase in risk of pre-eclampsia (16). This observation is responsible for the insight that trophoblastic tissue, and not a fetus, is the prerequisite for the development of pre-eclampsia.

Pregnancies arising from assisted reproductive techniques show increased risk of hypertensive disorders (69). This effect is seen in both singleton and twin gestations. The risk is greater for frozen-thawed cycles than for fresh cycles (65). In addition, as noted above, risk is increased when donor sperm is used, and decreases with the number of cycles of intra-uterine insemination with donor cycles (40). There is no difference in risk between *in vitro* fertilization and intra-cytoplasmic sperm injection (65).

The effect of HIV infection on risk has received a deal of attention with some authors suggesting a decrease in risk in women with HIV infection (70). This has been postulated to be as result of the immune-modulating effects of HIV infection. This finding has not been borne out by all investigations (33). In addition, the provision of anti-retroviral drugs to women with HIV, and the possible confounding effects is now impossible to separate from the effects of HIV. A large meta-analysis concluded that there is currently no evidence of HIV changing the risk for pre-eclampsia (71).

Many other factors have been investigated for a role in the development of pre-eclampsia. In some series, previous caesarean section (72), national economic disasters (73), obstetric cholestasis (74) and even ambient temperature around the time of conception (75) were found to have some weak association with pre-eclampsia. Although interesting in passing, these findings are unlikely to be helpful in either elucidating the pathogenesis or in predicting incidence of the disease.

2.1.5 Screening for pre-eclampsia

Given the many known risk factors for pre-eclampsia, the logical continuation would be to construct screening models for pre-eclampsia. Simple models based on history and identified risk factors are in use in the UK (23), and more complex models that include the use of biomarkers and ultrasound investigations have also been proposed and trialled (76).

The primary criticism against any attempt at screening is the observation that pre-eclampsia fails to meet several of Wilson and Jungner's criteria for screening (77). Specifically, the requirements for a recognisable latent period and understanding of the natural course of the disease are not definitively met.

In favour of screening is the existence of two well-studied preventive interventions for pre-eclampsia. The first of these is the use of low-dose aspirin. This has been shown to be of benefit in decreasing the risk for pre-eclampsia in women at high risk of pre-eclampsia based on clinical risk factors (78). The second is the use of supplementary calcium in women with low dietary calcium intake, or in women at high risk of developing pre-eclampsia (58).

The use of low-dose aspirin is more effective if initiated before 16 weeks' gestation. The odds ratio for developing pre-eclampsia and severe pre-eclampsia was 0.3 and 0.6 in one meta-analysis; this translated to a number needed to treat of 19 at-risk women to prevent one case of pre-eclampsia (79).

The Cochrane Library meta-analysis showed a relative risk reduction of 17% (78). The performance of using only maternal characteristics as a screening tool to determine risk has been quoted as including only 36% of women at risk of developing early pre-eclampsia with a false positive rate of 5% (80). Assuming a baseline rate of pre-eclampsia of 3%, this will translate to a number needed to treat of 29.

The difficulty in making recommendations based on the evidence can be illustrated by the recommendations for considering women to be at high risk for developing pre-eclampsia from two geographically neighbouring societies. The Society of Obstetricians and Gynaecologists of Canada name no less than 10 high and 13 moderate risk factors for developing pre-eclampsia (81). In contrast, the American Congress of Obstetricians and Gynecologists Task Force identifies only two risk factors that indicate the initiation of prophylactic aspirin (21).

The situation regarding the use of calcium is also not straightforward. There is a benefit in women with a low dietary calcium intake of less than 600mg/day(58), and supplementation is recommended by the World Health Organisation (82). The evidence for women with normal dietary calcium intake at high risk for pre-eclampsia is less convincing, and currently the use of calcium as a preventive agent for women with normal dietary intake of calcium is not recommended by any of the major societies.

A novel statistical method has been proposed to develop a competing-risks model for the prediction of pre-eclampsia (76). The calculation of risk is based on the assessment of first trimester uterine artery Doppler studies as well as measurement of mean arterial pressure, PlGF and pregnancy-associated plasma protein-A (PAPP-A). The performance of this combination of measurements in the competing-risks calculation model has been reported as a sensitivity of 93% for the development of pre-eclampsia before 34 weeks at a false-positive rate of 5% (76).

While this proposed model appears to have significant benefits over traditional methods of risk assessments, its cost-effectiveness and ultimate effect on pregnancy outcome is still to be determined and its routine use is not recommended by the American College of Obstetricians and Gynecologists in a committee opinion (83).

2.1.6 Aetiology of pre-eclampsia

Synthesizing the many identified risk factors into a coherent theory on the aetiology of pre-eclampsia remains a daunting prospect. There are however a few central observations that are incorporated into current understanding of the aetiology of the disease.

First of these is that the presence of a placenta is the pre-condition for the disease. Secondly, there is a strong association of failure of trophoblastic invasion and remodelling of the maternal myometrial spiral arteries in early pregnancy with the development of pre-eclampsia. Thirdly, the manifestations of the disease are the result of systemic endothelial dysfunction.

These observations form the basis of a two-stage model of development of pre-eclampsia (84). The first stage is characterised by abnormality of the transformation of the maternal spiral myometrial arteries by invading cytotrophoblasts. The second stage is characterised by systemic maternal vascular inflammation and endothelial dysfunction. This dysfunction of the maternal vascular bed results in the clinical features of organ system dysfunction (85).

The association of abnormality of maternal spiral artery invasion and remodelling was first published more than half a century ago (17). In normal human pregnancy, extra-placental cytotrophoblasts invade the decidua and myometrium and migrate into the vessel walls of the spiral arteries of the uterine placental bed. The muscular wall of these arteries are then degraded and the vessels are transformed into dilated, low-resistance flaccid vessels (86).

In some pre-eclamptic women, this process is incomplete, and the spiral artery walls remain muscular, so that the vessel lumen diameter remains the same (87). While the association of this incomplete remodelling with pre-eclampsia is well-described, its study is hampered by the difficulty of obtaining specimens for study, the tissues of the pregnant uterus not being safely available for biopsy. Animal models are not transferable to humans, as not all species display invasion and remodelling of the uterine spiral arteries (87).

The mechanism, by which abnormal invasion leads to the systemic effects of pre-eclampsia, is still not fully understood. Infarction is common in the placentae of pre-eclamptic women, and the placental production of several anti- and pro-angiogenic proteins are altered (88). The stimulus for these changes have been proposed to be variously due to placental hypoxia (89), placental hypoperfusion and reperfusion with resultant oxidative stress (85) or mechanical shear stress due to the increased velocity of blood flow through the narrow vessels (90).

2.1.7 Vascular endothelial growth factor family biomarkers and pre-eclampsia

A bewildering array of possible biomarkers for pre-eclampsia has been described, with more than 15 biomarkers proposed for screening and diagnostic use (91). The group of biomarkers currently most examined are related to the VEGF signalling protein family and their receptors. The possible causative role of these ligands and their proteins was first raised in 2003 based on work in animal models (92).

The VEGF system constitutes a family of signalling proteins. There are five variants of VEGF in humans, VEGF-A, -B, -C, -D, and PlGF. VEGF-A was the first described member, and stimulates angiogenesis. VEGF-B regulates embryonic angiogenesis, and VEGF-C and –D play a role in the regulation of lymphangiogenesis. There is no evidence of a role for VEGF-B, -C and –D in the development of pre-eclampsia (93).

There are three described receptors for VEGF. The first is VEGF-receptor-1, also known as fms-like tyrosine kinase-1 (FLT1). Fms-like tyrosine kinase is named for its similarity to the product of the *fms* gene, a membrane-bound colony stimulating factor-1 (CSF-1) receptor. The *fms* gene was first designated in 1982 (94) as a result of work in felines, describing the development of feline sarcoma as a result of infection with feline McDonough strain sarcoma virus, which carries genes for a variant of the *fms* gene (95).

VEGF-receptor-2 is also known as kinase domain region (KDR). VEGF-receptor-3 is also known as fms-like tyrosine kinase-4 (FLT4), and acts as a receptor for VEGF-C and -D. sFLT1 is a splice variant of VEGFR-1 without the membrane-bound portion of the receptor that thus functions as a circulating decoy receptor.

VEGF-A binds to both FLT1 and KDR, and causes increased vasodilation via prostacyclin and nitrous oxide production, angiogenesis and increased vascular permeability. Most of the cellular responses are elicited by binding KDR, with FLT1 thought to act mostly as a modulating receptor (96).

Soluble fms-like tyrosine kinase 1 is expressed in the placenta, and has been shown to be upregulated *in vitro* in a relative hypoxic environment (89). It has also been shown that early pregnancies that are destined for pregnancy loss have decreased serum levels of sFLT1 compared to pregnancies that progress (97).

The initial work describing a possible role for sFLT1 in the development of pre-eclampsia was done in a rat model. It was shown *in vitro* to cause endothelial dysfunction with vasospasm and increased vascular permeability. In pregnant rats increasing sFLT1 led to hypertension, proteinuria and glomerular endotheliosis that was rescued by the administration of exogenous VEGF (92).

Despite the promise of these biomarkers, there are still many questions unanswered. An argument has been made that sFLT1 meets the Bradford Hill criteria for causation (98). However, although the differences in the means between pre-eclamptic women and healthy controls meet a high level of statistical significance, there is significant overlap of the levels between healthy and pre-eclamptic women. Indeed, the only

validated clinical use of sFLT1 to date is in combination with PlGF levels, as a rule-in prognostic test (19). Its use has been mooted to distinguish other causes of hypertension, proteinuria and thrombocytopaenia from pre-eclampsia in pregnant women, but widespread clinical use is still elusive.

Many questions remain about the role of sFLT1 and sEng in pre-eclampsia. It has been shown that VEGF acts as a chemo-attractant for cytotrophoblast (99). Changes in the levels of sFLT1 appear to have an effect on the outcome of pregnancy long before invasion of the myometrial arteries take place (97). It may therefore be asked whether changes in the homeostasis system of the VEGF angiogenesis system is the cause, rather than the consequence of abnormal placentation.

Also, the translation of results of animal models to humans is not straightforward. There are physical differences in the expressed VEGF in rats and humans. In addition, the circulating concentrations of VEGF and sFLT1 between humans and rats are different by some orders of magnitude. When taking into account the equimolar binding of sFLT1 and VEGF or PlGF, sFLT1 is not in high enough concentrations to make a large difference to the free circulating levels of VEGF (93).

Finally, the sheer complexity of the VEGF system means that conclusions are not easy to draw. VEGF-A alone can be expressed as one of 7 isoforms of different molecular weights, and different affinities. In addition, depending on the splicing, isoforms may act as inhibitors rather than as agonists. Several other moieties also act as modulators of VEGF effects (93).

Despite the many unanswered questions, the central place of the VEGF signalling system in the development of pre-eclampsia is widely accepted, and sFLT1 and PlGF levels should be considered as additional outcome measures in studies of clinical pre-eclampsia.

2.2 Human papillomavirus

2.2.1 Biology of HPV

Papillomaviruses are non-enveloped double-stranded DNA-viruses. They comprise the family *Papillomaviridae*, and are further sub-classified into genus, species, type and subtype. Papillomaviruses that have humans as host are termed human papillomaviruses (HPV) (4).

HPV may infect cutaneous or mucosal squamous epithelium, although there is some overlap between types. Mucosal HPV types belong predominantly to the alpha-papillomavirus genus, and may be further classified as high or low risk, according to its ability to immortalise keratinocytes. Thirteen types of HPV are associated with carcinoma of the cervix, and are consequently termed high risk HPV (hrHPV) (3).

The HPV genome is a circular plasmid and comprises 10 genes, and expresses 8 major proteins. E1, E2, E4, E5, E6 and E7 are termed early proteins and are involved in modifying the cell cycle and replication of the host cell. L1 and L2 are late proteins and make up the viral capsid necessary for extracellular survival and transmission. (100). E1 and E2 regulate viral replication, while E4 plays a role in disrupting the host cell defences to enhance infectivity.

The early proteins E5, E6 and E7 interfere with the host cellular mechanisms, in particular those involved with regulation of cell replication and the cell cycle, which forms the basis of the carcinogenic potential of HPV. E5 is a small, pluri-potential protein that has been shown to interfere with many intracellular processes, including signalling growth factors (101).

E5 however is not necessary for oncogenesis, as this follows after viral DNA integration in the host cell DNA, where the E5 gene is often lost (102). E6 inhibits p53, a tumour suppressor gene that arrests the cell in G1 phase for DNA repair, and induces apoptosis if DNA repair does not take place (103). E7 binds to and inactivates Rb1, the product of the retinoblastoma gene-1. Rb1 is a tumour suppressor protein that arrests the cell cycle in G1, and deletion or inactivation of Rb1 is associated with retinoblastoma and other tumours (104).

2.2.2 Epidemiology of HPV

Given the importance of HPV to the development of cervical cancer, most studies of the prevalence have focussed on the prevalence of cervical HPV, in particular hrHPV. There is considerable variation of prevalence of HPV infection between geographical regions and age groups. It has been estimated that almost all sexually active women will be infected with at least one strain of HPV during her lifetime (105).

In South African populations, two recent studies tested the prevalence of cervical HPV in large samples from Cape Town and Pretoria, and compared it to both HIV infection and cervical cytology abnormalities. In the Cape Town study, an overall HPV prevalence of 25.4% was found among 9421 women (106). In Pretoria, an overall prevalence of 74.6% and an hrHPV prevalence of 54.3% was found among 1445 women (105).

The prevalence of HPV infection in pregnancy has not been determined in South African populations before. In the United States, an overall prevalence of HPV of 42% in pregnant women was found in one study, compared to 41% in non-pregnant controls (107).

2.2.3 Methods of testing for HPV

Multiple methods exist for the detection of HPV. A complicating factor in comparing research done on HPV is the variation in methods of testing. Different testing modalities may also differ in the range of subtypes that they can detect (108). The comparative performance of different testing modalities is not always known.

In general, formalin fixed, paraffin embedded tissue specimens can be tested using immunohistochemistry or in-situ hybridisation. Fresh specimens may be analysed using polymerase chain reaction (PCR) DNA amplification followed by hybridisation to detect different subtypes. Commercial assays assays differ in their sensitivity and in the range of HPV subtypes that they detect. Care must be taken in the collection and storage of specimens to avoid contamination and degradation of viral DNA leading to false positive and false negative results respectively (109).

In a small study, the performance of L1 antibody immunohistochemistry was compared to PCR testing. Immunohistochemistry detected HPV in 78.9% of the specimens that tested positive by PCR (110)

2.2.4 HPV and the placenta

In the light of the widespread prevalence of HPV in the female genital tract, the association of HPV with pregnancy has been scrutinised. There is evidence for an association for HPV in early pregnancy loss (6), premature rupture of the membranes (111) and premature labour (5).

HPV has also been shown to infect trophoblasts *in vitro* (5), and has been demonstrated in trophoblasts in spontaneously aborted products of conception (112). The observation that some infants have congenital HPV infection despite being delivered by Caesarean section (113) has led to attempts to detect HPV in the placenta and cord blood (114,115). Although demonstrated, it is rare. In one study from Belgium, chorionic villus sampling specimens were analysed for HPV using PCR, and yielded 2 positive out of 35 (115).

Another study from Finland of placentae delivered at term found 4.2% of 306 positive for HPV using nested PCR (114). A third study from Austria found 5.2% of 153 placentae positive for HPV using the Hybrid Capture II test (116).

2.3 HPV and pre-eclampsia

McDonnold *et al* in Texas found that cervical HPV infection at the time of entry into antenatal care is associated with a roughly doubled risk of developing pre-eclampsia (7). This intriguing finding, although proving association and not causation, raises the exciting possibility of a novel, preventable mechanism in the development of pre-eclampsia.

In a second study, Hung *et al* in Otago studied a large series of placentae from women with preterm labour, pre-eclampsia, gestational diabetes and healthy controls. They found a high prevalence of HPV in the placentae studied, as well as a strong association between placental HPV and pre-eclampsia, with all the placentae from pre-eclamptic women showing HPV (8).

The evidence however is conflicting, with previous studies finding no such association when testing mature placentae for HPV (5), or very little HPV in specimens collected from first trimester chorionic villus sampling (115).

The theoretical underpinning for an association between HPV and pre-eclampsia is the ability of HPV infection to influence VEGF expression. It has been noted that HPV can *in vitro* induce VEGF production in infected tissues (117). Other researchers have found that increased endometrial VEGF levels led to upregulation of s-FLT expression in the placenta in a murine model (118). Thus decidual HPV may lead to increased VEGF and in turn upregulation of placental s-FLT, causing the systemic maternal complications of pre-eclampsia.

Two separate circumstantial associations exist that provide further ground to suspect an association. The first observation is that usage of intra-uterine contraceptive devices are protective against cervical HPV infection and premalignant cervical lesions (119), and also against pre-eclampsia (120).

The second, perhaps more fanciful connection is between falling reported rates of pre-eclampsia in Australian populations (121), and the use of HPV vaccination in that country. Australia was the first large country to start the use of HPV vaccination in 2007 (122), and was also the first to introduce a national vaccination programme. It would appear that the falling trend in pre-eclampsia incidence predates the introduction of the vaccine, but the fall in incidence has been sustained subsequent to the start of vaccination programmes. However, with its good record-keeping and close surveillance of HPV, Australia may in future provide stronger evidence of correlation.

3. MATERIALS AND METHODS

3.1 Objectives

3.1.1 Primary objective

3.1.1.1 To determine if there is an association between the presence of HPV in the placenta at the time of delivery and clinically diagnosed pre-eclampsia.

3.1.2 Secondary objectives

3.1.2.1 To determine if there is an association between HPV infection in the cervix and clinically diagnosed pre-eclampsia.

3.1.2.2 To determine if an association exists between the presence of HPV in the placenta and serum sFLT1 and PlGF levels at delivery in all subjects.

3.1.2.3 To determine if an association exists between cervical HPV infection and increased serum sFLT1 and placental growth factor levels in all subjects.

3.1.2.4 To determine if a correlation exists in the presence of HPV, and subtype distribution, between cervical specimens taken prior to caesarean section and placental specimens in all subjects.

3.1.2.5 To determine if a correlation exists between HPV subtype distribution in cervical and placental specimens in all subjects.

3.1.2.6 To determine if brush cytology specimens of the decidual surface of the placenta and chorionic membrane have a diagnostic yield comparable to tissue sample obtained from a flash-frozen placental specimen in all subjects.

3.2 Methods

3.2.1 Definitions

3.2.1.1 Hypertension is defined as systolic blood pressure of more than 140mmHg or diastolic blood pressure more than 90mmHg on two occasions, six hours or more apart, or a single reading with systolic blood pressure more than 160mmHg or diastolic blood pressure more than 110mmHg.

3.2.1.2 Pre-eclampsia

Pre-eclampsia is defined as hypertension arising for the first time after 20 completed weeks of gestation, with the presence of 1+ or more protein on protein dipsticks on two separate samples at least six hours apart, or a urine protein:creatinine ratio > 30mg/mmol, or urinary protein excretion of more than 300mg over 24 hours.

3.2.2 Study design

The study was designed as a cross-sectional observational analytical case-control study to achieve objectives 3.1.1 (to determine if there is an association between the presence of HPV in the placenta at the time of delivery and clinically diagnosed pre-eclampsia) and 3.1.2.1 (to determine if there is an association between HPV infection in the cervix and clinically diagnosed pre-eclampsia). The outcome in question is pre-eclampsia, and the studied exposure is the overall, high- or low-risk subtype-specific presence of HPV in the placenta in objective 3.1.1 (to determine if there is an association between the presence of HPV in the placenta at the time of delivery delivery and clinically diagnosed pre-eclampsia) and cervix in objective 3.1.2.1 (to determine if there is an association between HPV infection in the cervix and clinically diagnosed pre-eclampsia) at the time of delivery.

In addition, for secondary objectives the data will be analysed as for a cross-sectional observational study to achieve objectives 3.1.2.2 (to determine if an association exists between the presence of HPV in the placenta and serum sFLT1 and PlGF

levels at delivery in all subjects) and 3.1.2.3 (to determine if an association exists between cervical HPV infection and increased serum s-Flt and placental growth factor levels in all subjects). The outcome here is serum levels of sFLT1, PlGF, and their ratio at delivery, compared to the exposures of placental (in objective 3.1.2.2) and cervical (in objective 3.1.2.3) HPV.

In a similar fashion the study was considered as a cross-sectional observational study to achieve objectives 3.1.2.4 (to determine if a correlation exists in the presence of HPV, and subtype distribution, between cervical specimens taken prior to caesarean section and placental specimens in all subjects) and 3.1.2.5 (to determine if a correlation exists between HPV subtype distribution in cervical and placental specimens in all subjects).

3.2.3 Sample size

The study was planned as a pilot study with a limited number of participants. With a sample size of 40 and a case to control ratio of 1:1, with significance at 0.05 and power at 0.8, probability of exposure amongst controls of 0.4, this sample was powered to detect an odds ratio of 8.0 or greater.

3.2.4 Study setting

The study is set in two academic hospitals associated with the University of the Witwatersrand Department of Obstetrics and Gynaecology, Chris Hani Baragwanath Academic Hospital and Charlotte Maxeke Johannesburg Academic Hospital. These two hospitals cater to the urban population of Soweto and Johannesburg, and act as tertiary referral centres for other hospitals in the region.

3.2.5 Study population

3.2.5.1 Case population

The case population consists of women diagnosed with pre-eclampsia meeting the defined diagnostic criteria, delivering singleton pregnancies by caesarean section with intact fetal membranes, in the study hospitals.

3.2.5.2 Control population

The control population consists of women with no other systemic morbidities delivering singleton pregnancies by caesarean section with intact fetal membranes.

3.2.5.3 Exclusion criteria

Exclusion criteria were known modifiable risk factors for pre-eclampsia, hypertension with proteinuria present before 20 weeks' gestation and women under the age of 18. Pre-existing diabetes mellitus, pre-existing hypertension, known anti-phospholipid syndrome, thrombophilia, renal and auto-immune disease and a family history of pre-eclampsia were considered as modifiable risk factors for pre-eclampsia. In addition, women who have received a vaccination to HPV, and women who conceived by artificial reproductive techniques were excluded from the study.

3.2.5.4 Participant recruitment

Women meeting inclusion criteria were identified from the theatre booking lists at CHBAH and CMJAH daily during the data collection period. The identified women were approached and counselled on the study, and offered the opportunity to participate.

3.2.6 Data collection

The data collection period ran from 1 December 2015 to 30 April 2016. Study data was collected and managed using the REDCap (Research Electronic Data Capture) electronic data capture tool hosted at the University of the Witwatersrand. REDCap is a secure, web-based application designed to support data capture for research studies. The data is encrypted and stored in an access-controlled environment in the Wits Data Center. After collection, identifying information will be removed from participant data. Each participant's data was assigned a unique study number. A record matching unique study numbers with identifying data is kept securely by the investigator.

3.2.7 Sample collection and storage

Informed consent was obtained from women meeting the inclusion criteria using the informed consent form (Appendix D) prior to caesarean section by the principal investigator. The data sheet (Appendix E) was captured manually and entered electronically using REDCap to record diagnostic and demographic data and exposure to other risk factors for pre-eclampsia.

Prior to caesarean section a cervical brush cytology specimen was obtained from participants using the standard method for collecting cervical smears, using a Cytobrush (NMS supplies, Johannesburg, South Africa). The specimens were stored in PreservCyt preservation solution (ThinPrep solution, Hologic, Marlborough, Massachussetts) and stored at between 2 and 8 degrees Celsius until testing at the testing laboratory according to the manufacturer's guidelines.

After delivery the placentae were collected and specimens prepared. Smear specimens were prepared by rinsing blood from the decidual surface of the placenta using tap water. The specimen was obtained using the Cytobrush. The decidual surface of the placenta was brushed from the centre of the placenta onto the edge of the decidual surface of the membranes, while rotating the brush so that the surface of the brush in contact with the placental surface was brushed in the direction of the placental edge.

After collection of the smear specimen, a full-thickness transmural placental specimen was collected. The specimens were flash-frozen and stored at -80 degrees Celsius until transfer to the testing laboratory (South African National Institute for Communicable Diseases STI and HIV section), where it was processed to obtain a 300mg protein sample.

In addition, prior to caesarean section, a blood sample was collected for the maternal serum s-Flt and Placental Growth Factor levels in a serum separator tube mark II (SST-II), centrifuged and frozen at -80 degrees Celsius until testing

3.2.8 Laboratory analysis

DNA was extracted from 120 specimens (40 cervical, 40 placental smear and 40 flash-frozen placental samples) using the AmpliLute Liquid Media Extraction Kit (Roche Molecular Systems, USA) according to the manufacturer's instructions. HPV genotyping was be performed on extracted DNA using the Linear Array HPV Genotyping Test (Roche Molecular Systems, USA).

This assay is a qualitative *in vitro* test that uses HPV DNA amplification by polymerase chain reaction (PCR) and nucleic acid hybridization for the detection of 13 high-risk (HR) HPV genotypes (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 24 low-risk (LR) HPV genotypes (6, 11, 26, 40, 42, 53, 54, 55, 61, 62, 64, 66, 67, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108). The probe for HPV type 52 is a mixed probe that cross-reacts with HPV-33, HPV-35 and HPV-58, therefore the presence of HPV-52 is only reported in the absence of the latter three types. The assay contains an additional primer pair that targets the human ß-globin gene (internal control) to provide a control for cell adequacy, extraction and amplification. The manufacturer's instructions are included as appendix F.

Maternal serum was tested for s-Flt and PIGF using the Roche Elecsys Pre-eclampsia Assay according to the manufacturer's guidelines (Appendix G).

3.2.9 Data analysis

3.2.9.1 Descriptive statistics

The incidence of placental and cervical HPV in case and control group was expressed as proportions and standard error (SE) calculated. All other binary variables (parity, previous pregnancy loss and HIV status) were expressed as proportions and standard errors calculated. HPV subtypes were grouped as binary variables, for any types, high-risk and low-risk types only. In addition, HPV status was sorted into a categorical variable of no infection, single subtype infection and multiple subtype infection.

Means and standard errors of continuous variables (age, gestation, birth weight, sFLT1 and PlGF levels) were calculated. Continuous variables were assessed for normality of distribution using histograms or skewness and kurtosis testing.

3.2.9.2 Inferential statistics

Binary variables were compared using Pearson's Chi squared test. Binary and continuous variables were tested for association using unpaired Student's t-test. Continuous variables were tested for association using Pearson's correlation. A two-sided p-value of 0.05 was used to determine significance.

The potentially confounding variables of age, HIV status, BMI, parity and previous pregnancy loss were assessed for association with the outcomes of clinical pre-eclampsia, sFLT1 and PlGF levels and corrected for by multiple regression.

3.2.9.3 Data tools

Data were analysed using Stata software (StataCorp. 2015. *Stata Statistical Software: Release 14*. College Station, TX: StataCorp LP).

3.3 Ethical considerations

Ethics review approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical). A copy of the approval certificate is attached as appendix A.

Because the presence of hrHPV in the cervix is a risk factor for cervical cytological abnormality, women were offered the opportunity to be informed of their test results if their cervical specimen tested positive for hrHPV. All women taking parted opted to be informed. Those who tested positive were contacted telephonically and provided with referral letters to their primary health care providers for cervical cytology smears and referral for abnormalities according to the South African National guidelines.

4. Results

4.1. Demographics, risk factors and clinical pre-eclampsia

4.1.1. Description of demographics and risk factors

Fifty women were identified as eligible for inclusion in the study and approached for consent. One declined to take part. Of the forty-nine who agreed to take part, one was excluded because of failed serum biomarker testing and eight were excluded because their cervical smear specimens were discarded before testing. Forty cases were thus included in the final analysis. The patient demographics are demonstrated in Table 4.1. 27 cases were recruited from Chris Hani Baragwanath Academic Hospital and 13 from Charlotte Maxeke Johannesburg Academic Hospital.

The mean age of participants was 27.6 years (95% CI, 25.7 - 29.5). The mean BMI was 31.1 kg/m^2 (29.1-33.1). The mean birth weight and gestation was 2696.6g (2415.6 - 2977.6) and 36.0 weeks (34.6 - 37.4) respectively. 26 (65%) women had previous pregnancies into the third trimester, i.e. were parous, and 9 (22.5%) women reported a previous non-viable pregnancy loss, including ectopic pregnancy, termination of pregnancy and miscarriage.

The HIV status of all the women were tested during their pregnancy and recorded. Twelve (30%) women were HIV positive. The women who tested HIV positive were all on anti-retroviral treatment. All continuous variables met the requirements for normality.

***Table 4.1**: Description of demographic and risk factor variables, including the association with clinical pre-eclampsia expressed as odds ratios [95% confidence interval(CI)] for binary variables or p-values (continuous variables)*

Variable	**Cases**	**Controls**	**Total**	**p-Value/OR**
Number	21	19	40	
Mean age, years [95%CI]	27.0 [23.9 -30.0]	28.3 [25.7 - 30.8)	27.6 [25.7 - 29.5]	p = 0.50 (Unpaired t-test)
By age group, n (%)				
20-25	9 (42.9%)	4 (21.1%)	13 (32.5%)	
26-30	6 (28.6%)	8 (42.1%)	14 (35.0%)	
31-35	2 (9.5%)	5 (26.3%)	7 (17.5%)	
36-40	4 (19.1%)	2 (10.5%)	6(15.0%)	
Mean BMI, kg/m^2 [95%CI]	31.2 [27.8 - 34.6]	30.9 [28.6 - 33.3]	31.1 [29.1 - 33.1]	P = 0.44 (Unpaired t-test)
BMI by group, n (%)				
20-24.99 kg/m^2	5 (23.8%)	1 (5.3%)	6 (15.0%)	
25-29.99 kg/m^2	3 (14.3%)	7 (36.8%)	10 (25.0%)	
30-34.99 kg/m^2	7 (33.3%)	9 (47.4%)	16 (40.0%)	
35-39.99 kg/m^2	4 (19.1%)	1 (5.3%)	5 (12.5%)	
>40 kg/m^2	2 (9.5%)	1 (5.3%)	3 (7.5%)	
Mean birthweight, g [95%CI]	2 232 [1 805 - 2 658]	3 210 [3 012 – 3 407]	2 696.6 [2 415 – 2 977]	p = < 0.01 (Unpaired t-test)
Birthweight by group				
<1500 g	6 (28.6%)	0 (0%)	6 (15.0%)	
1500-2499 g	6 (18.6%)	0 (0%)	6 (15.0%)	
>2500 g	9 (42.9%)	19 (100%)	28 (70%)	
Mean gestation, weeks [95%CI]	34.0 [31.7 - 36.2]	38.2 [37.1 - 39.3]	36.0 [34.6 - 37.4]	p = <0.01 (Unpaired t-test)
Gestation by group, n (%)				
< 28 weeks	1 (4.8%)	0 (0%)	1 (2.5%)	
28-34 weeks	7 (33.3%)	0 (0%)	7 (17.5%)	
34-37 weeks	6 (28.6%)	3 (15.8%)	9 (22.5%)	
37-41 weeks	7 (33.3%)	14 (73.7%)	21 (52.5%)	
>41 weeks	0 (0%)	2 (10.5%)	2 (5.0%)	
Parous, n (%)	13 (61.9%)	13 (68.4%)	26 (65.0%)	OR 0.8 [0.2 – 3.3] (Pearson's χ^2)
Previous pregnancy loss, n (%)	3 (14.3%)	6 (31.6%)	9 (22.5%)	OR 0.4 [0.1 – 2.1] (Pearson's χ^2)
HIV positive, n(%)	6 (28.6%)	6 (31.2%)	12 (30.0%)	OR 0.9 [0.2 – 4.2] (Pearson's χ^2)

4.1.2 Associations between demographics/risk factors

Variables were tested for association using Pearson's chi-squared test (for two binary variables), Student's t-test (for a binary and continuous variables) or Pearson's correlation (for two continuous variables). P-values for these measures of association are shown in table 2.

Table 4.2: *P-values for tests of association between demographic and risk factor variables, using Pearson's chi-squared test (χ^2) (for two binary variables), Student's t-test (t-test) (for a binary and continuous variable) or Pearson's correlation (pc) (for two continuous variables). Significant values in* ***bold****.*

Variable	**Age**	**BMI**	**Birth weight**	**Gestation**	**Parity**	**Previous pregnancy loss**
BMI	0.18 *(pc)*	-	-	-	-	-
Birth weight	0.85 *(pc)*	0.11 *(pc)*	-	-	-	-
Gestation	0.50 *(pc)*	0.09 *(pc)*	**<0.01** *(pc)*	-	-	-
Parity	**<0.01** *(t-test)*	**0.03** *(t-test)*	0.86 *(t-test)*	0.85 *(t-test)*	-	-
Previous pregnancy loss	**0.01** *(t-test)*	0.54	0.14	0.10 *(t-test)*	0.09 *(χ^2)*	-
HIV	**0.03** *(t-test)*	0.29	0.44	0.90 *(t-test)*	0.11 *(χ^2)*	0.28 *(χ^2)*

Age showed a significant association with parity with a mean age in parous women of 30.4 [95% CI, 28.2 – 32.6] years versus a mean of 22.4 [95% CI, 21.0 – 23.7] years in nulliparous women. A similar effect was seen with previous non-viable pregnancy loss, with women who reported pregnancy loss having a mean age of 32.0 [95% CI, 27.0 – 37.0] years, versus 26.3 [95% CI, 24.3 – 28.2] years in those without.

In addition, there was a significant association between HIV status and age, with a mean age of 30.8 [95% CI, 27.1 – 34.4] years in HIV positive women, and a mean age of 26.2 [95% CI, 24.0 – 28.4] in HIV negative women. Age showed no significant association with BMI, birthweight or gestation at birth.

BMI showed a significant association with parity, with parous women having a BMI of 32.7 [95% CI, 30.0 – 35.4] versus 28.1 [95% CI, 25.5 – 30.7) in nulliparous women. There were no significant associations between BMI and previous pregnancy loss or HIV. Birthweight and gestation trended towards a positive association with BMI when analysed with Spearman's correlation, without reaching statistical significance (p = 0.07 and 0.11).

Birthweight and gestation showed a high degree of correlation (p = <0.01). Neither showed association with parity, previous pregnancy loss or HIV status. There were no significant associations between parity and HIV status or previous pregnancy loss.

4.1.3 Demographic and risk factor variables and clinical pre-eclampsia

Mean patient age had a similar distribution between cases (27.0 years [95% CI, 23.9 – 30.0] and controls (28.3 years [95% CI, 25.7 – 30.8]).

The mean BMI between case and control groups was similar (cases 31.2kg/m^2 [95% CI, 27.8 - 34.6], controls 30.9kg/m^2 [95% CI, 28.6 - 33.3]). The median BMI was 30.5kg/m^2 with range 21.0 – 50.8kg/m^2.

The majority (65%) of women were parous (defined as having had at least one previous pregnancy of more than 26 weeks' gestation), with no significant difference (p = 0.67) between cases (60.9% [95% CI, 39.2 – 84.5%]) and controls (68.4% [95% CI, 45.4 – 91.4%]). Nine (22.5%) women had previous non-viable pregnancy loss with no significant difference (p = 0.19) between cases and controls.

There was no significant difference in prevalence of HIV between cases and controls (p = 0.84).

Clinical pre-eclampsia showed no significant associations between patient age (p = 0.50), BMI (p = 0.44), parity (p = 0.67), previous pregnancy losses (p = 0.19) and HIV status (p = 0.86). Adjusted odds ratios for clinical pre-eclampsia and HIV, previous pregnancy loss or multiparity as calculated using multiple logistic regression are shown in Table 4.3 below.

Table 4.3: *Adjusted odds ratios for developing clinical pre-eclampsia*

Risk factor	**aOR**	**[95% CI]**
HIV status	1.0	[0.2 - 4.1]
Previous pregnancy loss	2.7	[0.5 - 13.8]
Multiparity	1.1	[0.3 - 4.4]

Clinical pre-eclampsia was associated with a lower gestation (figure 1) and birth weight (figure 2) at delivery. In women with clinical pre-eclampsia mean birth weight was 2 232 [95% CI, 1 805 – 2 659] grammes and mean gestation 34.0 [95% CI, 31.1 – 36.2] weeks. In women without pre-eclampsia mean birth weight was 3 210 grams [95% CI, 3 012.7 – 3 407.3] and mean gestation 38.2 weeks [95% CI, 37.1 – 39.3]. These differences were statistically significant with p-values of <0.01 for both birth weight and gestation, illustrated in Figures 4.1 and 4.2 below.

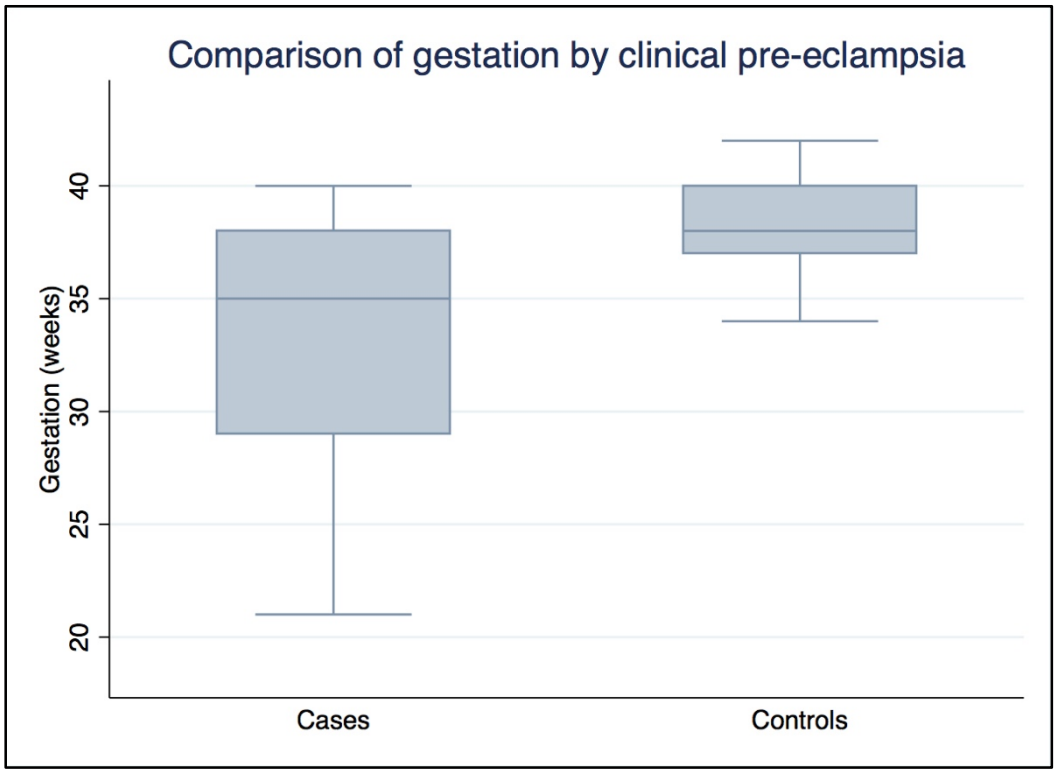

Figure 4.1: *Comparison of gestation between case and control groups*

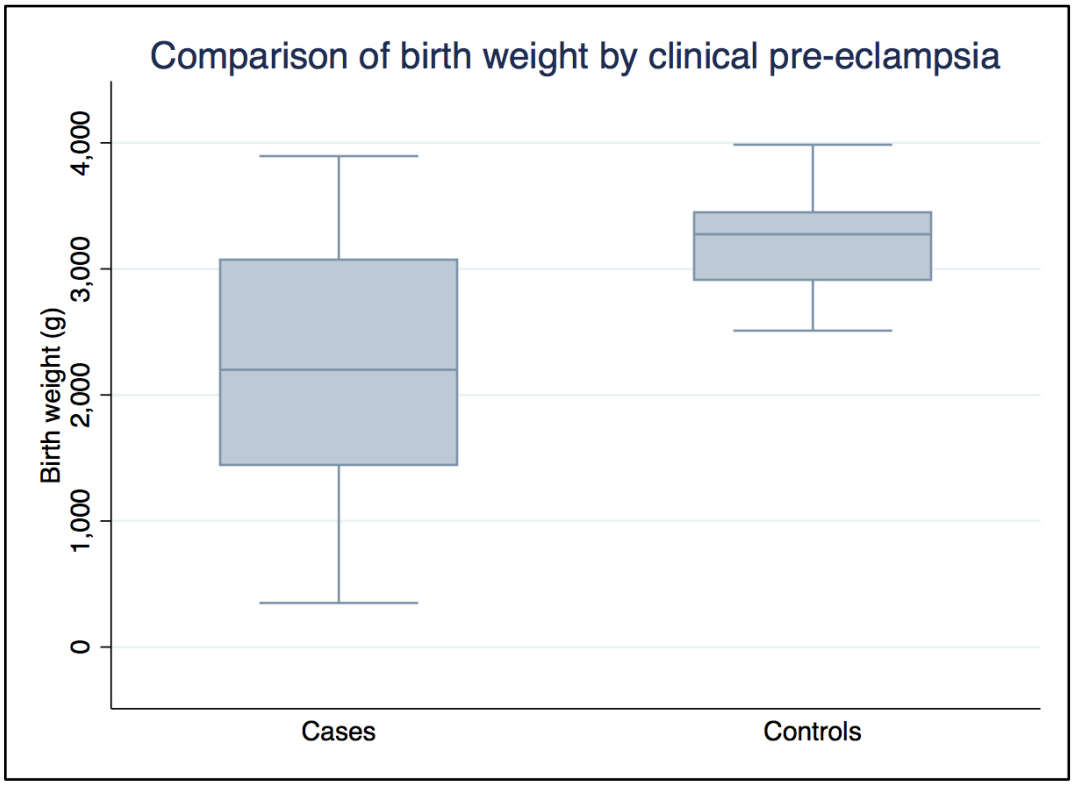

Figure 4.2: *Comparison of birth weight between case and control groups*

4.2 Demographics, risk factors and sFLT1 and PlGF levels

Table 4.4: *Comparison of mean serum levels of sFLT1 and PlGF and their ratio in case and control groups.*

	Cases	**Controls**	**Total**	**p-value**
Number	21	19	40	
Biomarkers *sFLT1(pg/ml)*	10 810.9 (8 256.8 – 13 364.9)	4 148.5 (3 120.5 – 5 176.5)	7 646.2 (5 906.3 – 9 386.1)	<0.01
PlGF (pg/ml)	114.4 (10.1 - 218.7)	270.6 (178.6 - 362.6)	188.6 (117.1 - 260.1)	0.03
s-Flt/PlGF ratio	379.2 (201.6 - 556.7)	41.9 (6.1 - 77.7)	219.0 (113.1 - 324.9)	<0.01

The described association between clinical pre-eclampsia and decreased PlGF and raised sFLT1 held true in this sample, with a high degree of statistical significance for both measures, as well as for the ratio between the two, as shown above in table 4. The quantile plots in Figures 4.3, 4.4 and 4.5 below illustrate this relationship graphically.

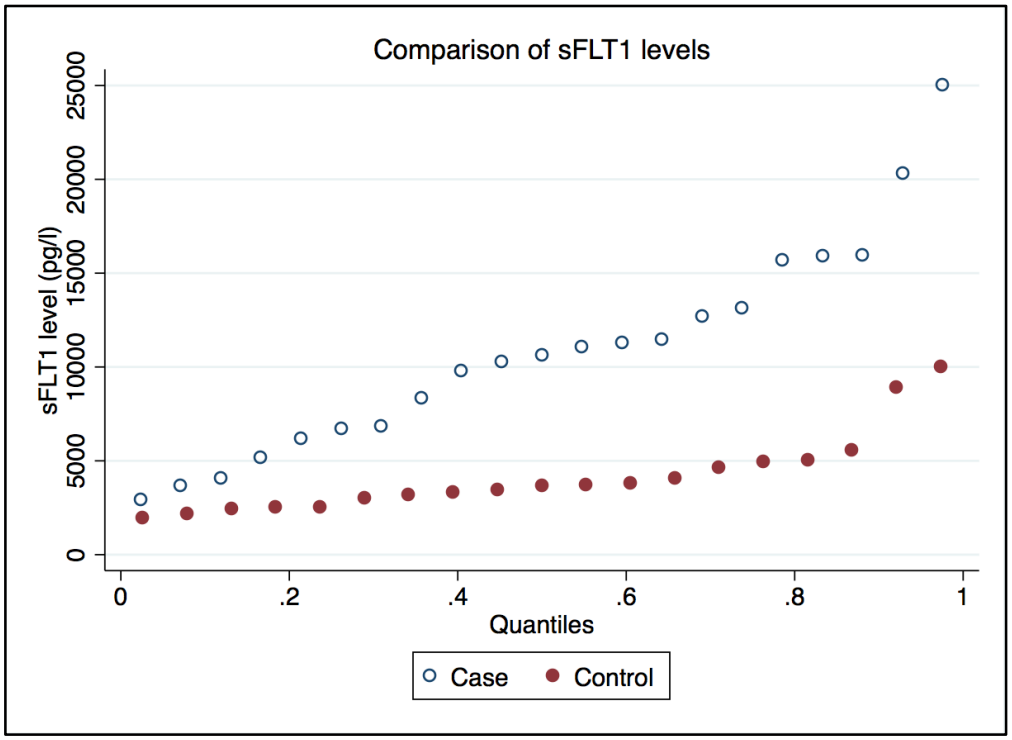

Figure 4.3: *Comparison sFLT1 level measurements between case and control groups*

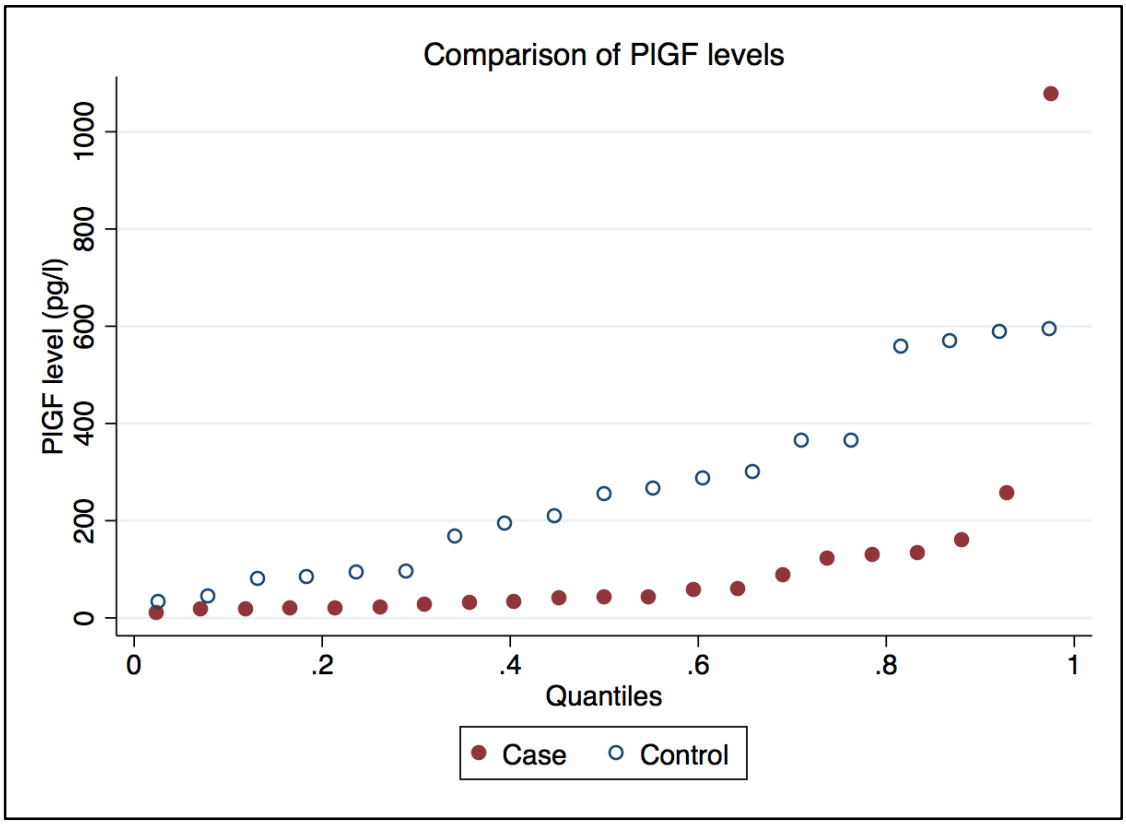

Figure 4.4: *Comparison of PlGF level measurements between case and control groups*

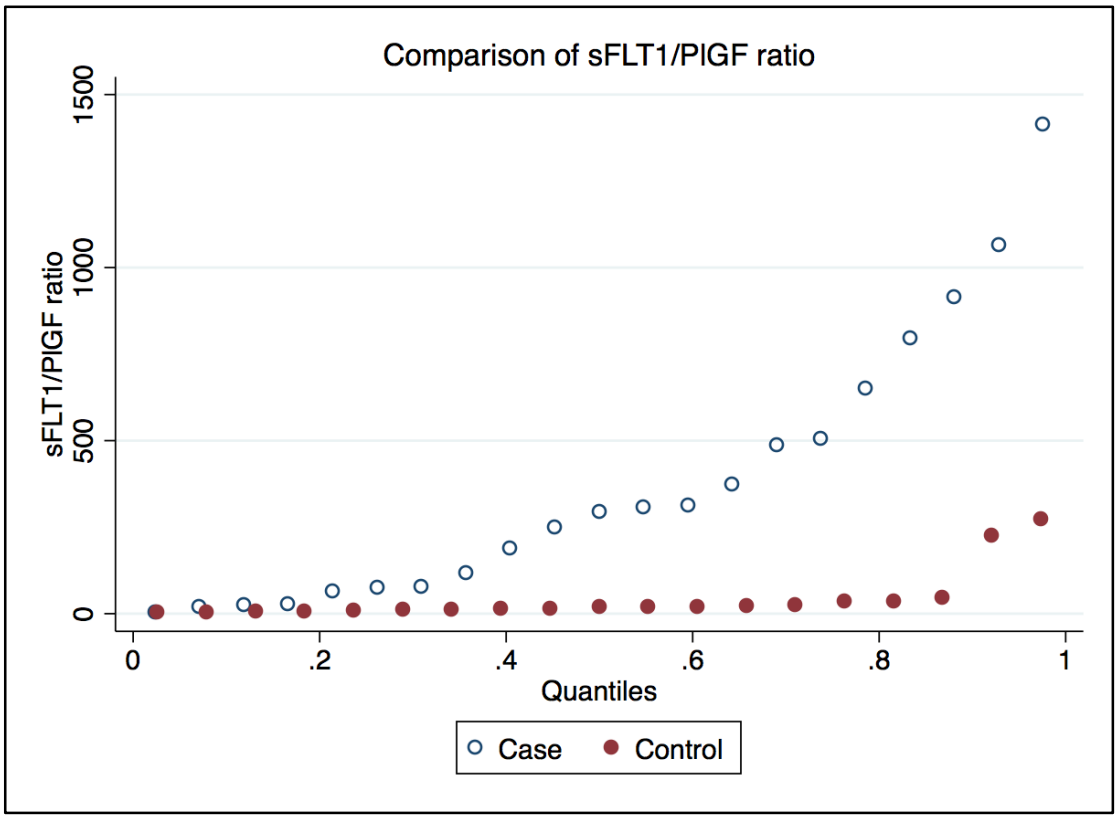

Figure 4.5: *Comparison of sFLT1/PlGF ratio between case and control groups*

Age showed a negative correlation with sFLT1 levels, but no significant effect on PlGF levels. BMI showed no correlation with either sFLT1 or PlGF levels. Gestation and birthweight had a negative correlation with both sFLT1 and PlGF levels and their ratio.

Table 4.5: *Testing for association between continuous variables and biomarkers using Pearson's correlation*

	sFLT1 levels (pg/ml)	**PlGF levels (pg/ml)**	**sFLT1/PlGF ratio**
Age	0.09	0.51	0.16
BMI	0.59	0.34	0.59
Gestation	<0.01	0.43	<0.01
Birth weight	<0.01	0.40	<0.01

Soluble fms-like tyrosine kinase-1 showed a trend towards lower levels in HIV positive women (6 310 pg/ml [95% CI, 3 485 – 9 135]) than in HIV negative women (8 219 pg/ml [95% CI, 5 972 – 10 465]), without statistical significance, with p-value 0.32. PlGF tended to be higher in HIV positive women (241.4 pg/ml [95% CI, 48.5 – 434.3]) than in HIV negative women (166.0 pg/ml [95% CI, 95.7 – 236.3]), again without reaching statistical significance.

Both multiparity and previous pregnancy loss had a negative association with sFLT1 and PlGF levels. This effect disappeared when comparing the means of the sFLT1/PlGF ratio.

Table 4.6: *Testing for association between binary variables and biomarkers using unpaired Student's t-test*

Variable	**Sflt1 (PG/ML)**		**PlGF (PG/ML)**		**Sflt1/PlGF RATIO**	
	Mean [95% CI]	P-value	Mean [95% CI]	P-value	Mean [95% CI]	P-value
HIV status						
Positive	6 309.9 [3 484.8 – 9 135.0]	0.32	241.35 [48.5 - 434.25]	0.34	141.3 [-11.6 - 294.2]	0.34
Negative	8 218.9 [9 5972.5 – 10 465.4]		165.97 [95.7 - 236.3]		252.3 [112.0 - 392.6]	
Parity						
Nulliparous	10 049.8 [6 967.2 - 13 132.6]	0.04	103.89 [41.6 - 166.16]	0.08	329.5 [83.36 575.65]	0.12
Multiparous	6351.96 [4 277.8 - 8 426.2]		234.19 [130.6 - 337.8]		159.46 [55.7 - 263.2]	
Pregnancy loss						
Yes	4 528.44 [2 299.1 - 6 757.8]	0.05	184.7 [45.8 - 323.7]	0.95	78.49 [-10.7 167.7]	0.15
No	8 551.38 [6 461.2 - 10 641.6]		189.696 [102.7 - 276.7]		259.76 [126.8 - 392.7]	

When separate scatterplots of sFLT1 against birthweight for cases and controls are compared, two different distributions of sFLT1 levels may be observed. The first shows lower birthweight with higher levels of sFLT1, while the second group is clustered in higher birthweight but with lower sFLT1 levels.

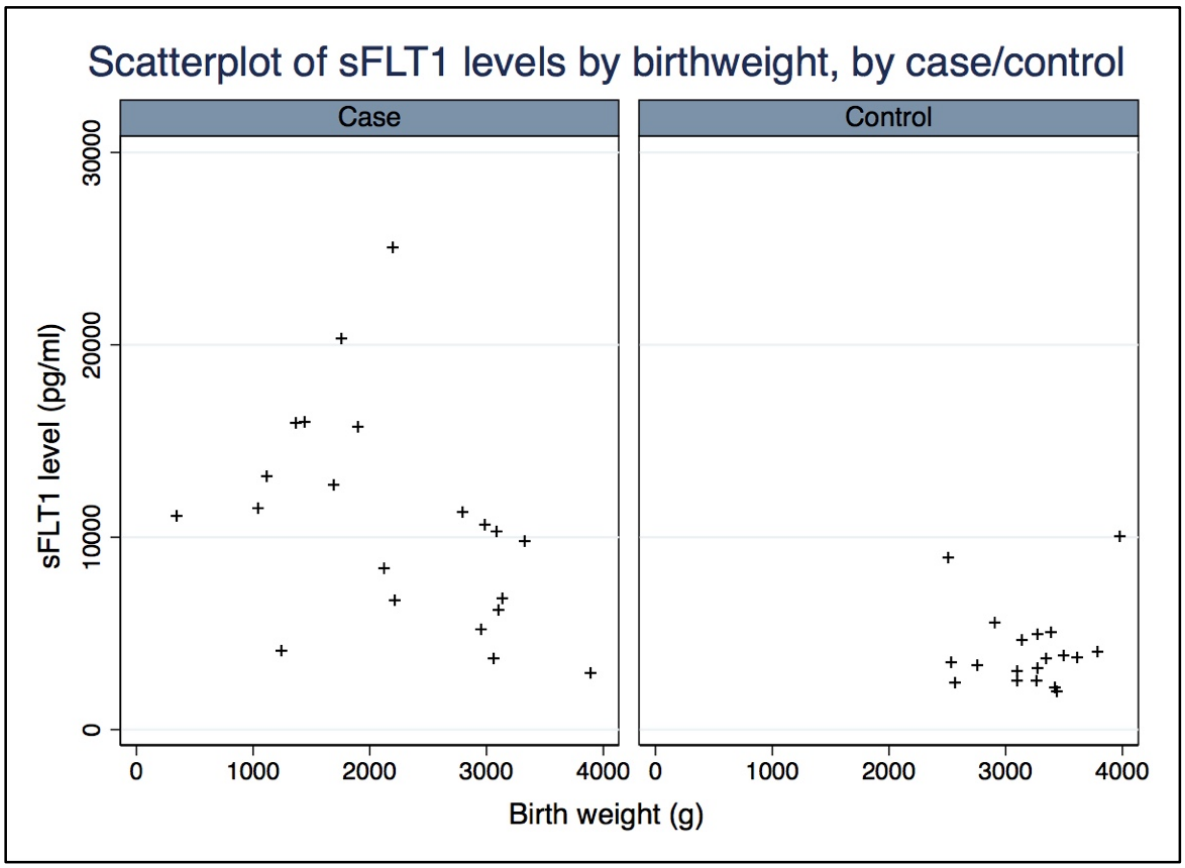

Figure 4.6: *sFLT1 level and birthweight, by case and control groups*

4.3 Detection of placental and cervical HPV

4.3.1 Placental HPV

None of the collected samples tested positive for any HPV types detectable by the Roche Linear Array. This included placental tissue samples, as well as placental decidual surface smear specimens. In all tests DNA detection control strips were positive, indicating that negative results were not due to assay failure.

As a result, no association between placental HPV and any of the patient characteristics, clinical pre-eclampsia or cervical HPV was found.

4.3.2 Cervical HPV

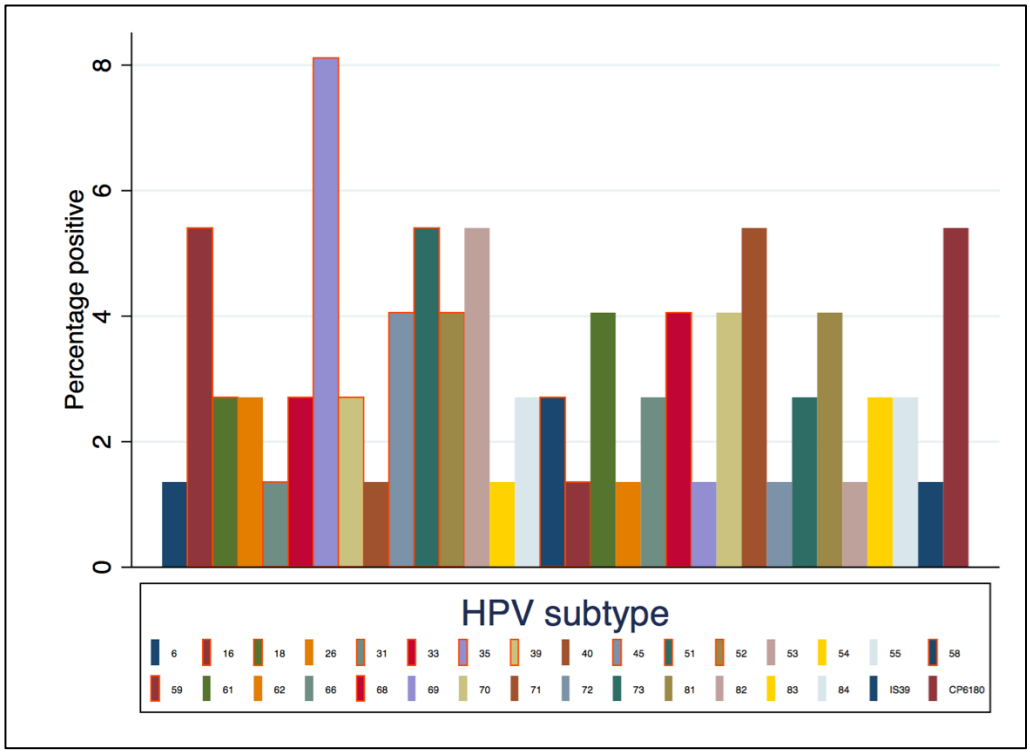

Figure 4.7: *HPV subtype prevalence, high-risk subtypes outlined in red.*

Of the 40 women in the study, 15 tested positive for both hrHPV and lrHPV subtypes. Five tested positive for hrHPV only and 5 tested positive for lrHPV only. Fifteen women tested negative for both hrHPV and lrHPV.

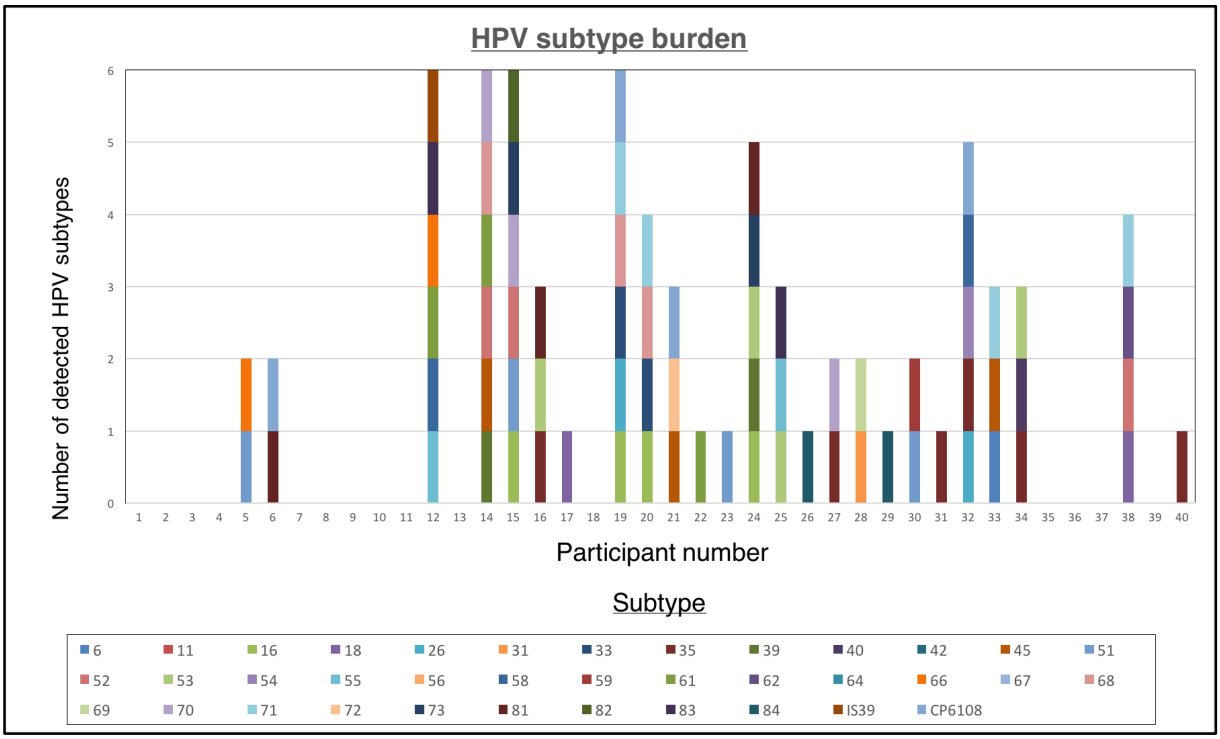

Figure 4.8: *Number of HPV subtypes by participant*

Of the 25 women who tested positive for cervical HPV, seven women had only one subtype while 18 had co-infection with more than one subtype. The variety of subtypes detected in the sample was wide with 32 out of the 37 possible subtypes detectable by the Linear Array detected in at least one specimen. Similarly, there were not any marked predominant subtypes, with the highest prevalence for one subtype with six out of 40 (15%) samples for HPV-35, a high-risk type. Only six out of 32 detected types showed a prevalence of 10% or more.

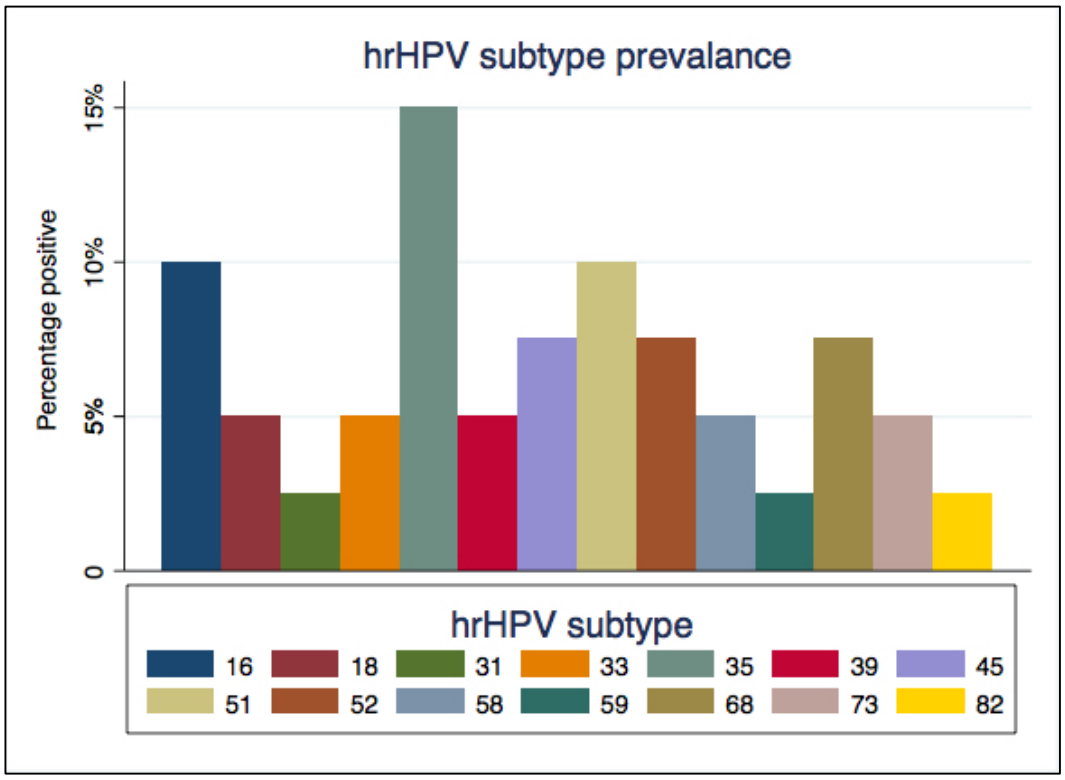

Figure 4.9: *High-risk HPV subtype prevalence*

Restricting attention to high-risk subtypes showed a similar picture. Distribution of high-risk subtypes was heterogenous with 14 separate high risk types detected (see figure 4.9*)*. HPV-35 was the most prevalent with 15%, but the majority of other types infected at least 5% of women.

Overall HPV detection showed no association with age (p-value = 0.402), BMI (p-value = 0.3243), gestation (p-value = 0.9061) or birth weight (p-value = 0.4978) using unpaired t-test, or with parity (p = 0.231), previous pregnancy loss (p=0.769) or HIV status (p=0.722) using Pearson's chi-squared test.

4.4 Association between cervical HPV and clinical pre-eclampsia

Table 4.7: *The association between cervical HPV and clinical pre-eclampsia*

	Cases	**Controls**	**Total**	**OR [95% CI] (p-value)**
Number	21	19	40	
Any HPV	11 (52.4%)	14 (74,7%)	25 (62.5%)	0.39 [0.1 - 1.8] (p=0.16)
hrHPV	10 (47.6%)	10 (52.6%)	20 (50.0%)	0.8 [0.2 – 3.4] (p = 0.75)
lrHPV	9 (42.9%)	11 (57.9%)	20 (50.0%)	0.5 [0.1 – 2.3] (p = 0.34)
Single subtype	1 (4.8%)	6 (32.6%)	7 (17.5%)	p = 0.70 (One-way ANOVA)
Multiple subtypes	10 (47.6%)	8 (42.1%)	18 (45.0%)	
No subtypes detected	10 (47.6%)	5 (26.3%)	15 (37.5%)	

When combining all HPV positive women, there is a trend towards lower prevalence in pre-eclamptic women than in controls, without reaching statistical significance (p=0.17). When hrHPV and lrHPV are analysed separately, no association is apparent. Similarly, if women with single subtype infection and co-infection are compared to negative women using one-way ANOVA testing, no significant trend emerges (p = 0.70).

4.5 Association between cervical HPV and sFLT1 and PlGF levels

When analysing the association between cervical HPV and the tested biomarkers some trends are revealed. Levels of sFLT1 tend to be higher in women who tested negative (9164 pg/ml [95% CI, 6 417 – 11 911]) than in women who tested positive (6 735.3 pg/ml [95% CI, 4 420 - 9 049]) for any cervical HPV, although this trend fell short of statistical significance (p = 0.17).

There was no significant difference in mean PlGF values between women testing positive (202.0 pg/ml [95% CI,104.1 – 301.8]) and women testing negative (164.6 pg/ml [95% CI, 53.9 – 275.3]) (p = 0.61). This is similar for the sFLT1/PlGF ratio, with positive women showing a mean of 201.9 [95% CI, 46.3 – 357.4] and negative women showing a mean of 247.4 [95% CI, 111.1 – 383.8] (p = 0.68). These relationships are illustrated in Figures 4.10 – 4.12.

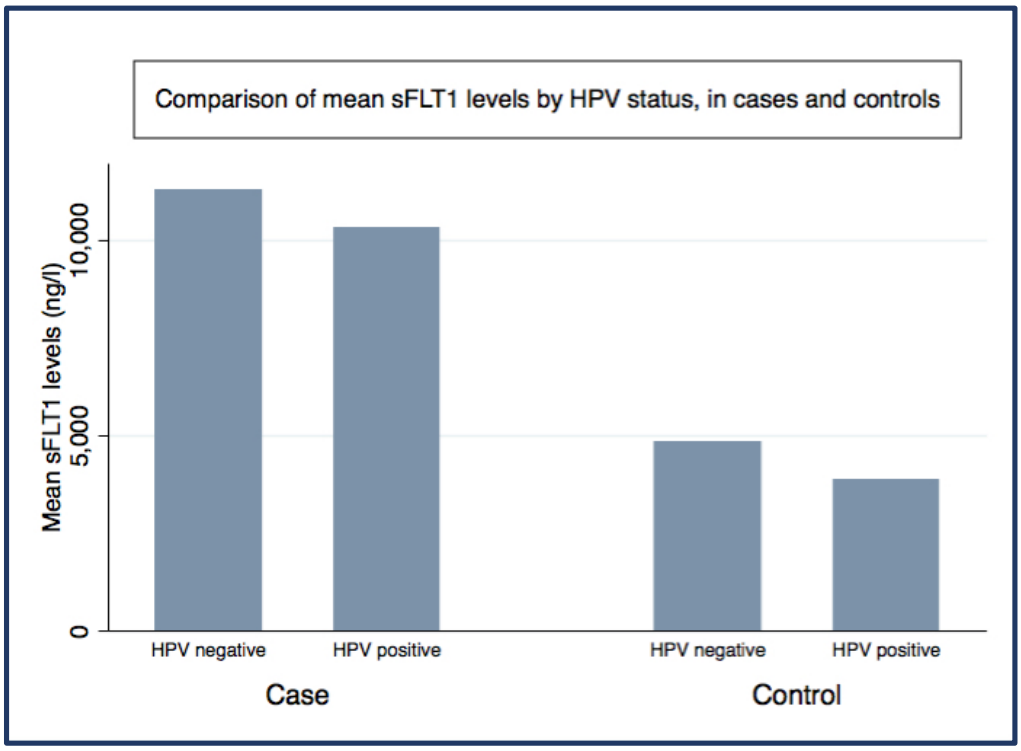

Figure 4.10: *sFLT1 levels by HPV status, cases versus controls*

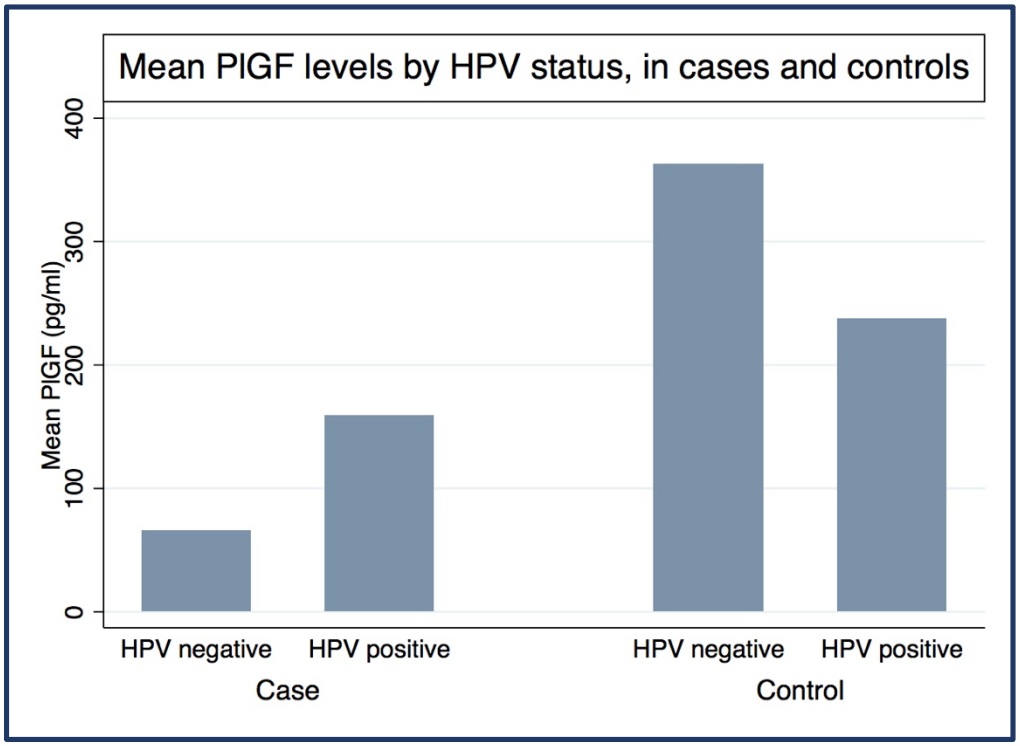

Figure 4.11: *PlGF levels by HPV status, cases versus controls*

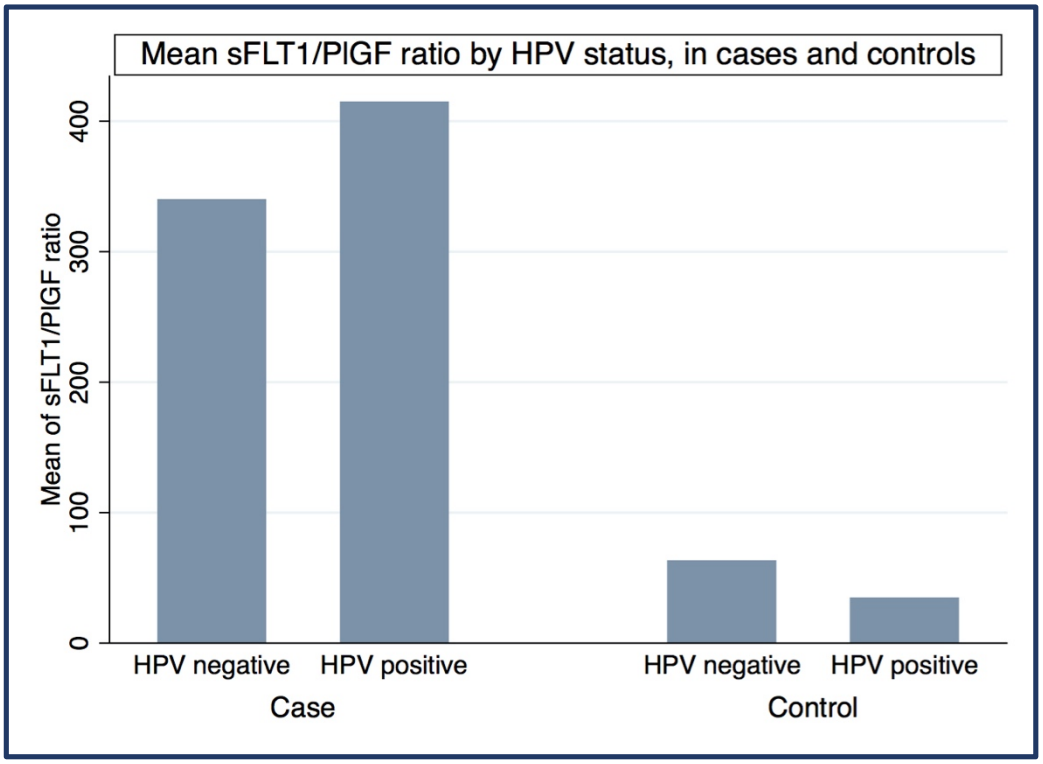

Figure 4.12: *sFLT1/PlGF ratio by HPV status, cases versus controls*

5. Discussion

5.1 Demographics, risk factors and clinical pre-eclampsia

The sample presented here was homogenous. The associations between age and parity and previous pregnancy loss can be explained by the higher likelihood for a woman to have had a previous pregnancy as she gets older. While the mean BMI in this group appears high, it should be borne in mind that these measurements were taken in the third trimester of pregnancy, when a reasonably expected weight gain during pregnancy of 10kg would add approximately 4kg/m^2 to the BMI of a 160cm-tall woman. The association between BMI and parity is expected and has been described (123). The overall prevalence of HIV in this sample of 30% is congruent with reported rates of prevalence of HIV in pregnancy in South Africa (124). The association between HIV status and age also reflects the increasing probability of contracting HIV the longer the duration of a women's reproductive activity. The association between birth weight and gestation is expected and unremarkable.

There was a similar distribution of demographics and risk factors between women with and without clinical pre-eclampsia. None of the non-modifiable risk factors for pre-eclampsia were significantly different between the groups. The negative association between gestation and birth weight and clinical pre-eclampsia reflects earlier intervention and delivery in women with pre-eclampsia.

Of interest is the similarity in distribution of parity between the groups, given that nulliparity is a described risk factor for pre-eclampsia (36). There is also no clear correlation between gestational age at delivery and parity in this sample.

5.2 Demographics, risk factors and biomarker levels

Biomarker levels correlated well with the presence of clinical pre-eclampsia. The one outlier with very high sFLT1 levels but without clinical pre-eclampsia was a woman with large obstructive fibroids that mandated delivery by caesarean section. The effects of fibroids on VEGF levels have been previously described (125), but the effects on sFLT1 levels are unknown.

A correlation between parity and previous pregnancy loss with increased sFlt1 levels was found. It is not possible to determine in this small sample whether the association is due to parity or age or to a combination of the two.

5.3 Detection of HPV

5.3.1 Detection of placental HPV

The finding of no HPV detection in any placentae, although unexpected, is significant. There is considerable variation in the reported literature regarding the prevalence of placental HPV, ranging from 4.2% (114) to 57% (8). Several factors can be considered to be responsible for this.

Firstly, the method of collection of the specimen is of particular interest. As demonstrated here, cervical HPV infection is very common. Viral particles were collected from the cervix in this study using a cervical brush which is atraumatic to tissues. It is therefore not inconceivable that the placenta may be contaminated with viral particles simply by passage through the cervix. While this may lead to detection of viral particles by PCR testing, it is unknown whether infection of trophoblast can take place after delivery of the placenta. In the studies that reported the prevalence of placental HPV (8, 114, 115) the route of delivery and incidence of rupture of membranes prior to delivery was not reported and it is uncertain whether that played a role in the detection of HPV.

Secondly, the method of testing may affect the findings. Previous studies which found low prevalence of HPV in the placenta made use of PCR testing (114, 115, 116). The study which found higher prevalence made use of the L1 antibody staining (8). There are of course two possible explanations for this. First would be that L1 antibody staining has a high level of interference and false positives. The second possibility is that PCR testing has an unacceptably low sensitivity. In the light of its high reliability and very high sensitivity (109) when used for cervical smear testing, this would appear unlikely.

Based on the evidence in this study, the conclusion is that there is no evidence of detectable levels of HPV DNA in placental tissue, and consequently no relationship between placental HPV and clinical pre-eclampsia, or serum levels of sFLT1 and PlGF.

5.3.2 Detection of cervical HPV

Cervical HPV is very common in this sample. This is the first description of cervical HPV prevalence in a pregnant population in South Africa. Using the same testing, the findings were very similar to that found in the earlier study by Richter *et al*. The overall rate of HPV infection was 62.5% in this study compared to 74.6% found by Richter *et al,* high-risk HPV infection 50% compared to 54.3% and the presence of multiple subtypes 45% compared to 52.7%. However, the rates of cervical cytological abnormality found by Richter *et al* was much lower (17.6%) than the rates of HPV infection (105).

This similarity with non-pregnant populations is congruent with findings from the United States of similar HPV prevalence rates between pregnant (42%) and non-pregnant women (41%) (107). A previous meta-analysis of 14 studies from Asia, Europe, North America and Australia found an overall rate of 16.8% against 12.3% in non-pregnant women, although with variations in prevalence in pregnant women from 9.6% in Italy to 37.1% in Mexico (126).

5.4 Cervical HPV and pre-eclampsia

No association between HPV detection and clinical pre-eclampsia could be demonstrated in this sample, and indeed, the observed values showed the opposite of the hypothesis with lower rather than higher prevalence of pre-eclampsia in women with cervical HPV. This observation differs from what is expected from the findings of McDonnold *et al*, where a positive association was found between implied cervical HPV infection and clinical pre-eclampsia. (7).

5.5 Cervical HPV and sFLT1 and PlGF

The relationship between cervical HPV and sFLT1 and PlGF levels was opposite to what was hypothesized, with consistent higher sFLT1 and lower PlGF. It remains to be determined whether this relationship would hold true in a larger sample, to reach statistical significance. Based on the findings here, with mean sLFT1 levels of 4 148 pg/l in the HPV negative group and 3 895 pg/l in the HPV positive group, with a standard deviation of 2 132 pg/l, and assuming an HPV prevalence of 62.5%, using a standard power calculation a group with more than 306 participants would be necessary to reach statistical significance.

The hypothesis that HPV would be associated with clinical pre-eclampsia and with raised levels of sFLT1 was thus not borne out. There are three assumptions that underpin this hypothesis that deserves re-examination.

McDonnold *et al* found that cervical cytological abnormality was associated with clinical pre-eclampsia (7). The first assumption is that cervical cytological abnormality is associated with cervical HPV infection. The corollary is that clinical pre-eclampsia must be associated with cervical HPV. The third assumption is that clinical pre-eclampsia is associated with raised sFLT1 levels.

The association between pre-eclampsia and sFLT1 levels stood up in this sample. The association between cervical HPV infection and cervical cytological abnormality on the other hand is worth considering. While it is generally accepted that HPV is the aetiological agent in abnormal cervical cytology, infection does not inevitably lead to

cytological changes, and indeed the majority of infections are cleared without neoplasia developing (105).

This poses the question whether a temporal relationship exists between HPV infection and effects on the VEGF system. HPV DNA detection does not imply integration into the cellular DNA, or cytological abnormality (105). It can be postulated that the presence of cytological abnormality changes the effects that HPV has on the developing pregnancy.

In addition, whether early HPV infection, which is associated with the expression of E5 (101), is associated with lower levels of sFLT1, whereas later stages of infection, associated with abnormal cervical cytology and where E5 expression has been lost (101), is associated with higher levels and more pre-eclampsia may be grounds for future research.

Whether these effects will also be demonstrable in the first trimester would be of interest. When one considers the known association between HPV and spontaneous miscarriage (6) and the known association between lower first trimester sFLT1 levels and miscarriage (97), this raises the possibility that its effects on sFLT1 levels may be the link between HPV and miscarriage. In addition, should such a link exist, the molecular mechanisms whereby HPV affects sFLT1 levels and VEGF remain to be elucidated.

In a thought experiment, its association with first trimester miscarriage and potentially protective role against pre-eclampsia raises the possibility of a symbiotic role for HPV in the human cervix. It is well known that historically the third trimester of pregnancy, in addition to childbirth, held very high morbidity and mortality in the era before modern obstetrics (24). Any agent that would first act as an abortifacient in the first trimester, when morbidity and mortality were lower, and secondly decrease the incidence of one of the major complications of pregnancy, would conceivably confer reproductive advantage.

The oncogenic effects of HPV would only have its deleterious effects on survival later, after its effects on reproduction. This stimulates further interesting questions on the full role of this ubiquitous virus in the human organism.

5.6 Conclusion

In conclusion, in the absence of any HPV in placental samples, no association was found between the presence of HPV in the placenta at delivery and clinical pre-eclampsia. Similarly, no association could be demonstrated between placental HPV and sFLT1 and PlGF levels, or with cervical HPV, or between different methods of testing the placenta.

There was no association between cervical HPV and clinical pre-eclampsia found. There was a non-significant trend towards lower sFLT1 levels in subjects with cervical HPV.

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7. APPENDICES

7. 1 Appendix A: Ethics approval

R14/49 Dr Francois Retief

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150854

NAME: (Principal Investigator)	Dr Francois Retief
DEPARTMENT:	Obstetrics and Gynaecology Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	The Association Between Incidence of Placental Human Papillomarivus Detection and Preeclampsia in Two Academic Hospitals
DATE CONSIDERED:	28/08/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Haroun Rhemtula
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/11/2015

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

7.2 Appendix B: Permission to conduct research from CHBAH

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

MEDICAL ADVISORY COMMITTEE
CHRIS HANI BARAGWANATH ACADEMIC HOSPITAL

PERMISSION TO CONDUCT RESEARCH

Date: 8 October 2015

TITLE OF PROJECT: The association between incidence of placental human papillomavirus detection and pre-eclampsia in two academic hospitals in Johannesburg

UNIVERSITY: Witwatersrand

Principal Investigator: F Retief

Department: Obstetrics and Gynaecology

Supervisor (If relevant): H Rhemtula; M Bothma

Permission Head Department (where research conducted): Yes

RECEIVED BY F. Retief
DATE: 19/10/2015

Date of start of proposed study: January 2016
Date of completion of data collection: December 2017

The Medical Advisory Committee recommends that the said research be conducted at Chris Hani Baragwanath Hospital. The CEO /management of Chris Hani Baragwanath Hospital is accordingly informed and the study is subject to:-

- Permission having been granted by the Human Research Ethics Committee of the University of the Witwatersrand.
- the Hospital will not incur extra costs as a result of the research being conducted on its patients within the hospital
- the MAC will be informed of any serious adverse events as soon as they occur
- permission is granted for the duration of the Ethics Committee approval.

.....
Recommended
(On behalf of the MAC)
Date: 08 October 2015

.....
Approved/Not Approved
Hospital Management
Date: 08/10/15

7.3 Appendix C: Permission to conduct research from CMJAH

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

CHARLOTTE MAXEKE JOHANNESBURG ACADEMIC HOSPITAL

Enquiries:
Mr. J. Maepa
Office of the Clinical Director
Tell: (011): 488-3365
Email:Johannes.maepa@gauteng.gov.za
07 October 2015

Dear Dr. F. Retief

STUDY TITLE: The association between Incidence of placental human papillomarivus detection and preeclampsia in udult woman giving birth in two academic hospitals in Johannesburg

Permission is granted for you to conduct the above recruitment activities as described in your request provided:

1. Charlotte Maxeke Johannesburg Academic Hospital will not anyway incur or inherit costs as result of the said study.
2. Your study shall not disrupt services at the study sites.
3. Strict confidentiality shall be observed at all times.
4. Informed consent shall be solicited from patients participating in your study.

Please liaise with the HOD and Unit Manager or sister in charge to agree on the dates and time that would suit all parties.

Kindly forward this office with the results of your study on completion of the research.

Supported/~~not supported~~

Dr. M.I. Mofokeng
Clinical Director
DATE: 7/10/2015

~~Approved~~/~~not approved~~

Ms. G. Bogoshi
Chief Executive Officer
DATE: 7/10/2015

7.4 Appendix D: Data collection instrument

Confidential

Preeclampsia Risk Factors

Please use the number printed at the top of the consent form and on the specimen bottle as the record number. All fields are mandatory.

Exclusion criteria

	yes	no
Pre-existing hypertension?	◯	◯
Pre-existing Diabetes Mellitus?	◯	◯
Pre-existing kidney disease?	◯	◯
Known thrombophilia?	◯	◯
Known anti-phospholipid syndrome?	◯	◯
Known auto-immune disease?	◯	◯
Multiple pregnancy?	◯	◯
History of pre-eclampsia in patient's mother?	◯	◯
Smoked during third trimester?	◯	◯
Use of infertility treatment in the conception of the index pregnancy?	◯	◯
Vaccinated against HPV?	◯	◯

Date _____

Study number _____
(Use the study number on the consent form and matching specimen kit)

Patient name _____

Age _____

Diagnostic Criteria

Diagnostic criteria for hypertension
◯ BP > 140/90, 2 occasions 6h apart
◯ BP >160/110

Proteinuria
☐ >1+ protein on dipstick
☐ u-PCR > 0.025
☐ du-Prot > 3g

Organ system dysfunction
◯ Yes
◯ No
(Includes CNS, respiratory, CVS, hepatic, haematological, and fetoplacental dysfunction)

Parity
◯ nulliparous
◯ multiparous

Exposure factors

Previous non-viable pregnancy loss?
◯ Yes
◯ No
(Includes TOP, first and second trimester miscariages)

Duration of cohabitation with father of index pregnancy
◯ < 1 year
◯ > 1 year
◯ unknown

History of preeclampsia in previous partners of the father of the index pregnancy
◯ no
◯ yes
◯ unknown

Miscellaneous risk factors

Height
_____
(In centimetres)

Weight
_____
(In kg)

BMI
_____

HIV status?
◯ negative
◯ positive
◯ unknown

On ARV's?
◯ Yes
◯ No

Previously had a pap smear?
◯ Yes
◯ No

Any previous abnormal smear result?
☐ none
☐ LSIL
☐ HSIL
☐ ASCUS
☐ Don't know

Had a colposcopy?
☐ No
☐ Yes but no LLETZ
☐ Yes and had LLETZ
☐ No but had cone biopsy

Investigator

Name of person completing
_____

7.5 Appendix E: Patient information sheet and consent form

PARTICIPANT INFORMATION DOCUMENT

Study title: The association between incidence of placental human papillomavirus detection and pre-eclampsia in adult women giving birth in two academic hospitals in Johannesburg

Good day, my name is Francois Retief and I would like to invite you to take part in a study I am conducting. I am a doctor in this hospital and I work in the maternity department. I am doing research on the causes of high blood pressure in pregnancy.

In this study I want to learn whether the presence of human papillomavirus (HPV) in the placenta is associated with a disease called pre-eclampsia. This is a disease that occurs in pregnant women that causes high blood pressure and passing of protein in the urine, and may cause severe organ damage and even death. HPV is a virus that infects the female reproductive tract and may cause warts but may also be present without any outward sign, and in some forms causes cancer of the cervix. In addition, I will be comparing two different ways of testing the placenta for HPV, and testing whether having HPV in your placenta changes some of the substances your placenta releases into your blood.

I would like to invite you to take part in this study. In this study I will be comparing the placentas of women with high blood pressure with the placentas of healthy women to detect human papillomavirus. After testing the placenta will be destroyed, the same as it would be if you didn't take part in the study. In addition, I will be testing your cervix for the papillomavirus, similar to undergoing a pap smear test. Your blood will also be tested for two substances made by the placenta that are increased in pre-eclampsia, namely soluble fms-like tyrosine kinase and placental growth factor.

If you agree to take part, your doctor will ask you some questions about your risk factors for pre-eclampsia, and look for some information in your file. This will take about five minutes of your time.

Before your caesarean section, your doctor will collect a blood sample from you. This blood will be tested for soluble fms-like tyrosine kinase and placental growth factor, two substances in your blood that is made by your placenta, and that are increased in women with pre-eclampsia. Also before your caesarean section the doctor will also do a cervical smear on your cervix. To do this they will pass a speculum into the vagina, and brush your cervix gently to obtain some of the cells lining your cervix. This will be done after you have received your anaesthetic, so you will experience no additional discomfort, and it holds no extra risk for you. When your baby has been delivered a piece of your placenta will be collected to test, as well as brushing your placenta to collect cells to test for the papillomavirus.

There is no risk to you or your baby in taking part in this study. Because the test on your cervix may reveal that you have an increased risk of developing cervical cancer in the future, doing this test may benefit you by discovering this earlier. The information we get from this study may benefit other women in the future.

You will be requested to provide your contact details, as well as the details of your preferred primary care provider (clinic or GP) so if your cervical smear test indicates increased risk, I will contact you with your result via your preferred means of communication, and provide you with a referral to your preferred health care facility.

If you prefer not to, you are under no obligation to take part in this study. If you choose not to take part, you and your baby will receive exactly the same care and treatment. You may decide at any time not take part any more. If you decide to withdraw from the study, the care and treatment you and your baby receives will not be affected in any way. If you wish to withdraw from this study, you can do so by contacting me at the phone number or email provided, and your information will be withdrawn from the study.

You will be provided with any additional information that becomes available during the course of the study, which may influence your decision to take part in the study.

You will not be paid to take part in this study, and it will not cost you anything to participate in this study.

Your personal information will be kept confidential and secret and will only be made available with your permission or as required by law. Any information that can identify you personally will be removed once your information has been collected. A separate record of study participants will be kept securely in order to be able to trace your information, should you wish to withdraw your consent to take part, and to contact you with your results, if they are positive.

You can contact me on phone 082 545 0768, or email 1017956@students.wits.ac.za, if you have any questions or if you wish to withdraw your consent. If you have any questions about your rights as a participant, or have any concerns about the conduct of the study, you may contact Prof Cleaton-Jones, Chairman of the University of the Witwatersrand Human Research Ethics Committee (Medical), which is an independent committee established to protect the rights of research participants, at 011 717 2301.

CONSENT FORM

I, ……., confirm that the information contained in this form has been explained to me in a language that I understand, and I am willing to take part in this study.

☐ I WOULD like to be informed if my test results show increased risk

☐ I WOULD NOT like to be informed if my test results show increased risk

My preferred contact details are:

……

……

……

My preferred health care facility is:

…….

I understand that I can withdraw this consent at any time.

Signed …….. at (place) …….. on (date) ……..

By (print name) …….

Witness ..……

Investigator ……..…….

7.6 Appendix F: Linear Array package insert

LINEAR ARRAY HPV Genotyping Test

FOR *IN VITRO* DIAGNOSTIC USE.

AmpliLute Liquid Media Extraction Kit	EXTRN	50 Tests	P/N: 03750540 190
LINEAR ARRAY HPV Genotyping Test	LA HPV GT	48 Tests	P/N: 04391853 190
LINEAR ARRAY Detection Kit	LA DK	96 Tests	P/N: 03378179 190
24-Well Tray with Lid		1 Each	P/N: 03140725 001

INTENDED USE

The LINEAR ARRAY HPV (Human Papilloma Virus) Genotyping Test is a qualitative *in vitro* test for the detection of Human Papilloma Virus in clinical specimens. The test utilizes amplification of target DNA by the Polymerase Chain Reaction (PCR) and nucleic acid hybridization and detects thirty seven anogenital HPV DNA genotypes [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39 and CP6108] in cervical cells collected in cobas® PCR Cell Collection Media or PreservCyt® Solution.

SUMMARY AND EXPLANATION OF THE TEST

Persistent infection with human papillomavirus (HPV) is the principal cause of cervical cancer and its precursor cervical intraepithelial neoplasia (CIN)[1-3]. The presence of HPV has been implicated in greater than 99% of cervical cancers, worldwide[3]. HPV is a small, non-enveloped, double-stranded DNA virus, with a genome of approximately 8000 nucleotides. There are more than 100 different genotypes of HPV, and approximately 40 different HPV genotypes that can infect the human genital mucosa. However, only a subset of these sexually-transmitted viral genotypes are associated with high-grade cervical dysplasia and cervical cancer[3-5]. These are termed high-risk HPV genotypes, while the low-risk HPV genotypes are often associated with benign low-grade intraepithelial lesions or condylomas. Sexually transmitted infection with HPV is extremely common, with estimates of up to 75% of all women experiencing exposure to HPV at some point[6]. The majority of HPV infections clear spontaneously, but persistence of a high-risk HPV is a significant risk factor for the development of cervical cancer.

In developed countries with cervical cancer screening programs, the Pap smear (named after Dr. George Papanicolaou) has been used since the mid-1950s as the primary tool to detect early precursors to cervical cancer. Although it has decreased the death rates due to cervical cancer dramatically in those countries, the Pap smear requires interpretation by highly trained cytopathologists and is a relatively inaccurate test with a high rate of false negatives. Cytological abnormalities observed in the Pap smear are primarily due to infection with HPV; however, various inflammatory or sampling variations can result in false positive Pap results. Triage of an abnormal Pap smear involves repeat testing, colposcopy, and biopsy. A histologically confirmed high-grade lesion must be surgically removed in order to prevent the development of invasive cervical cancer.

Papillomavirus is extremely difficult to culture *in vitro*, and not all patients infected with HPV have a demonstrable antibody response. Therefore, nucleic acid (DNA) testing by PCR is a sensitive and non-invasive method for determining the presence of an active cervical HPV infection. Reported applications of an HPV typing test include the evaluation of acquisition and clearance of specific HPV types[7]; monitoring type-specific persistence of genotypes associated with higher risk of cervical disease and cervical carcinogenesis[8]; effectiveness of excisional therapy[9], radiotherapy[10], or chemotherapy[11] towards HPV-associated lesions (test of cure); genotyping in selected screening programs pre- and post-vaccine introduction[12]; and facilitation of epidemiologic investigations regarding the natural history of HPV infections.

PRINCIPLES OF THE PROCEDURE

The LINEAR ARRAY HPV Genotyping Test is based on four major processes: specimen preparation; PCR amplification[13] of target DNA using HPV primers; hybridization of the amplified products to oligonucleotide probes; and detection of the probe-bound amplified products by colorimetric determination.

The Document Revision Information section is located at the end of this document.

The specimen preparation using the AmpliLute Liquid Media Extraction Kit yields HPV target DNA and human genomic DNA suitable for PCR amplification. The Master Mix reagent contains primers for the amplification of DNA from 37 HPV genotypes and the human ß-globin gene. The detection and genotype determination is performed using the denatured amplified DNA and an array of oligonucleotide probes that permit independent identification of individual HPV genotypes.

Specimen Preparation

HPV DNA is released by lysing cervical cell specimens under denaturing conditions at elevated temperatures. Lysis is performed in the presence of proteinase K, chaotropic agent and detergent and is followed by isolation and purification of DNA over a column and elution with elution reagent. The ß-globin gene is isolated concurrently and assesses cellular adequacy, extraction and amplification for each individually processed specimen.

PCR Amplification

Target Selection

The LINEAR ARRAY HPV Genotyping Test uses biotinylated primers to define a sequence of nucleotides within the polymorphic L1 region of the HPV genome that is approximately 450 base pairs long. A pool of HPV primers present in the Master Mix is designed to amplify HPV DNA from 37 HPV genotypes[14] including 13 high risk genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68)[3-5]. Capture probe sequences are located in polymorphic regions of L1 bound by these primers.

An additional primer pair targets the human ß-globin gene to provide a control for cell adequacy, extraction and amplification.

Target Amplification

AmpliTaq® Gold DNA Polymerase is utilized for "hot start" amplification of the HPV target DNA and the ß-globin control. First, the PCR reaction mixture is heated to activate AmpliTaq® Gold DNA polymerase, to denature the viral DNA and human genomic DNA and to expose the primer target sequences. As the mixture cools, the primers (both upstream and downstream) anneal to the target DNA. The AmpliTaq® Gold DNA polymerase, in the presence of Mg^{2+} and excess dNTPs, extends the annealed primers along the target templates to produce an approximately 450 base pair double-stranded HPV target DNA molecule or a 268 base pair ß-globin DNA molecule termed an amplicon. This process is repeated for a number of cycles, each cycle effectively doubling the amount of amplicon DNA. Amplification occurs only in the region of the HPV genome or ß-globin gene between the appropriate primer pair. The entire genome is not amplified.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the LINEAR ARRAY HPV Genotyping Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine[15], but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate in addition to deoxythymidine triphosphate in the Master Mix reagent; therefore, only amplicon contain deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by AmpErase enzyme prior to amplification of the target DNA. AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at the alkaline pH of Master Mix, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. AmpErase enzyme is inactive at temperatures above 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon. Following amplification, any residual enzyme is denatured by the addition of the Denaturation Solution, thereby preventing the degradation of any target amplicon.

Hybridization Reaction

Following PCR amplification, the HPV and the ß-globin amplicon are chemically denatured to form single-stranded DNA by the addition of Denaturation Solution. Aliquots of denatured amplicon are then transferred to the appropriate well of the typing tray that contains hybridization buffer and a single LINEAR ARRAY HPV Genotyping Strip that is coated with HPV and ß-globin probe lines. The biotin-labeled amplicon will hybridize to the oligonucleotide probes only if the amplicon contains the matching sequence of the complementary probe. In addition, the LINEAR ARRAY HPV Genotyping Strip is coated with one cross-reactive oligonucleotide probe that hybridizes with HPV genotypes 33, 35, 52 and 58. Amplicon containing closely matching sequences (only 1 to 3 mismatches) complementary to the probe will hybridize to this probe line.

Detection Reaction

Following the hybridization reaction, the LINEAR ARRAY HPV Genotyping Strip is stringently washed to remove any unbound material. Streptavidin-Horseradish Peroxidase Conjugate is then added to the strip. The Streptavidin-Horseradish Peroxidase Conjugate binds to the biotin-labeled amplicon hybridized to the oligonucleotide probes on the strip. The strip is washed to remove any unbound Streptavidin-Horseradish Peroxidase Conjugate and a substrate solution containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine (TMB) is added to each strip. In the presence of hydrogen peroxide, the bound streptavidin-horseradish peroxidase catalyzes the oxidation of TMB to form a blue colored complex, which precipitates at the probe positions where hybridization occurs. The LINEAR ARRAY HPV Genotyping Strip is then read visually by comparing the pattern of blue lines to the LINEAR ARRAY HPV Genotyping Test Reference Guide.

REAGENTS

AmpliLute Liquid Media Extraction Kit EXTRN **50 Tests**

P/N: 03750540 190

CAR 1 x 310 µg
(Carrier RNA)

Synthetic RNA, lyophilized
(Add AVE)

PK 1 x 1.25 mL
(Proteinase K)

Proteinase K, Serine Proteinase, tritirachium album

Xn  Proteinase K

Harmful

R: 36/37/38-42/43	Irritating to eyes, respiratory system and skin. May cause sensitization by inhalation and skin contact.
S: 23-24-26-36/37	Do not breathe spray. Avoid contact with skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing and gloves.

AVE 4 x 2 mL
(Elution Buffer)

RNase-free water
< 0.09% Sodium azide

AW2 1 x 13 mL
(Wash Buffer 2)

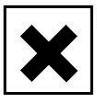
Tris-HCl buffer
< 0.09% Sodium azide
(Add absolute ethanol)

ATL 1 x 10 mL
(Tissue Lysis Buffer)

EDTA
≤ 10% Sodium dodecyl sulphate

AL 1 x 33 mL
(Lysis Buffer)

≤ 50% Guanidine HCl

Xn  25-50% Guanidine HCl

Harmful

R: 22-36/38	Harmful if swallowed. Irritating to eyes and skin.
S: 13-26-36-46	Keep away from food, drink and animal foodstuffs. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. If swallowed, seek medical advice immediately and show this container or label.

EXT 50 pcs
(Column Extenders, 3 mL)

VC 50 pcs
(VacConnectors)

ELT 50 pcs
(Elution Tubes, 1.5 mL)

CLM 50 pcs
(QIAamp® MinElute® Columns)

Silica membrane

LINEAR ARRAY HPV Genotyping Test LA HPV GT **48 Tests**
(P/N: 04391853 190)

HPV MMX 4 x 0.58 mL
(LINEAR ARRAY HPV Master Mix)

Tris buffer
Potassium chloride
< 0.02% AmpliTaq® Gold DNA Polymerase (microbial)
< 0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial)
< 0.001% dATP, dCTP, dUTP, dGTP, dTTP
< 0.001% Each of upstream and downstream primers (biotinylated)
0.06% Sodium azide

HPV Mg^{2+} 4 x 0.125 mL
(LINEAR ARRAY HPV Magnesium Solution)

< 1% Magnesium chloride
Amaranth dye
0.05% Sodium azide

HPV (+) C 4 x 0.5 mL
(LINEAR ARRAY HPV Positive Control)

Tris-HCl buffer
EDTA
< 0.002% Poly rA RNA (synthetic)
< 0.001% Non-infectious plasmid DNA (microbial) containing HPV sequences
< 0.001% Non-infectious plasmid DNA (microbial) containing human ß-globin sequences
0.05% Sodium azide

HPV (–) C 4 x 0.5 mL
(LINEAR ARRAY HPV Negative Control)

Tris-HCl buffer
EDTA
< 0.002% Poly rA RNA (synthetic)
0.05% Sodium azide

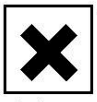
HPV Strip 4 x 12 Tests
(LINEAR ARRAY HPV Genotyping Strip)

Nylon strip coated with HPV DNA probes and 1 human ß-globin DNA probe (at high and low probe concentrations)

LINEAR ARRAY Detection Kit LA DK **96 Tests**
(P/N: 03378179 190)

DN 2 x 12 mL
(Denaturation Solution)

1.6% Sodium hydroxide
EDTA
Thymol blue

Xi  1.6% (w/w) Sodium hydroxide

Irritant

SDS 4 x 27 mL
(SDS Concentrate)

20% Sodium lauryl sulfate (SDS)
1% ProClin® 150 preservative

SSPE 2 x 160 mL
(SSPE Concentrate)

Sodium phosphate solution
Sodium chloride
EDTA
1% ProClin® 150 preservative

SA-HRP 2 x 2 mL
(Streptavidin-Horseradish Peroxidase Conjugate)

Streptavidin-horseradish peroxidase conjugate
ACES buffer
Sodium chloride
1% ProClin® 150 preservative

CIT 2 x 36 mL
(Citrate Concentrate)

Citrate solution

SUB A 3 x 160 mL
(Substrate A)

Citrate solution
0.01% Hydrogen peroxide
0.1% ProClin® 150 preservative

SUB B 3 x 40 mL
(Substrate B)

0.1% 3,3',5,5'-Tetramethylbenzidine (TMB)
40% Dimethylformamide (DMF)

T  40% (w/w) Dimethylformamide (DMF)

Toxic

R: 61-20/21-36	May cause harm to the unborn child. Harmful by inhalation and in contact with skin. Irritating to eyes.
S: 53-45	Avoid exposure – obtain special instructions before use. In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

WARNINGS AND PRECAUTIONS

A. **FOR *IN VITRO* DIAGNOSTIC USE.**

B. This test is for use with human cervical cells collected in cobas® PCR Cell Collection Media or PreservCyt Solution.

C. Do not pipet by mouth.

D. Do not eat, drink or smoke in laboratory work areas. Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and kit reagents. Wash hands thoroughly after handling specimens and test reagents.

E. Avoid microbial and DNA contamination of reagents when removing aliquots from reagent bottles. The use of sterile disposable pipets, DNA-free and DNase-free pipet tips is recommended.

F. Do not pool reagents from different lots or from different bottles of the same lot.

G. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

H. Do not use a kit after its expiration date.

I. Safety Data Sheets (SDS) are available on request from your local Roche office.

J. Workflow in the laboratory must proceed in a uni-directional manner, beginning in the Pre-Amplification Area and moving to the Post-Amplification (Amplification/Detection) Area. Pre-amplification activities must begin with reagent preparation and proceed to specimen preparation. Supplies and equipment must be dedicated to each pre-amplification activity and not used for other activities or moved between areas. Gloves must be worn in each area and must be changed before leaving that area. Equipment and supplies used for reagent preparation must not be used for specimen preparation activities or for pipetting or processing amplified DNA or other sources of target DNA. Post-amplification supplies and equipment must remain in the Post-Amplification Area at all times.

K. Specimens should be handled as if infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[16] and in the CLSI Document M29-A3[17]. Thoroughly clean and disinfect all work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in deionized or distilled water.

NOTE: Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.

L. **AW2**, **AVE**, **HPV MMX**, **HPV Mg^{2+}**, **HPV (–) C** and **HPV (+) C** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of water to prevent azide buildup.

M. Wear eye protection, laboratory coats and disposable gloves when handling **AL**, **PK**, **HPV MMX**, **HPV Mg^{2+}**, **DN**, **SA-HRP**, **SUB A**, **SUB B** and Working Substrate (mixed **SUB A** and **SUB B** reagent). Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills of these reagents occur, dilute with water before wiping dry.

N. **SUB B** and Working Substrate contain dimethylformamide which has been reported to be toxic in high oral doses and may be harmful to the unborn child. Skin contact, inhalation of fumes and ingestion must be avoided. If skin contact occurs, wash thoroughly with soap and water and seek medical advice immediately.

O. **AL** contains guanidine hydrochloride, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilled, clean with suitable laboratory detergent and water. If the spilled liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 0.5% sodium hypochlorite. Do not add bleach or acidic solutions directly to waste containing **AL**.

P. Glass or plastic containers that are under vacuum negative pressure have the potential to implode during use, which may result in personal injury. Eye protection is strongly recommended whenever glass or plastic containers are used under partial vacuum negative pressure to guard against injury. Only containers specifically designed for these applications should be used. Do not use a container for vacuum applications if it is not designed to withstand a vacuum; if the container is scratched, chipped or cracked; if the container is clamped in such a way as to induce stress; or if the container is being hand held.

Q. Use only LINEAR ARRAY HPV Master Mix with the LINEAR ARRAY HPV Genotyping Test. AMPLICOR® HPV Master Mix cannot be used with the LINEAR ARRAY HPV Genotyping Test.

STORAGE AND HANDLING REQUIREMENTS

A. ***Do not freeze reagents unless otherwise noted.***

B. Store the **CLM** and **PK** from the AmpliLute Liquid Media Extraction Kit at 2-8ºC. Store other reagents from the AmpliLute Liquid Media Extraction Kit at 2-25ºC. Once opened, any unused portion of **PK** can be stored up to 2 months at 2-8ºC and **ATL** and **AL** can be stored up to 2 months at 2-25ºC or until the expiration date, whichever comes first. Once reconstituted with **AVE**, **CAR** may be stored for up to 24 hours at 2-8ºC or aliquoted and stored at -20ºC for up to 2 months, or until the expiration date, whichever comes first. Once absolute ethanol is added to **AW2**, any unused portion may be stored at 2-25ºC for up to 2 months or until the expiration date, whichever comes first. Once the reconstituted **CAR** is added to **AL**, the working **AL** may be stored at 2-8ºC for up to 48 hours.

C. Store **HPV MMX** and **HPV Mg^{2+}** at 2-8ºC. Unopened, these reagents are stable until the expiration date indicated. Once opened, any unused portion must be discarded. Working Master Mix (prepared by the addition of **HPV Mg^{2+}** to **HPV MMX**) must be stored at 2-8ºC and used within 6 hours of preparation.

D. Store **HPV (–) C** and **HPV (+) C** at 2-8ºC. Unopened, these reagents are stable until the expiration date indicated. Once opened, any unused portion must be discarded.

E. Store **HPV Strip** at 2-8ºC. Unopened, this reagent is stable until the expiration date indicated. Once opened, any unused strips must be discarded.

F. Store **DN** at 2-25ºC. Unopened, **DN** is stable until the expiration date indicated. Once opened, **DN** is stable for 3 months at 2-25ºC (or until the expiration date, whichever comes first).

G. Store **CIT, SA-HRP, SUB A** and **SUB B** at 2-8ºC. Unopened, these reagents are stable until the expiration date indicated. Once opened, these reagents are stable for 1 month at 2-8ºC (or until the expiration date, whichever comes first).

H. Store **SDS** and **SSPE** at 2-8ºC. Unopened, these reagents are stable until the expiration date indicated. Once opened, these reagents are stable for 1 month at 2-8ºC (or until the expiration date, whichever comes first). A precipitate forms in **SDS** and **SSPE** during storage at 2-8ºC. Prior to use, warm at 53ºC ± 2ºC for a maximum of 30 minutes and mix thoroughly to dissolve the precipitated material. Working Hybridization and Wash Buffers, prepared by diluting **SDS** and **SSPE** with distilled or deionized water, should be stored at room temperature in a clean, closed container and are stable for 30 days from the date of preparation.

I. Working Conjugate must be freshly prepared each day by mixing **SA-HRP** with Working Ambient Wash Buffer and is stable for 3 hours at ambient temperature.

J. Working Substrate must be freshly prepared each day by mixing **SUB A** with **SUB B** and is stable for 3 hours at ambient temperature when protected from light. Do not expose **SUB A**, **SUB B** or Working Substrate to metals, oxidizing agents or direct light.

K. Working Citrate Buffer, prepared by diluting **CIT** with distilled or deionized water, should be stored at room temperature in a clean, closed container and is stable for 30 days from the date of preparation.

MATERIALS PROVIDED

A. AmpliLute Liquid Media Extraction Kit EXTRN
(P/N: 03750540 190)

CAR
(Carrier RNA)

PK
(Proteinase K)

AVE
(Elution Buffer)

AW2
(Wash Buffer 2)

ATL
(Tissue Lysis Buffer)

AL
(Lysis Buffer)

EXT
(Column Extenders, 3 mL)

VC
(VacConnectors)

ELT
(Elution Tubes, 1.5 mL)

CLM
(QIAamp® MinElute® Columns)

B. LINEAR ARRAY HPV Genotyping Test LA HPV GT
(P/N: 04391853 190)

HPV MMX
(LINEAR ARRAY HPV Master Mix)

HPV Mg^{2+}
(LINEAR ARRAY HPV Magnesium Solution)

HPV (+) C
(LINEAR ARRAY HPV Positive Control)

HPV (–) C
(LINEAR ARRAY HPV Negative Control)

HPV Strip
(LINEAR ARRAY HPV Genotyping Strip)

Reference Guide
(LINEAR ARRAY HPV Genotyping Test Reference Guide)

C. LINEAR ARRAY Detection Kit LA DK
(P/N: 03378179 190)

DN
(Denaturation Solution)

SDS
(SDS Concentrate)

SSPE
(SSPE Concentrate)

SA-HRP
(Streptavidin-Horseradish Peroxidase Conjugate)

CIT
(Citrate Concentrate)

SUB A
(Substrate A)

SUB B
(Substrate B)

D. 24-Well Tray with Lid
(P/N: 03140725 001)

MATERIALS REQUIRED BUT NOT PROVIDED

Pre-Amplification – Reagent Preparation Area

- For the Applied Biosystems Gold-plated 96-Well GeneAmp® PCR System 9700, use MicroAmp® Reaction Tubes (AB# N801-0533), Caps (AB# N801-0535 or AB# N801-0534), Tray/Retainers (AB# 403081) or Ready-Pak MicroAmp Assembly (AB# 403083) and Base (AB# N801-0531)
- Plastic resealable bag
- Eppendorf Multipette® Pipet*
- 1.0 mL or 1.25 mL Eppendorf Combitip® plus (sterile, individually wrapped)*
- Pipettors (capacity 50 µL to 125 µL)* with aerosol barrier or positive displacement, DNA and DNase-free tips
- Disposable gloves, powderless

Pre-Amplification – Specimen and Control Preparation Area

- Eppendorf Multipette Pipet*
- 1.0 mL and 5.0 mL Eppendorf Combitip plus (sterile, individually wrapped)*
- Pipettors (capacity 20 µL, 200 µL and 1000 µL)* with aerosol barrier or positive displacement DNA and DNase-free tips
- Sterile 2.0 mL screw cap tubes (Sarstedt 72.693.005 or equivalent)
- Tube racks (Sarstedt 93.1428 or equivalent)
- Absolute ethanol - meets ACS specifications
- Sterile polypropylene conical tubes; 15 mL and 50 mL: (Corning 430052 and 430290 or equivalent)
- Sterile, disposable serological pipets (5 mL, 10 mL and 25 mL)
- Pipet-Aid® (Drummond 4-000-100 or equivalent)
- Microcentrifuge (min. RCF 12,500 x g); Eppendorf 5415C, HERMLE Z230M, or equivalent
- Vortex mixer
- 56ºC ± 2ºC and 70ºC ± 2ºC dry heat blocks
- Vacuum manifold (e.g., QIAvac 24, cat no. 19403; QIAvac 24 Plus, cat no. 19413 with QIAvac Connecting System, cat no. 19419)

- Vacuum Source [e.g., a pump capable of providing vacuum of -800 to -900 mbar, KNF Neuberger LABOPORT® model UN840.3FTP (115 V, 60 Hz) or model N840.3FT.18 (230 V, 50 Hz), or QIAGEN Vacuum Pump P/N: 84000 (110 V, 60 Hz), 84010 (115 V, 60 Hz), or 84020 (230 V, 50 Hz)] with tubing to the manifold
- Vacuum Regulator (QIAGEN cat no. 19530), optional
- Collection Tubes (2 mL, QIAGEN cat no. 19201), optional
- Disposable gloves, powderless

Post-Amplification – Amplification/Detection Area

- Gold-plated 96-Well GeneAmp PCR System 9700 (Applied Biosystems, P/N: 4314878)
- Sterile disposable, polystyrene serological pipets (5 mL, 10 mL and 25 mL)
- Multichannel pipettor (capacity 100 µL)*
- Pipettors (capacity 20 µL, 200 µL and 1000 µL)* with aerosol barrier or positive displacement DNA and DNase-free tips
- Vortex mixer
- Distilled or deionized water
- 53ºC ± 2ºC shaking water bath capable of approximately 60 RPM (Bellco Hotshaker Plus, P/N: 774622110 (115 V, 60 Hz), or P/N: 774622220 (230 V, 50 Hz), or equivalent)
- 53ºC ± 2ºC water bath
- Orbital shaker capable of approximately 60 RPM
- Forceps, stainless steel (VWR #: 30033-042 or equivalent)
- Water/chemical/heat-resistant permanent ink pen (Sharpie® Industrial Super Permanent Marker, P/N: 13801 or equivalent)
- Vacuum aspiration apparatus with an appropriate liquid collection reservoir (PYREX® brand heavy walled filter flask, Corning P/N: 5340-2L, or equivalent)
- 12-channel needle stream splitter/aspirator (Art Robbins Instruments, P/N: 102-5020-12)
- 1 lb. lead ring weight (VWR #: 29700-004 or equivalent)
- 1 L, 2 L, 3 L storage bottles or flasks
- 100 mL, 250 mL, 500 mL beakers
- 100 mL, 250 mL, 500 mL, 1 L graduated cylinders
- Eppendorf Multipette Pipet*
- 50 mL Eppendorf Combitip plus (sterile, individually wrapped)
- 50 mL adapter Eppendorf Biopur® (P/N: 0030 069.480)
- RBS35 Tray Cleaning Solution (VWR #: PI27952 / Pierce #: 27952)
- Disposable gloves, powderless

* Pipettors should be accurate within 3% of stated volume. Aerosol barrier or positive displacement DNA and DNase-free tips must be used where specified to prevent specimen and amplicon cross-contamination.

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

NOTE: Handle all specimens as if they are capable of transmitting infectious agents.

A. Specimen Collection

Only cervical cell specimens collected in cobas® PCR Cell Collection Media or PreservCyt Solution have been validated for use with the LINEAR ARRAY HPV Genotyping Test. Follow the manufacturer's instructions for collecting cervical cell specimens into cobas® PCR Cell Collection Media or PreservCyt Solution.

B. Specimen Transport

Cervical cell specimens collected in cobas® PCR Cell Collection Media or PreservCyt Solution can be transported at 2-30ºC. Transportation of cervical cell specimens must comply with county, federal, state and local regulations for the transport of etiologic agents[18].

C. Specimen Storage

Cervical cell specimens collected in cobas® PCR Cell Collection Media or PreservCyt Solution may be stored at room temperature for up to 21 days or at 2-8ºC for up to 12 weeks.

INSTRUCTIONS FOR USE

NOTE: Allow all reagents to equilibrate to room temperature (15-30ºC) before use unless otherwise noted. Visually examine all reagents for sufficient reagent volume before beginning the test procedure.

NOTE: Cervical cell specimens must be at room temperature (15-30ºC) before use.

NOTE: Use pipettors with aerosol barrier or positive displacement tips where specified. Use extreme care to ensure selective amplification.

Run Size:

Each AmpliLute Liquid Media Extraction Kit contains reagents sufficient for 50 tests. Each LINEAR ARRAY Detection Kit contains reagents sufficient for 96 tests. Each LINEAR ARRAY HPV Genotyping Test contains reagents sufficient for four 12-test runs, which may be performed separately or simultaneously. At least one replicate each of the LINEAR ARRAY HPV Negative Control and the LINEAR ARRAY HPV Positive Control must be included in each test run of up to 22 specimens (see *"Quality Control"* section).

The Amplification Reagents are packaged in 12-test, single-use vials. LINEAR ARRAY HPV Negative Control and LINEAR ARRAY HPV Positive Control are packaged in single-use vials. The LINEAR ARRAY HPV Genotyping Strips are packaged in 12-test, single-use pouches. For the most efficient use of reagents, specimens and controls should be processed in batches that are multiples of 12.

Workflow:

The LINEAR ARRAY HPV Genotyping Test can be completed in one day or over two days. If the testing is to be completed in a single workday, follow the instructions in Parts A through D in order. If the testing is to be completed over 2 days, the procedure may be stopped after Specimen and Control Preparation (Part B) or after Amplification (Part C).

To perform specimen and control preparation on Day 1 and amplification and detection on Day 2, perform Steps B.1 through B.29 and store the processed specimens and controls as indicated in Step B.29. On Day 2, begin with Part A (Reagent Preparation), then thaw processed specimens and controls at room temperature and continue with Step B.30.

NOTE: Do not subject processed specimens and controls to more than 1 freeze/thaw cycle.

To complete specimen and control preparation and amplification on Day 1 and detection on Day 2, perform Parts A (Reagent Preparation), B (Specimen and Control Preparation) and C (Amplification) on Day 1 and store the denatured amplicon at 2-8ºC as indicated in Step C.6. Continue with Part D (Genotype Detection with the LINEAR ARRAY HPV Strip) on Day 2.

A. Reagent Preparation

Performed in: Pre-Amplification – Reagent Preparation Area

1. Determine the appropriate number of reaction tubes needed for specimen and control testing. Place the tubes in the MicroAmp tray and lock in place with retainer.
2. Prepare Working Master Mix by adding 125 µL of **HPV Mg^{2+}** to one vial of **HPV MMX**. It is not necessary to measure the volume of Master Mix. Add 125 µL **HPV Mg^{2+}** to the entire vial of **HPV MMX**. Recap the tube and mix well by inverting the tube 10-15 times. Do not vortex the Working Master Mix. The pink dye in **HPV Mg^{2+}** is used for visual confirmation that **HPV Mg^{2+}** has been added to **HPV MMX**. Discard remaining **HPV Mg^{2+}**.
3. Add 50 µL of Working Master Mix into each reaction tube using a Multipette pipet or a pipettor with an aerosol barrier or positive displacement tip. Do not cap the reaction tubes at this time.
4. Place the tray containing Working Master Mix and the appropriate number of reaction tube caps in a resealable plastic bag and seal the plastic bag securely. Move to the Pre-Amplification – Specimen and Control Preparation Area. Store the tray(s) containing Working Master Mix at 2-8ºC in the Pre-Amplification – Specimen and Control Preparation Area until specimen and control preparation is completed. Working Master Mix is stable for 6 hours at 2-8ºC in the reaction tubes sealed in the plastic bag.

B. Specimen and Control Preparation

Performed in: Pre-Amplification – Specimen and Control Preparation Area

1. Set the temperature of a dry heat block at 56ºC ± 2ºC.
2. Set the temperature of another dry heat block at 70ºC ± 2ºC.

3. Equilibrate reagents, specimens and controls to ambient temperature (at least 15 minutes). If a precipitate has formed in **ATL** or **AL**, dissolve by heating to 70ºC and agitating gently.

NOTE: Steps B.4-B.6 may be performed during the incubation of the specimens/controls with PK and ATL (Step B.10).

4. Dissolve the lyophilized **CAR** by adding 310 µL of **AVE**. Vortex for 10 seconds. Initial and date the vial.

NOTE: Dissolved CAR is stable at 2-8ºC for up to 24 hours or can be aliquoted and frozen at -20ºC for up to 2 months or until the expiration date, whichever comes first. Do not freeze-thaw the dissolved CAR more than 3 times.

5. Add 30 mL of absolute ethanol to **AW2**. Initial and date the bottle. Mix diluted **AW2** by shaking.

NOTE: Diluted AW2 may be stored at room temperature for up to 2 months or until the expiration date, whichever comes first.

6. Prepare Working **AL** by adding the appropriate volume of dissolved **CAR** to **AL** as shown in Table 1. Gently mix by inverting the tube 10 times. To avoid foaming, do not vortex.

Table 1
Preparation of Working AL

	Number of Specimens/Controls to be processed						
Reagents	**12**	**24**	**36**	**48**	**60**	**72**	**96**
CAR (mL)	0.04	0.07	0.10	0.13	0.16	0.20	0.25
AL (mL)	4.0	7.0	10.0	13.0	16.0	20.0	25.0

NOTE: Working AL is stable at 2-8ºC for up to 48 hours.

7. Label one 2 mL screw cap tube for each specimen and control to be processed.

NOTE: DO NOT use 1.5 mL screw cap tubes or any conical-shaped tubes as it will interfere with the heat transfer during incubation and may result in incomplete lysis.

8. Add 80 µL **ATL** to each tube.
9. Vortex each specimen and control for 10 seconds. Add 250 µL of each specimen and control to the appropriately labeled tube.
10. Add 20 µL **PK** to each tube. Cap the tubes and vortex for 10 seconds. Incubate the tubes at 56ºC ± 2ºC for 30 minutes in a dry heat block.
11. While the specimens and controls are incubating, assemble the QIAvac 24 (or QIAvac 24 Plus) vacuum manifold according to the QIAGEN vacuum manifold handbook. Remove one **CLM** for each specimen and control from the blister packages and label. Save the waste collection tubes for use in Step B.23. Open the lid on the **CLM** and insert into the **VC**. Insert the **EXT** into the **CLM**.
12. After the incubation at 56ºC ± 2ºC is complete, add 250 µL of Working **AL** to each tube. Cap the tubes and vortex for 10 seconds at maximum speed.

NOTE: A white precipitate may form when Working AL is added. The precipitate does not interfere with the specimen and control preparation procedure and will dissolve during the following incubation.

13. Incubate the tubes at 70ºC ± 2ºC for 15 minutes in a dry heat block. Vortex the specimens and controls occasionally throughout the incubation period.
14. After the incubation of specimens and controls at 70ºC ± 2ºC is complete, add 300 µL of absolute ethanol to each tube. Cap the tubes and vortex for 15 seconds at maximum speed.
15. Incubate the tubes at ambient temperature for 5 minutes. Pulse spin the tubes using a table-top centrifuge at maximum RPM.
16. Transfer the lysate from each tube into the corresponding **CLM**. Allow to incubate at least 1 minute.
17. Turn on the vacuum pump. Apply the vacuum to the manifold to remove **all** lysate and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook.

NOTE: If the lysate from an individual specimen or control has not completely passed through the membrane, place the CLM into a clean 2 mL collection tube, close the cap, and centrifuge at full speed for 1 minute or until the lysate has completely passed through. Additional 2 mL collection tubes can be purchased separately (see "MATERIALS REQUIRED BUT NOT PROVIDED").

18. Add 750 µL **AW2** to each **CLM**. Allow to incubate for at least 1 minute.
19. Turn on the vacuum pump. Apply the vacuum to the manifold to remove all liquid and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook.
20. Add 750 µL of absolute ethanol to each **CLM**. Allow to incubate for at least 1 minute.
21. Turn on the vacuum pump. Apply the vacuum to the manifold to remove all liquid and continue vacuum application for at least 1 more minute. Release the vacuum from the manifold as described in QIAGEN vacuum manifold handbook.
22. Carefully remove the **EXT** from each **CLM**. Discard the **EXT**.

NOTE: To avoid cross contamination, be careful not to contact the adjacent CLM while EXT are being removed.

23. Place each **CLM** into a waste collection tube (from Step B.11) and close the lid. Centrifuge the assemblies at maximum RPM for 3 minutes.
24. Label one **ELT** for each specimen and control.
25. Discard the waste collection tubes and place each **CLM** into the corresponding **ELT**.
26. Open the lid on each **CLM** and incubate at room temperature for 15 minutes.
27. Add 120 µL **AVE** to each **CLM**. Close each lid.

NOTE: Ensure that AVE is equilibrated to room temperature prior to adding to each CLM.

28. Incubate the assemblies at ambient temperature for 5 minutes and then centrifuge at maximum RPM for 1 minute.
29. Remove and discard the **CLM** from the assemblies and cap the tubes. Amplify the processed specimens and controls immediately or store at 2-8ºC or frozen at ≤ -20ºC. Processed specimens and controls may be stored at room temperature for up to 6 hours, at 2-8ºC for up to 7 days, or at -20ºC or colder for up to 8 weeks, with no more than one freeze-thaw cycle. More than one freeze-thaw cycle may result in loss of signal.
30. Add 50 µL of each processed specimen and control to the appropriate amplification tubes containing Working Master Mix. Use a new aerosol barrier or positive displacement tip for each specimen and control. Cap the amplification tubes.

NOTE: If processed specimens and controls are stored frozen, allow the frozen processed specimens and controls to thaw at room temperature. Once thawed, vortex each processed specimen and control vigorously for 10 seconds. If processed specimens and controls are stored at 2-8ºC, vortex each processed specimen and control vigorously for 10 seconds prior to addition to Working Master Mix.

NOTE: Amplification on the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 must begin within 45 minutes of processed specimen and control addition to the Working Master Mix.

31. Transfer the prepared specimens and controls in the amplification tray to the Amplification/Detection Area. The remainder of the processed specimens and controls may be stored according to the instructions in Step B.29.

C. Amplification

Performed in: Post-Amplification – Amplification/Detection Area

1. Place the Tray/Retainer assembly into the thermal cycler block.
2. Program the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 for the LINEAR ARRAY HPV Genotyping Test as follows:

HOLD Program:	2 min 50ºC
HOLD Program:	9 min 95ºC
CYCLE Program (40 Cycles):	30 sec 95ºC, 1 min 55ºC, 1 min 72ºC (Ramp rate = 50%)
HOLD Program:	5 min 72ºC
HOLD Program:	72ºC Indefinitely

In the CYCLE programs, set ramp rate to 50%. Assign the Method Name and the User Name as required. Consult the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 User's Manual for additional information on programming and operation of the thermal cycler.

3. Start the METHOD program. Set the "Ramp Speed" to "Max" and "Reaction Volume" to "100 µL" in the "Method Options" screen. Press START again. The program runs approximately 3 hours and 15 minutes.
4. Remove the tray from the thermal cycler within 4 hours of the start of the final HOLD program, place in the MicroAmp base and continue immediately with Step 5. Do not allow the reaction tubes to remain in the thermal cycler beyond 4 hours. ***DO NOT BRING AMPLIFIED SPECIMENS INTO THE PRE-AMPLIFICATION AREA. AMPLIFIED CONTROLS AND SPECIMENS SHOULD BE CONSIDERED A MAJOR SOURCE OF POTENTIAL CONTAMINATION.***
5. Remove the caps from the reaction tubes carefully to avoid creating aerosols of the amplification products. Immediately pipet 100 µL **DN** to the first column (or row) of reaction tubes using a multichannel pipettor with aerosol barrier tips and mix by pipetting up and down five times. For each column (or row), repeat this procedure using a fresh set of tips.
6. The denatured amplicon can be held at room temperature for no more than 5 hours before proceeding to Detection (Part D). If the detection reaction can not be performed within 5 hours, re-cap the tubes with new caps and store the denatured amplicon at 2-8ºC for up to 7 days.

D. Genotype Detection with the LINEAR ARRAY HPV Strip

Performed in: Post Amplification – Amplification/Detection Area

NOTE: Throughout the detection procedure, do not allow the specimens to splash from one tray well to another. Do not allow the water from the bath to splash into the tray wells. Do not stack the 24-well trays.

1. Warm all detection reagents to room temperature.
2. Pre-warm a water bath to 53ºC ± 2ºC.
3. Pre-warm the shaking water bath to 53ºC ± 2ºC at a shaking speed of approximately 60 RPM. Be sure that there is sufficient water in the bath to heat the 24-well tray, but not too much water such that it splashes into the 24-well tray. Water must be in contact with approximately 1/4 of exterior well depth or 0.5 cm of the 24-well tray.

NOTE: False positive results may occur if the water in the water bath is not in proper contact with the 24-well tray.

4. Prepare Working Hybridization Buffer as follows: Examine **SSPE** and **SDS** and, if necessary, warm to 53ºC ± 2ºC in a water bath to re-dissolve any precipitate. Add 100 mL **SSPE** to 388 mL of distilled or deionized water. Mix well. Add 12.5 mL **SDS** and mix well. The Working Hybridization Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Hybridization Buffer should be stored at room temperature in a clean container and is stable for 30 days.
5. Prepare Working Ambient Wash Buffer as follows: Examine **SSPE** and **SDS** and, if necessary, warm to 53ºC ± 2ºC in a water bath to re-dissolve any precipitate. Add 133 mL **SSPE** to 2520 mL of distilled or deionized water. Mix well. Add 13.3 mL **SDS** and mix well. The Working Ambient Wash Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Ambient Wash Buffer should be stored at room temperature in a clean container and is stable for 30 days.
6. Prepare Working Stringent Wash Buffer as follows: For each strip being tested, remove 5 mL of Working Ambient Wash Buffer (prepared in the previous step) and add it to an appropriately sized clean media bottle. (e.g., For a run of 24 strips, remove 120 mL of Working Ambient Wash Buffer and add to the media bottle.) Working Stringent Wash Buffer should be prepared fresh prior to each run.
7. Warm the Working Hybridization Buffer and Working Stringent Wash Buffer in the 53ºC ± 2ºC water bath for a minimum of 15 minutes. Leave the Working Hybridization Buffer and Working Stringent Wash Buffer in the water bath until use.
8. Prepare Working Citrate Buffer as follows: Examine **CIT** and, if necessary, warm to 53ºC ± 2ºC in a water bath to dissolve any precipitate. Add 25 mL **CIT** to 475 mL of distilled or deionized water. Mix well. The Working Citrate Buffer is sufficient for 100 LINEAR ARRAY HPV Genotyping Strips. Working Citrate Buffer should be stored at room temperature in a clean container and is stable for 30 days.
9. Remove the required number of LINEAR ARRAY HPV Genotyping Strips from the **HPV Strip** pouch using clean forceps.

10. With a water/chemical/heat-resistant permanent ink pen, label each **HPV Strip** with the appropriate specimen or control identification.
11. Place each strip with the probe lines facing upward into the appropriate well of the 24-well tray.
12. Add 4 mL of pre-warmed Working Hybridization Buffer into each well that contains a labeled strip.
13. Using a pipettor with an aerosol barrier or positive displacement tip, carefully pipet 75 µL of denatured amplicon into the appropriate well containing a labeled strip. Rock the tray gently between each addition. Use a new tip for each amplicon addition.
14. Cover the 24-well tray with the lid and place tray in the 53ºC ± 2ºC shaking water bath. Place a 1 lb. lead ring weight on the tray lid in order to hold the 24-well tray in place in the shaking water bath. Hybridize for 30 minutes at a shaking speed of approximately 60 RPM.
15. During hybridization (Step 14), prepare Working Conjugate as follows: Add 15 µL **SA-HRP** to 5 mL of Working Ambient Wash Buffer for each strip being tested. Mix well. Working Conjugate should be stored at room temperature in a clean container and is stable for 3 hours.
16. Remove the 24-well tray from the shaking water bath and remove the Working Hybridization Buffer from the wells by vacuum aspiration.
17. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock the 24-well tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.
18. Add 4 mL of pre-warmed Working Stringent Wash Buffer to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and return the 24-well tray to the 53ºC ± 2ºC shaking water bath. Place a 1 lb. lead ring weight on the tray lid in order to hold the 24-well tray in place in the shaking water bath. Incubate for 15 minutes at a shaking speed of approximately 60 RPM.
19. Remove the 24-well tray from the shaking water bath and remove the Working Stringent Wash Buffer from the wells by vacuum aspiration.
20. Add 4 mL of Working Conjugate to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker. Incubate for 30 minutes **at room temperature (15-30ºC)** at a shaking speed of approximately 60 RPM.
21. Remove the 24-well tray from the orbital shaker and remove the Working Conjugate from the wells by vacuum aspiration.
22. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock the 24-well tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.
23. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Wipe any condensation from the tray lid with a clean paper towel, place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 10 minutes at a shaking speed of approximately 60 RPM.
24. Remove the 24-well tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.
25. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 10 minutes at a shaking speed of approximately 60 RPM.
26. Remove the 24-well tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.
27. Add 4 mL of Working Citrate Buffer to each well containing a strip. Place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 5 minutes at a shaking speed of approximately 60 RPM.
28. Prepare Working Substrate as follows: Add 4 mL **SUB A** to 1 mL **SUB B** per strip being tested. Mix well. Working Substrate should be stored at room temperature, protected from exposure to direct light in a clean container and is stable for 3 hours.
29. Remove the 24-well tray from the orbital shaker and remove the Working Citrate Buffer from the wells by vacuum aspiration.
30. Add 4 mL of Working Substrate to each well containing a strip. Place the lid on the 24-well tray and place the 24-well tray on the room temperature orbital shaker for 5 minutes at a shaking speed of approximately 60 RPM.

31. Remove the 24-well tray from the orbital shaker and remove the Working Substrate from the wells by vacuum aspiration.
32. Add 4 mL of distilled or deionized water to each well containing a strip.
33. Remove the strips from the 24-well tray using clean forceps, place on a clean and dry surface and allow the strips to air dry for a minimum of one hour or up to 72 hours at room temperature prior to interpretation.

24-Well Tray Cleaning

Performed in: Post Amplification – Amplification/Detection Area

NOTE: The 24-well trays are disposable or may be re-used. To re-use the trays, this cleaning procedure must be followed after every use.

1. Prepare a 10% solution of RBS35 by adding 1 part RBS35 to 9 parts distilled or deionized water.
2. Fill each tray well with the 10% solution and let soak overnight at room temperature.
3. Rinse the tray **thoroughly** with distilled or deionized water.
4. Dry tray completely before use.

RESULTS

Ensure that the control results for the run are valid (see Quality Control Section). If the run is invalid, repeat the entire run (specimen preparation, amplification and genotype detection with the LINEAR ARRAY HPV strip).

For a valid run, interpret the LINEAR ARRAY HPV Genotyping Strip by placing the LINEAR ARRAY HPV Genotyping Test Reference Guide over the strip in the cut out area so that the HPV genotype reference lines appear on each side of the strip. Ensure that the black ink reference line (REF) on the guide is in alignment with the solid black line on the HPV strip. Record the positive visible bands and interpret the HPV and ß-globin (BG) results for each strip as follows:

HPV Result	BG Low Result	BG High Result	Interpretation
–	–	–	**Result Invalid.** HPV DNA, if present, could not be detected. The absence of BG High and BG Low results indicates inadequate specimen collection, processing, or the presence of inhibitors. Process another aliquot of the original specimen and repeat the test. If the original specimen is not available, a new specimen must be collected.
–	–	+	**Result Invalid.** HPV DNA, if present, may not have been detected. The absence of BG Low is suggestive of inadequate specimen collection, processing, or the presence of inhibitors. Process another aliquot of the original specimen and repeat the test. If the original specimen is not available, a new specimen must be collected.
–	+	+	**HPV DNA not detected.** A negative result does not preclude the presence of HPV infection because results depend on adequate specimen collection, processing, absence of inhibitors, and sufficient HPV DNA to be detected.
+	–	–	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The absence of BG Low and BG High is suggestive of inadequate specimen collection, processing, presence of inhibitors or competition with a high titer HPV target. Additional HPV genotypes that were not detected may be present.
+	–	+	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The absence of BG Low is suggestive of inadequate specimen collection, processing, presence of inhibitors, or competition with a high titer HPV target. Additional HPV genotypes that were not detected may be present.
+	+	+	**HPV DNA detected (Report genotypes).** Specimen is positive for the presence of HPV. The presence of additional HPV genotypes in the specimen cannot be completely ruled out.

Cross Reactive Probe Interpretation

The LINEAR ARRAY HPV Genotyping Strip contains a cross reactive probe that hybridizes with HPV genotypes 33, 35, 52 and 58. Positive band results for the probe should be interpreted as follows:

Band Result	Interpretation
33, 52/33/35/58	HPV 33*
35, 52/33/35/58	HPV 35*
58, 52/33/35/58	HPV 58*
52/33/35/58	HPV 52

* co-infection with HPV Genotype 52 cannot be ruled out by these test results.

QUALITY CONTROL

At least one replicate of the LINEAR ARRAY HPV Negative Control and one replicate of the LINEAR ARRAY HPV Positive Control must be processed with each run of up to 22 specimens. As with any new laboratory procedure, new operators should consider the use of additional positive and negative controls each time the test is performed until such time that a high degree of confidence is reached in their ability to perform the test procedure correctly. There are no requirements regarding the position of the controls in the MicroAmp tray.

Specimens and controls from separate specimen preparation runs may be amplified and detected at the same time. However, each separate specimen preparation run is validated individually by the set of controls included with the run. Therefore, it is possible to reject one run of specimens from a common amplification and/or detection run while accepting another run based upon the performance of the controls processed with those specimens.

All test specimens and controls prepared in the same run should be amplified and detected in adjacent positions in the thermal cycler and in the detection tray. The exact order of placement of these specimens and controls in the thermal cycler or detection tray is not critical.

Negative Control

The assay result of the LINEAR ARRAY HPV Negative Control must be no positive bands. If any positive bands are visible, the entire run is invalid. Repeat the entire process (Specimen and Control Preparation, Amplification and Genotype Detection with the LINEAR ARRAY HPV Strip). If the LINEAR ARRAY HPV Negative Control consistently yields results with positive bands, contact your local Roche office for technical assistance.

Positive Control

The assay result of the LINEAR ARRAY HPV Positive Control must yield a positive result interpreted as HPV 16, ß-Globin high, and ß-Globin low. The ß-Globin low band will be faint in comparison to the ß-Globin high band but must be visible by eye. If the LINEAR ARRAY HPV Positive Control does not yield this exact result, the entire run is invalid. Repeat the entire process (Specimen and Control Preparation, Amplification and Genotype Detection with the LINEAR ARRAY HPV Strip). If the LINEAR ARRAY HPV Positive Control consistently yields results with a different pattern of positive bands, contact your local Roche office for technical assistance.

PROCEDURAL PRECAUTIONS

As with any test procedure, good laboratory technique is essential to the proper performance of this assay. Due to the high analytical sensitivity of this test, extreme care should be taken to preserve the purity of kit reagents or amplification mixtures. All reagents should be closely monitored for purity. Discard any reagents that may be suspect.

PROCEDURAL LIMITATIONS

1. This test has been validated for use with human cervical cells collected in cobas® PCR Cell Collection Media or PreservCyt Solution. Testing of other specimen types may result in false negative or false positive results.
2. This test has been validated for use with the AmpliLute Liquid Media Extraction Kit only. Testing using other specimen extraction procedures may lead to incorrect results.
3. Reliable results are dependent on adequate specimen collection, transport, storage and processing procedures.
4. Detection of HPV is dependent on the number of virus genomes present in the specimen and may be affected by specimen collection methods, patient factors (i.e. age, presence of symptoms), and/or stage of infection.

5. False negative results may occur due to polymerase inhibition or cell inadequacy. The ß-globin amplification has been added to the LINEAR ARRAY HPV Genotyping Test to permit the identification of processed specimens containing substances that may interfere with PCR amplification and or inadequate cell collection.
6. The presence of AmpErase enzyme in the LINEAR ARRAY HPV Master Mix reduces the risk of amplicon contamination. However, contamination from HPV positive controls and specimens can be avoided only by good laboratory practices and careful adherence to the procedures specified in this insert.
7. Use of this product should be limited to personnel trained in the techniques of PCR.
8. Only the Applied Biosystems Gold-plated 96-Well GeneAmp PCR System 9700 has been validated for use with this product. No other thermal cycler, including GeneAmp PCR System 2400, GeneAmp PCR System 9600, or GeneAmp PCR System 9700 thermal cycler with aluminum block, can be used with this product.
9. Specimens containing Advantage-S® Bioadhesive Contraceptive Gel may interfere with the performance of the LINEAR ARRAY HPV Genotyping Test and may give false negative or invalid results.
10. Specimens containing greater than 3.5% (v/v) blood have been shown to inhibit PCR amplification and may give false negative results.
11. Identification of HPV genotypes 64 and 69 are based on LINEAR ARRAY HPV Genotyping Test results for HPV genotypes 64 and 69 plasmid DNA. HPV genotypes 64 and 69 were not detected in a clinical specimen during the performance evaluations.
12. Though rare, mutations within the highly conserved region of the viral genome covered by the LINEAR ARRAY HPV Genotyping Test primers and/or probes may result in the failure to detect a particular genotype.
13. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next; users perform method correlation studies in their laboratory to qualify technology differences.

INTERFERING SUBSTANCES

The following common feminine personal hygiene products, lubricants, anti-fungal creams and contraceptive gels have been shown not to interfere with the performance of the LINEAR ARRAY HPV Genotyping Test.

Table 2

Product Name	
Ortho Options™ Delfen® Vaginal Contraceptive Foam	Mycelex® 3 Antifungal Cream
Clotrimazole 3 Vaginal Cream	Vagi-Gard® Medicated Cream
Gyne-Lotrimin® 3 Vaginal Cream	Vagisil® Anti-itch Cream
Gynecort® 1% Hydrocortisone Anti-itch Cream	Yeast Gard™ Homeopathic Vaginal Suppositories
Vaginex® Hydrocortisone Formula	Norforms® Deodorant Suppositories
Betadine® Medicated Douche Concentrate	K-Y® Brand Jelly Personal Lubricant
Miconazole External Vulvar Cream	Vagisil® Intimate Lubricant
Monistat® 3 Combination Pack Vaginal Antifungal	

False negative and/or invalid results were observed for HPV positive samples containing Advantage-S Bioadhesive Contraceptive Gel at concentrations at 0.11 g/20 mL and greater.

False negative results were observed for HPV positive samples containing greater than 3.5% (v/v) blood.

No false negative or invalid results were observed for HPV positive samples containing endocervical or exocervical mucus.

PREVALENCE OF HPV GENOTYPES

The prevalence and distribution of HPV genotypes has been shown to vary according to geographic region, worldwide[19-21]. Additionally, several genotypes, e.g. 55, 64, and 69, appear to be novel genotypes reported at low prevalence and not associated with cervical cancer[20,22]. The most common HPV genotypes associated with cervical cancer in decreasing prevalence or frequency are types 16, 18, 45, 31; variability of frequency in geographic distribution has been found for most genotypes, including 33, 35, 39, 51, 52, 56, 58, 59 and 68.

Table 3
Reported Prevalence of HPV Genotypes in Control and Cervical Cancer Cases[19-21]

HPV Genotype	Prevalence (%)
16	7.5 – 56.0
18	2.3 – 22.1
45	2.4 – 7.9
58	0.2 – 5.4
53	0.0 – 5.2
33	1.0 – 4.4
31	2.0 – 4.2
52	0.5 – 4.2
62	4
54	0.0 – 3.6
39	0.0 – 3.3
61	0.0 – 3.2
35	0.4 – 2.7
56	0.2 – 2.5
66	0.0 – 2.4
84	2.3
6	0.3 – 2.3
81	0.1 – 2.3
70	0.0 – 2.3
59	0.0 – 2.1
CP6108	0.0 – 1.8
83	1.6
72	0.0 – 1.5
68	0.1 – 1.2
42	0.0 – 1.2
43	0.0 – 1.2
55	1.1
73	0.1 – 1.0
11	0.1 – 0.8
40	0.0 – 0.8
82	0.0 – 0.6
67	0.5
44	0.0 – 0.4
69	0.2
71	0.2
IS39	0.2
26	0.0 – 0.2
64	0.1
57	0

NON CLINICAL PERFORMANCE EVALUATION

A. Limit of Detection

The limit of detection was determined for 18 HPV genotypes (6, 16, 18, 26, 31, 33, 35, 39, 45, 51, 53, 56, 58, 59, 66, 68, 73 and 82). Plasmid DNA [quantitated by spectrophotometry (260 nm absorption)] for each genotype was diluted to at least 5 concentration levels using PreservCyt Solution containing 250 ng/mL of human genomic DNA as the diluent. Two operators each performed a minimum of 4 runs with each run consisting of at least 3 replicates per concentration level for a total of at least 24 replicates per level. For each of these 18 HPV genotypes, a Probit analysis was performed and the predicted 95% positive hit rate concentration for each genotype is shown in Table 4. In addition, the lowest concentration level with an observed ≥ 95% positive hit rate and at which all higher concentration levels had observed ≥ 95% positive hit rate is shown in Table 4.

Table 4
Limit of Detection Results for the LINEAR ARRAY HPV Genotyping Test

HPV Genotype	Probit Analysis Predicted 95% Hit Rate Concentration (c/mL)	Observed LOD Concentration ≥ 95% positive hit rate (c/mL)
6	2,319	2,000
16#	195	200
18#	580	1,300
26	2,935	6,000
31#	1,863	6,600*
33#	4,000	20,000**
35#	466	600
39#	1,367	1,500
45#	401	900
51#	181	260
53	256	400
56#	6,915	12,000
58#	185	250
59#	53	76
66	250	300
68#	848	900
73	165	300
82	8,089	20,000

\# Identified as high-risk genotypes associated with high-grade cervical dysplasia and cervical cancer[3-5].

* For genotype 31, > 95% hit rates were observed at 2,000; 1,300; 900 and 660 c/mL and 92% hit rates were observed at 3,300 and 3,000 c/mL.

** For genotype 33, > 95% hit rates were observed at 4,000 and 2,700 c/mL, a 92% hit rate was observed at 6,700 c/mL, and a 94% hit rate was observed at 13,000 c/mL.

B. Genotype Inclusivity: Plasmid DNA

Genotype inclusivity was determined by analysis of 36 HPV genotype plasmids (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108). Plasmid DNA for HPV genotype 52 was not available for testing. Inclusivity for HPV type 52 was demonstrated in studies with a clinical specimen (Section C). For HPV genotypes 6, 16, 18, 26, 31, 33, 35, 39, 45, 51, 53, 56, 58, 59, 66, 68, 73 and 82, inclusivity was demonstrated in the limit of detection study. The remaining 18 available HPV genotype plasmid DNA stocks were quantitated by spectrophotometry (260 nm absorption), diluted to 900 copies/mL in PreservCyt Solution, and then tested in replicates of six using two reagent lots. For any HPV genotype that was not detected at a 100% positivity rate at 900 copies/mL, additional testing was performed using increasing plasmid DNA concentrations until the 100% inclusivity level (n=6) for that genotype was determined. The results demonstrated that the LINEAR ARRAY HPV Genotyping Test can detect 36 HPV genotypes from HPV Plasmid DNA at various concentration levels dependent on the HPV genotype (Table 5).

Table 5
Genotype Inclusivity for the LINEAR ARRAY HPV Genotyping Test

HPV Genotype	Inclusivity Level* (copies/mL)	HPV Genotype	Inclusivity Level* (copies/mL)
6	2,319	59#	53
11	900	61	900
16#	195	62	900
18#	580	64	300,000
26	2,935	66	250
31#	1,863	67	30,000
33#	4,000	68#	848
35#	466	69	900
39#	1,367	70	900
40	70,000	71	900
42	30,000	72	900
45#	401	73	165
51#	181	81	900
53	256	82	8,089
54	900	83	900
55	900	84	900
56#	6,915	CP6108	900
58#	185	IS39	1,500

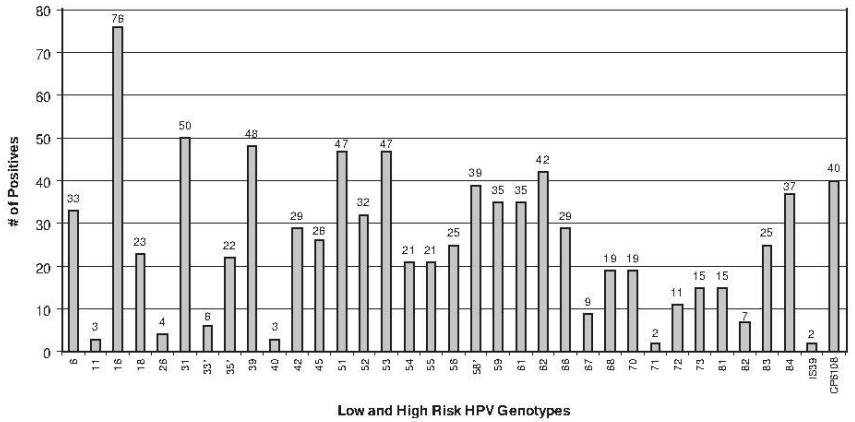
* Inclusivity Level equates to a 100% positive hit rate (n=6) at the concentrations listed except for HPV Genotypes 6, 16, 18, 26, 31, 33, 35, 39, 45, 51, 53, 56, 58, 59, 66, 68, 73 and 82, where the concentration listed is the Probit predicted 95% positive hit rate.

\# Identified as high-risk genotypes associated with high-grade cervical dysplasia and cervical cancer[3-5].

C. Genotype Inclusivity: Clinical Specimens

During the performance evaluations, a total of 451 clinical specimens tested HPV positive with the LINEAR ARRAY HPV Genotyping Test. 35 of the 37 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 66, 67, 68, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108) were detected in clinical specimens with the LINEAR ARRAY HPV Genotyping Test thereby demonstrating inclusivity. The distribution of these HPV genotypes is shown in Figure 1. Within the performance evaluations, HPV genotypes 64 and 69 were not detected in a clinical specimen.

Figure 1
Distribution of HPV Genotypes in LINEAR ARRAY HPV Positive Clinical Specimens (n=451) throughout the Performance Evaluations



* For 58 clinical specimens that tested positive for HPV 33, HPV 35, and/or HPV 58 DNA, co-infection with HPV genotype 52 could not be ruled out.

D. Reproducibility

Reproducibility was evaluated with 3 lots of reagents, 5 combinations of instruments (thermal cyclers and water baths), and 3 operators over a total of 15 runs. Each operator performed a total of 5 runs with each of the three lots of reagents and with one run being performed on each of the 5 combinations of instruments. A four member panel was prepared consisting of a pool of four HPV genotypes (16, 18, 31 and 45) in PreservCyt Solution containing 250 ng/mL of human genomic DNA. Panel members were prepared with each genotype at 2,000 copies/mL as well as at low (~3 to 4 times the LOD), medium (~10 to 15 times the LOD) and high (~100 to 150 times the LOD) concentrations. Each panel member was tested in triplicate using each reagent lot for each run. For HPV genotypes 16, 18, 31 and 45, a 99% positive hit rate was exhibited at 2,000 c/mL and at the low concentration. A 100% positive hit rate was exhibited for HPV genotypes 16, 18, 31 and 45 at the medium and high concentrations (Table 6). No significant run to run, lot to lot, operator to operator, or instrument to instrument variability was observed.

Table 6
Reproducibility for the LINEAR ARRAY HPV Genotyping Test

Panel Member	HPV 16 DNA (copies/mL)	HPV 18 DNA (copies/mL)	HPV 31 DNA (copies/mL)	HPV 45 DNA (copies/mL)	Number Tested	LINEAR ARRAY HPV Genotyping Test Results HPV Positive		
						Positive Results	%	95% CI
High	12,000	81,500	192,000	25,100	135	135	100%	98-100%
Medium	1,200	8,150	19,200	2,510	135	135	100%	98-100%
Low	360	2,445	5,760	753	135	133	99%	95-100%
2,000 (c/mL)	2,000	2,000	2,000	2,000	135	133	99%	95-100%

E. Analytical Specificity: Non-HPV Microorganisms

To assess the specificity of the test for exclusion of non-HPV microorganisms, the cultured bacteria, virus, protozoan or yeast listed in Table 7 were added into PreservCyt Solution containing 250 ng/mL of human genomic DNA and tested with the LINEAR ARRAY HPV Genotyping Test. The concentrations of microorganisms tested ranged between 3.5e2 to 2.0e3 IFU/mL for *C. trachomatis*, 1e6 PFU/mL for Herpes viruses, 8e5 cells/mL for *T. vaginalis*, 2e2 to 2e3 CFU/mL for *C. neoformans*, and 1.5e4 to 9.8e9 CFU/mL for the remaining microorganisms excluding *H. ducreyi, M. hominis* and *N. gonorrhoeae*, for which concentrations were unknown. The results indicated that the LINEAR ARRAY HPV Genotyping Test did not cross-react with a variety of viruses, bacteria, protozoa and yeast that could be present in cervical specimens.

Table 7
Non-HPV Microorganisms Tested

Achromobacter xerosis	*Eikenella corrodens*	*Mycobacterium avium*
Acinetobacter sp. genospecies 3	*Enterobacter cloacae*	*Mycoplasma hominis*
Actinomyces israelii	*Enterococcus faecalis*	*Neisseria gonorrhoeae*
Aerococcus viridans	*Erysipelothrix rhusiopathiae*	*Pasteurella maltocida*
Aeromonas hydrophila	*Escherichia coli*	*Pediococcus acidilactica*
Agrobacterium radiobacter	*Ewingella americana*	*Peptostreptococcus productus*
Alcaligenes faecalis	*Flavobacterium meningosepticum*	*Prevotella corporis*
Bacteriodes fragilis	*Gamella morbillorum*	*Proteus mirabilis*
Bifidobacillus longum	*Gardnerella vaginalis*	*Providencia stuartii*
Bifidobacterium adolescentis	*Haemophilus ducreyi*	*Pseudomonas aeruginosa*
Branhamella catarrhalis	*Herpes simplex* virus 1	*Rahnella aquatilis*
Brevibacterium linens	*Herpes simplex* virus 2	*Salmonella minnesota*
Candida albicans	*Kingella kingae*	*Serratia denitrificans*
Chlamydia trachomatis type D	*Klebsiella pneumoniae ss ozaenae*	*Staphylococcus aureus*
Chlamydia trachomatis type F	*Lactobacillus vaginalis*	*Streptococcus anginosus*
Chromobacter violaceum	*Lactococcus lactis cremoris*	*Streptococcus epidermidis*
Clostridium perfringens	*Legionella pneumophila*	*Treponema phagedenis*
Corynebacterium genitalium	*Leuconostoc paramesenteroides*	*Trichomonas vaginalis*
Cryptococcus neoformans	*Micrococcus luteus*	*Vibrio parahaemolyticus*
Deinococcus radiopugnans	*Moraxella osloensis*	*Yersinia enterocolitica*
Derxia gummosa	*Morganella morganii*	

F. Analytical Specificity: HPV Genotypes

The HPV genotype specificity of each HPV probe line on the HPV Strip was evaluated by testing high concentrations of HPV plasmid DNA for 36 HPV genotypes (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73, 81, 82, 83, 84, IS39 and CP6108). Plasmid DNA for HPV genotype 52 was not available for testing. The available HPV plasmid DNA stocks were quantitated by measuring the 260 nm absorbance on a spectrophotometer and diluted to 500,000 copies/mL in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0). Each HPV plasmid genotype was amplified and detected in duplicate using the LINEAR ARRAY HPV Genotyping Test. The results demonstrated HPV genotype specificity only to the expected probe line for the 36 HPV genotypes evaluated with plasmid DNA. In addition, specificity of HPV genotype 52 to the expected probe line was demonstrated in testing of a clinical specimen (genotype confirmed by sequencing) at an unknown HPV titer.

G. Sensitivity and Specificity for Detection of HPV from Clinical Specimens

The sensitivity and specificity of the LINEAR ARRAY HPV Genotyping Test for detecting high-risk HPV DNA from clinical specimens was assessed by testing 698 cervical specimens collected in PreservCyt Solution. For the scope of this study, HPV genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 were classified as high risk. 487 of these specimens were negative and 211 were positive for high-risk HPV by the Hybrid Capture® 2 High-Risk HPV DNA Test (HC2 HPV Test). The presence or the absence of high-risk HPV DNA in the specimens was determined by the concordance between the two tests. Discordant results were resolved by further analysis of the specimens using the AMPLICOR® HPV Test. Based on the consensus results for 2 out of the 3 assays, the true disposition for each clinical specimen was determined (i.e., True Positive for high-risk HPV DNA or True Negative for high-risk HPV DNA). Results for the LINEAR ARRAY HPV Genotyping Test are shown in Table 8. Out of the 485 clinical specimens that were True Negative for high-risk HPV DNA, 118 clinical specimens tested positive for low-risk

HPV DNA by the LINEAR ARRAY HPV Genotyping Test. The distribution of the low-risk HPV genotypes detected in these 118 clinical specimens is shown in Figure 2. The sensitivity and specificity for the presence of high-risk HPV DNA in cervical specimens collected in PreservCyt Solution was 96% and 99%, respectively, for the LINEAR ARRAY HPV Genotyping Test (Table 9).

Table 8
Clinical Specimen Results using the LINEAR ARRAY HPV Genotyping Test

LINEAR ARRAY HPV Genotyping Test Results*	Number of Clinical Specimens
True Positive	197
True Negative	485
False Negative	9
False Positive	3
Invalid	4
Total	698

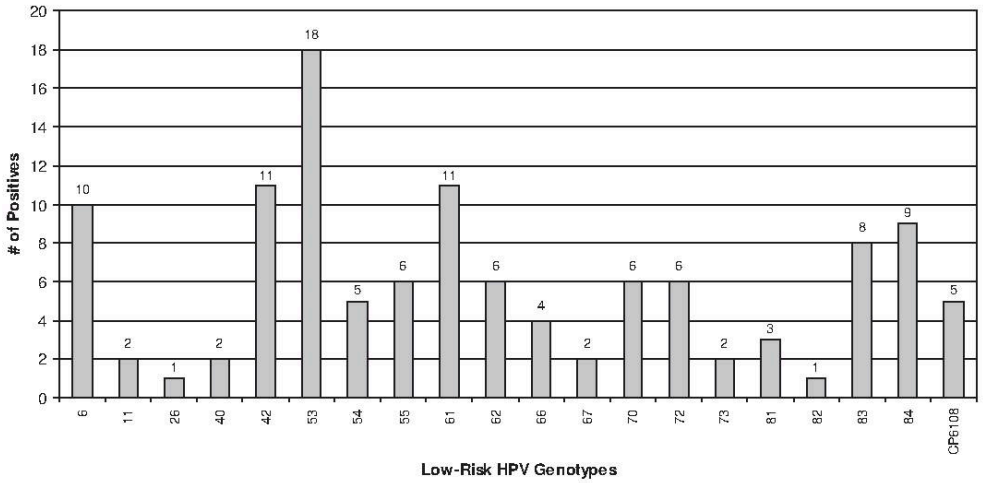
* A positive result means positive for High-Risk HPV DNA; specimens positive only for low-risk HPV DNA are classified as negative for this analysis.

Table 9
Specificity and Sensitivity of the LINEAR ARRAY HPV Genotyping Test for High-Risk HPV DNA

Parameter	Estimate	95% CI*
Sensitivity	96%	92 – 98%
Specificity	99%	98 – 100%

* 95% Wald confidence interval

Figure 2
Distribution of Low-Risk HPV Genotypes in Clinical Specimens that were Negative for High-Risk HPV DNA (n=118) in the Specificity and Sensitivity Evaluations



H. Whole System Failure

The whole system failure rate was determined for the LINEAR ARRAY HPV Genotyping Test by evaluating 135 test results for each of four HPV genotypes (16, 18, 31 and 45) at concentrations three times the limit of detection (limit of detection was defined as the Probit analysis predicted 95% positive hit rate). Test solutions containing plasmid DNA for the four HPV genotypes were prepared in PreservCyt Solution and also contained 250 ng/mL of human genomic DNA. Collectively, out of 540 total trials for the four HPV genotypes, there were two observed system failure events (HPV negative and ß-globin positive) for a Whole System Failure rate of 0.4%.

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Document Revision Information	
Doc Rev. 7.0 1/2010	The **WARNINGS AND PRECAUTIONS** section, **INTENDED USE**, **SPECIMEN COLLECTION**, **TRANSPORT AND STORAGE** section and **PROCEDURAL LIMITATIONS** section have been modified to include the following sample collection media: • cobas® PCR Cell Collection Media Hybrid Capture® 2 High Risk HPV DNA Test(TM) has been corrected to identify Qiagen as the manufacturer. Biopur® was added and ThinPrep® was removed from the trademarks section. IVDD symbol information previously contained on an ancillary card packaged in the kit has been added. The Distributed by affiliate addresses have been updated. Please contact your local Roche Representative if you have any questions.

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1/2010 04392116001-07

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CE

7.7 Appendix G: Elecsys sLFT1 and PlGF assay package inserts

PlGF

Human placental growth factor

cobas®

REF **05144671** 119 **100 tests**

• Indicates analyzers on which the kit can be used

Elecsys 2010	MODULAR ANALYTICS E170	**cobas e** 411	**cobas e** 601	**cobas e** 602
•	•	•	•	•

English

Intended use

Immunoassay for the in vitro quantitative determination of placental growth factor (PlGF) in human serum.
This assay is intended for use as an aid in the diagnosis of preeclampsia, in conjunction with the Elecsys sFlt-1 assay and other diagnostic and clinical information.
The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Note

Please note that the catalogue number appearing on the package insert retains only the first 8 digits of the licensed 11-digit Catalogue Number: 05144671190 for the Elecsys PlGF assay. The last 3 digits -190 have been replaced by -119 for logistic purposes.

Summary

Preeclampsia (PE) is a serious complication of pregnancy characterized by hypertension and proteinuria after 20 weeks of gestation. Preeclampsia occurs in 3-5 % of pregnancies and results in substantial maternal and fetal or neonatal mortality and morbidity. Clinical manifestations can vary from mild to severe forms. Preeclampsia associated with thrombocytopenia and liver enzyme elevation represents the HELLP-syndrome (hemolysis, elevated liver enzymes and low platelets).[1,2,3,4,5]
Preeclampsia appears to be due to the release of angiogenic factors from the placenta that induce endothelial dysfunction. Serum levels of PlGF (placental growth factor) and sFlt-1 (soluble fms-like tyrosine kinase-1, also known as VEGF receptor-1) are altered in women with preeclampsia. Moreover, circulating levels of PlGF and sFlt-1 can discriminate normal pregnancy from preeclampsia even before clinical symptoms occur. In normal pregnancy, the pro-angiogenic factor PlGF increases during the first 2 trimesters and decreases as pregnancy progresses to term. In contrast, levels of the anti-angiogenic factor sFlt-1 remain stable during the early and middle stages of gestation and increase steadily until term. In women who develop preeclampsia, sFlt-1 levels have been found to be higher and PlGF levels have been found to be lower than in normal pregnancy.[6,7,8,9]
The ratio of sFlt-1 to PlGF has been shown to be a better predictor of preeclampsia than either measure alone.[10] Placental endoglin, a member of the TGF-ß family is also upregulated in preeclampsia and released as soluble endoglin into the maternal circulation. Soluble endoglin has been shown to be increased in severe cases of preeclampsia.[11,12,13,14]
In summary, PlGF and sFlt-1 concentrations measured by immunoassay in maternal blood improve the diagnostic possibilities in preeclampsia which comprise clinical symptoms, proteinuria and uterine artery Doppler velocimetry.[15,16]
PlGF in cardiovascular diseases: PlGF can be detected in normal non-pregnant subjects at lower levels. Increased levels of PlGF can be found in patients with cardiovascular diseases as an indicator of micro- and macrovascular atherosclerosis and as a sign of pathological angiogenesis. In addition PlGF has been shown to be an independent predictor of cardiovascular morbidity and mortality in patients with type 1 und type 2 diabetes.[17,18,19,20,21,22,23,24]

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 50 µL of sample, a biotinylated monoclonal PlGF-specific antibody and a monoclonal PlGF-specific antibody labeled with a ruthenium complex[a] react to form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

[a] Tris(2,2'-bipyridyl)ruthenium(II)-complex ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL:
Streptavidin-coated microparticles 0.72 mg/mL; preservative.
R1 Anti-PlGF-Ab~biotin (gray cap), 1 bottle, 8 mL:
Biotinylated monoclonal anti-PlGF antibody (mouse) 0.6 mg/L; phosphate buffer 50 mmol/L, pH 6.0; preservative.
R2 Anti-PlGF-Ab~$Ru(bpy)_3^{2+}$ (black cap), 1 bottle, 8 mL:
Monoclonal anti-PlGF antibody (mouse) labeled with ruthenium complex 4.0 mg/L; phosphate buffer 50 mmol/L, pH 6.0; preservative.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.
Avoid foam formation in all reagents and sample types (specimens, calibrators, and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.
All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C.
Store the Elecsys PlGF reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.
Stability:

PIGF

Human placental growth factor

unopened at 2-8 °C	up to the stated expiration date
after opening at 2-8 °C	12 weeks
on the analyzers	6 weeks

Specimen collection and preparation
Only the specimens listed below were tested in a sufficient number and found acceptable.
Serum collected using standard sampling tubes or tubes containing separating gel.
Stable for 8 hours at 2-8 °C, 4 months at -20 °C. Freeze only once.
The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.
Centrifuge samples containing precipitates before performing the assay. Do not use heat-inactivated samples. Do not use samples and controls stabilized with azide.
Ensure the samples, calibrators, and controls are at ambient temperature (20-25 °C) before measurement.
Due to possible evaporation effects, samples, calibrators, and controls on the analyzers should be analyzed/measured within 2 hours.

Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided)
- REF 05144701190, PIGF CalSet, for 4 x 1 mL
- REF 05341787190, PreciControl Multimarker, for 3 x 2 mL each of PreciControl Multimarker 1 and 2
- General laboratory equipment
- Elecsys 2010, MODULAR ANALYTICS E170 or **cobas e** analyzer

Accessories for Elecsys 2010 and **cobas e** 411 analyzers:
- REF 11662988122, ProCell, 6 x 380 mL system buffer
- REF 11662970122, CleanCell, 6 x 380 mL measuring cell cleaning solution
- REF 11930346122, Elecsys SysWash, 1 x 500 mL washwater additive
- REF 11933159001, Adapter for SysClean
- REF 11706802001, Elecsys 2010 AssayCup, 60 x 60 reaction vessels
- REF 11706799001, Elecsys 2010 AssayTip, 30 x 120 pipette tips

Accessories for MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers:
- REF 04880340190, ProCell M, 2 x 2 L system buffer
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 12135027190, CleanCell M, 1 x 2 L measuring cell cleaning solution (for USA)
- REF 03023141001, PC/CC-Cups, 12 cups to prewarm ProCell M and CleanCell M before use
- REF 03005712190, ProbeWash M, 12 x 70 mL cleaning solution for run finalization and rinsing during reagent change
- REF 03004899190, PreClean M, 5 x 600 mL detection cleaning solution
- REF 12102137001, AssayTip/AssayCup Combimagazine M, 48 magazines x 84 reaction vessels or pipette tips, waste bags
- REF 03023150001, WasteLiner, waste bags
- REF 03027651001, SysClean Adapter M

Accessories for all analyzers:
- REF 11298500316, Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay
For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.
Resuspension of the microparticles takes place automatically prior to use. Read in the test-specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read, enter the 15-digit sequence of numbers.

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers: PreClean M solution is necessary.

Bring the cooled reagents to approx. 20 °C and place on the reagent disk (20 °C) of the analyzer. Avoid foam formation. The system **automatically** regulates the temperature of the reagents and the opening/closing of the bottles.

Calibration
Traceability: This method has been standardized against a commercially available PIGF assay.
Every Elecsys PIGF reagent set has a barcoded label containing the specific information required for calibration of the particular reagent lot. The pre-defined master curve is adapted to the analyzer using the PIGF CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer).

Renewed calibration is recommended as follows:
- after 1 month (28 days) when using the same reagent lot
- after 7 days (when using the same reagent kit on the analyzer)
- as required: e.g. quality control findings outside the defined limits

Quality control
For quality control, use PreciControl Multimarker.
Other suitable control material can be used in addition.
Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.
Follow the applicable government regulations and local guidelines for quality control.
Note: The controls are not barcode-labeled and therefore have to be

PIGF

Human placental growth factor

run like external controls. All values and ranges have to be entered manually. Please refer to the section "QC" in the operator's manual or to the online help of the instrument software.

Calculation
The analyzer automatically calculates the analyte concentration of each sample in pg/mL.

Limitations - interference
The assay is unaffected by icterus (bilirubin < 428 µmol/L or < 25 mg/dL), hemolysis (Hb < 0.311 mmol/L or < 0.5 g/dL), lipemia (Intralipid < 1500 mg/dL) and biotin (< 123 nmol/L or < 30 ng/mL). Criterion: Recovery within ± 15 % of initial value.
Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.
No interference was observed from rheumatoid factors up to a concentration of 600 IU/mL.
There is no high-dose hook effect at PlGF concentrations up to 100000 pg/mL.
In vitro tests were performed on 18 commonly used pharmaceuticals. No interference with the assay was found.
In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

Limitations - general
For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings. Values below 10 pg/mL are not reliable as the coefficient of variation between runs is > 20%.

Limits and ranges
Measuring range
3-10000 pg/mL (defined by the Limit of Detection and the maximum of the master curve). Values below 3 pg/mL are reported as < 3 pg/mL. Values above the measuring range are reported as > 10000 pg/mL.

Lower limits of measurement
Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ)

Limit of Blank	= 2 pg/mL
Limit of Detection	= 3 pg/mL
Limit of Quantitation	= 10 pg/mL

The Limit of Blank and Limit of Detection were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A requirements.
The Limit of Quantitation was determined using the result of functional sensitivity testing.
The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.
The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).
The Limit of Quantitation (functional sensitivity) is the lowest analyte concentration that can be reproducibly measured with a between-run coefficient of variation of ≤ 20 %. It has been determined using low concentration PlGF samples.
Note: In the USA, when reporting values below 10 pg/mL, the client report should be annotated with the following information. "Values below 10 pg/mL are not reliable as the between-run coefficient of variation is > 20 %."

Dilution
Generally not necessary due to the broad measuring range.

Expected values
Clinical studies are still ongoing. From a first data set of 524 serum samples collected from 280 singleton pregnancies with normal pregnancy outcome (i.e. no PE/HELLP, no intrauterine growth restriction) preliminary expected values were obtained. For each sample levels of sFlt-1 and PlGF were determined in parallel and sFlt-1/PlGF ratio was calculated. Weeks of gestation: defined as completed weeks of pregnancy beginning with the start of the last menstruation cycle.
The following results were obtained:

Percentile Elecsys sFlt-1 (pg/mL)

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	555	470	649	630	707	978	1671
50th perc.	1445	1459	1576	1449	1934	2972	4400
95th perc.	2361	2785	2944	3890	6688	9921	11324
N (visits)	40	44	82	98	105	78	77

Percentile Elecsys PlGF (pg/mL)

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	29.4	65.7	125	130	73.3	62.7	52.3
50th perc.	62.8	135	265	412	439	232	161
95th perc.	183	203	541	1108	1108	972	659
N (visits)	40	44	82	98	105	78	77

Percentile Elecsys sFlt-1/PlGF ratio

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	5.21	4.32	2.19	1.01	0.945	1.38	3.65
50th perc.	22.7	12.6	6.09	3.80	4.03	13.3	26.2
95th perc.	57.3	26.9	14.8	16.9	86.4	92.0	138
N (visits)	40	44	82	98	105	78	77

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data
Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision
Precision was determined using Elecsys reagents, samples and controls in a protocol (EP5-A2) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplication each for 21 days (n = 84). The following results were obtained:

PIGF

Human placental growth factor

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Elecsys 2010 and **cobas e** 411 analyzers					
		Repeatability[b]		**Intermediate precision**[c]	
Sample	Mean pg/mL	SD pg/mL	CV %	SD pg/mL	CV %
Human serum 1	112	1.12	1.0	4.55	4.1
Human serum 2	595	5.11	0.9	23.9	4.0
Human serum 3	4510	38.2	0.8	181	4.0
Human serum 4	9542	66.4	0.7	342	3.6
PreciControl MM[d]1	104	0.954	0.9	2.79	2.7
PreciControl MM2	1010	9.33	0.9	27.1	2.7

[b] Repeatability = within-run precision
[c] Intermediate precision = total precision
[d] MM = Multimarker

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers					
		Repeatability		**Intermediate precision**	
Sample	Mean pg/mL	SD pg/mL	CV %	SD pg/mL	CV %
Human serum 1	107	1.21	1.1	2.93	2.7
Human serum 2	563	6.81	1.2	14.9	2.6
Human serum 3	4255	46.2	1.1	104	2.4
Human serum 4	9150	83.7	0.9	253	2.8
PreciControl MM1	97.4	2.90	3.0	4.48	4.6
PreciControl MM2	978	29.6	3.0	39.9	4.1

Clinical sensitivity and specificity

In an external study using the Elecsys sFlt-1 and Elecsys PlGF assays in parallel on samples from 268 pregnant women with normal pregnancy outcome (no PE/HELLP, no intrauterine growth restriction) and 71 patients with PE/HELLP an optimal cut-off for the sFlt-1/PlGF ratio of 85 was determined. At this cut-off the sensitivity was calculated at 82 % and the specificity at 95 %. The area under the receiver operating characteristic (ROC) curve was 0.95. For a subcollective of 37 women with early-onset PE the sensitivity/specificity was calculated to be 89 %/97 % at the same cut-off. The area under the ROC curve was 0.97.

All pregnancies were singleton pregnancies. PE was defined as new onset of both hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) and proteinuria (> 0.3 g/24 h or dipstick ≥ 1+ if a 24 h urine collection could not be obtained) after week 20 of gestation. A PE-pregnancy was defined as early-onset PE if clinical signs of PE appeared before week 34 of gestation.
The data as obtained for all samples of cases (■) and controls (□) are shown in the scatter plots below.

Fig. 1: Elecsys sFlt-1 assay

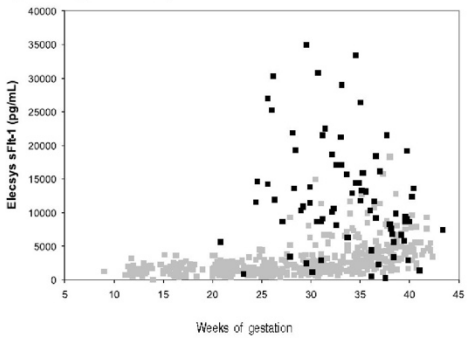


Fig. 2: Elecsys PlGF assay

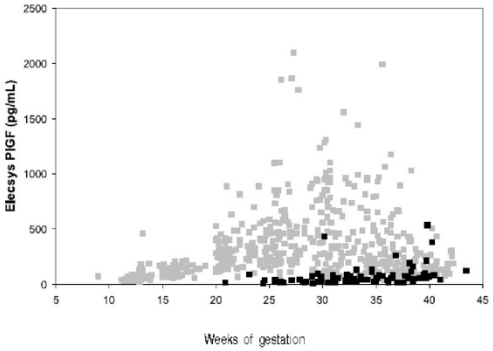
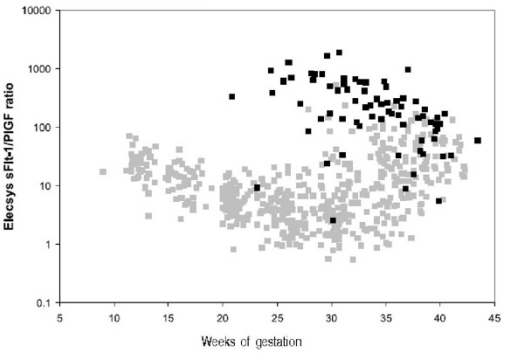


Fig. 3: Elecsys sFlt-1/PlGF ratio



PIGF

Human placental growth factor

Method comparison
A comparison of the Elecsys PIGF assay (y) with a commercially available PIGF assay (x) using clinical samples gave the following correlations (pg/mL):
Number of samples measured: 119

Passing/Bablok[25]	Linear regression
y = 1.07x + 0.144	y = 1.06x - 1.03
T = 0.930	r = 0.994

The sample concentrations were between approx. 9 and 850 pg/mL.

Analytical specificity
The monoclonal antibodies used are highly specific against human PIGF. The following cross-reactivities were found:

Substance	Max. concentration tested (pg/mL)	Cross-reactivity (%)
VEGF 165	10000	< 0.1
VEGF/PIGF-1 heterodimer	10000	< 4
Glycosylated recombinant human PIGF-2	5000	< 28

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For further information, please refer to the appropriate operator's manual for the analyzer concerned, the respective application sheets, the product information, and Method Sheets of all necessary components.
A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

PIGF

Human placental growth factor

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305, Mannheim, Germany
www.roche.com

sFlt-1

Soluble fms-like tyrosine kinase-1

cobas®

REF **05109523** 119 **100 tests**

•Indicates analyzers on which the kit can be used

Elecsys 2010	MODULAR ANALYTICS E170	**cobas e** 411	**cobas e** 601	**cobas e** 602
•	•	•	•	•

English

Intended use

Immunoassay for the in vitro quantitative determination of soluble fms-like tyrosine kinase-1 (sFlt-1) in human serum.
This assay is intended for use as an aid in the diagnosis of preeclampsia, in conjunction with the Elecsys PlGF assay and other diagnostic and clinical information.
The **e**lectro**c**hemi**l**uminescence **i**mmuno**a**ssay "ECLIA" is intended for use on Elecsys and **cobas e** immunoassay analyzers.

Note:

Please note that the catalogue number appearing on the package insert retains only the first 8 digits of the licensed 11-digit Catalogue Number: 05109523190 for the Elecsys sFlt-1 assay. The last 3 digits -190 have been replaced by -119 for logistic purposes.

Summary

Preeclampsia (PE) is a serious complication of pregnancy characterized by hypertension and proteinuria after 20 weeks of gestation.[1] Preeclampsia occurs in 3-5 % of pregnancies and results in substantial maternal and fetal or neonatal mortality and morbidity. Clinical manifestations can vary from mild to severe forms. Preeclampsia associated with thrombocytopenia and liver enzyme elevation represents the HELLP-syndrome (hemolysis, elevated liver enzymes and low platelets).[2,3,4,5,6]
Preeclampsia appears to be due to the release of angiogenic factors from the placenta that induce endothelial dysfunction. Serum levels of PlGF (placental growth factor) and sFlt-1 (soluble fms-like tyrosine kinase-1, also known as VEGF receptor-1) are altered in women with preeclampsia. Moreover, circulating levels of PlGF and sFlt-1 can discriminate normal pregnancy from preeclampsia even before clinical symptoms occur. In normal pregnancy, the pro-angiogenic factor PlGF increases during the first 2 trimesters and decreases as pregnancy progresses to term. In contrast, levels of the anti-angiogenic factor sFlt-1 remain stable during the early and middle stages of gestation and increase steadily until term. In women who develop preeclampsia, sFlt-1 levels have been found to be higher and PlGF levels have been found to be lower than in normal pregnancy.[7,8,9,10]
The ratio of sFlt-1 to PlGF has been shown to be a better predictor of preeclampsia than either measure alone.[11] Placental endoglin, a member of the TGF-β family is also upregulated in preeclampsia and released as soluble endoglin into the maternal circulation. Soluble endoglin has been shown to be increased in severe cases of preeclampsia.[12,13,14,15]
In summary, PlGF and sFlt-1 concentrations measured by immunoassay in maternal blood improve the diagnostic possibilities in preeclampsia which comprise clinical symptoms, proteinuria and uterine artery Doppler velocimetry.[16,17]

Test principle

Sandwich principle. Total duration of assay: 18 minutes.

- 1st incubation: 20 µL of sample, a biotinylated monoclonal sFlt-1-specific antibody, and a monoclonal sFlt-1-specific antibody labeled with a ruthenium complex[a] react to form a sandwich complex.
- 2nd incubation: After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin.
- The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell/ProCell M. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier.
- Results are determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

a) Tris(2,2'-bipyridyl)ruthenium(II)-complex) ($Ru(bpy)_3^{2+}$)

Reagents - working solutions

M Streptavidin-coated microparticles (transparent cap), 1 bottle, 6.5 mL: Streptavidin-coated microparticles 0.72 mg/mL; preservative.

R1 Anti-sFlt-1-Ab~biotin (gray cap), 1 bottle, 9 mL: Biotinylated monoclonal anti-sFlt-1 antibody (mouse) 0.5 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

R2 Anti-sFlt-1-Ab~$Ru(bpy)_3^{2+}$ (black cap), 1 bottle, 9 mL: Monoclonal anti-sFlt-1 antibody (mouse) labeled with ruthenium complex 1.0 mg/L; phosphate buffer 100 mmol/L, pH 7.2; preservative.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.
Avoid foam formation in all reagents and sample types (specimens, calibrators, and controls).

Reagent handling

The reagents in the kit have been assembled into a ready-for-use unit that cannot be separated.
All information required for correct operation is read in from the respective reagent barcodes.

Storage and stability

Store at 2-8 °C.
Store the Elecsys sFlt-1 reagent kit **upright** in order to ensure complete availability of the microparticles during automatic mixing prior to use.

Stability:

unopened at 2-8 °C	up to the stated expiration date
after opening at 2-8 °C	12 weeks
on all analyzers	6 weeks

Specimen collection and preparation

Only the specimens listed below were tested in a sufficient number and found acceptable.
Serum collected using standard sampling tubes or tubes containing separating gel.
Stable for 3 hours at 18-25 °C, 8 hours at 2-8 °C, 1 month at -20 °C.
Freeze only once.
The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.
Centrifuge samples containing precipitates before performing the assay. Do not use heat-inactivated samples. Do not use samples and controls stabilized with azide.
Ensure the samples, calibrators, and controls are at ambient temperature (20-25 °C) before measurement.
Due to possible evaporation effects, samples, calibrators, and controls on the analyzers should be analyzed/measured within 2 hours.

sFlt-1

Soluble fms-like tyrosine kinase-1

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Materials provided
See "Reagents - working solutions" section for reagents.

Materials required (but not provided)
- REF 05109531190, sFlt-1 CalSet, for 4 x 1 mL
- REF 05341787190, PreciControl Multimarker, for 3 x 2 mL each of PreciControl Multimarker 1 and 2
- General laboratory equipment
- Elecsys 2010, MODULAR ANALYTICS E170 or **cobas e** analyzer

Accessories for Elecsys 2010 and **cobas e** 411 analyzers:
- REF 11662988122, ProCell, 6 x 380 mL system buffer
- REF 11662970122, CleanCell, 6 x 380 mL measuring cell cleaning solution
- REF 11930346122, Elecsys SysWash, 1 x 500 mL washwater additive
- REF 11933159001, Adapter for SysClean
- REF 11706802001, Elecsys 2010 AssayCup, 60 x 60 reaction vessels
- REF 11706799001, Elecsys 2010 AssayTip, 30 x 120 pipette tips

Accessories for MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers:
- REF 04880340190, ProCell M, 2 x 2 L system buffer
- REF 04880293190, CleanCell M, 2 x 2 L measuring cell cleaning solution
- REF 03023141001, PC/CC-Cups, 12 cups to prewarm ProCell M and CleanCell M before use
- REF 03005712190, ProbeWash M, 12 x 70 mL cleaning solution for run finalization and rinsing during reagent change
- REF 03004899190, PreClean M, 5 x 600 mL detection cleaning solution
- REF 12102137001, AssayTip/AssayCup Combimagazine M, 48 magazines x 84 reaction vessels or pipette tips, waste bags
- REF 03023150001, WasteLiner, waste bags
- REF 03027651001, SysClean Adapter M

Accessories for all analyzers:
- REF 11298500316, Elecsys SysClean, 5 x 100 mL system cleaning solution

Assay
For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.
Resuspension of the microparticles takes place automatically prior to use.
Read in the test-specific parameters via the reagent barcode. If in exceptional cases the barcode cannot be read, enter the 15-digit sequence of numbers.

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers:
PreClean M solution is necessary.

Bring the cooled reagents to approx. 20 °C and place on the reagent disk (20 °C) of the analyzer. Avoid foam formation. The system **automatically** regulates the temperature of the reagents and the opening/closing of the bottles.

Calibration
Traceability: This method has been standardized against a commercially available sFlt-1 assay.
Every Elecsys sFlt-1 reagent set has a barcoded label containing specific information for calibration of the particular reagent lot. The predefined master curve is adapted to the analyzer using the sFlt-1 CalSet.

Calibration frequency: Calibration must be performed once per reagent lot using fresh reagent (i.e. not more than 24 hours since the reagent kit was registered on the analyzer).

Renewed calibration is recommended as follows:
- after 1 month (28 days) when using the same reagent lot
- after 7 days (when using the same reagent kit on the analyzer)
- as required: e.g. quality control findings outside the defined limits

Quality control
For quality control, use PreciControl Multimarker.
Other suitable control material can be used in addition.

Controls for the various concentration ranges should be run individually at least once every 24 hours when the test is in use, once per reagent kit, and following each calibration. The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits.
Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.
Follow the applicable government regulations and local guidelines for quality control.

Note: The controls are not barcode-labeled and therefore have to be run like external controls. All values and ranges have to be entered manually. Please refer to the section "QC" in the operator's manual or to the online help of the instrument software.

Calculation
The analyzer automatically calculates the analyte concentration of each sample in pg/mL.

Limitations - interference
The assay is unaffected by icterus (bilirubin < 427 µmol/L or < 25 mg/dL), hemolysis (Hb < 0.311 mmol/L or < 0.5 g/dL), lipemia (Intralipid < 1400 mg/dL) and biotin (< 123 nmol/L or < 30 ng/mL).
Criterion: Recovery within ± 15 % of initial value.
Samples should not be taken from patients receiving therapy with high biotin doses (i.e. > 5 mg/day) until at least 8 hours following the last biotin administration.
No interference was observed from rheumatoid factors up to a concentration of 600 IU/mL.
There is no high-dose hook effect at sFlt-1 concentrations up to 200000 pg/mL.
In vitro tests were performed on 18 commonly used pharmaceuticals.
No interference with the assay was found.
In rare cases, interference due to extremely high titers of antibodies to analyte-specific antibodies, streptavidin or ruthenium can occur. These effects are minimized by suitable test design.

Limitations - general
For diagnostics purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings. Values below 15 pg/mL are not reliable as the coefficient of variation between runs is > 20%.

Limits and ranges
Measuring range
10-85000 pg/mL (defined by the Limit of Detection and the maximum of the master curve). Values below 10 pg/mL are reported as < 10 pg/mL. Values above the measuring range are reported as > 85000 pg/mL.

Lower limits of measurement
Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ)

Limit of Blank	= 6 pg/mL
Limit of Detection	= 10 pg/mL
Limit of Quantitation	= 15 pg/mL

The Limit of Blank and Limit of Detection were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A requirements. The Limit of Quantitation was determined using the result of functional sensitivity testing.
The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.
The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples. The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).
The Limit of Quantitation (functional sensitivity) is the lowest analyte concentration that can be reproducibly measured with an intermediate precision CV of ≤ 20 %. It has been determined using low concentration sFlt-1 samples.

Dilution
Generally not necessary due to the broad measuring range.

sFlt-1

cobas®

Soluble fms-like tyrosine kinase-1

Please note
For linearity studies within the measuring range, samples may be diluted with human serum. The final concentration of the diluted sample must be > 5000 pg/mL.
The analyte sFlt-1 is known to be heterogeneous and this may give rise to non-linear dilution phenomena for certain individual samples.

Expected values
Clinical studies are still ongoing. From a first data set of 524 serum samples collected from 280 singleton pregnancies with normal pregnancy outcome (i.e. no PE/HELLP, no intrauterine growth restriction) preliminary expected values were obtained. For each sample levels of sFlt-1 and PlGF were determined in parallel and sFlt-1/PlGF ratio was calculated. Weeks of gestation: defined as completed weeks of pregnancy beginning with the start of the last menstruation cycle.
The following results were obtained:

Percentile Elecsys sFlt-1 (pg/mL)

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	555	470	649	630	707	978	1671
50th perc.	1445	1459	1576	1449	1934	2972	4400
95th perc.	2361	2785	2944	3890	6688	9921	11324
N (visits)	40	44	82	98	105	7	77

Percentile Elecsys PlGF (pg/mL)

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	29.4	65.7	125	130	73.3	62.7	52.3
50th perc.	62.8	135	265	412	439	232	161
95th perc.	183	203	541	1108	1108	972	659
N (visits)	40	44	82	98	105	7	77

Percentile Elecsys sFlt-1/PlGF ratio

	Weeks of gestation						
	10-14	15-19	20-23	24-28	29-33	34-36	37-delivery
5th perc.	5.21	4.32	2.19	1.01	0.945	1.38	3.65
50th perc.	22.7	12.6	6.09	3.80	4.03	13.3	26.2
95th perc.	57.3	26.9	14.8	16.9	86.4	92.0	138
N (visits)	40	44	82	98	105	78	77

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data
Representative performance data on the analyzers are given below.
Results obtained in individual laboratories may differ.

Precision
Precision was determined using Elecsys reagents, samples and controls in a protocol (EP5-A2) of the CLSI (Clinical and Laboratory Standards Institute): 2 runs per day in duplication each for 21 days (n = 84).
The following results were obtained:

Elecsys 2010 and **cobas e** 411 analyzers

		Repeatability[b]		Intermediate precision	
Sample	Mean pg/mL	SD pg/mL	CV %	SD pg/mL	CV %
Human serum 1	63.1	0.984	1.6	2.71	4.3
Human serum 2	589	4.79	0.8	13.8	2.3
Human serum 3	34516	359	1.0	1017	2.9
Human serum 4	79101	915	1.2	2933	3.7
PreciControl MM[c]1	107	1.59	1.5	4.05	3.8
PreciControl MM2	1080	15.2	1.4	42.4	3.9

b) Repeatability = within-run precision
c) MM = Multimarker

MODULAR ANALYTICS E170, **cobas e** 601 and **cobas e** 602 analyzers

		Repeatability		Intermediate precision	
Sample	Mean pg/mL	SD pg/mL	CV %	SD pg/mL	CV %
Human serum 1	65.5	0.849	1.3	2.12	3.2
Human serum 2	617	11.5	1.9	15.7	2.5
Human serum 3	34243	527	1.5	1148	3.4
Human serum 4	78677	1213	1.5	3054	3.9
PreciControl MM1	100	3.94	3.9	5.58	5.6
PreciControl MM2	988	26.3	2.7	35.0	3.5

Clinical sensitivity and specificity
In an external study using the Elecsys sFlt-1 and Elecsys PlGF assays in parallel on samples from 268 pregnant women with normal pregnancy outcome (no PE/HELLP, no intrauterine growth restriction) and 71 patients with PE/HELLP an optimal cut-off for the sFlt-1/PlGF ratio of 85 was determined. At this cut-off the sensitivity was calculated at 82 % and the specificity at 95 %. The area under the receiver operating characteristic(ROC) curve was 0.95. For a subcollective of 37 women with early-onset PE the sensitivity/specificity was calculated to be 89 %/97 % at the same cut-off. The area under the ROC curve was 0.97.

All pregnancies were singleton pregnancies. PE was defined as new onset of both hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg) and proteinuria (> 0.3 g/24 h or dipstick ≥ 1+ if a 24 h urine collection could not be obtained) after week 20 of gestation. A PE-pregnancy was defined as early-onset PE if clinical signs of PE appeared before week 34 of gestation.
The data as obtained for all samples of cases(■) and controls (□) are shown in the scatter plots below.

Fig. 1: Elecsys sFlt-1 assay

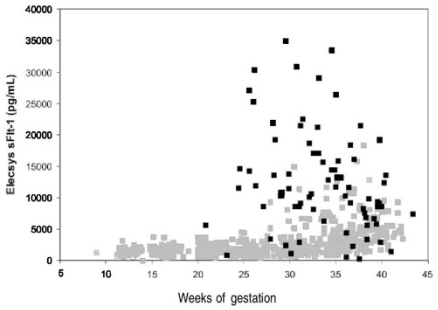

Fig. 2: Elecsys PlGF assay

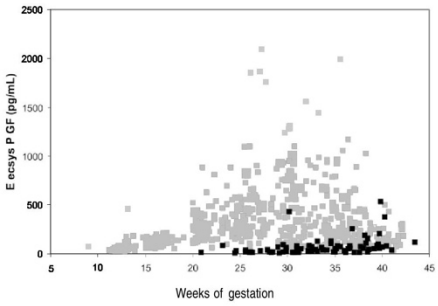

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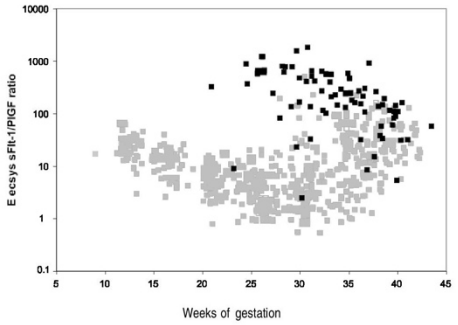
Fig. 3: Elecsys sFlt-1/PlGF ratio



Method comparison

A comparison of the Elecsys sFlt-1 assay (y) with a commercially available sFlt-1 assay (x) using clinical samples gave the following correlations (pg/mL):
Number of samples measured: 112

Passing/Bablok[18]	Linear regression
y = 1.11x - 47.3	y = 1.05x - 23.7
τ = 0.827	r = 0.952

The sample concentrations were between approx. 42 and 1860 pg/mL.

Analytical specificity

Recombinant Human VEGF-R1 extracellular domain is the only know immunogen specific for Elecsys sFLT-1 test.

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7.8 Appendix H: Plagiarism declaration

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SENATE PLAGIARISM POLICY: APPENDIX ONE

I Pieter Francois Retief (Student number: 1017956) am a student registered for the degree of Master of Medicine (Obstetrics & Gynaecology) in the academic year 2017.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
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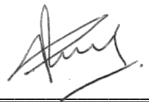
Signature:  Date: 23 January 2017

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