Supplementation with antioxidant vitamins C and E for the prevention of pre-eclampsia: a randomised controlled trial.

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LIST OF ABBREVIATIONS

ASSHP	Australasian Society for the Study of Hypertension in Pregnancy
CI	confidence intervals
DBP	diastolic blood pressure
FFQ	food frequency questionnaire
g	grams
HDL	high density lipoprotein
HELLP	haemolysis, elevated liver enzymes and low platelets
ISSHP	International Society for the Study of Hypertension in Pregnancy
IU	International Units
IUGR	intrauterine growth restriction
LDL	low density lipoprotein
MDA	malondialdehyde
mg	milligrams
mls	millilitres
mmHg	millimetres of mercury
NHBPEP	National High Blood Pressure Education Program
NHMRC	National Health and Medical Research Council
NNTB	numbers needed to treat to benefit
NNTH	numbers needed to treat to harm
OR	odds ratio
oxLDL	oxidised low density lipoprotein
PE	pre-eclampsia
PROM	prelabour rupture of membranes
PUFA	polyunsaturated fatty acid
RCT	randomised controlled trial
RDI	recommended dietary intake
ROS	reactive oxygen species
RR	relative risk
SBP	systolic blood pressure
SD	standard deviation
SGA	small for gestational age infant
SOD	superoxide dismutase
TBARS	thiobarbituric acid reacting substances
vs.	versus
WMD	weighted mean difference

ABSTRACT

Background: Oxidative stress has been implicated in the pathogenesis of pre-eclampsia. Supplementing women with the antioxidant vitamins C and E during pregnancy may help counteract oxidative stress and thereby reduce the risk of pre-eclampsia and its related complications.

Methods: 1,538 nulliparous women between 14^{+0} and 21^{+6} weeks' gestation were randomised to either supplementation with vitamin C and E (n=770) or placebo (n=768). Primary outcome measures were the risk of having a small for gestational age infant (birth weight < 10^{th} centile), pre-eclampsia and death or serious adverse outcome for the infant.

Results: Women in each treatment group were comparable for all important baseline maternal characteristics. No difference was seen between treatment groups for the risk of the infant being born small for gestational age (vitamin group 66 [8.6%] vs. placebo 71 [9.4%], Relative Risk (RR) 0.92, 95% Confidence Intervals (CI) 0.67 to 1.27, p=0.614) or the risk of pre-eclampsia (vitamin group 60 [7.8%] vs. placebo group 51 [6.6%], RR 1.17, 95% CI 0.82 to 1.68, p=0.383). Supplementation with vitamin C and E was associated with a 30 percent reduction in the relative risk of death or serious adverse outcome for the infant (vitamin group 54 [7.0%] vs. placebo group 77 [10.0%], RR 0.70, upper 95% CI <0.93, p=0.021, one-tailed test). This was associated with an absolute risk reduction of three percent, whereby 33 women would need to take vitamin C and E supplements in pregnancy in order for one infant to benefit. Mean head circumference at birth was greater for infants in the vitamin C and E group (vitamin group 34.6 cm [SD 1.7] vs. placebo group 34.4 cm [SD 1.9], mean difference 0.18 cm, 95% CI 0.00 to 0.36, p=0.047), and these infants also had a reduced risk of developing respiratory distress syndrome (vitamin group 1 [0.1%] vs. placebo group 9 [1.2%], RR 0.11, 95% CI 0.01 to 0.87, p=0.011).

Conclusion: Vitamin C and E supplementation cannot be recommended as a prophylaxis for pre-eclampsia for nulliparous women. The impact of supplementation on the risk of death or adverse health outcomes for the infant, including respiratory morbidity, requires further confirmation.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

ALICE RUMBOLD

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AUTHOR'S CONTRIBUTION

My involvement with the trial included the development and piloting of all of the questionnaires, and data forms, assisting with protocol revisions and Ethics applications, and all negotiations and subsequent correspondence with Herron Pharmaceuticals, Cognis and CSR regarding production of the trial vitamin and placebo tablets. I oversaw the tablet packing and drug changeover of expired tablets, whilst remaining blinded to treatment allocations.

The trial involved nine collaborating centres around Australia, four of which were in South Australia. A research assistant was employed at each site to undertake recruitment at that site and pregnancy outcome data collection. I was responsible for the training of research assistants at the South Australian and interstate sites and for the development of an information video for potential trial participants. A research coordinator was employed at the Women's and Children's Hospital to assist with the trial coordination, recruitment and administration. I had overall control of the day to day running of the trial throughout. Working along side the research coordinator, I assisted with recruitment of women at the Women's and Children's Hospital, as well as Modbury Public Hospital and The Queen Elizabeth Hospital, follow up of women at antenatal appointments, completion of antenatal and postnatal questionnaires over the phone, development of newsletters for women and staff, correspondence with research assistants undertaking the trial at interstate sites, and checking of questionnaires for data queries and query resolution.

I completed the abstraction of pregnancy outcome data from medical records for women recruited from the Women's and Children's Hospital, Modbury Public Hospital and The Queen Elizabeth Hospital, and checked all other data forms incoming from other sites for data queries and aided in query resolution. Where women delivered at a different site to their site of recruitment, I was responsible for obtaining access to their pregnancy outcomes and completion of data forms.

Under the supervision of Kristyn Willson, I undertook the statistical analyses.

1. LITERATURE REVIEW

1.1 Introduction

Pre-eclampsia is a major cause of maternal and infant morbidity and mortality worldwide. As the only definitive treatment is delivery of the infant, preventing pre-eclampsia continues to be a major focus of perinatal research. To date, no single intervention has emerged as an effective strategy for preventing pre-eclampsia. This may reflect the lack of understanding of the aetiology and pathogenesis of pre-eclampsia, which is yet to be characterised. Current theory implicates inadequate implantation, poor placental perfusion, immune maladaption, endothelial dysfunction and inflammation as underlying mechanisms contributing to the development of this syndrome. Oxidative stress coupled with overwhelmed antioxidant defence mechanisms has been proposed as a link between these events. Interventions targeting the oxidative stress pathway, such as dietary antioxidant vitamin supplementation, represent a 'promising' prophylaxis against pre-eclampsia and its related morbidities. The efficacy and safety of antioxidant vitamin use in pregnancy must be demonstrated before any treatment recommendations can be made.

1.2 Definition of pre-eclampsia

The literature surrounding classification and diagnosis of hypertensive disorders of pregnancy continues to reflect a lack of consensus, furthering controversies in counselling, management and documentation of immediate and remote outcomes (Brown et al 2001). Pre-eclampsia is a multi-system disorder, defined by the presence of hypertension and proteinuria arising in pregnancy. Pre-eclampsia falls into a spectrum of hypertensive diseases occurring in pregnancy, ranging from hypertension that antedates pregnancy (on which pre-eclampsia may be superimposed) to pregnancy specific hypertension alone or arising with multiple organ involvement (pre-eclampsia). The involvement of multiple maternal organs permits a diagnosis of the now recognised 'syndrome' of pre-eclampsia.

Four distinct categories have been proposed for classifying hypertensive disorders of pregnancy and these include gestational hypertension (de novo hypertension after 20 weeks' with no proteinuria), pre-eclampsia (de novo hypertension after 20 weeks' with proteinuria and/or other systemic changes), chronic hypertension (pre-existing hypertension or hypertension arising in early pregnancy) and pre-eclampsia superimposed on chronic hypertension (women with chronic hypertension and symptoms of pre-eclampsia) (Brown et al 2000; Report of the NHBPEP 2000). Controversy continues to surround diagnostic criteria for pre-eclampsia, particularly whether a broad range of criteria should be adopted to reflect the clinical presentation of pre-eclampsia or whether more restrictive criteria should be applied in keeping with previous definitions and knowledge about an already well defined group of women (Brown et al 2001).

The Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) defines pre-eclampsia as hypertension with the occurrence of a range of related maternal and fetal complications (Brown et al 2000). The ASSHP guidelines define hypertension in pregnancy as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg (confirmed by repeated readings four or more hours apart) and pre-eclampsia is diagnosed when hypertension arises after 20 weeks' gestation, in the presence of one or more of the following:

- Proteinuria (≥ 300 mg/24 hours or spot urine protein/creatinine ratio ≥ 30 mg/mmol)
- Renal insufficiency (serum/plasma creatinine ≥ 0.09 mmol/L or oliguria)
- Liver disease (raised serum transaminases and/or severe epigastric/right upper quadrant pain)

- Neurological problems (convulsions (eclampsia); hyperreflexia with clonus; severe headaches with hyperreflexia; persistent visual disturbances (scotomata))
- Haematological disturbances (thrombocytopenia; disseminated intravascular coagulation; haemolysis)
- Fetal growth restriction (birth weight less than the 10^{th} centile).

The final ASSHP requirement for a diagnosis is that the hypertension of pre-eclampsia has returned to normal within three months' postpartum.

In the United States of America, the National High Blood Pressure Education Program (NHBPEP) defines pre-eclampsia as de novo hypertension (systolic blood pressure 140 mmHg and/or diastolic blood pressure 90 mmHg) arising after 20 weeks' gestation plus proteinuria (urinary excretion of 0.3 g protein or higher in a 24 hour urine specimen) (Report of the NHBPEP 2000). While this definition is based only on two factors (hypertension and proteinuria), the authors suggest that in the absence of proteinuria, pre-eclampsia is highly suspected when there are concomitant renal, liver and neurological symptoms. Although the ASSHP and NHBPEP guidelines differ in their clinical definition of pre-eclampsia, both guidelines reflect the recognition of pre-eclampsia as a 'syndrome', with systemic involvement of the liver, kidneys, brain and the blood clotting system. The International Society for the Study of Hypertension in Pregnancy (ISSHP) has recommended the adoption of both of the classification schemas proposed by ASSHP and NHBPEP, noting their similar criteria for each category of hypertensive disease *except* pre-eclampsia (Brown et al 2001).

The notion of pre-eclampsia as a syndrome rather than a single disease state implies a heterogeneous disease that is consequently much harder to characterise and thus reliably define. This has implications for the reported incidence of pre-eclampsia and documentation of maternal and infant outcomes related to pre-eclampsia and highlights the importance for researchers to clearly state the diagnostic criteria used.

1.3 Outcomes for women with pre-eclampsia and their infants

Pre-eclampsia is a leading cause of maternal and infant morbidity and mortality worldwide. Women who develop pre-eclampsia are at increased risk of placental abruption, acute renal failure, pulmonary oedema, aspiration pneumonia, cerebrovascular haemorrhage, retinal detachment, liver rupture and death (Hallak 1999). While the majority of maternal deaths occur in developing countries (WHO 2004), pre-eclampsia continues to be a leading cause of pregnancy-related death in industrialised countries such as Australia (Slayter, Sullivan and King 2004), the United Kingdom (de Swiet 2000) and the United States (Berg et al 1996). In later life, women who had pre-eclampsia experience an increased risk of death from cardiovascular disease (Irgens et al 2001).

For the infant, intrauterine growth restriction (IUGR), preterm birth and perinatal mortality are all increased in the presence of pre-eclampsia. For women with an IUGR infant, pre-eclampsia is implicated in 12 percent of all preterm cases (Kramer 2000). IUGR carries significant health risks for the infant in the short and long term; it remains a primary cause of perinatal mortality where it is associated with 9.7 percent of all perinatal deaths (Department of Human Services 2003). In addition, maternal hypertensive disease alone is associated with just under five percent of perinatal deaths (Department of Human Services 2003). Infants born small for gestational age and surviving are at on-going risk of compromised health including poor childhood growth and development, delayed neurodevelopment and academic underachievement (McCowan et al 1999; Strauss 2000). Furthermore, as the only definitive treatment for pre-eclampsia is delivery, pre-eclampsia is a known antecedent in up to 19 percent of preterm births (Kramer 2000). Preterm birth is a leading cause of neonatal and infant mortality, primarily from respiratory distress syndrome (AIHW National Perinatal Statistics Unit 2004). Long term health problems for preterm survivors include chronic lung disease and lasting neurological disability (Donoghue and Cust 2000).

1.4 Risk factors and screening for pre-eclampsia

To date, there are no screening tests available for use in routine clinical practice that reliably predict pre-eclampsia. The incidence of pre-eclampsia has been reported at between two and 10 percent of all pregnancies and the disease will progress to eclampsia in one in 2,000 pregnant women (Wallenburg 2001). In general, hypertensive disorders in pregnancy including pre-existing hypertension, hypertension arising in pregnancy and superimposed pre-eclampsia complicate up to 10 percent of all births (Jacobs et al 2003). Pre-eclampsia is predominantly a disease of first pregnancies, where the incidence of severe pre-eclampsia has been reported as 15 times greater compared with the second pregnancy (MacGillivray 1958). Furthermore, there is a greatly reduced risk of pre-eclampsia in a second pregnancy if the first pregnancy was normotensive (Campbell, MacGillivray and Carr-Hill 1985). Coupled with primiparity, an increased risk of pre-eclampsia has been demonstrated when there is a change in paternity (Trupin, Simon and Eskenazi 1996). This however, has been challenged, and an increased interval between pregnancies has also been proposed (Trogstad et al 2001).

Several other factors have been associated with an increased risk of pre-eclampsia. These include a family history of pre-eclampsia, multiple pregnancy, obesity, renal disease, essential hypertension, diabetes, autoimmune disease (especially systemic lupus erythematosus and antiphospholipid syndrome), thrombophilia and severe alloimmunisation (Brown et al 2000). Similarly, the extremes of maternal age, infertility treatment, stress, urinary tract infection in pregnancy, structural congenital anomalies, and pregnancy conditions associated with large amounts of trophoblast such as hydrops fetalis, chromosomal abnormalities (trisomy 13, triploidy) and hydatiform mole, have also been associated with an increased risk (Dekker and Sibai 1998). The recognition and documentation of these risk factors has aided in investigating aetiologic and pathological mechanisms involved in the development of pre-eclampsia, however no particular risk factor has emerged as the primary target for prevention studies.

In addition to identifying risk factors, developing tests predictive of pre-eclampsia continues to be a major research focus. Screening tests have obvious clinical benefits, particularly in identifying and targeting women for closer surveillance and early management of any symptoms that may arise. To date, no universal test has emerged as an effective screening tool for use in routine clinical practice. Conde-Agudelo, Villar and Lindheimer (2004) have systematically reviewed cohort and cross-sectional studies assessing predictive tests for pre-eclampsia. Their review assessed 87 studies reporting on screening tests for high and low risk

populations including: uterine Doppler ultrasonography; 24-hour ambulatory blood pressure monitoring; placental and fetal peptides; renal dysfunction tests and markers of endothelial dysfunction and oxidative stress. The review demonstrated that for women at low risk of developing pre-eclampsia, the presence of anticardiolipin antibodies, bilateral diastolic notches during Doppler ultrasonography and a low urinary kallikrein to creatinine ratio had moderate predictive accuracy for the development of pre-eclampsia (i.e. pooled positive and negative likelihood ratio's in the order of 5-10 and 0.1-0.2 respectively). However as these screening tests were associated with only small increases in the probability of pre-eclampsia, the authors suggested that their use in clinical practice is limited. In addition, many of the tests had high technical requirements that may not be available in all settings. For women at high risk of pre-eclampsia, Doppler ultrasonography had low predictive accuracy (pooled positive and negative likelihood ratio's <3 and >0.4 respectively). For many of the screening tests evaluated, the small number of studies reporting these assessments precluded the authors from drawing any reliable conclusions about the tests.

The use of screening tests for pre-eclampsia continues to be confined to research purposes, often in highly specific populations. Moreover, the limited use and development of screening tests for routine clinical practice illustrates the current gaps in understanding about the causes of pre-eclampsia. Further research investigating the pathophysiology of the disease is clearly needed.

1.5 Aetiology and pathogenesis of pre-eclampsia

Pre-eclampsia is often referred to as the 'disease of theories', highlighting the complex aetiology of pre-eclampsia which is yet to be completely understood. Current theory on the pathogenesis of pre-eclampsia has focused on inappropriate placental implantation and development leading to reduced placental perfusion, oxidative stress, abnormal inflammatory responses and resulting endothelial dysfunction.

In healthy pregnancies, implantation is characterised by vast expansion of the spiral arteries, where invading cytotrophoblast replaces the endothelium for the length of the spiral arteries and into the inner third of the myometrium, creating elastic, low resistance vessels un-reactive to vasoactive stimuli (Kliman 2000). In women with pre-eclampsia, this vessel expansion and trophoblast invasion is abnormally reduced (Brosens, Robertson and Dixon 1972). There is limited trophoblast invasion of the decidual and to a greater extent myometrial segments (Meekins et al 1994). As a result the vessels remain small, lined with endothelium and contain smooth muscle responsive to vasoactive factors. Furthermore, the failure to convert spiral arterioles into low resistance vessels results in reduced placental perfusion. This poor placental perfusion is then speculated to mediate the release of factors that enter the maternal circulation, thus linking placental abnormalities with the systemic disease (Roberts and Redman 1993).

The impaired fetal placental vascularisation seen in women with pre-eclampsia has also been demonstrated in women giving birth to small for gestational age infants (Khong et al 1986) and in one third of women giving birth preterm (Alias et al 1993). These placental abnormalities occur early in pregnancy, however the clinical signs of pre-eclampsia do not usually manifest until much later in pregnancy. This suggests that while placental defects play a key role in the development of pre-eclampsia, other factors must be involved in the maternal systemic disease. Endothelial dysfunction secondary to impaired fetal placental vascularisation is proposed as the 'second stage' of the disease resulting in the clinical syndrome (Roberts and Hubel 1999).

Endothelial cells line the vasculature of the body and are involved in the regulation of vascular tone, platelet activation and aggregation, leukocyte adhesion and underlying smooth muscle cells. When endothelial cells become activated or injured, their normal functions can be impaired and replaced by the production of procoagulants, vasoconstrictors and mitogens (Roberts and Redman 1993). Evidence of impaired endothelial cell integrity and resulting

increased vascular permeability, was initially demonstrated in pre-eclampsia by the finding that Evans blue, a protein-bound dye, exited the vascular compartment at an increased rate in pre-eclamptic women when compared with healthy pregnant women (Campbell and Campbell, 1983). Further evidence of endothelial dysfunction has been characterised in a variety of studies in women with established pre-eclampsia, as evidenced by: elevated plasma concentrations of fibronectin (Stubbs, Lazarchick and Horger 1984); clotting factors such as von Willenbrand factor; tissue plasminogen activator and plasminogen activator inhibitor (Halligen et al 1994; Friedman et al 1995), all of which are produced upon endothelial activation or injury. Similarly, increased concentrations of endothelin, a vasoconstrictive peptide produced primarily by endothelial cells (Taylor et al 1990); vascular endothelial growth factor, an endothelial cell-specific mitogen (Hayman et al 1999) and leucocyte adhesion molecules such as vascular cell adhesion molecule, an indicator of leucocyte-endothelial cell interactions (Higgins et al 1998) have also been demonstrated in women with pre-eclampsia.

Activated and injured endothelial cells promote a pro-thrombotic state characterised by increased platelet aggregation and leucocyte endothelial cell adhesion and decreased fibrinolysis. Furthermore, women with pre-eclampsia fail to have the usual blunted response to all pressors occurring in normal pregnancy, and their sensitivity to pressors may in fact be increased. Impaired endothelium-dependent vasodilation has been demonstrated in the resistance arteries of women with pre-eclampsia when elicited by acetylcholine (McCarthy et al 1993). An increase in vascular endothelial permeability and endothelial cell injury and dysfunction promotes coagulation, vasoconstriction and hypertension. In pre-eclampsia this may occur initially in the uteroplacental bed and then subsequently in the maternal circulation.

Poor placental perfusion and endothelial dysfunction are proposed as key factors in the development of pre-eclampsia. While there is considerable evidence supporting these two findings, little is known about the mechanisms linking these stages together. One mechanism currently proposed is oxidative stress.

1.6 Oxidative stress

Oxidative stress refers to an imbalance in the generation of reactive oxygen species (oxidants or pro-oxidants) and the availability of antioxidants, where production of pro-oxidants is favoured. Reactive oxygen species (ROS) are either free radicals (molecules with at least one unpaired electron in their outer shell) or intermediates that are non-radical species but continue to be biologically active and have the ability to oxidise molecules (Diplock et al 1998). ROS associated damage occurs when free radicals remove electrons from surrounding molecules (i.e. DNA, proteins and lipids) in order to achieve stability in their outer shells (Halliwell 1996). ROS implicated in damage to the vascular system include superoxide (O_2^-), nitric oxide (NO^-), hydrogen peroxide (H_2O_2), peroxynitrite ($OONO^-$), lipid hydroperoxides (ROOH), the peroxyl radical (ROO^-) and hydroxyl-like radicals (OH) (Kojda and Harrison 1999). Hydrogen peroxide and peroxynitrite are produced from reaction products of the superoxide anion; hence superoxide is also a key source of other ROS (Fridovich 1986a).

ROS are formed endogenously by oxidative reactions. Enzymatic reactions that are likely sources of ROS include: the mitochondrial electron transport chain, xanthine oxidase, cyclooxygenase, lipoxygenase, NO synthase, haeme oxygenases, peroxidases, haemoproteins and NAD(P)H oxidases (Kojda and Harrison 1999). Most simply, a major source of superoxide radicals is the expenditure of atmospheric oxygen, where via the mitochondrial transport chain, it has been estimated that between one and three percent of the oxygen consumed by humans is subsequently involved in superoxide formation (Fridovich 1986b; Halliwell 1996).

ROS can also be generated in the process of lipid peroxidation, that is, peroxidase catalysed oxidation of lipids (lipoproteins, triglycerides and cholesterol), which can be initiated by other ROS in the first instance. For example, peroxyl radicals are generated when hydroxyl radicals remove hydrogen atoms from polyunsaturated fatty acids (PUFA's), which are present in cell membranes and in particular, low density lipoprotein particles (LDL's) (Reaven and Witztum 1996). As a result there is a loss of integrity of the original cell membrane resulting in oxidised LDL (oxLDL), coupled with the formation of other harmful radicals, which creates a vicious cycle of ROS generation and biological damage. ROS are also produced by the immune system, where they have important physiological functions. Phagocytic cells such as monocytes and neutrophils produce large amounts of superoxide or nitric oxide as part of their defence mechanisms and this can indicate intracellular activation. NAD(P)H oxidases

are known sources of superoxide in neutrophils as well as vascular smooth muscle and endothelial cells (Griendling, Sorescu and Ushio-Fukai 2000).

Antioxidants scavenge free radicals circulating in the body and in doing so inhibit peroxidation reactions. Antioxidants are loosely defined as "any substance that, when present in low concentrations compared to that of an oxidisable substrate, significantly delays or inhibits oxidation of that substrate" (Diplock et al 1998). Not surprisingly, arrays of antioxidant defence mechanisms exist. Antioxidants can be termed preventative antioxidants, which includes cellular and extracellular enzymes that inhibit peroxidase reactions, such as glutathione reductase, glutathione-S-transferase, glucose-6-phosphate dehydrogenase, superoxide dismutase (SOD) and catalase (Diplock et al 1998). Antioxidants can also be free-radical scavengers or chain breaking antioxidants, where they trap or decompose radicals or peroxides already present in the body, and these include vitamin C (ascorbic acid or ascorbate), vitamin E (the generic name for any compounds exhibiting the activity of alpha-tocopherol), carotenoids, glutathione, serum albumin and metabolites such as bilirubin and uric acid. While antioxidant enzymes are of prime importance for intracellular defences, non-enzymatic antioxidants are the major defence mechanism in the extracellular compartment. Antioxidants are thus important in maintaining cellular integrity, particularly cell membranes, and protecting enzymes and proteins from destruction by peroxides. In oxidative stress, antioxidant concentrations are overwhelmed from the increased consumption of free radicals.

Excessive formation of ROS may lead to the disruption of key physiological functions in the vascular compartment. In atherosclerosis, oxidative stress leading to the formation of oxLDL in the subendothelial space has been proposed as the key factor resulting in endothelial damage (Witztum 1994). Oxidised LDL and other lipid peroxides are highly reactive and damaging to enzymes, proteins and cell membranes by promoting further peroxidation reactions and disrupting endothelial function. While these findings are demonstrated in atherosclerosis, the hyperlipidemic profile seen in atherosclerosis of decreased high density lipoprotein (HDL) cholesterol, raised serum triglycerides and increased formation of small, dense LDL particles is seen also in women with pre-eclampsia (Hubel 1997a).

In pre-eclampsia, reduced placental perfusion is proposed to interact with maternal genetic, behavioural and/or environmental factors (i.e. diabetes, hypertension, obesity, diet) to generate oxidative stress (Roberts and Hubel 1999). Furthermore, maternal factors such as a dietary deficiency of antioxidants or a predisposition to small dense LDL formation may further predispose women to oxidative stress and therefore pre-eclampsia.

1.6.1 Oxidative stress in pre-eclampsia

Reduced placental and other organ perfusion with subsequent re-oxygenation is proposed as a primary source of ROS and subsequent oxidative stress in pre-eclampsia, primarily through the xanthine oxidase pathway. Xanthine oxidase production of oxygen-derived free radicals has been described in experimentally induced ischemic and reperfusion tissue injury (McCord 1985). In pregnancy, human umbilical endothelial cells cultured under hypoxic-reperfusion conditions show increased xanthine oxidase and dehydrogenase activity (Michiels et al 1992; Zhang et al 1998). Both catalyse ROS generation, and under hypoxic conditions the oxidase form is favoured. Xanthine oxidase reduces oxygen to superoxide, hence increased production of superoxide occurs with subsequent re-oxygenation. ROS resulting from reperfusion injury may be formed initially in the intervillous space, creating a state of placental oxidative stress and localised endothelial cell damage, however increased ROS production coupled with the formation of lipid peroxides and other organ damage may result in a more generalised state of oxidative stress and thus systemic endothelial damage.

Oxidative stress may also contribute to an imbalance between the production of prostacyclin and thromboxane-A_2, resulting in changes to blood flow regulation. A potent vasodilator, prostacyclin inhibits platelet aggregation and uterine contractility, increasing uteroplacental blood flow. In contrast, thromboxane-A_2 is a potent vasoconstrictor that stimulates platelet aggregation and uterine contractility leading to a decrease in uteroplacental blood flow (Walsh 1985). Endothelial cells produce prostacyclin and nitric oxide, which cooperate to inhibit platelet adhesion and aggregation. Prostacyclin, thromboxane-A_2 and other prostaglandins are formed from arachidonic acid via the cyclooxygenase pathway, and lipid peroxides are formed secondary to prostaglandin synthesis (Diplock et al 1998). During pregnancy, low concentrations of lipid peroxides stimulate cyclooxygenase and the formation of prostacyclin. At elevated lipid peroxide concentrations, prostacyclin production is decreased through inhibition of prostacyclin synthase and cyclooxygenase (Wang et al 1991a). Lipid peroxides do not alter thromboxane synthase. As such oxidative stress will favour thromboxane-A_2 production and may lead to a thromboxane-A_2/prostacyclin imbalance, resulting in reduced uteroplacental blood flow and increased platelet activation by dysfunctional endothelial cells (Wang et al 1991a). In pre-eclampsia, a thromboxane-A_2/prostacyclin imbalance may therefore further contribute to the reduced placental perfusion observed.

1.6.2 Circulating markers of oxidative stress

Much research has focussed on assessing markers of oxidative stress, in both women with established pre-eclampsia and to a lesser extent, women destined to develop pre-eclampsia prior to the onset of clinical symptoms. A range of markers of oxidative stress have been consistently assessed in women with pre-eclampsia, occurring initially with the observation of elevated lipid concentrations or hyperlipidemia (Konttinen, Pyoeraelae and Carpen 1964; Nelson, Zuspan and Mulligan 1966; Potter and Nestel 1979). Elevated concentrations of lipid hydroperoxides, thiobarbituric acid reacting substances (TBARS) including malondialdehyde (MDA) a lipid peroxidation metabolite, conjugated dienes (bond migration in the unsaturated fatty acid hydrocarbon chain resulting from lipid peroxidation), uric acid (a product of the xanthine/xanthine oxidase pathway), protein carbonyls (formed from direct damage of proteins) and isoprostanes are all markers of oxidative stress that can be measured in pregnancy. Concentrations of antioxidants can also be used as proxy markers of oxidative stress as they provide indirect support or lack thereof, for increased free radical induced lipid peroxidation and subsequent antioxidant consumption.

Wang and colleagues (1991b) have demonstrated that 'normal' pregnancy is characterised by increased serum lipid peroxidation measured by malondialdehyde concentrations, appearing in the first trimester and remaining stable throughout gestation to term. Parallel increases in vitamin E concentrations are seen in the first trimester and these continue to increase with advancing gestation, suggesting there is a gradual favouring of antioxidant capacity over lipid peroxidation. Similarly, increased plasma concentrations of prostacyclin have been demonstrated with increasing gestational age and a concurrent decrease in thromboxane-A_2 concentrations, both favouring vasorelaxation (Wang et al 1991b). These ratios of antioxidants/lipid peroxidation and prostacyclin/thromboxane-A_2 are positively correlated, leading to the view that in normal pregnancy the vasodilating actions of prostacyclin and the antioxidant capacity of vitamin E are progressively favoured with advancing gestation (Wang et al 1991b). In contrast, both mild and severe pre-eclampsia are characterised by decreased production of prostacyclin, and a resulting imbalance in the prostacyclin/thromboxane-A_2 ratio. Elevated lipid peroxides, as measured by malondialdehyde concentrations have also been demonstrated in women with mild and severe pre-eclampsia, and in severe cases decreased concentrations of vitamin E (Wang et al 1991a). These reports support the suggestion that pre-eclampsia involves imbalances in the prostacyclin/thromboxane-A_2 ratio and antioxidant/lipid peroxidation status, leading to vasoconstriction and endothelial and platelet damage. Progressive favouring of these imbalances has been demonstrated with increasing severity of disease (Wang et al 1991a).

In support of these findings, various reports have suggested that vitamin C is the first antioxidant defence mechanism damaged in pre-eclampsia (Mikhail et al 1994; Hubel et al 1997b). In the first instance water soluble antioxidants such as vitamin C may be consumed in response to free radical induced cell injury, ahead of lipid soluble antioxidants such as vitamin E, which may be decreased only in women with severe disease. Mikhail and colleagues (1994) demonstrated decreased blood ascorbate concentrations in women with mild and severe pre-eclampsia, and decreased alpha-tocopherol concentrations only in women with severe pre-eclampsia. Hubel and colleagues (1997b) found evidence of accelerated oxidation of ascorbate in the whole blood of women with pre-eclampsia relative to normal pregnancy, where ascorbate concentrations were 50 percent lower in women with pre-eclampsia. No changes in alpha-tocopherol concentrations were observed. Furthermore, Morris and colleagues (1998) demonstrated elevated vitamin E concentrations in the maternal blood when comparing pregnant and non-pregnant women, but found similar concentrations in women with pre-eclampsia when compared with normotensive pregnancies.

Madazli and colleagues (1999) report a positive correlation between increased plasma malondialdehyde and decreased vitamin C and E concentrations with increasing increments of diastolic blood pressure, suggesting a positive relationship between the degree of lipid peroxidation and severity of disease. Similarly, Hubel and colleagues (1996) have reported malondialdehyde concentrations fifty percent higher and positively correlated with increases in fasting serum triglycerides and free fatty acids in women with pre-eclampsia when compared with pregnant controls. No differences in HDL and LDL cholesterol concentrations were observed and all lipid concentrations decreased significantly in both groups within 48 hours postpartum, coupled with a decrease in malondialdehyde concentration. These findings support the presence of oxidative stress in conjunction with lipid abnormalities in pre-eclampsia. Elevated fasting serum free fatty acids and triglycerides have been demonstrated before 20 weeks' gestation in women who subsequently developed pre-eclampsia (Lorentzen et al 1994; Enquobahrie et al 2004) suggesting a degree of dyslipidemia even in early pregnancy in women destined to develop pre-eclampsia.

Few studies have assessed markers of oxidative stress prior to the onset of clinical symptoms of pre-eclampsia. Decreased plasma concentrations of vitamin C and increased concentrations of uric acid and isoprostanes have been demonstrated at 20 and 24 weeks' gestation in women who subsequently developed pre-eclampsia (Chappell et al 2002). Similarly, decreased plasma concentrations of vitamin E have also been demonstrated, when assessed in women at 28 weeks' gestation prior to the onset of clinical signs (Jendryczko and Drozdz 1989). While

these initial reports lend support for a pathological role for oxidative stress, further longitudinal studies are required to assess markers in early and mid pregnancy, prior to the onset of disease, in both women who develop pre-eclampsia and those who remain normotensive.

Women from a range of geographic locations and ethnic groups have been assessed for markers of oxidative stress in relation to the onset of pre-eclampsia. Reports to date have demonstrated a positive association between established pre-eclampsia and increased markers of oxidative stress via: (1) elevated plasma concentrations of malondialdehyde or diene conjugation (Maseki et al 1981; Hubel et al 1989; Uotila et al 1993a; Uotila et al 1993b; Kharb et al 1998; Orhan, Ozgunes and Beksac 2001; Ilhan, Ilhan and Simsek 2002) and/or (2) decreased concentrations of water and lipid soluble antioxidants including vitamin C and E (Bowen et al 1998; Sagol et al 1999; Kharb 2000; Panburana, Phuapradit, and Puchaiwatananon 2000; Palan et al 2001; Mohindra et al 2002; Zhang et al 2002; Zusterzeel et al 2002; Aydin et al, 2004; Palan et al 2004; Harma et al 2005); (3) increased plasma malondialdehyde and xanthine oxidase activity (Karabulut et al 2004); (4) increased plasma xanthine oxidase activity with a concurrent decrease in SOD and glutathione peroxidase activity (Yildirim et al 2004); (5) increased TBAR's in addition to increased glutathione peroxidase activity (Orhan et al 2003); (6) reduced toenail concentrations of selenium (Rayman, Dobe and Redman 2003) and (7) even by measuring ethene and volatile organic compounds in alveolar breath of women with pre-eclampsia (Zusterzeel et al 2002; Moretti et al 2004).

For the infant, increased maternal urinary concentrations of malondialdehyde measured at birth have been associated with decreased birth weight in term infants after adjustment for the potential confounding effects of maternal age, body mass index, diet and alcohol intake and smoking (Kim et al, 2005). Maternal blood concentrations of antioxidants have also been assessed in relation to infant growth parameters, where serum vitamin C concentrations assessed between 24 and 28 weeks' gestation have been positively associated with birth weight and length (Lee et al 2004). In this study, higher maternal serum concentrations of vitamin C and E were associated with higher birth weight and length. Others however, have shown no association between maternal serum vitamin E concentration and birth weight or the risk of having a small for gestational age (SGA) infant (Tamura et al 1997).

Term infants born SGA also demonstrate a degree of oxidative stress as measured by increased malondialdehyde concentrations and decreased concentrations of SOD, catalase and

glutathione in cord blood, when compared with appropriately grown term infants (Gupta et al 2004). Similarly, increased triglyceride concentrations in fetal cord plasma have been demonstrated in pre-eclamptic and IUGR pregnancies (Rodie et al 2004), however these concentrations were not correlated with maternal lipid concentrations or infant birth weight. The studies do however, indicate possible placental abnormalities including oxidative stress and perturbations in lipid transport in pre-eclamptic and IUGR pregnancies, and support the notion of generalised oxidative stress and dyslipidemia seen in pre-eclampsia and its related complications.

While there is evidence of elevated markers of oxidative stress with or without concomitant decreases in antioxidant concentrations occurring in pre-eclampsia, not all reports to date support this pattern of events. Uotila and colleagues (1993b) demonstrated increased lipid peroxidation and concomitant increases in antioxidants such as vitamin E and glutathione peroxidase in severe pre-eclampsia. Similarly, decreased plasma concentrations of oxLDL have been demonstrated in women with pre-eclampsia compared with pregnant controls matched for gestational age (Raijmakers et al 2004a). Higher concentrations of oxLDL would be expected in oxidative stress, however in this study the authors suggest that decreased concentrations may reflect rapid clearance of oxLDL by autoantibodies, which has been demonstrated previously in pre-eclampsia (Branch et al, 1994).

Morris and colleagues (1998) reported elevated markers of oxidative stress as indicated by isoprostanes, lipid hydroperoxides and malondialdehyde concentrations and elevated vitamin E concentrations in pregnant women when compared with non-pregnant controls. However they found no differences in these parameters between women with and without pre-eclampsia. Similarly, Ben-Haroush and colleagues (2002), found no difference in plasma concentrations of vitamin E measured prior to the onset of disease in women with pre-eclampsia and IUGR and in healthy pregnant controls. One explanation for the elevated vitamin E concentrations seen may be that both normal pregnancy and pre-eclampsia more so, are associated with hyperlipoproteinaemia. As vitamin E is transported in lipoproteins, this would result in increased vitamin E concentrations in the presence of increasing lipids such as in pre-eclampsia. These findings do however, lend support to the idea that even normal pregnancy is characterised by a mild state of oxidative stress.

Others have shown no differences in markers of oxidative stress between women with established pre-eclampsia and pregnant controls as measured by plasma and urinary isoprostanes and plasma tocopherols (Ishihara et al 2004). Llurba and colleagues (2004)

assessed a range of markers of oxidative stress in pre-eclamptic women and pregnant controls, and reported that some markers (increased lipid hydroperoxide and decreased vitamin C concentrations) supported a role for oxidative stress in pre-eclampsia, while differences or lack there of in other markers (MDA, plasma carbonyls, vitamin E, glutathione, SOD, glutathione peroxidase) did not.

Isoprostanes are considered to be the most reliable marker of lipid peroxidation and subsequently oxidative stress *in vivo* (Diplock et al 1998). Isoprostanes are a series of prostaglandin like compounds produced by free radical catalysed peroxidation of arachidonic acid bound to the phospholipid plasma membrane (Morrow et al 1990). Isoprostanes circulate in a free and lipoprotein-bound state in plasma and in a free state in urine and saliva. 8-epi-prostaglandin $F_{2\alpha}$ is the most abundant isoprostane product of free radical oxidation in plasma (McKinney et al 2000). Several reports have demonstrated increased blood and/or urinary concentrations of isoprostanes in women with established pre-eclampsia (Wickens et al 1981; Barden et al 1996; McKinney et al 2000). In contrast, Regan and colleagues (2001) found no evidence for increased lipid peroxidation in women with severe pre-eclampsia as measured by urinary $F_{2\alpha}$-isoprostanes. One explanation for these conflicting findings may be that urinary isoprostanes are a more accurate reflection of renal production of lipid peroxides or renal clearance of isoprostanes, where higher plasma concentrations may reflect decreased renal clearance. These findings highlight the need for research studies to assess both plasma and urinary isoprostane concentrations.

1.6.3 Placental markers of oxidative stress

The placenta is a potential source of lipid peroxides due to its high lipid concentration (Ogburn et al 1988). In a normal pregnancy lipid peroxides are produced by the placenta, however the degree of lipid peroxidation decreases with increasing gestational age (Sekiba and Yoshioka 1979). This may be explained by increased placental activity of antioxidant enzymes such as SOD and free radical scavengers including vitamin E. In pre-eclampsia, deficient placental production of antioxidant enzymes or overwhelmed antioxidant defences would result in increased lipid peroxidation and thus contribute to oxidative stress.

In the healthy placenta, Jauniaux and colleges (2000) have correlated increases in blood flow from 10 weeks' gestation with increases in oxygen tension and expression and activity of the antioxidant enzymes glutathione peroxidase and catalase. Overall, the activity and expression of the antioxidant enzymes increased with gestational age. The authors suggest that in early

pregnancy, increasing oxygen tension is associated with a degree of oxidative stress, primarily through generation of superoxide radicals via the mitochondrial electron-transport chain. As an adaptive solution, increased expression and activity of antioxidant defences results. They hypothesise that where the antioxidant defence mechanisms are not well developed, oxidative stress may promote syncytiotrophoblast damage. This results in poor perfusion initially, as well as contributing to the oxidative stress associated with placental under perfusion and subsequent re-oxygenation, and thus the development of pre-eclampsia. In support of these findings, Pavan and colleagues (2004) have also demonstrated that oxLDL inhibits trophoblast cell invasion in first trimester placental samples. These studies in healthy pregnancies suggest that in pre-eclampsia, perturbed antioxidant defences may contribute to poor implantation and the poor placental perfusion, leading to oxidative stress and a further compromised antioxidant defence system.

In established pre-eclampsia, increased lipid peroxides and thromboxane-A_2 have been documented in the placenta (Wang, Walsh and Kay 1992) as have decreased tissue concentrations of vitamin E, SOD, and glutathione peroxidase (Wang and Walsh 1996; Vanderlelie et al 2005). Impaired antioxidant gene expression has also been demonstrated, with decreased messenger ribonucleic acid (mRNA) expression of SOD and glutathione peroxidase found in pre-eclamptic placentas (Wang and Walsh 1996). Decreased placental concentrations of vitamin C, glutathione peroxidase, SOD and glutathione transferase have also been shown in pre-eclamptic placentas, accompanied by increased thiobarbituric acid reacting substances (Mutlu-Turkoglu et al 1998; Madazli et al 2002). Furthermore, decreased placental concentrations of glucose-6-phosphate dehydrogenase have also been demonstrated in women with pre-eclampsia (Poranen et al 1996); however in this study, no differences in placental vitamin E concentration, glutathione peroxidase or glutathione-S-transferase were reported. The presence of oxidative stress localised in the placenta of women with established pre-eclampsia and severe gestational hypertension has also been reported by several groups when assessed by elevated isoprostane concentrations (Gulmezoglu et al 1996; Gratacos et al 1998; Walsh et al 2000), the presence of peroxynitrite (Many et al 2000) or oxidative protein or deoxyribonucleic acid (DNA) damage (Zusterzeel et al 2000; Wiktor et al 2004). In contrast, others have reported increased glutathione peroxidase activity in women with pre-eclampsia and the HELLP syndrome (haemolysis, elevated liver enzymes, low platelets) (Knapen et al 1999).

Term healthy placental samples cultured in low oxygen conditions (2% O_2) demonstrate increased concentrations of lipid peroxides including malondialdehyde (Li et al 2005),

supporting the notion that placental under perfusion mediates a state of oxidative stress. Furthermore, Many and colleagues (2000) have shown increased xanthine oxidase/dehydrogenase concentrations and increased xanthine oxidase activity in the invasive cytotrophoblasts in women with pre-eclampsia. Decreased SOD activity, which is required to scavenge superoxide and increased formation of peroxynitrite, were also observed in these cells and in villous vessels. Increased placental generation of superoxide by NAD(P)H oxidase has also been demonstrated in women with severe early onset pre-eclampsia (Raijmakers et al 2004b). These findings lend further support for the placental role in generating harmful reactive oxygen species, and in pre-eclampsia this may occur primarily via the xanthine oxidase and NAD(P)H oxidase pathways.

In vitro studies involving pre-eclamptic and normal placentas perfused with exogenous vitamin C and/or vitamin E, report decreased lipid peroxidation after vitamin C perfusion in the pre-eclamptic placentas (Poranen et al 1998). This study demonstrated no effect of perfusion on lipid peroxidation markers in normal placentas and in fact, the median concentrations of lipid peroxides after perfusion with either vitamin C or vitamin E increased in normal placentas, suggesting a pro-oxidant effect. No effect on lipid peroxidation was seen in pre-eclamptic placentas after perfusion with vitamin E. In a similar study, pre-treatment of term human fetal membranes (amnion and chorion) with vitamin C and E prevented damage from the addition of hypochlorous acid, a source of reactive oxygen species (Plessinger, Woods and Miller 2000). Treatment with these antioxidants prevented damage to the amniotic epithelium and collagen present in the amnion-chorion at all dosages of hypochlorous acid exposure, suggesting there may be potential therapeutic significance for prelabour rupture of membranes (PROM). Vitamin C deficiency has been linked to PROM in the past (Wideman et al 1964; Casanueva et al 1995) and these studies support the proposal of an oxidative stress model in the pathogenesis of PROM and preterm PROM (Plessinger, Woods and Miller 2000).

Research to date suggests that even normal pregnancy may be characterised by mild oxidative stress, where the increased serum lipids observed in normal pregnancy increase the chance of polyunsaturated fatty acids succumbing to peroxidative damage from ROS. However in normal pregnancy, increased lipid peroxidation is counteracted by sufficient antioxidant defence mechanisms. Pre-eclampsia reflects an imbalance in lipid peroxidation and antioxidant status occurring in the placenta and maternal systemic circulation, where antioxidant defence mechanisms are overwhelmed by increased production of pro-oxidants.

1.6.4 Oxidative stress and endothelial cell activation

The initial finding that serum from women with pre-eclampsia is cytotoxic to endothelial cells (Rodgers et al 1988) highlighted the possibility that a circulating factor in the vascular system may be responsible for endothelial damage. Endothelial damage from oxidative stress may occur via several pathways, however the mechanisms linking endothelial activation and oxidative stress are unclear. Reactive oxygen species, oxidised LDL, free fatty acids and lipid peroxides produced in the placenta may cause direct damage to endothelial cell membranes and thus endothelial cell integrity, by promoting peroxidation reactions. Furthermore ROS may influence vascular tone either indirectly by inactivating nitric oxide, reducing the release of prostacyclin, or directly by contracting smooth muscles (Hubel et al 1989). Increased deportation of syncytiotrophoblastic microvillous fragments or membrane particles have also been reported in pre-eclampsia (Cockell et al 1997; Knight et al 1998), and as a by-product of oxidative stress damage to the trophoblast, they may interact with the endothelium. Alternatively, activation of neutrophils and monocytes passing through the uteroplacental circulation by exposure to reactive oxygen species may lead to interactions with endothelial cells and resulting damage (Roberts and Cooper 2001; Mellembakken et al 2002).

Inflammatory markers including the cytokines Tumor Necrosis Factor-alpha (TNF-α), Interleukin-1 (IL-1) and Interleukin-6 (IL-6) have been shown to be elevated in plasma of women with pre-eclampsia (Greer et al 1994) and this may result in altered lipid metabolism by promotion of fatty acid synthesis in adipocytes (Dekker and Sibai 2001). Similarly, increased surface expression of adhesion molecules CD11b and CD64 on monocytes and granulocytes, and an increased presence of intracellular ROS in granulocytes, monocytes and lymphocytes have been demonstrated in healthy pregnant women and more so in pre-eclamptic women (Sacks et al 1998). Whether the exaggerated immune response seen in pre-eclampsia characterised by leucocyte activation and the release of a range of pro-inflammatory markers, predates endothelial activation is not entirely clear, however leukocyte activation and adhesion may further promote endothelial damage.

To date, a number of factors occurring in response to placental insufficiency and oxidative stress are likely candidates for endothelial activation and damage. Most likely it is a combination of factors such as lipid peroxides, ROS, syncytiotrophoblastic fragments and the induction of an excessive inflammatory response, that contribute to endothelial activation and resulting dysfunction and thus the maternal clinical syndrome.

1.6.5 Summary

Markers of oxidative stress have been consistently demonstrated in women with established pre-eclampsia, as evidenced by decreased plasma concentrations of antioxidants such as vitamin C and E and increased concentrations of markers of lipid peroxidation including malondialdehyde and isoprostanes. Similarly, pre-eclamptic women exhibit increased placental activity of ROS generating enzymes such as xanthine oxidase/dehydrogenase and NAD(P)H oxidase, coupled with decreased capacity of antioxidant defence mechanisms including superoxide dismutase and glutathione peroxidase activity, as well as reduced concentrations of vitamin C and E. There is limited evidence of such markers occurring prior to the onset of clinical signs, in women destined to develop pre-eclampsia.

The current research investigating the pathophysiology of pre-eclampsia is limited by the lack of longitudinal studies assessing markers of oxidative stress in early and mid pregnancy in women who develop pre-eclampsia. To date, most studies are cross-sectional, assess limited numbers of biochemical markers, and involve small groups of women with established disease that are compared with normotensive women or non-pregnant controls at one time point or one stage of the disease. These factors may lead to a biased perception of the clinical presentation and pathophysiology of pre-eclampsia. In contrast, longitudinal studies usually result in a low prevalence of pre-eclampsia in the study population with only small numbers of women with severe disease. Cross sectional studies also yield less information about the pathogenesis of pre-eclampsia as they are unable to distinguish between markers associated with the pathogenesis of the disease and markers that may be secondary to the onset of the disease. Furthermore, the small numbers of women in many of the reported studies necessitate huge expected differences for any statistically meaningful effect. Few studies report power calculations demonstrating that any differences in effect size can be shown adequately with the number of women assessed. Further appropriately designed longitudinal studies are required.

The previous research has also been hampered by differing methodologies and the use of unrefined techniques. Assessments of lipid peroxidation such as diene conjugation and the simple thiobarbituric acid test are unspecific (Diplock et al 1998), which may explain some of the conflicting findings to date. Newer and more expensive methods such as urinary and plasma isoprostane concentration are more specific, and the findings of studies assessing these markers continue in part to demonstrate a role of oxidative stress and lipid peroxidation in pre-eclampsia. There is still a continued need for the use of common standards, common techniques reproduced between groups, and the recognition that samples can be influenced by

auto-oxidation *in vitro*. For example, samples stored in the absence of an antioxidant can result in raised concentrations of lipid peroxidation products, which may reflect an increased susceptibility of pre-eclamptic plasma to oxidation *in vitro* rather than *in vivo* (Morris et al 1998).

To date, there is still no 'gold standard', single and specific measurement of lipid peroxidation. The field is further confused by the potential impact of dietary sources of lipid peroxides and their degradation products, which may be generated through various cooking processes (Diplock et al 1998). Similarly, decreased concentrations of antioxidants may reflect dietary deficiencies rather than higher requirements imposed by lipid peroxidation. Few studies have concurrently investigated the impact of dietary sources of antioxidants or peroxides in relation to plasma or placental concentrations.

1.7 Dietary intake of antioxidants

The impact of dietary intake of antioxidants and the risk of pre-eclampsia and its related complications has not been adequately assessed. While the decreased concentrations of antioxidants demonstrated in women with established pre-eclampsia may reflect increased consumption during oxidative stress or even deficient antioxidant defence mechanisms, dietary deficiencies of antioxidants may also contribute to an increased risk of oxidative stress. A small number of studies have assessed dietary intake of antioxidants such as vitamin C and E during pregnancy and the risk of pre-eclampsia, using a range of methodologies, and the results to date are conflicting.

When systematically reviewing the literature assessing dietary intake of micronutrients and hypertensive disorders of pregnancy, few studies were identified that specifically assessed women's diet. Zhang and colleagues (2002) in their study of 109 women with pre-eclampsia and 259 pregnant controls, reported women with the lowest quartile of intake for vitamin C and in the lowest decile of plasma vitamin C concentration experienced a 3.8 fold increase in the risk of pre-eclampsia (Odds ratio (OR) 3.8, 95% Confidence Intervals (CI) 1.7 to 8.8). Assessments were made using a semi-quantitative food frequency questionnaire (FFQ) with adjustments for maternal age and parity, pre-pregnancy body mass index and energy intake. Similarly, Australian women assessed using a FFQ in mid to late pregnancy were found to have an increased risk of hypertensive disease, including pre-eclampsia and gestational hypertension, when they had a dietary vitamin E intake in the lowest quartile (Relative risk (RR) 1.75, 95% CI 1.11 to 2.75) (Rumbold, Maats and Crowther 2005).

In contrast, others have demonstrated that in late pregnancy women with late onset pre-eclampsia have increased dietary consumption of vitamin E as well as increased plasma concentrations compared with pregnant controls, when assessed with a FFQ (Schiff et al, 1996). Similarly, Morris and colleagues (2001), using a 24-hour dietary recall, did not find any association between dietary intake of any micronutrients in early to mid pregnancy and the risk of hypertensive disorders of pregnancy, during a one-off assessment of 4,589 nulliparous women enrolling for a randomised controlled trial of calcium supplementation in pregnancy. Moreover, while dietary intake of antioxidants has been positively associated with infant birth weight for intakes of vitamin C (Mathews, Yudkin and Neil 2000) and vitamin E (Lagiou et al 2005) in early pregnancy, after adjustments were made for confounding factors the positive correlations reported were weak.

An increased risk of pre-eclampsia has been associated with a high second trimester intake of energy, sucrose and polyunsaturated fatty acids (PUFA's) (Clausen et al 2001). In their study of 3,133 Norwegian pregnant women, Clausen and colleagues (2001) found no associations between the development of pre-eclampsia and non-energy providing antioxidant nutrients such as vitamin C and E. High energy intakes were postulated to contribute to hyperlipidemia in women already at risk for dyslipidemia. Similarly, the high intake of free fatty acids consumed by women who later developed pre-eclampsia, was attributed to the potential for PUFA's to have pro-oxidant effects by acting as a substrate for oxidation, which lends indirect support for a role of oxidative stress in the development of pre-eclampsia.

The relationship between dietary intakes of antioxidants such as vitamin C and vitamin E and pre-eclampsia remains unclear. Few studies have assessed micronutrient intake in addition to maternal plasma micronutrient concentrations. The literature to date is complicated by the use of differing methodologies, both validated and unvalidated, for assessing dietary intake in their respective populations, including 24-hour recall, semi-quantitative food frequency questionnaires and 7-day weighed dietary records. For many of the current studies, women's diet was assessed during a one-off assessment in early or late pregnancy, which doesn't account for any dietary changes in pregnancy or any influence of hyperemesis, where women were assessed in early pregnancy. Similarly, few studies adjust for potentially confounding factors including smoking, which is known to increase vitamin C requirements. No firm conclusions can be drawn from the literature to date regarding the dietary intake of antioxidants and the risk of pre-eclampsia and its related complications.

1.8 Vitamin C and E

Primates including humans are unable to synthesise vitamin C as they lack the enzyme gulonolactone oxidase (Institute of Medicine 2000), necessitating vitamin C intake from dietary sources. The recommended dietary intake (RDI) of vitamin C for Australian women is 30 mg per day, which is increased to 60 mg per day during pregnancy (2^{nd} and 3^{rd} trimester only) and lactation (NHMRC 1989). For men, the RDI is 40 mg. The RDI is based on the amount required to prevent vitamin C deficiency or scurvy in most individuals. Recommended intakes are often higher for smokers due to increased catabolism of vitamin C in these individuals (Levine et al 1995). Similarly, the RDI is higher for women during pregnancy and lactation to account for maternal losses to the fetus and in breast milk. Vitamin C is actively transported across the placenta (Streeter and Rosso 1981).

Vitamin C or ascorbate is a powerful reducing agent and free radical scavenger in the aqueous phase. As an electron donor, ascorbic acid is involved in hydroxylation reactions, where it is a cofactor for eight enzymes involved in the formation of connective tissue, tyrosine metabolism, carnitine synthesis, drug metabolism via the cytochrome P-450 oxidase system (detoxification of the liver), steroid and peptide hormone synthesis and catecholamine synthesis (Institute of Medicine 2000). Vitamin C is also important for iron metabolism where it is involved in the reduction of iron for mobilisation from body stores and reduction of iron prior to absorption from the gut. In both cases iron must be reduced from the ferric (Fe^{+++}) to ferrous (Fe^{++}) state, and prior to absorption, ascorbic acid reduces non-haem iron thus increasing the bioavailability of dietary non-haem iron (Hallberg, Brune and Rossander-Hulthen, 1987). Ascorbic acid may also play a role in the metabolism of folate, although the exact mechanism is unclear (Lewis et al 1982).

As an antioxidant in the aqueous phase, ascorbic acid interacts with free radicals to form the relatively non-reactive free radical intermediate semidehydroascorbic acid (Levine et al 1995). By donating electrons ascorbic acid becomes oxidised, resulting in the reduction of another substance or oxidant. Ascorbic acid directly scavenges ROS including superoxide, peroxyl and peroxynitrite (Frei, Stocker and Ames 1989; Whiteman and Halliwell 1996). Vitamin C may also play a role in mediating the bioavailability of nitric oxide (NO), a potent vasodilator. After production, NO is either used immediately in endothelial vasodilation or stored in complexes known as S-nitrosothiols (Scorza et al 1997). Ascorbate and the thiol group of serum albumin are the plasma components primarily involved in the release of NO from S-nitrosothiols (Scorza et al 1997).

While vitamin C functions primarily as an antioxidant, pro-oxidant effects have been demonstrated *in vitro*. High dosages of ascorbic acid have been associated with increased mutagenicity in cells *in vitro*. Ascorbic acid induced breakdown of lipid hydroperoxides and the resulting production of genotoxic compounds such as α-β-unsaturated aldehyde compounds (4-oxo-2-nonenal and 4-hydroxy-2-nonenal) which can cause DNA lesions, has been demonstrated in cells cultured in an ascorbate containing medium (Lee, Oe and Blair 2001). At present however, there is no evidence of *in vivo* pro-oxidant effects of ascorbic acid, particularly damage to DNA from ascorbate-induced mutagenicity (Carr and Frei, 1999). Furthermore, *in vivo* biological structures are protected by efficient free radical scavenging systems and repair systems, of which vitamin C is involved.

Vitamin E is the generic name given to eight lipid soluble and plant-derived compounds, four are referred to as tocopherols (alpha, beta, gamma, delta) and four are known as tocotrienols (alpha, beta, gamma, delta). These compounds share a common 6-chromanol ring structure, but differ in the side chain and number of methyl substitutes, and all exhibit the biological activity of alpha-tocopherol (α-tocopherol) to a varying degree (Institute of Medicine 2000). Confusion around the term vitamin E exists due to the range of compounds it has been used to refer to, however vitamin E in general refers to all compounds that exhibit the activity of α-tocopherol, which is the most biologically active form. In nature, only one stereoisomer of α-tocopherol exists (RRR-α-tocopherol or d-α-tocopherol) and in nutritional supplements naturally derived α-tocopherol can be esterified to prolong stability and shelf-life at room temperatures (resulting in RRR-α-tocopherol acetate or succinate). Synthetic production of α-tocopherol (all rac-α-tocopherol) results in the production of seven other stereoisomers in addition to the RRR-α-tocopherol, four of which are not able to be maintained in human plasma, resulting in synthetically derived α-tocopherol having approximately half the biological activity of natural source α-tocopherol (Institute of Medicine 2000). Similarly, after ingestion, all forms of vitamin E are absorbed in the gut, however the liver preferentially favours the RRR-α-tocopherol form (Traber et al 1994), where it is incorporated in very low density lipoproteins (VLDL) and thus LDL in circulation. The amounts of α-tocopherol ingested from the diet or via supplementation are presented either in International Units (IU) or milligrams (mg), where 1 IU equates to 0.67 mg for naturally derived vitamin E and 0.45 mg for synthetically derived vitamin E (Institute of Medicine 2000).

For women, the RDI for vitamin E is 7 mg, which is unchanged for pregnancy but increased to 9.5 mg due to increased requirements during lactation (NHMRC 1989). For men the RDI

is 10 mg primarily due to increased energy intake. Without supplementation it is proposed that the maximum vitamin E intake from foods would not exceed 30 mg per day, unless wheat germ oil became a major dietary ingredient (NHMRC 1989). Vitamin E deficiency is rarely reported and complicated by the lack of any overt symptoms of deficiency. However, given the lipid soluble nature of vitamin E, deficiency may be predisposed in those individuals with fat malabsorption disorders or in preterm infants.

Vitamin E and more specifically α-tocopherol, acts as an antioxidant in the lipid phase of cell membranes, helping to prevent oxidation of phospholipid fatty acids and stabilise cell membranes. Unlike vitamin C, a specific role for vitamin E in a required metabolic function has not been found, suggesting that vitamin E functions primarily as a non-specific chain-breaking antioxidant (i.e. intercepts radicals which would otherwise bind with adjacent fatty acid side chains) (Institute of Medicine 2000). Alpha-tocopherol scavenges the peroxyl radical protecting PUFA's present in membrane phospholipids and plasma lipoproteins from oxidative damage (Burton, Joyce and Ingold 1983). In lipid membranes, peroxyl radicals preferentially react with tocopherol to form lipid hydroperoxides and the tocopherol radical. From here, the tocopherol radical can then be reduced by other antioxidants (particularly vitamin C or glutathione) to form α-tocopherol; react with another tocopherol radical to form non-reactive products; undergo further reduction to tocopheryl quinone, or theoretically, could go on to act as a pro-oxidant and oxidize other lipids (Institute of Medicine 2000). Hence under certain circumstances tocopherol may act as a pro-oxidant, although these reactions have not been demonstrated *in vivo*.

Tocopherol radicals generated in the inhibition of lipid peroxidation can be quenched at the lipid water interface by the donation of an electron from ascorbic acid, thus regenerating vitamin E and forming the ascorbyl radical. This synergistic relationship between vitamin C and vitamin E has been widely demonstrated. Ascorbic acid aids in the regeneration of α-tocopherol by reducing the tocopherol radical to α-tocopherol (Packer, Slater and Willson 1979). The ascorbate radical produced in this process can then be converted back to ascorbate or ascorbic acid by a glutathione dependent enzyme. This relationship where vitamin E is recycled at the expense of vitamin C may account for the absence of overt vitamin E deficiency reported in healthy adults. Similarly, it may represent a transfer of free radical load from the lipid phase to the aqueous phase where there are enzymatic antioxidant defences available to counteract ROS (Diplock et al 1998).

1.8.1 Optimal intakes of vitamin C and E

The optimal vitamin C and E requirements for humans is unknown. For vitamin C, the current recommended intakes are based on the prevention of experimentally induced scurvy plus a margin of error, tissue saturation (the amount ingested which results in urinary excretion of vitamin C), and the amount required to replace vitamin C lost in catabolism (Levine et al 1995). For vitamin E, few studies have involved any pharmacokinetic studies on vitamin E plasma saturation, bioavailability and excretion. The relatively few reports of vitamin E deficiency further compromise formulation of required dietary intake of vitamin E in relation to its antioxidant functions. As with most recommended intakes, the amount needed to prevent deficiency may not be the amount needed for optimum human health and in particular the amount needed for specific functions, for example antioxidant defences.

In pharmacokinetic studies involving vitamin C depleted individuals supplemented with vitamin C in the range of 30 mg to 2,500 mg per day, Levine and colleagues (1996) demonstrated complete tissue saturation occurred when male volunteers ingested 1,000 mg per day. These findings have also been replicated in women (Levine et al 2001). Direct antioxidant effects of vitamin C and E have been demonstrated in observational studies of male and female individuals supplemented with 1,000 mg vitamin C and 800 IU vitamin E for ten days, as demonstrated by decreased production of TBARS *in vitro* when lipoproteins isolated from these individuals were subjected to copper-induced oxidation (Rifici and Khadachadurian 1993). Other studies have demonstrated reductions in plasma malondialdehyde concentrations in healthy male and female individuals, after supplementation with either 500, 750 or 1,000 mg per day vitamin C and/or 200, 400, 600 or 800 IU vitamin E, for fourteen days (Naidoo and Lux 1998). In this study, the earliest significant reduction in plasma malondialdehyde concentrations occurred after four weeks supplementation with 500 mg vitamin C and 400 IU vitamin E. However, in all of these studies, comparisons were made within individuals, prior to and after vitamin supplementation, not compared with control subjects. For vitamin E alone, Devaraj and colleagues (1997) conducted a randomised placebo controlled trial involving 79 healthy participants 19 of whom were female, and demonstrated decreased lipid peroxide concentration in individuals supplemented with 400 IU per day when compared to individuals supplemented with a placebo. Dosages in this study ranged from 100 to 800 IU vitamin E daily for eight weeks, and during this time period no side effects were reported by any individuals.

Few studies have assessed measures of oxidative stress in women supplemented with antioxidants during pregnancy. However in a pilot matched cohort study involving five women at risk of preterm birth, women supplemented with 1,000 mg vitamin C in addition to 167.8 mg (250 IU) vitamin E per day for a median of three days had reduced plasma malondialdehyde concentrations at delivery when compared with pre-treatment concentrations and pregnant controls matched for gestational age (Bolisetty et al 2002). In a randomised controlled trial of a multivitamin preparation containing small amounts of vitamin C (60 mg) and vitamin E (10 mg) initiated in mid pregnancy (from 14 weeks' gestation), no differences were found in the concentrations of TBARS, and markers of protein and amino acid oxidation between the multivitamin and placebo groups (Hininger et al 2004). These studies demonstrate that the amount of both vitamin C and vitamin E required to have antioxidant effects *in vivo* is well above the current recommended daily amount, and in particular for vitamin E, well above the amount able to be ingested from a 'normal healthy diet'. Dosages reported to have antioxidant effects start at 500 mg per day for vitamin C, with plasma saturation occurring at 1,000 mg per day. For vitamin E, effective antioxidant dosages occur from 400 IU. These amounts must be obtained from dietary supplementation.

1.8.2 Safety and adverse effects of vitamin C supplementation

Vitamin C is often referred to as a non-toxic substance. Few side effects have been reported at dosages of up to ten times the current recommended dietary intake (Bendich 1997). The safety and tolerance of vitamin C supplementation in humans has been extensively reviewed. Bendich (1997) reviewed the results of 22 double-blind placebo controlled intervention studies and eight single blind and/or non-placebo-controlled intervention studies involving vitamin C supplementation. These studies involved individuals ingesting dosages of vitamin C up to six grams per day over a long period of time, in some cases for up to five years. Overall few consistent and reproducible side effects were reported. In fact, there was a beneficial effect of vitamin C on increasing iron stores in individuals with low iron status, while not increasing stores in iron-replete individuals. However, because ascorbic acid enhances absorption of non-haem iron and aids in the mobilisation of iron stores in the body, individuals suffering from diseases which increase iron stores such as haemochromatosis are often recommended to limit their ascorbic acid intake (Diplock et al 1998). Only one trial involving supplementation with 10 grams of vitamin C for five days, reported side effects. In this trial two of the total 15 participants experienced diarrhoea.

The safety of vitamin C supplementation in pregnancy is not well established. Early reports of "rebound scurvy" or vitamin C deficiency in infants of women with high vitamin C intake, led to the suggestion that pregnant women ingesting high dosages of vitamin C may predispose their infants to scurvy due to increase turnover of vitamin C (Cochrane 1965). The original studies in this area were conducted in a small number of guinea pigs, and to date the findings have not been demonstrated in other animal models or in humans. However, there is still limited evaluation of the safety of vitamin C use in pregnancy.

1.8.3 Safety and adverse effects of vitamin E supplementation

Because vitamin E is lipophilic, high dosage supplementation may result in increased storage in muscle, liver and adipose tissue. Like vitamin C, the safety of vitamin E supplementation has also been extensively reviewed. Bendich and Machlin (1993) systematically reviewed randomised and non-randomised controlled trials investigating vitamin E dosages between 250 and 2,400 IU per day for durations ranging from 28 days to 4.5 years. They concluded that vitamin E has a very low toxicity in humans due to the absence of any consistent side effects reported. In particular, no side effects were reported by any of the participants themselves, and analyses measuring haematological, hepatic, thyroid and immune functions and lipid profiles were unchanged in the vitamin E supplemented groups. However, uncontrolled evaluation of vitamin E supplementation has reported an association between supplementation and blood clotting, particularly prothrombin time, when in the presence of low vitamin K concentrations (Corrigan and Ulfers 1981). In the review of trials, this decreased platelet adhesion was seen as a benefit, in reducing the risk of thrombosis, however two studies reviewed reported greater postoperative bleeding (Bendich and Machlin 1993). High dosage vitamin E supplementation has also been associated with an immunosuppressive effect by the finding of lowered leucocyte action in men given daily 222 mg alpha-tocopherol for 3 weeks (Prasad 1980). However, these findings were not confirmed in any of the trials reviewed by Bendich and Machlin (1993). The relationship between vitamin E and blood clotting factors is unclear, however in the presence of clotting abnormalities such as vitamin K deficiency (which may be induced by warfarin anticoagulant therapy, for example), vitamin E may act to increase clotting time and is thus contraindicated in these individuals (Corrigan and Ulfers 1981).

Of concern, a recent systematic review of trials evaluating vitamin E supplementation in 135,967 men and non-pregnant women demonstrated an increase in all cause mortality in individuals supplemented with $\geq$400 IU vitamin E per day for at least one year (RR 1.04,

95% CI 1.04 to 1.07) (Miller et al 2005). This review encompassed trials of vitamin E supplementation in individuals either at risk of or with established cardiovascular disease, with Alzheimers or early onset Parkinson's disease, institutionalised elderly individuals as well as the general population. Many of the included trials assessed vitamin E in conjunction with other nutritional supplements or agents. Of note, trials where there were less than ten deaths reported were excluded, based on the assumption that mortality data may not have been by the smaller trials. However excluding such trials creates the possibility of introducing systematic bias into the review, and limits the generalisability of the review findings.

While the findings of this review highlight the need for controlled evaluation of any vitamin E supplementation, they cannot be generalised to healthy adults including pregnant women, and for those individuals supplemented with lower dosages or short term vitamin E supplementation (less than one year). The review does however, illustrate the large body of literature assessing antioxidant supplementation for preventing or delaying cardiovascular disease and other adult onset diseases. While the effect of antioxidants on cardiovascular disease and related complications remain controversial, several of these large studies of antioxidants have demonstrated the safety of supplementation. For example, the Heart Protection Study (Heart Protection Study Collaborative Group 2002) assessed vitamin E (600 mg/day), vitamin C (250 mg/day) and beta-carotene (20 mg/day) supplementation for a period of five years in 20,536 individuals at high risk of cardiovascular events in the United Kingdom. The HPS found no benefit for supplementation on mortality from or incidence of vascular disease or any other major outcomes, however it did demonstrate the safety of the supplements, with no adverse effects reported.

While vitamin E supplementation in adults has been assessed widely, few studies have assessed the safety of vitamin E supplementation in pregnancy, or more specifically in early pregnancy. One prospective cohort study has reported on the use of high dosage vitamin E supplementation in early pregnancy and the risk of congenital malformations (Boskovic et al 2005). Eighty-two women in the vitamin E group took vitamin E supplements by choice in a range of doses ($\geq$400 IU to 1200 IU per day) as a part of a "healthy lifestyle" program, and all women indicated that they took vitamin E during the first trimester, i.e. during organogenesis. These women were compared with 130 self-selected pregnant controls. One major malformation (omphalocele) was reported in the vitamin E group, however this rate was comparable to the overall population incidence of birth defects. Infants of mothers in the vitamin E group had a significantly lower birth weight than controls (3,173 $\pm$ 367 grams versus 3,417 $\pm$ 565 grams, p=0.0015), however there was no difference in the proportion of

infants with a birth weight <2,500 grams. No differences were reported for any other pregnancy outcomes including spontaneous abortion, stillbirth and preterm birth, between women taking vitamin E and the self-selected controls. While this study provides some initial reassurance, there is still limited evaluation of the safety of vitamin E supplementation in pregnancy. Further controlled evaluation is required to established safe dosages in pregnancy.

1.8.4 Systematic review of vitamin C and E supplementation in pregnancy

Two Cochrane systematic reviews were undertaken to evaluate the use of vitamin C supplements and vitamin E supplements in pregnancy. These reviews entitled "*Vitamin C supplementation in pregnancy*" and "*Vitamin E supplementation in pregnancy*" respectively are published in full in the Cochrane Library (Rumbold and Crowther 2005a; Rumbold and Crowther 2005b). Detailed descriptions of the methodology and findings of these reviews can be found on the Cochrane Library website (The Cochrane Library, Issue 2, 2005).

Both reviews separately confirm that the current data are too few to determine if vitamin C supplementation and/or vitamin E supplementation, alone or combined with other supplements, are beneficial or harmful during pregnancy. The numbers of women involved in the current trials is small (766 in total in the vitamin C supplementation review and 566 in total in the vitamin E supplementation review). In both reviews however, vitamin C supplementation and vitamin E supplementation appeared to reduce the risk of pre-eclampsia, however substantial statistical heterogeneity was detected in the results. Heterogeneity is an indication that substantial variability exists in the treatment effects seen between trials, more than what would be expected by chance alone (Green and Higgins 2005). Pre-specified subgroup analyses were undertaken to assess the impact of differences in dosages, gestation at onset of treatment, prior dietary intake of the respective vitamin(s), women's risk status for adverse pregnancy outcomes and the use of the vitamin combined with other supplements. The data however, were too few to reliably assess the impact of any of these subgroups, and thus explain the heterogeneity seen. Of concern, vitamin C supplementation appeared to increase the risk of preterm birth. One trial involving 56 women reported acne, transient weakness and skin rashes as side effects of vitamin C and E supplementation. No other adverse effects or side effects were reported. To date, the data are too few to produce any reliable conclusions about any benefits or harms of supplementation with vitamin C and vitamin E in pregnancy, emphasising the need for further evaluation in pregnancy. The full systematic review of antioxidant supplementation in pregnancy for the prevention of pre-eclampsia is presented in Chapter Two of this thesis.

1.8.5 Summary

As antioxidants, vitamin C and vitamin E (alpha-tocopherol) act in a synergistic relationship in the aqueous and lipid phase, protecting against oxidative damage from reactive oxygen species. Both must be obtained via dietary intake or supplementation. The safety of vitamin C and vitamin E supplementation has been demonstrated in adults, by the systematic review of supplementation trials, which have failed to demonstrate consistent side effects across a range of dosages. Few studies however, have assessed the safety of using these supplements during pregnancy. Despite this, current safe "upper tolerable limits" of ingestion of these vitamins in pregnancy have been set at 2,000 mg per day for vitamin C (based on the potential for diarrhoea) and 1,000 mg (1490 IU) per day for vitamin E (based on the potential for increased tendency to haemorrhage) by the Institute of Medicine in the United States of America (2000). Further evaluation of the safety of antioxidant vitamin use in pregnancy is required to confirm these recommendations. Similarly, assessments of the safety of interventions in pregnancy should always involve long-term follow up of both mothers and infants, beyond hospital discharge. To date, no studies have assessed any potential long-term benefits or harms for the mother or exposed children.

1.9 Conclusions

Markers of oxidative stress and lipid membrane damage have been consistently reported in women with established pre-eclampsia, supporting a role for oxidative stress in the pathogenesis of pre-eclampsia. Therapeutic interventions targeting oxidative stress, such as dietary antioxidant vitamin supplementation, represent a promising prophylaxis against pre-eclampsia and its related complications. Any new therapy needs careful and controlled evaluation and any benefits need to be weighed against potential harms. The best evidence to form the basis of clinical recommendations will be provided by systematic review of completed and reported randomised controlled trials.

2. SYSTEMATIC REVIEW OF ANTIOXIDANTS FOR THE PREVENTION OF PRE-ECLAMPSIA

2.1 Introduction

Supplementing women with antioxidant agents during pregnancy may help to prevent or delay the onset of pre-eclampsia and its related complications. Before any treatment recommendations can be made however, evidence for the safety and efficacy of antioxidant use during pregnancy is required. Recommendations should be based on the best available evidence, taking into account the level, quality, relevance and strength of the evidence. Ideally, treatment recommendations should be based on Level 1 evidence, which the National Health and Medical Research Council (NHMRC) of Australia defines as the systematic review of all relevant randomised controlled trials (Figure 2.1) (NHMRC 1999).

Level I	Evidence obtained from a systematic review of all relevant randomised controlled trials.
Level II	Evidence obtained from at least one properly designed randomised controlled trial.
Level III-1	Evidence obtained from well-designed pseudo-randomised controlled trials (alternate allocation or some other method).
Level III-2	Evidence obtained from comparative studies with concurrent controls and allocation not randomised (cohort studies), case-control studies, or interrupted time series with a control group.
Level III-3	Evidence obtained from comparative studies with historical control, two or more single-arm studies, or interrupted time serious without a parallel control group.
Level IV	Evidence obtained from case series, either post-test or pre-test and post-test.

Figure 2.1 Designation of the levels of evidence used by the Health Advisory Committee of the National Health and Medical Council of Australia (NHMRC 1999).

This 'hierarchy of evidence' denotes that Level I evidence, the best available evidence, is synthesised from the systematic review of high quality randomised trials assessing biological, clinical and patient-relevant outcomes (NHMRC 1999). Systematic reviews encompass an objective overview of primary studies, using meta-analytical statistical methods to summarise the results of individual studies to produce more precise, evidence-based recommendations about health care interventions. The hallmarks of a systematic review are the use of explicit

and reproducible methodology, including *a priori* explicit statements about the objectives, materials and methods (Greenhalgh 1997). The Cochrane Collaboration (The Cochrane Collaboration 2005), an international non-profit organisation, is responsible for producing and disseminating systematic reviews of health care interventions, via preparation, regular updating and publishing of systematic reviews in the Cochrane Library (The Cochrane Library 2005). The Cochrane Central Register of Controlled Trials (CENTRAL), published in the Cochrane Library, provides the most comprehensive source of all records related to controlled trials, including conference proceedings, non-English citations and citations not indexed in databases such as MEDLINE and EMBASE (Green and Higgins 2005).

To assess the available evidence for antioxidant supplementation in pregnancy for preventing pre-eclampsia and its related complications, a systematic review of all trials assessing antioxidant supplementation for the prevention of pre-eclampsia was undertaken for the Pregnancy and Childbirth Collaborative Review Group of the Cochrane Collaboration.

2.2 Cochrane systematic review: "Antioxidants for preventing pre-eclampsia"

This systematic review entitled "*Antioxidants for preventing pre-eclampsia*" is published as a protocol in the Cochrane Library (Rumbold et al 2003), and the full review is currently under editorial review.

2.2.1 Description of the review

2.2.1.1 Objectives

The objective of this review was to determine the effectiveness and safety of any antioxidant supplementation during pregnancy on the risk of pre-eclampsia; small for gestational age infants; stillbirth and neonatal death; maternal and neonatal morbidity; long term development of the child and side effects and adverse events. If antioxidants were found to be effective, an additional objective was to determine which of these agents were best, what was the ideal dose, and to compare antioxidants with other interventions.

2.2.1.2 Criteria for inclusion of studies

Studies considered for inclusion in the review were all randomised and quasi-randomised controlled trials comparing one or more antioxidants with either placebo or no antioxidants

during pregnancy for the prevention of pre-eclampsia, and trials comparing one or more antioxidants with another, or with other interventions. Trials were included if the primary aim of the study was to prevent pre-eclampsia or if the primary aim was otherwise but the outcome of pre-eclampsia was reported by the authors.

The following interventions were considered:

1. Comparisons of any antioxidant/s (any dosage regimen) with either placebo or no antioxidant/s.
2. Comparisons of one or more antioxidant with other antioxidant/s.
3. Comparisons of antioxidant/s with other interventions.
4. Comparisons of one or more antioxidants with other agents compared with placebo or no antioxidant/s, other antioxidants or other interventions.

All types of antioxidants, including enzymes that inhibit or retard the production of oxidative substances or free radical scavengers that interact with free radicals, were included. Antioxidants were classified into vitamins (such as vitamin C, vitamin E and beta-carotene), minerals (such as selenium) and non-vitamin antioxidants (such as glutathione peroxidase, catalase, superoxide dismutase).

2.2.1.3 Participants and subgroups

The types of participants considered included pregnant women considered to be at low, moderate or high risk of developing pre-eclampsia. Women were classified into subgroups based on:

1. Their level of risk at trial entry of developing pre-eclampsia:

a) High risk, defined as one or more of the following: previous severe pre-eclampsia, diabetes, chronic hypertension, renal disease or autoimmune disease;

b) Moderate/low risk, defined as any other risk factors, in particular first pregnancy, a mild rise in blood pressure and no proteinuria, positive roll-over test, abnormal uterine artery Doppler scan, multiple pregnancy, a family history of pre-eclampsia, maternal age less than 20 years and known thrombophilia.

c) When the risk was unclear or unspecified, women were classified as moderate/low risk.

2. Their gestational age at randomisation: before or greater than or equal to 20 weeks' gestation.

2.2.2 Health outcomes specified in the review

2.2.2.1 Primary outcomes

1. Pre-eclampsia: onset before or after 34 weeks' gestation, variously defined by the authors.
2. Severe pre-eclampsia: including HELLP syndrome and imminent eclampsia.
3. Preterm birth and very preterm birth: less than 37 weeks' gestation or less than 34 weeks' gestation.
4. Small for gestational age infants: whenever possible, defined as less than the third centile, otherwise the most extreme centile reported.
5. Baby death (stillbirth, neonatal or infant death).

2.2.2.2 Secondary outcomes

For the mother: death up to six weeks' postpartum; elective delivery (induction of labour or elective caesarean section); severe hypertension; caesarean section (emergency plus elective); bleeding episodes (such as abruption of the placenta, antepartum haemorrhage, postpartum haemorrhage, need for transfusion); measures of serious maternal morbidity (including eclampsia, liver failure, renal failure, disseminated intravascular coagulation, stroke); and maternal views of care.

For the child: gestational age at birth; birth weight; Apgar score less than four at five minutes; respiratory distress syndrome; chronic lung disease; bleeding episodes (such as intraventricular haemorrhage, periventricular leucomalacia); bacterial sepsis; necrotising enterocolitis; retinopathy of prematurity; disability during childhood (such as cerebral palsy, intellectual disability, hearing disability and visual impairment); and poor childhood growth.

For mother and child: side effects and adverse effects of antioxidants sufficient to stop supplementation; side effects and adverse effects of supplementation not sufficient to stop supplementation.

Use of health service resources for the woman: antenatal hospital admission; visits to day care units; use of intensive care, ventilation and dialysis.

Use of health service resources for the infant: admission to special care/intensive care nursery; use of mechanical ventilation; length of stay in hospital; as well as developmental and special needs after discharge.

2.2.3 Search strategy for identification of studies

The Cochrane Pregnancy and Childbirth Group trials register was searched by contacting the Trials Search Coordinator (June 23rd, 2004), in accordance with the Cochrane Collaboration recommendations (Green and Higgins 2005). The Cochrane Pregnancy and Childbirth Group's trials register is maintained by the Trials Search Co-ordinator and contains trials identified from:

1. Quarterly searches of the Cochrane Central Register of Controlled Trials (CENTRAL);
2. Monthly searches of MEDLINE;
3. Hand searches of 30 journals and the proceedings of major conferences;
4. Weekly current awareness search of a further 37 journals.

Details of the search strategies for CENTRAL and MEDLINE, the list of hand searched journals and conference proceedings, and the list of journals reviewed via the current awareness service can be found in the 'Search strategies for identification of studies' section within the editorial information about the Cochrane Pregnancy and Childbirth Group.

Trials identified through the searching activities described above are given a code (or codes) depending on the topic. The codes are linked to review topics. The Trials Search Co-ordinator searches the register for each review using these codes rather than keywords.

In addition, we searched the Cochrane Central Register of Controlled Trials (The Cochrane Library, Issue 3, 2004) using the terms pregnan*, preeclamp*, pre-eclamp*, antioxidant*.

MEDLINE and Current Contents were searched using the strategy:

1. "Antioxidants" / all subheadings
2. "Ascorbic-Acid"/ all subheadings
3. "Vitamin-E" / all subheadings
4. "Beta-Carotene" / all subheadings
5. "Selenium" / all subheadings
6. "Glutathione peroxidase" / all subheadings
7. "Superoxide dismutase" / all subheadings
8. "Catalase" / all subheadings
9. "Pregnancy" / all subheadings
10. "Pre-Eclampsia" / all subheadings
11. "Pregnancy complications" / all subheadings

12. #1 or #2 or #3 or #4 or #5 or #6 or # 7 or #8

13. #12 and (#9 or #10 or #11)

14. "Controlled-clinical-trials" / all subheadings

15. "Randomized-Controlled-Trials" / all subheadings

16. #14 or #15

17. #13 and #16

2.2.4 Methods of the review

Two reviewers (Alice Rumbold and Caroline Crowther) assessed potentially eligible trials for their suitability for inclusion in the review. Decisions regarding inclusion were made separately and results compared. Any disagreement was resolved through discussion. It is desirable to involve more than one reviewer in the assessment of potentially eligible trials to prevent the exclusion of reports that may in fact be relevant to the review (Edwards et al 2002), and to ensure that decisions regarding the inclusion of studies are based on judgements that can be reproduced (Green and Higgins 2005). Essentially, the use of two reviewers helps to minimise biased assessments of both the relevance and validity of articles (Green and Higgins 2005).

Data were extracted by the two reviewers using an agreed format, and again discrepancies resolved through discussion. Data were entered into Review Manager (RevMan) software (RevMan 2003) and double checked. Once decisions about the inclusion of each trial were made, the validity of each included trial was assessed according to the criteria outlined in the Cochrane Handbook (Green and Higgins 2005). Trials were assessed with a grade allocated to each trial on the basis of allocation concealment: A (adequate), B (unclear) or C (inadequate). Where the method of allocation concealment was unclear, attempts were made to contact authors to provide further details.

Blinding, completeness of follow up and use of placebo were assessed for each outcome using the following criteria (Table 2.1):

Table 2.1 Criteria for assessing blinding, completeness of follow up and use of placebo

Rating	Blinding	Completeness of follow up	Use of placebo
A	Double blind, neither investigator, outcome assessor or participant knew or were likely to guess the allocated treatment	Less than three percent of participants excluded	Placebo controlled
B	Single blind, either the investigator or the participant knew the allocation. Or, the trial is described as double blind, but side effects of one or other treatment mean that it is likely that for a significant proportion (≥20%) of participants the allocation could be correctly identified	Three to 9.9% of participants excluded	Unclear if placebo controlled
C	No blinding, both investigator and participant knew (or were likely to guess) the allocated treatment	10 to 19.9% of participants excluded	No placebo control
D	Unclear	If not possible to present the data by all participants analysed or if ≥20% of participants excluded	-

Statistical analyses were carried out using the RevMan software (RevMan 2003), with results presented as summary relative risks (RR). Tests of heterogeneity between trials were applied to assess the significance of any differences between trials (I^2 ≥50%), and possible causes of any heterogeneity were explored. The statistic used to determine heterogeneity was the I^2, which describes the "percentage of the variability in effect estimates that is due to heterogeneity rather than sampling error (chance)" (Green and Higgins 2005). For the I^2 statistic, a value of 50 percent or greater is considered substantial heterogeneity (Green and Higgins 2005). Summary relative risks were calculated using a fixed effects model. If heterogeneity was detected, subgroup analyses for the main outcomes were performed by risk of pre-eclampsia, gestational age at trial entry, type of antioxidant used, antioxidant dosage, use of antioxidants alone or in combination with other agents and prior dietary intake of antioxidants. Heterogeneity that was not explained by subgroup analyses was modelled using random effects analysis.

Subgroup analyses based on antioxidant dosage were undertaken according to the dosage of vitamin C, as there was a range of vitamin C dosages used in the included studies. For vitamin C, maximum circulating concentrations of vitamin C in the body have been show to result from taking vitamin C in doses ranging from 400 mg (Levine 2001) to 1,000 mg (Levine 1996). For vitamin E, an oral dose of 400 IU has been shown to have antioxidant effects

(Devaraj et al 1997). Little is known about effective pharmacological doses of lycopene and selenium, and trials involving these interventions (Han and Zhou 1994; Sharma et al 2003) were included as 'unspecified' in the analyses.

All included trials were included in the initial analyses and sensitivity analyses were carried out to explore the effect of trial quality. This involved analysis based on an A, B, C or D rating of allocation concealment, blinding of assessment of outcome and placebo control. The results of high quality studies were compared with those of poorer quality studies, where studies rated A were compared with those rated B or C.

Our pre-specified subgroup analyses for the primary outcomes were based on comparing:
(a) Women who are at low/moderate or high risk of pre-eclampsia;
(b) Women randomised in the first rather than the second half of pregnancy (before or after 20 weeks' gestation);
(c) The type of antioxidant supplement(s);
(d) The dosage of the antioxidant supplement(s) (above, within or below accepted pharmacological range);
(e) Whether antioxidants were given alone or in combination with other agents;
(f) Women who have low or adequate dietary antioxidant(s) intake (where applicable) before trial entry.

If antioxidants were effective, further analyses aimed to determine which of these agents were best, what was the ideal dose, and to compare antioxidants with other interventions.

2.2.5 Description of studies

Seven trials involving 6,082 women were included in the review, and many of the trials were reported in serial publications (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000; Sharma et al 2003; Steyn et al 2002, 2003).

Four of these trials enrolled women at "high risk of pre-eclampsia" (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; Rivas-Echeverria et al 2000). In the three studies that listed the criteria for determining high risk women, the criteria varied considerably. They included: previous pre-eclampsia, chronic hypertension, insulin requiring diabetes mellitus or multiple gestation (Beazley et al 2002, 2005); abnormal uterine artery Doppler at 18-22

weeks' gestation or a history in the preceding pregnancy of pre-eclampsia necessitating delivery before 37 weeks' gestation, eclampsia or HELLP syndrome (Chappell et al 1999); or nulliparity, previous pre-eclampsia, obesity, hypertension, less than 20 years old, diabetes, nephropathy, mean arterial pressure above 85 mmHg, positive roll-over test, black race, family history of hypertension or pre-eclampsia, twin pregnancy and poor socioeconomic conditions (Rivas-Echeverria et al 2000).

The timing of commencement of supplementation differed widely between trials. One trial enrolled women between 14 and 20 weeks' gestation (Beazley et al 2002, 2005), while others enrolled women between 16 and 20 to 22 weeks' gestation (Chappell et al 1999; Sharma et al 2003), below 24 weeks' gestation (People's League of Health 1942, 1946), below 26 weeks' gestation (Steyn et al 2002, 2003); below 29 weeks' gestation (Rivas-Echeverria et al 2000) and one trial merely stated "during late pregnancy" (Han and Zhou 1994).

2.2.5.1 Interventions

The antioxidant interventions assessed included supplementation with vitamin C alone (Steyn et al 2002, 2003), vitamin C and vitamin E alone (Beazley et al 2002, 2005; Chappell et al 1999), vitamin C and vitamin E in addition to fish oil and aspirin (Rivas-Echeverria et al 2000), multivitamin containing vitamin C (People's League of Health 1942, 1946), selenium (Han and Zhou 1994) and lycopene (Sharma et al 2003). For the trials involving supplementation with vitamin C, the daily dosages varied from 100 mg to 1,000 mg. For vitamin E, all of the trials used the same dosage of daily 400 IU. For selenium, the daily dose was 100 mg and for lycopene the daily dose was four milligrams.

2.2.5.2 Primary outcomes

All of the trials reported pre-eclampsia, either as pre-eclampsia (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000; Sharma et al 2003; Steyn et al 2002, 2003), toxaemia (People's League of Health 1942, 1946) or pregnancy induced hypertension (PIH) (Han and Zhou 1994). Two trials (Chappell et al 1999; Sharma et al 2003) defined pre-eclampsia according to the definitions specified by the International Society for the Study of Hypertension in Pregnancy (ISSHP) (two recordings of diastolic blood pressure of 90 mmHg at least 4 hours apart plus proteinuria defined as excretion of 300 mg or more in 24 hours or two readings of 2+ or higher on dipstick analysis) and the oldest trial defined pre-eclampsia as "hypertension occurring with albuminuria" (People's League of Health 1942, 1946). Four trials did not specify how pre-eclampsia was defined (Beazley et al 2002, 2005; Han and

Zhou 1994; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003). No trials categorised the timing of onset of pre-eclampsia (either prior to or after 34 weeks' gestation) or reported severe pre-eclampsia. Three trials reported preterm birth (Beazley et al 2002, 2005; Chappell et al 1999; Steyn et al 2002, 2003), no trials reported very preterm birth and three trials reported small for gestational age infants as birth weight less than the 10^{th} centile (Beazley et al 2002, 2005; Chappell et al 1999; Sharma et al 2003). Stillbirth was reported by three trials (Chappell et al 1999; People's League of Health 1942, 1946; Steyn et al 2002, 2003) and neonatal death reported by two trials (People's League of Health 1942, 1946; Steyn et al 2002, 2003). No trials reported infant death.

2.2.5.3 Secondary outcomes

No trials reported maternal death, severe hypertension, caesarean section or maternal views of care. Maternal bleeding episodes were reported as placental abruption by one trial (Chappell et al 1999) and antepartum haemorrhage including placental abruption by another trial (Steyn et al 2002, 2003). Induction of labour was reported by one trial (Steyn et al 2002, 2003); eclampsia by one trial (Sharma et al 2003); gestational age at birth by two trials (Beazley et al 2002, 2005; Sharma et al 2003) and birth weight by three trials (Beazley et al 2002, 2005; Han and Zhou 1994; Sharma et al 2003). For infant outcomes, no trials reported Apgar score less than four at five minutes, respiratory distress syndrome, chronic lung disease, bleeding episodes, bacterial sepsis, necrotising enterocolitis, retinopathy of prematurity, disability during childhood or poor childhood growth.

No trials reported any maternal or infant side effects, either sufficient or not sufficient to stop supplementation, and no trials reported data on use of health service resources for the woman. One trial reported duration of hospitalisation for the infant (Steyn et al 2002, 2003).

One trial reported hypertension (Steyn et al 2002, 2003) however no other information was provided.

2.2.5.4 Excluded trials

Thirteen studies were excluded from the review. Five studies were excluded as women involved had established pre-eclampsia at trial entry (Anthony et al 1996; Gulmezoglu, Hofmeyer and Oosthuisen 1997; Morrison, O'Brien and Micklewright 1984; Sawhney et al 2000; Sikkema, Franz and Bruinse 2002); three studies were excluded as the interventions assessed were not considered to have direct antioxidant properties (Ferguson 1955; Herrera

1993; Marya, Rathee and Manrow 1987); two studies were not randomised (Bolisetty et al 2002; Power, Fraser and Gibson 2000); two studies did not report any clinically meaningful outcomes or pre-eclampsia (Pawlowicz et al 2000; Pressman et al 2003) and one study (Chaudhuri 1969) had greater than 20 percent losses to follow up.

2.2.5.5 Methodological quality of included studies

Randomisation and allocation concealment

Formal randomisation was reported in three trials by use of third party randomisation (Chappell et al 1999; Sharma et al 2003; Steyn et al 2002, 2003), that is, women were allocated to each group either by an individual not directly involved in the research or via telephone or computer allocation. The degree of allocation concealment for these three trials was therefore adequate. For three trials (Beazley et al 2002, 2005; Han and Zhou 1994; Rivas-Echeverria et al 2000), the degree of allocation concealment was unclear, as no information was provided about the method of randomisation or allocation concealment. One trial (People's League of Health 1942, 1946) was quasi-randomised, with women allocated treatments according to alternate lists and allocation concealment was therefore inadequate.

Blinding

Two trials explicitly stated that women, caregivers and researchers were blinded to treatment allocations (Chappell et al 1999; Sharma et al 2003; Steyn et al 2002, 2003). One trial stated "double-blind" in the text (Beazley et al 2002, 2005) while the other trial used the term "triple-blind" in the text (Rivas-Echeverria et al 2000). The degree of blinding if any, was unclear for two trials (Han and Zhou 1994; People's League of Health 1942, 1946).

Completeness of follow up

Two trials either reported outcomes for all randomised women according to treatment allocation (Chappell et al 1999; Sharma et al 2003) and another three did not mention any losses to follow up (Han and Zhou 1994; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003). In the two remaining trials, losses to follow up included nine women (8%) (Beazley et al 2002, 2005) and 622 women (11%) (People's League of Health 1942, 1946).

Use of placebo

Six trials used a placebo control (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; Rivas-Echeverria et al 2000; Sharma et al 2003; Steyn et al 2002, 2003). The contents of the placebo used were explicitly stated by two trials as microcrystalline cellulose

and soya bean oil (Chappell et al 1999) and soya bean oil and bees wax (Sharma et al 2003). No details of the placebo preparation were disclosed for the remaining four trials (Beazley et al 2002, 2005; Han and Zhou 1994; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003). The one quasi-randomised trial did not use a placebo control (People's League of Health 1942, 1946).

2.2.6 Results

2.2.6.1 Pre-eclampsia

All seven trials involving 6,082 women comparing any antioxidants with placebo or no antioxidants reported pre-eclampsia (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000; Sharma et al 2003; Steyn et al 2002, 2003). Supplementation with any antioxidants during pregnancy compared with control or placebo was associated with a 39 percent reduction in the relative risk of pre-eclampsia (RR 0.61, 95% CI 0.50 to 0.75), when using a fixed effects model (Figure 2.2). When the quasi-random study (People's League of Health 1942, 1946) was excluded, supplementation with any antioxidants was associated with a 54 percent reduction in the relative risk of pre-eclampsia (RR 0.46, 95% CI 0.32 to 0.66, six trials, 1,061 women). No trials categorised pre-eclampsia according to the timing of onset, and no trials reported severe pre-eclampsia.

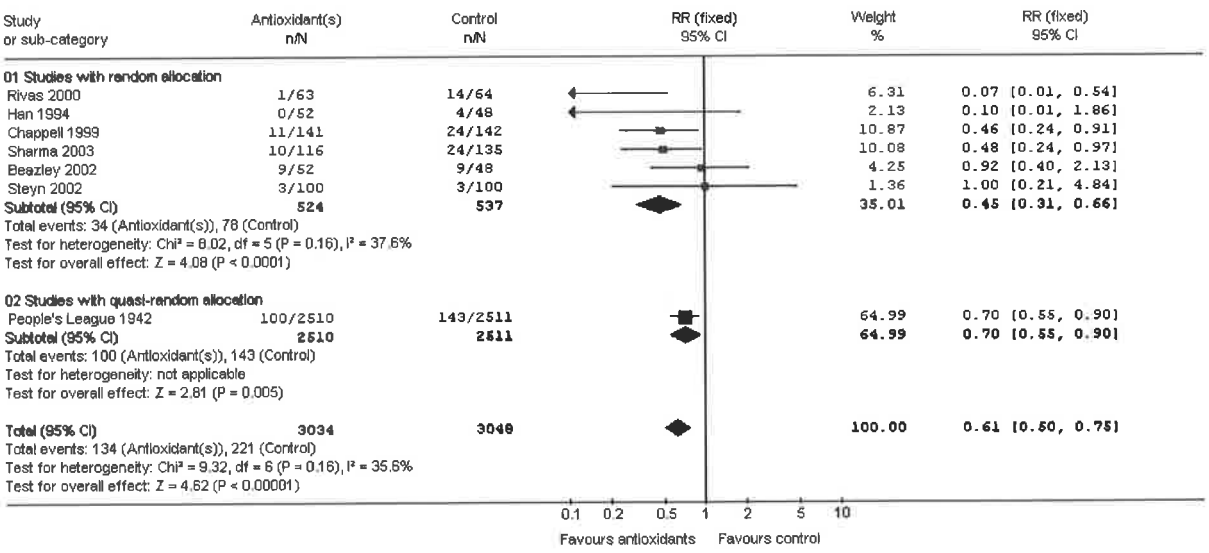

Figure 2.2 Meta-analyses of the effect of any antioxidants and the risk of pre-eclampsia (subgrouped on random or quasi–random allocation).

2.2.6.2 Preterm birth

Three trials involving 583 women (Beazley et al 2002, 2005; Chappell et al 1999; Steyn et al 2002, 2003) reported preterm birth (<37 weeks' gestation) in a format for inclusion in the review. None of these trials were quasi-randomised. Women supplemented with any antioxidants compared with placebo were at increased risk of giving birth preterm (RR 1.38, 95% CI 1.04 to 1.82) (Figure 2.3). No trials reported data for very preterm birth (<34 weeks' gestation).

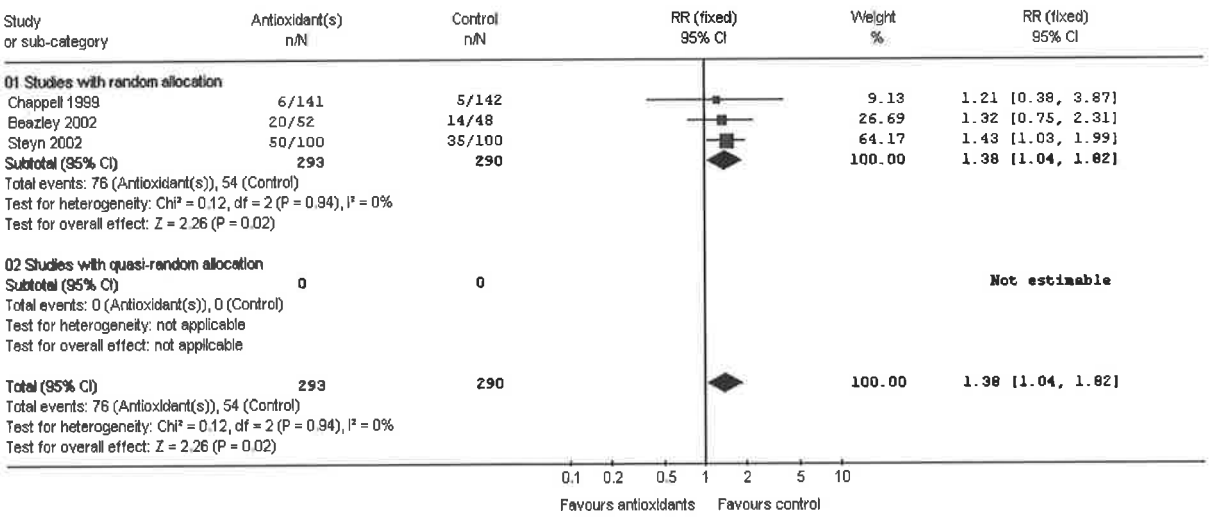

Figure 2.3 Meta-analyses of the effect of any antioxidants and the risk of preterm birth (subgrouped on random or quasi–random allocation).

2.2.6.3 Small for gestational age infants

Three trials involving 634 women (Beazley et al 2002, 2005; Chappell et al 1999; Sharma et al 2003) reported birth weight less than the 10th centile. None of these trials were quasi-randomised. Women supplemented with antioxidants compared with placebo had a reduced risk of having a small for gestational age infant (RR 0.64, 95% CI 0.47 to 0.87) (Figure 2.4).

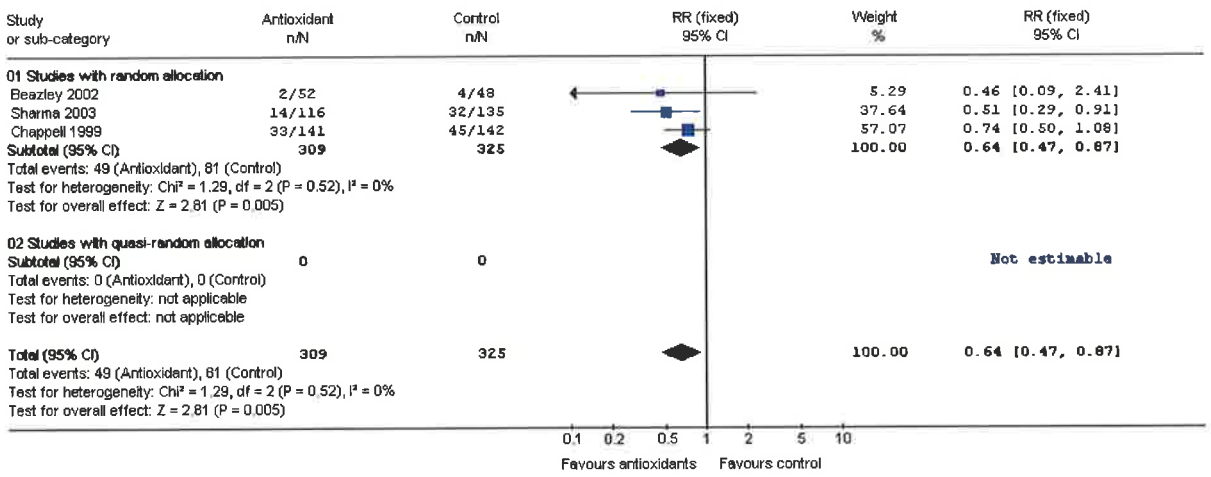

Figure 2.4 Meta-analyses of the effect of any antioxidants and the risk of small for gestational age infants (subgrouped on random or quasi–random allocation).

2.2.6.4 Baby death

Three trials involving 5,504 women (Chappell et al 1999; People's League of Health 1942, 1946; Steyn et al 2002, 2003) reported stillbirth. No difference was demonstrated in the risk of having a stillbirth between women supplemented with antioxidants compared with control (RR 0.83, 95% CI 0.59 to 1.17) (Figure 2.5). When the quasi-randomised trial (People's League of Health 1942, 1946) was excluded from the analyses, no difference was seen for the risk of stillbirth (RR 1.00, 95% CI 0.18 to 5.76, two trials, 483 women).

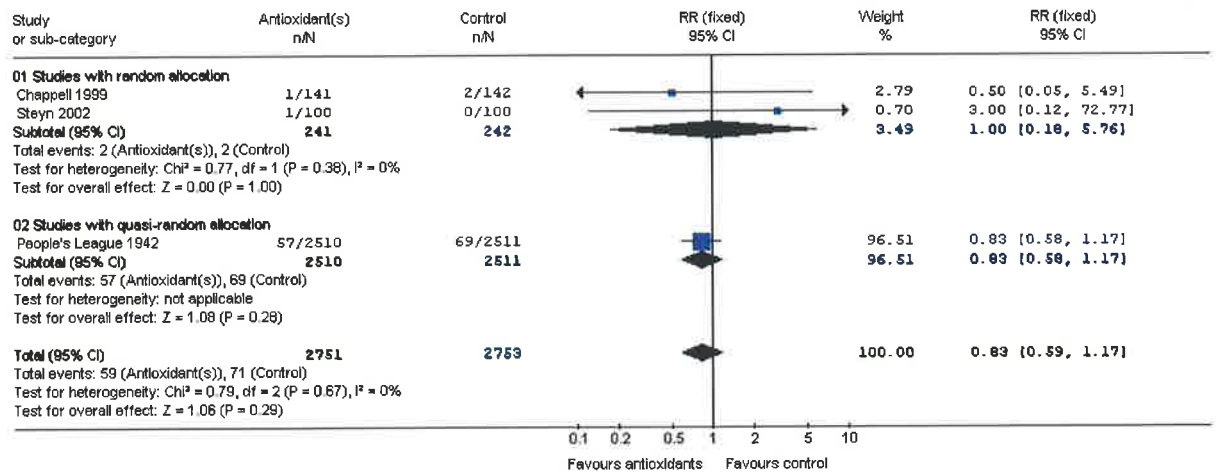

Figure 2.5 Meta-analyses of the effect of any antioxidants and the risk of stillbirth (subgrouped on random or quasi–random allocation).

Two trials involving 5,076 women reported neonatal death (People's League of Health 1942, 1946; Steyn et al 2002, 2003). No difference was seen for the risk of neonatal death between women supplemented with any antioxidants compared with control (RR 1.33, 95% CI 0.81 to

2.19) (Figure 2.6). Similarly, no difference was seen for the risk of neonatal death when the quasi-randomised trial (People's League of Health 1942, 1946) was excluded from the analyses (RR 0.69, 95% CI 0.12 to 4.03, one trial, 181 women).

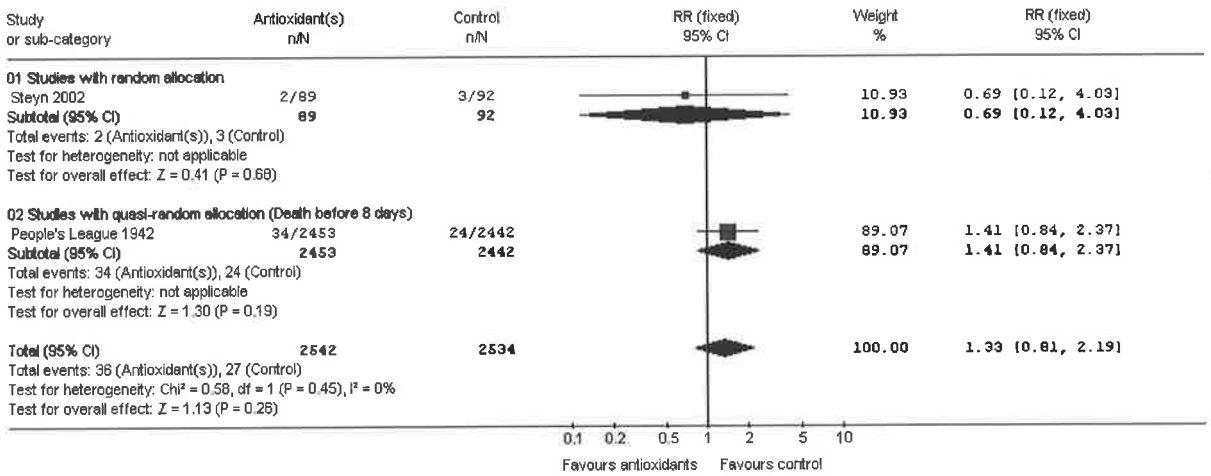

Figure 2.6 Meta-analyses of the effect of any antioxidants and the risk of neonatal death (subgrouped on random or quasi–random allocation).

2.2.6.5 Secondary outcomes

Antioxidant supplementation compared with control or placebo was associated with a greater mean birth weight (Weighted Mean Difference (WMD) 91.83 g, 95% CI 11.55 to 172.11, three trials, 451 women (Beazley et al 2002, 2005; Han and Zhou 1994; Sharma et al 2003)). There was insufficient evidence for reliable conclusions about the potential effects on any other outcomes including the need for induction of labour (RR 2.00, 95% CI 0.51 to 7.78, one trial, 200 women (Steyn et al 2002, 2003)); the risk of eclampsia (RR 0.38, 95% CI 0.02 to 9.54, one trial, 251 women (Sharma et al 2003)); gestational age at birth (WMD 1.01 weeks, 95% CI 0.56 to 1.46, two trials, 351 women (Beazley et al 2002, 2005; Sharma et al 2003)) or neonatal length of stay in hospital (WMD 1.30 days, 95% CI -0.28 to 2.88, one trial, 181 women (Steyn et al 2002, 2003)). Significant heterogeneity was demonstrated for the risk of bleeding episodes. No significant difference was demonstrated between women supplemented with antioxidants compared with placebo for the risk of bleeding episodes (RR 1.58, 95% CI 0.08 to 31.47, two trials, 483 women (Chappell et al 1999; Steyn et al 2002, 2003), using a random effects model.

2.2.7 Sensitivity analyses based on Trial Quality

Assessments of the treatment effects were made for the primary outcomes based on trial quality. Only three trials (Chappell et al 1999; Sharma et al 2003; Steyn et al 2002, 2003) fulfilled all of the criteria for a high quality trial, that is they were rated A for allocation concealment; women, caregivers and research staff were blinded to treatment allocation; had less than three percent of participants excluded and were placebo controlled. Four trials were not rated high quality (Beazley et al 2002, 2005; Han and Zhou 1994; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000), and were excluded from the analyses. Women receiving antioxidant supplementation compared with placebo had a significantly reduced risk of pre-eclampsia for trials rated high quality (RR 0.51, 95% CI 0.32 to 0.80, three trials, 734 women) (Figure 2.7).

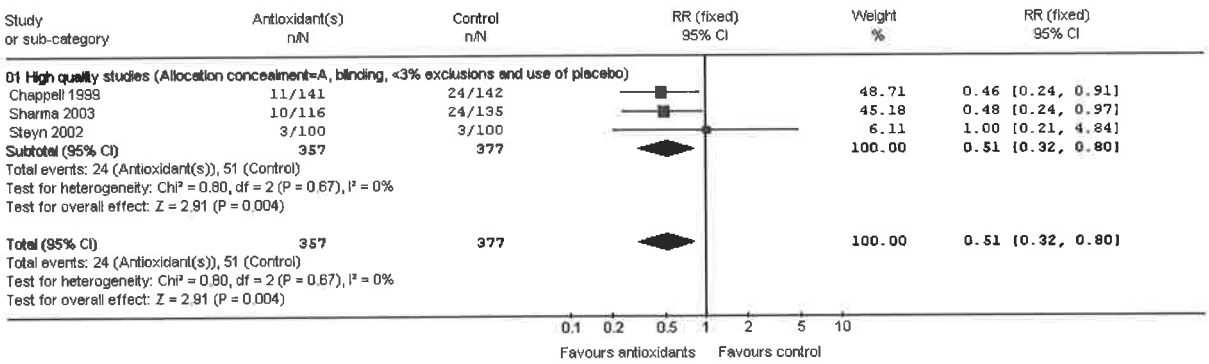

Figure 2.7 Meta-analyses of the effect of any antioxidants and the risk of pre-eclampsia (high quality trials only).

Women supplemented with antioxidants compared with placebo had an increased risk of giving birth preterm for trials rated high quality (RR 1.40, 95% CI 1.02 to 1.93, two trials, 483 women (Chappell et al 1999; Steyn et al 2002, 2003)) (Figure 2.8).

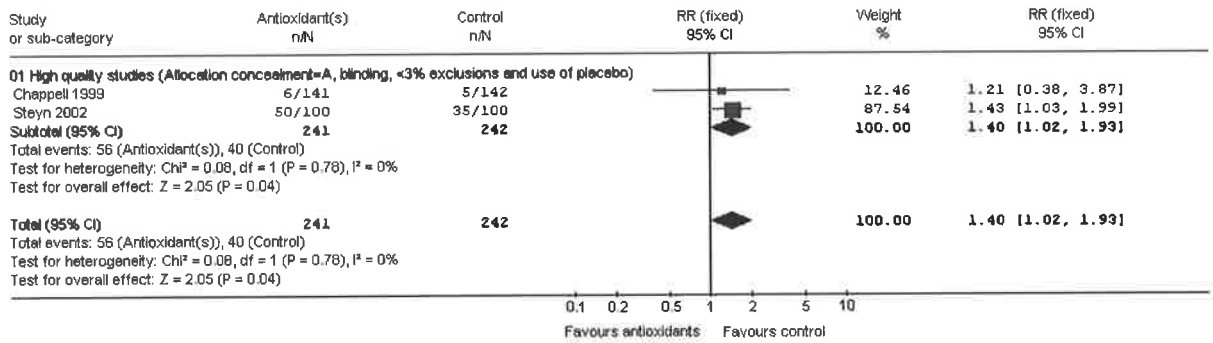

Figure 2.8 Meta-analyses of the effect of any antioxidants and the risk of preterm birth (high quality trials only).

Women receiving antioxidants compared with placebo were less likely to have a small for gestational age infant for trials rated high quality (RR 0.65, 95% CI 0.47 to 0.89, two trials, 534 women (Chappell et al 1999; Sharma et al 2003)) (Figure 2.9).

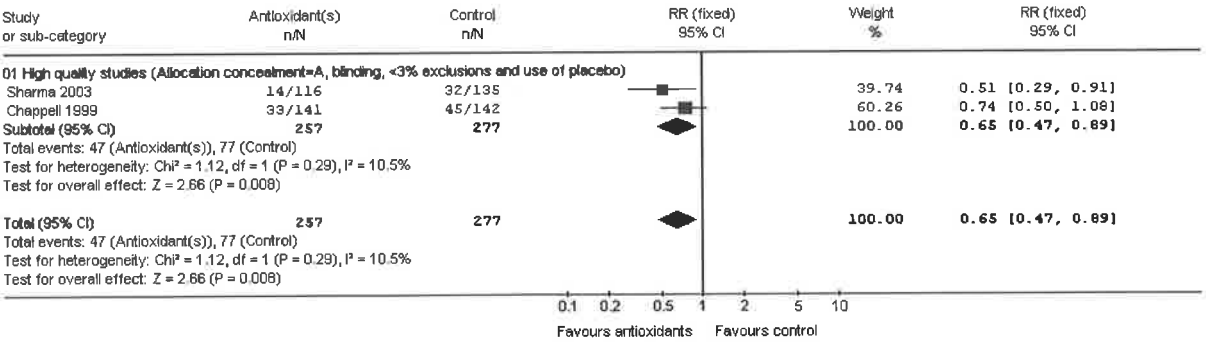

Figure 2.9 Meta-analyses of the effect of any antioxidants and the risk of small for gestational age infants (high quality trials only).

No difference was seen between women supplemented with antioxidants compared with placebo for the risk of stillbirth (RR 1.00, 95% CI 0.18 to 5.76, two trials, 483 women (Chappell et al 1999; Steyn et al 2002, 2003)) or neonatal death (RR 0.69, 95% CI 0.12 to 4.03, one trial, 181 women (Steyn et al 2002, 2003)), for trials rated high quality.

2.2.8 Subgroup analyses

2.2.8.1 Women's level of risk at trial entry of developing pre-eclampsia

Assessments of the treatment effects were made for the primary outcomes based on women's risk of developing pre-eclampsia at trial entry. Four trials (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; Rivas-Echeverria et al 2000) enrolled women who were at "high risk of pre-eclampsia". However, according to the criteria pre-specified in the review, three trials (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000) included women in both the high and moderate/low risk categories. These studies are grouped as moderate/high risk in this review, as they included high and moderate risk women. Nevertheless, most women actually recruited to these studies were probably high risk, as 19 percent in the control group developed pre-eclampsia, compared with six percent for the studies enrolling moderate/low risk women. The fourth trial (Han and Zhou 1994) did not specify the inclusion criteria, and was classified as moderate/low risk. Four trials enrolled women classified as moderate/low risk (Han and Zhou 1994; People's League of Health 1942, 1946; Sharma et al 2003; Steyn et al 2002, 2003). Comparisons were made between treatment

groups for women classified as moderate/low risk and moderate/high risk. The results for the primary outcomes are listed in Table 2.2.

Table 2.2 Primary outcomes (sub-grouped on risk status for pre-eclampsia)

Outcome	No. of trials	Antioxidant group	Control group	RR	95% CI	Statistical heterogeneity
Moderate/ Low risk						
Pre-eclampsia	4	117/2,778	181/2,794	0.65	0.52 to 0.82	I^2=0%
Preterm birth	1	50/100	35/100	1.43	1.03 to 1.99	N/A
SGA	1	14/116	32/135	0.51	0.29 to 0.91	N/A
Stillbirth	2	58/2,610	69/2,611	0.84	0.60 to 1.19	I^2=0%
Neonatal death	2	36/2,542	27/2,534	1.33	0.81 to 2.19	I^2=0%
Moderate/ High Risk						
Pre-eclampsia	3	21/256	47/254	0.44	0.27 to 0.71	I^2=67.6%
Preterm birth	2	26/193	19/190	1.29	0.78 to 2.15	I^2=0%
SGA	2	35/193	49/190	0.72	0.49 to 1.04	I^2=0%
Stillbirth	1	1/141	2/142	0.50	0.05 to 5.49	N/A
Neonatal death	0	-	-	-	-	-

SGA= small for gestational age infant, RR= relative risk, CI= confidence intervals, N/A= not applicable
I^2 = measure of statistical heterogeneity

2.2.8.1.1 Antioxidants for women at moderate/high risk of pre-eclampsia

No trials included women that could be exclusively categorised according to our high risk criteria defined as one or more of the following: previous severe pre-eclampsia, diabetes, chronic hypertension, renal disease or autoimmune disease. However, three trials included women in both the high and low risk categories. For trials including women with moderate/high risk status, women receiving any antioxidants compared with control or placebo had a reduced risk of pre-eclampsia (RR 0.44, 95% CI 0.27 to 0.71, three trials, 510 women (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000)). The magnitude of the treatment effect was greater than for the trials enrolling women at moderate/low risk. No difference was seen between women supplemented with antioxidants compared with control for trials including women with moderate/high risk status for the outcomes preterm birth (RR 1.29, 95% CI 0.78 to 2.15, two trials, 383 women (Beazley et al 2002, 2005; Chappell et al 1999), small for gestational age infants (RR 0.72, 95% CI 0.49 to 1.04, two trials, 383 women (Beazley et al 2002, 2005; Chappell et al 1999)) or stillbirth (RR 0.50, 95% CI 0.05 to 5.49, one trial, 283 women (Chappell et al 1999)). No data were available for the risk of neonatal death for moderate/high risk women.

2.2.8.1.2 Antioxidants for women at moderate/low risk of pre-eclampsia

In the trials including women at moderate/low risk, women supplemented with any antioxidants compared with control or placebo had a reduced risk of pre-eclampsia (RR 0.65, 95% CI 0.52 to 0.82, four trials, 5,572 women (Han and Zhou 1994; People's League of Health 1942, 1946; Sharma et al 2003; Steyn et al 2002, 2003)) and having a small for gestational age infant (RR 0.51, 95% CI 0.29 to 0.91, one trial, 251 women (Sharma et al 2003)). Conversely, women at moderate/low risk and supplemented with any antioxidants compared with placebo were at increased risk of giving birth preterm (RR 1.43, 95% CI 1.03 to 1.99, one trial, 200 women (Steyn et al 2002, 2003)). No difference was seen in the risk of stillbirth (RR 0.84, 95% CI 0.60 to 1.19, two trials, 5,221 women (People's League of Health 1942, 1946; Steyn et al 2002, 2003)) or neonatal death (1.33, 95% CI 0.81 to 2.19, two trials, 5,076 women (People's League of Health 1942, 1946; Steyn et al 2002, 2003)).

2.2.8.2 Women's gestational age at trial entry

Assessments of the treatment effects were made for the primary outcomes based on women's gestational age at trial entry. Two trials (Beazley et al 2002, 2005; Sharma et al 2003) enrolled women less than 20 weeks' gestation, one trial (Han and Zhou 1994) enrolled women after 20 weeks' gestation, and four trials (Chappell et al 1999; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003) enrolled women both prior to and after 20 weeks' gestation. The results for the primary outcomes are detailed in Table 2.3.

Table 2.3 Primary outcomes (sub-grouped on gestational age at trial entry)

Outcome	No. of trials	Antioxidant group	Control group	RR	95% CI	Statistical heterogeneity
Entered into study < 20 weeks'						
Pre-eclampsia	2	19/168	33/183	0.61	0.36 to 1.04	I^2=26.2%
Preterm birth	1	20/52	14/48	1.32	0.75 to 2.31	N/A
SGA	2	16/168	36/183	0.50	0.29 to 0.87	I^2=0%
Stillbirth	0	-	-	-	-	-
Neonatal death	0	-	-	-	-	-
Entered into study ≥ 20 weeks'						
Pre-eclampsia	1	4/52	11/48	0.34	0.11 to 0.98	N/A
Preterm birth	0	-	-	-	-	-
SGA	0	-	-	-	-	-
Stillbirth	0	-	-	-	-	-
Neonatal death	0	-	-	-	-	-
Entered into study both < and ≥ 20 weeks'						
Pre-eclampsia	4	115/2,814	184/2,817	0.63	0.50 to 0.79	I^2=52.8%
Preterm birth	2	56/241	40/242	1.40	1.02 to 1.93	I^2=0%
SGA	1	33/141	45/142	0.74	0.50 to 1.08	N/A
Stillbirth	3	59/2,751	71/2,753	0.83	0.59 to 1.17	I^2=0%
Neonatal death	2	36/2,542	27/2,534	1.33	0.81 to 2.19	I^2=0%

SGA= small for gestational age infant, RR= relative risk, CI= confidence intervals, N/A= not applicable
I^2 = measure of statistical heterogeneity

2.2.8.2.1 Gestation < 20 weeks' at trial entry

No benefit was seen for antioxidants and the risk of pre-eclampsia for women enrolled at less than 20 weeks' gestation (RR 0.61, 95% CI 0.36 to 1.04, two trials, 351 women (Beazley et al 2002, 2005; Sharma et al 2003)), although given the small number of women involved, these results should be interpreted with caution. Furthermore, women supplemented with antioxidants were at a decreased risk of having a small for gestational age infant if they were enrolled at less than 20 weeks' gestation (RR 0.50, 95% CI 0.29 to 0.87, two trials, 351 women (Beazley et al 2002, 2005; Sharma et al 2003)). No difference was seen for the risk of preterm birth (RR 1.32, 95% CI 0.75 to 2.31, one trial, 100 women (Beazley et al 2002, 2005)) and no data were available for the outcomes stillbirth and neonatal death.

2.2.8.2.2 Gestation ≥ 20 weeks' at trial entry

Women supplemented with antioxidants were at a reduced risk of pre-eclampsia if they were enrolled after 20 weeks' gestation (RR 0.34, 95% CI 0.11 to 0.98, one trial, 100 women (Han and Zhou 1994)), however given the small number of women involved, these results again, should be interpreted with caution. No data were available for the outcomes preterm birth, small for gestational age infants, stillbirth or neonatal death.

2.2.8.2.3 Gestation both prior to and greater or equal to 20 weeks' at trial entry

Women supplemented with antioxidants in the trials enrolling women both prior to and after 20 weeks' gestation had a reduced risk of pre-eclampsia (RR 0.63, 95% CI 0.50 to 0.79, four trials, 5,631 women (Chappell et al 1999; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003)). Conversely, these women appeared to have an increased risk of preterm birth (RR 1.40, 95% CI 1.02 to 1.93, two trials, 483 women (Chappell et al 1999; Steyn et al 2002, 2003)). No difference was seen for the risk of small for gestational age infants, stillbirth or neonatal death, in the trials enrolling women both prior to and greater or equal to 20 weeks' gestation.

2.2.8.3 The type of antioxidant(s)

Each trial was classified according to the type of antioxidant being assessed, either vitamins, minerals or non-vitamin antioxidants. Six trials (Beazley et al 2002, 2005; Chappell et al 1999; People's League of Health 1942, 1946; Rivas-Echeverria et al 2000; Sharma et al 2003; Steyn et al 2002, 2003) assessed vitamin antioxidants and one trial (Han and Zhou 1994) assessed a mineral antioxidant. As the only trial assessing a mineral antioxidant was small (n=100), the data were too few to make any meaningful comparisons, and subgroup analyses based on antioxidant type were not performed.

2.2.8.4 The dosage of antioxidant(s)

Each trial was classified according to the dose of the antioxidant being above, within or below accepted pharmacological range for antioxidant effects, where information on pharmacological ranges is available. For this review, comparisons were made for the trials involving vitamin C supplementation where there was a range of dosages between the trials. For vitamin C, four trials used a dosage within expected pharmacologic range (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003) and one

trial (People's League of Health 1942, 1946) used a dosage below expected pharmacological range. The results for the primary outcomes are detailed in Table 2.4.

Table 2.4 Primary outcomes (sub-grouped on dosage of antioxidant)

Outcome	No. of trials	Antioxidant group	Control group	RR	95% CI	Statistical heterogeneity
High dose (within pharmacological range)						
Pre-eclampsia	4	24/356	50/354	0.47	0.30 to 0.75	I^2=55.3%
Preterm birth	N/A	-	-	-	-	-
SGA	N/A	-	-	-	-	-
Stillbirth	2	2/241	2/242	1.00	0.18 to 5.76	I^2=0%
Neonatal death	1	2/89	3/92	0.69	0.12 to 4.03	N/A
Low dose (below pharmacological range)						
Pre-eclampsia	1	100/2,510	143/2,511	0.70	0.55 to 0.90	N/A
Preterm birth	N/A	-	-	-	-	-
SGA	N/A	-	-	-	-	-
Stillbirth	1	57/2,510	69/2,511	0.83	1.58 to 1.17	N/A
Neonatal death	1	34/2,453	24/2,442	1.41	0.84 to 2.37	N/A
Unspecified						
Pre-eclampsia	2	14/168	35/183	0.43	0.24 to 0.78	I^2=0%
Preterm birth	N/A	-	-	-	-	-
SGA	N/A	-	-	-	-	-
Stillbirth	0	-	-	-	-	-
Neonatal death	0	-	-	-	-	-

SGA= small for gestational age infant, RR= relative risk, CI= confidence intervals, N/A= not available
I^2 = measure of statistical heterogeneity

Women receiving any antioxidants compared with control or placebo had a reduced risk of pre-eclampsia regardless of whether vitamin C was given within pharmacological range (RR 0.47, 95% CI 0.30 to 0.75, four trials, 710 women (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000; Steyn et al 2002, 2003)), below pharmacological range (RR 0.70. 95% CI 0.55 to 0.90, one trial, 5,021 women (People's League of Health 1942, 1946)) or where the pharmacological range of the antioxidant assessed was unspecified (RR 0.43, 95% CI 0.24 to 0.78, two trials, 351 women (Han and Zhou 1994; Sharma et al 2003)).

No difference was seen in the risk of stillbirth or neonatal death, regardless of whether vitamin C was given in a dosage within pharmacological range or below pharmacological range. For the outcomes preterm birth and small for gestational age infants, all of the included

trials were either within pharmacological range (Beazley et al 2002, 2005; Chappell et al 1999) or the range is unknown (Sharma et al 2003), therefore subanalyses based on dosage were not able to be performed.

2.2.8.5 Use of antioxidants combined with other agents

Assessments of the treatment effects were made based on whether the antioxidant preparations were given alone or in combination with other agents. Five trials (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; Sharma et al 2003; Steyn et al 2002, 2003) supplemented women with antioxidants alone. Two trials supplemented women with antioxidants combined with other agents including other vitamins and minerals (People's League of Health 1942, 1946) and aspirin and fish oil (Rivas-Echeverria et al 2000). The results for the primary outcomes are listed in Table 2.5.

Table 2.5 Primary outcomes (sub-grouped on antioxidant combination)

Outcome	No. of trials	Antioxidant group	Control group	RR	95% CI	Statistical heterogeneity
Antioxidants alone						
Pre-eclampsia	5	37/461	71/473	0.54	0.36 to 0.80	I^2=0%
Preterm birth	N/A	-	-	-	-	-
SGA	N/A	-	-	-	-	-
Stillbirth	2	2/241	2/242	1.00	0.18 to 5.76	I^2=0%
Neonatal death	1	2/89	3/92	0.69	0.12 to 4.03	N/A
Antioxidants combined with other agents						
Pre-eclampsia	2	101/2,573	143/2,511	0.64	0.50 to 0.82	I^2=80.0%
Preterm birth	N/A	-	-	-	-	-
SGA	N/A	-	-	-	-	-
Stillbirth	1	57/2,510	69/2,511	0.83	0.58 to 1.17	N/A
Neonatal death	1	34/2,453	24/2,442	1.41	0.84 to 2.37	-

SGA= small for gestational age infant, RR= relative risk, CI= confidence intervals, N/A= not available
I^2 = measure of statistical heterogeneity

Women supplemented with antioxidants alone compared with control or placebo had a reduced risk of pre-eclampsia (RR 0.54, 95% CI 0.36 to 0.80, five trials, 934 women (Beazley et al 2002, 2005; Chappell et al 1999; Han and Zhou 1994; Sharma et al 2003; Steyn et al 2002, 2003)). Similarly, women supplemented with antioxidants combined with other agents compared with control or placebo had a reduced risk of pre-eclampsia (RR 0.64, 95% CI 0.50

to 0.82, two trials, 5,148 women (People's League of Health 1942, 1946; Rivas-Echeverria et al 2000)), although there was significant heterogeneity detected in this comparison.

For preterm birth and the risk of small for gestational age infants, all of the included trials supplemented women with antioxidants alone, therefore subanalyses based on use of antioxidants combined with other agents were not able to be performed for these outcomes. No difference was seen in the risk of stillbirth or neonatal death for women supplemented with antioxidants alone compared with placebo or for women supplemented with antioxidants combined with other agents compared with control.

2.2.8.6 Women's dietary antioxidant intake before or at trial entry

Only one trial (People's League of Health 1942, 1946) reported information about women's dietary intake at trial entry. Approximately half of the women in this trial had a dietary vitamin C deficiency. There are therefore insufficient data for any reliable conclusions about any possible differences in the effect of antioxidants based on dietary intake at trial entry.

2.2.9 Discussion

Women allocated antioxidants, either alone or in combination with other supplements, rather than placebo or no antioxidant had a significantly reduced risk of developing pre-eclampsia or having a small for gestational age baby. There were insufficient data for any reliable conclusions about the possible impact of the pre-specified subgroups comparing women's risk status for pre-eclampsia, or to provide guidance on the optimal type and dosage of antioxidant(s) or timing of supplementation.

Much of the current data come from poor quality studies, mainly the quasi-randomised study. The analyses using high quality studies include just 734 women, however the effects of antioxidants were consistent across poor and high quality studies, where antioxidants were associated with a reduced risk of pre-eclampsia in the high quality studies. By far the largest trial included in the review (People's League of Health 1942, 1946) was quasi-randomised, included multiple nutritional interventions and involved women in wartime conditions. For these women, the impact of supplementation with a multivitamin on their general nutrition cannot be ignored. In this study, approximately half of all women had a dietary vitamin C deficiency at trial entry. Antioxidants did not appear to be more beneficial for these women. The multivitamin assessed in this study also contained small amounts of calcium, other minerals and fish oil. It is therefore unclear whether the treatment effects observed are due

exclusively to the antioxidant component (vitamin C) or even at all, given the low dosage of vitamin C used (100 mg) and the known benefits of calcium supplementation on the risk of pre-eclampsia (Atallah, Hofmeyr and Duley 2002). Similarly, one other trial assessed antioxidants in combination with aspirin and fish oil (Rivas-Echeverria et al 2000). Again, aspirin is associated with a reduced risk of pre-eclampsia (Duley et al 2003), which further limits the ability to directly attribute the treatment effects seen to antioxidants. Further studies are needed to directly establish a relationship between antioxidants given alone during pregnancy and the risk of pre-eclampsia.

Of concern, antioxidant supplementation was associated with a moderate increase in the risk of preterm birth. This finding may have been influenced by one trial (Steyn et al 2002, 2003), which assessed vitamin C supplementation alone in women already at high risk of giving birth preterm. The trial was stopped early after an interim analysis demonstrated an increase in the risk of preterm birth, however the authors noted that there was no corresponding increase in adverse neonatal outcome. This trial received the greatest weighting in the analyses for preterm birth. Further information on the effect of antioxidant supplementation on the risk of preterm birth is warranted.

There are insufficient data for reliable conclusions to be made about the effect, if any, of antioxidants on the risk of stillbirth, neonatal death or substantiative measures of morbidity for the baby. Few trials reported data for the secondary outcomes in this review. Only one small trial reported eclampsia and there were no data reported for other measures of serious maternal morbidity, maternal death, the timing of onset of pre-eclampsia or the severity of hypertension. Similarly there was no information on long-term development of the child or on the use of health care resources for the mother. Side effects or adverse events were not reported by any trials.

Although antioxidants appear to reduce the risk of pre-eclampsia, much more information is required about other possible benefits and hazards before there can be any reliable conclusions made about whether such therapy is, overall, worthwhile.

2.3 Conclusions

The current best available evidence about antioxidant supplementation in pregnancy is promising, however most of the data are contributed from poor quality studies. Women supplemented with antioxidants during pregnancy had a reduced risk of developing pre-

eclampsia and having a small for gestational age baby. However they also appeared to be at increased risk of preterm birth. A summary of the level, quality, relevance and strength of the current evidence synthesised from the review is presented below.

2.3.1 Current level of evidence for use of antioxidant supplements in pregnancy

There is Level 1 evidence supporting any antioxidant supplementation in pregnancy for the prevention of pre-eclampsia and having a small for gestational age infant. Antioxidant supplementation was associated with a 39 percent reduction in the relative risk of pre-eclampsia and 36 percent reduction in the relative risk of having a small for gestational age infant. Of concern, antioxidant supplementation was associated with a moderate increase in the risk of preterm birth. While the review may have been influenced by the inclusion of one trial involving women at high risk of preterm birth, further careful evaluation of the effects of antioxidants and the risk of preterm birth is clearly required. None of the included trials reported any serious adverse effects. However the adverse effects of supplementation warrant further consideration as few trials provided any data. Any future trials should collect information on side effects and adverse effects detected to enable an accurate evaluation of the safety of antioxidant use during pregnancy.

2.3.2 Quality of the evidence for use of antioxidants supplements in pregnancy

Only three trials involving 734 women were rated high quality. Further large good quality studies are required to confirm the reduced risks of pre-eclampsia and small for gestational age infants seen. Moreover, further high quality trials are required, with sufficiently large numbers of participants to permit a wider evaluation of all relevant maternal, infant and childhood outcomes.

2.3.3 Relevance of the evidence for use of antioxidant supplements in pregnancy

Two of the trials included in the review were undertaken in countries traditionally classed as having developing economies (low or low-middle income), including India and South Africa. Two trials were conducted in the United Kingdom, and the larger of these trials was carried out during the Second World War. The remaining two small trials involved women living in the United States of America or Venezuela. Given the well-established disparity in maternal and infant health outcomes between countries classed as developing compared with developed, the current findings from the systematic review of trials cannot be confidently

relayed across all clinical settings. Moreover to date, there have been no randomised controlled trials assessing antioxidants during pregnancy in an Australian population.

Few of the included trials reported on many of the relevant health outcomes specified in the review. Furthermore, as the majority of trials included involved only small numbers of women, these trials were clearly underpowered to detect serious maternal and infant morbidities and perinatal mortality. For the trials assessing vitamin C and vitamin E supplementation specifically, all trials involved either women at high risk of pre-eclampsia or preterm birth. The impact of antioxidant supplementation in pregnancy, particularly vitamin C and vitamin E for women at lower risk of pre-eclampsia is unknown and warrants further evaluation. Future trials are therefore needed to determine whether antioxidants may be of benefit to women in different settings and to women at high and low risk of pre-eclampsia.

2.3.4 Strength of the evidence for use of antioxidant supplements in pregnancy

The reduction in risk of pre-eclampsia found was substantial, with supplementation with any antioxidants, alone or with other agents, associated with an almost halving in the risk of pre-eclampsia, and a similar reduction in the risk of having a small for gestational age infant. However, a direct causality cannot be determined due to the use of other agents combined with the antioxidant preparations. Several of these non-antioxidant co-interventions assessed including aspirin and calcium, have individually been associated with moderate reductions in the risk of pre-eclampsia and its related complications. It therefore remains unclear, whether the treatment effects observed are exclusively or even at all due to antioxidants or the combination of antioxidants with other agents.

2.3.5 Implications for practice

There is not enough evidence on the safety and efficacy of antioxidant supplementation during pregnancy to recommend routine use in pregnancy for all women in all geographical and clinical settings.

2.3.6 Implications for research

Further research is required to confirm the effect of antioxidants on pre-eclampsia and its related complications and on more substantive clinical outcomes. Further high quality trials of sufficient statistical power are needed to confirm a direct casual relationship between antioxidants and a reduced risk of pre-eclampsia and its related complications. Further research must also address whether antioxidants are beneficial for women at lower risk of pre-

eclampsia, for Australian women and women in other developed settings, and whether women with a dietary deficiency of antioxidants may be more likely to benefit from antioxidant supplementation. If antioxidants are confirmed as an effective prophylaxis against pre-eclampsia, future trials must also determine the optimum dosage, type of antioxidant and timing of supplementation.

Further research is required to address the following priority research questions relating to antioxidant supplementation in pregnancy:

1. Does antioxidant supplementation alone in pregnancy prevent pre-eclampsia and its related complications for those women:
   a) With differing risk status for pre-eclampsia (i.e. high risk or low risk status for pre-eclampsia)?
   b) In all settings (developed and developing)?
   c) With a poor dietary intake of the antioxidant(s) (where applicable) and with adequate dietary intake of the antioxidant(s)?

2. Furthermore, if antioxidant supplementation is proven to be effective in preventing pre-eclampsia and its related complications, the women most likely to benefit (i.e. according to risk status and dietary intake) and the optimal type of antioxidant (vitamin, minerals, non-vitamin or a combination), dosage and timing will need to be established.

3. VITAMIN C AND E SUPPLEMENTATION FOR THE PREVENTION OF PRE-ECLAMPSIA – A RANDOMISED CONTROLLED TRIAL

3.1 Introduction

Large high quality randomised trials assessing antioxidant supplementation in pregnancy are clearly needed. This chapter describes the results from a double-blind randomised placebo controlled trial of vitamin C and vitamin E supplementation during pregnancy in nulliparous women for the prevention of pre-eclampsia, small for gestational age infants and adverse infant and maternal outcomes.

3.2 Study aims and hypotheses

The specific aims of this clinical trial were to assess the effect of vitamin C and E supplementation in nulliparous women on:

1. The likelihood of the infant being born small for gestational age.
2. The development of pre-eclampsia.
3. The risk of serious complications for the infant.
4. The risk of serious complications for the woman.

The primary hypotheses of the study were that vitamin C and E supplementation from 14 weeks' gestation in nulliparous women:

1. Reduces the incidence of small for gestational age infants defined as birth weight below the 10^{th} centile for gestation and fetal sex on standardised birth weight charts (Roberts and Lancaster 1999).
2. Reduces the incidence of pre-eclampsia as defined by the Australasian Society for the Study of Hypertension in Pregnancy (ASSHP) (Brown et al 2000) as hypertension (systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure (Korotkoff V) ≥ 90 mmHg on two occasions four hours or more apart) and the onset after 20 weeks' gestation of one or more of the following:-
    - proteinuria - ≥ 300 mg/24 hours or spot urine protein/creatinine ratio ≥ 30 mg/mmol
    - renal insufficiency - serum/plasma creatinine ≥ 0.09 mmol/L or oliguria
    - liver disease - raised serum transaminases and/or severe epigastric/right upper quadrant pain
    - neurological problems - convulsions (eclampsia); hyperreflexia with clonus; severe headaches with hyperreflexia; persistent visual disturbances (scotomata)

- haematological disturbances - thrombocytopenia; disseminated intravascular coagulation; haemolysis
- fetal growth restriction (birth weight < 10^{th} centile for gestational age).

3. Reduces the risk of death or serious adverse outcome for the infant defined as one or more of:- fetal death after trial entry, occurring either between 14^{+0} and 19^{+6} weeks' gestation or at or after the 20^{th} week of pregnancy; death of a live born infant prior to hospital discharge; severe intrauterine growth restriction; severe respiratory distress syndrome; chronic lung disease; intraventricular haemorrhage grade three or four; cystic periventricular leucomalacia; retinopathy of prematurity grade three or four; necrotising enterocolitis; Apgar score less than four at five minutes; seizures at < 24 hours age or requiring two or more drugs to control; hypotonia for at least two hours; stupor, decreased response to pain or coma; tube feeding for four or more days; care in neonatal intensive care unit for greater than four days; use of ventilation for ≥ 24 hours.

The primary hypothesis for the effect of vitamin C and E supplementation on the risk of death or serious infant outcome was one tailed, as we did not expect vitamin C and E supplementation to increase the risk of adverse outcomes for the infant.

The secondary hypotheses were that vitamin C and E supplementation from 14 weeks' gestation in nulliparous women reduces the risk of adverse outcomes for the woman up to six weeks' postpartum, defined by maternal death, or one or more of the following serious complications:- pulmonary oedema; eclampsia; stroke; thrombocytopenia; oliguria; renal insufficiency; preterm prelabour rupture of membranes; adult respiratory distress syndrome; cardiac arrest; respiratory arrest; placental abruption; abnormal liver function; haemolysis; coagulopathy; major postpartum haemorrhage; postpartum pyrexia; deep vein thrombosis or pulmonary embolus requiring anticoagulant therapy.

3.3 Methods

A multi-centred randomised, double blind, placebo controlled trial comparing supplementation with vitamin C and vitamin E versus a microcrystalline cellulose placebo in nulliparous women was undertaken at nine maternity centres around Australia, between December 2001 and May 2005. The study protocol was approved by the Research and Ethics Committee at all nine collaborating centres.

3.3.1 Participants

Inclusion criteria: those eligible for the study were all nulliparous women presenting to the antenatal clinic at the collaborating centre with a singleton pregnancy, between 14 and 21 weeks' plus six days gestation, with a normal blood pressure at trial entry, and who were expecting to give birth at the collaborating centre. Participating women gave informed, written consent.

Exclusion criteria: women not eligible for the study included those with any of the following: multiple pregnancy, life threatening fetal anomaly on ultrasound, known thrombophilia, chronic renal failure, haemochromatosis, on heparin, warfarin or antihypertensive therapy or with a contraindication to vitamin C or E therapy. Contraindications to vitamin C therapy included women with haemochromatosis due to the risk of iron overload. Contraindications to vitamin E therapy included those on warfarin due to the possibility for vitamin E to potentiate warfarin effectiveness.

3.3.2 Randomisation, treatment pack preparation and allocation

3.3.2.1 Generation of random number sequence

An individual not involved in the recruitment of study participants prepared the randomisation schedule. The schedule was based on a computer generated random number list using balanced variable blocks, stratified by collaborating centre and gestational age (< 18 weeks' or ≥ 18 weeks'). The unit of randomisation was the individual woman.

3.3.2.2 Treatment pack preparation

All trial tablets were packed and bottled at the coordinating centre in Adelaide and treatment packs were prepared for each collaborating centre. Study treatment packs contained four white plastic bottles sealed with a celloseal wad and child resistant cap to prevent tampering. Each treatment pack contained either the antioxidant vitamin C and vitamin E medication or the placebo medication.

The vitamin C and E tablets and the placebo tablets were prepared by Herron Pharmaceuticals Pty Ltd, specifically for the trial. Vitamin E was sourced from Cognis Australia Pty Ltd and for vitamin C, from CSR Food Ingredients Pty Ltd. Herron Pharmaceutical Pty Ltd provided the microcrystalline cellulose required for the placebo preparations, and were responsible for manufacturing the combined vitamin C and E tablets and ensuring the placebo preparations

were identical to the active tablets. The active vitamin C and E tablets had a shelf life of two years, resulting in the need for several changeovers of medication to prevent tablet expiry. During the entire progression of the trial, five batches of combined vitamin C and E tablets and eight batches of placebo tablets were prepared and sent to the coordinating centre. Certificates of analysis were provided for each batch of tablets and each batch was tested individually for quality control and assurance by Herron Pharmaceuticals Pty Ltd.

Treatment codes detailing the study number and treatment group allocation were kept in a locked cabinet not accessible by members of the study team directly involved in recruitment, clinical care, data collection or analysis. Treatment packs were prepared by placing individual study numbers on opaque bottles containing either the vitamin C and E or placebo tablets. Each bottle was labelled with a study number, the text "250 mg vitamin C and 100 IU vitamin E tablets or placebo tablets" and had instructions for taking four tablets daily. Concealment of allocation was maintained by ensuring that all bottles were labelled by a staff member not involved with treatment allocation. Correct labelling was then checked by a second person. Prior to allocation, treatment packs were stored in a cool dry environment.

3.3.3 Treatment group allocation

Participants were allocated to either the vitamin C and E group or the placebo group. Randomisation was undertaken through the Central Telephone Randomisation Service at the Maternal Perinatal Clinical Trials Unit, Department of Obstetrics and Gynaecology, the University of Adelaide. Using this service, each woman was allocated a study number and the correctly numbered treatment pack corresponding with the woman's s*tudy number* was then given to her by the research staff. In some cases, treatment packs were mailed out to women.

3.3.4 Recruitment procedures

Recruitment commenced in December 2001. Eligible women were identified and approached by a member of the research team in the antenatal clinics. They were provided with information about the trial and encouraged to take it home and discuss the trial information with relevant family and/or friends. For those women interested in participating, written signed consent was obtained. The consent form was then copied for the records at the collaborating centre, a copy was placed in the woman's medical records, and the original was sent to the coordinating centre.

3.3.5 Treatment Schedules

The choice of supplementation and dosage are similar to that used in the trial conducted by Chappell and colleagues (1999).

Vitamin C and E treatment: Women in the vitamin C and E group took four combined tablets of 250 mg vitamin C (as ascorbic acid) and 100 IU vitamin E (as d-alpha-tocopherol succinate) coated to mask the characteristic tart taste of ascorbic acid. The total daily dose of vitamin C was therefore 1,000 mg and for vitamin E, 400 IU.

Placebo treatment: Women in the placebo group took four tablets containing microcrystalline cellulose that were similarly coated and identical in appearance to the combined vitamin C and vitamin E tablets.

Women were given four bottles of tablets regardless of their gestational age at entry to the trial. This provided women with enough tablets for the entire length of the pregnancy (based on the assumption that the maximum time spent actively taking the tablets would be between 14^{+0} and 42^{+0} weeks' gestation).

Women were asked to swallow the tablets whole without crushing or chewing them, to maintain blinding to the treatment allocation. The dosing recommendations were for women to take two tablets in the morning and two tablets in the evening, however if women chose to, all of the tablets could be taken at one time. Women remained with the same treatment allocation throughout their pregnancy and were asked to take the tablets from the date of trial entry until delivery. On the back of the tablet bottles a calendar grid was provided to assist with tablet compliance, and women were asked to cross over each day they took the tablets. Each treatment pack contained written instructions about taking the tablets and asked women not to "double up" if they missed a daily dose.

Upon entering the trial, women were advised not to take any other antioxidant supplements, however a multivitamin preparation that provided a daily intake of $\leq$200 mg vitamin C or $\leq$ 50 IU vitamin E was permitted. This included the majority of supplements commercially available for pregnant women in Australia. All babies in the study were recommended to have intramuscular vitamin K after birth, due to the possibility for vitamin E to potentiate the effects of vitamin K deficiency.

3.3.6 Assessment of compliance with the trial Treatment Schedules

Women were asked to record the days tablets were taken by marking off a calendar grid on the individual tablet bottles, and in addition, to note down any side effects of treatment. Information on compliance with the trial medications was assessed by direct questioning using the questionnaires sent to women at 28 weeks' gestation and postnatally. At these times women were asked about the mode of tablet taking (twice daily, once daily in the morning, once daily at night, other (please specify)); how often they missed the trial tablets (never, occasionally, frequently, other (please specify)) and how many times a week they missed all of the trial tablets (0, 1-2, 3-4, >4, other (please specify)). The questionnaire asked women to tick the main reasons why they missed taking all of their trial tablets (have not missed any tablets, have just forgotten to take them, didn't have them with me, have been feeling unwell, tablets too large, too many tablets, too much effort required, other (please specify)). Potential side effects were assessed at these times by asking women if they had experienced any of the following in the previous eight weeks: nausea, vomiting, abdominal pain, diarrhoea, fatigue, weakness, other (please specify) or none of the above.

Where possible, women were seen at their antenatal visits, to provide a prompt about the tablet taking and any outstanding questionnaires. Women were asked to bring in their tablet pack at delivery and these tablet packs were then sent to the coordinating centre. Trial newsletters were sent to women at 24, 32 and 36 weeks' gestation. The purpose of these newsletters was to inform participants about the progress of the trial, increase compliance with the trial medications by providing tips and strategies for remembering to take the tablets, and to prompt the return of any outstanding questionnaires.

3.3.7 Care during the Antenatal Period, Labour and Postnatal Stay

The care women and their infants received was standard practice at each collaborating centre. Surveillance for hypertension was made using standardised measurements for blood pressure at the routinely scheduled antenatal visits (typically at 18, 24, 28, 32 and 36 weeks' gestation, then weekly until birth), during hospital admission for the birth, during the in-patient postnatal period and during any unscheduled antenatal visits or antenatal hospitalisations. The guidelines for measurement of blood pressure were those recommended by the ASSHP (Brown et al 2000). Diastolic blood pressure was taken as the Korotkoff phase V unless this was zero when Korotkoff phase IV is used. If the woman's blood pressure was elevated, urinalysis for proteinuria was recommended following the hospital protocol at each collaborating centre.

3.3.8 Primary outcomes

The effect of vitamin C and E supplementation in women from 14 weeks' gestation throughout pregnancy was assessed by the following:

1. **Incidence of small for gestational age infants** defined as birth weight below the 10^{th} centile for gestation and fetal sex on standardised birth weight charts (Roberts and Lancaster 1999).
2. **Incidence of pre-eclampsia** as defined by the ASSHP (Brown et al 2000) as hypertension (systolic blood pressure of ≥ 140 mmHg and/or diastolic blood pressure (Korotkoff V) of ≥ 90 mmHg on at least two occasions four or more hours apart) arising after 20 weeks' gestation and the onset after 20 weeks' gestation of one or more of:
   - proteinuria - ≥ 300 mg/24 hours or spot urine protein creatinine ratio ≥ 30 mg/mmol
   - renal insufficiency – serum/plasma creatinine ≥ 0.09 mmol/L or oliguria (< 30 mls of urine per hour for six or more hours)
   - liver disease – raised serum transaminases (aspartate aminotransferase (AST) or alanine aminotransferase (ALT) > 50 IU/L) and/or severe epigastric/right upper quadrant pain
   - neurological problems – convulsions (eclampsia); hyperreflexia with clonus; severe headaches with hyperreflexia; persistent visual disturbances (scotomata)
   - haematological disturbances – thrombocytopenia (platelet count < 100,000/mm^3); disseminated intravascular coagulation (international normalised ratio (INR) > 1.5 or activated partial thromboplastin time (APTT) > 5 seconds longer than laboratory reference or fibrinogen < 1g/L); haemolysis (lactate dehydrogenase (LDH) > 500 IU/L and/or fragmentocytes on peripheral blood smear)
   - fetal growth restriction (birth weight < 10^{th} centile for gestational age).
3. **Incidence of death or serious adverse outcome for the infant** to include one or more of:- fetal death after trial entry, occurring either between 14^{+0} and 19^{+6} weeks' gestation or at or after the 20^{th} week of pregnancy; death of a live born infant prior to hospital discharge; severe intrauterine growth restriction (< 3^{rd} centile for gestational age); severe respiratory distress syndrome (defined as Mean Airway Pressure > 10 mmHg and/or FiO_2 cm H_20 ≥ 0.80); chronic lung disease (defined as need for oxygen at 36 weeks' postconceptual age for those infants born < 32 weeks' gestation, or oxygen required on day 28 for those infants born ≥32 weeks' gestation); intraventricular haemorrhage grade three or four; cystic periventricular leucomalacia; retinopathy of prematurity three or four; necrotising enterocolitis; Apgar score less than four at five minutes; seizures at < 24 hours age or requiring two or more drugs to control; hypotonia for at least two hours; stupor, decreased response to pain or coma; tube feeding for four or more days; care in the

neonatal intensive care unit greater than four days; use of ventilation for ≥ 24 hours. These definitions of serious infant outcomes are based on the definitions for adverse outcomes used by the Australian New Zealand Neonatal Network (Donoghue and Cust 2000) and from those considered by experts as important measures of morbidity at or beyond term (Hannah et al 1992).

3.3.9 Secondary outcomes

Severe adverse outcomes for the woman up to six weeks' postpartum, as defined by maternal death, or one or more of the following serious complications: pulmonary oedema; eclampsia (defined as a seizure without any other known cause in a women with pregnancy-induced hypertension or pre-eclampsia); stroke (defined as acute neurological deficit > 24 hours); thrombocytopenia (two or more platelet counts < 100,000/mm^3); renal insufficiency (defined as oliguria (< 30 mls of urine per hour six or more hours) or serum creatinine ≥ 0.09 mmol/L); adult respiratory distress syndrome; cardiac arrest; respiratory arrest; placental abruption (defined as abdominal pain and bleeding before birth associated with a retroplacental clot at delivery); abnormal liver function (defined as AST/ALT ≥ 50 IU/L); coagulopathy (defined as INR > 1.5 or APTT > 5 seconds longer than laboratory reference or fibrinogen < 1g/L)); preterm prelabour rupture of membranes; major postpartum haemorrhage (defined as ≥ 1,500 mls or use of blood transfusion); postpartum pyrexia (defined as ≥ 38.5°C on two occasions > 24 hours apart); pneumonia; deep vein thrombosis or pulmonary embolus requiring anticoagulant therapy.

Secondary outcomes for the infant after randomisation - gestational age at birth, preterm birth (defined as less than 37 weeks' gestation), very preterm birth (defined as less than 34 weeks' gestation), extremely preterm birth (defined as less than 28 weeks' gestation), weight, length and head circumference at birth, placental weight, birth weight less than 2,500 g, birth weight less than 1,500 g, need for resuscitation at birth, Apgar scores less than seven at five minutes, admission to and length of stay in the neonatal intensive care unit, incidence and severity of respiratory distress syndrome, use of and duration of mechanical ventilation, cerebrovascular haemorrhage on early cranial ultrasound, periventricular leucomalacia on later cranial ultrasound, need for oxygen therapy at 28 days' or more of life, use of postnatal steroids, use of antibiotics in the first 48 hours' of life, proven systemic infection in the first 48 hours' of life, use of antibiotics after the first 48 hours' of life, proven systemic infection after the first 48 hours' of life, number of episodes of proven infection, use of surfactant, nitric oxide for respiratory support, need for ionotropic support, air leak syndrome,

retinopathy of prematurity, patent ductus arteriosis requiring treatment, thrombocytopenia or neonatal encephalopathy (Sarnat stage 1, 2 or 3) (Sarnat and Sarnat, 1976).

It is appreciated that a trial of this size may not be able to demonstrate a difference between treatment groups for some of the secondary and subsidiary endpoints but the information may allow comparison with women and infants studied in other trials in a systematic review or suggest other beneficial or adverse effects of treatment that require further controlled evaluation.

3.3.10 Subsidiary outcomes

Subsidiary outcomes for the women by the following events after randomisation - clinical chorioamnionitis requiring intrapartum antibiotics; need for antenatal hospitalisation or day care admission for preterm labour; tocolytic use; prenatal corticosteroid use; development of gestational diabetes (defined as two hour oral glucose tolerance test result ≥ 7.8 mmol/L); prelabour rupture of membranes (preterm or term); need for induction of labour and reasons; complications of delivery including pyrexia, bleeding due to placenta praevia or abruptio, labour augmentation or meconium stained liquor; mode of delivery; perineal trauma and the level of trauma; use of postpartum antibiotics; maternal postnatal stay for seven or more days; breast feeding at hospital discharge; potential side effects; women's views on their participation and subsidiary outcomes related to hypertensive disease including the need for antenatal hospitalisation or day care admission for hypertension, need for induction of labour for hypertension, use of antihypertensives, use of magnesium sulphate.

Data on hypertensive disease and proteinuria were collected to allow the application of various definitions of hypertensive disease in pregnancy (Davey and MacGillivray 1986, Brown et al 2000, Report of the NHBPEP 2000) that are less open to clinical interpretation and error (Brown et al 2001, Higgins and de Swiet 2001). Assessing pre-eclampsia in the context of these definitions permits comparisons with other trials, past and present, reporting on internationally used definitions of pre-eclampsia. These data are presented in Chapter Four of this thesis.

Gestational hypertension - defined as either:

a) one measurement of diastolic blood pressure of 110 mmHg or more; or
b) two consecutive measurements of diastolic blood pressure of 90 mmHg or more, four hours or more apart.

Pre-eclampsia - defined as gestational hypertension and either:

a) one 24 hour urine collection of 0.3 g proteinuria or more; or

b) two random clean catch specimens of urine with 2+ protein (1 g) as measured by dipstick.

Severe gestational hypertension - (defined as two recordings of diastolic blood pressure of 110 mmHg or more, four hours or more apart, or one recording of diastolic blood pressure of at least 120 mmHg).

Severe pre-eclampsia - (defined as pre-eclampsia with either severe pregnancy induced hypertension or severe proteinuria (defined as one 24 hour urine collection of 3 g protein or more or two random clean catch specimens of urine with 3+ protein or more as measured by dipstick).

3.3.11 Data collection

3.3.11.1 Pregnancy outcome data

Pregnancy outcome data and neonatal data were abstracted from the medical records at each collaborating centre by the research assistant blinded to the woman's treatment allocation. The *Delivery Form* was completed after the woman gave birth. The *Antenatal/Postnatal Form* was completed after six weeks' postpartum and the *Neonatal Data Form* completed for live born infants after discharge of both mother and baby from hospital, or both forms were completed at 12 weeks' postpartum if either or both were still in hospital. All delivery, antenatal/postnatal and neonatal data forms were checked, and where there were any queries about the data forms, the forms were further checked by the maternal fetal medicine specialist (Caroline Crowther) and/or the neonatologist on the trial steering group (Ross Haslam), blinded to the treatment allocation.

3.3.11.2 Trial questionnaires

Women completed a semi-quantitative food-frequency questionnaire (FFQ) (Willett et al 1985) at trial entry to determine baseline intakes of dietary antioxidants and to assess whether vitamin C and E supplementation would be more beneficial for women with lower dietary antioxidant intakes. The FFQ asks about the average frequency of intake of over 100 foods as well as information about type, dose and duration of use of vitamin and mineral supplements. This FFQ has been shown to accurately assess vitamin C and E intakes in the general American population (Willet et al 1985; Jacques et al 1993) and during pregnancy in American Caucasian women (Brown et al 1996).

At trial entry women completed a patient entry form asking about their family history of hypertension and pre-eclampsia, use of dietary supplements and demographic information. At 28 weeks' gestation, women were asked to complete a questionnaire assessing compliance, side-effects and use of dietary supplements. Postnatally, women completed a questionnaire asking about compliance, side effects, their perceived treatment group allocation and their views on participating in the trial. Where questionnaires were posted to women, all women received a reply paid free post envelope to facilitate return of the questionnaires. Furthermore, questionnaires that were not returned within two weeks were followed up with a phone call prompt, and where needed, questionnaires were reposted to women.

Women, their caregivers, outcome assessors, clinical investigators and myself were all blinded to the treatment allocations until all study analyses had been completed. All information obtained from the study remained strictly confidential. Data were stored in a locked filing cabinet or on password protected computer file, and accessible only by the study team.

3.3.12 Sample size

Sufficient women were randomised to provide reliable evidence about the effects of vitamin C and E supplementation for women and their infants. The incidence of the infant dying or having a serious adverse outcome was the principal endpoint of the trial. **A trial of 1,540 women, allowing for three percent losses to follow-up, has at least 80 percent power to detect a statistically significant difference at an alpha level of 0.05 (*one-tailed*) in reducing the risk of death or serious adverse infant outcome by 45 percent from 6.5 percent to 3.6 percent with vitamin C and E supplementation.**

Calculations are based on data from the Australian Collaborative Trial of calcium supplementation in pregnancy (Crowther et al 1999), data from the Clinical Information Services at The Women's and Children's Hospital, Adelaide and the randomised trial of antioxidants in women at increased risk of pre-eclampsia (Chappell et al 1999). Losses to follow-up were expected to be minimal, based on the previous experience in the Australian calcium supplementation trial where study outcome data were available for all women and their infants recruited (Crowther et al 1999).

The trial was planned with a sample size of 1,870 women. A trial of 1,870 women, with allowances for three percent losses to follow up, has 80 percent power to detect a reduction in

the risk of death or serious adverse infant outcome by 40 percent, from 6.5 percent to 3.9 percent (alpha level 0.05, *one tailed*). At the onset of this project this appeared to be a realistic target. However, due to the time constraints of a PhD Candidature (four years), coupled with the slower than anticipated recruitment rate, and long lag time from recruitment to birth and completion of the data collection for all women randomised, a decision was made to write up the findings for 1,540 women for the purpose of presentation in this thesis. This number of women was chosen to allow for minimal losses of power for detecting differences in the primary outcomes.

The trial has now completed its target recruitment of 1,870 women, although final data collection of women is not expected to be completed until September 2005. The results of this trial will not be presented other than in this thesis, until the full data are available.

A trial of 1,540 women has 80 percent power to detect the following differences in the other specified primary and secondary endpoints at an alpha level of 0.05 (*two-tailed*) (Table 3.1).

Table 3.1 Detectable changes in clinical outcomes with a sample size of 1,540 women

Other clinical outcomes	Rate in placebo group (%)	% Change detectable	Expected rate in Vitamin C and E group (%)
Small for gestational age (birth weight < 10th centile)	12.0	37.0	7.6
Pre-eclampsia (defined according to ASSHP criteria)	10.0	40.0	6.0
Serious adverse outcome for the woman	6.8	48.5	3.5

3.3.13 Statistical analyses

All statistical analyses were performed on an intention to treat basis according to treatment allocation at randomisation, using Stata Version 8 (Stata Corporation, 2003). The initial analyses examined the baseline characteristics of all women randomised to the trial, to ensure prognostic factors were balanced between the treatment and placebo groups. All binary outcomes were analysed using Chi-squared tests and Fisher's exact test (where the number of observations in the cell was $\leq$5), and Relative Risks (RR) and 95% Confidence Intervals (CI) were calculated for the major study outcomes. The numbers needed to treat to benefit

(NNTB) or the numbers needed to treat to harm (NNTH) are presented for all statistically significant comparisons. Differences were considered to be statistically significant where the probability value (p value) was less than or equal to 0.05. For our principal endpoint death or serious adverse infant outcome, one tailed p values and the upper 95 percent confidence interval are presented in accordance with our one-tailed hypothesis and sample size calculation undertaken for this outcome.

For outcomes on a continuous scale, where the data were normally distributed, comparisons between the treatment groups were made using the Student's T-test, and for skewed data, non-parametric tests were used. For the infant growth parameters birth weight, length and head circumference, z-scores were calculated using data from the United Kingdom as a reference population (Freeman et al 1995), which was amended to include length data for births' less than 32 weeks' gestation, using Australian birth length percentiles published by Beeby and colleagues (1996).

Pre-specified subgroup analyses were undertaken to evaluate the effect of the following factors on the treatment effects: baseline dietary vitamin C and vitamin E intake (below or equal to and above the RDI); maternal smoking status at trial entry; family history of pre-eclampsia or gestational hypertension; gestational age at randomisation and women's compliance with the trial medications.

3.3.14 Adverse Events Committee

Deaths of infants enrolled in the trial were reviewed by a multidisciplinary adverse event committee blinded to treatment allocation, which classified the cause of death.

3.4 Recruitment, flow of participants and maternal baseline characteristics

Over the entire study period, 6,034 women were approached and counselled by study research personnel about the trial. This figure represents approximately 81 percent of the total potentially eligible population (n=7,438). Of these, 1,538 (26%) women consented to the trial and were randomised to either the vitamin C and E group (n=770) or the placebo group (n=768). Pregnancy outcome data were available for all women randomised and there were no losses to follow up.

Most women were recruited from the combined South Australian sites, with the Women's and Children's Hospital in South Australia recruiting the highest number of women (422, 27%). The breakdown of recruitment according to each collaborating centre is listed in the Appendix (Table 8.1). The flow of eligible women approached, those declining and the main reasons, the number of women randomised, their treatment allocation, gestational age strata and pregnancy outcome are listed in Figure 3.1.

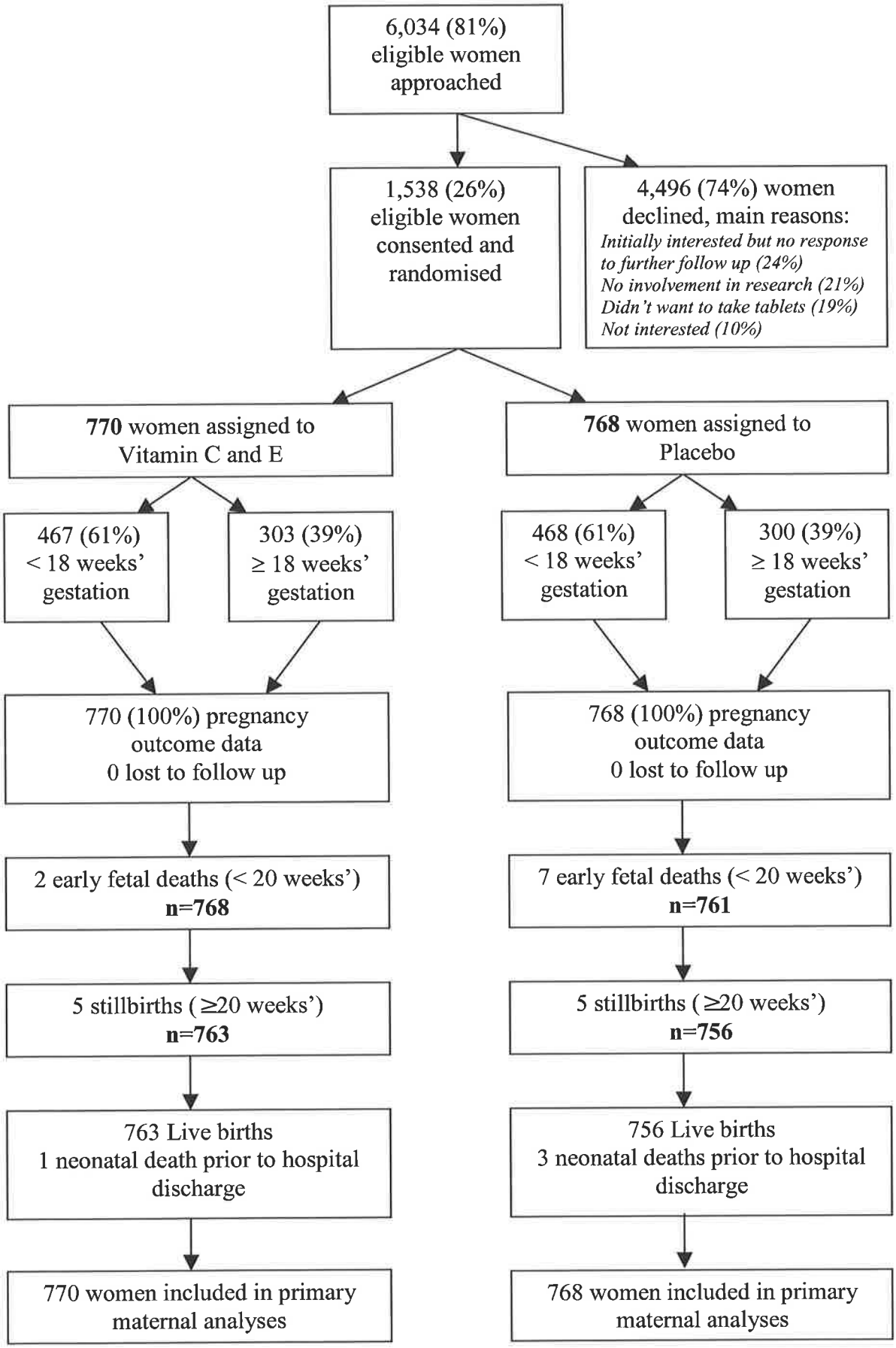

Figure 3.1 Randomisation, Treatment and Follow up of all participants.

3.4.1 Unblinding of treatment group allocation

The treatment code was unmasked for one woman during the study period. In this instance, a request was made by the clinicians involved in the care of a woman with multiple organ failure whose baby was stillborn at 36 weeks' gestation. The treatment group allocation (in this instance the placebo group) was given to the clinician by a member of the study team not directly involved in the trial.

3.4.2 Maternal characteristics at trial entry

Women in the vitamin C and E group and the placebo group were comparable for all important baseline characteristics (Table 3.2), including maternal age, gestational age at randomisation, gestational age women planned to start the trial tablets, systolic and diastolic blood pressure, body mass index (BMI) at antenatal hospital booking and the number of women with a previous pregnancy of less than 20 weeks' duration. The mean age of women involved in the trial was 26 years, and the mean gestation at enrolment was 17 weeks'. Women's demographic characteristics were also comparable between treatment groups, where most women in the trial were Caucasian, non smokers, in a married or de-facto relationship and had some higher education or training.

Just over 20 percent of women in each treatment group had a previous medical condition recorded in their medical records. The type of medical condition varied widely and was most commonly Asthma (vitamin group 62 women [8.3%] vs. placebo group 47 [6.3%]). A full list of the previous medical conditions reported is detailed in the Appendix (Table 8.2). As expected, few women had a previous medical condition related to gynaecological complications and less than 1 percent of women had epilepsy, previous kidney disease, previous hypertension requiring antihypertensive medication or were diabetic at trial entry.

Table 3.2 Maternal characteristics at trial entry in the vitamin and placebo groups

Maternal demographic characteristics		Vitamin C & E n=770	%	Placebo n=768	%
Maternal age, yrs*		26	6	26	6
Gestational age at randomisation, wks*		17	2	17	2
Gestational age planned to start trial tablets, wks*		18	2	18	2
Blood pressure at randomisation, mmHg					
	Systolic*	110	10	110	10
	Diastolic*	66	8	66	8
Blood pressure ≥130/80 mmHg		24	3	20	3
Body mass index at hospital booking*		25	6	25	5
Previous pregnancies ≤ 20 weeks'		217	28	233	30
Use of assisted reproductive technologies in this pregnancy		22	3	17	2
Maternal Race	Caucasian	732	95	725	94
	Asian	25	3	26	3
	Aboriginal/TSI	4	<1	6	<1
	Polynesian/Maori	4	<1	3	<1
	Other/Unknown	5	0.6	8	1.0
Smoking status	Non-smoker	491	65	490	65
	Quit in pregnancy[#]	100	13	120	16
	Smoker	164	22	143	19
	Unknown	15	2	15	2
Marital status	Never married	153	20	159	21
	Married/de facto	608	79	593	77
	Widowed/separated/divorced	3	<1	8	1
	Unknown	6	<1	8	1
Level of education	<Year 10	64	8	66	9
	Year 10 or equivalent	75	10	85	11
	High School	211	27	194	25
	TAFE Diploma	176	23	191	25
	University studies	227	30	216	28
	Unknown	17	2	16	2
Any previous medical condition		177	23	164	21
If yes, type:	Gynaecological	22	3	22	3
	Epilepsy	10	1	11	1
	Kidney disease	4	<1	5	<1
	Hypertension (req. medication)	5	<1	3	<1
	Diabetes	3	<1	2	<1

Figures are n, % or * mean, standard deviation (SD)
[#]before first antenatal visit
TSI= Torres Strait Islander

3.4.3 Women's family history of hypertension and pre-eclampsia

Women were asked about their family history of hypertension, pre-eclampsia and gestational hypertension (Table 3.3). Women's reports of their family history of hypertensive disease including pre-eclampsia were similar between treatment groups. Almost half of all women indicated they had a family history of hypertension (vitamin group 351 [46%] vs. placebo group 349 [45%]), primarily their mother and/or father. Close to 20 percent of all women further indicated a family history of pre-eclampsia or gestational hypertension (vitamin group 155 [20%] vs. placebo group 143 [19%]), again most commonly in their mothers. These findings compare with much lower rates documented in women's medical records relating to family history of hypertension (vitamin group 279 [36%] vs. placebo group 275 [36%]) and pre-eclampsia (vitamin group 78 [10%] vs. placebo group 77 [10%]). As expected, few women had any family history of eclampsia, epilepsy, thromboembolism or thrombophilia.

Table 3.3 Women's family history of hypertensive disease

Family history		Vitamin C & E n = 770	%	Placebo n = 768	%
Family history of hypertension	Yes	351	46	349	45
	No	300	39	293	38
	Unsure	104	13	111	15
	Missing	15	2	15	2
Family members with hypertension	Mother	185	24	169	22
	Father	123	16	135	18
	Sister	31	4	26	3
	Brother	7	1	10	1
	Other (i.e. grandparents)	97	13	86	11
Family history of PE or GH	Yes	155	20	143	19
	No	348	45	354	46
	Unknown	252	33	256	33
	Missing	15	2	15	2
Family members with PE or GH	Mother	89	12	94	12
	Sister	44	6	34	4
	Aunt	19	2	24	3
	Other (i.e. grandmother, cousin)	20	3	23	3
Any family history of eclampsia		4	<1	8	1
Any family history of epilepsy		18	2	17	2
Any family history of thromboembolism		20	3	20	3
Any family history of thrombophilia		1	<1	1	<1

Figures are n, %
PE = pre-eclampsia, GH = gestational hypertension

3.4.4 Women's use of dietary supplements

Women were asked about their use of dietary supplements before and during the current pregnancy (Table 3.4). There were no differences in the use of dietary supplements between treatment groups. Overall, close to half of all women reported taking a dietary supplement before pregnancy (vitamin group 310 [40%] vs. placebo group 323 [42%]), and over 80 percent of all women reported taking a dietary supplement before or at trial entry (vitamin group 624 [81%] vs. placebo group 628 [82%]). The most common supplements taken were multivitamins, folic acid alone or a combined folic acid and iron/vitamin C supplements.

Table 3.4 Use of dietary supplements before pregnancy and in early pregnancy

Use of dietary supplements	Vitamin C & E		Placebo	
	n=770	%	n=768	%
Dietary supplements used prior to pregnancy	310	40	323	42
Dietary supplements used at trial entry	624	81	628	82
Type of dietary supplement at or before trial entry				
Multivitamin (with or without folic acid)	369	48	369	48
Folic acid alone	349	45	334	43
Folic acid with iron and/or vitamin C	67	9	81	10
Iron formula (including iron + vitamin C)	33	4	36	5
Vitamin B Complex/Morning sickness formula	35	4	30	4
Vitamin C alone	28	4	27	3
Calcium	25	3	17	2
Fish oil/Evening Primrose Oil	4	<1	9	1
Zinc	2	<1	5	<1
Herbal	7	1	5	<1
'Antioxidant preparation'	1	<1	3	<1
Vitamin E	3	<1	2	<1
Selenium	0	0	1	<1
Probiotics	1	<1	1	<1
Other	6	<1	2	<1
Not available*	13	2	15	2

Figures are n, %
* data are missing

3.4.5 Women's dietary intake of vitamin C and E at trial entry

Women's self reported dietary intakes of vitamin C and vitamin E at trial entry were highly variable (Table 3.5). Median vitamin C intakes from diet alone and combined diet and vitamin supplements were similar between treatment groups (median total vitamin C intake per day; vitamin group 197.3 mg [interquartile range (IQR) 173.7] vs. placebo group 199.8 mg [IQR 184.0]). Similarly, median vitamin E intakes from both diet alone and diet and supplements were also comparable between treatment groups (total vitamin E intake per day; vitamin group 8.6 mg [IQR 8.3] vs. placebo group 8.3 mg [IQR 9.1]). Women's dietary intakes were classified as either equal to and above or below the RDI for vitamin C (60 mg per day) or

vitamin E (7 mg per day) (NHMRC 1989). Approximately five percent of women in both treatment groups had a daily vitamin C intake below the RDI (Table 3.5). For vitamin E, the number of women with a daily intake below the RDI was substantially higher, with close to one third of all women having a daily intake below the RDI. The numbers of women with low intakes of vitamin C or vitamin E were comparable between treatment groups.

Table 3.5 Median dietary intakes of vitamin C and E at trial entry

Dietary intakes	Vitamin C & E n=770		Placebo n=768	
Vitamin C without supplements (diet alone), mg	153.5	106.6	150.9	117.8
Vitamin C total (diet and supplements), mg	197.3	173.7	199.8	184.0
Vitamin C intake below RDI (60 mg)*	31	4	37	5
Vitamin E without supplements (diet alone), mg	6.3	3.2	6.4	3.5
Vitamin E total (diet and supplements), mg	8.6	8.3	8.3	9.1
Vitamin E intake below RDI (7 mg)*	242	35	263	38
Not available**	85	11	76	10

Figures are median, interquartile range or *n, %
** data are missing
RDI = recommended dietary intake

3.4.6 Women's use of dietary supplements at 28 weeks' gestation

When assessed again at 28 weeks' gestation, sixty percent of women in each treatment group reported taking a dietary supplement since joining the trial (vitamin group 463 [60%] vs. placebo group 463 [60%]) (Table 3.6). The types of dietary supplements taken were again similar to those reported at trial entry, where the most common type of supplements taken were multivitamins, folic acid alone, iron alone and combined folic acid and iron/vitamin C.

Table 3.6 Use of dietary supplements assessed at 28 weeks' gestation

	Vitamin C & E		Placebo	
	n=770	%	n=768	%
Use of dietary supplements since trial entry	463	60	463	60
Type of dietary supplement since trial entry				
Multivitamin (with or without folic acid)	281	36	270	35
Folic acid alone	124	16	125	16
Iron formula (including iron + vitamin C)	75	10	70	9
Folic acid with iron and/or vitamin C	57	7	62	8
Calcium	35	4	27	3
Vitamin B Complex/Morning sickness formula	17	2	21	3
Vitamin C	18	2	21	3
Fish oil/Evening Primrose Oil	11	1	9	1
Herbal	7	1	8	1
Minerals	1	<1	2	<1
Probiotics	0	0	2	<1
Zinc	1	<1	1	<1
Vitamin E	1	<1	0	0
Other	2	<1	1	<1
Not available*	51	7	66	9

Figures are n, %
* data are missing

3.5 Women's compliance with the trial tablets

3.5.1 Progress with the tablets from trial entry to 28 weeks' gestation

When assessed at 28 weeks' gestation, women's progress with the trial tablets did not differ between the treatment groups (Table 3.7). Furthermore, 647 women (84%) in the vitamin C and E group and 666 women (87%) in the placebo group indicated they were still taking the tablets at 28 weeks' gestation. No differences were seen in the mode of tablet taking, how often women missed taking their tablets and the number of tablet doses women missed. For all women in the trial the mode of tablet taking varied widely. Approximately half of all women took the trial tablets either "all in the morning" (vitamin group 247 [32%] vs. placebo group 245 [32%], p=0.941) or in the recommended mode of "two in the morning and two at night" (vitamin group 183 [24%] vs. placebo group 203 [26%], p=0.228). By 28 weeks' gestation, 123 women (16%) in the vitamin C and E group and 102 women (13%) in the placebo group reported they were no longer taking the trial tablets (p=0.135).

When asked how often they missed the trial tablets, the majority of women reported they either "never missed" (vitamin group 120 [15%] vs. placebo group 120 [16%], p=0.982) or "occasionally missed" (vitamin group 409 [53%] vs. placebo group 410 women [53%], p=0.916). Fifty-two women (7%) in the vitamin C and E group and 43 women (5%) in the placebo group reported they had "never taken" any of the trial tablets up until 28 weeks' gestation (p=0.347) and slightly fewer women reported that they had stopped taking the

tablets at or before 28 weeks' gestation (vitamin group 28 [4%] vs. placebo group 24 [3%], p=0.579).

When asked to quantify the average number of times per week the trial tablets were not taken, the majority of women indicated they missed "1-2 times per week" (vitamin group 285 [37%] vs. placebo group 294 [38%], p=0.608), followed by "none" (vitamin group 156 [20%] vs. placebo group 160 [21%], p=0.781) or "less than once a week" (vitamin group 97 [13%] vs. placebo group 84 [11%], p=0.312).

Table 3.7 Progress with trial tablets from trial entry to 28 weeks' gestation

	Vitamin C & E		Placebo		p value
	n=770	%	n=768	%	
Mode of tablet taking					
Twice a day	183	24	203	26	0.228
All in morning	247	32	245	32	0.941
All in evening	120	15	113	15	0.634
All at lunchtime	10	1	5	<1	0.135
All at once, time varied	12	2	10	1	0.672
Varied times	22	3	16	2	0.328
Other	2	<1	7	1	0.094
No longer taking	123	16	102	13	0.135
How often women missed the trial tablets					
Never missed	120	15	120	16	0.982
Occasionally missed	409	53	410	53	0.916
Frequently missed	107	14	100	13	0.615
Not taking at or before 28 weeks'	28	4	24	3	0.579
Unknown	3	<1	5	<1	0.720
Never taken	52	7	43	5	0.347
Average number of missed doses per week					
None	156	20	160	21	0.781
<1 per week	97	13	84	11	0.312
1-2	285	37	294	38	0.608
3-4	33	4	39	5	0.462
>4	87	11	72	9	0.215
Other	8	1	10	1	0.631
Not taking	53	7	43	6	0.298
Not available*	51	7	66	9	0.145

Figures are n, %
* data are missing

For those women who missed some of the doses of trial tablets, the most common reason for missing the tablets was "have just forgotten to take them" (vitamin group 410 [54%] vs. placebo group 413 [53%], p=0.471) (Table 3.8). There were no differences between the treatment groups for any of the reasons indicated why the tablets were missed. Few women indicated they missed the trial tablets for reasons relating to the actual tablets including "too

many tablets" (vitamin group 46 [5%] vs. placebo group 36 [6%], p=0.308) or the "tablets too large" (vitamin group 28 [3%] vs. placebo group 21 [4%], p=0.354).

Table 3.8 Reasons for missing trial tablets from trial entry to 28 weeks' gestation

Reason	Vitamin C & E n=770	%	Placebo n=768	%	p value
Have not missed any	115	15	117	15	0.723
Have just forgotten to take them	410	54	413	53	0.471
Didn't have them with me	106	15	116	14	0.349
Feeling unwell	86	10	79	11	0.684
Too many tablets	46	5	36	6	0.308
Tablets too large	28	3	21	4	0.354
Too much effort required	24	3	21	3	0.713
Other reasons*	131	16	122	17	0.688
Not available**	50	9	66	6	0.119

Figures are n, %
* includes those women who chose to stop taking the tablets altogether
** data are missing

3.5.2 Progress with the trial tablets after 28 weeks' gestation

Women's progress with the trial tablets in late pregnancy (after 28 weeks' gestation) is detailed in Table 3.9. Five-hundred and ninety-six women (77%) in the vitamin C and E group and 624 women (81%) in the placebo group indicated they were taking the tablets after 28 weeks' gestation. No difference was seen in the mode of tablet taking between the treatment groups. Like earlier in pregnancy, most women were taking the trial tablets either "all in the morning" (vitamin group 232 [30%] vs. placebo group 234 [30%], p=0.885) or "two in the morning and two at night" (vitamin group 157 [20%] vs. placebo group 176 [23%], p=0.229). By the end of pregnancy, 174 women (23%) in the vitamin C and E group and 144 women (19%) reported that they were no longer taking the trial tablets late in pregnancy (p=0.062).

There were no differences between treatment groups in women's reports of how often they missed taking the trial tablets. Most women reported they either "never missed" (vitamin group 97 [13%] vs. placebo group 116 [15%], p=0.155) or "occasionally missed" (vitamin group 377 [49%] vs. placebo group 357 [46%], p=0.331). Like in earlier pregnancy, the majority of women indicated they missed taking the trial tablets on average "1-2 times per week" (vitamin group 203 [26%] vs. placebo group 207 [27%], p=0.794), followed by "none" (vitamin group 131 [17%] vs. placebo group 136 [18%], p=0.719) or "less than once a week"

(vitamin group 132 [17%] vs. placebo group 130 [17%], p=0.910). Significantly more women (152, 20%) in the vitamin C and E group when asked about missed trial doses reported that they had either never taken or had stopped taking all of the trial tablets compared with women in the placebo group (119, 15%) (p=0.029).

Table 3.9 Progress with trial tablets after 28 weeks' gestation

	Vitamin C & E		Placebo		p value
	n=770	%	n=768	%	
Mode of tablet taking					
Twice a day	157	20	176	23	0.229
All in morning	232	30	234	30	0.885
All in evening	114	15	104	13	0.477
All at lunchtime	5	<1	8	1	0.401
All at once, time varied	8	1	6	<1	0.595
Varied	16	2	18	2	0.723
Other	5	<1	4	<1	0.741
Not taking after 28 weeks	174	23	144	19	0.062
How often women missed the trial tablets					
Never missed	97	13	116	15	0.155
Occasionally missed	377	49	357	46	0.331
Frequently missed	58	8	75	10	0.119
Occasionally missed until last month of pregnancy, when missed frequently	9	1	5	<1	0.285
Never taken/stopped taking	171	22	141	18	0.061
Average number of missed doses per week					
None	131	17	136	18	0.719
<1 per week	132	17	130	17	0.910
1-2	203	26	207	27	0.794
2-3	2	<1	5	<1	0.254
3-4	34	4	50	6	0.071
>4	36	5	37	5	0.895
Missed 1-2 per week until last month of pregnancy, then missed frequently	14	2	8	1	0.200
Other	6	1	2	<1	0.157
Never taken/stopped taking	**152**	**20**	**119**	**15**	**0.029**
Not available*	58	7	74	10	0.141

Figures are n, %
* data are missing

When asked about the main reasons for missing the trial tablets late in pregnancy, the most common reason indicated was "have just forgotten to take them" (vitamin group 392 [51%] vs. placebo group 390 [51%], p=0.646) (Table 3.10). There were no differences between the treatment groups for any of the reasons indicated why the tablets were missed. Again, very few women indicated they missed the trial tablets for reasons relating to the actual tablets including "too many tablets" (vitamin group 15 [1%] vs. placebo group 10 [2%], p=0.347) or the "tablets too large" (vitamin group 28 [3%] vs. placebo group 21 [4%], p=0.354). Just over 10% of women indicated they "have not missed any" trial tablets after 28 weeks' gestation (vitamin group 94 [13%] vs. placebo group 103 [12%], p=0.370).

Table 3.10 Reasons for missing trial tablets after 28 weeks' gestation

Reason	Vitamin C & E		Placebo		p value
	n=770	%	n=768	%	
Have not missed any	94	13	103	12	0.370
Have just forgotten to take them	392	51	390	51	0.646
Didn't have them with me	70	8	64	9	0.703
Feeling unwell	47	8	58	6	0.208
Too many tablets	26	2	15	3	0.098
Tablets too large	15	1	10	2	0.347
Too much effort required	23	2	17	3	0.381
Other reasons (including withdrawn)	204	23	183	26	0.346
Not available*	57	10	74	7	0.117

Figures are n, %
* data are missing

3.5.3 Overall compliance

Information collected at the 28 week and postnatal assessments relating to women's progress with the trial tablets was compiled to give an overall compliance assessment (Table 3.11). Compliance was based on how often women missed their tablets (never missed or occasionally missed) and the average number of times women missed taking all of their trial tablets (missed 1-2 times per week or less), which equates with trial tablets being taken for approximately 80 percent of the required time. Approximately 70 percent of all women were compliant from the time they entered the trial to 28 weeks' gestation (vitamin group 544 women [71%] vs. placebo group 549 [71%], RR 0.98, 95% CI 0.93 to 1.05, p=0.718). There was no difference in the number of women compliant at this stage between the treatment groups. From 28 weeks' gestation to delivery slightly fewer women were classed as compliant, however again, there was no difference between the treatment groups (vitamin group 480 [62%] vs. placebo group 482 [63%], RR 0.99, 95% CI 0.92 to 1.07, p=0.864). On the whole, there was no difference in overall compliance between the treatment groups, where 457 women (59%) in the vitamin C and E group and 459 women (60%) in the placebo group were compliant for the entire pregnancy (RR 0.99, 95% CI 0.91 to 1.0, p=0.868).

Table 3.11 Overall compliance in the vitamin and placebo groups

	Vitamin C & E		Placebo		RR	95% CI	p value
	n=770	%	n=768	%			
Compliance from trial entry to 28 weeks' gestation	544	71	549	71	0.98	0.93 to 1.05	0.718
Compliance from 28 weeks' gestation to delivery	480	62	482	63	0.99	0.92 to 1.07	0.864
Overall compliance	457	59	459	60	0.99	0.91 to 1.08	0.868

Figures are n, %
RR = relative risk, CI = confidence intervals

3.6 Primary outcomes

No difference was seen in the risk of having a small for gestational age (SGA) infant between treatment groups, where the incidence was 8.6 percent (66 infants) in the vitamin C and E group and 9.4 percent (71 infants) in the placebo group (RR 0.92, 95% CI 0.67 to 1.27, p=0.614) (Table 3.12). For pre-eclampsia, there was no difference in the overall risk of pre-eclampsia (vitamin group 60 [7.8%] vs. placebo group 51 [6.6%], RR 1.17, 95% CI 0.82 to 1.68, p=0.383), or whether pre-eclampsia was detected either prior to 34 weeks' or at or greater than 34 weeks' gestation. Supplementation was associated with a 30 percent reduction in the relative risk of death or serious adverse outcome for the infant (vitamin group 54 [7.0%] vs. placebo group 77 [10.0%], RR 0.70, upper 95% CI <0.93, p=0.021). This corresponds with an absolute risk reduction of three percent, whereby 33 women would need to take the vitamins during pregnancy in order for one infant to benefit (NNTB=33). When all lethal congenital abnormalities and terminations of pregnancy were excluded from death or serious adverse infant outcome, vitamin C and E supplementation was associated with a 28 percent relative risk reduction or a 2.5 percent absolute risk reduction in death or serious adverse infant outcome (vitamin group 52 [6.8%] vs. placebo group 71 [9.3%], RR 0.73, upper 95% CI <0.97, p=0.041, NNTB=39).

Table 3.12 Primary study outcomes in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)	66	8.6	71	9.4	0.92	0.67 to 1.27	0.614
Pre-eclampsia	60	7.8	51	6.6	1.17	0.82 to 1.68	0.383
Detected < 34 weeks'	6	0.8	4	0.5	1.50	0.42 to 5.28	0.753
Detected ≥ 34 weeks'	54	7.0	47	6.1	1.14	0.78 to 1.67	0.479
Death or serious adverse outcome for the infant	**54**	**7.0**	**77**	**10.0**	**0.70**	**<0.93**	**0.021***

Figures are n, %
RR = relative risk, CI = confidence intervals
*one tailed p value and upper 95% confidence interval

No difference was seen in the risk of early fetal death (<20 weeks' gestation), stillbirth or neonatal death prior to hospital discharge between treatment groups (Table 3.13). When all perinatal losses were combined (including early fetal death, stillbirth and neonatal death), there were fewer deaths in the vitamin C and E group overall, however this was not statistically significant (vitamin group 8 [1.0%] vs. placebo 15 [2.0%], RR 0.53, 95% CI 0.23 to 1.25, p=0.140). When all lethal congenital abnormalities and terminations of pregnancy were excluded from the total infant death outcome, no differences were seen between

treatment groups, where 6 (0.8%) perinatal losses occurred in the vitamin C and E group and 9 (1.2%) in the placebo group (RR 0.66, 95% CI 0.24 to 1.85, p=0.297). The causes of perinatal death according to the amended Whitfield (Whitfield et al 1986) and amended Wigglesworth (Wigglesworth, 1980) classification systems are listed in Table 3.14.

Table 3.13 Perinatal losses in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Early fetal death*	2	0.3	7	0.9	0.28	0.06 to 1.37	0.108
Stillbirth	5	0.7	5	0.7	0.99	0.29 to 3.41	1.000
Neonatal death prior to hospital discharge	1	0.1	3	0.4	0.33	0.03 to 3.17	0.374
Any fetal or neonatal death	8	1.0	15	2.0	0.53	0.23 to 1.25	0.140

Figures are n, % * includes pregnancy terminations
RR = relative risk, CI = confidence intervals

Table 3.14 Amended Whitfield classification of fetal and neonatal deaths

	Vitamin C & E n=770	%	Placebo n=768	%
Early fetal death < 20 weeks' gestation (total)	**2**	**0.3**	**7**	**0.9**
Fetal demise – cause unknown	1	0.1	3	0.4
Congenital abnormality				
Central Nervous System				
Anencephaly (termination of pregnancy)	0	0.1	1	0.1
Encephalocele (termination of pregnancy)	0	0	1	0.1
Multiple abnormalities (termination of pregnancy)	0	0	1	0.1
Other: Amniotic band	0	0	1	0.1
Termination of pregnancy (maternal psychosocial reasons)	1	0.1	0	0
Stillbirth (total)	**5**	**0.7**	**5**	**0.7**
Intrauterine growth restriction *(normally formed stillbirth)**	1	0.1	2	0.3
Fetal abnormality - Chromosomal *(congenital malformation)** (termination of pregnancy for Trisomy 18)	1	0.1	0	0
Spontaneous preterm *(condition associated with prematurity)**	1	0.1	0	0
Unexplained intrauterine death *(normally formed stillbirth)**	2	0.2	1	0.1
Bacterial infection: E. Coli *(normally formed stillbirth)**	0	0	1	0.1
Cause Other: intraventricular haemorrhage with fetal thrombotic vasculopathy *(normally formed stillbirth)**	0	0	1	0.1
Neonatal death (total)	**1**	**0.1**	**3**	**0.4**
Extreme prematurity *(condition associated with prematurity)**	1	0.1	0	0
Congenital abnormality *(congenital malformation)**				
Central nervous system	0	0	1	0.1
Other congenital abnormality (diaphragmatic hernia)	0	0	1	0.1
Gastrointestinal – necrotising enterocolitis *(condition associated with immaturity)**	0	0	1	0.1

Figures are n, %
* Amended Wigglesworth classification of death in italics and parentheses

Excluding all perinatal losses, fewer infants in the vitamin C and E group had any serious adverse outcomes when compared with infants in the placebo group however the difference was not statistically significant (vitamin group 46 [6.0%] vs. placebo group 62 [8.2%], RR 0.73, 95% CI 0.51 to 1.06, p=0.097) (Table 3.15). When the individual outcomes included in the composite endpoint of infant death or serious adverse outcome were reported separately, there were no statistically significant differences for any of the outcomes between treatment groups.

Table 3.15 Serious adverse infant outcome in the vitamin and placebo groups

Outcome	Vitamin C & E n=763	%	Placebo n=756	%	RR	95% CI	p value
Severe adverse infant outcome	46	6.0	62	8.2	0.73	0.51 to 1.06	0.097
Severe IUGR(< 3rd centile)	19	2.5	26	3.4	0.72	0.40 to 1.30	0.275
Severe RDS	1	0.1	2	0.3	0.50	0.05 to 5.45	0.623
Chronic lung disease*	1	0.1	4	0.5	0.25	0.03 to 2.21	0.216
Intraventricular haemorrhage grade 3 or 4	1	0.1	1	0.1	0.99	0.06 to 15.81	1.000
Cystic periventricular leucomalacia	0	0	1	0.1		-	0.498
ROP requiring treatment	0	0	1	0.1		-	0.498
Necrotising enterocolitis	0	0	2	0.3		-	0.248
Apgar score <4 at 5 mins	3	0.4	2	0.3	1.49	0.25 to 8.87	1.000
Seizures at <24 hours age or requiring 2 or more drugs to control	3	0.4	1	0.1	2.97	0.31 to 28.51	0.625
Hypotonia for at least 2hrs	7	0.9	7	0.9	0.99	0.35 to 2.81	0.986
Stupor, decreased response to pain or coma	4	0.5	3	0.4	1.32	0.30 to 5.88	1.000
Tube feeding ≥4 days	20	2.6	31	4.1	0.64	0.37 to 1.11	0.110
Care in NICU >4 days	8	1.1	11	1.5	0.72	0.29 to 1.78	0.476
Ventilation for ≥24 hrs	11	1.4	17	2.2	0.64	0.30 to 1.36	0.242

Figures are n, %
RR = relative risk, CI = confidence intervals, IUGR = intrauterine growth restriction, RDS = respiratory distress syndrome, ROP = retinopathy of prematurity, NICU = neonatal intensive care unit
*defined as need for oxygen at 36 weeks' postconceptual age for those infants born < 32 weeks' gestation, or oxygen required on day 28 for those infants born ≥32 weeks' gestation

3.7 Secondary maternal outcomes

No difference was seen in women's risk of death or serious adverse outcome between treatment groups (vitamin group 74 [9.6%] vs. placebo group 59 [7.7%], RR 1.25, 95% CI 0.90 to 1.73, p=0.179) (Table 3.16). When the individual outcomes included in the composite outcome of death or serious maternal adverse outcome were reported separately, women in the vitamin C and E group had a statistically significant increase in the risk of abnormal liver

function (vitamin group 18 [2.3%] vs. placebo group 6 [0.8%], RR 2.99, 95% CI 1.19 to 7.50, p=0.014, NNTH=67). Of these women with abnormal liver function, the majority (15 women) were diagnosed with pre-eclampsia, and a further five women had obstetric cholestasis (vitamin group 3 [0.4%] vs. placebo group 2 [0.3%], RR 1.50, 95% CI 0.25 to 8.93, p=0.656). No other differences were seen between the treatment groups for any of the individual maternal outcomes. There were no maternal deaths or any women with eclampsia, stroke, adult respiratory distress syndrome or cardiac or respiratory arrest in either of the treatment groups.

Table 3.16 Death or serious adverse maternal outcome in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Death or serious maternal adverse outcome	74	9.6	59	7.7	1.25	0.90 to 1.73	0.179
Maternal death	0	0	0	0	-		-
Pulmonary oedema	1	0.1	1	0.1	1.00	0.06 to 15.9	1.000
Eclampsia	0	0	0	0	-		-
Stroke	0	0	0	0	-		-
Thrombocytopenia	7	0.9	2	0.3	3.49	0.73 to 16.75	0.178
Renal insufficiency	10	1.3	5	0.7	1.99	0.69 to 5.80	0.196
Adult respiratory distress syndrome	0	0	0	0	-		-
Cardiac arrest	0	0	0	0	-		-
Respiratory arrest	0	0	0	0	-		-
Placental abruption	3	0.4	1	0.1	2.99	0.31 to 28.70	0.625
Abnormal liver function	**18**	**2.3**	**6**	**0.8**	**2.99**	**1.19 to 7.50**	**0.014**
Coagulopathy	4	0.5	6	0.8	0.66	0.19 to 2.35	0.547
Preterm PROM	22	2.9	20	2.6	1.09	0.60 to 1.99	0.761
Major postpartum haemorrhage	21	2.7	22	2.9	0.95	0.53 to 1.72	0.870
Postpartum pyrexia	9	1.2	6	0.8	1.50	0.53 to 4.18	0.439
Pneumonia	2	0.3	2	0.3	1.00	0.14 to 7.06	1.000
Deep vein thrombosis	0	0	1	0.1	-		0.499
Pulmonary embolus	0	0	1	0.1	-		0.499

Figures are n, %
RR = relative risk, CI = confidence intervals, PROM = prelabour rupture of membranes

3.8 Secondary infant outcomes

3.8.1 Preterm birth

For all births occurring after 20 weeks' gestation, there was no difference in the gestational age at birth between treatment groups (median, weeks; vitamin group 40.0 [range 24-42] vs.

placebo group 40 [range 21-42], p=0.706) (Table 3.17). Approximately 6 percent of all births were preterm (< 37 weeks' gestation), however no difference was seen in the risk of preterm birth between treatment groups (vitamin group 48 [6.2%] vs. placebo group 52 [6.8%], RR 0.91, 95% CI 0.62 to 1.34, p=0.645). Similarly, the risk of very preterm birth (< 34 weeks' gestation) or extremely preterm birth (< 28 weeks' gestation) did not differ between the treatment groups.

Table 3.17 Preterm birth in the vitamin and placebo groups

Outcome	Vitamin C & E		Placebo		RR	95% CI	p value
	n=768	%	n=761	%			
Gestational age at birth*	40	24-42	40	21-42	-		0.706
Preterm birth (< 37 wks)	48	6.2	52	6.8	0.91	0.62 to 1.34	0.645
Very preterm (< 34 wks)	13	1.7	13	1.7	0.99	0.46 to 2.12	0.981
Extremely preterm (< 28 wks)	4	0.8	5	0.6	0.79	0.21 to 2.94	0.728

Figures are n, % or * median, range
RR= relative risk, CI=confidence intervals

3.8.2 Infant growth parameters

Infants of mothers in the vitamin C and E group had a greater head circumference at birth when compared with those in the placebo group (mean, cm; vitamin group 34.6 [SD 1.7] vs. placebo group 34.4 [SD 1.9], mean difference 0.18 cm, 95% CI 0.00 to 0.36, p=0.047) (Table 3.18). Moreover, infants in the vitamin C and E group had a greater head circumference z-score (vitamin group –0.06 [1.11] vs. placebo group –0.19 [1.18], mean difference –0.12, 95% CI 0.01 to 0.24, p=0.035). No differences were seen for any other growth parameters at birth including weight and length and their z-scores, the number of infants with a birth weight less than the 10th centile or 3rd centile for gestational age, or birth weight less than 2,500 or 1,500 grams. No differences were seen in placental weight between treatment groups, however placental weight was not available for over half of all women in each treatment group.

Table 3.18 Birth weight and other growth parameters in the vitamin and placebo groups

Outcome	Vitamin C & E n=763		Placebo n=756		TE	95% CI	p value
Birth weight (grams)	3413.6	516.6	3398.9	568.4	14.6	-42.2 to 71.5	0.613
Birth weight z-scores	-0.02	0.03	-0.05	0.97	0.03	-0.06 to 0.13	0.535
Length at birth (cm)[†]	50.5	3.0	50.4	2.7	0.10	-0.19 to 0.39	0.498
Length z-scores[†]	0.01	1.16	0.00	1.18	0.01	-0.11 to 0.13	0.857
Head circumference (cm)[‡]	**34.6**	**1.7**	**34.4**	**1.9**	**0.18**	**0.00 to 0.36**	**0.047**
Head circumference z-scores[‡]	**-0.06**	**1.11**	**-0.19**	**1.18**	**0.12**	**0.01 to 0.24**	**0.035**
Placental weight[¶]	597.9	133.4	587.9	153.3	10.0	-11.37 to 31.4	0.358
Birth weight < 10th centile*	66	8.6	71	9.4	0.92	0.67 to 1.27	0.614
Birth weight < 3rd centile*	19	2.5	26	3.4	0.72	0.40 to 1.30	0.275
Birth weight < 2,500g*	36	4.7	34	4.5	1.05	0.66 to 1.66	0.837
Birth weight < 1,500g*	6	0.8	7	0.9	0.85	0.29 to 2.52	0.768

Figures are mean, standard deviation and mean difference, 95% CI around the mean difference or * n, %
[†]n=749 (97%) in vitamin C and E group and n=734 (97%) in placebo group
[‡]n=750 (98%) in vitamin C and E group and n=745 (98%) in placebo group
[¶]n= 346 (45%) in vitamin C and E group and n= 352 (46%) in placebo group
TE= treatment effect, CI= confidence intervals

3.9 Subsidiary infant outcomes

Other subsidiary infant outcomes are shown in Table 3.19. No overall difference in the need for resuscitation at birth was observed, however fewer infants in the vitamin C and E group required bag and mask resuscitation (vitamin group 56 [7.3%] vs. 85 [11.1%], RR 0.66, 95% CI 0.45 to 0.91, p=0.010). Significantly fewer infants in the vitamin C and E group had respiratory distress syndrome (vitamin group 1 [0.1%] vs. placebo group 9 [1.2%], RR 0.11, 95% CI 0.01 to 0.87, p=0.011), with the difference primarily occurring in those infants with moderate disease severity (vitamin group 0 [0%] vs. placebo group 5 [0.6%], p=0.034). No difference was seen however in the need for and duration of mechanical ventilation (vitamin group 11 [1.4%] vs. placebo group 17 [2.3%], RR 0.64, 95% CI 0.30 to 1.36, p=0.242).

No other differences were observed between treatment groups for any other subsidiary outcomes for the infant including Apgar score less than seven at five minutes of age; admission to and duration of stay in the neonatal intensive care unit; any cerebrovascular haemorrhage or periventricular leucomalacia; the need for oxygen therapy after 28 days of life; use of postnatal steroids; suspected or confirmed infection or the use of antibiotics; use of surfactant; need for nitric oxide for respiratory support; need for ionotropic support; air leak syndrome; retinopathy of prematurity; patent ductus arteriosis requiring treatment; thrombocytopaenia; major congenital malformation or neonatal encephalopathy (at any Sarnat

stage). Details of all congenital malformations in live born infants in each treatment group are detailed in the Appendix (Table 8.3).

Table 3.19 Subsidiary outcomes for the infant in the vitamin and placebo groups

Outcome	Vitamin C & E n=763	%	Placebo n=756	%	RR	95% CI	p value
Resuscitation at birth	237	30.8	251	32.7	0.94	0.81 to 1.09	0.423
O2 alone	179	23.2	169	22.0			
Bag and mask	**56**	**7.3**	**85**	**11.1**	**0.66**	**0.48 to 0.91**	**0.010**
IPPV via ETT	8	1.0	10	1.3			
Drugs given	18	2.3	18	1.6			
Apgar scores <7 at 5 minutes	14	1.8	8	1.1	1.73	0.73 to 4.11	0.205
Admission to NICU	21	2.8	26	3.4	0.80	0.45 to 1.41	0.440
Length of stay (days)*	3	4	2.5	5	-		0.441
Respiratory Distress Syndrome	**1**	**0.1**	**9**	**1.2**	**0.11**	**0.01 to 0.87**	**0.011**
Mild RDS	0	0	2	0.3	-		0.127
Moderate RDS	0	0	5	0.6	-		0.034
Severe RDS	1	0.1	2	0.3	1.5	0.67 to 3.33	0.505
Mechanical ventilation	11	1.4	17	2.3	0.64	0.30 to 1.36	0.242
Duration, hrs**	0	0-772	0	0-2304	-		0.240
Intraventricular haemorrhage	2	0.3	2	0.3	0.99	0.14 to 7.01	1.000
Periventricular leucomalacia	0	0	1	0.1	-		0.498
Oxygen therapy ≥28 days	2	0.3	6	0.8	0.33	0.07 to 1.64	0.178
Use of postnatal steroids	1	0.1	3	0.4	0.33	0.03 to 3.17	0.372
Infection							
Suspected infection ≤48hrs	83	10.9	96	12.7	0.86	0.65 to 1.13	0.271
Proven infection ≤48hrs	0	0	2	0.3	-		0.248
Use of antibiotics ≤48hrs	37	4.9	40	5.3	0.92	0.59 to 1.41	0.695
Suspected infection > 48hrs	25	3.3	20	2.6	1.24	0.69 to 2.21	0.468
Proven infection > 48hrs	2	0.3	4	0.5	0.50	0.09 to 2.70	0.450
Use of antibiotics > 48hrs	18	2.4	16	2.1	1.11	0.57 to 2.17	0.749
No. episodes of infection**	0	0-1	0	0-2	-		0.274
Use of surfactant	2	0.3	6	0.8	0.33	0.07 to 1.63	0.177
NO for respiratory support	0	0	1	0.1	-		0.498
Need for ionotropic support	0	0	4	0.5	-		0.061
Air leak syndrome	2	0.3	1	0.1	1.98	0.18 to 21.81	1.000
Retinopathy of prematurity	1	0.1	5	0.7	0.20	0.02 to 1.69	0.123
PDA requiring treatment	2	0.3	4	0.5	0.49	0.09 to 2.70	0.450
Thrombocytopenia	2	0.3	3	0.4	0.66	0.11 to 3.94	0.686
Major congenital malformation	8	1.0	12	1.6	0.66	0.27 to 1.61	0.357
Neonatal encephalopathy	3	0.4	1	0.1	2.97	0.31 to 28.51	0.625
Sarnat stage 1	2	0.3	1	0.1			
Sarnat stage 2	0	0	0	0			
Sarnat stage 3	1	0.1	0	0			

Figures are n, % or *median, interquartile range or ** median, range
RR = relative risk, CI = confidence intervals, NICU = neonatal intensive care unit, RDS = respiratory distress syndrome, U/S = ultrasound, NO = nitric oxide, PDA = patent ductus arteriosis

As stated earlier, infants in the vitamin C and E group had a reduced risk of developing respiratory distress syndrome. When all neonatal respiratory outcomes were analysed (Table 3.20), there was no difference in the risk of any neonatal lung disease or the respiratory disease severity between treatment groups (any neonatal lung disease: vitamin group 52 [6.8%] vs. placebo group 53 [7.0%], RR 0.97, 95% CI 0.67 to 1.41, p=0.881). Similarly there were no other differences in any other respiratory complications including non-specific respiratory distress, meconium aspiration syndrome, apnoea, respiratory complications resulting from a congenital abnormality, persistent pulmonary hypertension or other neonatal lung disease.

Table 3.20 Neonatal respiratory outcomes in the vitamin and placebo groups

Outcome	Vitamin C & E n=763	%	Placebo n=756	%	RR	95% CI	p value
Any neonatal lung disease	52	6.8	53	7.0	0.97	0.67 to 1.41	0.881
Mild	42	5.5	37	4.9			
Moderate	9	1.2	13	1.7			
Severe	1	0.1	3	0.4			0.560#
Main respiratory diagnosis							
Non specific respiratory distress	45	5.9	38	5.0	1.17	0.77 to 1.78	0.455
Respiratory distress syndrome	**1**	**0.1**	**9**	**1.2**	**0.11**	**0.01 to 0.87**	**0.011**
Meconium aspiration	1	0.1	2	0.3	0.49	0.04 to 5.45	0.623
Apnoea	0	0	1	0.1	-		0.498
Congenital abnormality	1	0.1	0	0	-		1.000
Persistent pulmonary hypertension	0	0	2	0.3	-		0.248
Other	4	0.5	1	0.1	3.96	0.44 to 35.38	0.374
Central asphyxial failure	1	0.1	0	0			
Air Leak/Pneumothorax/ Pneumomediastinum	3	0.4	0	0			
Pulmonary hypoplasia	0	0	1	0.1			

Figures are n, %
RR = relative risk, CI = confidence intervals
#overall comparison including all levels of disease severity between treatment groups

3.10 Subsidiary maternal outcomes

Fewer women in the vitamin C and E group had clinical chorioamnionitis requiring intrapartum antibiotics and pyrexia in labour when compared with the placebo group however these findings did not reach statistical significance (chorioamnionitis, vitamin group 3 [0.4%] vs. placebo group 9 [1.2%], RR 0.33, 95% CI 0.09 to 1.22, p=0.091; pyrexia, vitamin group 8 [1.0%] vs. placebo group 17 [2.2%], RR 0.47, 95% CI 0.20 to 1.08, p=0.069) (Table 3.21). There were no differences for any of the other subsidiary outcomes for women in the vitamin C and E group compared with the placebo group including hospitalisation for preterm labour, use of tocolytics or prenatal corticosteroids, the development of gestational diabetes, any

prelabour rupture of membranes (preterm or term), need for induction of labour and reasons, complications of delivery, mode of delivery, perineal trauma, use of postpartum antibiotics, maternal postnatal stay beyond seven days or the number of women who were exclusively breastfeeding at hospital discharge.

Table 3.21 Subsidiary outcomes for women in the vitamin and placebo groups

Outcome		Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Clinical chorioamnionitis**		3	0.4	9	1.2	0.33	0.09 to 1.22	0.091
Antenatal hospitalisation for preterm labour		18	2.3	21	2.7	0.85	0.46 to 1.59	0.621
Tocolytic use		9	1.2	12	1.6	0.75	0.32 to 1.77	0.506
Prenatal corticosteroids use		21	2.7	25	3.3	0.84	0.47 to 1.48	0.543
Gestational diabetes#		28	3.6	30	3.9	0.93	0.56 to 1.54	0.781
Any PROM (preterm or term)		116	15.1	119	15.5	0.97	0.77 to 1.23	0.815
Term PROM		94	12.2	99	12.9	0.95	0.73 to 1.23	0.686
Induction of labour		262	34.0	233	30.3	1.12	0.97 to 1.30	0.122
Main reason:	Post term	111	14.4	98	12.8	1.13	0.88 to 1.45	0.344
	Hypertension	61	7.9	45	5.9	1.35	0.93 to 1.96	0.110
	IUGR	7	0.9	12	1.6	0.58	0.23 to 1.47	0.246
	Fetal distress	1	0.1	3	0.4	0.33	0.03 to 3.19	0.374
	Other	92	12.0	85	11.1	1.08	0.82 to 1.42	0.589
Complications of delivery								
Pyrexia		8	1.0	17	2.2	0.47	0.20 to 1.08	0.069
Bleeding due to placental praevia		0	0	2	0.3	-		0.249
Bleeding due to placental abruption		1	0.1	4	0.5	0.25	0.03 to 2.22	0.218
Augmentation		306	39.7	276	35.9	1.10	0.97 to 1.26	0.124
Meconium stained liquor		143	18.6	154	20.1	0.93	0.75 to 1.14	0.462
Mode of delivery								
NVD		406	52.7	406	52.9	1.00	0.91 to 1.10	0.957
Caesarean section		203	26.4	200	26.0	1.01	0.86 to 1.20	0.886
Forceps		65	8.4	49	6.4	1.32	0.93 to 1.89	0.123
Ventouse		88	11.4	102	13.3	0.86	0.66 to 1.12	0.270
Vaginal breech		6	0.8	4	0.5	1.50	0.42 to 5.28	0.528
Prelabour caesarean section		42	5.4	40	5.2	1.05	0.69 to 1.60	0.830
Perineal trauma sutured		379	49.2	378	49.2	1.00	0.90 to 1.11	0.999
Episiotomy		151	19.6	156	20.3	0.97	0.79 to 1.18	0.731
Tear		242	31.4	241	31.4	1.00	0.86 to 1.16	0.984
Degree of perineal trauma								
1		53	6.9	56	7.3			0.799
2		297	38.6	298	38.8			
3		26	3.4	20	2.6			
4		3	0.4	4	0.5			
Postpartum antibiotics		100	13.0	119	15.5	0.84	0.65 to 1.07	0.159
Maternal postnatal stay ≥ 7days		15	2.0	21	2.7	0.71	0.37 to 1.37	0.308
Exclusive breast feeding at hospital discharge		623	81.8	617	82.1	0.99	0.95 to 1.04	0.840

Figures are n, %

** requiring intrapartum antibiotics

#2 hour glucose tolerance test result ≥ 7.8 mmol/L

RR = relative risk, CI = confidence intervals, IUGR = intrauterine growth restriction, PROM = prelabour rupture of membranes, NVD = normal vaginal delivery

3.10.1 Medications and health service utilisation

Use of antihypertensive medications was greater in the vitamin C and E group (40 [5.2%]) compared with the placebo group (24 [3.1%]) (RR 1.66, 95% CI 1.01 to 2.73, p=0.042), however no difference was seen in the use of magnesium sulphate (vitamin group 10 [1.3%] vs. placebo group 6 [0.8%], RR 1.67, 95% CI 0.61 to 4.55, p=0.317) (Table 3.22). No difference was seen in the need for antenatal hospitalisation admission or day care admission for hypertension or induction of labour for hypertension between treatment groups.

Table 3.22 Use of medications and health services for hypertensive disease

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Use of antihypertensives	**40**	**5.2**	**24**	**3.1**	**1.66**	**1.01 to 2.73**	**0.042**
Antenatally	22	2.9	15	1.9			
Intrapartum	16	2.1	12	1.6			
Postnatally	22	2.9	13	1.7			
Use of $MgSO_4$	10	1.3	6	0.8	1.67	0.61 to 4.55	0.317
Hypertension requiring antenatal hospitalisation or antenatal day care admission	82	10.6	70	9.1	1.17	0.86 to 1.58	0.313
Induction of labour for hypertension	61	7.9	45	5.9	1.35	0.93 to 1.96	0.110

Figures are n, %
RR= relative risk, CI=confidence intervals, $MgSO_4$=magnesium sulphate

Details of the use of health services resources for all women and their infants are presented in Table 3.23. Fewer infants in the vitamin C and E group were admitted to an observation nursery (133 [17.4%]) compared with the placebo group (162 [21.4%]) (RR 0.81, 95% CI 0.61 to 1.00, p=0.049). No other differences were seen for measures of health service utilisation for the mother including antenatal hospital, day care admission, mean maternal postnatal stay, and postnatal readmission. For the infant, there were no difference in the mean postnatal stay, admission and length of stay in the level two nursery, admission and length of stay in the neonatal intensive care unit or the duration of mechanical ventilation.

Table 3.23 Use of health services for all women in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95%CI	p value
Maternal							
Any antenatal hospitalisation	156	20.3	138	18.0	1.13	0.91 to 1.38	0.253
No. of days admitted*	2	3	2	3	-		0.548
Antenatal day care admission	116	15.1	103	13.4	1.12	0.88 to 1.44	0.353
No. of admissions*	1	1	1	1	-		0.115
Postnatal stay (days)*	3	2	3	2	-		0.372
Readmission after discharge	27	3.5	30	3.9	0.89	0.54 to 1.49	0.678
For puerperal infection	5	0.7	6	0.8	0.83	0.25 to 2.71	0.859
Infant	**n=763**		**n=756**				
Postnatal stay* (days)	4	2	4	2	-		0.273
Neonatal nursery admission							
Observation Nursery	**133**	**17.4**	**162**	**21.4**	**0.81**	**0.66 to 1.00**	**0.049**
Duration of admission*(hrs)	4	6	5	6	-		0.945
Level 2 Nursery	100	13.1	97	12.8	1.02	0.79 to 1.32	0.873
Duration of admission* (days)	4	5	3	4.5	-		0.429
Level 3 /Intensive Care Unit	21	2.7	26	3.4	0.80	0.45 to 1.41	0.439
Duration of admission** (days)	0	0-74	0	0-94	-		0.441
Duration of mechanical ventilation (hours)**	0	0-772	0	0-2304	-		0.240

Figures are n, % or * median, interquartile range or **median, range
RR = relative risk, CI = confidence intervals

3.11 Potential side effects

3.11.1 Self reported side effects from trial entry to 28 weeks' gestation

There was no difference in any self-reported side effects between the treatment groups when assessed at 28 weeks' gestation (Table 3.24). When women were asked to indicate if they had experienced any symptoms of illness from our predetermined list, the most commonly expressed symptom was fatigue (vitamin group 248 [32%] vs. placebo group 224 [29%], RR 1.08, 95% CI 0.93 to 1.25, p=0.310). When women were given the opportunity to indicate "other" symptoms, the most commonly reported complaints were heartburn (vitamin group 21 [3%] vs. placebo group 13 [2%], RR 1.57, 95% CI 0.79 to 3.12, p=0.189), constipation (vitamin group 10 [1%] vs. placebo group 7 [1%], RR 1.39, 95% CI 0.53 to 3.64, p=0.497) and headaches (vitamin group 7 [1%] vs. placebo group 11 [1%], RR 0.62, 95% CI 0.24 to 1.59, p=0.316). Women were not asked if they believed the symptoms were potential side effects of the tablets, they were merely asked to report if they had experienced them.

Table 3.24 Self reported side effects from trial entry to 28 weeks' gestation

Self assessed side effects	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
None	318	41	294	38	1.05	0.94 to 1.19	0.384
Fatigue	248	32	224	29	1.08	0.93 to 1.25	0.310
Nausea	112	14	134	17	0.81	0.65 to 1.02	0.078
Vomiting	92	12	90	12	1.00	0.76 to 1.31	0.981
Abdominal pain	87	11	108	14	0.78	0.60 to 1.02	0.070
Diarrhoea	72	9	69	9	1.02	0.74 to 1.39	0.914
Weakness	69	9	52	7	1.29	0.92 to 1.83	0.141
Heartburn	21	3	13	2	1.57	0.79 to 3.12	0.189
Constipation	10	1	7	1	1.39	0.53 to 3.64	0.497
Headaches	7	1	11	1	0.62	0.24 to 1.59	0.316
Other	64	9	64	9	0.98	0.70 to 1.36	0.881
Not available*	50	6	66	9			0.119

Figures are n, %
* data are missing, RR = relative risk, CI = confidence intervals

3.11.2 Self reported side effects after 28 weeks' gestation

When asked again about symptoms of illness in late pregnancy, more women in the vitamin C and E group reported "abdominal pain" when compared with women in the placebo group (vitamin group 52 [7%] vs. placebo group 31 [4%], RR 1.63, 95% CI 1.06 to 2.51, p=0.024) (Table 3.25). There were no other differences in self reported side effects between the treatment groups.

Table 3.25 Self reported side effects from 28 weeks' gestation until birth

Self assessed side effects	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
None	378	49	361	47	1.02	0.92 to 1.12	0.708
Fatigue	178	23	183	24	0.95	0.79 to 1.13	0.547
Nausea	108	14	98	13	1.07	0.83 to 1.38	0.586
Vomiting	68	9	66	9	1.00	0.73 to 1.38	0.986
Abdominal pain	**52**	**7**	**31**	**4**	**1.63**	**1.06 to 2.51**	**0.024**
Diarrhoea	66	9	60	8	1.07	0.77 to 1.49	0.688
Weakness	48	6	32	4	1.46	0.94 to 2.25	0.086
Heartburn	14	2	19	2	0.72	0.36 to 1.42	0.337
Headaches	3	<1	6	<1	0.49	0.12 to 1.94	0.297
Constipation	5	<1	1	<1	4.87	0.57 to 41.55	0.109
Other	69	9	63	8	1.07	0.77 to 1.47	0.700
Not available*	57	7	74	10			0.117

Figures are n, %
* data are missing, RR = relative risk, CI = confidence intervals

3.12 Women's views on their participation

Women's views about their involvement in the trial were sought to permit a wider evaluation of the feasibility of vitamin C and E supplementation in pregnancy. Women who stopped taking the trial tablets early were asked about the main reasons for stopping, and all women were asked about what they may have liked or disliked about participating in the trial during their pregnancy.

3.12.1 Women who stopped the trial medications early

Throughout the trial, 217 women in the vitamin C and E group (28%) and 196 women in the placebo group (25%) indicated they stopped taking the trial tablets before delivery (p=0.239) (Table 3.26). Most of these women took the trial medications for only a few weeks, where the mean gestational age women stopped the trial medications was 19.3 weeks' (SD 9.9) in the vitamin C and E group and 18.5 weeks' (SD 10.8) in the placebo group (p=0.517). The reasons for discontinuing the trial tablets are listed in Table 3.28. There were no differences between the treatment groups for any of the reasons indicated, with common reasons for stopping indicated as "too much effort required" (vitamin group 70 [9%] vs. placebo group 51 [7%], p= 0.074) or simply that they would "prefer not to continue" (vitamin group 31 [4%] vs. placebo group 36 [5%], p=0.525).

Table 3.26 Number of women stopping the trial medications

Reason	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Stopped taking tablets	217	28	196	25	1.10	0.94 to 1.30	0.239
Gestational age stopped tablets*	19.3	9.9	18.5	10.8	-		0.517
Reasons for stopping							
Nothing disliked	2	<1	3	<1	0.66	0.11 to 3.97	0.652
Prefer not to continue	31	4	36	5	0.86	0.54 to 1.37	0.525
Too much effort required	70	9	51	7	1.37	0.97 to 1.94	0.074
Felt anxious about not knowing treatment allocation	6	<1	6	<1	1.00	0.32 to 3.08	0.996
Contact with study personnel	1	<1	0	0	-		0.318
Tablets too large	19	2	16	2	1.18	0.61 to 2.28	0.613
Length of time required to take tablets	3	<1	5	<1	0.60	0.14 to 2.49	0.476
Partner/Family worried about trial participation	15	2	7	1	2.14	0.88 to 5.21	0.087
Other individual reasons	151	20	139	18	1.08	0.88 to 1.33	0.449

Figures are n, % or *mean, standard deviation
RR=relative risk, CI=confidence interval

3.12.2 What women liked about being involved in the trial

When women were asked what they liked about their involvement with trial, the vast majority indicated that they liked "assisting with research to help others like me" (vitamin group 581 [75%] vs. placebo group 570 [74%], p=0.754) (Table 3.27). There were no differences between treatment groups for women's likes about the study.

Table 3.27 What women liked about being in the trial

	Vitamin C & E		Placebo		p value
	n=770	%	n=768	%	
Extra attention from study personnel	34	4	29	4	0.593
Knowing that I could be helping my baby	304	39	297	39	0.952
Assisting with research to help others like me	581	75	570	74	0.754
My contact with the study personnel	52	7	46	6	0.624
Study explanation and information	73	9	68	9	0.783
Other individual reasons	38	5	48	6	0.214
i.e. Study newsletters	7	1	7	1	0.996
Liked nothing	43	6	35	5	0.418
Not available*	57	7	74	10	0.117

* data are missing

3.12.3 What women disliked about being involved in the trial

When women were asked what they disliked about the trial (Table 3.28), most responses related to the tablet taking including "remembering to take or actually taking the tablets" (vitamin group 95 [12%] vs. placebo group 78 [10%]) or "tablets too large" (vitamin group 87 [11%] vs. placebo group 70 [9%], p=0.208). Approximately half of all women in each group indicated they disliked nothing about the trial (vitamin group 373 [48%] vs. placebo group 387 [50%], p=0.194). There were no differences in women's dislikes about their involvement in the trial, between the treatment groups.

Table 3.28 What women disliked about being in the trial

	Vitamin C & E n=770	%	Placebo n=768	%	p value
Felt anxious about not knowing treatment allocation	57	7	57	7	0.880
Contact with study personnel	1	<1	0	0	0.324
Being randomised meant I had no say in the treatment group I received	28	4	26	3	0.860
Tablets too large	87	11	70	9	0.208
Too many tablets	72	9	53	7	0.105
Other reasons including:	186	24	170	22	0.492
Remembering to take or taking the tablets	95	12	78	10	
Too many tablets	50	6	48	6	
Trial questionnaires	9	1	9	1	
Potentially on a placebo/not knowing	6	<1	7	1	
Taste and/or size of tablets	5	<1	0	0	
Potential risks	5	<1	1	<1	
Perceived side effects from tablets	4	<1	9	1	
Other individual reasons	12	2	18	2	
Nothing disliked	373	48	387	50	0.194
Not available*	57	7	74	10	0.117

Figures are n, %
* data are missing

3.12.4 Women's views on their perceived treatment group and future participation

When asked about their perceived treatment allocation, only 79 women in the vitamin C and E group (10%) and 97 women in the placebo group (13%) correctly identified their treatment group allocation (p=0.144) (Table 3.29). This provides some reassurance that women remained blinded to their treatment allocation throughout pregnancy. Similar numbers of women incorrectly identified their treatment group allocation (vitamin group 102 [13%] vs. placebo group 90 [12%], p=0.365). Most women however, were unsure whether they were taking the vitamin or placebo tablets (vitamin group 525 [68%] vs. placebo group 502 [65%], p=0.241). Overall there were no differences in women's perception of their treatment group allocation.

When asked about their participation in the study, overall 80 percent of women indicated they would participate in the study again (vitamin group 613 [80%] vs. placebo group 616 [80%], p=0.116) (Table 3.29). Even more women indicated they would recommend the study to other pregnant women in their position (vitamin group 675 [98%] vs. placebo group 651 [85%], p=0.485). There were no differences between treatment groups for women's views on participating in the study.

Table 3.29 Women's views on their treatment allocation and future participation

	Vitamin C & E n=770	%	Placebo n=768	%	p value
Correctly identified treatment group	79	10	97	13	0.144
Incorrectly identified treatment group	102	13	90	12	0.365
Unsure of treatment group	525	68	502	65	0.241
Would participate in future studies					
Yes	613	80	616	80	0.116
No	100	13	78	10	
Would recommend the study to other pregnant women					
Yes	675	88	651	85	0.485
No	38	5	43	6	
Not available*	57	7	74	10	0.117

Figures are n, %
* data are missing

3.13 Pre-specified subgroup analyses

Pre-specified subgroup analyses were performed on the primary and secondary outcomes to investigate the impact of prognostic factors on the observed treatment effects. Each factor was considered for its potential to influence either women's risk of pre-eclampsia, or the potential effectiveness of vitamin C and E supplementation. The subgroups pre-specified were: baseline dietary vitamin C and vitamin E intake (below or equal to and above the RDI); family history of pre-eclampsia or gestational hypertension; maternal smoking status at trial entry; gestational age at randomisation and women's compliance with the trial medications. Comparisons were made between treatment groups in each subgroup and treatment effects were also compared between subgroups (between group p-value).

3.13.1 Dietary intake of vitamin C at trial entry

At trial entry, 31 women in the vitamin C and E group (4%) and 37 women in the placebo group (5%) had a daily vitamin C intake below the RDI (60 mg). Dietary information was not available for 85 women in the vitamin C and E group (11.0%) and 76 women in the placebo group (9.9%), and these women were not included in the subgroup analyses. For all of the subgroup analyses based on women's intake of vitamin C at trial entry, no significant differences were seen between the treatment groups for any of the primary or secondary outcomes (Table 3.30).

Table 3.30 Primary and secondary outcomes (sub-grouped by vitamin C intake)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
< RDI (< 60 mg)	4/31	12.9	7/35	20.0	0.65	0.21 to 1.99	0.521
≥RDI (≥60 mg)	55/644	8.5	56/648	8.6	0.99	0.69 to 1.41	0.948
Between groups							0.480
Pre-eclampsia							
< RDI	5/31	16.1	3/37	8.1	1.99	0.51 to 7.67	0.454
≥RDI	49/651	7.5	42/653	6.4	1.17	0.79 to 1.74	0.438
Between groups							0.459
Detected < 34 weeks'							
< RDI	2/31	6.4	1/37	2.7	2.39	0.23 to 25.09	0.588
≥RDI	3/651	4.6	3/653	4.6	1.00	0.20 to 4.95	1.000
Between groups							0.550
Detected ≥ 34 weeks'							
< RDI	3/31	9.7	2/37	5.4	1.79	0.32 to 10.04	0.653
≥RDI	46/651	7.1	39/653	6.0	1.18	0.78 to 1.79	0.424
Between groups							0.647
Death or serious adverse outcome for the infant							
< RDI	4/31	12.9	8/37	21.6	0.60	<1.50	0.270
≥RDI	44/651	6.7	60/653	9.2	0.73	<1.01	0.064
Between groups							0.724
Death or serious adverse outcome for the woman							
< RDI	2/31	6.4	4/37	10.8	0.60	0.12 to 3.04	0.681
≥RDI	65/651	10.0	49/653	7.5	1.33	0.93 to 1.90	0.113
Between groups							0.346

Figures are n, %
RR = relative risk, CI = confidence intervals
RDI=recommended dietary intake

3.13.2 Dietary intake of vitamin E at trial entry

At trial entry, 242 women in the vitamin C and E group (35%) and 263 women in the placebo group (38%) had a daily vitamin E intake below the RDI (7 mg). Dietary information was again not available for 85 women in the vitamin C and E group (11%) and 76 women in the placebo group (10%), and these women were not included in the subgroup analyses. Like the subgroup analyses based on women's vitamin C intake, no differential treatment effects were seen according to women's intake of vitamin E at trial entry (Table 3.31).

Table 3.31 Primary and secondary outcomes (sub-grouped by vitamin E intake)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
< RDI (< 7 mg)	18/241	7.5	25/258	9.7	0.77	0.43 to 1.38	0.377
≥RDI (≥7 mg)	41/434	9.4	38/425	8.9	1.06	0.69 to 1.61	0.798
Between groups							0.388
Pre-eclampsia							
< RDI	26/242	10.7	21/263	8.0	1.34	0.78 to 2.33	0.286
≥RDI	28/440	6.4	24/427	5.6	1.13	0.67 to 1.92	0.645
Between groups							0.657
Detected < 34 weeks'							
< RDI	3/242	1.2	1/263	0.4	3.26	0.34 to 31.10	0.354
≥RDI	2/440	0.4	3/427	0.7	0.65	0.11 to 3.85	0.682
Between groups							0.269
Detected ≥ 34 weeks'							
< RDI	23/242	9.5	20/263	7.6	1.25	0.70 to 2.22	0.445
≥RDI	26/440	5.9	21/427	4.9	1.20	0.69 to 2.10	0.519
Between groups							0.923
Death or serious adverse outcome for the infant							
< RDI	19/242	7.8	31/263	11.8	0.67	<1.05	0.091
≥RDI	29/440	6.6	37/427	8.7	0.76	<1.13	0.153
Between groups							0.717
Death or serious adverse outcome for the woman							
< RDI	26/242	10.7	21/263	8.0	1.34	0.78 to 2.33	0.286
≥RDI	41/440	9.3	32/427	7.5	1.24	0.80 to 1.93	0.334
Between groups							0.826

Figures are n, %
RR = relative risk, CI = confidence intervals
RDI=recommended dietary intake

3.13.3 Family history of hypertensive disorders in pregnancy

One hundred and fifty women in the vitamin C and E group (20%) and 143 women (19%) in the placebo group reported a family history of pre-eclampsia or gestational hypertension. Data were not available regarding family history for two percent of all women (vitamin group 15 [2%] vs. placebo group 15 [2%]) and these women were not included in the subgroup analyses. There were no differences between treatment groups for any of the outcomes when women were grouped on their family history of hypertensive disorders in pregnancy (Table 3.32).

Table 3.32 Primary and secondary outcomes (sub-grouped by family history)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
Family history	13/155	8.4	15/141	10.6	0.79	0.39 to 1.60	0.509
No history or unsure	51/593	8.6	55/605	9.1	0.95	0.66 to 1.36	0.765
Between groups							0.653
Pre-eclampsia							
Family history	16/155	10.3	15/143	10.5	0.98	0.50 to 1.92	0.962
No history or unsure	44/600	7.3	36/610	5.9	1.24	0.81 to 1.90	0.316
Between groups							0.476
Detected < 34 weeks'							
Family history	3/155	1.9	1/143	0.7	2.77	0.29 to 26.30	0.624
No history or unsure	3/600	0.5	3/610	0.5	1.01	0.21 to 5.02	1.000
Between groups							0.476
Detected ≥ 34 weeks'							
Family history	13/155	8.4	14/143	9.8	0.86	0.42 to 1.76	0.673
No history or unsure	41/600	6.8	33/610	5.4	1.26	0.81 to 1.97	0.301
Between groups							0.368
Death or serious adverse outcome for the infant							
Family history	8/155	5.1	15/143	10.5	0.49	<0.99	0.066
No history or unsure	46/598	7.7	56/610	9.2	0.83	<1.14	0.199
Between groups							0.253
Death or serious adverse outcome for the woman							
Family history	16/155	10.3	14/143	9.8	1.05	0.53 to 2.08	0.879
No history or unsure	58/600	9.7	43/610	7.0	1.37	0.94 to 2.00	0.100
Between groups							0.508

Figures are n, %
RR = relative risk, CI = confidence intervals

3.13.4 Smoking status at trial entry

At trial entry, 164 (22%) women in the vitamin C and E group indicated they were smokers compared with 143 (19%) women in the placebo group. For the remaining women, most were non smokers (vitamin group 491 [65%] vs. placebo group 490 [65%]) or indicated they were smokers but had quit smoking in the current pregnancy before the first antenatal visit (vitamin group 100 [13%] vs. placebo group 120 [6%]). Data were not available on smoking status for two percent of all women (vitamin group 15 [2.0%] vs. placebo group 15 [2.0%]) and these women were not included in the subgroup analyses. There were no differences in any of the outcomes between treatment groups for any of the subgroup based on women's smoking status (Table 3.33).

Table 3.33 Primary and secondary outcomes (sub-grouped by smoking status)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
Non smoker	34/487	7.0	38/488	7.8	0.90	0.57 to 1.40	0.631
Quit in pregnancy*	8/99	8.1	5/118	4.2	1.91	0.64 to 5.64	0.263
Smoker	22/162	13.6	27/140	19.3	0.70	0.42 to 1.18	0.180
Between groups							0.262
Pre-eclampsia							
Non smoker	45/491	9.2	35/490	7.1	1.28	0.84 to 1.96	0.247
Quit in pregnancy*	6/100	6.0	4/120	3.3	1.80	0.52 to 6.20	0.518
Smoker	9/164	5.5	12/143	8.4	0.65	0.28 to 1.51	0.315
Between groups							0.285
Detected < 34 weeks'							
Non smoker	3/491	0.6	1/490	0.2	2.99	0.31 to 28.68	0.624
Quit in pregnancy*	1/100	1.0	0/120	0	-		0.454
Smoker	2/162	1.2	3/143	2.1	0.58	0.10 to 3.43	0.667
Between groups							0.239
Detected ≥ 34 weeks'							
Non smoker	42/461	8.5	34/490	6.9	1.23	0.80 to 1.90	0.344
Quit in pregnancy*	5/100	5.0	4/120	3.3	1.50	0.41 to 5.44	0.735
Smoker	7/164	4.3	9/143	6.3	0.68	0.26 to 1.77	0.426
Between groups							0.492
Death or serious adverse outcome for the infant							
Non smoker	30/491	6.1	36/490	7.3	0.83	<1.23	0.259
Quit in pregnancy*	4/100	4.0	12/120	10.0	0.40	<1.01	0.072
Smoker	20/164	12.2	23/143	16.1	0.76	<1.21	0.207
Between groups							0.485
Death or serious adverse outcome for the woman							
Non smoker	50/491	10.1	35/490	7.1	1.42	0.94 to 2.15	0.090
Quit in pregnancy*	8/100	8.0	9/120	7.5	1.07	0.43 to 2.66	0.890
Smoker	16/164	9.7	13/143	9.1	1.07	0.53 to 2.15	0.842
Between groups							0.719

Figures are n, %
* quit in pregnancy before first antenatal visit
RR = relative risk, CI = confidence intervals

3.13.5 Gestational age at trial entry

The randomisation schedule developed for this trial was stratified according to women's gestational age at trial entry (< 18 weeks' or ≥18 weeks' gestation) to ensure equal numbers of women in each treatment group started the trial tablets either earlier or later in pregnancy. Equal numbers of women in the treatment groups were randomised < 18 weeks' (vitamin group 467 [61%] vs. placebo group 468 [61%]) and ≥18 weeks' gestation (vitamin group 303 [39%] vs. placebo group 300 [39%]). For women randomised < 18 weeks' gestation and in the vitamin C and E group, there was a reduced risk of their infant dying or having a serious

adverse health outcome (vitamin group 31/467 [6.6%] vs. placebo group 46/468 [9.8%], RR 0.67, 95% CI <0.97, p=0.048). No statistically significant difference was seen in the risk of death or serious adverse infant health outcome between treatment groups for women randomised after 18 weeks' gestation, indicating that earlier initiation of therapy may be more beneficial for women and their infants. For the remaining primary and secondary outcomes, there were no differences between the treatment groups regardless of whether women were randomised prior to or after 18 weeks' gestation (Table 3.34).

Table 3.34 Primary and secondary outcomes (sub-grouped by gestational age at trial entry)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
< 18 weeks'	37/462	8.0	43/459	9.4	0.85	0.56 to 1.30	0.464
≥ 18 weeks'	29/301	9.6	28/297	9.4	1.02	0.62 to 1.67	0.931
Between groups							0.589
Pre-eclampsia							
< 18 weeks'	42/467	9.0	34/468	7.3	1.24	0.80 to 1.91	0.333
≥ 18 weeks'	18/303	5.9	17/300	5.7	1.04	0.55 to 1.99	0.886
Between groups							0.674
Detected < 34 weeks'							
< 18 weeks'	4/467	0.8	1/468	0.2	4.00	0.45 to 35.73	0.178
≥ 18 weeks'	2/303	0.7	3/300	1.0	0.66	0.11 to 3.92	0.217
Between groups							0.207
Detected ≥ 34 weeks'							
< 18 weeks'	38/467	8.1	33/468	7.0	1.15	0.74 to 1.81	0.531
≥ 18 weeks'	16/303	5.3	14/300	4.7	1.13	0.56 to 2.28	0.729
Between groups							0.963
Death or serious adverse outcome for the infant							
< 18 weeks'	**31/467**	**6.6**	**46/468**	**9.8**	**0.67**	**<0.97**	**0.049**
≥ 18 weeks'	23/303	7.6	31/300	10.3	0.73	<1.13	0.150
Between groups							0.807
Death or serious adverse outcome for the woman							
< 18 weeks'	50/467	10.7	38/468	8.1	1.32	0.88 to 1.97	0.176
≥ 18 weeks'	24/303	7.9	21/300	7.0	1.13	0.64 to 1.99	0.667
Between groups							0.665

Figures are n, %
RR = relative risk, CI = confidence intervals

3.13.6 Blood pressure at trial entry

At trial entry three percent of women in each treatment group had a randomisation blood pressure ≥130/80 mmHg. For the subgroup of women with a blood pressure < 130/80 mmHg

at randomisation, the risk of death or adverse infant outcome was lower in the vitamin C and E group (vitamin group 52/746 [7.0%] vs. placebo group 76/748 [10.2%], RR 0.69, 95% CI <0.91, p=0.017). No clear difference was seen for the risk of death or serious adverse infant outcome between treatment groups for women with a randomisation blood pressure ≥130/80 mmHg, however the number of women and the event rate in this subgroup was small. For all of the other subgroup analyses based on women's blood pressure at randomisation, no differences were seen between treatment groups for any other outcomes (Table 3.35). Caution must be taken when interpreting these subgroup analyses due to the small numbers of women in the subgroup with a blood pressure ≥130/80 mmHg.

Table 3.35 Primary and secondary outcomes (sub-grouped by blood pressure at trial entry)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
≥ 130/80 mmHg	0/23	0.0	1/19	0.5	-		0.465
< 130/80 mmHg	66/740	8.9	70/736	9.5	0.94	0.68 to 1.29	0.694
Between groups							-
Pre-eclampsia							
≥ 130/80 mmHg	5/24	20.8	1/20	5.0	4.17	0.53 to 32.8	0.198
< 130/80 mmHg	55/746	7.4	50/748	6.7	1.10	0.75 to 1.59	0.603
Between groups							0.213
Detected < 34 weeks'							
≥ 130/80 mmHg	1/24	4.2	0/20	0.0	-		1.000
< 130/80 mmHg	5/746	0.7	4/748	0.5	1.25	0.34 to 4.65	0.753
Between groups							-
Detected ≥ 34 weeks'							
≥ 130/80 mmHg	4/24	16.7	1/20	5.0	3.33	0.40 to 27.48	0.356
< 130/80 mmHg	50/746	6.7	46/748	6.1	1.09	0.74 to 1.60	0.663
Between groups							0.306
Death or serious adverse outcome for the infant							
≥ 130/80 mmHg	2/24	8.3	1/20	5.0	1.70	<11.74	0.570
< 130/80 mmHg	**52/746**	**7.0**	**76/748**	**10.2**	**0.69**	**<0.91**	**0.017**
Between groups							0.459
Death or serious adverse outcome for the woman							
≥ 130/80 mmHg	4/24	16.7	4/20	20.0	0.83	0.24 to 2.91	1.000
< 130/80 mmHg	70/746	9.4	55/748	7.3	1.28	0.91 to 1.79	0.156
Between groups							0.519

Figures are n, %
RR = relative risk, CI = confidence intervals

3.13.7 Compliance with the trial tablets

Overall, 60 percent of women in each treatment group were compliant for the entire length of the study. For women who were compliant, vitamin C and E supplementation was associated with a reduced risk of their infant dying or having a serious adverse health outcome, however this difference was not statistically significant (vitamin group 27/457 [5.9%] vs. placebo group 40/459 [8.7%], RR 0.68, 95% CI <1.00, p=0.066) (Table 3.36). No statistically significant difference was seen between treatment groups for those women who were not compliant. There were no other differences in any of the primary or secondary study outcomes between treatment groups for those women who were compliant or those women classed as non-compliant. Of concern, the increased risk of abnormal liver function found in the main analyses remained for women who were compliant (vitamin group 12 [2.6%] vs. placebo group 2 [0.4%], RR 6.02, 95% CI 1.36 to 26.8, p=0.007) however no difference was observed in abnormal liver function for those women who were not compliant (vitamin group 6 [1.9%] vs. placebo group 4 [1.3%], RR 1.48, 95% CI 0.42 to 5.20, p=0.752). Moreover, women in the vitamin C and E group who were compliant were more likely to develop renal insufficiency (vitamin group 8 [1.7%] vs. placebo 1 [0.2%], RR 8.03, 95% CI 1.01 to 63.98, p=0.021), however no difference was observed between groups for those women not compliant (vitamin group 2 [0.6%] vs. placebo group 4 [1.3%], RR 0.49, 95% CI 0.09 to 2.68, p=0.448).

Table 3.36 Primary and secondary outcomes (sub-grouped by compliance)

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Small for gestational age infants (< 10th centile)							
Compliant	39/457	8.5	44/459	9.6	0.89	0.59 to 1.34	0.579
Non compliant	27/306	8.8	27/297	9.1	0.97	0.58 to 1.61	0.908
Between groups							0.796
Pre-eclampsia							
Compliant	37/457	8.1	32/459	7.0	1.16	0.74 to 1.83	0.519
Non compliant	23/313	7.3	19/309	6.1	1.19	0.66 to 2.14	0.551
Between groups							0.940
Detected < 34 weeks'							
Compliant	4/457	0.9	4/459	0.9	1.00	0.25 to 3.99	1.000
Non compliant	2/313	0.6	0/309	0.0	-		0.499
Between groups							-
Detected ≥ 34 weeks'							
Compliant	33/457	7.2	28/459	6.1	1.18	0.73 to 1.92	0.496
Non compliant	21/313	6.7	19/309	6.1	1.09	0.60 to 1.99	0.776
Between groups							0.836
Death or serious adverse outcome for the infant							
Compliant	27/457	5.9	40/459	8.7	0.68	<1.00	0.066
Non compliant	27/313	8.6	37/309	12.0	0.72	<1.07	0.107
Between groups							0.858
Death or serious adverse outcome for the woman							
Compliant	44/457	9.6	31/459	6.7	1.42	0.92 to 2.21	0.113
Non compliant	30/313	9.6	28/309	9.1	1.06	0.65 to 1.73	0.822
Between groups							0.375

Figures are n, %
RR = relative risk, CI = confidence intervals

3.14 Discussion

This is the first large clinical trial evaluating antioxidant vitamin supplementation during pregnancy in a population of nulliparous women, a recognised risk factor for pre-eclampsia. Supplementation with vitamin C and E during pregnancy did not prevent women developing pre-eclampsia or reduce the risk of their infant being born small for gestational age. Vitamin C and E supplementation was however, associated with a 30 percent relative risk reduction in the infant dying or having a serious adverse health outcome. This corresponds with an absolute risk reduction of three percent. The choice of outcomes contributing to this composite neonatal endpoint have been recommended by experts as important measures of morbidity in preterm (Donoghue and Cust 2000) and term infants (Hannah et al 1992). They include rare neonatal outcomes with both short and long term adverse health sequelae including death or severe physical or neuro-developmental disability. Complementary to the benefits seen for the risk of death or serious adverse infant outcome, for all surviving infants, maternal vitamin C and E supplementation was associated with a 1.8 millimetre increase in head circumference at birth, and a reduced risk of developing respiratory distress syndrome.

3.14.1 Mechanisms of action for improved infant health

The beneficial effect of vitamin C and E supplementation on infant health outcomes was unexpected given the lack of effect of supplementation on the risk of pre-eclampsia or the risk of being small for gestational age. This raises the question of how maternal vitamin C and E supplementation may be influencing the risk of death or adverse infant outcomes if not through the anticipated pathways of a reduction in the risk of pre-eclampsia, fetal growth restriction or a reduction in iatrogenic preterm birth. Oxidative stress has been implicated in many of the disorders common to preterm infants including respiratory distress syndrome, chronic lung disease, intraventricular haemorrhage, periventricular leucomalacia, retinopathy of prematurity, necrotising enterocolitis and bronchopulmonary dysplasia (Saugstad 1988; Saugstad 2001). In the current study, although most of the outcomes contributing to the composite adverse neonatal endpoint were lower in the vitamin C and E group, there were no statistically significant differences between treatment groups for any of these outcomes. This trial was not powered to detect small differences in these outcomes individually and for many of the outcomes, the numbers of infants in each group was small (< 5). The difference between treatment groups appeared to be in the outcomes chronic lung disease, the need for ventilation for 24 or more hours and in the requirement for tube feeding for four or more days. There were no differences between treatment groups in the number of infants born preterm (< 37 weeks' gestation), very preterm (< 34 weeks' gestation) or extremely preterm (< 28 weeks'

gestation) that may account for the difference observed in death or serious adverse infant outcome.

Maternal supplementation in late gestation with vitamin C (500 mg) and vitamin E (400 IU) in similar doses to those used in this study has been shown to correlate with amniotic fluid and chorioamnion vitamin concentrations respectively (Pressman et al 2003). Moreover, a small pilot matched cohort study of antioxidant supplementation given to women (n=5) at risk of preterm birth demonstrated reduced malondialdehyde concentration in mothers at birth, and a trend towards reduced cord blood malondialdehyde concentrations at birth (Bolisetty et al 2002). It therefore may be possible that maternal supplementation with antioxidant vitamins increases the antioxidant status of at risk infants, and thus results in a reduced risk of morbidities associated with oxidative stress, such as respiratory distress syndrome.

Trials of postnatal supplementation in preterm infants have been undertaken assessing antioxidants to improve health. The Cochrane systematic review of randomised controlled trials of vitamin E supplementation in preterm infants included 26 studies involving over 1,500 infants (Brion, Bell and Raghuveer 2003). Vitamin E supplementation was associated with a reduced risk of intraventricular haemorrhage and for very low birth weight infants, a reduced risk of severe retinopathy and blindness. For all infants however, supplementation was associated with an increased risk of sepsis. There was insufficient evidence about the best dose of vitamin E for use in supplementation, although the authors cautioned against high dose supplementation, which was associated with the greatest increase in the risk of sepsis. Furthermore, as many of the trials included in the review were undertaken in the 1980's or earlier, few extremely preterm infants were included, and many of the trials predated the use of laser photo coagulation for the prevention of blindness, which is widely available for preterm infants today. The authors therefore called for further research investigating any potential benefits of supplementation in extremely low birth weight infants, the most at risk group for diseases of prematurity.

Few studies have assessed postnatal vitamin C supplementation in preterm infants. One small trial (Darlow et al 2005) assessed three regimes of vitamin C supplementation in 119 preterm infants in the first 28 days of life (low dose supplementation throughout [LL], low supplementation for 10 days and then high dose for the remaining days [LH], high dose supplementation throughout [HH]). There were no statistically significant differences between the three treatment regimes for the primary outcomes including chronic lung disease, days of oxygen therapy, and retinopathy of prematurity, however the proportion of surviving infants

(19%) in the highest dose [HH] vitamin C group requiring oxygen at 36 weeks' postmenstrual age was half that of the infants (41%) on the low regime [LL] (p=0.06) (Darlow et al 2005). It should also be noted however, that of the six infants who died in this trial, all had significantly higher pre-randomisation plasma vitamin C concentrations when compared with surviving infants.

How maternal vitamin C and E supplementation may be causing a reduction in death or adverse neonatal health outcomes remains unclear given the lack of consistent benefits demonstrated with postnatal antioxidant supplementation in preterm infants. However, maternal antioxidant status and infant antioxidant status at birth may still prove to be a predictor of neonatal outcome. A trial of postnatal selenium supplementation (Darlow et al 2000), another antioxidant, demonstrated no benefit for very low birth weight infants for any neonatal outcomes, however low maternal and infant pre-randomization selenium status was associated with an increased risk of respiratory morbidity. The authors highlighted the need for further research investigating maternal selenium supplementation in the second half of pregnancy and any potential impact on neonatal outcomes.

In preterm infants, low antioxidant status at birth (as measured by the microlitre plasma required to inhibit lipid peroxidation) has been associated with an increased risk of mortality and bronchopulmonary dysplasia (Silvers, Gibson and Powers 1994; Silvers et al 1998). In these studies however, low antioxidant status was associated with high plasma vitamin C concentrations, suggesting a possible pro-oxidant effect of vitamin C. Like in the trial by Darlow and colleagues (2005), higher plasma vitamin C at birth was also demonstrated in infants who died compared with those with a good outcome, although these findings were diminished after adjusting for gestational age. The cause of the high plasma vitamin C in these infants is unknown. Maternal plasma vitamin C concentrations were not assessed in the studies by Silvers and colleagues (1994, 1998), making it difficult to assess how the infant plasma concentrations may relate to, if at all, maternal plasma concentration and vitamin C intake. Darlow and colleagues (2005) speculate the high vitamin C status observed in these infants may be more likely to be a marker of neonatal insult rather than a contributing factor to the infants' death, due to the observance of no adverse pro-oxidant effects in their trial. The relationship between plasma vitamin C and other antioxidant status at birth and the risk of morbidity and mortality in preterm infants remains unclear, however further careful evaluation is warranted.

It is also unclear how these findings relate to term infants, the majority of infants in the current study. It was not feasible to assess infant vitamin C and E status in all 1,538 infants in this trial due to the limitations of research funding. Further research investigating the relationship between maternal and infant plasma antioxidant status in term infants and the relationship with neonatal health outcomes is clearly warranted.

In this trial, infants in the vitamin C and E group had a significantly increased head circumference at birth when compared with infants in the placebo group. This finding remained significant when head circumference z-scores were compared between treatment groups, which effectively adjust for any differences between treatment groups in the gestational age at delivery and infant sex. The difference between treatment groups was in the order of 1.8 millimetres or a 0.12 difference in mean z-scores. The clinical relevance of the magnitude of this difference is unclear. Birth dimensions including low birth weight, length and ponderal index have been associated with the onset of adult disease including cardiovascular disease and diabetes (Barker 1998). In addition to these birth dimensions, small head circumference at birth has been linked with subnormal intelligence and psychological performance (Lundgren et al 2001). These studies highlight the growing body of research implicating infant growth status at birth and the risk of adult onset diseases.

The potential mechanism behind an increase in head circumference and how vitamin C and E may influence this is unclear. Errors in measurement may have occurred to account for the difference seen, however the randomisation process undertaken in this trial would imply that any measurement errors are evenly distributed between treatment groups. Animal studies in diabetic rats (induced by the injection of streptozotocin), have demonstrated fewer congenital malformations and late resorptions in pregnant rats supplemented with vitamin C from prior to mating when compared with non-supplemented controls (Simán and Eriksson 1997). In these studies, fetal brain weight as a proportion of fetal body weight was also greater in the vitamin C supplemented diabetic group. Animal studies have also demonstrated that antioxidants may be important for myelination. *In vitro* studies where the culture medium is depleted of antioxidants including vitamin C and E, demonstrate a loss of myelination of dorsal root ganglion neurons in the rat model (Podratz, Rodriguez and Windebank 2004). The addition of vitamin C and vitamin E respectively induced partial myelination, with complete restoration of myelination occurring with the addition of a combination of antioxidants. While these findings cannot be inferred to healthy pregnant women and their infants, they highlight the possibility that maternal vitamin C and E supplementation may have the potential to influence fetal brain size and thus head circumference. Whatever the mechanism, the

differences seen in this study, however small, underline the need for long term follow up of infants enrolled in this trial to assess childhood development. This follow up is currently underway, where all women and infants are being assessed at four months' and 18 months' postpartum.

3.14.2 Impact of supplementation on the risk of pre-eclampsia

Our results do not support an association between antioxidant supplementation and a reduced risk of pre-eclampsia. Indeed, they support the reverse of this proposition, where the incidence of pre-eclampsia was slightly higher in the vitamin C and E group, and more women in the vitamin C and E group required antihypertensive medications. The findings are in contrast to the majority of trials included in the systematic review presented in Chapter Two of this thesis. There are several factors that may limit the effectiveness of vitamin C and E supplementation in pregnancy. These factors relate to women's compliance with the tablets, the dosage of vitamin C and/or vitamin E used, the intervention itself (i.e. vitamin C and E alone), the length of time the supplements were taken, and the population in which the intervention was tested, and all are discussed below.

Approximately sixty percent of women in each treatment group were compliant for the entire length of the pregnancy. This was ascertained by direct questioning on the trial questionnaires at two separate time points (28 weeks' gestation and postnatally). Although the accuracy of women's accounts of their compliance may have been increased by a second assessment such as direct pill counts, this was not a feasible option in this study, as not all treatment packs for all women were returned and able to be assessed. Moreover, there is no gold standard assessment for measuring compliance with trial medications. Assessing compliance by direct pill counts, the use of blister packs or measuring plasma vitamin C and E concentrations over the course of pregnancy is costly, and these measures were not possible within the budget for this trial, coupled with the limited evidence that such measures actually improve patient compliance.

Women's compliance in this study is comparable with other supplementation trials involving nulliparous women, including the calcium to prevent pre-eclampsia (CPEP) trial from the United States (Levine et al 1997). The CPEP trial asked women to take four tablets per day, two in morning and two at night, the same protocol as the current study, and subjected potential participants to a pre-trial compliance assessment where only those women compliant with a weeks' supply of tablets were allowed to continue in the trial. Compliance in the

calcium group was 64 percent and for the placebo group, 67 percent. In our study, approximately 28 percent of women in the vitamin C and E group and 25 percent in the placebo group stopped taking the trial medications before birth. This compares directly with the Australian Calcium Trial (ACT) (Crowther et al 1999), which supplemented nulliparous women with three tablets of calcium per day or placebo. In this trial 27 percent of women stopped taking the trial medications during pregnancy. Consequently, the level of compliance observed in this study is consistent with other supplementation trials during pregnancy. It is unlikely however, that a lack of compliance in this study is solely responsible for the lack of treatment effects seen for the risk of pre-eclampsia and other maternal outcomes. Our pre-specified subgroup analyses based on compliance failed to demonstrate any greater treatment effect of vitamin C and E supplementation in women who were compliant for approximately 80 percent of the required time. These women did not have a reduced risk of pre-eclampsia, nor was there a trend in this direction.

The dosages used in this trial were based on the trial by Chappell and colleagues (1999) which demonstrated a halving in the incidence of pre-eclampsia with vitamin C and E supplementation in women at high risk of pre-eclampsia. This included women with either an abnormal uterine artery Doppler at 18 to 22 weeks' gestation or a history in the preceding pregnancy of pre-eclampsia necessitating delivery before 37 weeks' gestation, eclampsia or HELLP syndrome. Increasing the dosages of vitamin C beyond the 1,000 mg per day assessed in this trial would come at a cost of decreased bioavailability (Levine et al 1996) and thus potentially effectiveness. For vitamin E, doses of 400 IU per day have been shown to be effective at preventing low density lipoprotein oxidation (Devaraj et al 1997). There is limited evidence that higher dosages (above 400 IU per day) are more effective and in the absence of evidence about the safety of using higher doses in pregnancy, should be avoided. It is therefore unlikely that altering the dosages of vitamins in this study would have altered the treatment effects or lack thereof seen.

The dietary assessment undertaken in this study indicated that approximately five percent of all women had a daily intake of vitamin C below the recommended amount. For vitamin E, this was reported for up to one third of all women. The pre-specified subgroup analyses based on women's dietary intakes, demonstrated no greater treatment effect for women with either a vitamin C or vitamin E intake below the recommended amount. However caution must be taken in interpreting these subgroups as the numbers of women in the deficient subgroups were small. Recommended dietary intakes (RDI's) represent the amount required to meet the nutritional needs for most healthy individuals. RDI's do however, include a generous

allowance for variations in metabolism and individual needs, so much that the RDI's exceed the actual nutrient requirements for most individuals (NHMRC, 1989). For women in our study, it would therefore appear that the majority had sufficient dietary intakes of vitamin C and E upon entering the trial. Results of this trial would consequently suggest that vitamin C and E supplementation may not be beneficial for women with adequate nutritional intakes.

Furthermore, only one quarter of all women counselled about this trial gave consent and were randomised. Therefore there is a possibility for selection bias, where women in this study may have had better general health, including dietary intakes of vitamin C and vitamin E, than other nulliparous women. It is therefore possible that women in this trial may have had less potential to benefit than the majority of the nulliparous population, who did not consent to being involved in this trial.

Parallels can be drawn with the trials assessing calcium supplementation for the prevention of pre-eclampsia. The Cochrane systematic review of trials of calcium supplementation (Atallah, Hofmeyer and Duley 2002) demonstrated substantial benefits associated with calcium supplementation and the risk of pre-eclampsia, however significant heterogeneity was detected in the results. Women most likely to benefit from calcium supplements appeared to be those at high risk of pre-eclampsia and those with a low baseline calcium intake. It has subsequently been suggested that calcium supplementation needs further confirmation in trials designed to increase the intake in those with a calcium deficiency rather than to obtain a pharmacologic, perhaps non-nutritional, effect in individuals with an adequate calcium intake (Villar and Belizan 2000). This may also be true of dietary antioxidants. The results of this study therefore cannot be inferred to populations at risk of or with established nutritional deficiency. The impact of supplementation in these women is unknown and requires evaluation.

It may also be possible that any potential treatment effects associated with vitamin C and E supplementation may have been diluted due to the high usage of multivitamin and other dietary supplements containing vitamin C and E, calcium and other micronutrients, in our study population. Over 80 percent of women at trial entry reported using a dietary supplement, and at 28 weeks' gestation, 60 percent of women still reported dietary supplement use. Women in this study were not recommended to take any dietary supplements, nor are we aware that hospital guidelines at each collaborating centre recommend the use of dietary supplements, with the exception of periconceptional folic acid. The high usage of dietary supplements in pregnancy in this study is therefore surprising.

Medication use throughout pregnancy has been assessed in other Australian primiparous and multiparous women, where the reported use of folic acid supplements was 63 percent in the first trimester and for multivitamins the average usage across the semesters of pregnancy was 28 percent (Henry and Crowther 2000). Compared with these findings, the apparent increase in the use of dietary supplements in pregnancy observed in this study (excluding folic acid) is alarming, in the absence of any new evidence supporting their use.

Periconceptional folic acid supplementation is currently the only supplement with a clear evidence base supporting its use in early pregnancy for the prevention of neural tube defects (Lumley et al 2001). While many of the women in this study reported taking folic acid (over 50%), multivitamins were also commonly taken. There is limited evidence for the safety and efficacy of the use of multivitamin supplements or any other dietary supplements in pregnancy, either for women with a dietary micronutrient deficiency or for women with presumed adequate intakes. There is a clear need to evaluate the safety of using these preparations in pregnancy. These findings also highlight the need for health care providers to continue to monitor and record women's use of dietary supplements in pregnancy. Often these preparations can be costly for women (for example typically between AUD$17 to AUD$25 for one month's supply of a pregnancy formulated multivitamin supplement), however it is clear that women are prepared to take these supplements despite the lack of information about potential health benefits or adverse effects. Further research investigating the reasons why women take these supplements during pregnancy is therefore warranted.

In the antioxidant review described in Chapter Two of this thesis, only two of the seven trials included assessed vitamin C and E supplementation alone or even at all. These trials contributed data from less than 400 women. Other included studies assessed vitamin C alone (one trial); vitamin C, vitamin E, aspirin and fish oil (one trial), multivitamin containing vitamin C (one quasi-randomised trial) and the remaining trials assessed other antioxidants either selenium or lycopene. The review findings could not establish if the treatment effects seen were directly related to the vitamin C and E interventions, or the combination of vitamin C and E with co-interventions including aspirin and fish oil or whether the other antioxidants or other agents assessed were responsible for the observed treatment effects. The results of this study, the largest study of vitamin C and E supplementation in pregnancy to date, would support the latter two propositions. The use of a combination of interventions including vitamin C and E with agents such as aspirin, fish oil and calcium may be more likely to be responsible for the treatment effects seen in the review, and further investigation into the use of co-interventions would be appropriate. Furthermore, the findings from this study cannot be

extrapolated to other antioxidant preparations (for example lycopene and selenium), and further evaluation of other antioxidant preparations is still warranted.

The average gestational age women joined this trial was 17 weeks'. Our pre-specified subgroup analyses based on women's gestation at trial entry (< 18 weeks' or ≥18 weeks) did not show any differences in the treatment effect size for women in these two subgroups. It is therefore unlikely that the effectiveness of this intervention would be dependent on earlier initiation of therapy. However, as most women entered the trial at 17 weeks' gestation, when placentation is almost complete, whether initiating this therapy in the first trimester or even before conception would have a more direct effect on placental oxidative stress and thus the risk of pre-eclampsia is unknown and warrants further investigation. However before initiation of therapy prior to 12 weeks' is undertaken, more information is required about the safety of using these vitamins in early pregnancy.

Women included in the trials of vitamin C and E supplementation in pregnancy to date, have been at high risk of pre-eclampsia and other adverse pregnancy outcomes such as preterm birth. Our study included nulliparous women, a known risk factor for pre-eclampsia, however women in this trial were at lower risk of pre-eclampsia than women in the antioxidant trials reported to date. This is evidenced by the higher incidence of pre-eclampsia in the placebo arms of the trials to date (generally in the order of 17%) compared with the six percent seen in women in the placebo group of this trial. Our study has demonstrated that vitamin C and E supplementation is not an effective prophylaxis for pre-eclampsia in nulliparous women. Moreover it was not beneficial for women in this study with additional risk factors for pre-eclampsia, including a family history of pre-eclampsia or gestational hypertension, as demonstrated in our pre-specified subgroup analyses.

It remains to be established whether antioxidant supplementation is beneficial for women at higher risk of pre-eclampsia, including those women with a history of pre-eclampsia in a previous pregnancy. This requires further evaluation in larger studies and this is the subject of several large clinical trials currently underway including the Vitamins in Pregnancy trial (United Kingdom, Prof. L Poston), involving 2,400 high risk women (soon to be completed with over 90 percent of their target recruitment reached as of March 2005) and The Diabetes and Pre-eclampsia Intervention Trial (United Kingdom, Dr D McCance), involving 756 women with Type 1 Diabetes (completion of recruitment is expected by the end of 2007). We are also aware of an antioxidant trial being undertaken in developing countries in conjunction with the World Health Organisation (Dr J Villar) (recruitment is on-going). A similar trial to

the one presented in this thesis is also currently underway in the United States of America, the Combined Antioxidants and Pre-eclampsia Prediction Study (United States of America, Prof J Roberts, NICHD MFMU Network) involving 10,000 nulliparous women (currently with over a quarter of the target recruitment completed as of March 2005).

We await with interest the findings of these larger trials, given the disparity between the results of this trial and the findings of the antioxidant trials published to date. Given the lack of effect on prevention of pre-eclampsia seen in our study, it is possible that the treatment effects seen in the initial trials of antioxidants are more likely to be due to chance than a true biological effect. Small trials are in general underpowered to detect all but large differences in outcomes, and statistically significant differences reported tend to overestimate true treatment effects when assessed in larger trials. In the antioxidant review presented in Chapter Two, excluding the quasi-randomised trial assessing multivitamin supplementation, the largest trial included involved 283 women (Chappell et al et al 1999). Clearly all of the small trials of vitamin C and E supplementation included were underpowered to detect clinically relevant differences in adverse perinatal outcomes and may in fact represent an overestimation of the true effects if any, of vitamin C and E supplementation in pregnancy.

There may also be potential for publication bias, where the majority of small trials published to date have reported positive findings. Publication bias has been suggested to be responsible for the findings presented in the initial systematic reviews of antiplatelet agents, particularly aspirin, for the prevention of pre-eclampsia, which demonstrated dramatic risk reductions with antiplatelet therapy (Duley et al 2003). For many of the outcomes in these reviews however, the funnel plots were asymmetrical highlighting the possibility that small negative trials of antiplatelet agents have never been published. The Cochrane systematic review of antiplatelet agents for preventing pre-eclampsia published in 2003 (Duley et al 2003) included data from over 36,500 women. The review demonstrated small to moderate reductions in the risk of pre-eclampsia and its related complications with low dose aspirin therapy, however the benefits were much smaller than initially suggested from the early systematic reviews and trials published in the area. It remains plausible that small trials of antioxidant supplementation in pregnancy may have been undertaken but not published to date, due to no effect or negative findings associated with supplementation.

3.14.3 Safety of vitamin C and E supplementation in pregnancy

Our results clearly demonstrate that vitamin C and E supplementation during pregnancy is not associated with any harmful effects for the infant. The incidence of spontaneous miscarriage or early fetal death, stillbirth or neonatal death was not increased in the vitamin C and E group, indeed, infants in this group were at lower risk of death or serious adverse health outcomes.

For women however, supplementation was associated with an increase in abnormal liver function as measured by raised serum transaminases or severe epigastric pain. This observation was strengthened when the comparisons were restricted to compliant women only, where supplementation was associated with a highly significant increase in the risk of abnormal liver function and renal insufficiency. These findings may be related to the occurrence of more women in the vitamin C and E group self-reporting abdominal pain in late pregnancy, when compared with women in the placebo group.

The cause of these findings is unknown. Systematic review of trials of high dose vitamin C and E supplementation outside of pregnancy have failed to demonstrate any adverse effect of supplementation on liver function (Bendich and Machlin 1993; Bendich 1997). Similarly, of the limited evidence from trials of vitamin C and E supplementation in pregnancy, no increased risk of adverse liver function has been reported. However it remains unclear whether any of these trials in adults or pregnant women, assessed or reported liver function. In our study, the assessment of liver function was not a routine antenatal test, and was most commonly requested in women with symptoms of pre-eclampsia or obstetric cholestasis. It therefore remains unclear whether supplementation truly impacted adversely on liver function in those women whose liver function was never assessed. Alternatively, it may reflect vitamin C and E supplementation altering the disease progression in women with established pre-eclampsia. Caution must be taken however, in interpreting the results of tests that were not performed on all trial participants. These findings do highlight the need to demonstrate the safety of vitamin C and E supplementation and assess liver function in all women enrolled in trials that are sufficiently powered to detect potentially serious adverse effects.

3.14.4 Women's views on participating in this research

Assessing women's views on participating in research is an important component of any health care evaluation. Women in this trial were very positive about their involvement. Eighty percent of all women indicated they would participate in the study again, and even more

would recommend the study to pregnant women in a similar situation. This demonstrates that women are prepared to be involved in supplementation trials in pregnancy, and also suggests that if efficacy was demonstrated, vitamin C and E supplementation may be a feasible intervention. As expected, most dislikes about the trial indicated by women related to the size of the tablets and the number of tablets required to be taken in the study period. It must be acknowledged that participants in this trial were healthy women in their first on-going pregnancy, often with limited knowledge or experience of pre-eclampsia or other adverse pregnancy outcomes. Accordingly, asking these women to take four tablets per day for up to six months of their pregnancy, relied heavily on women's altruism and the desire to participate in research. Given this, any future supplementation trials and clinical practice recommendations should continue to evaluate and take into account women's preferences regarding the intervention.

Few women, less than 10 percent, were able to correctly identify their treatment group allocation, with most women indicating that they were unsure of their treatment group. This provides some reassurance that women remained blinded to their treatment allocation throughout the study. It provides further reassurance that women's responses were not influenced by knowledge of their treatment allocation, and that clinicians involved in the care of these women were unlikely to have knowledge about women's treatment allocation.

3.14.5 Statistical power of this trial

Sufficient numbers of women in this study were randomised to be able to detect expected clinically meaningful differences in perinatal outcomes. For all of the outcomes however, the observed differences were smaller than the differences initially postulated in our sample size calculations. This may have impacted on the ability to detect any differences in outcomes, particularly for pre-eclampsia and the risk of having a small for gestational age infant. For pre-eclampsia, the proposed sample size was based on the assumption that the rate of pre-eclampsia in the placebo group would be 10 percent. This was based on the incidence of pre-eclampsia reported in the placebo arm of the Australian Calcium Trial (Crowther et al 1999), which was conducted in a similar population of women as the current study (nulliparous women in South Australia and Queensland). This was an overestimation of the expected incidence of pre-eclampsia, where in the current study, the rate of pre-eclampsia in the placebo arm was 6.6 percent, and 7.8 percent in the vitamin C and E group. As such, while this is still the largest trial of vitamin C and E supplementation to date, this study is

underpowered to detect small reductions in the risk of pre-eclampsia if such differences truly exist.

Consideration must also be given to the large number of outcomes reported in this study. Multiple comparisons were undertaken in our pre-specified primary and subgroup analyses, which increases the risk of making a type 1 error, that is, finding a difference when there is no true biological difference between treatment groups. Furthermore, due to the large sample size of this study, small differences between treatment groups are more likely to be statistically significant with larger numbers of women involved. This will occur regardless of whether the difference between groups is regarded as clinically relevant. Caution must be taken when inferring any treatment effects seen in this study where there are multiple comparisons and subgroup analyses.

3.15 Conclusions

Infants of women supplemented with vitamin C and vitamin E during pregnancy were at a significantly reduced risk of dying or developing a serious adverse health outcome when compared with infants in the placebo group. In order to prevent one infant dying or developing a serious adverse health outcome, a total of 33 women would need to take vitamin C and E supplements throughout their pregnancy. Additional benefits were seen for the infants in the vitamin C and E group including an increased head circumference at birth and a reduced risk of developing respiratory distress syndrome.

Conversely, this study did not demonstrate any beneficial effects of supplementation on the risk of having a small for gestation age infant, developing pre-eclampsia or other maternal health outcomes. Furthermore, women in the vitamin C and E group experienced an increased risk of abnormal liver function, although the cause of this remains unclear and warrants further investigation. The absolute increase in risk was small, indicating that for 67 women taking vitamin C and E supplements during pregnancy, one woman would develop abnormal liver function tests.

3.15.1 Implications for practice

Vitamin C and E supplementation cannot be recommended as a prophylaxis for pre-eclampsia in nulliparous women. Although the impact of supplementation on infant death or serious adverse health outcomes, as well as measures of growth and respiratory morbidity appears

promising, further confirmation of these findings is required before vitamin C and E can be recommended for routine clinical practice.

3.15.2 Implications for research

Further evaluation of the impact of supplementation on measures of infant health status is warranted, with particular focus on infant growth and respiratory outcomes. Similarly, assessing the relationships between supplementation and maternal and infant antioxidant status, and infant health outcomes should be a research priority, and would aid in the interpretation of the results of the current study.

There is insufficient evidence about the impact of supplementation on the risk of adverse perinatal outcomes for women at high risk of pre-eclampsia, or with a dietary deficiency of antioxidants. Future trials are still required to address these questions. Similarly, the results of this trial cannot be inferred for all antioxidant types or the use of antioxidants with co-interventions like aspirin, fish oil and calcium, and further evaluation of these treatment regimens would seem appropriate.

Any future trials of vitamin C and E supplementation should involve assessment of maternal liver function, to further examine potential harmful effects of supplementation on liver function. All future research should include long term follow up of all children enrolled to determine any long term benefits or harms of supplementation on later childhood development.

Women in this trial reported high usage of dietary supplements in pregnancy, commonly multivitamin supplements. Evidence on the safety and efficacy of using these dietary supplements in pregnancy is required and should be the subject of further research. Assessing women's beliefs and reasons for using these supplements would also be appropriate.

4. HYPERTENSIVE DISORDERS OF PREGNANCY

4.1 Introduction

Controversy continues to surround the most appropriate definition of pre-eclampsia, whether the definition should reflect the multi-system nature of the syndrome of pre-eclampsia, or whether it should be confined strictly to proteinuric hypertension, to permit comparisons about an already well-defined group of women. In the current study, information on any hypertensive disease in pregnancy was collected to permit the application of different definitions of pre-eclampsia, and thus permit comparisons with other trials and research. Table 4.1 describes the incidence of hypertensive disorders of pregnancy, when applying criteria to define pre-eclampsia proposed by the ASSHP (Brown et al 2000), the NHBPEP (Report of the NHBPEP 2000) and by Davey and MacGillivray (1986). These three definitions were chosen to highlight the range of definitions currently available, as well as those used internationally.

4.1.1 Gestational hypertension

The incidence of gestational hypertension in this study population ranged from nine to 13 percent in each treatment group (Table 4.1). No difference was seen in the risk of gestational hypertension between treatment groups regardless of whether the definition was based on ASSHP and NHBPEP criteria (systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg confirmed on repeated readings) or that proposed by Davey and MacGillivray based on diastolic blood pressure alone (one measurement of DBP ≥ 110 mmHg or two consecutive measurements of DBP's ≥ 90 mmHg, four hours or more apart).

4.1.2 Pre-eclampsia

4.1.2.1 ASSHP criteria – the "inclusive definition"

The ASSHP criteria defines pre-eclampsia on the presence of hypertension occurring with either: proteinuria, renal insufficiency, liver disease, neurological problems, haematological disturbances or fetal growth restriction (Brown et al 2000). No difference was seen between treatment groups when pre-eclampsia was defined according to the ASSHP (vitamin group 60 [7.8%] vs. placebo group 51 [6.6%], RR 1.17, 95% CI 0.82 to 1.68, p=0.383) (Table 4.1).

4.1.2.2 Proteinuric hypertension – the "restrictive definition"

The NHBPEP report (Report of the NHBPEP 2000) employs a more restrictive definition based on gestational hypertension and proteinuria alone, with proteinuria defined as one 24 hour urine collection of 0.3 g proteinuria or more (or two random clean catch specimens or urine with 2+ (1 g) protein as measured by dipstick). No difference was seen in the risk of pre-eclampsia between treatment groups when defined according to the NHBPEP (vitamin group 18 [2.3%] vs. placebo group 14 [1.8%], RR 1.28, 95% CI 0.64 to 2.56, p=0.479) (Table 4.1). Similarly, when using the definitions proposed by Davey and MacGillivray (1986) based on gestational hypertension (using DBP values only) and one 24 hour urine collection of 0.3 g proteinuria or more or two random clean catch specimens or urine dipstick with 2+ (1 g) protein, there was no difference in the risk of pre-eclampsia between treatment groups.

Unlike the ASSHP definition, the NHBPEP report and the definition proposed by Davey and MacGillivray do not include criteria for the diagnosis of proteinuria using the spot urine protein creatinine ratio. However in the current study, for those women with proteinuria, by far the majority were assessed using the spot urine protein creatinine ratio (67 women, 4.3%), rather than a 24 hour urine collection (16 women, 1%) or even dipstick urinalysis (25 women, 1.6%). When the criteria outlined in the NHBPEP Report were expanded to include a spot urine protein creatinine ratio (i.e. spot urine protein/creatinine ratio ≥ 30 mg/mmol), women in the vitamin C and E group had a statistically significant increase in the risk of pre-eclampsia (vitamin 43 [5.6%] vs. placebo group 25 [3.3%], RR 1.71, 95% CI 1.05 to 2.78, p=0.026) (Table 4.1). Using Davey and MacGillivray's definition with the addition of the spot urine protein creatinine ratio, there was a trend towards an increased risk of pre-eclampsia in the vitamin C and E group however this did not reach statistical significance (vitamin group 31 [4.0%] vs. placebo group 19 [2.5%] RR 1.63, 95% CI 0.93 to 2.85, p=0.086) (Table 4.1).

4.1.2.3 Clinical label of "pre-eclampsia"

When assessing women's medical records, there was a trend for more women in the vitamin C and E group being labelled as "pre-eclamptic" when compared with women in the placebo group (vitamin group 45 [5.8%] vs. placebo group 30 [3.9%], RR 1.50, 95% CI 0.95 to 2.35) however this did not reach statistical significant (p=0.078) (Table 4.1).

4.1.3 Severe hypertensive disease

No difference was seen between the treatment groups in the risk of severe gestational hypertension, either using the NHBPEP definitions or those proposed by Davey and MacGillivray (based on DBP alone) (Table 4.1). For severe pre-eclampsia, there were no differences between the treatment groups for the risk of severe pre-eclampsia defined as either severe gestational hypertension (SBP 160 mmHg or DBP 110 mmHg or two DBP's ≥ 110 mmHg, four or more hours apart) or severe proteinuria (one 24 hour urine collection of 3 g protein or more or two random clean catch specimens or urine with 3+ protein or more as measured by dipstick). However when the definition of severe proteinuria included the spot protein/creatinine ratio (i.e. one 24 hour urine collection of 3 g protein or more *or* two random clean catch specimens or urine with 3+ protein or more as measured by dipstick *or* spot urine protein/creatinine ratio ≥ 300 mg/mmol), women in the vitamin C and E group had an increased risk of severe pre-eclampsia (vitamin group 24 [3.1%] vs. placebo group 11 [1.4%], RR 2.17, 95% CI 1.07 to 4.41, p=0.027).

Table 4.1 Hypertensive disease and severity in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Gestational hypertension[1]	102	13.2	99	12.9	1.03	0.79 to 1.33	0.836
Gestational hypertension[2]	71	9.2	70	9.1	1.01	0.74 to 1.38	0.942
Pre-eclampsia (ASSHP criteria)	60	7.8	51	6.6	1.17	0.82 to 1.68	0.383
Clinical diagnosis of pre-eclampsia in medical records	45	5.8	30	3.9	1.50	0.95 to 2.35	0.078
Pre-eclampsia[3]	18	2.3	14	1.8	1.28	0.64 to 2.56	0.479
Pre-eclampsia[4]	13	1.7	12	1.6	1.08	0.50 to 2.35	0.845
Pre-eclampsia[5]	**43**	**5.6**	**25**	**3.3**	**1.71**	**1.05 to 2.78**	**0.026**
Pre-eclampsia[6]	31	4.0	19	2.5	1.63	0.93 to 2.85	0.086
Severe gestational hypertension[7]	28	3.6	20	2.6	1.40	0.79 to 2.46	0.244
Severe gestational hypertension[8]	6	0.8	3	0.4	1.99	0.50 to 7.95	0.506
Severe pre-eclampsia[9]	5	0.6	7	0.9	0.75	0.26 to 2.14	0.385
Severe pre-eclampsia[10]	9	1.2	7	0.9	1.28	0.48 to 3.42	0.403
Severe pre-eclampsia[11]	**24**	**3.1**	**11**	**1.4**	**2.17**	**1.07 to 4.41**	**0.027**

Figures are n, % RR = relative risk, CI = confidence intervals
ASSHP = Australasian Society for the Study of Hypertension in Pregnancy

[1]defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg confirmed on repeated readings (four or more hours apart) (Brown et al 2000, Report of the NHBPEP 2000)

[2]defined as one measurement of diastolic blood pressure ≥ 110 mmHg or two consecutive measurements of diastolic blood pressures ≥ 90 mmHg, four or more hours apart (Davey and MacGillivray, 1986)

[3]defined as gestational hypertension[1] and one 24 hour urine collection of 0.3 g proteinuria or more or two random clean catch specimens or urine dipstick with 2+ (1 gm) protein (Report of the NHBPEP 2000)

[4]defined as gestational hypertension[2] with one 24 hour urine collection of 0.3 g proteinuria or more or two random clean catch specimens or urine dipstick with 2+ (1 gm) protein (Davey and MacGillivray, 1986)

[5]defined as gestational hypertension[1] with one 24 hour urine collection of 0.3 g proteinuria or more or two random clean catch specimens or urine with 2+ (1 gm) protein as measured by dipstick or spot urine protein/creatinine ratio ≥30 mg/mmol (expansion of NHBPEP criteria)

[6] defined as gestational hypertension[2] with one 24 hour urine collection of 0.3 g proteinuria or more or two random clean catch specimens or urine with 2+ (1 gm) protein as measured by dipstick or spot urine protein/creatinine ratio ≥30 mg/mmol (expansion of Davey and MacGillivray criteria)

[7]defined as systolic blood pressure ≥ 160 mmHg and/or diastolic blood pressure ≥ 110 mmHg confirmed on repeated readings (four or more hours apart) (Report of the NHBPEP 2000)

[8]defined as two recordings of diastolic blood pressure of ≥ 110 mmHg, four ore more hours apart, or one recording of diastolic blood pressure of at least 120 mmHg (Davey and MacGillivray, 1986)

[9]defined as pre-eclampsia with either severe pregnancy induced hypertension[8] or severe proteinuria (defined as one 24 hour urine collection of 3 g protein or more or two random clean catch specimens or urine with 3+ protein or more as measured by dipstick) (Davey and MacGillivray, 1986)

[10]defined as pre-eclampsia with either severe pregnancy induced hypertension[8] or severe proteinuria (defined as one 24 hour urine collection of 3 g protein or more or two random clean catch specimens or urine with 3+ protein or more as measured by dipstick or spot urine protein/creatinine ratio ≥ 300 mg/mmol) (expansion of Davey and MacGillivray criteria)

[11]defined as either severe pregnancy induced hypertension[7 or 8] or severe proteinuria (defined as one 24 hour urine collection of 3 g protein or more or two random clean catch specimens or urine with 3+ protein or more as measured by dipstick or spot urine protein/creatinine ratio ≥ 300 mg/mmol) (expansion of NHBPEP and Davey and MacGillivray criteria)

4.1.4 Proteinuria

Further analyses were undertaken to explore any differences in the proteinuria assessments, due to the observed differences in pre-eclampsia risk according to the inclusive or restrictive definitions used and due to the differences in proteinuria assessments (Table 4.2). When exploring any variation in the surveillance for proteinuria between treatment groups, there was no difference in the number of women who were assessed for proteinuria at any stage in pregnancy (vitamin group 155 [20.1%] vs. placebo group 159 [20.7%], RR 0.97, 95% CI 0.80 to 1.18, p=0.780). For those women assessed, no difference was demonstrated in the number of women with proteinuria as assessed by dipstick (vitamin group 12 [1.6%] vs. placebo group 13 [1.7%], RR 0.92, 95% CI 0.42 to 2.00, p=0.835) or 24 hour urine collection (vitamin group 10 [1.3%] vs. placebo group 6 [0.8%], RR 1.66, 95% CI 0.61 to 4.55, p=0.317). A trend was observed with more women in the vitamin C and E group diagnosed with proteinuria based on the spot urine protein/creatinine ratio (vitamin group 41 [5.3%] vs. placebo group 26 [3.4%], RR 1.57, 95% CI 0.97 to 2.54, p=0.062), however this did not reach statistical significance.

No differences were demonstrated between treatment groups in the risk of severe proteinuria for any of the assessments based on dipstick, 24 hour urine collection or the spot protein/creatinine ratio. However, when all of the proteinuria assessments were combined to give a composite outcome of any proteinuria, regardless of the method of assessment, significantly more women in the vitamin C and E group had proteinuria (vitamin group 48 [6.2%] vs. placebo group 30 [3.9%], RR 1.59, 95% CI 1.02 to 2.49, p=0.038). No difference was seen between treatment groups in the number of women with any proteinuria but without concomitant hypertension.

Table 4.2 Proteinuria assessments in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Proteinuria assessed	155	20.1	159	20.7	0.97	0.80 to 1.18	0.780
Proteinuria							
Two random clean catch urine specimens with ≥ 2+ (1 gm) protein measured by dipstick	12	1.6	13	1.7	0.92	0.42 to 2.00	0.835
One 24 hour urine collection ≥ 0.3 g/L proteinuria	10	1.3	6	0.8	1.66	0.61 to 4.55	0.317
Spot urine protein/creatinine ratio ≥30 mg/mmol	41	5.3	26	3.4	1.57	0.97 to 2.54	0.062
Heavy (severe) proteinuria							
Two random clean catch urine specimens with ≥ 3+ (3 gm)	3	0.3	6	0.8	0.50	0.13 to 1.99	0.342
One 24 hour urine collection ≥ 3.0 g/L proteinuria	1	0.1	2	0.3	0.50	0.05 to 5.49	0.624
Spot urine protein/creatinine ratio ≥300 mg/mmol	4	0.5	4	0.5	1.00	0.25 to 3.97	1.000
Any proteinuria*	**48**	**6.2**	**30**	**3.9**	**1.59**	**1.02 to 2.49**	**0.038**
Any proteinuria without hypertension	5	0.6	5	0.6	1.00	0.29 to 3.43	0.997

Figures are n, %
*Includes proteinuria assessed by dipstick *or* 24 hour urine collection *or* spot urine protein /creatinine ratio, with or without concomitant increases in blood pressure

Further analyses were also undertaken to explore the criteria resulting in the diagnosis of pre-eclampsia using the ASSHP definition (Table 4.3). As stated earlier, there was no overall difference in the risk of pre-eclampsia between treatment groups when using the ASSHP diagnostic criteria. For those women with pre-eclampsia defined by the ASSHP, more women in the vitamin C and E group had concomitant proteinuria (42/60 [70.0%]) compared with women in the placebo group [24/51 [47.1%]) (RR 1.49, 95% CI 1.06 to 2.08, p=0.014). Conversely, 18 women in the vitamin C and E group (18/60 [30.0%]) and 27 women in the placebo group (27/51 [52.9%]) were diagnosed with pre-eclampsia without the occurrence of any proteinuria (RR 0.57, 95% CI 0.35 to 0.90, p=0.014). When assessing the other criteria used to diagnose pre-eclampsia, women with pre-eclampsia in the vitamin C and E group were more likely to have abnormal liver function (13/60 [21.7%]) compared with pre-eclamptic women in the placebo group (2/51 [3.9%]) (RR 5.52, 95% CI 1.31 to 23.34, p=0.010). No other differences were seen in the risk of renal insufficiency, oliguria, severe epigastric pain, convulsions, hyperreflexia, clonus, severe headaches, visual disturbances, thrombocytopenia, coagulopathy, haemolysis or fetal growth restriction for women diagnosed with pre-eclampsia in the vitamin C and E group and the placebo group.

Table 4.3 ASSHP pre-eclampsia criteria in the vitamin and placebo groups

Outcome	Vitamin C & E n=770	%	Placebo n=768	%	RR	95% CI	p value
Pre-eclampsia (ASSHP criteria)	60	7.8	51	6.6	1.17	0.82 to 1.68	0.383
Any proteinuria*	**42/60**	**70.0**	**24/51**	**47.1**	**1.49**	**1.06 to 2.08**	**0.014**
No proteinuria	**18/60**	**30.0**	**27/51**	**52.9**	**0.57**	**0.35 to 0.90**	**0.014**
Renal insufficiency	8/60	13.3	3/51	5.9	2.27	0.63 to 8.10	0.220
Oliguria	2/60	3.3	1/51	2.0	1.70	0.16 to 18.21	1.000
Abnormal liver function	**13/60**	**21.7**	**2/51**	**3.9**	**5.52**	**1.31 to 23.34**	**0.010**
Severe epigastric pain	10/60	16.7	3/51	5.9	2.83	0.82 to 9.74	0.136
Convulsions	0/60	0.0	0/51	0.0	-		-
Hyperreflexia	17/60	28.3	12/51	23.5	1.20	0.64 to 2.28	0.566
Clonus	10/60	16.7	8/51	15.7	1.06	0.45 to 2.49	0.889
Severe headaches	13/60	21.7	7/51	13.7	1.58	0.68 to 3.65	0.278
Visual disturbances	10/60	16.7	13/51	25.5	0.65	0.31 to 1.36	0.253
Thrombocytopenia	4/60	6.7	1/51	2.0	3.40	0.39 to 29.46	0.233
Coagulopathy	1/60	1.7	0/51	0.0	-		1.000
Haemolysis	3/60	5.0	1/51	2.0	2.55	0.27 to 23.77	0.623
Fetal growth restriction	8/60	13.3	10/51	19.6	0.68	0.29 to 1.59	0.371

Figures are n, %
*Includes proteinuria assessed by dipstick *or* 24 hour urine collection *or* spot urine protein /creatinine ratio
RR = relative risk, CI = confidence intervals
ASSHP = Australasian Society for the Study of Hypertension in Pregnancy

4.2 Discussion

The findings presented in this Chapter clearly highlight the continued need for consensus statements on the definition of pre-eclampsia. Depending on the individual definition used, the incidence of pre-eclampsia in this study ranged from 1.7 to 7.8 percent in the vitamin C and E group and 1.6 to 6.6 percent in the placebo group. This has obvious huge implications for the clinical management of women, the use of health services, determining whether treatment is effective and the prevention of adverse perinatal outcomes. Moreover it limits the comparison of women between studies particularly when definitions are not specified, ultimately contributing to the lack of clarity and understanding about the pathophysiology of the disease. The diversity in the incidence of pre-eclampsia based on varying definitions seen in this study would suggest that researchers are inevitably studying different populations of women depending on their set definitions. This needs to be recognised when comparing the results of studies, and in particular, when trials are included in systematic reviews.

Not surprisingly, the highest incidence of pre-eclampsia occurred using the ASSHP definition when compared with the more restrictive definition of proteinuric hypertension. Little is

known however about the population of women who are diagnosed with pre-eclampsia using the broader ASSHP criteria but who do not meet the criteria for proteinuric hypertension. It would appear that their perinatal outcomes may be worse, given that the ASSHP criteria include serious maternal adverse outcomes such as liver disease and renal insufficiency. Further research is warranted investigating the demographic characteristics and pregnancy outcomes of these women, compared with those women strictly with hypertension and proteinuria alone.

In this study, no differences between treatment groups were observed in the risk of gestational hypertension, however defined. Of concern however, when defined according to proteinuric hypertension including the spot protein creatinine ratio, vitamin C and E supplementation appeared to increase the risk of pre-eclampsia. No statistically significant differences were seen between treatment groups for the other definitions of pre-eclampsia, although the incidence of pre-eclampsia was often higher in the vitamin C and E group. The mechanism behind this potential treatment effect is unclear. Antioxidants have long been proposed to have pro-oxidant properties, although there is insufficient information about these effects in humans. Pro-oxidant effects of vitamin C have been demonstrated *in vitro*, more so in the presence of iron, however *in vivo* studies have consistently failed to demonstrate such effects (Carr and Frei 1999). It is possible however, that in the current study, which involved a population of women with adequate nutritional intakes of vitamin C and E, supplementation resulted in excess amounts of these vitamins leading to pro-oxidant effects. If true, this would contribute to oxidative stress and thus potentially increase the risk of pre-eclampsia.

Alternatively as the greatest difference between the treatment groups appeared to be in onset of proteinuria when assessed using the spot protein creatinine ratio, it may be possible that vitamin C and E are influencing creatinine formation or creatine clearance. Decreased formation or clearance of creatinine would yield higher protein creatinine ratios as seen in this study, and thus result in significant proteinuria. The findings may also be related to the properties of the protein/creatinine assessment itself. Vitamin C has been shown to interfere with enzymatic methods for the determination of creatinine concentrations (Siest et al 1978; Weber and van Zanten 1991). It is therefore possible that the elevated protein creatinine ratios seen in this study may be influenced by the presence of false positive results. No differences were seen between treatment groups when proteinuria was assessed from a 24 hour urine collection, or dipstick urinalysis, however the number of women assessed with these methods was small compared with those assessed with the spot protein creatinine ratio.

There is disagreement about the accuracy of the spot protein creatinine assessment. In Australia no data currently exists on the extent of usage of this proteinuria assessment, however from the current study, it would appear that this test is now commonly undertaken at the expense of the 24 hour urine save. While the accuracy and validity of the spot protein creatinine ratio in detecting proteinuria has been demonstrated in some studies (Saudan et al 1997; Robert et al 1997), other studies have demonstrated that the ratio is a poor predictor of proteinuria in hypertensive women (Al et al 2004) and normotensive women (Haas et al 2003). Of concern, it would appear that the introduction of this test into Australian antenatal hospitals has occurred without appropriate evaluation, and at the expense of the more accurate 24 hour urine collection. Further testing and refining of this proteinuria assessment is warranted in the Australian setting. Moreover, assessing the impact of the introduction of this test on the detection of proteinuria, and in the context of a cost benefit analysis would seem appropriate.

The impact of any potential inaccuracies in detecting proteinuria using this assessment on the findings from this study is unclear. This would be better answered had all women in this study been assessed for proteinuria by a 24 hour urine save and the spot protein creatinine ratio. This was not possible in the current study, which relied on the individual protocols at each collaborating centre for proteinuria surveillance and assessment. Nor was this the focus of the current study, however these findings do highlight the need for further research evaluating the accuracy of the protein creatinine assessment, and the potential for vitamin C to falsely influence creatinine values determined in this assessment.

Women in the vitamin C and E group appeared to be at increased risk of pre-eclampsia based on the proteinuric hypertension definition. However no corresponding increases in adverse neonatal outcomes was observed in this group, indeed the exact reverse was apparent. Fewer infants in the vitamin C and E group died or experienced a serious adverse health outcome, as presented in Chapter Three of this thesis. The disparity between these findings is difficult to interpret. As discussed earlier, the incidence of pre-eclampsia in the two groups may be influenced by the mode of proteinuria assessment. However it must also be considered that the findings may be due to the play of chance alone. Multiple comparisons were undertaken in these analyses assessing hypertensive disease in the context of the different definitions, which increases the risk of type 1 error, falsely finding an association or difference when there is truly no effect. Moreover, the differences between treatment groups for all of the pre-eclampsia definitions in this study were small, however the large sample size of this study

will detect small significant differences between the treatment groups regardless of whether they may be clinically relevant.

Of those women diagnosed with pre-eclampsia using the ASSHP criteria, more women in the vitamin C and E group had abnormal liver function tests. This was true for women with or without hypertension as discussed in Chapter Three of this thesis. Of all the women diagnosed with pre-eclampsia, only one was diagnosed as pre-eclamptic based on hypertension and abnormal liver function alone, the remaining women either had proteinuria or at least one of the other criteria defined by the ASSHP. This would therefore not suggest that abnormal liver function potentially associated with vitamin C and E supplementation is contributing to the incidence of pre-eclampsia in this study. This continued finding of abnormal liver function in the vitamin C and E group warrants further investigation, as this is the only study to date demonstrating a potentially adverse effect on liver function during pregnancy with vitamin C and E supplementation.

While the lack of effect on or in fact an increased risk of pre-eclampsia observed in this study may appear disappointing, it does echo the findings of many of the antioxidant trials assessing adult diseases such as atherosclerosis. Trials assessing antioxidants and cardiovascular disease to date have produced mixed findings. It would seem that supplementation with antioxidants like vitamin C, vitamin E and beta-carotene either have no effect on the risk of cardiovascular disease morbidity or mortality but no adverse effects (The Heart Outcomes Prevention Evaluation Study Investigators 2000; Heart Protection Study Collaborative Group 2002), or in some populations may in fact be associated with more harm than good, with excess all-cause mortality demonstrated in a recent systematic review of vitamin E supplementation trials (Miller et al 2005). Pre-eclampsia and atherosclerosis share the common disease pathways of inflammation and oxidative stress, and furthermore women with pre-eclampsia have an excess risk of mortality from cardiovascular events in later life (Irgens et al 2001). Therefore in the context of the antioxidant trials in adults and their lack of effectiveness in preventing or delaying the onset of cardiovascular disease, the failure to demonstrate an association between vitamin C and E and a reduced risk of pre-eclampsia seen in this study is appropriate.

4.3 Conclusions

Antioxidants are not an effective strategy for preventing pre-eclampsia in nulliparous women, either when pre-eclampsia is defined according to proteinuric hypertension or the broader criteria specified by the ASSHP. The findings presented in this Chapter clearly demonstrate

that defining pre-eclampsia based on criteria related to the "syndrome" of pre-eclampsia or based solely on proteinuric hypertension results in vast differences in the incidence of pre-eclampsia, and thus the management of women with hypertension in pregnancy. While there is now some consensus on blood pressure measurement techniques and the definitions of hypertensive disorders of pregnancy excluding pre-eclampsia, it is obvious from this study that further agreement is required. Clarification is clearly needed about the assessment of proteinuria in pregnancy as well as the inclusion of restrictive or broader diagnostic criteria.

4.3.1 Implications for practice

However defined, antioxidants cannot be recommended as a prophylaxis for pre-eclampsia in nulliparous women. Moreover, antioxidants may have contributed to the development of pre-eclampsia in these women, however the mechanism behind this treatment effect is unclear. The absolute risk difference in the incidence of pre-eclampsia between treatment groups is small, in the order of two percent, indicating that with 43 women taking vitamin C and E supplements in pregnancy, one extra case of pre-eclampsia may occur.

4.3.2 Implications for research

Further research evaluating the accuracy of the spot protein creatinine ratio for detecting proteinuria, and the potential for vitamin C to influence the results of this assessment is warranted. Assessing the clinical and demographic characteristics of women meeting the different criteria for a diagnosis of pre-eclampsia would provide further insight into the pathophysiology of the disease and this is warranted. Similarly, assessing the impact of the adoption of the ASSHP criteria on the incidence of pre-eclampsia and adverse perinatal outcomes would seem appropriate.

Any future research should clearly state the criteria used to define pre-eclampsia to permit comparisons between studies and to facilitate inclusion in systematic reviews.

5. UPDATED SYSTEMATIC REVIEW OF ANTIOXIDANTS FOR THE PREVENTION OF PRE-ECLAMPSIA

5.1 Introduction

Evidence for the safety and efficacy of antioxidant use during pregnancy is required in order to formulate treatment recommendations. These recommendations should be based on the best available evidence, the systematic review of all relevant randomised controlled trials. Chapter Two of this thesis described the systematic review of all trials of antioxidant supplementation in pregnancy either for the prevention of pre-eclampsia or where supplementation occurred and the authors reported pre-eclampsia. This chapter describes an updated systematic review incorporating the data from women in the trial presented in Chapter Three of this thesis (ACTS 2005).

5.2 Objectives and Methods of the review

The objective of this review was to determine the effectiveness and safety of any antioxidant supplementation during pregnancy on the risk of pre-eclampsia; small for gestational age infants; stillbirth and neonatal death; maternal and neonatal morbidity; long term development of the child and side effects and adverse events. The criteria for including studies, participants and subgroups, and methods of the review are described in detail in Chapter Two.

5.3 Results

Eight trials involving 7,620 women are included in this review. Significant heterogeneity of results was detected for the outcomes pre-eclampsia, gestational age at birth and neonatal length of stay in hospital. The data were too few to categorise women into subgroups to adequately explain the heterogeneity seen. However factors contributing to the heterogeneity appeared to be women's risk status, the combination of antioxidants with other agents and the size of the trial, with smaller treatment effects reported in the larger trials. For all of the comparisons where heterogeneity was detected, a random effects model was used.

Assessments of the treatment effects were also made for the primary outcomes based on trial quality. Four trials involving 2,272 women (ACTS 2005; Chappell et al 1999; Sharma et al 2003; Steyn et al 2002, 2003) fulfilled all of the criteria for a high quality trial, that is they were rated A for allocation concealment; women, caregivers and research staff were blinded to treatment allocation; had less than three percent of participants excluded and were placebo controlled.

5.3.1 Pre-eclampsia

5.3.1.1 All trials

Supplementation with any antioxidants during pregnancy compared with control or placebo was associated with a 34 percent reduction in the relative risk of pre-eclampsia (RR 0.66, 95% CI 0.45 to 0.97, eight trials, 7,620 women) (Figure 5.1). When the one quasi-random study was excluded, this risk difference was no longer statistically significant (RR 0.60, 95% CI 0.34 to 1.06, seven trials, 2,599 women). Only one trial (ACTS 2005) reported data relative to the onset of pre-eclampsia and severity of disease, hence the data were too few to make reliable conclusions about the impact of supplementation on these outcomes. Data for these outcomes are presented in Chapter Three of this thesis.

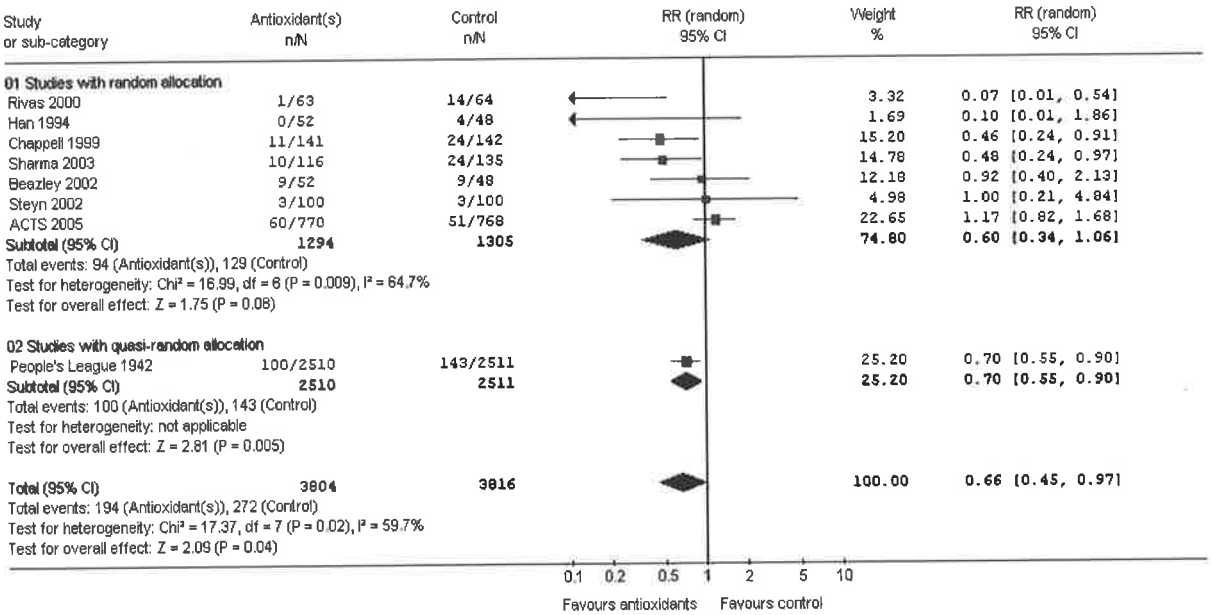

Figure 5.1 Meta-analyses of the effect of any antioxidants and the risk of pre-eclampsia (subgrouped on random or quasi–random allocation).

5.3.1.2 High quality trials only

When the analyses were restricted to high quality studies only, no clear difference was seen in the relative risk of pre-eclampsia between women supplemented with any antioxidants compared with placebo (RR 0.71, 95% CI 0.39 to 1.28, four trials, 2,272 women).

5.3.2 Preterm birth

5.3.2.1 All trials

Four trials involving 2,112 women (ACTS 2005; Beazley et al 2002, 2005; Chappell et al 1999; Steyn et al 2002, 2003) reported preterm birth (< 37 weeks' gestation) in a format for

inclusion in the review. None of these trials were quasi-randomised. No difference was seen in the risk of preterm birth between women supplemented with any antioxidants compared with placebo (RR 1.15, 95% CI 0.92 to 1.45) (Figure 5.2). Only one trial (ACTS 2005) reported data for very preterm birth (< 34 weeks' gestation), hence the data were too few to produce reliable conclusions. Data for this outcome are presented in Chapter Three of this thesis.

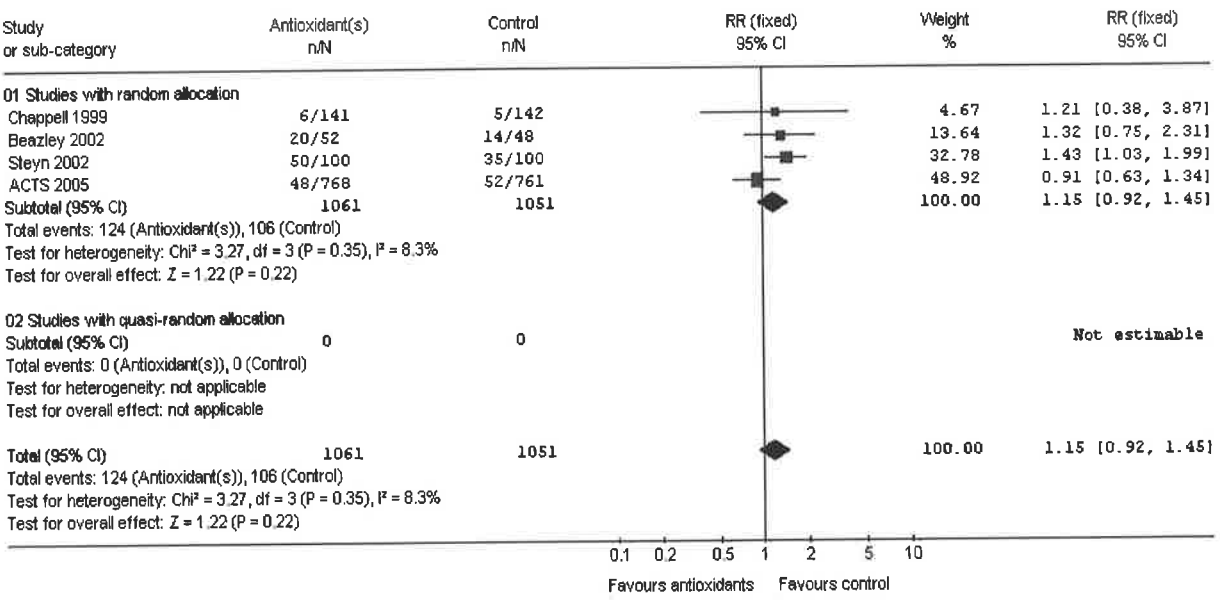

Figure 5.2 Meta-analyses of the effect of any antioxidants and the risk of preterm birth (subgrouped on random or quasi–random allocation).

5.3.2.2 High quality trials only

No difference was seen in the risk of preterm birth between women supplemented with any antioxidants compared with placebo, for high quality trials only (RR 1.13, 95% CI 0.88 to 1.44, three trials, 2,012 women).

5.3.3 Small for gestational age infants

5.3.3.1 All trials

Four trials involving 2,172 women (ACTS 2005; Beazley et al 2002, 2005; Chappell et al 1999; Sharma et al 2003) reported birth weight less than the 10th centile. None of these trials were quasi-randomised. Women supplemented with antioxidants compared with placebo had a reduced relative risk of their infant being small for gestational age (RR 0.78, 95% CI 0.62 to 0.97) (Figure 5.3).

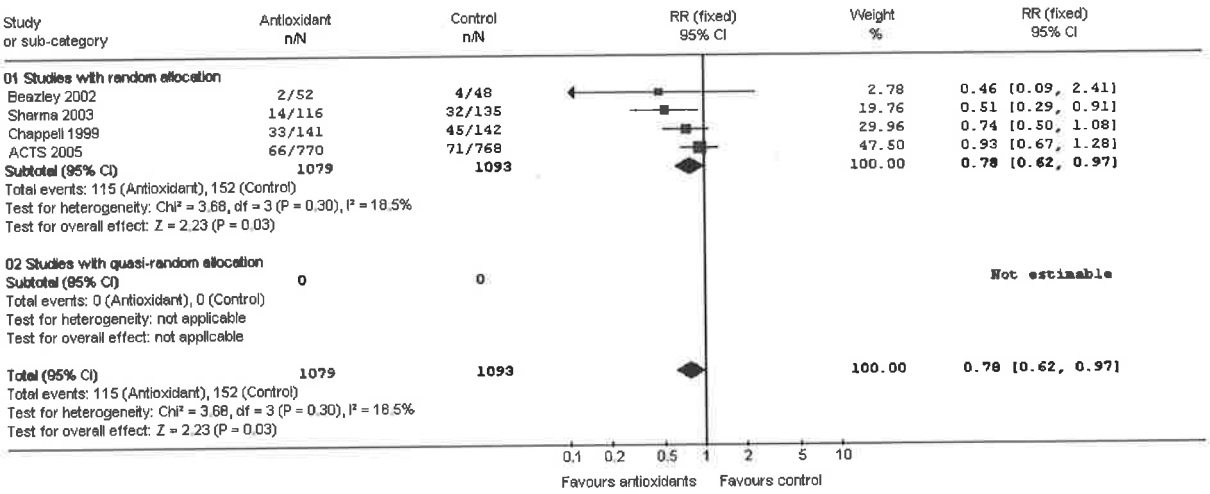

Figure 5.3 Meta-analyses of the effect of any antioxidants and the risk of small for gestational age infants (subgrouped on random or quasi–random allocation).

5.3.3.2 High quality trials only

The reduction in the risk of having a small for gestational age infant remained for women supplemented with any antioxidants in high quality trials (RR 0.78, 95% CI 0.63 to 0.98, three trials, 2,072 women).

5.3.4 Baby death

5.3.4.1 All trials

Four trials involving 7,033 women (ACTS 2005; Chappell et al 1999; People's League of Health 1942, 1946; Steyn et al 2002, 2003) reported stillbirth. No difference was seen in the risk of having a stillbirth between women supplemented with antioxidants compared with control for all trials (RR 0.84, 95% CI 0.61 to 1.17) or when the quasi-randomised trial was excluded from the analyses (RR 1.00, 95% CI 0.36 to 2.73, three trials, 2,012 women) (Figure 5.4).

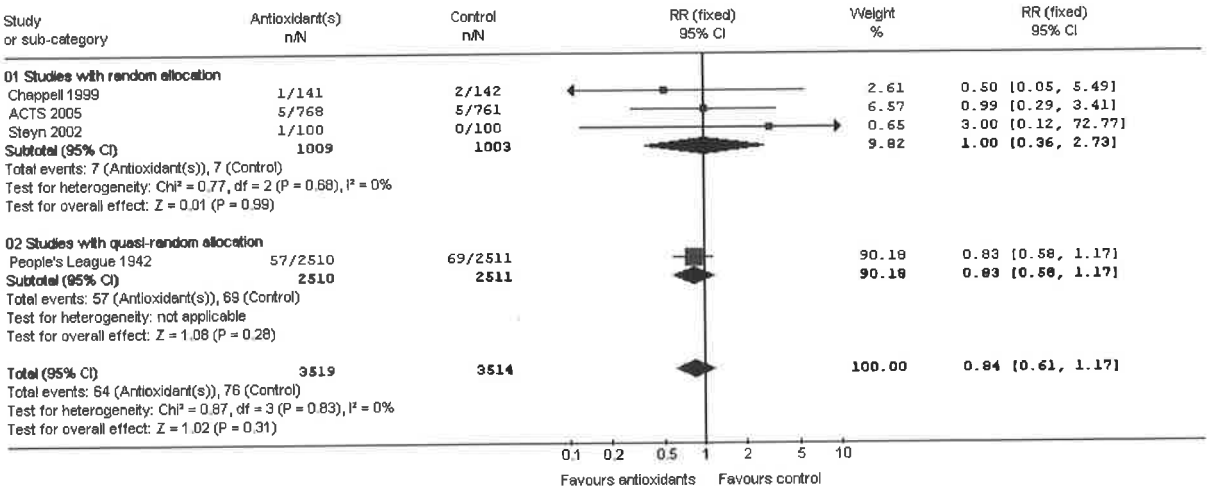

Figure 5.4 Meta-analyses of the effect of any antioxidants and the risk of stillbirth (subgrouped on random or quasi–random allocation).

Three trials involving 6,595 women reported neonatal death (ACTS 2005; People's League of Health 1942, 1946; Steyn et al 2002, 2003). No difference was seen for the risk of neonatal death between women supplemented with any antioxidants compared with control for all trials (RR 1.23, 95% CI 0.76 to 1.99) or when the quasi-randomised trial was excluded from the analyses (RR 0.51, 95% CI 0.13 to 2.00, two trials, 1,700 women) (Figure 5.5).

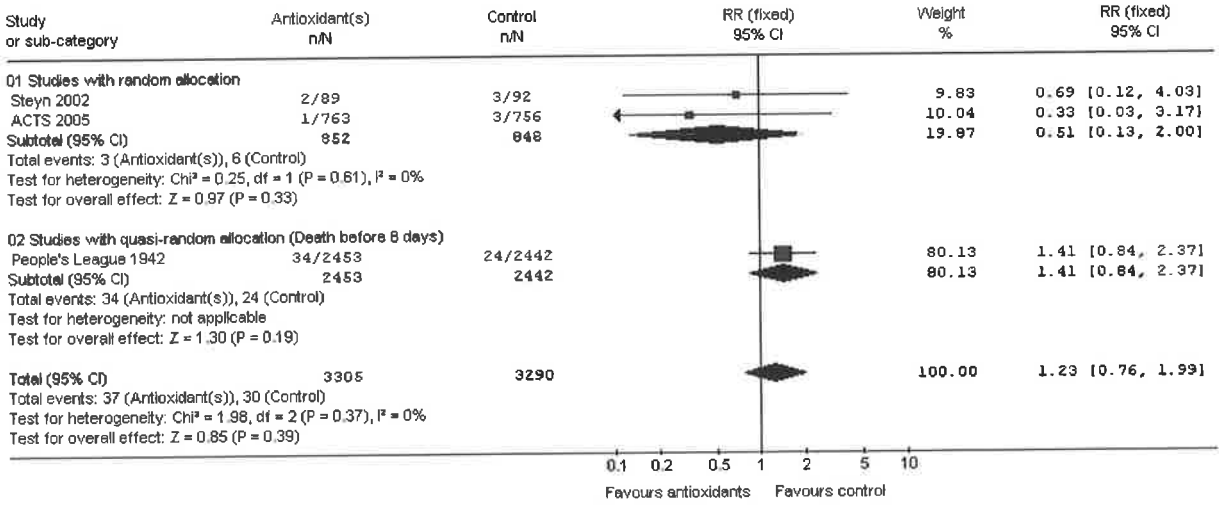

Figure 5.5 Meta-analyses of the effect of any antioxidants and the risk of neonatal death (subgrouped on random or quasi–random allocation).

5.3.4.2 High quality trials only

For the high quality trials only, no difference was seen between women supplemented with any antioxidants compared with placebo for the risk of stillbirth (RR 1.00, 95% CI 0.36 to 2.73, three trials, 2,012 women) or neonatal death (RR 0.51, 95% CI 0.13 to 2.00, two trials, 1,700 women).

5.3.5 Secondary outcomes

There were no differences between women supplemented with antioxidants compared with control or placebo for any of the secondary outcomes including: the need for induction of labour, the risk of bleeding episodes, eclampsia, mean birth weight, gestational age at birth or neonatal length of stay in hospital (Table 5.1). For the remaining secondary outcomes including maternal death; severe hypertension; caesarean section (emergency or elective); other bleeding episodes including antepartum haemorrhage, postpartum haemorrhage and the need for transfusion; other measures of serious maternal morbidity including liver failure, renal failure, disseminated intravascular coagulation and stroke; side effects and adverse effects of supplementation; maternal use of health service resources and maternal views of care, only one trial (ACTS 2005) reported these outcomes, and these are presented in Chapter Three of this thesis. Similarly, the secondary infant outcomes Apgar score less than four at five minutes; respiratory distress syndrome; chronic lung disease; bleeding episodes (such as intraventricular haemorrhage, periventricular leucomalacia); bacterial sepsis; necrotising enterocolitis; retinopathy of prematurity and infant use of health service resources were reported by one trial only (ACTS 2005) and are discussed in Chapter Three of this thesis.

Table 5.1 Secondary outcomes in the antioxidant and control groups

Outcome	No. of trials	Antioxidant group	Control group	RR	95% CI	Statistical heterogeneity
Induction of labour	2	268/879	236/868	1.13	0.98 to 1.31	$I^2 = 0.0$
Bleeding episodes (placental abruption)	3	11/1011	5/1010	1.98	0.33 to 11.75	$I^2 = 49.3\%$
Eclampsia	2	0/886	1/903	0.39	0.02 to 9.42	N/A
Birth weight* (grams)	4	983	987	39.12	-6.05 to 84.29	$I^2 = 26.0\%$
Gestational age at birth* (weeks)	3	938	951	0.42	-0.45 to 1.29	$I^2 = 86.9\%$
Neonatal length of stay in hospital*	2	852	848	0.36	-1.15 to 1.86	$I^2 = 67.9\%$

*weighted mean difference
RR= relative risk, CI= confidence intervals, N/A= not applicable
I^2 = measure of statistical heterogeneity

5.3.6 Other subgroup analyses

Predefined subgroup analyses were undertaken based on women's risk status, women's gestational age at trial entry, the dosage of antioxidants used, the use of antioxidants with other agents and women's dietary antioxidant intake at trial entry. Many of the subgroups for these comparisons contained only one trial, meaning that the data were too few to make any meaningful and reliable comparisons about these subgroups. For the subgroup analyses on

women's risk status, no statistically significant difference was seen for antioxidant supplementation and the risk of pre-eclampsia for women at moderate/low risk of pre-eclampsia (RR 0.76, 95% CI 0.50 to 1.15, five trials, 7,110 women (ACTS 2005; Han and Zhou 1994; People's League of Health 1942, 1946; Sharma et al 2003; Steyn et al 2002, 2003)). Similarly, no statistically significant difference was seen for women at moderate/high risk of pre-eclampsia (RR 0.44, 95% CI 0.16 to 1.22, three trials, 510 women (Beazley et al 2002, 2005; Chappell et al 1999; Rivas-Echeverria et al 2000)) however the magnitude of effect was much greater than for women at moderate/low risk.

5.4 Discussion

This review summarises data for over 7,600 women, supplemented with a variety of antioxidants including vitamin C alone, vitamin C and E, vitamin C and E combined with aspirin and fish oil, a multivitamin containing vitamin C, lycopene and selenium. Women allocated any antioxidants, either alone or in combination with other supplements, rather than placebo or no antioxidant had a reduced risk of developing pre-eclampsia or having a small for gestational age infant. No clear difference was seen between women supplemented with antioxidants for the risk of preterm birth, stillbirth or neonatal death.

In this review, supplementation with antioxidants during pregnancy was associated with a moderate reduction (34%) in the relative risk of pre-eclampsia. This corresponds with an absolute risk reduction of four percent, whereby 25 women would need to take antioxidants during pregnancy to prevent one case of pre-eclampsia. The confidence intervals around the relative risk point estimate are wide and suggest that the true effect could be as large as a 55 percent reduction or as little as a three percent relative risk reduction. By far the largest trial included in this review is still the quasi-randomised trial of multivitamin supplementation. By removing this trial from the analyses, the difference seen for the risk of pre-eclampsia associated with antioxidant supplementation was no longer statistically significant, although the point estimate was still for a reduction. Similarly, when the analyses were restricted to high quality studies only (2,272 women), no statistically significant differences were seen for the risk of pre-eclampsia or preterm birth, stillbirth or neonatal death.

Antioxidant supplementation was associated with a 22 percent relative risk reduction in the infant being born small for gestational age. This represents an absolute risk reduction of three percent, requiring 33 women to take antioxidants during pregnancy to prevent one infant being born small for gestational age. When only high quality trials were assessed, the

difference between treatment groups for the risk of having a small for gestational age infant remained.

No other differences were seen between women supplemented with antioxidants compared with control for any other outcomes, including preterm birth. The review described in Chapter Two of this thesis indicated that women supplemented with antioxidants may be at increased risk of preterm birth. However with the addition of the trial presented in this thesis, no clear difference was seen between treatment groups, which provides some reassurance that preterm birth is not increased with antioxidant supplementation. There is still limited information about the impact of supplementation on the timing of onset of pre-eclampsia or the severity of hypertension or measures of serious maternal or infant morbidity or maternal death. The trial presented in this thesis is the only trial to report these outcomes to date. Moreover, there is currently is no information about the impact of antioxidant supplementation on the long-term development of the child.

Significant statistical heterogeneity was detected for all of the pre-eclampsia comparisons and the outcomes gestational age at birth and neonatal length of stay in hospital. The greatest factor contributing to the heterogeneity seen appeared to be size of the trial, with the two large trials reporting small effects compared with the smaller trials which appeared to report large treatment effects. This review also includes women with a wide range of clinical risk, which may have contributed to the heterogeneity seen. The majority of the smaller trials included women deemed at high risk of pre-eclampsia who may have had greater potential to benefit. Conversely, the larger trials tended to include women at moderate/low risk of pre-eclampsia who may have had less potential to benefit. The pre-specified subgroup analyses on women's risk status, indicated that women at high risk of pre-eclampsia may be more likely to benefit from antioxidant supplementation, however the numbers of women included in the high risk trials was small. Furthermore, the subgroup analyses based on risk status could not explain all of the heterogeneity seen.

Other factors which may be contributing to heterogeneity in results were the use of antioxidants with co-interventions like aspirin and fish oil, the type of antioxidant(s) (vitamin C and E versus lycopene or selenium), the dosage of the antioxidant(s), and whether women had an adequate or deficient dietary intake of antioxidants. The data were too few to adequately categorise trials based on these factors, as the majority of trials included assessed vitamin C and E in similar dosages. Only one trial (People's League of Health 1942, 1946), reported that a significant proportion of women included in the trial had a dietary vitamin

deficiency at trial entry. The data are therefore too few to reliably determine whether antioxidants may be of more benefit to women with a dietary antioxidant deficiency prior to and in early pregnancy.

There may also be potential for publication bias as highlighted in Chapter Three of this thesis. When the effect size is plotted against the standard error, a measure of the sample size of each trial, the funnel plot generated for pre-eclampsia is asymmetrical (Figure 5.6). This is consistent with the occurrence of publication bias where small trials of antioxidant supplementation with negative findings may not have been published. Confirmation of this may be found in the finding that the majority of the small trials published to date report positive findings. As such, there still remains a possibility that small trials with negative findings may exist but have not been reported or they were not identified for inclusion in this review. Funnel plot asymmetry can also be influenced by heterogeneity in the results, such as clinical differences between study populations, as discussed earlier or methodological heterogeneity, for example the inclusion of the large quasi-randomised trial.

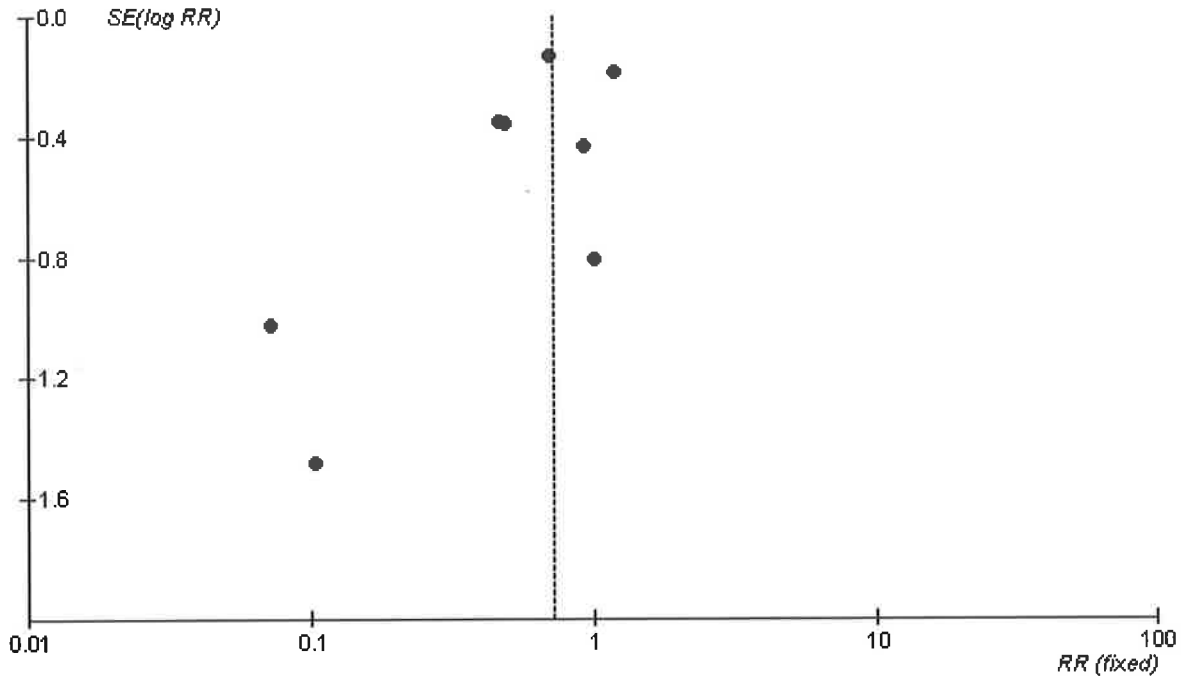

Figure 5.6 Funnel plot of the effect of any antioxidants and the risk of pre-eclampsia.

5.5 Conclusions

Although antioxidants appear to reduce the risk of pre-eclampsia, substantial heterogeneity was detected for all of the comparisons. As such, antioxidants cannot be recommended as a prophylaxis for pre-eclampsia for all women. Much more information is required to determine

what women are most likely to benefit, as well as the optimal antioxidant type and dosage. Furthermore, although antioxidants appear to reduce the risk of having a small for gestational age infant, further confirmation is required before any reliable conclusions can be made about whether such therapy is, overall, worthwhile.

5.5.1 Implications for practice

Antioxidants cannot be recommended for routine use in pregnancy for all women. There is insufficient evidence to determine whether antioxidants may be beneficial for women at high risk of pre-eclampsia and other adverse pregnancy outcomes, or for those women with a dietary insufficiency of antioxidants at trial entry.

5.5.2 Implications for research

Further research is required to confirm whether maternal antioxidant supplementation can prevent infants being born small for gestational age, or whether supplementation may be beneficial for women at high risk of pre-eclampsia or with a dietary deficiency of antioxidants. If antioxidants are confirmed to be beneficial during pregnancy, future trials must also determine the optimum dosage, type of antioxidant and timing of supplementation. This may be best answered by reviewing data from individual women, therefore undertaking an individual patient data review would seem appropriate. There are currently several large on-going randomised controlled trials assessing antioxidant supplementation in pregnancy. Further confirmation from these studies will help identify which women are more likely to benefit.

6. SUMMARY CONCLUSIONS

6.1 Conclusions from the randomised controlled trial of vitamin C and E supplementation during pregnancy

The randomised controlled trial presented in this thesis was designed to evaluate whether supplementing nulliparous women from mid pregnancy, with vitamin C and vitamin E, prevented their infants being small for gestational age, prevented pre-eclampsia and prevented the occurrence of other serious maternal and infant adverse health outcomes. This is the largest trial of vitamin C and E supplementation in pregnancy to date.

This trial has shown that supplementation with the antioxidants vitamin C and E does not prevent pre-eclampsia or small for gestational age infants in nulliparous women. Furthermore for women, supplementation appeared to increase the risk of abnormal liver function. Vitamin C and E supplementation was associated with health benefits for the infant. Fewer infants in the vitamin C and E group died or experienced a serious adverse health outcome. Moreover, surviving infants in the vitamin C and E group had an increased head circumference at birth and at a reduced risk of developing respiratory distress syndrome. While these findings require further confirmation from larger trials, they represent potentially clinical meaningful differences in serious adverse neonatal outcomes. These findings also highlight the need for long term follow up of all surviving infants in order to assess any longer term benefits of maternal vitamin C and E supplementation. Follow up of women and infants in this trial is currently underway.

Women in this trial were very positive about their experiences of the trial. By far the majority would participate in similar research and recommend the study to other pregnant women in a similar situation. These findings highlight the fact that women in their first on-going pregnancy are willing to be involved in supplementation trials and other similar research projects.

6.1.1 Implications for practice

Vitamin C and E cannot be recommended as a prophylaxis for pre-eclampsia in nulliparous women. There is insufficient evidence about the potential benefits of these and other antioxidants for women at high risk of pre-eclampsia or with a dietary deficiency of antioxidants. Vitamin C and E supplementation was associated with a reduced risk of the infant dying or having a serious adverse health outcome. Further confirmation of the impact

of supplementation on infant growth, respiratory morbidity and other measures of morbidity from the current on-going trials of antioxidants is required, so that all the benefits of supplementation can be weighed against potential harms, including abnormal maternal liver function.

6.1.2 Implications for future research

A number of areas for future research have been identified from the findings presented in this thesis.

1. This trial demonstrated that supplementing women with vitamin C and E during pregnancy was associated with a greater head circumference at birth for their infant. Future research must be undertaken to assess any longer term health benefits or harms of supplementation on infant growth and development.

2. Women in the vitamin C and E group in this trial appeared to have an increased risk of abnormal liver function. The impact of vitamin C and E supplementation during pregnancy on liver function and status needs further investigation.

3. Women in this trial reported high usage of dietary supplements during pregnancy. Further research assessing women's beliefs around using dietary supplements would be appropriate, and evaluation of the safety and efficacy of these dietary supplements is clearly needed.

4. Further evaluation of the surveillance and assessment of proteinuria in pregnancy is warranted, particularly the accuracy of the spot protein creatinine ratio in the presence of vitamin C supplementation.

6.2 Conclusions from the systematic review of supplementation with any antioxidants during pregnancy for the prevention of pre-eclampsia

Antioxidants cannot be recommended for routine use in pregnancy for all women. There is insufficient evidence to determine whether antioxidants may be beneficial for women at high risk of pre-eclampsia and other adverse pregnancy outcomes, or for those women with a dietary insufficiency of antioxidants at trial entry. The impact of supplementation on the risk of small for gestational age infants required further confirmation.

The systematic review presented in Chapter Two of this thesis highlighted the following research priorities. The contribution of the trial presented in this thesis to these priority areas is discussed below.

Does antioxidant supplementation alone in pregnancy prevent pre-eclampsia and its related complications for women:

1. With differing risk status for pre-eclampsia (i.e. high risk or low risk status)?

This trial has shown that supplementation with the antioxidants vitamin C and E does not prevent nulliparous women developing pre-eclampsia or their infant being born small for gestational age. This trial was not designed to address the question of whether vitamin C and E supplementation is beneficial for women at high risk of pre-eclampsia. Further evaluation of the impact of supplementation in high risk women is the subject of several large ongoing randomised trials in the United Kingdom and the United States of America.

2. In all settings (developed and developing)?

Antioxidant vitamins C and E are not an effective intervention for Australian nulliparous women in a developed setting.

3. With a poor dietary intake of the antioxidant(s) or with adequate dietary intake of the antioxidant(s)?

The majority of women in this trial had adequate dietary intakes of vitamin C and E. No overall benefit was seen for the risk of pre-eclampsia in these women and no additional benefit was seen for the small number of women with a dietary intake of vitamin C or vitamin E below the recommended amount at trial entry.

With the completion and reporting of the current on-going trials assessing antioxidant supplementation in pregnancy and the follow up of children involved in these trials, the benefits of supplementation for the risk of pre-eclampsia and infant growth and development will be weighed against the potential harms, so that reliable conclusions can be made about whether such therapy is, overall, worthwhile.

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8. APPENDIX: ADDITIONAL TABLES

Table 8.1 Number of women recruited at each collaborating centre

Coordinating centre	Number of women recruited	
	n	%
Women's and Children's Hospital, SA	422	27
Mater Mother's Hospital, QLD	382	25
Lyell McEwin Health Service, SA	183	12
Royal North Shore, NSW	105	7
The Royal Women's Hospital, VIC	97	6
Modbury Public Hospital, SA	89	6
The Royal Women's Hospital, QLD	88	6
The Queen Elizabeth Hospital, SA	87	6
The Townsville Hospital, QLD	85	5
TOTAL	1538	100

Figures are n, %
SA= South Australia, QLD= Queensland, NSW= New South Wales, VIC=Victoria

Table 8.2 Women's previous medical history

Previous medical condition		Vitamin C & E		Placebo	
		n	%	n	%
Any previous medical condition		177	23.0	164	21.3
If yes, type:	**Asthma**	62	8.1	47	6.1
	Gynaecological	22	2.9	22	2.9
	Psychiatric	10	1.3	19	2.5
	Epilepsy	10	1.3	11	1.4
	Anaemia/Thalassaemia	8	1.0	10	1.3
	Cardiac	9	1.2	6	0.8
	Kidney disease	4	0.5	5	0.7
	Thyroid	4	0.5	5	0.7
	Blood clotting/Antibody disorder	6	0.8	3	0.4
	Hypertension**	5	0.7	3	0.4
	Urinary tract infections	5	0.7	3	0.4
	Migraine	0	0	3	0.4
	Diabetes	3	0.4	2	0.3
	Connective tissue disorder	1	0.1	2	0.3
	High cholesterol/hyperlipidemia	1	0.1	1	0.1
	Digestive system	6	0.8	1	0.1
	Haemochromatosis carrier	2	0.3	0	0
	Other	23	3.0	24	3.1

Figures are n, %

Table 8.3 Classifications of congenital malformations in live born infants

Congenital malformation category†	Specifics	n	%
Vitamin C & E group (n=763)			
Cardiovascular system (2)	Coarctation of the aorta, VSD, PFO, bicuspid aortic valve	1	0.13
	Primum AVSD and pulmonary stenosis	1	0.13
Gastrointestinal system	Duodenal atresia	1	0.13
	Duplication cyst	1	0.13
	Anal stenosis	1	0.13
Urinary system	Unilateral renal agenesis	1	0.13
	Single kidney	1	0.13
Respiratory system	Diaphragmatic hernia	1	0.13
Total		**8**	**1.05**
Placebo group (n=756)			
Cardiovascular system (5)	Patent ductus arteriosis	1	0.13
	VSD	3	0.40
	Congenital heart disease (TGA)	1	0.13
Urinary system (3)	Cystocele (duplex right kidney)	1	0.13
	Ectopic kidney	1	0.13
	Crossed fused renal ectopia	1	0.13
Respiratory system (1)	Diaphragmatic hernia*	1	0.13
Nervous system (1)	Hydrocephalus*	1	0.13
Gastro-intestinal system (1)	Cleft Palate	1	0.13
Metabolic Disorders (1)	Congenital hypothyroidism	1	0.13
Total		**14**	**1.85**

Figures are n, %
*Neonatal death

†Classifications according to the South Australian Birth Defects Register (2005).