A COMPARISON OF CALCIUM LEVELS IN

PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANCIES

IN A LOW DIETARY CALCIUM SETTING

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Dissertation submitted in part fulfilment of the requirements of the degree Master of Medicine in Obstetrics and Gynaecology of the University of Cape Town.

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DECLARATION

I, Dominic Giles Dudley Richards, hereby declare that the work contained in this dissertation is my original work and work by others has been acknowledged as such.

This study was completed while a registrar in the Department of Obstetrics and Gynaecology at the University of Cape Town.

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DECLARATION BY SUPERVISORS

This research which Dr Dominic Richards has untaken and the presentation of this dissertation were supervised by Zephne van der Spuy, Professor of Obstetrics and Gynaecology of the University of Cape Town, South Africa and Stephen Lindow, Senior Lecturer of the Department of Obstetrics and Gynaecology, University of Hull, United Kingdom and Honorary Associate Professor of Obstetrics and Gynaecology of the University of Cape Town, South Africa.

We are satisfied that this is Dr Richards' original work and that this dissertation should be submitted in part fulfilment of the requirements for the degree Master of Medicine in Obstetrics and Gynaecology of the University of Cape Town.

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ABSTRACT

Background

Pre-eclampsia is a leading cause of maternal mortality and morbidity in South Africa. At present this disease cannot be prevented and many interventions to reduce the incidence of pre-eclampsia have been investigated. Calcium supplementation of pregnant women at high risk of developing pre-eclampsia has been shown to be of some benefit in reducing the incidence of the disease, with the greatest benefit seen in low dietary calcium settings. While serum calcium is an unreliable indicator of chronic calcium status, hair analysis is an accurate and well documented method of determining long-term micronutrient status.

Objective

To examine differences in serum and hair concentrations of calcium and magnesium between women with pre-eclampsia and normotensive matched-controls.

Design

An observational case-controlled study conducted at the Maternity Centre of Groote Schuur Hospital and Mowbray Maternity Hospital.

Population

Any woman above the age of eighteen years who had delivered a single, live infant in a period not exceeding 96 hours from birth to enrolment was eligible to be recruited. A case was defined as any woman with pre-eclampsia in the index pregnancy. A control was defined as any woman with a healthy, normotensive pregnancy. Pre-eclampsia was defined according to the Davey and MacGillivray classification of Hypertensive Disorders of Pregnancy. Exclusion criteria included women who had not received antenatal care, multiple pregnancies, eclampsia, any form of antenatal vitamin or mineral supplementation other than iron or folic acid and any pre-existing disorder that could affect blood pressure, proteinuria or calcium metabolism

Methods

Two hundred and fourteen women were recruited to the study. Ninety six women with pre-eclampsia were matched to normotensive controls by ethnicity, age ± 2 years, gestational age at delivery ± 3 weeks and gravidity. Each participant completed a dietary questionnaire. A sample of venous blood and scalp hair was taken from each participant. Serum calcium and magnesium concentrations were determined by the National Health Laboratory Service. Hair calcium and magnesium levels were measured by inductively coupled plasma mass spectrometry at the Department of Chemistry of the University of Hull.

Main outcome measures

Hair calcium and magnesium levels and serum calcium and magnesium concentrations between women with pre-eclampsia and matched-controls were assessed. The impact of HIV infection on serum and hair calcium and magnesium levels was also investigated.

Results

There was no significant difference in hair calcium level between women with pre-eclampsia and matched-controls (1241 ppm, 331 – 4654 *vs*. 1146 ppm, 480 – 4136, p = 0.5). These findings were the same in the HIV negative and HIV positive groups. There was no correlation between serum calcium and hair calcium levels. There was no difference in hair magnesium levels between women with pre-eclampsia and matched-controls in the total group and by HIV status. Diet and socio-economic status in the two groups were similar.

Conclusion

Women with pre-eclampsia showed no difference in chronic calcium status compared to matched-controls. While the benefit of supplementing women with calcium who are at high risk of developing pre-eclampsia is generally attributed to correcting a nutritional deficiency, an alternative explanation would be that calcium supplementation has a pharmacological effect which modifies the pathological processes underlying pre-eclampsia.

LIST OF ABBREVIATIONS

AT1	-	Angiotensin II type 1
AZT	-	Zidovudine
BMI	-	Body mass index
B2	-	Bradikinin type 2
DEXA	-	Dual emission x-ray absorpitometry
eNOS	-	Endothelial nitric oxide synthase
Fms	-	a gene that encodes a macrophage growth factor receptor
Flt1	-	*Fms*-like tyrosine kinase 1
GPH	-	Gestational proteinuric hypertension
HAART	-	Highly active anti-retroviral therapy
HIV	-	Human immunodeficiency virus
HDP	-	Hypertensive disorders of pregnancy
ICP-MS	-	Inductively coupled plasma mass spectrometry
mmol/l	-	Millimoles per litre
NVP	-	Nevirapine
NO	-	Nitric oxide
PGI_2	-	Prostacyclin

ppm	-	Parts per million in one gram
PTH	-	Parathyroid hormone
PTHrP	-	Parathyroid hormone-related protein
sFLT1	-	Soluble *fms*-like tyrosine kinase 1
TxA_2	-	Thromboxane
WHO	-	World Health Organisation
1,25-OHD	-	1,25 dihydroxycholecalciferol
25-OHD	-	25 hydroxycholecalciferol

CHAPTER 1. INTRODUCTION AND LITERATURE REVIEW

Pre-eclampsia, which is classed in the group Hypertensive Disorders of Pregnancy (HDP), is a leading cause of maternal and perinatal morbidity and mortality in South Africa and many developing countries.[1] The exact pathogenesis of the disease remains unclear, making preventative interventions and management difficult and often unsuccessful. Studies have investigated the relationship between calcium and HDP, specifically pre-eclampsia, and have shown that oral calcium supplementation from twenty weeks gestation may reduce the incidence, or at least the severity of the disease.[2] Calcium is thought to counteract at the molecular level the mechanisms that are responsible for increased vascular tone and vasospasm, which cause elevated blood pressure in pre-eclampsia. The exact mechanism of action is, however, unknown. While the evidence surrounding calcium supplementation is conflicting, the most beneficial effect of this intervention is seen in calcium deficient populations.[2]

To date there is no literature investigating the long-term calcium status of pre-eclamptic women within our clinical environment in South Africa. This was confirmed by performing a Medline search. A single study performed in Durban, South Africa over two decades ago, demonstrated significantly lower mean serum calcium levels in black women with eclampsia compared to pregnant and non pregnant-controls.[3] This study was limited to a single ethnic group and women with eclampsia, and to date has not been repeated in different clinical groups.

Assessing the long-term calcium status rather than only serum levels of mothers with pre-eclampsia in a low calcium intake population may reveal that these patients are chronically calcium deficient. If this is found to be true, initiating replacement therapy as a potential way of modifying the presentation of pre-eclampsia will warrant serious consideration.

1.1 Epidemiology of pre-eclampsia

Globally 2 – 8% of all pregnancies are complicated by pre-eclampsia making it one of the most common obstetric disorders requiring hospital admission.[4] In South Africa HDP are the most common direct cause of maternal death. The National Committee for Confidential Enquiry into Maternal Deaths found HDP to be the primary cause in 15.7% of deaths over the period 2005-2007.[5] Eighty three percent of these patients had either pre-eclampsia (27.8%) or eclampsia (55.3%) as the primary obstetric cause. Pre-eclampsia and eclampsia account for 25% of stillbirths and neonatal deaths in developing countries, and contribute significantly to preterm delivery and small for gestational age infants.[6]

It is apparent that pre-eclampsia may have serious health implications extending far beyond the reproductive period and the affected pregnancy. Norwegian studies have shown that women who have had pre-eclampsia and associated preterm delivery have an increased risk of dying from cardiovascular causes or developing end-stage renal disease.[7,8] Women with eclampsia may develop more cognitive failure some years after the affected pregnancy when compared with unaffected women.[9]

1.2 Diagnosis of pre-eclampsia

Pre-eclampsia is a progressive disease causing widespread endothelial damage that usually presents as a syndrome with *de novo* hypertension and significant proteinuria after 20 weeks gestation. Pre-eclampsia is categorised under gestational proteinuric hypertension (GPH) according to the Davey and MacGillivray classification of the hypertensive disorders of pregnancy.[10] GPH is defined as hypertension and significant proteinuria developing in pregnancy, labour, or the puerperium in a previously normotensive aproteinuric woman. Hypertension is diagnosed when the diastolic blood pressure is ≥ 110 mmHg on any one occasion or when the diastolic blood pressure is ≥ 90 mmHg on two or more consecutive occasions ≥ 4 hours apart. Proteinuria is considered significant when a 24-hour urine collection contains ≥ 300 mg of protein or when two clean-catch-midstream or catheter specimens of urine which are collected ≥ 4 hours apart have ≥ 2+ protein on reagent strip or sulphosalicylic acid test. This definition is more robust than others and was designed for clinical use to maximise sensitivity as it is safer to over-diagnose the condition than to fail to recognise it.

While most cases of pre-eclampsia manifest as hypertension with concurrent proteinuria, it is not always the case as the pattern of organ system involvement may vary. A woman with pre-eclampsia might present with fetal manifestations of the disease including growth restriction, oligohydramnios or abnormal umbilical artery Doppler flow before developing hypertension or proteinuria. If pre-eclampsia is not recognised or inadequately managed it may advance to eclampsia, a disorder which is characterised by seizures and is considered a hypertensive emergency. Both disorders may have severe immediate implications for the

mother and the fetus. While the definitive treatment is delivery of the pregnancy, the challenge of management is to decide at what gestation to deliver the fetus to maximise a good neonatal outcome while not jeopardising maternal health.

1.3 Pathogenesis of pre-eclampsia

The exact pathogenesis of pre-eclampsia is unclear and most studies recruit subjects with established disease rather than prior to clinical onset. In 2005 Noris *et al* published an extensive review which attempted to reconcile the abnormalities that occur at the feto-placental level in pre-eclampsia with the clinical features of maternal disease.[11] Research performed more recently highlights the important role of endothelium-derived hyperpolarising factor in the pathogenesis of the disease.[12]

1.3.1 The feto-placental level

During placental development there are two waves of trophoblast invasion, the second wave beginning around the sixteenth week. In pre-eclampsia, it is postulated that failure of invasion of the endovascular trophoblast, the so-called second wave, is the precipitant of abnormal placentation which leads to poor placental perfusion, subsequent ischaemia and pre-eclampsia. More recent research suggests that failure of vascular invasion may begin earlier than previously believed.[13]

Increased levels of arginase II in trophoblast cells in placentas of pre-eclamptic pregnancies result in lower L-arginine levels which in turn stimulate endothelial nitric oxide synthase (eNOS) to produce reactive oxygen species and reduced levels of nitric oxide. Both these effects impair vascular invasion, thus decreasing placental perfusion leading to placental hypoxia. Decreased NO levels in pre-eclampsia reduce the activity of metalloproteinase 2 and 9 which may impair trophoblast invasion and alter maternal endothelial behaviour.[14]

Placentas of pregnancies that develop pre-eclampsia produce increased levels of *fms*-like tyrosine kinase (Flt1), an endothelial growth factor, in response to restricted blood flow and hypoxia of the placenta.[15] Most Flt1 is soluble (sFlt1) and is able to leave the cell membrane and circulate freely in blood. sFlt1 binds to the Flt1 receptor on cytotrophoblast cells and prevents binding of vascular endothelial growth factor and placental growth factor. This deprives the feto-placental unit and maternal vasculature of essential maintenance signals and leads to defective cytotrophoblast differentiation, impaired angiogenesis and defective placentation; all characteristics of pre-eclampsia.[16]

1.3.2 The maternal level

In pre-eclampsia the normal pregnancy shift in the balance between the eicosanoids thromboxane (TxA_2), a potent vasoconstrictor, and prostacyclin (PGI_2) a potent vasodilator, is reversed favouring TxA_2. This causes vasoconstriction and mimics an inflammatory endothelial response.[17]

Vascular responsiveness to angiotensin II is increased in pre-eclampsia, not due to an increase in serum levels of angiotensin II but from the formation of highly sensitive heterodimers between bradykinin type 2 (B2) receptors and angiotensin II type 1 (AT1) receptors. Angiotensin II is a potent vasoconstrictor and stimulation of the B2-AT1 heterodimer receptor will increase blood pressure significantly.[18]

The combined effect of reduced NO levels, increased sFlt1, TxA_2 mediated vasoconstriction, increased sensitivity to angiotensin II and endothelial inflammation on the maternal vasculature leads to vasospasm and increased endothelial permeability. Hypertension, non-dependant skin oedema, cerebral oedema, proteinuria and ischaemia of organs such as the kidneys, liver and heart are manifestations of these changes which together contribute to the maternal syndrome characteristic of pre-eclampsia.

1.3.3 Endothelium-derived hyperpolarising factor

Numerous abnormalities in metabolic and cellular pathways have been identified as potential contributors to the endothelial dysfunction typical of pre-eclampsia. To date no single pathway has been shown universally to explain the pathophysiology of the disease. Recent research has found that alterations in endothelium-derived hyperpolarising factor (EDHF), a vascular relaxing factor, might have a significant influence in the development of pre-eclampsia.[12] More recent thinking attributes endothelial adaptations in normal pregnancy to alterations in EDHF pathways in addition to the well established changes in NO

synthesis and vascular sensitivity to NO, however the EDHF mediated component of vasoconstriction in pre-eclampsia appears to outweigh the NO component.[12]

1.4 Prevention and screening of pre-eclampsia

In an attempt to prevent pre-eclampsia, studies have targeted certain of the pathophysiological mechanisms already outlined. In 2006 "The Vitamin C and vitamin E in pregnant women at risk for pre-eclampsia trial" supplemented over one thousand women with high doses of vitamins C and E in an effort to counteract oxidative stress in pre-eclampsia. No significant reduction in pre-eclampsia was found at the doses given and the study was prematurely terminated.[19] In 2009 the World Health Organisation (WHO) published a similar multicentre double-blinded randomised controlled trial of 1365 women from populations with low nutritional status in developing countries. Groote Schuur Hospital, Cape Town was included as a collaborator.[20] Using the same dose of vitamins C and E as the 2006 trial, this study found that the risk of pre-eclampsia was not reduced by supplementation.

Pregnant women at high risk for developing pre-eclampsia have been treated with low-dose aspirin as a preventative intervention. Aspirin inhibits the synthesis of platelet TxA_2 without affecting the production of vascular PGI_2.[21] A 2007 Cochrane review favoured the use of antiplatelet drugs, including aspirin and dipyridamole, for the prevention of pre-eclampsia citing a 17% risk reduction of developing the disease, 8% reduction in delivery before 37

weeks, 10% reduction in small-for-gestational-age babies and 14% reduction in neonatal death.[22]

Two studies in 2006 and 2007 investigated the effect of supplementing pregnant women with L-arginine where the diagnosis of pre-eclampsia had already been made.[23,24] While this intervention seemed to prolong the interval from diagnosis to delivery, these studies are underpowered and definitive recommendations cannot yet be made.

A systematic review by Hofmeyr *et al* published in 2010 in the Cochrane Collaboration reviewed thirteen randomised controlled trials which included 15 730 women.[25] It concluded that supplementing women with 1 gram of calcium during pregnancy versus placebo reduced both the average risk of developing hypertension in pregnancy and the average risk of developing pre-eclampsia. The greatest reduction in hypertension and pre-eclampsia was seen in women at high risk of developing hypertensive disease in pregnancy and those with a low baseline calcium intake.

1.5 Calcium metabolism

Calcium is the most abundant mineral in the body and is essential in almost all human biological processes. Ninety nine percent is found in the skeleton and the remaining 1% in muscle, soft tissue and plasma.[26] Calcium exists in the blood in one of three forms: ionised and physiologically active, protein-bound (mostly to albumin) and physiologically inactive, or

in complexed forms. Calcium in extracellular fluid and bone is in equilibrium. The biological activity of serum calcium is determined by the ionised and physiologically active component, and a serum level of 1.2 – 1.4 mmol/l is maintained in normal circumstances.[27] The laboratory determination of calcium measures only total serum calcium. This value is largely dependent on the protein-bound portion of calcium and therefore must be corrected for albumin levels. The corrected calcium level allows for adjustment of the total calcium level in situations where serum albumin levels are abnormally high or low.

Corrected calcium (mmol/l) = measured total Ca (mmol/l) + 0.02 (40 - serum albumin [g/l]), where 40 represents the average normal albumin level in g/l.[27]

The daily adult intake of calcium averages around 1000 mg, which matches the suggested value for women aged 19 to menopause by the WHO in 2004.[28] The WHO suggests a calcium intake during the last trimester of pregnancy of 1200 mg per day. These values are based on North American and Western European data, but it is acknowledged that figures may be different in developing countries. The WHO report recognises the concept of cultural and nutritional environments, allowing for variation in individual calcium intake and population-specific calcium status. Calcium status may be altered by Human Immunodeficiency Virus (HIV) infection as malnutrition and HIV infection are strongly associated. Malnutrition is listed as one of the major complications of HIV infection and it may occur early or late in the disease process.[29]

Physiological states such as puberty, pregnancy and menopause as well as vitamin D levels, age and calcium binders in food affect the absorption rate of calcium. Only 25% to 35% of ingested calcium is actually absorbed by vitamin D mediated active transport in the small intestine.[26] Calcium is excreted in urine, faeces, skin, hair and nails. Renal excretion is increased during pregnancy and by high protein and salt intake. While bone is the ultimate calcium reservoir, there is continuous flux of calcium between the gut, extracellular fluid, kidney and bone under the influence of vitamin D, parathyroid hormone (PTH) and calcitonin. Thyroid, adrenal, sex steroid and growth hormones also influence calcium metabolism.

Ninety nine percent of inactive vitamin D_3 (cholecalciferol) is produced by the photochemical conversion of 7-dehydrocholesterol in the skin, the remainder is ingested in fish oils, eggs, butter and liver either in the D_3 or D_2 (ergocalciferol) form. Intestinal absorption is facilitated by bile salts as is the case with fat soluble vitamins (A, D, E and K). Inactive vitamin D_3 is hydroxylated in the liver to 25-hydroxycholecalciferol (25-OHD) which is the dominant circulating form of the vitamin. The enzyme, 1α-hydroxylase, which in the non-pregnant state is only found in the kidney, is activated by PTH and it hydroxylates 25-OHD to 1,25 dihydroxycholecalciferol (1,25-OHD). The prime function of 1,25-OHD, the active form of vitamin D, is to maintain plasma calcium and phosphate levels. It achieves this by stimulating bone resorption, increasing renal reabsorption of calcium and phosphate and inducing synthesis of intestinal brush border proteins to enhance intestinal calcium and phosphate absorption.[30]

PTH release is stimulated in response to a reduction in ionised calcium to sustain the narrow range of calcium concentration in extracellular fluid. In bone it stimulates osteoclasts to secrete collagenase which increases metabolic destruction of bone matrix thus mobilising calcium and phosphate. In the kidney, PTH decreases phosphate reabsorption in the proximal tubule, while increasing calcium reabsorption in the distal tubule. PTH also increases the activity of 1α-hydroxylase in the kidney, so increasing the production of activated vitamin D (1,25-OHD). Calcium mobilised from bone far exceeds the capacity for renal reabsorption, which explains paradoxical hypercalciuria in the presence of low ionised calcium. Calcitonin is released in response to high ionised calcium levels by parafollicular cells of the thyroid. It directly inhibits bone breakdown, and indirectly inhibits vitamin D and PTH mediated bone resorption.[30]

Serum calcium and magnesium levels are related as they usually move in the same direction and both exert an effect on PTH release. This effect on PTH release has been shown in a study where humans received intravenous injections of sodium chloride (as a control), magnesium sulphate, magnesium pyrrolidone carboxylate and calcium gluconate at one week intervals. Plasma concentrations of PTH were unchanged after sodium chloride and magnesium pyrrolidone carboxylate. Both magnesium sulphate and calcium gluconate produced a 30% decrease in plasma PTH levels, though the duration of PTH decrease was longer after calcium injection (2 hours) than magnesium injection (45 minutes).[31]

Table 1.1 Function of hormones involved in calcium metabolism	
1,25 dihydroxycholecalciferol (1,25 OHD)	• Stimulates bone resorption • Increases renal reabsorption of calcium and phosphate • Increases intestinal calcium and phosphate absorption
Parathyroid hormone (PTH)	In bone: • Mobilises calcium and phosphate In kidneys: • Increases 1α-hydroxylase activity • Decreases phosphate absorption in proximal tubule • Increases calcium reabsorption in distal tubule
Calcitonin	• Directly inhibits bone breakdown • Indirectly inhibits vitamin D and PTH mediated bone resorption

1.6 Changes in calcium metabolism during pregnancy

The fetus acquires a total of 30 – 40 g of calcium from the mother through active placental transport, 20% of this during the first and second trimesters and 80% during the third where 250 – 300 mg of calcium is incorporated into the fetal skeleton each day.[32] To compensate for this loss to the fetus, the mother needs to increase calcium intake. If exogenous calcium sources are inadequate, maternal bone is mobilised to meet fetal calcium demands. In addition to the requirements of the fetus, calcium intake in early pregnancy must also provide the necessary amount of calcium to strengthen the maternal skeleton in anticipation of increasing body mass as gestation continues.

Maternal total serum calcium levels decrease secondary to haemodilution and a reduction in serum albumin, most notably between 28 – 32 weeks when the circulating plasma volume is at a maximum. The physiologically important ionised fraction however remains constant and seems to remain within the normal range throughout pregnancy in well-nourished women.[32] While a reduction in renal excretion would seem an effective manner to conserve maternal calcium in pregnancy, 24 hour urinary calcium excretion is greater in pregnancy; a consequence of increased intestinal absorption, increased renal calcium load and increased glomerular filtration rate.

The major pregnancy adaptation in calcium metabolism is a two–fold increase in the absorbed fraction of intestinal calcium from ±25% to 50% of the pre-pregnancy fraction of ingested calcium. This is generated by increasing 1α-hydroxylase activity and so doubling 1,25-OHD levels.[32] This increase in 1,25-OHD is seen as early as 12 weeks gestation when fetal calcium needs are minimal, possibly to increase maternal bone density in preparation for the demands of the fetus in the third trimester.

The increase in 1α-hydroxylase activity seen during pregnancy is not due to PTH. PTH levels decrease during pregnancy to low-normal range in the first trimester and steadily reach the mid-normal range closer to term. Rather, the increase in renal 1α-hydroxylase activity during pregnancy is driven by estradiol and probably progesterone, prolactin, human placental lactogen, growth hormones and parathyroid hormone-related protein (PTHrP).

The decidua and feto-placental unit also produce 1α-hydroxylase and contribute a small amount to 1,25-OHD production.

PTHrP describes a family of hormones produced in almost all maternal tissues, and in pregnancy by the placenta and fetus, and is so named due to a segment of amino acids which exhibit some similarity to PTH. Parathyroid hormone related proteins have profound effects on a number of physiological processes including proliferation, differentiation and death of certain cell lines as well as calcium metabolism. During pregnancy PTHrP plays a diverse role: the amino-terminal peptides stimulate skeletal calcium resorption and 1α-hydroxylase activity, the mid-molecular portion enhances placental calcium transfer and the carboxy-terminal peptides inhibits osteoclast activity, protecting the maternal skeleton.[32]

Calcitonin levels are increased during pregnancy, with some contribution from placental production, and are thought to protect the maternal skeleton from the calcium demands associated with pregnancy.[32]

Studies of bone metabolism to assess calcium metabolism indirectly during pregnancy are not useful. Alkaline phosphatase, a biochemical marker which indicates bone formation is produced by the placenta and levels are unreliable in pregnancy. Radiological investigations such as dual emission x-ray absorpitometry (DEXA) to measure bone density are damaging to the fetus, and to investigate bone changes in this manner would require repeated exposures.[32]

1.7 Rationale behind calcium supplementation in pre-eclampsia

At present because of a lack of knowledge about the etiology of pre-eclampsia, it appears that this condition cannot be prevented. There have been many interventional trials aimed at reducing the incidence or at least the severity of the disease. While most of these trials have had little success, oral calcium supplementation of pregnant women at high risk of developing pre-eclampsia may be of some benefit.

Research on the relationship between calcium and HDP is found in the literature as early as 1954.[33] The relationship between calcium intake and the incidence of pre-eclampsia was first described in 1980 where a population group in Guatemala who had a high calcium diet were found to have a low incidence of pre-eclampsia and eclampsia.[34] Subsequent epidemiological and clinical studies led to the hypothesis that calcium supplementation during pregnancy may reduce the incidence of HDP in women with low dietary calcium. Calcium supplementation is an attractive intervention as it is low-cost, readily available, easy to administer and safe for mother and fetus.

The 2007 systematic review of Hofmeyer *et al* included twelve selected studies investigating calcium supplementation and divided participants into three categories according to dietary calcium intake: adequate, low and not specified.[2] Subjects were given between 1.5 and 2 g of oral calcium daily throughout pregnancy, although the gestational age at which supplementation started varied between studies. The primary outcome measured was the relative risk of developing pre-eclampsia. The pooled data in each category showed a

reduction in the relative risk, with a combined risk reduction of 52% of developing the disease. The most significant reduction was seen in those subjects at high risk for developing pre-eclampsia and those with low dietary calcium. There was no significant risk reduction in the group with adequate dietary calcium. This review supports the use of calcium supplementation in pregnancy to reduce the risk of developing pre-eclampsia in women with low dietary intake. The Cochrane review undertaken by Hofmeyer *et al* published in 2010 reported similar findings, with a combined risk reduction of 65% of developing pre-eclampsia.[25] A criticism of the 2007 systematic review was that the outcome seemed to have been swayed by the smaller studies. The two larger studies which incorporated 12 648 of the 15 206 participants (83.2%) in the review showed little benefit from calcium supplementation.

1.8 Potential mechanisms of action of calcium treatment in pre-eclampsia

Calcium supplementation has been shown not to affect markers of endothelial damage and is believed to modify vascular tone and thus reduce hypertension via certain mechanisms.[35]

- Calcium supplementation lowers PTH release and reduces smooth muscle intracellular calcium which promotes vasodilatation.
- Endothelial nitric oxide synthase (eNOS) is a calcium dependent enzyme: increasing calcium levels facilitates production of endothelial NO which promotes vasodilatation.[36]
- Calcium supplementation may indirectly affect smooth muscle function by increasing magnesium levels which reduces vascular tone.[37]

These effects of calcium appear to blunt the degree of vasoconstriction secondary to pre-eclampsia and reduce the severity of hypertension. This may slow the progression of the disease and reduce hypertensive related complications so allowing the time interval from diagnosis to delivery to be prolonged which is advantageous to the fetus.

1.9 Hair analysis as an assessment of calcium status

Serum measurement of calcium levels is a poor indication of calcium status for a number of reasons:

- calcium occurs in ionised, protein-bound and complexed forms in blood plasma and the physiologically active ionised fraction is difficult to measure,
- serum calcium is influenced by protein concentration as albumin is its principal carrier protein, about 50% of total plasma calcium is bound to albumin,
- there is continuous and rapid flux of calcium between bone, cells and blood plasma,
- ionised calcium is sensitive to acid-base balance.

Subtle changes in calcium status and calcium intake are not easily detectable using serum measurements. To determine if pre-eclamptic women in a low calcium intake setting are more likely to be calcium deficient than normotensive controls, the long-term calcium status must be investigated.

The use of hair as a substrate to test for the presence, or to determine the concentration, of elements, micronutrients and chemicals is a method that has long been recognised. Hair

analysis is a useful tool to screen for toxic metals such as mercury and illicit drugs such as cocaine, and to measure the concentration of micronutrients which are incorporated into the hair. The effect of supplementing pregnant women with calcium, iron and zinc has been reflected in hair concentrations using X-ray fluorescence spectrometry.[38] Chronic lead exposure and long-term selenium status have both been investigated by determining hair concentration of these metals.[39,40]

During hair formation in the matrix cell, elements from the blood are incorporated at a constant rate. Once hair is formed it is separated from the body's internal metabolism thereby withdrawing these elements from dynamic equilibrium. This allows chronic mineral surplus or deficiency to be determined even if serum concentrations have returned to normal. Calcium is incorporated into hair, where it remains stable and can provide an indication of long-term status, and so will be an index of general nutritional status over time.[40]

Hair sample collection is painless, causes no harm to the study subject and specimens are stable when stored or transported and do not deteriorate over time. External contamination may complicate hair analysis. Contamination may be caused by grooming methods such as shampooing, hair styling, bleaching and colouring treatments or from environmental contaminants such as dust and moisture.[41] Thorough washing of the hair samples in the laboratory to remove the bulk of contaminants is an initial step prior to analysis.

Quantification of calcium and magnesium in hair samples can be performed using inductively coupled plasma mass spectrometry (ICP-MS). This specific method of analysis is utilised by the Department of Chemistry of the University of Hull and its suitability has been already evaluated for numerous substances including selenium and mercury.[42] The advantage of using ICP-MS is that it can quantify the concentration of an element present at trace levels in hair and perform simultaneous multi-element determinations.

1.10 Summary

- Pre-eclampsia and eclampsia are leading causes of maternal mortality and morbidity in South Africa. While much research has been done to investigate interventions to reduce the incidence of pre-eclampsia, at present it seems that it cannot be prevented.
- Calcium supplementation of pregnant women at risk of developing pre-eclampsia may be of some benefit as it has been shown to reduce the incidence and the severity of the disease. While the evidence surrounding calcium supplementation is conflicting, the most beneficial effect is seen in women living in low dietary calcium settings.
- Serum calcium concentration is affected by various factors and is therefore an unreliable indicator of chronic calcium status. Hair analysis is an accurate and well documented method of determining chronic nutritional status.
- By comparing hair calcium levels between women with pre-eclampsia and normal pregnancies, more information about the relationship between calcium and pre-eclampsia in a low dietary calcium setting may be obtained.

CHAPTER 2. METHODS

2.1 Aim of study

The aim of this study was to determine hair and serum levels of calcium and magnesium in women with pre-eclampsia and to compare these levels to normotensive pregnant controls. A further objective was to describe any effect that HIV infection might have on calcium and magnesium levels by dividing pre-eclamptic cases and normotensive controls into HIV positive and HIV negative groups and comparing the calcium and magnesium levels in hair and serum between these two groups.

2.2 Recruitment

This case controlled study was conducted in the Maternity Centre at Groote Schuur Hospital and Mowbray Maternity Hospital in Cape Town, South Africa between January and November 2010. Any woman above the age of eighteen years with pre-eclampsia as defined by Davey and MacGillivray, who had delivered a live infant in a period not exceeding 96 hours from birth was eligible for enrolment in the study.[10] Any healthy woman who had delivered a live infant in a period not exceeding 96 hours from birth to enrolment was eligible to be recruited as a control. Control participants were selected by matching them against recruited cases using ethnicity, gravidity, age ± 2 years and gestational age at delivery ± 3 weeks as criteria. Stringent inclusion and exclusion criteria were applied *(Table 2.1)*.

Table 2.1 Inclusion and exclusion criteria		
	CASES	**CONTROLS**
INCLUSION CRITERIA	≥ 18 years of age Pre-eclamptic[10] Singleton pregnancy	≥ 18 years of age Normotensive and aproteinuric Singleton pregnancy
EXCLUSION CRITERIA	Unbooked pregnancy Eclampsia Multiple pregnancy Intrauterine death Antenatal vitamin or mineral supplementation Chronic hypertension Diabetes mellitus Renal disease Known disorders of metabolism or digestion Known thyroid or adrenal disease Bleaching of hair during pregnancy Eating disorders	

Women's case records were screened and those who were considered for inclusion into this study were approached by the interviewer who explained the nature, purpose, advantages and disadvantages of this study. Possible exclusion criteria were identified from information provided on the antenatal card and by a few direct questions asked of the candidate by the interviewer. Written consent was obtained from each eligible candidate.

Each participant was given an information brochure written in English, Afrikaans or Xhosa which provided a simple explanation of the study and contact details for the principal investigator and the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (*Annexure A*). Participants were also provided with a carbon copy of the signed consent form (*Annexure B*).

2.3 Questionnaire

With the assistance of a trained interviewer from the Reproductive Medicine Unit of the Department of Obstetrics and Gynaecology, each participant provided answers to a questionnaire *(Annexure C*) which was completed by the interviewer. The questionnaire comprised six sections: demographic details, booking details of the index pregnancy, delivery details, general medical and health details, a dietary questionnaire and details relating to hair care.

Participants were asked to classify themselves into a population group rather than have the interviewer assign a population group. HIV status was obtained from the voluntary counselling and testing information performed at booking and noted on the antenatal card. Gestational age was determined with the best obstetric estimate, using menstrual history, symphysis-fundal height at the booking visit or ultrasound scan, where available. Blood pressure and urine protein measurements were recorded from patients' observation charts to confirm the diagnosis of pre-eclampsia. Four blood pressure readings taken four hours apart over twelve hours and four measurements of urinary protein using dipstix taken four

hours apart were documented by the interviewer. In cases where women presented to hospital with normal blood pressure and no detectable protein on their initial urine dipstix, further urine protein testing was discontinued. If magnesium sulphate was prescribed as part of the management of pre-eclampsia, this was recorded and blood samples were obtained at least twelve hours after cessation of magnesium sulphate therapy. Body mass index was calculated as the booking weight in kilograms divided by the square of the height in meters. Hair colour of participants was determined by themselves.

2.4 Biological samples

A sample of venous blood and scalp hair was taken from each participant by the interviewer once the questionnaire had been completed.

2.4.1 Hair collection and analysis

Approximately 0.5g of hair was cut from the sub-occipital region of the head of each participant in an identical manner using sterile surgical-grade steel scissors. The hair was cut as close to the scalp as possible to ensure that the most recent growth was included in the sample. In samples where the hair was longer than 4 centimetres, the cut end was identified. Each hair sample was placed into a numbered, clean polythene bag, excess air was expelled and the bag was sealed. Samples were stored in a dry, dark area at room temperature until collection was complete. All the hair samples were couriered together to the Department of Chemistry, University of Hull in the United Kingdom. The entire laboratory process was supervised by Dr R Knight of the Department of Chemistry,

University of Hull and the methodology described to measure calcium and magnesium in hair is one which he has developed.[42]

Hair samples were taken from the polythene bags and any sellotape or labels were removed. Samples were added with 2 millilitres of nitric acid to Teflon microwave vessels and slow room-temperature digestion was allowed to begin. The vessel was then sealed and heated in a microwave to 200 degrees Celsius for 10 minutes. When cooled to room temperature the digest was diluted by weight with pure water. Sample and digest weights were used to calculate the dilution factor of each sample. The digests were analysed using the Perkin Elmer Elan DRC II inductively coupled plasma mass spectrometer instrument and all digests were analysed in a single batch to avoid inter-assay variation. All available isotopes for calcium and magnesium were included in the analysis method. The concentration of calcium and magnesium in the original solids that were provided were calculated by multiplying the measured concentration of each element by the dilution factor and results were given in parts per million per gram (ppm). Parts per million per gram represents an actual amount of calcium or magnesium in the hair. Hair calcium and magnesium measurements were not converted into millimoles per litre as this would reflect a derived parameter in an artificially made solution.

2.4.2 Blood collection and analysis

Five millilitres of venous blood was taken in a sterile manner from the antecubital fossa of each subject. The area was prepared with isopropyl alcohol and in most cases a tourniquet

was not used. In those cases where a tourniquet was necessary, a needle was inserted into the vein then the tourniquet was removed and blood was withdrawn after waiting for a period of five to ten seconds. This was done to avoid any disturbance in albumin concentration that may occur when a tourniquet is applied for a period exceeding one minute. Blood was placed into BD Vacutainer SST II Advance tubes and processed by the National Health Laboratory Service at Groote Schuur Hospital within twelve hours of taking the specimen. Where patients had been treated with magnesium sulphate to prevent eclamptic seizures, blood was taken after a minimum of 12 hours had passed since the treatment had been stopped to ensure complete excretion of exogenous magnesium, thus providing a more accurate reflection of endogenous magnesium concentration.[43]

The serum calcium, magnesium and albumin concentrations were measured and the corrected calcium level was calculated by the laboratory. All tests were performed using the Roche Modular P analyser. Individual test specifics have been listed *(Table 2.2*). Corrected calcium was calculated in millimoles per litre (mmol/l) using the formula: corrected calcium = measured total Ca (mmol/l) + 0.02 (40 - serum albumin [g/l]).[27]

Table 2.2 Laboratory analysis of calcium, magnesium and albumin

Test and normal range	Reagent	Assay	Intra-assay variation		Inter-assay variation	
			CV (%)	Mean	CV (%)	Mean
Calcium 2.05-2.56 mmol/l	o-cresolphthalien complexone	colorimetric	0.9	2.11 mmol/l	1.6	2.09 mmol/l
Magnesium 0.65-1.10 mmol/l	xylidyl blue	colorimetric	1.2	0.81 mmol/l	1.4	0.95 mmol/l
Albumin 35 – 52 g/l	bromcresol green	colorimetric	0.4	410 µmol/l	1.7	486 µmol/l

2.5 Data analysis

Data were entered into Microsoft® Excel version 2007. Statistical analyses were performed using Stata® 11.1 (StataCorp LP, 4905 Lakeway Drive, College Station, TX 77845, USA). The Shapiro-Wilk test was used to assess if the distribution of data was normal. Student's-t and Wilcoxon rank-sum tests were used to compare means and medians between two groups respectively. Chi squared tests were used to compare proportions, and where cell frequencies were less than 5 subjects, Fisher's exact test was employed.

2.6 Ethical consideration

This study was approved by the Research Committee of the Department of Obstetrics and Gynaecology and then submitted to the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town, reference 016/2010 *(Annexure D)*. Permission to recruit participants was obtained from the heads of the Obstetric Units. This study was performed in accordance with the World Medical Association Declaration of Helsinki of 2008.[44]

CHAPTER 3. RESULTS

3.1 Recruitment

Between January and November 2010, 264 recently delivered women from Mowbray Maternity Hospital and Groote Schuur Hospital were asked to participate in this study. Thirty six women (14.6%) elected not to participate for a variety of reasons.

- Thirteen declined venepuncture.
- One was apprehensive of providing a hair sample.
- Seven were unable to communicate in any of the three languages used in the study.
- One felt she was in too much pain after surgery to participate.
- Three said that they were not interested in the study.
- Two were too tired to participate.
- Nine women declined without providing a reason.

Of the 228 (86.4%) women who agreed to participate fourteen (5.3%) were excluded; one did not fulfil the diagnostic criteria for pre-eclampsia and 13 had taken calcium-containing supplements during the pregnancy.

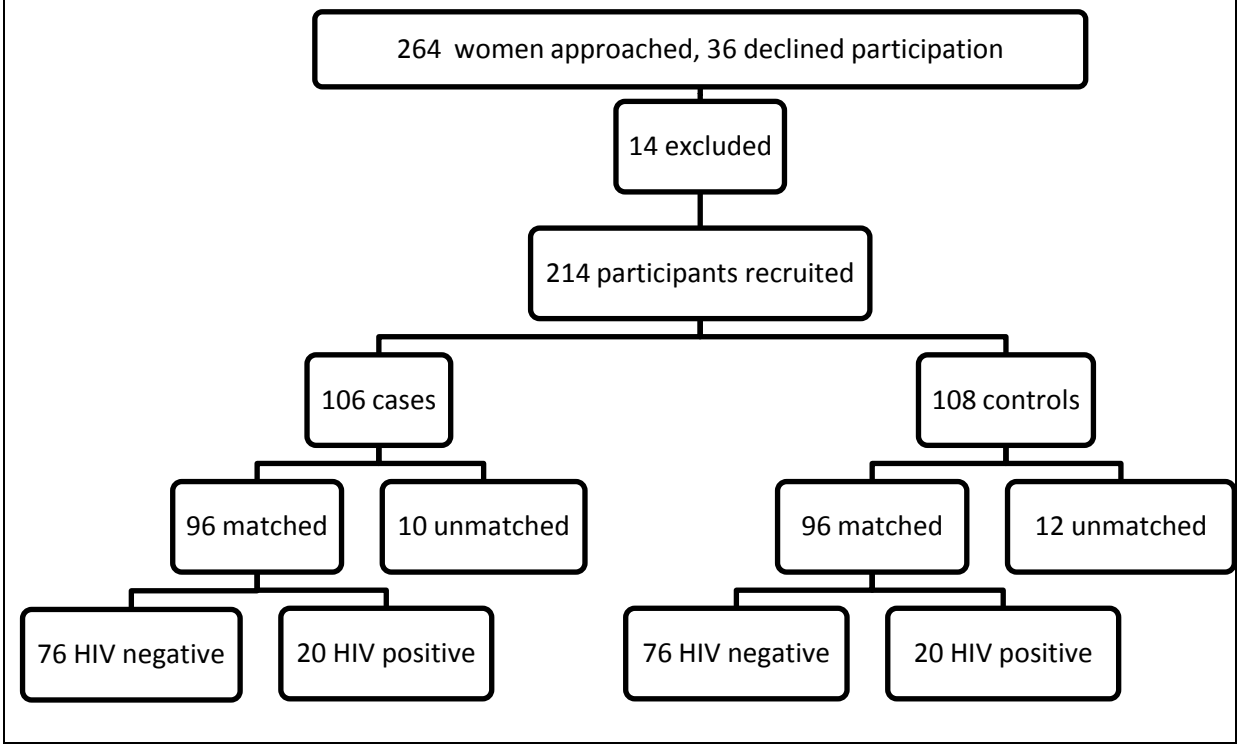
In total 214 women were recruited. 106 had pre-eclampsia (“cases”) and 108 were matched-controls. Of these recruits 166 were HIV negative and 48 were HIV positive. Ninety six of the cases were matched to controls according to ethnicity, age ± 2 years, gestational age at delivery ± 3 weeks and gravidity. Ten cases and 12 controls that were recruited could not be matched and were not included in the analysis. During the initial recruitment phase, women with pre-eclampsia and women who were potential controls were recruited in

batches and were not immediately matched resulting in an excess of 22 participants. One hundred and ninety two participants contributed to the final analysis of whom 152 were HIV negative and 40 were HIV positive (*Figure 3.1*).

All cases were confirmed as meeting the diagnostic criteria for pre-eclampsia according to the Davey and MacGillivray classification and as defined in the study protocol.[10]

Data were analysed in three groups: the total group (all women with pre-eclampsia and their corresponding matched-controls) and two sub-analyses of HIV positive cases and matched-controls and HIV negative cases and matched-controls.

Figure 3.1 Study profile



3.2 Demographics and socio-economic indicators

There was no statistically significant difference in age by case-control status overall or by HIV status. The youngest participant was 18 years old and the oldest 37 years of age (*Table 3.1*).

Table 3.1 Age (years), *median and range*

Total			HIV negative			HIV positive		
Case N=96	Control N=96	*p*	Case N=76	Control N=76	*p*	Case N=20	Control N=20	*p*
24 18-36	23.5 18-37	0.7	23 18-36	23 18-37	0.9	27 19-35	26 19-36	0.5

There was no difference in gravidity between cases and controls in each of the three groups. The majority of participants (79.17%) were primigravid (*Table 3.2*).

Table 3.2 Gravidity

Gravidity	Total		HIV negative		HIV positive	
	Case N=96 n (%)	Control N=96 n (%)	Case N=76 n (%)	Control N=76 n (%)	Case N=20 n (%)	Control N=20 n (%)
1	76 (79.2)	76 (79.2)	61 (80.3)	61 (80.3)	15 (75.0)	15 (75.0)
2	14 (14.6)	14 (14.6)	11 (14.5)	11 (14.5)	3 (15.0)	3 (15.0)
3	6 (6.2)	6 (6.2)	4 (5.2)	4 (5.2)	2 (10.0)	2 (10.0)
Total	96 (100.0)	96 (100.0)	76 (100.0)	76 (100.0)	20 (100.0)	20 (100.0)

The total group comprised 85 matched pairs (88.5%) who were black African and 11 matched pairs (11.5%) who were mixed-race. No white or Indian women were recruited to the study. In the HIV negative group 65 pairs (85.5%) were black African and 11 pairs (14.5%) were coloured. All 20 pairs in the HIV positive group were black African.

The education level of participants, monthly household income, household water supply and the type of dwelling in which they lived were used as socio-economic indicators in this study and were compared in each of the groups (*Table 3.3*).

Table 3.3 Socio-economic parameters[#]

Category	Total			HIV negative			HIV positive		
	Cases N=96 n (%)	Controls N=96 n (%)	*p*	Cases N=76 n (%)	Controls N=76 n (%)	*p*	Cases N=20 n (%)	Controls N=20 n (%)	*p*
Education									
- nil formal	1 (1)	1 (1)		0 (0)	0 (0)		1 (5)	1 (5)	
- ≤ grade 10	25 (26)	23 (24)		21 (28)	20 (26)		4 (20)	3 (15)	
- < grade 12	36 (38)	27 (28)	0.4	29 (38)	16 (21)	0.047	7 (35)	11 (55)	0.8
- grade 12	30 (31)	36 (38)		23 (30)	31 (41)		7 (35)	5 (25)	
- post-secondary	4 (4)	9 (9)		3 (4)	9 (12)		1 (5)	0 (0)	
Monthly income in Rands[##] (median, range)	2500 (250-17000) (n = 56)	2900 (240-38000) (n = 58)	0.3	2500 (250-17000 (n = 41)	3000 (240-38000 (n = 41)	0.3	3000 (700-5000) (n = 15)	2750 (1300-6000) (n = 17)	0.8
Water supply									
- domestic	50 (52)	50 (52)		39 (51)	32 (42)		11 (55)	18 (90)	
- shared tap	45 (47)	46 (48)	1.0	36 (48)	44 (58)	0.2	9 (45)	2 (10)	0.03
- well	1 (1)	0 (0)		1 (1)	0 (0)		0 (0)	0 (0)	
Dwelling									
- formal	52 (54)	56 (58)	0.7	43 (57)	50 (66)	0.3	9 (45)	6 (30)	0.5
- informal	44 (46)	40 (42)		33 (43)	26 (34)		11 (55)	14 (70)	

[#] Statistical significance of socio-economic parameters where cells contain < 5 is assessed using Fisher's exact test
[##] 78 participants were unable to supply their monthly income

The level of education was divided into five categories to assist analysis: no formal education, formal education not beyond completion of grade 10, formal education beyond grade 10 but not completing grade 12, formal education to completion of grade 12 and post-secondary education. There was no statistically significant difference in education level between women with pre-eclampsia and matched-controls in the total and HIV positive groups. In the HIV negative group there was a statistically significant difference in the distribution of level of education between women with pre-eclampsia and matched-controls as more matched-controls (53%) had completed grade 12 or beyond than women with pre-eclampsia (34%).

Monthly household income was determined as the combined net income of all people who contributed to the participant's household each month. The figure was often estimated by the participant. There was no statistically significant difference in monthly household income between women with pre-eclampsia and matched-controls in the three groups, however 78 participants were unable to provide this information.

There was no statistically significant difference in the type of water supply in the total group and the HIV negative group. In the HIV positive group there were statistically significantly more matched-controls who received domestic water supply than in the pre-eclamptic group.

Formal dwellings were classified as buildings made of brick, wood or concrete. Homes constructed from corrugated iron, asbestos, plastic or other materials were classified as informal dwellings. There was no statistically significant difference in the type of dwellings in which participants lived in all of the three groups.

Level of education, monthly household income and type of dwelling between women with pre-eclampsia and matched-controls were compared by HIV status. There were statistically significant differences in the level of education ($p = 0.008$) and type of dwelling ($p = 0.005$) in the matched-control groups. Fewer HIV positive matched-controls completed grade 12 or beyond than HIV negative matched-controls and more HIV positive matched-controls lived in informal dwellings than HIV negative matched-controls. There was no statistically significant difference in monthly household income between HIV negative and HIV positive women with pre-eclampsia ($p = 1.0$) or matched-controls ($p = 0.6$).

3.3 Medical considerations, HIV parameters and gestational age

In the HIV negative group no women with pre-eclampsia reported concurrent medical problems while 2 matched-controls suffered from asthma. In this same group three women with pre-eclampsia had been treated for tuberculosis in the past. In the matched control group 2 women had been previously treated for tuberculosis and one woman had received treatment for peptic ulcer disease.

In the HIV positive group no women with pre-eclampsia reported having a current medical condition other than HIV infection and 1 matched control was taking anti-tuberculosis treatment at the time of recruitment. Five women with pre-eclampsia and 1 matched-control had been previously treated for tuberculosis in the HIV positive group.

The degree of immune system compromise in HIV infection was analysed using the absolute CD_4 count. The specific antenatal anti-retroviral regime that was received by participants was described. The absolute CD_4 count of participants was divided into two categories: those less than 200 x 10^6/l and those equal to or above 200 x 10^6/l. According to the national clinical guideline instituted in 2004 and in use at the time of recruitment, a CD_4 value of less than 200 x 10^6/l was an indication to commence antenatal highly active anti-retroviral therapy (HAART). (This critical CD_4 value has since changed to 350 x 10^6/l with the institution of the latest national guideline of 2010).[45] Pregnant women not eligible for HAART received monotherapy zidovudine (AZT) during the pregnancy and a single dose of nevirapine (NVP) immediately before delivery. There was no statistically significant difference in distribution between the CD_4 categories in HIV positive women with pre-eclampsia and matched HIV positive controls (*Table 3.4*). The majority of participants in the pre-eclamptic group received monotherapy AZT and single-dose NVP, while in the matched control group the use of HAART and monotherapy AZT and NVP was more evenly distributed.

Table 3.4 HIV positive CD_4 count and anti-retroviral (ARV) therapy			
	Cases N=20 n(%)	Controls N=20 n(%)	
CD4 < 200	4 (20)	5 (25)	p= 1.0
CD4 ≥ 200	16 (80)	15 (75)	
HAART	4 (20)	9 (45)	
Monotherapy AZT with NVP	15 (75)	10 (50)	
No ARV therapy	1 (5)	1 (5)	

All participants in this study had booked their pregnancy and received antenatal care. There was no statistically significant difference in mean gestational age at booking between women with pre-eclampsia and matched-controls in each of the three groups that were analysed. In the total group and the HIV negative group, differences in mean gestational age at delivery between women with pre-eclampsia and matched-controls were statistically significant. While the difference in mean gestational age at delivery in the HIV positive group was similar to difference observed in the total group and the HIV negative group, it did not reach statistical significance (*Table 3.5*).

Table 3.5 Gestational age (in weeks) at booking and delivery												
	Total				HIV negative				HIV positive			
	Case N=98	Control N=96	Diff	*p*	Case N=76	Control N=76	Diff	*p*	Case N=20	Control N=20	Diff	*p*
Booking Mean ± SD	20.88 ±6.49	21.77 ±6.80	0.90	0.4	21.32 ±6.81	22.43 ±6.06	1.11	0.3	19.15 ±4.88	19.25 ±8.90	0.10	1.0
Delivery Mean ± SD	37.35 ±2.38	38.36 ±2.01	1.01	<0.05	37.61 ±2.32	38.59 ±2.00	0.99	<0.05	36.40 ±2.44	37.50 ±1.82	1.10	0.1

3.4 Dietary parameters

To compare the nutritional status and dietary calcium intake of the three groups that were analysed in this study a number of variables were investigated (*Table 3.6, 3.7*). Body mass index (BMI) was used as a crude indicator of nutritional status and to identify those participants who were underweight. Dietary intake of calcium-rich foods such as fish, dairy products, green vegetables, fruit, nuts, beans and rooibos tea and calcium-fortified foods such as bread and cereals were analysed. Dietary factors that might affect calcium absorption such as red meat, coffee, Ceylon tea, alcohol and cigarette smoking were also included in the analysis.

There was no difference in BMI between women with pre-eclampsia and matched-controls in the three groups that were analysed. One participant in the control arm of the HIV positive group was underweight with a BMI of less than 18.5 kg/m^2. There were no differences in consumption of calcium-rich foods including fish, milk, yoghurt, green vegetables, nuts, beans and rooibos tea in each of the three groups. In the HIV negative group cheese, a dairy product rich in calcium, was consumed by 15.8% of matched-controls and only 2.6% of women with pre-eclampsia. This difference was not statistically significant. None of the HIV positive participants consumed cheese during their pregnancy. Fruit was consumed by 57.9% of women with pre-eclampsia and 73.7% of matched-controls in the HIV negative group. This difference reached statistical significance in both the HIV negative group and the total group.

Table 3.6 Dietary parameters

(where results have not been given in the form n (%), the alternative unit of measurement has been indicated in brackets)

	Total			by HIV status					
				HIV negative			HIV positive		
	Case N=96 n (%)	Control N=96 n (%)	*P*	Case N=76 n (%)	Control N=76 n (%)	*p*	Case N=20 n (%)	Control N=20 n (%)	*p*
Body mass index (kg/m^2)[###] *(median and range)*	28.6 (18.8-52.5)	28.4 (17.9-51.5)	0.9	28.5 (18.8-522.5)	28.8 (19.9-51.5)	0.7	29.0 (23.1-39.1)	27.0 (17.9-39.1)	0.6
Servings of fish									
- daily	4 (4.2)	3 (3.1)		2 (2.6)	1 (1.3)		2 (10.0)	2 (10.0)	
- > once a week	29 (30.2)	28 (29.2)		23 (30.3)	19 (25.0)		6 (30.0)	9 (45.0)	
- < once a week	56 (58.3)	57 (59.4)	1.0	45 (59.2)	49 (64.5)	0.8	11 (55.0)	8 (40.0)	0.8
- never	7 (7.3)	8 (8.3)		6 (7.9)	7 (9.2)		1 (5.0)	1 (5.0)	
Servings of red meat									
- daily	6 (6.2)	4 (4.2)		4 (5.3)	3 (4.0)		2 (10.0)	1 (5.0)	
- > once a week	44 (45.8)	45 (46.9)		40 (52.6)	38 (50.0)		4 (20.0)	7 (35.0)	
- < once a week	37 (38.5)	40 (41.7)	0.8	29 (38.2)	32 (42.1)	0.9	8 (40.0)	8 (40.0)	0.7
- never	9 (9.4)	7 (7.3)		3 (4.0)	3 (4.0)		6 (30.0)	4 (20.0)	
Coffee, daily									
- drinkers	46 (47.9)	41 (42.7)		42 (55.3)	29 (38.2)		4 (20.0)	12 (60.0)	
- non-drinkers	50 (52.1)	55 (57.3)	0.6	34 (44.7)	47 (61.8)	0.51	16 (80.0)	8 (40.0)	0.02
Ceylon tea, daily									
- drinkers	30 (31.2)	23 (24.0)		25 (32.9)	20 (26.3)		5 (25.0)	3 (15.0)	
- non-drinkers	66 (68.8)	73 (76.0)	0.3	51 (67.1)	56 (73.7)	0.5	15 (75.0)	17 (85.0)	0.7
Rooibos tea, daily									
- drinkers	31 (32.8)	31 (32.3)		25 (32.9)	24 (31.6)		6 (30.0)	7 (35.0)	
- non-drinkers	65 (67.7)	65 (67.7)	1.0	51 (67.1)	52 (68.4)	1.0	14 (70.0)	13 (65.0)	1.0
Milk, daily									
- drinkers	35 (36.5)	47 (49.0)		30 (39.5)	41 (53.9)		5 (25.0)	6 (30.0)	
- non-drinkers	61 (63.5)	49 (51.0)	0.1	46 (60.5)	35 (46.1)	0.1	15 (75.0)	14 (70.0)	0.7
Cheese, daily									
- consumers	2 (2.1)	12 (12.5)		2 (2.6)	12 (15.8)		0	0	
- non-consumers	94 (97.9)	84 (87.5)	0.01	74 (97.4)	64 (84.2)	0.01	0	0	
Yoghurt, daily									
- consumers	14 (14.6)	18 (18.8)		9 (11.8)	15 (19.7)		5 (25.0)	3 (15.0)	
- non-consumers	82 (85.4)	78 (81.2)	0.4	67 (88.2)	61 (80.3)	0.2	15 (75.0)	17(85.0)	0.4
Green vegetables, daily									
- consumers	48 (50.0)	48 (50.0)		37 (48.7)	35 (46.1)		11 (55.0)	13 (65.0)	
- non-consumers	48 (50.0)	48 (50.0)	1.0	39 (51.3)	41 (53.9)	0.7	9 (45.0)	7 (35.0)	0.5
Nuts, daily									
- consumers	13 (13.5)	13 (13.5)		12 (15.8)	12 (15.8)		1 (5.0)	1 (5.0)	
- non-consumers	83 (86.5)	83 (86.5)	1.0	64 (84.2)	64 (84.2)	1.0	19 (95.0)	19 (95.0)	1.0
Fruit, daily									
- consumers	59 (61.5)	74 (77.1)		44 (57.9)	56 (73.7)		15 (75.0)	18 (90.0)	
- non-consumers	37 (38.5)	22 (22.9)	0.03	32 (42.1)	20 (26.3)	0.04	5 (25.0)	2 (10.0)	0.4
Beans. daily									
- consumers	10 (10.4)	8 (8.3)		7 (9.2)	5 (6.6)		3 (15)	3 (15)	
- non-consumers	86 (89.6)	88 (91.7)	0.6	69 (90.8)	71 (93.4)	0.5	17 (85)	17 (85)	1.0
Bread (slices per day) *(mean, range)*	4 0 - 16	4 0 - 24	0.9	4 0 - 8	4 0 - 24	0.4	4 0 - 16	4 0 - 6	.06
Cereal, daily									
- consumers	47 (49.0)	44 (45.9)		34 (44.7)	37 (48.7)		13 (65)	7 (35)	
- non-consumers	49 (51.0)	52 (54.1)	0.7	42 (55.3)	39 (51.3)	0.6	7 (35)	13 (65)	0.06

[###] Body mass index could not be calculated for 8 cases and 13 controls.

Coffee, Ceylon tea and red meat are products that have been shown to impair calcium absorption in the gastrointestinal tract, however published literature is conflicting. There were no statistically significant differences in coffee, Ceylon tea and red meat consumption between women with pre-eclampsia and matched-controls in all three groups, except in the HIV positive group where fewer women with pre-eclampsia consumed coffee each day (20.0%) compared to their matched-controls (60.0%). While bread and cereals are not naturally high in calcium, many are fortified with calcium by producers. There were no differences in the consumption of bread and cereal in the three groups that were analysed.

Table 3.7 Alcohol and cigarette use during pregnancy

	Total			by HIV status					
				HIV negative			HIV positive		
	Case N=96 n (%)	Control N=96 n (%)	*P*	Case N=76 n (%)	Control N=76 n (%)	*p*	Case N=20 n (%)	Control N=20 n (%)	*p*
Alcohol during pregnancy									
- drinkers	5 (5.2)	1 (1.0)	0.1	4 (5.3)	1 (1.3)	0.2	1 (5)	0	0.3
- non-drinkers	91 (94.8)	95 (99.0)		72 (94.7)	75 (98.7)		19 (95)	20 (100)	
Cigarettes during pregnancy									
- smokers	6 (6.3)	8 (8.3)	0.6	6 (7.9)	8 (10.5)	0.6	0	0	
- non-smokers	90 (93.7)	88 (91.7)		70 (92.1)	68 (89.5)		0 (100)	20 (100)	

While alcohol does not directly affect calcium metabolism, alcohol abuse and dependence are associated with poor diet; there was no statistically significant difference in alcohol consumption between the groups in the study. Smoking impairs calcium absorption in the gut; there was no statistically significant difference in this study in the proportion of

participants who smoked. Fourteen of 192 women (7.3%) who participated in this study were smokers and six (3.1%) consumed alcohol during their pregnancy.

3.5 Blood and hair analysis

Blood and hair levels of calcium and magnesium have been tabulated (*Tables 3.8, 3.9*). Twelve serum assays were not performed due to laboratory oversight. While unique laboratory forms requesting serum calcium, magnesium and albumin concentrations and a calculation of corrected calcium were used for this study, twelve individual results were not reported by the laboratory. These errors have been indicated in brackets. Serum calcium and magnesium concentration was reported in millimoles per litre. Hair calcium and magnesium content was calculated in parts per million in one gram (ppm) of hair and was not converted into an alternative unit of measurement.

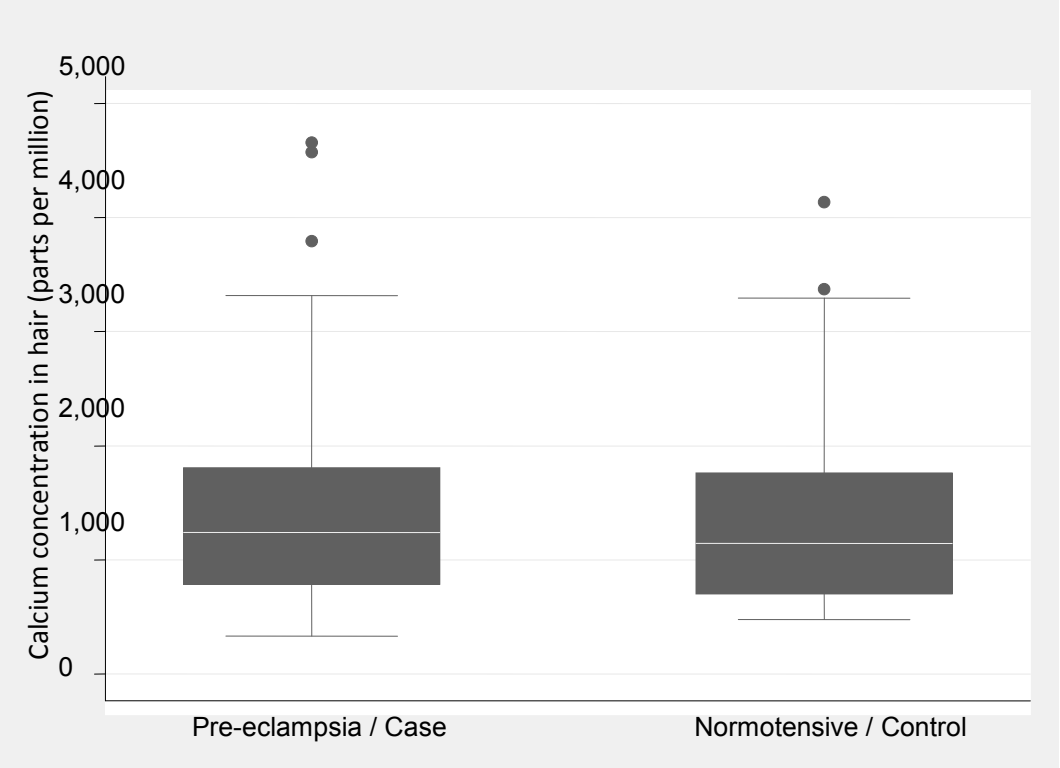
Table 3.8 Calcium and magnesium levels in serum and hair of the total group

	Cases N=96	Controls N=96	*p*
Calcium			
- Serum, corrected (mean ± SD) (normal range: 2.05–2.56 mmol/l)	2.23 ± 0.09 mmol/l n=95 (1 missing result)	2.26 ± 0.08 mmol/l n=93 (3 missing results)	0.04
- Hair (median, range)	1241 ppm, 331 - 4654 n=96	1146 ppm, 480 - 4136 n=96	0.5
Magnesium			
- Serum[####] (mean ± SD) (normal range: 0.65–1.10 mmol/l)	0.87 ± 0.21 mmol/l n=94 (2 missing results)	0.72 ± 0.07 mmol/l n=90 (6 missing results)	<0.01
- Hair (median, range)	117 ppm, 33 - 896 n=96	106 ppm, 18 - 819 n=96	0.4

[####]Magnesium data were not normally distributed and were transformed in order to perform parametric statistics using 1/(square of Mg). The untransformed means have been reported.

In the total group the mean serum calcium concentration of women with pre-eclampsia was statistically significantly lower than matched-controls. Only one woman, a matched control in the HIV negative group, had a serum calcium concentration below the lower limit of normal (2.05 – 2.56 mmol/l) accepted by the National Health Laboratory Service. There was no significant difference in the concentration of hair calcium between women with pre-eclampsia (median 1241 ppm, range 331 – 4654) and their matched-controls (median 1146 ppm, range 480 – 4136) (p = 0.5) (*Figure 3.2*).

Figure 3.2 Box plots of hair calcium concentration by case-control status of the total group



Eighty-six of ninety-two cases were treated with intravenous magnesium sulphate to offer primary protection against eclamptic seizures. The mean serum magnesium level of women with pre-eclampsia was significantly greater than matched-controls ($p < 0.01$) in the total group. There was no difference in the level of magnesium in hair between women with pre-eclampsia and their matched-controls in the total group.

Table 3.9 Calcium and magnesium levels in serum and hair by HIV status

	HIV negative			HIV positive		
	Cases N=76	Controls N=76	*P*	Cases N=20	Controls N=20	*P*
Calcium						
- Serum (corrected) (mean ± SD) (normal range: 2.05–2.56 mmol/l)	2.24 ± 0.09 mmol/l n=75 (1 error)	2.27 ± 0.08 mmol/l n=73 (3 errors)	0.03	2.22 ± 0.08 mmol/l n=20	2.23 ± 0.10 mmol/l n=20	0.7
- Hair (median, range)	1162 ppm, 479 - 4136 n=76	1168 ppm, 331 - 4654 n=76	0.1	1098 ppm, 498 - 3231 n=20	1443 ppm, 532 - 2378 n=20	0.1
Magnesium						
- Serum[####] (mean ± SD) (normal range: 0.65–1.10 mmol/l)	0.87 ± 0.22 mmol/l n=74 (2 errors)	0.71 ± 0.07 mmol/l n=70 (6 errors)	<0.01	0.87 ± 0.18 mmol/l n=20	0.72 ± 0.07 mmol/l n=20	<0.05
- Hair (median, range)	117 ppm, 33 - 896 n=76	104 ppm, 17 - 667 n=76	0.2	122 ppm, 37 - 76 n=20	153 ppm, 25 - 819 n=20	0.6

[####]Magnesium data were not normally distributed and were transformed in order to perform parametric statistics using 1/(square of Mg). The untransformed means have been reported.

In the HIV negative group, differences that were observed in calcium and magnesium levels in serum and hair between women with pre-eclampsia and their matched-controls were

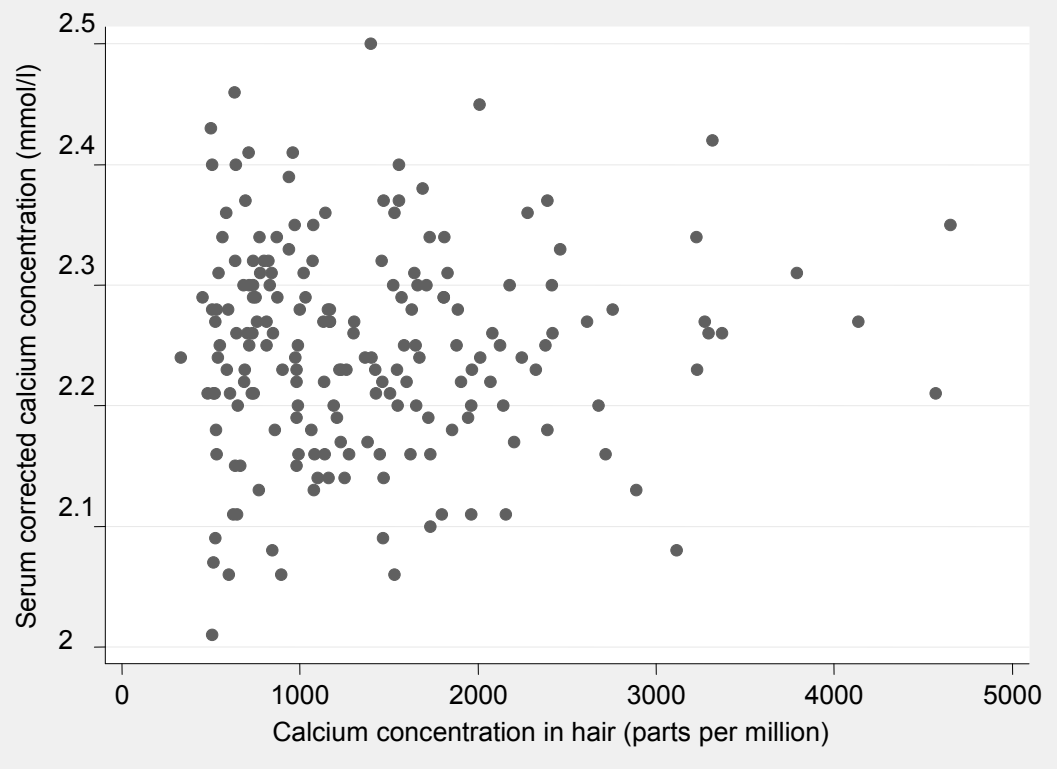
similar to those found in the total group. The differences in serum calcium and magnesium both reached statistical significance.

In the HIV positive group there were no statistically significant differences in serum corrected calcium, hair calcium and hair magnesium levels. The only difference that achieved statistical significance in this group was serum magnesium concentration where women with pre-eclampsia showed higher levels than their matched-controls. Seventeen of twenty HIV positive women with pre-eclampsia received magnesium sulphate therapy.

Serum calcium and magnesium concentrations and hair calcium and magnesium levels were compared between HIV negative and HIV positive women with pre-eclampsia. There were no statistically significant differences in these parameters between the two groups. There were no statistically significant differences in these same parameters when HIV negative and HIV positive matched-controls were compared.

The possibility of a correlation between corrected calcium concentration in serum and hair calcium concentration was also investigated and is graphically represented (*Figure 3.3*). There was no statistically significant correlation between corrected calcium concentration in serum and hair calcium concentration using Spearman's rank correlation co-efficient, calculated at -0.0076 (p = 0.92). There was also no statistically significant correlation between serum and hair magnesium concentrations using Spearman's rank correlation co-efficient, -0.0274 (p = 0.7121).

Figure 3.3 Scatter plot of hair calcium concentration and serum corrected calcium concentration



3.6 Summary of results

- One hundred and ninety two participants (96 matched pairs) were included in the analysis.
- There was no statistically significant difference in age and gravidity in any of the groups.
- Socio-economic indicators between the groups were similar, except in the HIV negative group where more matched-controls had received education to the

completion of grade 12 or beyond, and in the HIV positive group more matched-controls received domestic water supply than women with pre-eclampsia.

- More HIV positive matched-controls lived in informal dwellings and had poorer levels of education than HIV negative matched-controls.
- There was no difference in monthly household income by HIV status.
- There was no difference in CD_4 count between HIV positive women with pre-eclampsia and matched-controls.
- Mean gestational age at booking did not differ between pre-eclamptic women and matched-controls in the three groups.
- Mean gestational age at delivery was significantly less in women with pre-eclampsia in the total and HIV negative groups but not in the HIV positive group.
- Diet in the total, HIV negative and HIV positive groups was similar except in the HIV negative group where matched-controls consumed more cheese and fruit than women with pre-eclampsia. In the HIV positive group significantly more bread and cereal was consumed by women with pre-eclampsia while more coffee was consumed by matched-controls.
- Alcohol and cigarette use were similar in all groups.
- Serum corrected calcium levels were significantly less in women with pre-eclampsia in the total and HIV negative groups compared with matched controls, while there was no difference in the HIV positive group.
- There was no difference in hair calcium concentration between women with pre-eclampsia and matched-controls in the total, HIV negative and HIV positive groups.
- There was no correlation between serum and hair calcium levels.

- Serum magnesium levels were statistically significantly higher in pre-eclamptic women in all three groups.
- There were no differences in hair magnesium concentration between women with pre-eclampsia and matched controls in any of the three groups.
- No correlation was found between serum and hair magnesium levels.

CHAPTER 4. DISCUSSION

The relationship between pre-eclampsia and calcium supplementation has been extensively studied.[2,3,24,34,46] While the current evidence may not be completely convincing, supplementing pregnant women with oral calcium is a simple and cheap intervention which is potentially beneficial and with almost no risk to the mother and fetus. The literature surrounding calcium supplementation is extensive but conflicting. Studies have opposing results and differ in many aspects including size, population (from developed and developing countries and from different income groups and socio-economic status), primary outcomes and reference values used to measure the primary outcomes, all making comparison difficult.[46,47]

The mechanism by which calcium prevents pre-eclampsia however remains unclear. Pre-eclampsia has a high prevalence in developing countries including South Africa, India and many in South America and it is in these countries where the greatest effect of calcium supplementation has been seen in reducing the risk of developing the disease. A possible explanation may be that calcium status and the need for supplementation in pregnancy varies due to geographic location and the diet of a specific cultural or ethnic group within that area.[48] Widespread poverty in developing countries contributes to poor calcium intake and so an association between developing countries, insufficient dietary calcium and a high prevalence of pre-eclampsia has been made.

Serum calcium is a poor indicator of chronic calcium status as it is in constant flux with calcium in bone and cells, it exists in ionized, protein-bound and complexed forms and the result that is obtained is the present value which changes rapidly and not a reflection of the calcium level over time. Hair is a stable, easily accessible and reliable indicator of long-term calcium status as uptake of calcium via the hair follicle is dependent on calcium available in the serum. While the use of hair as a substrate to investigate trace elements is not a new concept, the technique employed by the Department of Chemistry of the University of Hull has been refined and standardised and uses the latest technology allowing accurate and reliable hair analyses to be performed.

To date ours is the first study to investigate the long-term calcium status of women with pre-eclampsia. While there is no recognised normal range of hair calcium, values can be used as a means to compare long-term calcium status between groups as has been done in this study. Our study has assessed the long-term calcium status of women with pre-eclampsia in a low calcium intake population and compared this to normotensive participants.

4.1 Recruitment, demographics and gestational age at booking and delivery

Women who suffered from any disease that could complicate the investigators making a clear diagnosis of pre-eclampsia, or from any condition that might alter calcium metabolism were excluded from the study. It was important that an accurate diagnosis of pre-eclampsia

was made in each case that was recruited and in situations where there was doubt the patient was not recruited.

By matching women with pre-eclampsia using age, gravidity, race and gestational age to normotensive women, no difference in demographic measurement was found between the matched groups. This was regarded as important as total body calcium levels may differ in women of varying ages and gestational age and calcium intake may differ between ethnic groups because of dietary customs.[48] Relatively few white and Indian women attend our antenatal service which does explain the absence of these ethnic groups in our study.

It is common for women in the Western Cape to attend their first booking visit well into the second trimester and the mean gestational age at booking for all participants in the study was 21 ± 6.64 weeks. There were no unbooked patients in our study. There was no difference in gestational age at booking in each of the three matched groups. Women with HIV infection booked for antenatal care earlier than HIV negative women. Earlier detection of pregnancy and referral from a HIV clinic, knowledge of their HIV status, of the risks it poses to them and their baby during pregnancy and the possibly of accelerated access to anti-retroviral medication may have motivated these women to present themselves earlier for antenatal care.

Differences in gestational age reached statistical significance in the total and HIV negative groups with woman suffering from pre-eclampsia delivering approximately one week earlier

than matched-controls. This result was not surprising as women who are diagnosed with pre-eclampsia after 34 weeks gestation at Mowbray Maternity Hospital or Groote Schuur Hospital are delivered immediately. The mean gestational age at delivery of women with pre-eclampsia in the total and HIV negative groups was greater than 37 weeks which indicates that most cases in the study had late onset of the disease.

4.2 Socio-economic status

Socio-economic status was crudely assessed using level of education, monthly household income, water supply and type of dwelling (formal or informal) as parameters. In the total group there was no difference in any of these parameters. This similarity between the two groups makes it possible to assume that women with pre-eclampsia and matched-controls had equal opportunity to access similar diets and calcium intake. Even though there was no statistically significant difference in monthly household income in any of the groups, the range in income was vast. In the HIV positive group the upper limit of monthly income was 6 000 South African Rand while the upper income of the total group ranged between 17 000 and 38 000 South African Rand. This difference may be a result of the small numbers in this group rather than a significant finding. Nearly 41% of the participants in our study were unable to provide their monthly household income which does influence the accuracy of these findings.

HIV positive matched-controls were of poorer socio-economic status than HIV negative matched-controls as they showed less advanced education and had poorer housing. There

was no difference in monthly household income between the HIV negative and HIV positive group which supports the assumption that each group had equal opportunity to access similar diets.

4.3 Diet

The dietary questionnaire that was used provided a broad dietary history of each participant. This questionnaire has been used in a previous study investigating nutritional status by hair analysis and was also used in a 2010 study conducted by the Department of Obstetrics and Gynaecology of the University of Cape Town which investigated the relationship between selenium and recurrent pregnancy loss.[40] It is a user-friendly tool and patients are usually confident in the answers that they provide. While the questionnaire does not focus on detailing exact quantities of food, it does indicate definite deficiencies where they exist. During statistical analysis it was found that the median value of the quantity of certain foodstuffs that were consumed was often zero and this made presentation of the data difficult. Data were converted from a continuous to a dichotomous variable (consumers or non-consumers) for most foodstuffs to facilitate the analysis. A criticism of manipulating data in this manner is that it obscures exact quantities of foods that are consumed which may cause detail to be lost during analysis.

The dietary parameters measured in our study looked at foods rich in calcium, foods that have been fortified with calcium and foods that impair calcium absorption from the gut. Generally there was little difference in diet composition between women with pre-

eclampsia and their matched-controls. This was an important finding as it supported the fact that any possible difference in hair or serum calcium levels between women with pre-eclampsia and their matched-controls could not be attributed to dietary differences. While statistically more HIV negative matched-controls ate cheese than HIV negative women with pre-eclampsia, the absolute values are small with only 7% of all participants in the study eating cheese. This may be because cheese is an expensive product or attributed to cultural differences that exist in diet. Differences in diet in the HIV positive group were seen in coffee, bread and cereal and while these reached statistical significance, this group had small numbers and so it is important to view these results with some reserve. It is reassuring to see that only 6 women drank alcohol and 14 smoked cigarettes during their pregnancy. Antenatal counselling and national campaigns focussed on the harmful effects of alcohol and cigarettes may possibly be having some impact.

4.4 HIV infection

The influence of HIV infection on nutritional status is recognised and extensively published. Unique to HIV infection, the "wasting syndrome" was defined in 1987 by the Centre for Disease Control as ≥ 10% loss of usual body weight associated with chronic diarrhoea, fever and asthenia with no detectable cause of wasting other than HIV infection.[49] Loss of body weight may occur independently of the degree of immune suppression, however, weight changes in pregnancy are difficult to quantify as it is a condition associated with weight gain.[50] Insufficient caloric and nutrient intake associated with malnutrition in HIV infection may alter calcium intake and long-term calcium status.

In the HIV positive group 22.5% of women had AIDS as they met the criterion of an absolute CD_4 count of less than 200 x 10^6/l and these women might be expected to have some nutritional deficiencies. In our study, calcium and magnesium levels in serum and hair did not differ between women with pre-eclampsia by HIV status. There was also no difference in serum and hair calcium and magnesium levels in matched-controls by HIV status. This indicates that there was no chronic difference in calcium and magnesium status between these two groups. Reasons that there was no difference in calcium and magnesium hair levels between the HIV negative and positive groups might be that a difference was obscured by the small numbers in the HIV positive group or a factor of the independent relationship between the degree of immune suppression and weight loss.[50]

The 2009 national prevalence of HIV infection among pregnant women in South Africa was 29.4% and in the Western Cape was 16.9%.[51] Among 192 participants 40 HIV positive women (20.8%) were included in our study which is in accordance with regional and national figure. Analysis of this small group did complicate the interpretation of the results as small variations often reached statistical significance. There was no difference in CD_4 count between women with pre-eclampsia and matched-controls and only four women with pre-eclampsia and nine matched-controls received HAART at the time of recruitment.

4.5 Serum and hair calcium and magnesium levels

It is evident in the current literature that the chronic calcium status of women recruited to studies investigating the effect of calcium supplementation on pre-eclampsia is not

investigated prior to commencing supplementation.[25,46,47,52] The calcium intake of populations under investigation in these studies is assessed by measuring the daily dietary calcium content or performing a diet survey. Little is known about the long-term calcium status of these women.

Serum calcium concentration is not an indication of long-term calcium status and therefore should probably not be used to investigate chronic calcium status. Our study found that serum calcium concentrations were lower in woman with pre-eclampsia than in matched-controls in the total and HIV negative groups. Although this difference was statistically significant its clinical relevance is difficult to assess given that all participants, with the exception of one HIV negative matched-control, had serum calcium levels within the normal range. It may be possible to explain why women with pre-eclampsia had significantly lower serum calcium concentrations than matched-controls in our study by the inhibitory effect that magnesium sulphate has on PTH levels and so on acute calcium fluxes.[43]

Hair calcium levels showed no difference between women with pre-eclampsia and matched-controls in any of the three groups which supports the null hypothesis that there is no difference on the long-term calcium status between women with pre-eclampsia and matched-controls. Neither socio-economic status nor dietary intake are thought to have had any influence in the outcome of the hair results as there were almost no differences in these parameters between woman with pre-eclampsia and matched-controls.

Serum magnesium was higher in women with pre-eclampsia than matched-controls in all three of the groups and this finding was unexpected. Ninety three percent of women with pre-eclampsia received magnesium sulphate therapy and had a significantly higher mean serum magnesium level (0.88 mmol/l ± 0.22) than women with pre-eclampsia who did not receive magnesium sulphate (0.74 mmol/l ± 0.08). There was no statistically significant difference in serum magnesium level between women with pre-eclampsia who did not receive magnesium sulphate therapy (0.74 mmol/l ± 0.08) and the total matched-control group (0.72 mmol/l ± 0.07). The average half-life of intravenously administered magnesium is 5.2 hours and we were advised that 12 hours after stopping intravenous magnesium sulphate therapy the serum magnesium concentration would return to pre-treatment values.[43] In participants who received magnesium sulphate, blood was drawn to measure serum calcium and magnesium concentrations after a period of not less (and often much longer) than 12 hours following completion of magnesium sulphate therapy to ensure complete excretion of exogenous magnesium. Our findings however indicate that this was not achieved. Most probably residual exogenous magnesium contributed to higher serum levels in women with pre-eclampsia. In future studies the interval between magnesium sulphate therapy and venepuncture to measure serum magnesium levels will need to be extended.

There was no significant difference in hair magnesium levels between women with pre-eclampsia and matched-controls in the three groups. This finding supports the opinion that the difference in serum magnesium level was due to acute magnesium sulphate therapy and not because of nutritional deficiency or chronic low intake.

While a normal range for the concentration of calcium and magnesium in hair has not been established, it was important to compare the results of our study with the existing literature. In 1999 Leung *et al* investigated the effect of calcium supplementation on pregnant women who were calcium deficient by performing serial hair calcium level measurements.[38] Deficiency was defined in this study as a serum calcium value of less than 90 µg/l (2.25 mmol/l). Hair calcium levels of non-pregnant controls and pregnant women who did not receive supplementation were also determined. The mean hair calcium concentration in 58 non-pregnant Chinese women was 1566 ± 266 ppm, and in 52 Chinese women in the third trimester of pregnancy who did not receive calcium supplementation was 946 ± 352 ppm. The serum calcium value used by this study to define calcium deficiency lies well within the normal range (2.05 – 2.56 mmol/l) used by the National Health Laboratory Service and could therefore not be applied to the population used in our study. All participants in our study delivered in the third trimester and median hair calcium levels ranged between 1098 – 1443 ppm which corresponds to the values reported in non-supplemented women in the third trimester by Leung *et al*.

A study in the United States of America published in 1974 investigated the normal range of certain elements in the pubic hair of 42 pregnant women just prior to delivery and found the range of calcium to be 188 – 4900 ppm and magnesium to be 10 – 101 ppm.[41] Pubic hair is less affected by grooming and cosmetic treatments and is probably a superior substrate upon which to perform analysis. Pubic hair was not collected in this study due to the sensitive nature of sample collection and because many pregnant women shave prior to birthing. The hair calcium results in our study fall within the range of values reported in this

study, however our hair magnesium results are slightly higher. A small study performed in North America investigated trace elements in human hair and reported a mean magnesium concentration of 54 ppm in 7 non-pregnant women which again differs from our hair magnesium results.[53] These differences in long-term magnesium status are not explained by our study.

A study conducted in Sweden established a positive correlation between calcium content in drinking water and hair calcium levels in women. Serum calcium concentrations were not investigated in this study. Significantly higher hair calcium levels were found in those women drinking water from an alkaline well which had a greater calcium concentration than water from an acidic well.[54] This study highlights the influence that the calcium content of drinking water has on chronic calcium status. In the alkaline well the mean water calcium concentration was 61.0 mg/l and the median hair calcium level in these women was 1290 ppm (range 231 – 5630). The mean calcium concentration in the water from the acidic well was 9.8 mg/l and the median hair calcium level of women drinking from this well was 283 ppm (range 50 – 2840).

Cape Town receives drinking water from 8 different supplies. The average values of calcium content in these 8 supplies between June 2007 and May 2010 ranged from 11.8 to 38.9 mg/l with a median value of 16.15 mg/l.[55] Cape Town water calcium values are much closer to the values found in the acidic well in the Swedish study while the range of hair calcium

levels in our study are more similar to those values found in women drinking from the alkaline well which had much higher calcium content.

Cape Town is an area of low calcium intake, however, the hair calcium values in our study are similar to the values obtained in these hair calcium studies performed in America, China and Sweden.[38,41,53] A study to compare the long-term calcium status between pregnant women in Cape Town and in developed countries would be necessary to determine if these two groups do have similar chronic calcium levels.

In summary, our study has found chronic calcium status to be no different between women with pre-eclampsia and matched-controls which raises the question: is the beneficial effect of calcium supplementation in pregnancy on reducing the risk of developing pre-eclampsia attributable to a pharmacological effect rather than the correction of a nutritional deficiency?

CHAPTER 5. CONCLUSIONS

Our study has found that women in Cape Town with pre-eclampsia showed no difference in calcium status when compared to matched normotensive pregnant women, using hair calcium analysis as an indication of long-term calcium status. Calcium intake, socio-economic factors that influence dietary calcium intake and factors that affect intestinal absorption of calcium were comparable between pre-eclamptic women and matched-controls. There was no correlation between hair and serum levels of calcium or of magnesium.

Much of the research on calcium supplementation of pregnant women to reduce the risk of pre-eclampsia is based on a belief that women who develop the disease are calcium deficient, usually because of poverty and poor diet. The findings of our study do not support this belief. An alternative explanation would be that the benefit of calcium supplementation in reducing the risk of developing pre-eclampsia is attributed to a pharmacological effect; that calcium may modify the pathological processes underlying pre-eclampsia.

Currently the PIERS (Pre-eclampsia Integrated Estimate of RiSk) study group, based in Canada, is conducting a multicentre international study. One arm of the study will investigate the effect of pre-pregnancy calcium supplementation of high risk women on the risk of developing pre-eclampsia in their next pregnancy. Four institutions in South Africa, including the University of Cape Town, are participating in the study. The supposition is that by correcting a potential calcium deficiency prior to pregnancy, implantation, placental

development and early pregnancy would occur in women with improved calcium levels and this may influence the incidence of pre-eclampsia. Nonetheless, if this study finds that pre-pregnancy supplementation is beneficial, this question still remains: is this effect of calcium supplementation on the risk of developing pre-eclampsia solely the result of correcting a nutritional deficiency?

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ANNEXURE A: Study information brochure

HAIR CALCIUM ANALYSIS IN PRE-ECLAMPSIA: CASE

Thank you for the time taken to help with this study.

Pre-eclampsia is a problem in pregnancy that affects many South African women. It is characterised by raised blood pressure and may affect both mother and baby. Little is known about how to prevent this condition. Previous studies have found that giving calcium tablets to women at high risk for developing pre-eclampsia may reduce their chance of developing the disease, or make it less severe. Because you have had pre-eclampsia in your pregnancy we wish to investigate the amount of calcium and magnesium in your blood and hair.

We would like you to complete a questionnaire with an interviewer, and to provide a 10 millilitre (2 teaspoons) blood sample and a small sample of scalp hair. It should not take more than 20 minutes of your time. All details and results will remain anonymous, it will be impossible to identify you from the data provided on the data collection booklet.

The blood taken from you will be tested for calcium and magnesium levels at the Groote Schuur Hospital National Health Laboratory Service. The hair specimen will be safely transported to the United Kingdom where the calcium and magnesium levels will be determined. No other tests will be performed on either of these specimens.

Advantages of participation

There are no personal advantages. You will be contributing to a collection of evidence which may benefit your future pregnancies or those of other women. Your help may improve maternal and child health by allowing us to develop more information about preventing pre-eclampsia.

Disadvantages of participation

You would have a small sample of hair cut from the back of your scalp which should not be noticeable and a sample of blood taken. This will not have any negative effects except the discomfort of venepuncture.

Investigators

Principal investigator	Dominic Richards
Supervisor	Professor Zephne van der Spuy

Both contactable at the Department of Obstetrics and Gynaecology, Old Main Building, Groote Schuur Hospital, Observatory, 7925. Tel: 27 21 404 6020.

Ethics approval

The study has been approved by the Research Ethics Committee of the Faculty of Health Sciences of the University of Cape Town. Their contact details are as follows:
Faculty of Health Sciences
Research Ethics Committee
E-52-23 Old Main Building, Groote Schuur Hospital
Observatory, 7925
Tel: 27 21 406 6492 Fax: 27 21 406 6411.

Your written permission is required before participation.

We appreciate your willingness to give of your time and to assist with this study.

ANNEXURE B: Consent form

HAIR CALCIUM ANALYSIS IN PRE-ECLAMPSIA

CASE STUDY NUMBER:

I _____, agree to participate in a study investigating calcium and magnesium levels in pre-eclamptic mothers by means of completing a questionnaire and giving a sample of my blood and hair. I understand that my HIV status will be recorded with the rest of my antenatal history. I confirm that the nature of the study has been explained to me in my language of choice and that I was given the opportunity to ask questions. I understand that I may withdraw from this study at any point without compromising my health care.

I understand that any personal information gathered from the questionnaire will be treated in a confidential manner and will only be used for the purpose of the study. I acknowledge that I will receive no feedback from the questionnaire and that my participation is voluntary.

I accept that I will receive no reimbursement for my participation. Choosing not to participate will not affect the standard of care or treatment that I will receive thereafter.

Signed at _____ on this the _____ day of _____ 2010.

SIGNATURE	SIGNATURE	SIGNATURE
NAME	NAME	NAME
PARTICIPANT	**INVESTIGATOR**	**WITNESS**

ANNEXURE C: Data sheet and questionnaire

HAIR CALCIUM ANALYSIS IN PRE-ECLAMPSIA

STUDY NUMBER:	*Case / Control*

SECTION A: DEMOGRAPHIC AND PREGNANCY INFORMATION

HOSPTAL NUMBER			***DATE OF INTERVIEW***		
GENERAL INFORMATION			AGE		
IN WHAT POPULATION GROUP DO YOU CLASSIFY YOURSELF?	BLACK	COLOURED	WHITE	INDIAN	OTHER *(SPECIFY)*:
AREA OF RESIDENCE FOR PAST 6 MONTHS	*(SPECIFY)*				
PREGNANCY BOOKING INFORMATION			DATE OF BOOKING		
GESTATIONAL AGE AT BOOKING					
BLOOD PRESSURE SBP/DBP			URINE ANALYSIS		
HIV STATUS	POSITIVE	NEGATIVE	IF POSITIVE CD_4 COUNT:		
BLOOD AND RHESUS D GROUPING			RPR/VDRL & TITRE		
			FTA / TPHA		
INFORMATION AT DELIVERY			DATE OF ADMISSION		
REASON FOR ADMISSION					
GESTATION AT DELIVERY			BIRTHWEIGHT		
DATE OF DELIVERY			APGAR SCORE	1 MIN	5 MIN
BLOOD PRESSURE SBP/DBP	ON ARRIVAL	4 HOURS LATER	8 HOURS LATER	12 HOURS LATER	
URINE PROTEIN SULPHOSALICYLIC ACID TEST	ON ARRIVAL	4 HOURS LATER	8 HOURS LATER	12 HOURS LATER	
URINE PROTEIN COLLECTION	YES	NO	RESULT (g/24 hours):		
MAGNESIUM SULPHATE GIVEN	YES		NO		
BIOCHEMCIAL ABNORMALITIES					
MODE OF DELIVERY	VAGINAL DELIVERY	FORCEPS DELIVERY	VACUUM DELIVERY	C/SECTION (INDICATION):	

SECTION B: DIETARY QUESTIONAIRRE

1	Age (years)		
2	Weight at booking (kg)		
3	Height (m)		
4	Body mass index at booking (kg/m^2)		
5	What is your town and country of birth?		
6.1	For how many months/years have you been living in Cape Town?	Y	M
6.2	If less than 6 months, where were you previously living?		
6.3	For how long had you been residing there?	Y	M
7	What is your marital status? *1. Single 2. Married 3. Divorced 4. Widow 5. Co-habitating*		
8	Into which population group do you classify yourself? *1. Black African 2. Coloured 3. Indian 4. White 5. Other (specify)*		
9 .1	What is the highest level of education attained by you?	.1	
.2	attained by your spouse/partner?	.2	
10.1	What is your occupation?	.1	
.2	What is the occupation of your spouse/partner?	.2	
11	What is your household income (gross per month)?		
12.1	How many adults live in your household?	.1	
12.2	How many of them contribute to the income of the household?	.2	
13	What type of dwelling do you live in? *1. Brick 2. Wood 3. Asbestos 4. Thatched 5. Concrete 6. Corrugated iron 7. Mixed (plastic, cardboard) 8. Other (specify)*		
14	What type of roofing do you have? *1. Asbestos 2. Thatched 3. Corrugated iron 4. Concrete 5. Tile 6. Other (specify)*		
15	Is your area of residence neighbouring or in the vicinity of a zone that is *1. agricultural 2. industrial (specify) 3. neither of these?*		
16	How long have you lived in the area in question 15?	Y	M

17	Are you currently suffering from a medical condition? *1. Yes (specify condition/s and duration of illness)* *2. No*	
18	Have you ever previously suffered from a recognised medical condition/s? *1. Yes (specify condition/s and duration of illness* *2. No*	
19	Are you taking any medication for the above condition/s? *1. Yes (type, daily dose, duration of use)* *2. No*	
20	Do you regularly use diuretics? *1. Yes (type, daily dose, duration of use 2. No*	
21	Do you regularly use laxatives? *1. Yes (type, daily dose, duration of use 2. No*	
22	Do you use nutritional supplements? *1. Yes (type, duration of use 2. No*	
23	Are you on a special diet? *1. Yes (specify type of diet and duration) 2. No*	
24	Have you gained weight in the last six months? *1. Yes (specify how many kilograms) 2. No*	
25	Have you lost weight in the last six months? *1. Yes (specify how many kilograms) 2. No*	
26	How often do you eat fish in an average week over the last 6 months? *1. Daily 2. More than once a week 3. Less than once a week 4. Never*	
27	How often do you eat red meat in an average week over the last 6 months? *1. Daily 2. More than once a week 3. Less than once a week 4. Never*	
28	How often do you eat chicken in an average week over the last 6 months? *1. Daily 2. More than once a week 3. Less than once a week 4. Never*	
29	What is your daily consumption of:	
.1	Coffee (cups/day)	.1
.2	Ceylon tea (cups/day)	.2
.3	Rooibos tea (cups/day)	.3
.4	White bread (slices/day)	.4
.5	Brown bread (slices/day)	.5
.6	Wholewheat bread (slices/day)	.6
.7	Cereals (bowls/day)	.7
.8	Cheese (helpings/day)	.8

.9	Nuts (tablespoons/day)	.9
.10	Fruit (pieces/day)	.10
.11	Chocolate (bars/day)	.11
.12	Cream (helpings/day)	.12
.13	Milk (cups/day)	.13
.14	Beans (helpings/day)	.14
.15	Potato (helpings/day)	.15
.16	Green leafy vegetables (helpings/day)	.16
.17	Yoghurt	.17
30	What type of food do you particularly dislike? Specify.	
31	What is your consumption of:	
.1	Alcohol (units/wk) (1 unit = 250 ml beer/1 glass wine)	.1
.2	Cigarettes (number/day)	.2
.3	Tobacco (grams/day)	.3
.4	Snuff (times used/day)	.4
32	How do you cook your food? *1. Open wood fire 2. Gas/electric cooker 3. Open coal fire 4. Paraffin stove 5. Other (specify)*	
33	What type of cooking vessels do you use? *1. Aluminium 2. Steel 3. Iron 4. Pottery*	
34	What type of water supply does your household have? *1. Stand pipe 2. Domestic supply 3. Well*	
35	What is your natural hair colour? *1. Blonde 2. Brown 3. Black 4. Red*	
36	What brand of hair shampoo do you use?	
37	Do you use a hair colouring agent? *1. Yes (what brand and for how long) 2. No*	
38	Please specify any hair sprays, setting lotions or dressings that you routinely use on your hair.	
39	Have you had any of the following hair treatments in the past 6 months? *1. Permanent wave 2. Straightening 3. Bleaching 4. Head lice treatment 5. Other (specify)*	
40	Do you use any anti-dandruff shampoo? *1. Yes (specify brand and duration of use) 2. No*	

ANNEXURE D: Approval of research ethics committee

UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone |021| 406 6338 • Facsimile |021| 406 6411
e-mail: [illegible]

15 January 2010

REC REF: 016/2010

Dr D Richards
Obstetrics & Gynaecology

Dear Dr Richards

PROTOCOL TITLE: A PILOT STUDY TO COMPARE CALCIUM LEVELS IN PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANCIES IN A LOW DIETARY CALCIUM SETTING

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above mentioned study.

Approval is granted for one year until 20 January 2011.

Please submit an annual progress report if the research continues beyond the approval period. Alternatively, please submit a brief summary of your findings so that we can close our records.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

This serves to confirm that the University of Cape Town Research Ethics Committee complies to the Ethics Standards for Clinical Research with a new drug in patients, based on the Medical Research Council (MRC SA), Food and Drug Administration (FDA-USA), International Convention on Harmonization Good Clinical Practice (ICH GCP) and Declaration of Helsinki guidelines.

The Research Ethics Committee granting this approval is in compliance with the ICH Harmonised Tripartite Guidelines File Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part: 50, 56 and 312.

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938