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SELENIUM LEVELS AND RECURRENT PREGNANCY LOSS: IS THERE AN ASSOCIATION?

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This is to certify that the work contained in this dissertation is the original work of the candidate and work by others has been acknowledged as such.

This work was carried out while a registrar in the Department of Obstetrics and Gynaecology, Faculty of Health Sciences, University of Cape Town in partial fulfilment of the requirements for the degree MMed (O&G).


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ABSTRACT:

Introduction:

Miscarriage is the commonest complication of pregnancy and affects 12-31% of all conceptions. About 1% of all couples trying to conceive will have recurrent pregnancy loss (RPL). Several causes for RPL have been documented and these include chromosomal abnormalities, PCOS, thrombophilias and anatomical anomalies such as cervical incompetence. In many couples the aetiology of the pregnancy loss is often not defined but nutritional deficiencies have been postulated as possible causes. In particular selenium deficiency is associated with reproductive failure in animals and, more recently, in some human studies. This study was undertaken to assess the selenium levels in women with RPL without an identified cause.

Methods:

Twenty four patients with RPL and 24 controls who had a successful obstetric history and who were matched for age and ethnicity were recruited. A detailed history was obtained and hair samples were collected and analysed for selenium content by inductively coupled plasma mass spectrometry.
Results:

The control subjects had a higher mean income and had completed more years of education compared with the patients. There was no significant difference in the intake of selenium-rich foods between the 2 groups. The patients, however, consumed significantly more fruit, cheese, potatoes and chocolate than the controls. Selenium levels in both groups were low, when compared to our available reference ranges (1.41-1.85ppm) and similar in the groups. Patients had a mean of 1.052ppm compared with 0.912ppm in controls (p=0.67).

Conclusions:

While there were significant differences in the 2 groups with regard to resources, education and diet, selenium levels were low in both groups. Selenium deficiency is therefore present in patients and controls and is not present only in the women with RPL.
ABBREVIATIONS:

Bi  Bismuth
Cl  Confidence interval
COC Combined oral contraceptives
DRCII Dynamic reaction cell
Ga  Galium
GnRH Gonadotropin releasing hormone
H⁺ Hydrogen
HbA1C Glycosylated haemoglobin
hCG Human chorionic gonadotropin
ICPMS Inductively coupled plasma mass spectrometry
IUCD Intra-uterine contraceptive device
LH Luteinising hormone
NO Nitric oxide
O₂ Oxygen
ONOO Peroxynitrate
OR Odds ratio
PCO Polycystic ovaries
PFA Paraformaldehyde
PPB Parts per billion
PPM Parts per million
RPL Recurrent pregnancy loss
RSD Relative standard deviation
SD Standard deviation
UK United Kingdom
v/v volume/volume
WHO World Health Organisation
INTRODUCTION AND LITERATURE REVIEW:

Miscarriage is the most frequent complication of pregnancy and one of the commonest gynaecological problems encountered in practice. It is a major cause of maternal morbidity and mortality in South Africa and contributes to 30% of deaths from "pregnancy related sepsis", and is directly responsible for 3.5% of all maternal deaths. (1)

DEFINITION:

Miscarriage is defined as the ending of an intrauterine pregnancy before the fetus is viable. In South African law, viability is defined as 28 weeks after the last menstrual period. This current definition of viability was promulgated in 1963 and needs amendment in the light of modern neonatal care. (1)

The World Health Organization (WHO) defines miscarriage as "the expulsion or extraction from its mother of an embryo or fetus weighing 500g or less," while recurrent pregnancy loss is defined as "the loss of three or more consecutive pregnancies before 20 weeks gestation."(2) Studies indicate that recurrent miscarriage affects 1% of all couples trying to conceive.(3-6)

INCIDENCE:

Most studies which assess the incidence of miscarriage report that 12 to 31% of all conceptions, first and second trimester, result in spontaneous miscarriage.(7-10) The wide variation between these figures may reflect the methods used to diagnose pregnancy.
A study done at Columbia University, New York, used a very sensitive urinary human chorionic gonadotropin (hCG) assay, capable of detecting levels as low as 0.01ng per millilitre, to assess the incidence of early pregnancy loss. A control group consisting of 28 women who had undergone tubal ligation was used to establish this level. The criterion for diagnosis of early pregnancy was a level above 0.025ng per millilitre. Healthy women intending to become pregnant were recruited. Daily early morning urine specimens from 221 women were collected and a total of 707 menstrual cycles were studied. The prevalence of total early pregnancy loss, which was defined as loss before 24 weeks, was quoted at 31%. A total of 22% of all pregnancies detected by hCG measurement ended before the pregnancy was clinically detected.\(^{(10)}\)

A woman's reproductive history has been shown to be a good predictor of future reproductive performance. A prospective study done in the Department of Obstetrics and Gynaecology in the University of Cambridge assessed the influence of past reproductive performance on the risk of spontaneous miscarriage. Women intending to conceive were recruited from the outpatient clinics. A total of 630 women were recruited and women reported as soon as their period was missed. Pregnancy was diagnosed with serum hCG measurements and abdominal ultrasound confirmed the pregnancy before eight weeks. The authors concluded that in primigravidas and women with a previous live birth, the incidence of miscarriage was 4 to 5%. The overall risk of miscarriage after one previous miscarriage was 19%, similar to other studies.\(^{(7-10)}\) In clinically recognizable pregnancies, the incidence of miscarriage before 20 weeks was 12%, 24% after 2 miscarriages, 30% after 3 losses and 40 to 50% after 4 losses. They concluded miscarriage risk was directly linked to the outcome of a woman's previous pregnancies.\(^{(8)}\)

The risk of miscarriage decreases after the demonstration of a fetal heartbeat but increases if there is bleeding. This was demonstrated in a study done at the University of Pittsburgh, Pennsylvania, where 347
women were recruited in the first trimester between 6 and 14 weeks and followed up. Ultrasound was used to confirm a first trimester pregnancy. Two groups were identified comprising women with bleeding or without bleeding. The rate of miscarriage in women who presented with bleeding was 12.7% and 4.2% in the second group. (11)

A retrospective study done at the Department of Medical Genetics, Montreal Children’s Hospital, analysed data collected over 10 years from 1952 to 1962. The data were collected from family histories and included details about previous pregnancies. The frequency of miscarriage was reported as 14.7%. (12)

Data collected from the national health registry, covering the entire Danish population from 1978 to 1992, were reviewed and pregnancy outcomes were analysed. A total of 634,272 women and 1,221,546 pregnancy outcomes was included and in this population the overall rate of miscarriage was 13.5%. (13)

A number of studies agree that the incidence of recurrent pregnancy loss (RPL) is about 1% in all couples trying to conceive. (6,9,12) The observed incidence of RPL is significantly higher than expected by chance alone. As mentioned above, the risk of miscarriage is directly linked to previous pregnancy outcomes. If miscarriage always occurred randomly, and as 12-14.7% of all clinically recognised pregnancies miscarry, then we can expect 0.4% of women to miscarry in three consecutive pregnancies. (8,11,12,14) Studies quote the incidence of recurrent miscarriage at 1%, thereby indicating that there may be specific causes contributing to the aetiology of recurrent miscarriages. (6,15)

In a survey done by Roman at the Epidemiological Monitoring Unit, London School of Hygiene and Tropical Medicine, 2786 women doctors were asked to report retrospectively on their pregnancies. The women listed all their pregnancies and all the details that they could remember. Women who had outcomes other than ectopic pregnancy, singleton
pregnancies, stillbirth or spontaneous miscarriage were excluded. The primary outcomes were live birth, stillbirth, ectopic pregnancy, induced abortion and spontaneous miscarriage. This study quoted that 1% of all respondents reported 3 or more consecutive miscarriages.\(^{(9)}\)
ETIOLOGY OF SPONTANEOUS MISCARRIAGE:

The etiology of pregnancy losses may be divided into first trimester and second trimester causes. First trimester miscarriages are more common while late miscarriages are often related to an identifiable pathology.

GENETIC CAUSES:

It is suggested that chromosomal abnormalities contribute to miscarriage and are responsible for up to 50 to 60% of spontaneous miscarriages occurring in the 1st trimester.(16,17) Boue et al, from Paris, reviewed the cytogenetic data from 1500 first trimester miscarriages over 5 years. The authors found that 921(61.2%) of all these miscarriages had demonstrable chromosomal abnormalities, of which 35(3.9%) had structural abnormalities. They reported that only one third of the abnormalities were transmitted from a parent and concluded that nearly all these chromosomal aberrations were a result of errors at the time of gametogenesis and fertilisation.(17)

Research by Strobino et al suggested that patients who suffer from RPL lose their pregnancies at a later gestation than those with sporadic miscarriages. They recruited 3755 women over 5 years, from all the New York City hospitals, who came to hospital seeking help for miscarriage. Those selected were gravida three or more and matched for age +/- 2 years. One group (cases) consisted of “repeaters”, defined as any woman who reported three or more miscarriages. Two control groups were identified, those with less than three miscarriages (sporadic) and those with no previous miscarriages and at least two live births. It was not specified if the miscarriages were consecutive, nor mentioned if the
same paternal genes contributed to all miscarriages. Fetal karyotype was obtained where possible. The frequency of normal karyotype was greater among repeaters (82%), compared to sporadics (66.8%) [Odds ratio = 2.4 (95% confidence interval: 1.2-4.7)]. Of the repeaters, 41.5% miscarried after 13 weeks compared to 29.7% of the sporadics.\[18\]

Advancing maternal age increases the risk of miscarriage as a result of chromosomally abnormal conceptions and decreased ovarian function. The authors of the Danish study (Nybo Anderson et al) reported that the risk of fetal loss increased with maternal age. At age 22 to 24, the risk of miscarriage was 8.9%, 15% at age 30-34, 24.6% at 35-39 years, 50% at age 42 and 74.7% in women over 45 years of age.\[13\]

Data collected from the European Study of Infertility and Subfecundity, a large multicentre study from 1991 to 1993, reflected 14 population based samples from Denmark, Germany, Italy and Spain. The original aim was to evaluate the frequency of, and risk factors for, subfecundity and infertility. Women between the ages of 25 and 44 were recruited and, in the questionnaire, the details of the male partner were also included. These data were then used to assess the effect of maternal and paternal age on the risk of miscarriage. Authors reported that the risk of miscarriage is highest in couples where the woman is 35 and older and the man 40 and older, [OR: 6.73(95% CI: 3.50-12.95) and 3.38 (95% CI: 1.76-6.47) respectively.]\[19\]

Balanced translocations and reciprocal translocations are the most common inborn parental chromosomal abnormality. Balanced translocations involve one parent who carries a normal genetic content but has some piece of genetic material inappropriately attached to another.

Balanced translocations can be reciprocal or Robertsonian. Robertsonian translocations occur when the short arms of 2 acrocentric chromosomes fuse. The resulting karyotype leaves 45 chromosomes since the 2 chromosomes have fused. The individual is phenotypically
normal. Inversions are chromosomal rearrangements in which a segment of a chromosome is reversed from end to end. It occurs when a single chromosome undergoes breakage and rearranges itself.

Researchers at The University of Quebec, Canada analysed data from 22 199 couples experiencing recurrent pregnancy losses (RPL). A database was generated from the information on cytogenetic studies done in couples with recurrent pregnancy losses. This included data on 16 721 couples. Information on parental major chromosome abnormalities defined as Robertsonian translocations, reciprocal translocations, inversions, and sex chromosome aneuploidy, was extracted. Balanced translocations represented the largest group of chromosome abnormalities in women with RPL with 1.3% of the individuals affected. Robertsonian translocations occurred in 0.66% of all cases and Inversions in 0.19%. The authors concluded that one carrier was found in 4.7% of the couples with RPL.(20)

A case control study from Japan reported that in 1309 women with a history of 2-20 consecutive first trimester miscarriages, the proportion of normal karyotype rate decreased significantly with the number of previous spontaneous miscarriages.(21)

There is a link between X-linked chromosomal abnormalities and pregnancy loss. Lanasa et al, from the University of Pittsburgh, USA, compared 105 women with RPL to 101 controls who had a successful pregnancy and no pregnancy loss. They studied X-linked inactivation patterns. Patients with RPL showed a statistically significant increase in the frequency of skewed X chromosome inactivation (P=0.0005). This suggests that X-linked disorders are closely related to RPL of female offspring but uncommonly result in loss of male offspring.(22)

While more than half of sporadic first trimester miscarriages have a genetic cause, however it is more difficult to establish if genetic causes are more common in RPL as compared to sporadic miscarriage.
ANATOMICAL CAUSES:

The incidence of anatomical abnormalities among patients with RPL is quoted as ranging from 1.8% to 37.6% with a mean overall incidence of 12.6%. This is a very wide range and is probably a reflection of the methods used to diagnose an abnormality and possibly even the criteria used to define RPL. The methods used to diagnose uterine abnormalities include ultrasound, hysterosalpingography (HSG), sonohysterography (SHG), laparoscopy and hysteroscopy. A comparative study by Soares et al concluded that HSG is superior to other methods.

It is difficult to assess the impact that uterine malformations have on pregnancy loss and RPL. Researchers from Valencia, Spain screened 680 parous women with a normal reproductive history. They found that 3% had a Mullerian abnormality. Of these 90% had septate uteri, 5% had a uterine didelphys and 5%, a bicornuate uterus. In a further study 127 women with untreated uterine abnormalities who had 342 pregnancies were reviewed by the same group. There were 226 live births, 131 term deliveries, 35 preterm deliveries and 60 miscarriages. The study did not compare early and late miscarriages.

Pregnancy outcome, reported in a Finnish study, in patients with uterine abnormalities was studied in 182 women with proven uterine abnormalities who had not received any treatment. Of the 182 women, 126 conceived. A total of 265 pregnancies were recorded. The incidence of miscarriage was 29%, 16.5% had preterm labour and 55.5% a term delivery and the fetal survival rate was 66%, similar to Acien's study which is discussed later. The authors concluded that the best survival rate was in women with a septate uteri (86% survival), followed by bicornuate uteri (50%) with the worst outcome in unicornuate uteri (40%).

Acien compared the reproductive outcomes of 176 women with untreated uterine malformations to 28 patients with a normal uterus.
Among the 176 women there were 40 arcuate, 49 bicornuate, 17 bicornis-bicollis, 15 didelphys, 24 unicollinate, 14 subseptus and 17 septate uteri. The rate of miscarriage in the study group was 36%. Conception was achieved in 142 women with uterine abnormalities and 26 women with normal uteri. The primary outcome was neonatal survival of more than 7 days and only 53% of women with uterine abnormalities achieved this compared to 89% of controls. Seven day survival was 40% in the bicornuate group, 45% in those with arcuate uteri, 59% septate uteri and 70% in bicornis–bicollis, didelphys, unicollinate and septate uteri. In the study group, 383 pregnancies were documented with a miscarriage rate of 36% (91.3% first trimester and 8.7% second trimester), compared with 8% among controls. Term deliveries were also calculated and this was lower in the study group, at 44%, compared to 85% in controls. The authors also compared the delivery rates in the study group to the subjects’ subsequent pregnancies and found a similar term delivery rate of 45%.²⁸

Cervical incompetence is a recognised anatomical cause of RPL. Outside pregnancy the diagnosis is made on clinical history of repeated second trimester losses, painless cervical dilatation, minimal bleeding, ballooning and rupture of the membranes, followed by preterm delivery with the fetus often alive during labour and delivery. During pregnancy, however, the use of transvaginal ultrasound demonstrating serial shortening of the cervix can help to make the diagnosis.²⁹ A survey by Drakeley and co-workers, reviewed the database from The Liverpool Women’s Hospital Miscarriage Clinic from 1991 to 1996. A total of 636 patients were identified of which 158 had 2nd trimester miscarriages. Within this subgroup 8% were reported to have cervical incompetence.³⁰
ENDOCRINE FACTORS:

Uncontrolled diabetes mellitus may cause RPL. The literature suggests that well controlled diabetes is not a cause for RPL. This was demonstrated by Mills et al, from the National Institute of Child Health and Human Development (Bethesda), who prospectively recruited 386 women with diabetes mellitus and compared the pregnancy outcomes to 432 healthy women. Women were recruited within 21 days of conception. The rate of miscarriage in the diabetics was 16.1% compared to 16.2% in the control group.

They also analysed the biochemical parameters of diabetic women who had miscarriages and reported that these women had a higher fasting blood glucose and post-prandial glucose. Of those women with poor glucose control, for every increase of the glycosylated haemoglobin (HbA1C) by 1 standard deviation above the normal range, the risk of miscarriage increased by 3.1%. (31)

Hanson et al reported an increase in pregnancy loss in Swedish women with poor diabetic control. This was a prospective nationwide study from 1982 to 1985. A total of 532 type 1 diabetics were compared to 222 healthy controls. The HbA1C was measured in all women before 9 weeks of gestation. They reported a pregnancy loss rate of 7.7% in the diabetic group compared to 7.2% in the control group. In a sub-analysis of patients with an HbA1C greater than 10.1%, there was a significant increase in pregnancy loss (p < 0.001). (32)

There is poor evidence to support the theory that the incidence of thyroid disease is increased in patients with RPL. Li and his co-workers in Sheffield studied thyroid function in 144 women and concluded that the incidence of thyroid disease in patients with RPL was the same as...
the general public. These authors suggest that only symptomatic women be screened.\(^{(33)}\)

**OVARIAN CAUSES:**

**LUTEAL PHASE DEFECT:**

The term "luteal phase defect" describes a state where the corpus luteum is not producing sufficient progesterone. Progesterone from the corpus luteum is needed for the maintenance of early pregnancy prior to the luteo-placental shift in progesterone production. It was postulated that luteal phase defects, resulting in inadequate progesterone levels, may contribute to early pregnancy loss. The incidence of luteal phase defect in RPL is difficult to establish. Fritz suggests that luteal phase defects affect 23-60% of women with RPL.\(^{(34)}\)

The literature on luteal phase defects is contradictory. A meta-analysis by Goldstein et al of six trials in which exogenous progesterone was administered in patients with RPL failed to show any benefit\(^{(35)}\). In contrast the meta-analysis of 3 randomised controlled trials in which progesterone was used in women with RPL by Daya showed a statistically significant increase in pregnancies that went beyond 20 weeks (OR: 3.09 and CI:1.28-7.24).\(^{(36)}\)

**POLYCYSTIC OVARY SYNDROME:**

The incidence of polycystic ovaries (PCO) is significantly higher in women with RPL compared to the incidence in the general population. There are conflicting data regarding the role of PCO in the aetiology of RPL. The incidence of PCO in the general population ranges from 17-
22%. Botis and co-workers assessed the incidence of PCO in 1078 women, and reported an incidence of 17% (95% CI 14-19).\(^{37}\)

The prevalence of PCO was studied in 2199 women with RPL. This study based the diagnosis of PCO on ultrasound, both transabdominal and transvaginal, findings of an enlarged ovarian volume >9ml, more than 10 cysts of 2-10mm and an increased density of the stroma. The prevalence of PCO was 40.7%. The live birth rate in this group was 60.9% compared to 58.5% in the women without PCO suggesting that PCO is not a predictor of miscarriage.\(^{38}\)

It was hypothesized that the link between RPL and PCO may be elevated LH secretion, causing premature resumption of the 2nd meiotic division of the oocyte with the oocyte being fertilized at an inappropriate stage in development. LH may cause asynchronous endometrial maturation resulting in defective implantation. Regan’s group suggested that raised serum LH levels in the follicular phase may therefore be predictive of a poor pregnancy outcome.\(^{38-40}\)

Regan’s original study aimed to assess the link between pre-pregnancy follicular phase LH and pregnancy outcome. One hundred and ninety three women with regular menstrual cycles who wished to conceive were recruited. A total of 147 women had LH levels below 10 IU/L and 46 women had LH levels above 10 IU/L. Of the women with elevated LH levels, the rate of miscarriage was 65% compared to 12% in the group with normal levels suggesting an increased risk of miscarriage in women with elevated pre-conception follicular phase LH.\(^{40}\)

Clifford demonstrated in a randomised placebo-controlled trial that pre-pregnancy suppression of luteinising hormone (LH) does not improve live birth rate. One hundred and six women, known with polycystic ovaries and LH hypersecretion with more than three first trimester miscarriages, were randomised before conception for suppression with luteinising hormone releasing hormone analogue followed by low dose ovulation induction and luteal phase progesterone, or spontaneous
ovulation with or without (placebo) luteal phase progesterone. The results showed conception (80% vs. 82%) and live birth rates (65% vs. 76%), in the suppression group and luteal support group respectively.\(^{[41]}\)

**NUTRITIONAL FACTORS:**

Malnutrition is a global problem. In developing countries, the main contributing factor is a scarcity of food or chronic ill health. In contrast, in the developed world, malnutrition often appears to be self inflicted either secondary to poor diet, inappropriate weight reducing diets or eating disorders.\(^{[42]}\)

The reproductive axis is closely linked to a woman’s nutritional status.\(^{[43]}\) Energy expenditure is highest in times of pregnancy and lactation and therefore it is plausible that fecundity is reduced in times of scarce nutrition where the woman will conserve energy for survival. The body will try to redirect energy expenditure to essential processes such as cell maintenance, neural activity and thermoregulation. This process also means that fat storage is not a priority during these times.\(^{[44]}\)

The effect of malnutrition on pregnancy was studied after World War II. Some cities in the Netherlands were subject to severe food shortages while other cities were less affected. This provided a unique opportunity to determine the impact of nutritional deprivation on pregnancy. Stein and Susser reported that in pregnancies conceived during the famine, the babies were born with a lower birth weight, highlighting the fact that undernutrition early in pregnancy has a significant negative effect
on the growing fetus and that the pregnancy will usually continue despite the mother's suboptimal nutrition state.\textsuperscript{[45]}

Micronutrient deficiencies might contribute to pregnancy loss and supplementation may reduce the prevalence of miscarriage. Nutritional factors are potentially reversible, as evidenced by the work done by Wynn and Wynn, who observed that during the Dutch Hunger Winter, malnutrition was a major cause of low birth weight and that refeeding reversed many of these effects.\textsuperscript{[46]}

Wade and Jones, scientists from the University Of Massachusetts, hypothesised that any activity or condition that limits the availability of oxidizable fuels (e.g. under-eating, excessive energy expenditure, diabetes mellitus) can inhibit gonadotropin releasing hormone (GnRH) and LH secretion thereby decreasing female fertility. They theorised that metabolic fuel availability is detected by cells in the caudal hind brain. These neurons in turn produce Neuropeptide Y and catecholamines and project signals to the forebrain where they directly inhibit GnRH neuron activity.\textsuperscript{[47]} These theories suggest that energy balance as opposed to "fatness" regulates reproductive function.

The effect of undernutrition on pregnancy has been widely studied; however there is paucity of data related to its role in miscarriage and there is no agreement in the literature that undernutrition can lead to pregnancy loss. The Department of Psychiatry at the University of Massachusetts recruited 54 women with anorexia and bulimia nervosa. These women were followed prospectively and pregnancy outcome was assessed. There were 82 pregnancies of which 46 (56\%) were live births, 25 (31\%) were therapeutic abortions and 11(13\%) were spontaneous miscarriages. The prevalence of spontaneous miscarriage in this study is similar to the incidence of spontaneous miscarriage in the general public.\textsuperscript{[48]} Another study, done at The Virginia Institute of Psychiatry and Behavioural Genetics, recruited 66 women with anorexia nervosa from their clinics and compared them to 98 controls randomly selected
from the community. The study concluded that the rate of miscarriage was higher in women with anorexia (27%) compared to 13% in the control group.\(^{(49)}\)

**MICRONUTRIENTS:**

There are ten known trace elements namely iron, zinc, mercury, copper, selenium, molybdenum, manganese, chromium, cobalt and iodine. Copper and mercury are considered poisons. There is very little data on these elements with regards to RPL, but selenium, zinc, copper, iron and mercury may be linked to pregnancy loss.

**IRON:**

Studies have shown a link between iron deficiency and low birth weight and preterm delivery. There are no studies implicating iron and miscarriage per se. The effects of Iron deficiency in pregnant women were compared in anaemic and non anaemic women in a study at The University of New Jersey. Serum ferritin of 12ug/l defined anaemia among 800 women attending antenatal care. The risk of preterm labour was reported as double that of controls and the risk of low-birth weight as three times greater.\(^{(50)}\) Whether this is a reflection of general malnutrition is not clear. There is no available evidence linking iron deficiency with miscarriage.

**ZINC:**

Zinc is one of the biological trace elements. Zinc deficiency impairs angiotensin converting enzyme activity. Zinc deficiency may lead to diabetes and chromosomal abnormalities in hamsters\(^{(51)}\) and animal studies show a high incidence of congenital malformation and
teratogenicity (mostly neural tube defects) associated with deficiency. Human studies support these findings. In women with acrodermatitis enteropathica, an inborn error of zinc metabolism, fetal malformations (especially neural tube defects) are frequent. Solten and Jenkins, compared 54 maternal and fetal blood samples from pregnancies with fetal abnormalities to controls. They found a significant difference in both maternal and fetal zinc levels.

**COPPER:**

Copper deficient pregnant rats are apparently protected against morbidity because estrogen alters the sub-cellular distribution of copper in the liver and increases circulating copper. There are scanty data on the effect of copper on RPL in humans. Alebic and Frkovic, from the Institute of Public Health in Kresimirova, Croatia, analysed 319 blood samples from women with pregnancy complications specifically spontaneous miscarriage, threatened miscarriage, missed miscarriage and preterm labour. Of these samples 176 were taken from women with spontaneous miscarriage in their first and second trimester. Serum copper levels were compared to laboratory reference values, not controls. The study reported no significant difference in the miscarriage group.

**MERCURY:**

A study from Hull University, UK, evaluated mercury levels in maternal and fetal hair samples in relation to placement of dental amalgam. This study did not use miscarriage as an outcome but showed a significantly higher level of mercury in women with dental amalgam. A case control study at The Finnish Institute of Occupational Health, Finland, compared workers exposed to dental amalgam to those not exposed. Information on pregnancy histories and occupational exposure was
obtained using a questionnaire. Pregnancy outcome of 222 cases, defined as women who had miscarriages, were compared to 498 controls with live births. Exposure was assessed by an occupational hygienist from history and a questionnaire. The difference between the two groups was not statistically significant. OR 1.3 (CI 0.6 to 2.5).{63}

**SELENIUM:**

Selenium first attracted medical interest in 1930 when it was found to cause poisoning of the livestock that grazed in areas with soils containing high selenium levels.{64} In 1957 Schwartz and Foltz reported that small amounts of selenium prevented liver necrosis in vitamin E deficient rats, a finding indicating that selenium was an essential nutrient and not only a toxin.{65} Subsequently, deficiencies of selenium and vitamin E were shown in several economically important nutritional diseases of cattle, sheep, pigs and poultry.{66}

In a study published in Science in 1973, this element was shown to be a constituent of the enzyme glutathione peroxidase.{67} Selenium is a key component of a number of functional selenoproteins required for normal health. The best known of these is the antioxidant glutathione peroxidase which removes hydrogen peroxide and other damaging lipid and phospholipid hydroxides generated by free radicals and other oxygen derived species. A balance between pro-oxidants and anti-oxidants is critical for survival and functioning of aerobic organisms. Oxidative stress occurs when an imbalance occurs and leads to cell damage.

Lipid hydroperoxides impair the structure and function of cell membranes and cause coagulation abnormalities by decreasing the production of prostacyclin while increasing the production of thromboxane, resulting in a predisposition to thromboembolic events. Selenium behaves as a peroxynitrate scavenger and is capable of
removing reactive oxygen species (hydroperoxides and oxidized lipoproteins) that can break down to free radicals resulting in cellular damage. Peroxynitrate is produced by the reaction between nitric oxide and superoxide. \[ O_2^+ \text{ NO} = \text{ONOO}^- \]. Inflammatory cells, such as macrophages and neutrophils, produce large amounts of both nitric oxide and superoxide which in turn rapidly form peroxynitrate. Peroxynitrate is stable, but when a proton (H\(^+\)) is added to it, it decays to a nitrate, forming peroxynitrous acid. This is unstable and highly reactive and yields oxygen free radicals. This may, in fact, be protective to the body in minimal amounts because it is toxic to bacteria. If, however, there is overproduction of peroxynitrate or a failure of the body to remove it, then this results in tissue damage. Peroxynitrate is also a potent inflammatory mediator. It is capable of causing vasoconstriction and platelet aggregation causing damage to the endothelial cells.

Selenium is a trace element that is obtained from bread, cereals, fish, poultry and meat. The selenium concentration in grain and meat reflects the selenium content in the soil. In 1995 the selenium content of over 700 food samples was determined. This sample selection represented 100 different food types available in the United Kingdom. The samples were prepared for cooking and eating and then analysed. The highest selenium content was found in Brazil nuts (245ug/100g), kidney (146ug/100g), crab meat (84ug/100g) and liver (42ug/100g).\(^{68}\)

The literature suggests that there is a wide geographical variation in selenium content of soil. In countries where there is a deficiency, for example in some areas in China, selenium deficiency is associated with an endemic cardiomyopathy called Keshans disease. In 1979 the Keshans Disease Research Group reported that selenium supplementation prevented cardiomyopathy among children living in low selenium areas.\(^{69}\)
In 1989 the recommended daily allowance of selenium was established and again reviewed in 2000. Women need 55ug/day and an additional 20ug/day in pregnancy and lactation.\(^{(70)}\) The World Health Organisation also issued dietary recommendations regarding selenium supplementation. \(^{(71)}\)

A small study from The Ludwig Rydygier Medical University in Poland investigated serum glutathione and glutathione peroxidase levels in women with spontaneous miscarriage. Forty women with miscarriage (spontaneous, not recurrent), were recruited: 22 had been in their first pregnancies, 10 with one previous live child and no miscarriage, 3 had one other miscarriage and the remaining women had more than 1 live child with no previous miscarriage. Two control groups were identified; firstly, 36 healthy pregnant women matched for age and gestational age and secondly 28 age matched, healthy non-pregnant women. Glutathione and glutathione peroxidase levels were measured in whole blood and plasma. The study reported that selenium levels were lower in women with miscarriage and in healthy pregnant women versus the non-pregnant group. Red blood glutathione levels did not differ between pregnant women versus the non-pregnant women. Glutathione levels were higher in the miscarriage group versus the control pregnant group and red cell plasma glutathione peroxidase activity was also lower in women with miscarriage.\(^{(72)}\) This study suggests that decreased glutathione and glutathione peroxidase levels may contribute to the aetiology of miscarriage and therefore selenium deficiency may play a role in pregnancy loss.

In an observational study, performed at The Department Of Obstetrics and Gynaecology at the Singleton Hospital, West Glamorgan (UK), the association between spontaneous first trimester miscarriage and serum selenium levels was evaluated. Forty women with first trimester miscarriage were compared to 40 healthy non-pregnant women and 40 healthy ante-natal clinic attendees. Serum selenium levels demonstrated that compared to non pregnant women, pregnant women
had lower selenium levels \( (P<0.0001) \). In women with miscarriage, the selenium levels were lower than the healthy pregnant controls \( (P=0.0054) \).\(^{(73)}\)

The literature mostly agrees that there is an association between spontaneous miscarriage and selenium deficiency. The possibility that a dietary deficiency of selenium could lead to recurrent miscarriage is therefore also a plausible theory. The West Glamorgan study was followed by another study in the same population in women with RPL. This was a small study and 12 women with RPL with no identifiable cause were recruited. Two control groups of 25 women with spontaneous first trimester miscarriage and 25 non pregnant controls without a history of miscarriage were identified. The mean and median serum selenium levels were lower in the RPL group versus non pregnant controls and the result was statistically significant. What was interesting was the finding that the selenium level in the spontaneous miscarriage group was lower than that of the RPL group although the small numbers make it difficult to draw definite conclusions.\(^{(74)}\)

Kocek et al, working in Turkey, measured serum selenium levels in 20 women with first trimester RPL, 20 age-matched non-pregnant healthy controls, 20 women with spontaneous first trimester miscarriage, and 20 healthy pregnant women. All women with RPL had been investigated and no cause of miscarriage was identified. All non-pregnant controls had at least one live child and no miscarriage. They reported a lower selenium level in women who were pregnant versus non-pregnant women and a further decrease in selenium levels in women with RPL.\(^{(75)}\)

The findings in this study are similar to the other quoted studies and suggest an association between selenium deficiency and RPL. Similar results were obtained in an Indian study from Hyderabad where twenty women were selected, where a known cause for RPL was excluded, and compared to 20 healthy non-pregnant women with at least 1 live birth. They found lower levels of selenium in the RPL group and the results reached statistical significance.\(^{(76)}\)
Only one study has not found an association between RPL and selenium deficiency. A study from the University of Glasgow recruited 20 women with RPL and compared them to 47 healthy parous non-pregnant controls which included women with one previous miscarriage. There was no statistical difference between the selenium levels of the two groups. This study may be criticised for the choice of the control group which included women who had a history of a previous miscarriage.\(^{77}\)

A case-control study from Hull University analysed both hair and serum samples in 18 women with one or more successful pregnancy with no miscarriages and compared them to 26 women with a history of RPL. The serum samples failed to show a difference in selenium levels, however the hair samples showed a significant mean reduction in selenium in the RPL group.\(^{78}\) Of interest, there was no difference in nutritional intake between the two groups. This may suggest that factors other than nutritional deficiency may contribute selenium deficiency and hence to RPL. A review of the literature shows a dearth of information from Africa and South Africa.

**AIM OF THE STUDY:**

A number of women with RPL in our clinic have no identifiable cause for their pregnancy failure. Given the data on selenium, the findings of other studies\(^{74,78}\) and the suggested selenium deficiency in the Western Cape, we undertook this study to determine whether selenium deficiency was present in women with RPL and to compare this group to women with uncomplicated obstetric/pregnancy history.

In view of the proven value of determining micronutrients and other substances in hair, we decided to use hair as it would reflect chronic/long-term nutritional state. We are fortunate in having access to the technology developed to analyse hair at the Department Of Chemistry, University Of Hull.
METHODS:

It was our aim to assess the association between miscarriage and selenium levels in our population by analysing of hair samples to determine chronic selenium intake in a way similar to that sought from the Hull study.\(^{(78)}\)

Subjects:

Two groups were selected. The first group consisted of patients with a history of RPL who had no known diagnosis contributing to pregnancy loss, and a second group of women without a history of miscarriage or other pregnancy complications and at least one live birth.

The patients were recruited from the Reproductive Failure Clinic at Groote Schuur Hospital (GSH), which is a tertiary referral hospital. Patients are referred to this clinic if they fulfil the criteria for RPL. These referrals include women with a history of three consecutive miscarriages at any gestation, first or second trimester, or a history of two second trimester miscarriage.

Before recruitment to our study, the patients were investigated for possible causes of RPL. Only women without an identified cause for RPL were included into the study. All patients were interviewed by a registrar at their first visit and a detailed history taken about their pregnancies. Included in this history was paternal history, diagnosis of previous pregnancies, where these patients sought help, management and gestational age at miscarriage. If further details of the pregnancy were available e.g. histology or karyotype, then such data were reviewed and documented. A history of systemic illness and endocrinopathies
was also recorded. Family history included a history of thromboembolic
disease and inherited genetic abnormalities.

A thorough clinical examination was performed. All patients had a
pelvic ultrasound to screen for anatomical defects and ovarian
morphology to exclude polycystic ovaries. When an anatomical defect
was suspected then hysterosalpingography and/or hysteroscopy was
performed for confirmation. Patients were screened for thyroid disease
using serum TSH and hyperprolactineamia was excluded by measuring
prolactin levels. A haematological screen included a full blood count,
blood group and rhesus screening. Women had a screening test for
syphilis [VDRL (Venereal disease research laboratory)] and rubella
immunity. Those with an unknown HIV status were offered counselling
and testing. HIV positive patients were not excluded from the study.
Patients were screened for antiphospholipid antibody syndrome and
thrombophilia. Chromosomal analysis was offered to women if
indicated.

Each control was matched to an index patient for age plus or minus
two years and for ethnicity. Controls were recruited from any women
attending outpatients or family planning clinics, staff and other women
who fulfilled the inclusion criteria. No pregnant or lactating women were
included. Women, who had been on any form of hormonal
contraception, including the levonorgestrel intra-uterine system, over
the previous six months, were excluded. The inclusion and exclusion
criteria are outlined in table 1.
### TABLE 1.

<table>
<thead>
<tr>
<th>INCLUSION CRITERIA</th>
<th>EXCLUSION CRITERIA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CASES</strong></td>
<td><strong>CONTROLS</strong></td>
</tr>
<tr>
<td>Non pregnant women</td>
<td>Non pregnant women</td>
</tr>
<tr>
<td>3 consecutive 1st trimester miscarriage or 2 second trimester miscarriage</td>
<td>No history of miscarriage</td>
</tr>
<tr>
<td>No live births after miscarriages</td>
<td>At least one successful pregnancy.</td>
</tr>
<tr>
<td>No diagnosis for RPL established</td>
<td>N/A</td>
</tr>
<tr>
<td>Not on hormonal contraception</td>
<td>Not on hormonal contraception</td>
</tr>
<tr>
<td><strong>CASES</strong></td>
<td><strong>CONTROLS</strong></td>
</tr>
<tr>
<td>Currently pregnant or lactating</td>
<td>Currently pregnant or lactating</td>
</tr>
<tr>
<td>Cause for RPL identified, e.g. thrombophilia, uterine abnormality etc.</td>
<td>Any underlying medical condition that may cause RPL</td>
</tr>
<tr>
<td>Use of hormonal contraception in the preceding 6 months</td>
<td>Use of hormonal contraception in the preceding 6 months</td>
</tr>
<tr>
<td>Not living in Cape Town for more than 2 years</td>
<td>Not living in Cape Town for more than 2 years</td>
</tr>
</tbody>
</table>

A datasheet was completed for each recruit with the assistance of a trained interviewer. Specific details were sought aimed at determining factors that could influence the outcome of our study. These included demographic details, habits and reproductive history aiming to establish basic characteristics among all subjects. We also asked about types of dwelling, roofing, hair treatments and hair products, proximity to industrial areas as this may indicate contaminated hair samples.
thereby influencing the results. Data on contraceptive usage, specifically hormonal contraceptives which is discussed later, was requested as sex steroids have been shown to increase glutathione peroxidase activity. A detailed dietary history helped us ascertain dietary differences between the two groups. A copy of the data sheet is attached as annexure 3.

**SAMPLE COLLECTION, PREPARATION AND ANALYSIS:**

Sample collection and preparation:

Hair has been proven to be of value in studying selenium and other micronutrients.\(^{78,85-87}\) Samples were obtained in the same way from all women. The newest hair was cut close to the skin just above the hair line with sharp surgical scissors. These samples were tied proximally indicating the newest hair growth and stored at room temperature in a plastic container. All samples were couriered to the University of Hull, Department of Chemistry, where the preparation and analysis were performed and reported. The laboratory methodology described here has been supplied by Dr. R. Knight, who performed the analyses.

Sample measurement:

The hair samples provided were taken from the polythene bags in which they were transported and any sellotape or labels removed from the hair. The hair was weighed then washed to remove surface debris, such as dust and adhesive from the sellotape, and oils which may contain surface hair treatments. This involved a quick rinse with lipsol detergent in pure water, then 3 washes in acetone. The hair was then air dried overnight at 40°C.
Depending on the sample weight, the hair was added to a Teflon digestion vessel and Nitric acid added. After a time to allow the hair to be completely wetted by the acid and slow room temperature digestion to begin at least, or being soaked overnight at best, the vessel was sealed. Digestion was then completed using the Microwave digestion system (CEM MARS Xpress) at high temperature. When cooled to room temperature the digest was diluted by weight in pure water.

Ideally, a dilution factor, (i.e. final weight of digest divided by the original weight of the hair) of 100 or 200 would be the target, keeping the acid strength to about 15 to 20%v/v.

Large samples, over about 0.1g, were digested in XP1500 microwave vessels, made of TFM Teflon, into 5ml nitric acid (Romil SpA grade) at 200°C for 20 minutes. Digestion is then complete, yielding no residues or colour in the solution. Twelve samples can be heated this way, the control vessel having the sample with the highest weight of the set.

Smaller samples were digested in 7ml PFA Teflon microdigestion vessels with screw caps, loaded into the XP1500 vessels on spacers with 5ml water to allow a build-up of pressure in the microvessels slightly higher than atmospheric pressure. It is not possible to control the temperature or pressure in the microvessels so a temperature of 100°C in the water of the control vessel is the only reliable way to make sure the samples are heated sufficiently without bursting the screw caps which would release the digest. While not being digested at the ideal 200°C temperature, digests using the microvessels can be mainly free of residues and are usually transparent yellow or pale yellow, indicating an incomplete digest. Samples were held at 100°C for 20 minutes. Eleven samples can be heated this way, one per XP1500 vessel. The control vessel has 5ml water only, and it’s temperature was measured by an infrared immersion probe and vapour pressure monitored by a pressure transducer attachment.
Analysis

The digests, or any samples requiring further dilutions, were analysed on the Perkin Elmer Elan DRCII ICPMS instrument. All available isotopes for Selenium were included in an analysis method. The liquid stream was mixed online with an internal standard containing 10 (parts per billion) [ppb] Ga and Bi to allow compensation for small differences in viscosity of the samples due to slight differences in the acid content. The internal standard also monitors the stability of the instrumental conditions during the analysis run.

The element of interest (in this case selenium), was calibrated at 0, 1, 5, 10, 20ppb by the analysis of mixed element solutions suitably diluted from 1000 parts per million (ppm) certified concentrated solutions (Romil PrimAg Xtra). Calibration lines of response against concentration were acceptably linear. Blank solutions of 2% Nitric acid were analysed to allow the calculation of detection limits, defined as 3 times the standard deviation of the mean of several blanks, each measurement being in triplicate to allow calculation of mean, standard deviation and RSD.

Ethics:

Permission to undertake this study was granted by the Ethics Committee of the Faculty of Health Science of the University of Cape Town. (Annexure 1).

Statistical Analysis:

Data were entered into Microsoft Excel and analysed using SPSS 17 (SPSS Corporation Chicago, Illinois, USA). The distributions of
individual variables were summarized using means, medians and proportions, as appropriate.

Bivariate analysis used paired T test, Wilcoxon signed-rank test and McNemar’s chi-square test to take into account the matched nature of patients and controls. All statistical tests were two sided at α=0.05.
RESULTS:

DEMOGRAPHY:

A total of 24 women was recruited to each group (patients and controls). All of the patients that were invited agreed to participate, while in the control group, out of 31 potential subjects, 7 were unsuitable and 24 satisfied all criteria and all agreed to participate. All participants gave written informed consent.

All the women recruited to this study had been living in South Africa and specifically in Cape Town for at least two years. In the RPL group, 18 (75%), were born and grew up in Cape Town. Of the other 6 (25%) women, 4 (16.66%) were born in the Eastern Cape, 1 (4.16%) in Durban and 1 (4.16%) in De Aar. Among women in the control group, 15(62.5%), were born and grew up in Cape Town, 2 (8.3%) were from the Eastern Cape, 2 (8.3%) from Kimberley 1(4.16%) from Malawi, 1 (4.16%) from Namibia, 1(4.16%) from Durban and the remaining three from towns in the Western Cape.

Cases and controls were matched for ethnicity. Each group had (17) 70.8% participants of Coloured origin, (6) 25% Black African and (1) 4.2% Indian. Table 2 presents the general characteristics of the two groups. The mean ages were 32.5 years (RPL) and 33 years (control group).
TABLE 2. GENERAL CHARACTERISTICS.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=24</td>
<td>n=24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age(years)</td>
<td>32.54 (5.62)</td>
<td>33 (5.87)</td>
<td>0.775</td>
</tr>
<tr>
<td>BMI</td>
<td>31.9 (21.8)</td>
<td>28.5 (6.3)</td>
<td>0.473</td>
</tr>
<tr>
<td>Education (years)</td>
<td>9.96(3.12)</td>
<td>14.85(4.23)</td>
<td>0.374</td>
</tr>
<tr>
<td>Gravidity (median)</td>
<td>4 (2-8) (median/range)</td>
<td>2 (1-5) (median/range)</td>
<td>0.001</td>
</tr>
<tr>
<td>Miscarriages (median)</td>
<td>3.5 (2-7) (median/range)</td>
<td>0 (0-0) (median/range)</td>
<td>0.001</td>
</tr>
<tr>
<td>Weight gain(kg)</td>
<td>1.92(3.43)</td>
<td>3.21(3.66)</td>
<td>0.214</td>
</tr>
<tr>
<td>Weight loss</td>
<td>0.979(1.79)</td>
<td>0.67(1.60)</td>
<td>0.87</td>
</tr>
<tr>
<td>Household income/Rands per month</td>
<td>R. 6147.55 (8022.6)</td>
<td>R. 14761 (11351.44)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

Values are given as mean (SD)/median (range)

The significant differences between the two groups were obstetric outcomes, as would be expected and household income. The weight and height of the two groups showed no difference with a body mass index (BMI) [weight (kg) divided by height squared] of 31.9 and 28.5 in the RPL and control group respectively [P=0.473] and there was no difference in weight gain or loss between the 2 groups given a p value of 0.214 ad 0.87 respectively. The mean number of miscarriages was 3.7 in the RPL group and no miscarriages in the control group.

Figure 1 demonstrates the difference in income between the two groups. The controls, represented by the squares, showed an income of
almost three times that of patients, represented by circles, with the median household income of R3450.00 and R12850.00 for the cases and controls respectively.

![Income Comparison Chart](chart.png)

**FIGURE 1. INCOME COMPARISON**

The majority (n=16) of patients earned under R5000.00. Education levels appear different, but did not achieve statistical significance, with mean schooling of 9.96 years for patients and 14.85 years for controls (p=0.374). Comparison of dwelling showed 79.166% (19) patients and 83.333% (20) controls lived in brick houses, 2 (8.333%) patients versus one (4.166%) control in corrugated iron homes, 1 (4.1666%) patient vs. 2 (8.333%) controls in wood houses, 2 (8.333%) patients lived in informal shelters and one (4.166%) control in a concrete wall building.
COMPARISON OF FOOD INTAKE AND HABITS:

Patients appeared to have a deficient diet, compared to controls, eating larger amounts of starch (potato) and chocolate. They also however appeared to eat more fruits and cheese. All these differences achieved statistical significance.

Comparison in selenium rich foods namely nuts, liver and kidney, did not show any difference between the groups after analysis. Statistically significant differences, highlighted in Table 3, included consumption of cheese (4.85 [sd.9.3] vs. 0.44 [sd.0.64] P=0.02), fruit (4.88 sd.[6.9] vs. 1.46 [1.06] P=0.025), chocolate (1.9 [sd.2.65] vs. 0.54 [sd.0.72] P=0.016) and potato (4.58 [sd.4.9] vs. 0.98[sd.0.93] P=0.001). The average number of alcohol units drunk per day, per group, which we assumed was consumed out of pregnancy, was 0.65 (range:0-6 units/day) in the RPL group and 0.38(range:0-6 units/day) in the controls (P=0.957). Intake of other food and drink was comparable between the groups. Neither alcohol consumption nor cigarette smoking achieved statistical significance as shown in table 3.
TABLE 3. FOOD, DRINK AND HABITS.

<table>
<thead>
<tr>
<th></th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol (units/day)</td>
<td>0.65(1.7)</td>
<td>0.38(1.24)</td>
<td>0.957</td>
</tr>
<tr>
<td>Bread (slices/day)</td>
<td>3.22(1.98)</td>
<td>3.04(1.98)</td>
<td>0.757</td>
</tr>
<tr>
<td>Cereals (bowls/day)</td>
<td>0.58(0.71)</td>
<td>0.63(0.49)</td>
<td>0.528</td>
</tr>
<tr>
<td>Ceylon tea (cups/day)</td>
<td>1(2.1)</td>
<td>1.04(1.3)</td>
<td>0.921</td>
</tr>
<tr>
<td>Cheese (helping/week)</td>
<td>4.85(9.3)</td>
<td>0.44(0.64)</td>
<td>0.026</td>
</tr>
<tr>
<td>Chocolate (bars/week)</td>
<td>1.9(2.65)</td>
<td>0.54(0.72)</td>
<td>0.016</td>
</tr>
<tr>
<td>Coffee (cups / day)</td>
<td>1.79 (2.4)</td>
<td>2(1.5)</td>
<td>0.73</td>
</tr>
<tr>
<td>Cream (helpings/week)</td>
<td>0.08 (0.28)</td>
<td>0.04(0.20)</td>
<td>0.575</td>
</tr>
<tr>
<td>Fruit (pieces/week)</td>
<td>4.88(6.9)</td>
<td>1.46(1.06)</td>
<td>0.025</td>
</tr>
<tr>
<td>Liver/kidney (helping/week)</td>
<td>0.56(1.4)</td>
<td>0.08(0.24)</td>
<td>0.129</td>
</tr>
<tr>
<td>Nuts (grams/day)</td>
<td>10.4(29.3)</td>
<td>7.92(17.4)</td>
<td>0.728</td>
</tr>
<tr>
<td>Peas/beans(helpings/day)</td>
<td>0.91(1.9)</td>
<td>0.71(0.53)</td>
<td>0.627</td>
</tr>
<tr>
<td>Potato (helping/week)</td>
<td>4.58(4.9)</td>
<td>0.98(0.93)</td>
<td>0.001</td>
</tr>
<tr>
<td>Rooibos tea (cups/day)</td>
<td>0.71(1.04)</td>
<td>0.42(0.97)</td>
<td>0.317</td>
</tr>
<tr>
<td>Smoking (cigarettes/day)</td>
<td>5.17(7.33)</td>
<td>2.58(4.78)</td>
<td>0.550</td>
</tr>
</tbody>
</table>

*Values are given as mean (SD).*

In Table 4, the proportion of weekly fish, red meat and chicken consumption between the two groups are compared. The data were originally divided into four categories: daily, more than once a week, less than once a week and never. These have subsequently been converted into binary variables: more than once a week and less than
once a week, to allow for easier statistical analysis. As shown, the fish intakes in both the RPL and control groups fall into the second category of less than one helping a week. The intake in the two groups was comparable and no statistical differences were identified (p=1.0).

**TABLE 4. INTAKE OF FISH, RED MEAT AND CHICKEN.**

<table>
<thead>
<tr>
<th></th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RED MEAT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than once a week</td>
<td>16(66.7)</td>
<td>14(58.3)</td>
<td>0.80</td>
</tr>
<tr>
<td>Less than once a week</td>
<td>8(33.3)</td>
<td>10(41.7)</td>
<td></td>
</tr>
<tr>
<td><strong>CHICKEN</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than once a week</td>
<td>22(91.7)</td>
<td>18(75.0)</td>
<td>0.29</td>
</tr>
<tr>
<td>Less than once a week</td>
<td>2(8.3)</td>
<td>6(25.0)</td>
<td></td>
</tr>
<tr>
<td><strong>FISH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>More than once a week</td>
<td>8(33.3)</td>
<td>9(37.5)</td>
<td>1.0</td>
</tr>
<tr>
<td>Less than once a week</td>
<td>16(66.7)</td>
<td>15(62.5)</td>
<td></td>
</tr>
</tbody>
</table>

Values are given as n (%).

Red meat was eaten more than once a week by 16(66.7%) women in the RPL group and 14(58.3%) women in the control group (P=0.80). The weekly intake of chicken was high in both groups but slightly higher in the RPL group (91.7%) versus (75%) in the controls though this value did not reach statistical significance (P=0.29).
HAIR TREATMENTS AND PRODUCTS:

All women were asked about the use of hair products because of their selenium content, and they were requested to specify the types of hair treatments they had used over the preceding six months. The questionnaire focused on specific treatments such as: 1 = Permanent wave; 2 = Straightening; 3 = Bleaching; 4 = Lice treatment; 5 = None. Women who had other treatments specified these in this question and only one was mentioned by one control subject who had her hair braided.

The RPL and control groups were comparable with no statistical difference. Figure 2 shows an average of 66.7% of controls had no hair treatments, and included in this group was one woman who braided her hair. In the RPL group, 54.2% had no treatments. 33.3% and 20.8% of women in the controls and RPL groups respectively had their hair straightened. The remaining 12.5% in both groups had bleached their hair.

**FIGURE 2.**
The use of hair products is summarized in table 5. All women were asked about the use of anti-dandruff shampoo and hair colouring agents. In the RPL group 62.5% used hair colouring agents, including **one patient who had used henna within the preceding 6 months**, compared with 45.8% in the control group (P=0.424). More women in the control group (41.7%) used anti-dandruff shampoo compared to RPL group (25%)[P=0.344]. A review of shampoos used by our subjects revealed that none contained selenium.

**Table 5:** Hair treatment and anti-dandruff use.

<table>
<thead>
<tr>
<th>Hair products</th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colouring</td>
<td>15 (62.5)</td>
<td>11 (45.8)</td>
<td>0.424</td>
</tr>
<tr>
<td>Anti dandruff shampoo</td>
<td>6 (25.0)</td>
<td>10 (41.7)</td>
<td>0.344</td>
</tr>
</tbody>
</table>

*Value given as n (%).*

**MEDICAL HISTORY:**

The medical history of participants in the two groups is shown below in Table 6. There were more women 8(33.3%) with medical conditions in the RPL group. Within this group, 1 had hypertension and asthma, 1 only asthma, 1 only hypertension, 1 reported sinusitis, 2 suffered from non insulin dependent diabetes mellitus, 1 reported being HIV positive and one was treated for cervicitis.

In the control group only 2 women (8.3%) suffered from medical conditions, one with hypertension on treatment and one with cardiac disease who was not on treatment. Three women in each group used
laxatives (p=1.0) and the hypertensive patients in both groups used only diuretics p=1.0.

**TABLE 6: Medical and drug history.**

<table>
<thead>
<tr>
<th>Medical history</th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medical condition present</td>
<td>8 (33.3)</td>
<td>2 (8.3)</td>
<td>0.109</td>
</tr>
<tr>
<td>On treatment</td>
<td>2 (8.3)</td>
<td>1 (4.2)</td>
<td>0.70</td>
</tr>
<tr>
<td>Laxative usage</td>
<td>3 (12.5)</td>
<td>3 (12.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>Diuretic usage</td>
<td>2 (8.3)</td>
<td>1 (4.2)</td>
<td>1.00</td>
</tr>
</tbody>
</table>

*Value given as n (%).*

**PAST HISTORY OF CONTRACEPTIVE USE:**

Past contraceptive history is summarized in table 7 indicating previous use of combined oral contraceptives (COC), depot progestogens and the copper intrauterine device. None of the women recruited for this study had used hormonal products within the preceding six months.

**TABLE 7: Previous contraceptive use.**

<table>
<thead>
<tr>
<th>Past Contraceptive use</th>
<th>Recurrent miscarriage</th>
<th>Control</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Past oral contraceptive pill usage</td>
<td>9 (37.5)</td>
<td>11 (45.8)</td>
<td>0.774</td>
</tr>
<tr>
<td>Past depot progestogens</td>
<td>13 (54.2)</td>
<td>9 (37.5)</td>
<td>0.344</td>
</tr>
<tr>
<td>Copper IUCD</td>
<td>0 (0)</td>
<td>7 (29.2)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*Value given as n (%).*

The two groups were similar in the past use of COC and depot progestogens, with p=0.774 and p=0.344 respectively. In contrast more of the controls used the copper IUCD (p=0.001).
Selenium levels in hair.

Table 8 represents our findings of selenium levels in hair. The mean selenium content was 1.86ppm (parts per million) and 0.96ppm in cases and controls respectively. (p= 0.74) There was no significant difference between the two groups.

**TABLE 8:**

<table>
<thead>
<tr>
<th></th>
<th>Selenium(ppm) Mean (SD)</th>
<th>Selenium(ppm) Median (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL</td>
<td>1.86 (4.06)</td>
<td>0.80 (4.06)</td>
</tr>
<tr>
<td>CONTROL</td>
<td>0.96 (0.75)</td>
<td>0.68 (0.75)</td>
</tr>
</tbody>
</table>

\[ p=0.74 \]

**COMPARISON OF HAIR SELENIUM**

**FIGURE 3:**
Figure 3 demonstrates the mean selenium levels in each group. Many patients had mean concentrations within the normal reference range while controls often fell well below normal reference levels. A comparison of the median levels shows a smaller difference between the two groups with 0.80 ppm for patients and 0.68 for controls. A table including each subject’s levels is included in annexure 2. One patient (RPL) had used henna products in her hair which is rich in selenium. Her selenium hair level was out of the range of all the other samples. She was excluded from the analysis of hair selenium levels.

Table 9: Comparison without the outlier:

<table>
<thead>
<tr>
<th></th>
<th>Selenium (ppm)</th>
<th>Median</th>
<th>Range</th>
<th>Reference Range (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Selenium Mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPL</td>
<td>1.052 (0.95)</td>
<td>0.799</td>
<td>0.19-4.15</td>
<td>1.41-1.85</td>
</tr>
<tr>
<td>CONTROLS</td>
<td>0.912 (0.72)</td>
<td>0.68</td>
<td>0.43-3.76</td>
<td>1.41-1.85</td>
</tr>
</tbody>
</table>

$p=0.67$

FIGURE 4: Selenium medians and interquartile range
Table 10: Interquartile range without the outlier.

Figure 3 demonstrates that there is an outlier with a very high selenium concentration of 20.4ppm. Figure 4 and table 10 illustrate the interquartile ranges. A review of the outlier's questionnaire revealed that she was the only subject who had used henna in the preceding six months. The patient fell into the low income category and dietary history did not show any difference in any food consumption. Having realized this, the single spurious reading was removed and the data re-analysed. The results, depicted in table 9, do not alter the analysis and the difference between the controls and patient groups remains non-significant. (p=0.67)
DISCUSSION:

The aetiology of RPL is multi-factorial and may include nutritional deficiencies. Selenium deficiency has been previously implicated in RPL.\(^{74,78}\) We attempted to ascertain if selenium may be deficient in our population with the hope that, if identified, replacement therapy would prove helpful.

Osrin et al. showed that antenatal micronutrient supplementation compared to only iron and folate supplement in Nepalese women was associated with increased birthweight. They included 65ug selenium in their cocktail of supplements in addition to: vitamin A, E, D, B1, B2, niacin, B6, B12, folic acid, vitamin C, iron, Zinc, copper and iodine. The mean birthweight was 64g greater in the supplement group than among controls.\(^{79}\) A similar study by Christian et al, with almost identical interventions, in the south of Nepal found no difference in outcome but their supplements did not include selenium.\(^{80}\) It is theoretically possible that selenium supplementation improved outcome in the first group. Healthcare in South Africa like Nepal is economically under-resourced, and malnutrition and/or undernutrition are among the major health challenges. Nutritional deficiency is potentially reversible and therefore more research is needed in this area.

Hair samples were chosen for analysis to give an indication of long term selenium concentrations, as this offers a good indicator of prolonged exposure. Forensic science has utilised hair for analysis of chronic drug exposure, pesticide exposure and post-mortem toxicology for decades and therefore the methods utilized are validated and reproducible.\(^{81-84}\) Hair was used by Al-Kunani et al. in a similar study on selenium levels in RPL in the UK. We were fortunate to be able to collaborate with the Department of Obstetrics and Gynaecology and the
Department of Chemistry, Hull University, where this methodology was validated and where our samples were analysed.\(^{(78)}\)

Concentrations of micronutrients and trace elements including selenium have been successfully analysed from hair samples.\(^{(88-91)}\)

These studies confirm the value of hair analysis in studies such as our assessment.

Selenium and other trace element concentrations may be affected by hair products such as anti-dandruff shampoo which may contain either selenium sulphide or pyrithione zinc as the active ingredient. We reviewed the constituents of all the hair products as reported by the manufacturers and used by the women recruited to our study and neither had these additives included. Our sample review showed, to our knowledge, that only two local commercial shampoos contain these ingredients, namely Head and Shoulders Intensive and Selsun Blue, and neither was used by the controls or the subjects.

Those who were invited to participate had lived in Cape Town for at least two years, suggesting that, between the two groups, exposure to environmental factors, water and food intake could be matched. Patients attending the Reproductive Failure Clinic (the study group) are often referred to GSH from surrounding public health centres, or from private referrals. The women recruited as controls were from a more diverse background and included hospital staff, many whom may access private hospitals.

The groups differed in the family incomes, and comparison revealed that RPL patients earned almost four times less the median income of the control subjects (R3450 vs. R12850.00 respectively). Analysis of the controls shows that not everyone had a high income as 9(37.5\%) of the control subjects earned less than R10 000.00pm and 4(16.66\%) earned less than R5000.00pm. Education also reflects this difference with mean schooling of 9.96 years(RPL) and 14.85 years (controls) respectively. This reflects that the control group were better educated
and had access to a higher income which may have impacted on nutritional status. Controls were matched with cases for ethnicity and age but were recruited from among patients and staff and proved to be on average better educated and with higher incomes.

The difference in socioeconomic factors may have influenced the study outcome. Although not intended, the difference between the groups may mean that controls had more access to dietary information, enabling them to eat healthier. They may also have had numerous lifestyle advantages which possibly impacted on pregnancy outcome in ways that were not defined in this study.

The level of selenium in South African foods has not been analysed but a U.K. study suggested that selenium containing foods include bread, cereals, fish, poultry and meat, while the highest content is found in liver, kidney and nuts.\textsuperscript{(92)} Comparison between our two groups, showed no difference in their intake, which does not suggest any particular dietary improvement in the controls in terms their economic and educational advantage.

We matched for ethnicity on the premise that different ethnic groups may prefer different foods, for example, in our study 70.8\% of women in each group were of coloured origin, however less than 33.3\% of women ate fish more than once a week.

Estrogens may have an effect on selenium concentrations, as evidenced by studies done by Massafra and co-workers in Siena, Italy, who demonstrated a positive correlation between mean estradiol and glutathione peroxidase activity,\textsuperscript{(93)} and in earlier work by the same author showed increased anti-oxidant activity in women using combined oral contraceptives.\textsuperscript{(94)} For this reason we excluded women with recent pregnancies or hormonal contraceptive use. None of the women recruited to our study had utilized hormonal contraception in the preceding 6 months.
Selenium available in the diet is subject to the availability in the soil. Van Ryssen reviewed the literature on the selenium status of grazing herbivores in different South African regions and suggested that selenium is deficient in many regions. The Western Cape, more specifically, was sited to have less than adequate concentrations of selenium. However it appears from this study that RPL subjects were getting adequate selenium in their diet.

Razagui and Haswell, established a reference range for hair selenium in the Department of Chemistry, Hull University, of 1.41-1.85 ug/gm (identical to parts per million[ppm]). To our knowledge, these are the only reference values available using hair as the primary sample. However, it must be stated that, the values were obtained from healthy women who had given birth within the preceding 48 hours. It is not ideal to use this as a reference range in our study group, as our subjects were not postpartum, and postpartum selenium concentrations are lower than those in non-pregnant women. If these reference ranges are applied, and one assumes that non pregnant women should have higher levels, the results from our study actually show that the mean selenium concentration among RPL patients (1.86ppm), and controls fell below this range. Bearing in mind that these reference values are taken from postpartum women, our population group does have low selenium concentrations.

The median selenium concentrations are probably a better indicator of selenium in both groups because of the wide range of results found in the patient group (0.19-20.42). If the median selenium levels are analysed, the concentrations are very similar, with patient concentrations of 0.80ppm and controls of 0.68ppm. These levels show that both groups fell below the normal range of 1.41-1.84ppm, suggesting that our population is indeed selenium deficient.

Among the cases, only one patient has an isolated elevated selenium concentration of 20.4ppm. Her history showed that she falls within the
lower income group with a household income of R3000.00 per month. Comparison of dietary history with other patients reveals that there is no difference in her eating pattern or in the intake of foodstuffs, specifically selenium rich foods. Her history however reveals having used henna on her hair over the prior 6 months. Henna (Lawsonia inermis) produces a protein binding dye that contains selenium.\(^{(96)}\) This outlier was subsequently removed and the data reanalysed, but the difference between the two groups remained statistically non-significant (\(p=0.67\))

Work undertaken, by Adams et al, in one of the largest agricultural research centres in the U.K, the Rothamsted Research Centre showed that the selenium intake through wheat in the U.K. was low. A total of 180 different types of wheat from the national grain surveys showed that, based on wheat selenium concentrations, the daily selenium intake was 6.4ug, which is 10 times lower than the recommended daily intake of 55ug.\(^{(97)}\) If the normal reference ranges of 1.41-1.85ppm are applied to the Hull study, it appears that the selenium concentration, in all their subjects (0.14ug/g in patients and 0.34ug/g in controls), is low. The selenium concentrations in our South African population were much higher than the U.K. population but still below recommended levels.

It appears as though there was no difference in the diets between the U.K. and the South African groups but perhaps some may exist, for example the mean intake of liver and kidney, foods with very high selenium concentrations, was higher for our patients versus controls (\(p=0.129\)) for cases and controls respectively. These findings are different to those found by Al-Kunani and co-workers where more women in the control group ate these selenium rich foods (\(p<0.05\)). It is possible that in our study population, liver and kidney were eaten as an alternative to red meat as the latter is much more expensive. As the numbers recruited were small, deductions from the food questionnaire
probably do not reflect the populations that the groups were meant to represent.

The results illustrate that patients ate significantly more potato, cheese, chocolate and fruit (Table 3). These foodstuffs are relatively low in selenium but may still influence hair selenium concentrations. Fruit and potatoes are grown in South Africa, where a kilogram of potatoes can be bought for R10.00 and most fruits can be bought for +/-R1.50, making them cheap and accessible. South African towns have numerous hawkers who sell fruit, sweets and chocolates at the roadside targeting people in our patient population, compared to the control group who are more likely to purchase a snack/meal at the hospital cafeteria.

Our study showed that patients ate more potato than the control group and this finding is most likely of little relevance. A fungus, Phytophthora infestans, was identified as the cause for potato blight in the Irish Famine of 1844 and although there is no evidence in the literature that there is a direct link to miscarriage however the possibility that this fungus may cause miscarriage should be investigated.

The association between listeriosis and pregnancy loss was explored by Jamshidi et al. at the Department of Obstetrics and Gynaecology, Hormozgan University of Medical Sciences, Iran. They recruited 250 women with spontaneous miscarriage and compared them to 200 women who had healthy pregnancies. All women were screened for serum Lysteria Monocytogenes antibody. The authors reported a higher incidence of listeriosis in the study group, with 89 (35.6%) of patients versus 35 (17.5%) of the control group testing positive, for L. monocytogenes antibody ($p = 0.001$).\(^{98}\)

The consumption of cheese in our RPL group was significantly higher than the controls. Unpasteurised dairy products and certain soft cheeses can be a source of listeria monocytogenes and women should
avoid them in pregnancy. We are not certain whether the cheese consumption occurred during pregnancy although this is a possibility.

Our study may be underpowered as only 24 cases and controls were recruited and this may account for the results. A power study was not done prior to the study which was based on numbers used in a similar study from the University of Hull where 26 cases and 18 controls were recruited.\textsuperscript{78} A counter-argument may be entertained suggesting that, in our study, power does not explain the lack of statistical significance and perhaps a much larger sample would be needed to detect small differences between the two groups given that the point estimates of the median selenium levels between the two groups are very similar.
CONCLUSION:

Undernutrition and malnutrition are recognised as having a potential impact on reproductive performance. The women who attend our Reproductive Failure Clinic often do not have an identified cause for their pregnancy loss, which makes future management and counselling difficult. Nutritional deficiencies have been identified as causes of failure of both fertility and fecundity. The animal work makes a compelling case for a role for selenium in successful reproduction and recent studies in women with RPL have suggested that selenium deficiency may contribute to RPL. Our study was undertaken in an attempt to identify the selenium status in women with both successful reproductive histories and those with RPL of unknown cause. Using hair as the tissue for study offers an opportunity to identify chronic/long term selenium status.

The 2 groups studied had different dietary habits, but not with regard to selenium rich foods, and different financial resources. No difference between the groups was identified in selenium status and both groups had evidence of low levels. Very few women had adequate levels of selenium. Obviously in the controls, the levels may have been different during their successful pregnancies but there is no way of ascertaining this and it seems likely that their diet was fairly constant over time.

The patients consumed significantly more potatoes and there is the theoretical possibility that infection with potato blight can contribute to pregnancy loss. This is not an association which has been considered or explored in our clinical practice in the past. Women are usually advised to avoid unpasteurised dairy products in pregnancy because of the risk
of Listeriosis which is a risk factors for miscarriage. Possibly the increased cheese ingestion in the patient group may warrant further investigation.

While we recognize the selenium deficiency present in our whole study population, we cannot comment on whether this played a role in the RPL. Replacement of selenium may contribute to a successful pregnancy outcome in the future. We lack data in South Africa on the selenium content of foodstuffs and have had to use UK standards for this study. We do recognize that selenium deficiency in soil and therefore in agricultural products has been documented in South Africa.

Micronutrient deficiency has been documented in a number of reproductive disorders and in a population with absolute and relative nutritional compromise, we must remain aware of the possible impact. This initial study presents the need for pursuing further research in this field and investigating the potential for nutritional supplementation.
REFERENCES:


(32) Hanson U, Persson B, Thunell S. Relationship between haemoglobin A1C in early type 1 (insulin-dependent) diabetic pregnancy and the occurrence of


(64) Moxon AL, Rhian MA. Selenium poisoning. Physiol Rev 1943;23:305-307.


Annexure 1: ETHICS APPROVAL:
Dear Prof van der Spuy,

PROJECT TITLE: MATERNAL AND FETAL SCALP HAIR MINERAL LEVELS IN HEALTHY AND COMPLICATED PREGNANCIES

Thank you for your letter to the Research Ethics Committee dated 13th March 2007.

I have pleasure in informing you that the Ethics Committee has granted approval to recruit a further 25 women into the above mentioned study.

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 Harmonisation 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 E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Parts 50, 56 and 312.

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

Please quote the REC. REF in all your correspondence.

Yours sincerely,

[signature]

PROF M BLOCHMAN
CHAIRPERSON, UCT HUMAN ETHICS
Dear Professor Blockman

REC REF: 041/2006

PROJECT TITLE: MATERNAL AND FETAL SCALP HAIR MINERAL LEVELS IN HEALTHY AND COMPPLICATED PREGNANCIES

Last year we received permission to extend our studies to include an assessment of lead levels in the hair of mothers and their newborn babies.

This work, as with all our other studies on micronutrients measured in hair, is done in collaboration with Dr Stephen Lindow of the University of Hull. The assessment of lead in hair was done in collaboration with several countries in Africa which use leaded petrol, the UK where petrol is unleaded and India. South Africa was included because we were going through a transition changing from leaded to unleaded petrol.

Subsequently we have suggested that we should repeat the study a year after recruiting the first 25 subjects. Women who deliver now have conceived and gone through their entire pregnancy exposed only to unleaded petrol. We are the only country that can offer this transition and it certainly may provide important information about the impact or otherwise of leaded petrol on mothers and their babies.

I should appreciate it, if you would give us permission to recruit a further 25 women from our maternity services, under exactly the same conditions as before to add a comparative group to our studies.

Yours sincerely

ZEPHNE M VAN DER SPUY

"OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
24 October 2006

REC REF: 041/2006

Prof ZM Van der Spuy
Dept of Obstetrics and Gynaecology
Medical School

Dear Prof Van der Spuy

PROJECT TITLE: SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSIS.

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

I have pleasure in informing you that the Ethics Committee has approved the above mentioned study as an amendment.

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

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 Harmonisation 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 E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

Please quote the REC. REF in all your correspondence.

Yours sincerely,

PROF. M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS
26 September 2006

Dr Marc Blockman
The Chairman
Research Ethics Committee
Faculty of Health Sciences
University of Cape Town

Email: Mblockman@uctgsh1.uct.ac.za

Dear Dr Blockman

REC REF: 041/2006
PROJECT TITLE: SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSES.

We originally obtained consent to proceed with this study in 1994 and this consent has recently been renewed with some amendments to the original submission. Our original application dealt with a series of pregnancy complications and the examination of nutritional factors in reproductive health.

We think it is essential that this study should be extended into the area of male infertility, also a major factor in Reproductive Health Care. I attach an amendment for this study which will deal with the investigation of men with infertility.

I should appreciate it if it could be reviewed by your Committee and approved as an amendment. If, however, you wish me to submit a completely new application, I will, of course, do so. All the laboratory methodologies remain the same as does the general study design which involves a questionnaire, obtaining a sample of hair and, in some cases, a sample of semen.

Thank you very much for your assistance

Yours sincerely

ZEPHNE M VAN DER SPUY

"OUR MISSION is to be an outstanding teaching and research university educating for life and addressing the challenges facing our society."
SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSES

[REC REF: 041/2006]

INTRODUCTION
Male factor infertility is a major contributing factor to couple infertility. It is estimated that abnormal semen parameters may contribute to failure to conceive in about 30-40% of couples who present with infertility. In about 20% of these couples a male factor maybe the only cause of infertility.

It is recognised that numerous nutritional factors may impact on spermatogenesis and therefore on fertility potential. These have, however, been inadequately investigated and very few forms of therapy are available for men which could potentially modify spermatogenesis or improve semen quality.

A number of factors have been associated with male infertility and these include deficiencies in zinc and selenium. If nutritional factors were implicated in semen abnormalities, then once these have been identified, intervention studies with appropriate replacement should offer a therapeutic possibility in this group of patients. At present, often the only feasible therapy is Assisted Reproductive Technology which is not accessible to many of our patients.

We have had a long-standing collaboration with Dr Stephen Lindow at the University of Hull and have already collaborated on a number of studies investigating nutritional factors in women with complicated pregnancies. Dr Lindow has done several additional studies including investigating the impact of amalgam use in dentistry on mercury levels in women and their infants and a comparison of selenium levels in women with miscarriage compared with those with healthy pregnancy outcomes. We are presently collaborating with him on a study of lead levels in patients exposed to leaded and unleaded petrol and nutritional factors in women with recurrent miscarriage compared to those with healthy pregnancies. It is proposed this study would be a continuation of our collaboration.

Aims of the proposed study:

It is proposed this study will carry out preliminary investigations aimed at ascertaining whether scalp hair mineral concentrations in men with an abnormal semen analyses vary from those in healthy men with normal semen parameters. Scalp hair is recognised as valuable biopsy material in the field of forensic toxicology. During its growth cycle the matrix cell and papilla of the hair follicle
undergoes intense metabolic activity, producing on average 0.2 to 0.5 mm of hair per day. This developing hair is exposed to the metabolic environment and from this accumulates a variety of compounds. As the hair matures, it's keratinous outer layer effectively encapsulates within it compounds, including mineral elements, of nutritional significance.

Methods:

Scalp hair samples will be obtained from men who present to our Reproductive Medicine Unit with couple infertility. Men who have abnormal semen analyses will be recruited. Hair will be obtained from the back of their scalp, as described in the original Ethics Committee Submission. A questionnaire will be completed with the assistance of one of the investigators in our Unit.

Chemical analysis of the hair will be carried out in the laboratory facilities available at the University of Hull, as described in the original submission.

All men, with the appropriate clinical presentation, will be approached and formal consent will be obtained from them for participation in this study. We hope that this study will offer us possible explanation for at least some of the abnormalities responsible for male infertility.

A control group will be recruited from among men who are either healthy semen donors in our service or who have a normal semen analysis and have recently fathered a child. These men may be recruited from among the fathers of the women who deliver within our service and would have to consent to a single semen analysis. It is proposed to match the controls and subjects according to ethnic group and age.

Because this is a pilot study, we hope in the first instance to study 50 affected and 50 control subjects. The results of the study will then inform the design of any future research which is done in this field.
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Because this is a pilot study, we hope in the first instance to study 50 affected and 50 control subjects. The results of the study will then inform the design of any future research which is done in this field.
REFERENCES


CONSENT FORM - CLINIC PATIENTS

SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSIS

[REC REF: 041/2006]

Over the past few years we have conducted a number of studies reviewing the nutritional status of women with pregnancy complications. Hair provides a good record of nutrition over a fairly long period of time and we have asked mothers to allow us to take a sample of the hair for analysis of nutritional factors. This hair has been analysed in a special laboratory at the University of Hull, UK and has given us the information on the nutrition of women with both healthy and complicated pregnancies. All this work has been done in collaboration with Dr Stephen Lindow of the University of Hull.

There is now a large body of evidence suggesting that nutritional factors may well impact on semen production and sperm quality in men and may affect fertility. We are hoping to assess the nutritional status of men who present to our clinic complaining of infertility and who are found to have an abnormal semen analysis.

We wish to invite you to participate in this study because you have a normal semen analysis and have fathered a healthy child. You are obviously under no obligation to participate in this study and declining to do so will not affect your management within our service.

Advantages of participation:

There are no personal advantages. You will be contributing to a body of information which may aid couples with infertility in the future but will not help you personally.

Disadvantages of participation:

We will have a small sample of hair cut from your hairline at the back of your scalp and a single blood sample taken. This should not have any negative effect.

Ethics approval:

This study has been approved by the Research Ethics Committee of the University of Cape Town.

"OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
CONSENT FORM – CLINIC PATIENTS

I .................................................................................................................. consent to participating in the study which has been described to me in a language of my choice.

I understand this study will involve the following:

1. Removal of a sample of scalp hair from the back of my scalp (about 500 to 1000mg)
2. Completion of a short questionnaire.
3. Obtaining a single sample of blood.

I am aware that the results of these investigations will not be available for a considerable length of time and will not necessarily be made known to me.

No costs will be incurred by me by participation in this study.

I am satisfied that the investigator has explained the study to me fully and allowed me an opportunity to ask questions.

Signed: ...........................................................

Name: ...........................................................

Hospital No.(if relevant) ...........................................................

Investigator ...........................................................

Date: ...........................................................
CONSENT FORM - CONTROL SUBJECTS

SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSIS

[REC REF: 041/2006]

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There is now a large body of evidence suggesting that nutritional factors may well impact on semen production and sperm quality in men and may affect fertility. We are hoping to assess the nutritional status of men who present to our clinic complaining of infertility and who are found to have an abnormal semen analysis.

We wish to invite you to participate in this study. This will involve completing a questionnaire – with the help of one of the research team – and allowing us to cut a sample of scalp hair from your hairline and to take a single sample of blood. In addition we request a semen sample from you for analysis.

You are under no obligation to participate in this study and declining to do so will not affect your management within our service.

Advantages of participation:
There may be no personal advantages. You will be contributing to a body of evidence which may influence management of men with infertility in the future and, in this way may personally advantage you although this is not certain and is, of course, dependent on the outcome of the study.

Disadvantages of participation:
We will have a small sample of hair cut from the hairline at the back of your scalp, a single blood sample taken and a semen analysis performed. This should not have any negative effect.

Ethics approval:
This study has been approved by the Research Ethics Committee of the University of Cape Town.

"OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
CONSENT FORM - CONTROL SUBJECTS

I ........................................................................................................ consent to participating in the study which has been described to me in a language of my choice.

I understand this study will involve the following:

1. Removal of a sample of scalp hair from the back of my scalp (about 500 to 1000mg)

2. Completion of a short questionnaire.

3. Obtaining a single sample of blood.

4. Obtaining a semen sample.

I am aware that the results of these investigations will not be available for a considerable length of time and will not necessarily be made known to me.

No costs will be incurred by me by participation in this study.

I am satisfied that the investigator has explained the study to me fully and allowed me an opportunity to ask questions.

Signed: ...........................................................................

Name: ..............................................................................

Hospital No. (if relevant) .......................................................

Investigator ...........................................................................

Date: .......................................................................................
CONSENT FORM - CLINIC PATIENTS

SCALP HAIR MINERAL LEVELS IN MEN PRESENTING WITH ABNORMAL SEMEN ANALYSIS

[REC REF: 041/2006]

Over the past few years we have conducted a number of studies reviewing the nutritional status of women with pregnancy complications. Hair provides a good record of nutrition over a fairly long period of time and we have asked mothers to allow us to take a sample of the hair for analysis of nutritional factors. This hair has been analysed in a special laboratory at the University of Hull, UK and has given us the information on the nutrition of women with both healthy and complicated pregnancies. All this work has been done in collaboration with Dr Stephen Lindow of the University of Hull.

There is now a large body of evidence suggesting that nutritional factors may well impact on semen production and sperm quality in men and, may affect fertility. We are hoping to assess the nutritional status of men who present to our clinic complaining of infertility and who are found to have an abnormal semen analysis.

We wish to invite you to participate in this study because you have a normal semen analysis and have fathered a healthy child. You are obviously under no obligation to participate in this study and declining to do so will not affect your management within our service.

Advantages of participation:

There are no personal advantages. You will be contributing to a body of information which may aid couples with infertility in the future but will not help you personally.

Disadvantages of participation:

We will have a small sample of hair cut from your hairline at the back of your scalp and a single blood sample taken. This should not have any negative effect.

Ethics approval:

This study has been approved by the Research Ethics Committee of the University of Cape Town.
CONSENT FORM - CLINIC PATIENTS

I ........................................................................ consent to participating in the study which has been described to me in a language of my choice.

I understand this study will involve the following:

1. Removal of a sample of scalp hair from the back of my scalp (about 500 to 1000mg)

2. Completion of a short questionnaire.

3. Obtaining a single sample of blood.

I am aware that the results of these investigations will not be available for a considerable length of time and will not necessarily be made known to me.

No costs will be incurred by me by participation in this study.

I am satisfied that the investigator has explained the study to me fully and allowed me an opportunity to ask questions.

Signed: ............................................................

Name: ............................................................

Hospital No.(if relevant) ............................................................

Investigator ............................................................

Date: ............................................................

“OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society.”
14 September 2006

REC REF: 041/2006

Prof Z Van Der Spuy
Obstets & Gynae

Dear Prof Van Der Spuy

PROJECT TITLE: MATERNAL AND FETAL SCALP HAIR MINERAL LEVELS IN ABRUPTIO PLACENTAE AND OTHER COMPLICATIONS

Thank you for your letter to the Research Ethics Committee dated 18 August 2006.

It is a pleasure to inform you that the Ethics Committee has approved permission to recruit a control group of subjects into the above-mentioned study.

The consent form for control subjects-recurrent miscarriage study and consent form for patients-recurrent miscarriage study are approved.

Please add the REC details to the informed consent document.

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 Harmonisation 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 E6: Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) and FDA Code Federal Regulation Part 50, 56 and 312.

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

Please quote the REC. REF in all your correspondence.
Yours sincerely

[Signature]

DR. M. BLOCKMAN
CHAIRPERSON, HSF HUMAN ETHICS

kmjedi
18 August 2006

Dr Marc Blockman
The Chairman
Research Ethics Committee
Faculty of Health Sciences
University of Cape Town

Email: Mblockman@uctgh1.uct.ac.za

Dear Dr Blockman

Rec Ref: 041/2006
Project Title: Maternal and Fetal Scalp Hair Mineral Levels in Abruptio Placentae and other complications.

Our initial application to the Ethics Committee included four groups of patients who were to be studied – women and their infants with abruptio placentae, patients with miscarriage, women and their infants with preterm labour and with term uncomplicated pregnancies.

We are now proceeding with the study reviewing hair mineral levels in women who have had recurrent miscarriages. I enclose, for inclusion in the file on this study, a copy of the informed consent form. I should also appreciate it if we could get permission to recruit a control group of subjects from among healthy women who have only had uncomplicated pregnancies and are either attending routine Family Planning Clinics or Gynaecology Clinics for screening (e.g. pap smear).

We continue to work in collaboration with Dr Stephen Lindow, our colleague at the University of Hull and he has already done preliminary work in patients with miscarriage which was published in 2001 (British Journal of Obstetrics and Gynaecology 107:1094-1097).

This study will include obtaining hair samples from patients and controls and a serum sample from each subject for measurement of mineral levels including selenium, zinc and magnesium. Deficiencies of these minerals have all been associated with reproductive failure.

I should appreciate it if this letter could be acknowledged and approval given for us to include control subjects in our study. We hope to recruit at least 50 control subjects and 50 women with recurrent miscarriage for our studies.

Yours sincerely

ZEPHNE M VAN DER SPUY

"Our mission is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
MATERNAL AND FETAL SCALP HAIR MINERAL LEVELS IN HEALTHY AND COMPLICATED PREGNANCIES

CONSENT FORM – CONTROL SUBJECTS – RECURRENT MISCARRIAGE STUDY

REC REF: 041/2006

Over the past few years we have conducted a number of studies, in collaboration with our colleagues at the University of Hull in the UK, reviewing the nutritional status of women with pregnancy complications. Hair provides a good record of nutrition over a very long period of time and we have asked mothers to allow us to take a sample of their hair for analysis of nutritional factors. This has been analysed in a special laboratory at the University of Hull and has given us information on the nutrition of women with both healthy and complicated pregnancies.

All this work has been done in collaboration with Dr Stephen Lindow of the University of Hull. It is hoped that ultimately this information will allow us to identify nutritional needs in women and possibly design appropriate interventions which will avoid some pregnancy complications.

In this current study we are investigating the nutritional status of women who have had recurrent miscarriages and comparing them to women who have only had a healthy outcome to pregnancy. In preliminary studies, Dr Lindow identified some nutritional deficiencies in women in the UK and there is always a possibility that correcting these deficiencies may lead to an improved pregnancy outcome.

We wish to invite you to participate in this study. This will involve completing a questionnaire – with the aid of one of the research team – and allowing us to cut a sample of scalp hair from the back of your head, at the hairline and take a blood sample for analysis from you.

You have always had a healthy pregnancy outcome and for this reason we would hope to compare your nutritional levels with those of women in whom the pregnancy outcome has not been successful. It is hoped that this will give us information on how to improve health during the pregnancy and perhaps prevent miscarriage.
You will be a volunteer for this study and are under no obligation to participate. Declining to do so will not affect your management within our service.

Advantages of participation:

There are no personal advantages. You will be contributing to a body of evidence which may influence the management of women in the future. This research is at a very early stage and will not benefit you personally. You have always had healthy pregnancies and you will be contributing to knowledge in the field of pregnancy management without any personal benefits.

Disadvantages of participation:

A small sample of hair will be cut from the back of your scalp and your hairline and you will have a single sample of blood taken. The only discomfort will be that of venepuncture.

Ethics approval:

This study has been approved by the Research Ethics Committee of both the University of Cape Town and the University of Hull.
I .............................................................. consent to participating in the study which has been described to me in a language of my choice.

I understand this study will involve the following:

4. Removal of a sample of scalp hair from the back of my scalp.

5. Completion of a short questionnaire.

6. Obtaining a single sample of blood.

I understand the purpose of this study is to investigate possible nutritional factors which may impact on pregnancy outcome.

I am aware that the results of these investigations will not be available for a considerable length of time and will not necessarily be made known to me.

No costs will be incurred by me by participation in this study.

I am satisfied that the investigator has explained the study to me fully and allowed me an opportunity to ask questions.

Signed:  ........................................................................................................

Name:  ........................................................................................................

Hospital No.(if relevant)  ..............................................................................

Investigator  ..................................................................................................

Date:  .........................................................................................................

"OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
MATERNAL AND FETAL SCALP HAIR MINERAL LEVELS IN HEALTHY AND COMPLICATED PREGNANCIES

CONSENT FORM – PATIENTS – RECURRENT MISCARRIAGE STUDY

REC REF: 041/2006

Over the past few years we have conducted a number of studies, in collaboration with our colleagues at the University of Hull in the UK, reviewing the nutritional status of women with pregnancy complications. Hair provides a good record of nutrition over a very long period of time and we have asked mothers to allow us to take a sample of their hair for analysis of nutritional factors. This has been analysed in a special laboratory at the University of Hull and has given us information on the nutrition of women with both healthy and complicated pregnancies.

All this work has been done in collaboration with Dr Stephen Lindow of the University of Hull. It is hoped that ultimately this information will allow us to identify nutritional needs in women and possibly design appropriate interventions which will avoid some pregnancy complications.

In this current study we are investigating the nutritional status of women who have had recurrent miscarriages and comparing them to women who have only had a healthy outcome to pregnancy. In preliminary studies, Dr Lindow identified some nutritional deficiencies in women in the UK and there is always a possibility that correcting these deficiencies may lead to an improved pregnancy outcome.

We wish to invite you to participate in this study. This will involve completing a questionnaire – with the aid of one of the research team – and allowing us to cut a sample of scalp hair from the back of your head, at the hairline and to take a single sample of blood for analysis from you.

You have already had several miscarriages and are presently attending our Reproductive Failure Clinic and for this reason we invite you to participate in this study. You are under no obligation to participate and declining to do so will not affect the management within our service.

"OUR MISSION is to be an outstanding teaching and research university, educating for life and addressing the challenges facing our society."
Advantages of participation:

There are no personal advantages. You will be contributing to a body of evidence which may influence the management of women in the future. If you have had recurrent miscarriages then there is a possibility we may identify a nutritional factor which possibly could be corrected. This research is, however, at a very early stage and may well not benefit you personally.

Disadvantages of participation:

A small sample of hair will be cut from the back of your scalp at your hairline and you will have a single sample of blood taken. The only discomfort will be that of venepuncture.

Ethics approval:

This study has been approved by the Research Ethics Committee of both the University of Cape Town and the University of Hull.
I ............................................................................................................ consent to participating in the study which has been described to me in a language of my choice.

I understand this study will involve the following:

1. Removal of a sample of hair from the back of my scalp.
2. Completion of a short questionnaire.
3. Obtaining a single sample of blood.

I understand the purpose of this study is to investigate possible nutritional factors which may impact on pregnancy outcome.

I am aware that the results of these investigations will not be available for a considerable length of time and will not necessarily be made known to me.

No costs will be incurred by me by participation in this study.

I am satisfied that the investigator has explained the study to me fully and allowed me an opportunity to ask questions.

Signed: ............................................................................................................

Name: .............................................................................................................

Hospital No.(if relevant) ....................................................................................

Investigator ....................................................................................................

Date: .............................................................................................................

"OUR MISSION is to be an outstanding teaching and research university,
educating for life and addressing the challenges facing our society."
Annexure 2:

<table>
<thead>
<tr>
<th>RPL</th>
<th>CONTROLS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.807</td>
<td>0.543</td>
</tr>
<tr>
<td>0.465</td>
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<tr>
<td>0.815</td>
<td>0.615</td>
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<tr>
<td>2.191</td>
<td>1.016</td>
</tr>
<tr>
<td>0.525</td>
<td>0.767</td>
</tr>
<tr>
<td>1.678</td>
<td>2.054</td>
</tr>
<tr>
<td>4.150</td>
<td>0.831</td>
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<td>1.219</td>
<td>3.764</td>
</tr>
<tr>
<td>0.441</td>
<td>0.812</td>
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<tr>
<td>3.248</td>
<td>0.433</td>
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<tr>
<td>0.862</td>
<td>0.609</td>
</tr>
<tr>
<td>20.419</td>
<td>2.042</td>
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<tr>
<td>0.885</td>
<td>0.637</td>
</tr>
<tr>
<td>0.643</td>
<td>0.637</td>
</tr>
<tr>
<td>0.302</td>
<td>0.545</td>
</tr>
<tr>
<td>0.799</td>
<td>0.774</td>
</tr>
<tr>
<td>0.550</td>
<td>0.466</td>
</tr>
<tr>
<td>0.831</td>
<td>1.557</td>
</tr>
<tr>
<td>0.382</td>
<td>0.594</td>
</tr>
<tr>
<td>0.189</td>
<td>0.552</td>
</tr>
<tr>
<td>0.527</td>
<td>1.103</td>
</tr>
<tr>
<td>0.635</td>
<td>0.738</td>
</tr>
<tr>
<td>1.256</td>
<td>0.535</td>
</tr>
<tr>
<td>0.800</td>
<td>0.512</td>
</tr>
</tbody>
</table>

Selenium concentrations in subjects recruited.
Annexure 3: QUESTIONNAIRE
REC REF: 041/2006

STUDY NUMBER:

PATIENT/CONTROL

DATE OF INTERVIEW:

RECURRENT MISCARRIAGE STUDY:
MICRONUTRIENTS IN SCALP HAIR AND SERUM

NAME:

HOSPITAL NUMBER:

DATE OF BIRTH:
PATIENT GROUP:

INCLUSION CRITERIA:

1. Three consecutive miscarriages (gestational age <24 weeks).
2. No live births after the miscarriages.
3. Investigations for recurrent pregnancy loss normal, i.e.
   - Chromosomal studies in both parents;
   - Anatomical study (hysteroscopy or hystero-salpingogram);
   - Immunological tests (anti-phospholipid syndrome);
   - Thrombophilia screen (protein C & S deficiency);
   - Endocrine (PCOS).

EXCLUSION CRITERIA:
1. Currently pregnant.
2. Cause for recurrent pregnancy loss identified.

CONTROL GROUP:

INCLUSION CRITERIA:

1. No previous miscarriage.
2. At least one healthy live birth.
3. At least 6 months post-partum.

EXCLUSION CRITERIA:
1. Currently pregnant.
2. Currently lactating.
3. Use of hormonal contraception or hormonal replacement therapy in the last 6 months.

TO DO:
1. Take a sample of scalp hair from the back of the participant’s scalp (about 500 – 1000mg).
2. Take 5ml venous blood from the participant.
3. Complete confidential questionnaire with participant.
RECURRENT MISCARRIAGE STUDY QUESTIONNAIRE (CONFIDENTIAL):

1. Age (yrs):

2. Weight (kg):

3. Height (cm):

4. BMI:

5. No. of pregnancies (gravidity):

6. No. of miscarriage/s:
   (pregnancy loss <20 weeks)

7. No. of still births:

8. No. of ectopic pregnancies:

9. No. of terminations of pregnancy:

10. What is your town and country of birth? _____________________

11. For how many months/years have you been living in Cape Town?

   11.1 If you have lived in Cape Town for less than 6 months, where were you previously living? ______________________________

   11.2 How long had you been residing there for? ________________

12. What is your marital status?
   1 = single  2 = married  3 = divorced  4 = widow
   5 = co-habiting

13. Population group
   1 = Black African  2 = Coloured  3 = Indian  4 = White
   5 = other

14. Highest level of education attained (including tertiary level): __________________________

   14.1 Number of years of formal education

15. What is your occupation? __________________________

16. What is the occupation of your spouse/partner? __________________________

17. What is your household income (gross per month)? __________________________

18. How many adults live in your household? __________________________
19. 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 ___________________________ (please specify)

20. What type of roofing do you have?

1 = asbestos  
2 = thatched  
3 = corrugated iron  
4 = concrete  
5 = tile  
6 = other ___________________________ (please specify)

21. Is your area of residence neighbouring or in the vicinity of a zone that is:

1 = agricultural  
2 = industrial ___________________________ (please specify)  
3 = other ___________________________ (please specify)

22. How many months/years have you lived in the area?

23. Are you suffering from a recognised medical condition at the moment? (e.g. hypertension, diabetes, etc)

1 = yes  
2 = no

23.1 If yes, please specify condition/s and duration of illness

24. Have you ever in the past suffered from a recognised medical condition?

1 = yes  
2 = no

24.1 If yes, please specify condition/s and duration of illness

25. Are you taking any medication for the above condition/s?

1 = yes  
2 = no

25.1 If yes, please specify

  g. type
  h. dose/day
  i. duration of use
26. Do you regularly use diuretics?

1 = yes 2 = no

26.1 If yes, please specify

   a. type
   b. dose/day
   c. duration of use

27. Do you regularly use laxatives?

1 = yes 2 = no

27.1 If yes, please specify

   a. type
   b. dose/day
   c. duration of use

28. Are you using/have you in the past used the oral contraceptive pill?

1 = yes 2 = no

28.1 If yes, please specify

   a. type
   b. dose/day
   c. duration of use

29. Are you using/have you in the past used depot hormonal contraception?

1 = yes 2 = no

29.1 If yes, please specify

   a. type
   b. duration of use

30. Do you have/have you previously used intrauterine contraceptive device?

1 = yes 2 = no

30.1 If yes, please specify

   a. type
   b. duration of use
31. Do you regularly use nutritional supplements?

1 = yes  2 = no

31.1 If yes, please specify

a. type
b. duration of use

32. Are you on a special diet?

1 = yes  2 = no

32.1 If yes, please specify

a. Sliming
b. High fibre
c. Other, please specify
d. How long have you been on it?

33. Have you lost weight in the past 6 months?

1 = yes  2 = no

33.1 If yes, how many kilograms

34. Have you gained weight in the past 6 months?

1 = yes  2 = no

34.1 If yes, how many kilograms

35. Do you have an eating disorder?

1 = yes  2 = no

35.1 If yes, please specify

36. How often do you eat fish in an average week over the last 6 months?

1 = daily  2 = >once a week  3 = <once a week  4 = never

37. How often do you eat red meat in an average week over the last 6 months?

1 = daily  2 = >once a week  3 = <once a week  4 = never

38. How often do you eat chicken in an average week over the last 6 months?

1 = daily  2 = >once a week  3 = <once a week  4 = never
39. What is your daily consumption of:

<table>
<thead>
<tr>
<th>Item</th>
<th>Consumption Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>39.1 coffee (cups/d)</td>
<td></td>
</tr>
<tr>
<td>39.2 ceylon tea (cups/d)</td>
<td></td>
</tr>
<tr>
<td>39.3 rooibos tea (cups/d)</td>
<td></td>
</tr>
<tr>
<td>39.4 bread (slices/d)</td>
<td></td>
</tr>
<tr>
<td>39.5 cereals (bowls/d)</td>
<td></td>
</tr>
<tr>
<td>39.6 cheese (helpings/d)</td>
<td></td>
</tr>
<tr>
<td>39.7 nuts (grams/d)</td>
<td></td>
</tr>
<tr>
<td>39.8 fruit (pieces/d)</td>
<td></td>
</tr>
<tr>
<td>39.9 chocolate (bars/d)</td>
<td></td>
</tr>
<tr>
<td>39.10 cream (helpings/d)</td>
<td></td>
</tr>
<tr>
<td>39.11 liver or kidney (helpings/d)</td>
<td></td>
</tr>
<tr>
<td>39.12 potato (helpings/d)</td>
<td></td>
</tr>
<tr>
<td>39.13 peas or beans (helpings/d)</td>
<td></td>
</tr>
<tr>
<td>39.14 fruit (helpings/d)</td>
<td></td>
</tr>
</tbody>
</table>

40. What type of food do you particularly dislike? Please specify

41. What is your consumption of:

<table>
<thead>
<tr>
<th>Item</th>
<th>Consumption Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>41.1 alcohol (units/wk)</td>
<td></td>
</tr>
<tr>
<td>(1 unit = 1/2 pint beer, 1 glass of wine)</td>
<td></td>
</tr>
<tr>
<td>41.2 cigarettes (#/day)</td>
<td></td>
</tr>
<tr>
<td>41.3 tobacco (grams/day)</td>
<td></td>
</tr>
<tr>
<td>41.4 snuff</td>
<td></td>
</tr>
</tbody>
</table>

42. How do you cook your food?

<table>
<thead>
<tr>
<th>Method</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>open wood fire</td>
<td>1</td>
</tr>
<tr>
<td>gas/electric cooker</td>
<td>2</td>
</tr>
<tr>
<td>open coal fire</td>
<td>3</td>
</tr>
<tr>
<td>paraffin stove</td>
<td>4</td>
</tr>
<tr>
<td>other</td>
<td>5</td>
</tr>
</tbody>
</table>
43. What type of cooking vessels do you use?
1 = aluminium 2 = steel 3 = iron 4 = pottery

44. What type of water supply does your household have?
1 = stand pipe 2 = domestic supply 3 = well

45. What is your natural hair colour?
1 = blonde 2 = brunette 3 = black 4 = red

46. What brand of hair shampoo do you use? ______________________

47. Do you use an anti-dandruff treatment?
1 = Yes 2 = No

48. Do you use a hair colouring agent?
1 = Yes 2 = No

49. Please specify any hair sprays, setting lotions, or dressings that you routinely use on your hair:
__________________________________________

50. Have you had any of the following hair treatment/s in past 6 months?
1 = permanent wave 2 = straightening 3 = bleaching
4 = head lice treatment 5 = other ___________________(please specify)