Antioxidant vitamin and carotenoid levels in relation to risk of preeclampsia and small for gestational age birth

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Abstract

Preeclampsia and small-for-gestational-age (SGA) birth are two common, related conditions in pregnancy associated with maternal, fetal, and neonatal morbidity and mortality; both have a heterogeneous etiology. Preeclampsia is a hypertensive disorder that affects 2-8% of pregnancies which is typically diagnosed in the presence of high blood pressure and excess protein in the urine during the latter half of pregnancy. It is a major cause of preterm birth and maternal mortality worldwide. SGA birth, usually defined as a birth weight below the 10^{th} percentile for gestational age and sex, is related to preeclampsia, since both conditions may involve placental dysfunction and often occur in the same pregnancy. Furthermore, they share a number of risk factors and biomarkers, including elevated markers of oxidative stress. Oxidative stress results from an increased production of reactive oxygen species or a deficient antioxidant response. Hence, it has been hypothesized that low levels of antioxidant micronutrients in the maternal circulation during pregnancy may predispose women to preeclampsia and SGA birth. In recent years, the effectiveness of antioxidant supplements, mainly vitamin C and E in preventing preeclampsia, has been assessed in several randomized trials. Such results were disappointing as the supplements not only did not decrease the risk of preeclampsia or SGA, in some instances they were associated with adverse outcomes. Those trials, however, evaluated few supplementation regimens and mostly in select, high-risk populations. Given that antioxidant micronutrient levels are potentially modifiable in pregnancy, the overarching objective of this thesis was to better understand their association with preeclampsia and SGA, which should inform future clinical trials of antioxidant interventions, if warranted. We sought to fill the knowledge gap regarding whether antioxidant micronutrient levels are systematically altered in pregnancies affected by preeclampsia and SGA.

The first manuscript is a systematic review of observational studies that have measured maternal antioxidant micronutrient levels in association with preeclampsia or SGA. We found that most of the studies reviewed measured antioxidant levels in women who were already diagnosed with preeclampsia; hence, it was not possible to determine if any associations observed were a cause or an effect of preeclampsia. While the pooled results indicated that blood levels of vitamins A, C, and E were lower in women with preeclampsia, publication bias was

suggested, and confounding was poorly addressed in most studies. Fewer studies addressed associations with SGA, but those studies had a lower risk of bias and a greater focus on measurement of biomarkers in the second trimester of pregnancy. The studies found that vitamin A was mostly similar, whereas vitamin C was consistently lower, in SGA cases versus controls. Results were mixed for vitamin E, particularly depending on whether the authors adjusted for lipid levels in their analysis. The second and third manuscripts are case-control studies, nested within a large population-based cohort of pregnant women in which antioxidant levels were measured from blood samples collected in the second trimester. Therefore, blood measurements were less likely to be affected by the outcomes which had not yet occurred at the time of sampling. In both studies, we addressed the correlated nature of these nutrients, derived from similar dietary sources, and the potential for non-linear associations or threshold effects. When examining SGA birth at term, we found that higher levels of carotenoid antioxidants in the second trimester were associated with a reduced risk of SGA. Unexpectedly, we also found that higher levels of retinol were associated with an increased risk; we speculate that underlying placental dysfunction may be responsible for this observation. When we investigated preeclampsia, we found that higher levels of lutein were associated with a significantly reduced risk of preeclampsia. Few prior studies have measured lutein in association with preeclampsia, and no previous trials have evaluated lutein supplementation for prevention of preeclampsia or SGA. If this association is confirmed in future prospective studies, lutein supplementation may be a target for preeclampsia prevention trials. The work in this thesis points to potential benefits of vitamins C and carotenoids for prevention of preeclampsia and SGA.

Résumé

La pré-éclampsie et la naissance de bébés petits pour l'âge gestationnel sont des conditions courantes liées à la grossesse, tous deux associées à la morbidité et mortalité maternelles, fœtales et néonatales, et ayant une étiologie hétérogène. La pré-éclampsie est un trouble d'hypertension qui affecte 2-8% des grossesses, typiquement diagnostiquée par la présence d'une tension artérielle élevée et d'un excès de protéines dans l'urine pendant la deuxième moitié de la grossesse. C'est une cause majeure de la prématurité et de mortalité maternelle dans le monde. La naissance de bébés petits pour l'âge gestationnel, habituellement défini comme un poids à la naissance sous le 10^{e} percentile pour l'âge gestationnel et le sexe, est relié à la pré-éclampsie puisque ces deux conditions pourraient impliquer une dysfonction placentaire et sont souvent présentes lors d'une même grossesse. De plus, elles partagent bon nombre de facteurs de risques et de biomarqueurs, incluant une élévation de marqueurs de stress oxydatif. Le stress oxydatif résulte d'une production accrue de dérivés réactifs de l'oxygène ou d'une réponse déficiente d'antioxydants. L'hypothèse qu'un bas niveau de micronutriments antioxydants dans la circulation sanguine maternelle pourrait prédisposer les femmes pour la pré-éclampsie et la naissance de bébés petits pour l'âge gestationnel a ainsi été émise. L'efficacité de suppléments d'antioxydants, principalement les vitamines C et E, a récemment été évaluée avec plusieurs essais randomisés. Les résultats ont été décevants puisque non seulement les suppléments d'antioxydants n'ont pas réduit le risque de pré-éclampsie ou de la naissance de bébés petits pour l'âge gestationnel, mais dans certains cas, ils étaient associés à des effets indésirables. Cependant, ces essais randomisés ont évalué peu de régimes de supplémentation, principalement dans des populations à haut risque. Étant donné que les niveaux de micronutriments antioxydants sont potentiellement modifiables lors de la grossesse, l'objectif général de cette thèse était d'améliorer notre compréhension de leur association avec la pré-éclampsie ou la naissance de bébés petits pour l'âge gestationnel afin d'informer, s'il y a lieu, les prochains essais cliniques. Nous avons cherché à combler le déficit de connaissances quant à savoir si les niveaux de micronutriments antioxydants sont systématiquement modifiés dans les grossesses affectées par la pré-éclampsie et la naissance de bébés petits pour l'âge gestationnel.

Le premier manuscrit est une revue systématique des études observationnelles mesurant les niveaux maternels de micronutriments antioxydants en association avec la pré-éclampsie ou la naissance de bébés petits pour l'âge gestationnel. Nous avons trouvé que la majorité des études ont mesuré les niveaux d'antioxydants chez des femmes qui avaient déjà été diagnostiquées avec la pré-éclampsie; il était donc impossible de déterminer si les associations observées étaient une cause ou un effet de la pré-éclampsie. Bien que les résultats combinés indiquaient que les niveaux sanguins de vitamines A, C et E étaient plus bas chez les femmes avec pré-éclampsie, la présence d'un biais de publication a été suggérée et le bais de confusion était inadéquatement adressé dans la plupart des études. Un moins grand nombre d'études ont adressé l'association avec la naissance de bébés petits pour l'âge gestationnel, mais le risque de biais était moins élevé chez celles-ci et elles portaient une plus grande attention à la mesure de biomarqueurs lors du second trimestre de la grossesse. Ces études ont trouvé que le niveau de vitamine A était similaire alors que le niveau de vitamine C était uniformément plus bas chez les mères ayant donné naissance à des bébés petits pour l'âge gestationnel comparé aux contrôles. Les résultats étaient partagés pour la vitamine E, dépendant particulièrement de l'ajustement des analyses pour les taux de lipide. Les deuxième et troisième manuscrits sont des études cas-témoin emboîtés dans une large cohorte représentative de la population des femmes enceintes, dans laquelle les niveaux d'antioxydants étaient mesurés avec des échantillons sanguins récoltés lors du second trimestre. Par conséquent, les mesures de sang étaient moins susceptibles d'être affectées par les résultats qui n'avaient pas encore eu lieu au moment de l'échantillonnage. Dans les deux études, nous avons abordé la nature corrélée de ces nutriments, dérivés de sources alimentaires similaires, ainsi que la possibilité d'une association non-linéaire ou d'un effet de seuil. En examinant la naissance de bébés petits pour l'âge gestationnel, nous avons trouvé que des niveaux élevés d'antioxydants caroténoïdes lors du second trimestre étaient associés à une réduction du risque de naissance de bébés petits pour l'âge gestationnel. Inopinément, nous avons aussi trouvé que des niveaux de rétinol élevés étaient associés avec un risque accru; nous spéculons que cette observation pourrait être due à une dysfonction placentaire sous-jacente. En examinant la pré-éclampsie, nous avons trouvé que des niveaux élevés de lutéine étaient associés à un risque significativement réduit de pré-éclampsie. Peu d'études ont mesuré la lutéine en association avec la pré-éclampsie et aucun essai clinique n'a évalué la supplémentation de lutéine pour prévenir la pré-éclampsie et la naissance de bébés petits pour l'âge gestationnel. Si

cette association est confirmée par de futures études prospectives, la supplémentation de lutéine pourrait être une cible pour les essais clinique de prévention de pré-éclampsie. Le travail dans cette thèse soulève les bénéfices potentiels des vitamines C et des caroténoïdes pour la prévention de la pré éclampsie et la naissance de bébés petits pour l'âge gestationnel.

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Statement of Originality

The work contained in this thesis makes an original contribution to the study of the association between antioxidant levels in pregnancy and preeclampsia and SGA birth and, indirectly, to evaluating the potential role of antioxidant supplementation to prevent these conditions.

Our systematic review (Manuscript 1) is the first, to our knowledge, that has sought out all observational epidemiologic studies that form the evidence base that would justify clinical trials of antioxidant supplementation interventions. Our review captured studies of many micronutrients that have been considered as possible targets for intervention during pregnancy to prevent preeclampsia and SGA. One published systematic review and meta-analysis of the observational literature focused more narrowly on vitamins C and E and did not investigate sources of heterogeneity (Gupta *et al.* 2009). Our review focuses on vitamins A, C, E and carotenoids with the goal to determine if systematic differences between cases of preeclampsia or SGA and controls are consistently reported in the literature. We sought to understand sources of potential bias and heterogeneity in the reviewed literature through critical appraisal and using meta-analysis and meta-regression techniques. We also assessed whether reverse causation may have been responsible for any observed differences reported by comparing the literature across pregnancy trimesters. Therefore, this work makes an original contribution in that it is comprehensive in scope in terms of exposures, outcomes, and study designs reviewed, as well as analysis methods that explore sources of heterogeneity and bias. Our review is the first to identify that publication bias, as well as individual study biases (e.g. differences in the mean gestational age at blood sampling between cases and controls), are likely to have overestimated the link between antioxidant levels and preeclampsia. Furthermore, the review suggests that reverse causation could be responsible for the associations observed.

In Manuscript 2, we report a nested case-control study of antioxidant levels in mid-pregnancy and SGA birth. It is the largest study of the topic to date and assesses a broad panel of antioxidant biomarkers. We focus on non-preeclamptic, term SGA to avoid confounding by preeclampsia or preterm birth. We use rigorous methods to analyze biomarker data and account

for the correlated nature of these biomarkers. This study also makes an original contribution to the perinatal literature.

In Manuscript 3, we report a nested case-control study of antioxidants and preeclampsia. Based on our systematic review, very few studies measured antioxidant levels before clinical diagnosis of preeclampsia, and even fewer took steps to address confounding. Further, no studies considered whether antioxidant levels were related to the timing of onset of preeclampsia. We were able to show through two separate analyses that antioxidant levels may be more strongly associated with early-onset preeclampsia. However, we could not rule out reverse causation as responsible for the pattern of findings we observed. This was an original application of survival analysis to case-control data, as was our approach of weighting the subjects to reconstruct the cohort from which they were sampled. This study makes an original contribution to knowledge about the role of antioxidants in preeclampsia. Although we cannot determine whether the association is due to a true (protective/harmful) effect of antioxidants or reverse causation, we provide suggestive evidence of reverse causation, whereas other studies have only speculated that it could be at play.

While I have received guidance and feedback from my committee members and co-authors on substantive, methodological, and statistical aspects of this thesis, the conception, execution, and drafting of the work in this thesis were my own.

Contribution of Authors

Manuscript 1

Cohen JM, Beddaoui M, Kramer MS, Platt RW, Basso O, Kahn SR. Maternal antioxidant levels in pregnancy and risk of preeclampsia and small-for-gestational age birth: a systematic review and meta-analysis. Prepared for *Obstetrics & Gynecology*

I conceived and designed the review, developed the methods, conducted the database searches, carried out all statistical analyses, prepared tables and figures, interpreted the results, and drafted the manuscript. Margaret Beddaoui, a research coordinator for Susan Kahn, was the second reviewer. We both reviewed all titles and abstracts of records retrieved in database searches and assessed the selected full-text articles for inclusion. We both performed data extraction, article appraisal, and data entry. Dr. Kahn was involved throughout the review process and was routinely consulted on the design, methods, and interpretation of the results. Drs. Kramer, Platt, and Basso provided feedback on protocol drafts and assisted in interpretation of the results. All co-authors critically revised the manuscript.

Manuscript 2

Cohen JM, Kahn SR, Platt RW, Basso O, Evans RW, Kramer MS. Small-for-gestational-age birth and maternal plasma antioxidant levels in mid-gestation: a nested case–control study. *BJOG* 2015; DOI: 10.1111/1471-0528.13303. [Epub ahead of print]

I conceived the design of the present analyses, developed the methods, undertook statistical analysis, prepared tables and figures, interpreted the results and drafted the manuscript. Drs. Kramer and Kahn designed the original cohort study and its nested case-control components, oversaw data collection, and decided on the biomarkers to measure in consultation with Dr. Evans, who oversaw the antioxidant biomarker analyses. Dr. Platt provided statistical guidance and substantive expertise. Dr. Basso provided input on the design, including sensitivity analyses. All authors critically reviewed drafts of the manuscript and approved the final version.

Manuscript 3

Cohen JM, Kramer MS, Platt RP, Basso O, Evans RW, Kahn SR. The association between maternal antioxidant levels in mid-pregnancy and preeclampsia. Prepared for submission to *Epidemiology*.

I conceived the design of the present study, developed the methods, undertook statistical analysis, prepared tables and figures, interpreted the results, and drafted the manuscript. Drs. Kahn and Kramer were involved in conception and design of the original cohort study, as well as selection of cases and controls, and oversaw data collection. Dr. Evans oversaw the antioxidant biomarker analyses. Dr. Platt provided statistical guidance. Dr. Basso provided input on the design and interpretation of the results. All authors critically reviewed drafts of the manuscript and approved the final version.

List of Abbreviations

AGA: appropriate for gestational age

ACOG: American College of Obstetricians and Gynecologists

BMI: body mass index

BP: blood pressure

CHS: Canadian Hypertension Society

CI: confidence interval

FFQ: food frequency questionnaire

GA: gestational age

HELLP (syndrome): hemolysis, elevated liver enzymes, low platelet count

HDL: high-density lipoprotein

HIC: high-income country

HPLC: high-performance liquid chromatography

ICC: intraclass correlation coefficient

IUGR: intrauterine growth restriction

LDL: low-density lipoprotein

LMIC: low- or middle-income country

MD: mean difference

NOS: Newcastle-Ottawa Scale

OR: odds ratio

PE: preeclampsia

RCT: randomized controlled trial

RRR: relative risk ratio

SD: standard deviation

SES: socioeconomic status

SGA: small for gestational age

SMD: standardized mean difference

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Chapter 1. Introduction

Preeclampsia is a poorly understood and heterogeneous multisystem disorder that affects 2-8% of pregnancies worldwide.[1] Although it can be diagnosed from the 20th week of pregnancy, most cases occur near term. Preeclampsia is characterized by the presence of high blood pressure and protein in the urine or other adverse conditions.[2-4] It may also affect the brain, lungs, kidney, and liver. Pregnant women with preeclampsia are at risk for severe complications, including seizures (eclampsia), multi-organ failure, stroke, and death.[5] Risks to the fetus include stillbirth, preterm birth, and suboptimal growth resulting in small-for-gestational-age (SGA) birth.[6] No single proposed disease model has been able to account for the heterogeneity in preeclampsia presentation and outcomes. It is thought that different presentations may suggest different underlying causes.[7,8]

Compromised fetal growth, even in the absence of preeclampsia, is another common and related complication of pregnancy. Small-for-gestational-age (SGA) birth is defined as the birth of an infant at less than the 10th percentile of gestational-age- and sex-specific-birth weight, according to an appropriate population reference. SGA birth is often the result of intrauterine growth restriction (IUGR), also known as fetal growth restriction (FGR), but also includes some constitutionally small infants. As indicated above, preeclampsia is associated with reduced birth weight and increased risk of SGA birth, particularly at preterm ages.[9,10] Infants born SGA are at increased risk of neonatal morbidity and mortality.[11,12] SGA birth is associated with impaired neurodevelopment,[13-15] more rapid postnatal growth,[16] and adult chronic disease.[17]

Both preeclampsia and SGA birth are well documented to involve a heightened degree of oxidative stress, which is characterized by an imbalance between high exposure to internal or external (environmental) oxidants and available antioxidant defenses.[18] Markers of oxidative stress such as lipid hydroperoxides, malondialdehyde, and total oxidant status are systematically elevated in both conditions.[19-23] Levels of antioxidant vitamins may be associated with oxidative stress, either because there is insufficient supply, or because depletion of reserves may arise as a consequence of heavy exposure to oxidants. Many previous studies have found that levels of antioxidant vitamins are lower in women with preeclampsia and SGA birth. Case-control studies have typically recruited women at the time of delivery, and many of these studies did not include

controls of comparable gestational ages or make adjustment for differences in gestational age at the time of biomarker measurement. Furthermore, in studies using a traditional case-control design, the outcome has already manifested, so it is unclear if the associations with levels obtained after diagnosis reflect a causal role for antioxidant levels in the conditions, or effects of the outcome itself.

Based on in vitro studies, animal experiments, and on observational studies in humans suggesting that women with preeclampsia have reduced antioxidants levels, researchers initiated several randomized controlled trials (RCTs) of antioxidant supplementation to prevent preeclampsia, mostly in women at high risk for the disorder. Unfortunately, despite several large efforts, including a multi-country World Health Organization RCT,[24] meta-analyses of those trials have concluded that antioxidant supplement interventions studied to date (mainly of vitamins C and E) do not reduce the risk of preeclampsia.[25-27] However, limited interventions and populations have been studied in RCTs to date, and it is unclear if observational studies consistently support the premise that antioxidant levels are lower in women with preeclampsia and/or SGA birth.

The main objective of my thesis is to provide rigorous new evidence bearing on the potential protective roles of antioxidants against preeclampsia and SGA birth. I aimed to overcome some of the methodologic limitations of previous observational studies in this field, particularly the potentially confounding differences in gestational age at sampling between cases and controls. I also sought to explore the potential role of other antioxidants in the etiology of preeclampsia and SGA that have not yet been assessed in RCTs. My thesis comprises three manuscripts.

An important goal of the thesis is to synthesize the published literature that provides the basis for clinical trials of antioxidant supplementation for prevention of preeclampsia. The first manuscript, "Maternal antioxidant levels in pregnancy and risk of preeclampsia and SGA birth: a systematic review and meta-analysis" (*in preparation)*, provides a systematic review of the published evidence bearing on the association between various antioxidants at different times in pregnancy with the risk of preeclampsia and SGA birth. By reviewing reported associations of various antioxidant biomarkers across pregnancy and trying to explain heterogeneity of results, we aimed to identify knowledge gaps and provide more definitive evidence as to whether or not

antioxidant supplementation may still be a useful prevention strategy. We hope to achieve a better understanding of how supplements should be used: identify the relevant subpopulation, choose the correct antioxidant, and initiate supplementation at a time in pregnancy when it could change the course of the disorder.

The second manuscript, "Small-for-gestational-age birth and maternal plasma antioxidant levels in midgestation: a nested case-control study" (*BJOG: An International Journal of Obstetrics and Gynaecology, in press)*, examines the association between levels of several antioxidant biomarkers at 24-26 weeks of gestation and the risk of SGA birth at term.

The third manuscript, "Maternal plasma antioxidant levels and their association with the risk and timing of onset of preeclampsia" (*in preparation)* assesses the relationship between individual antioxidants and the risk of preeclampsia. We also hypothesized that antioxidant levels in the second trimester may be associated with timing of onset of preeclampsia, with the idea that higher antioxidant levels could prevent oxidative stress and hence delay onset or prevent preeclampsia entirely. In exploratory analyses, we stratified early- and late-onset cases and also used a methodologically innovative approach to apply survival analysis to a case-control study setting. We used inverse-probability of sampling weights to reconstruct a representation of the cohort from which the cases and controls were drawn.

My thesis makes a contribution to the perinatal literature by organizing and contributing new findings to the available literature on the association between antioxidant levels in pregnancy and preeclampsia and SGA birth. It also addresses some antioxidants that have not previously been evaluated in trials. In the next chapter, I present the background material that relates to the three manuscripts.

Chapter 2. Background

This thesis addresses potential shared etiologic contributors to two important, interrelated but enigmatic complications of pregnancy: preeclampsia and small-for-gestational-age (SGA) birth. As discussed below, preeclampsia and SGA birth share a number of risk factors and may share some aspects of underlying pathophysiology. Both conditions and SGA in particular, have heterogeneous etiology. In subsequent manuscripts, we will investigate specific biomarkers to help understand shared and disparate components of these two conditions.

2.1 Preeclampsia

Preeclampsia has been variously defined according to international guidelines. It is usually characterized by new onset of hypertension or worsening of chronic hypertension after 20 weeks of gestation. Until recently, most guidelines required proteinuria to be present, but gradually this is changing.[4,28] Preeclampsia is a heterogeneous condition involving many organ systems, and the most recent guidelines recognize that many cases of preeclampsia may present without proteinuria.[28-30] The most recent definition of preeclampsia, according to the Canadian Hypertension Society guidelines requires hypertension (sBP ≥140 mmHg and/or dBP ≥ 90 mmHg) with proteinuria (0.3 g/day or 1+ dipstick), adverse conditions (e.g. headache/visual symptoms, epigastric pain), or severe complications (e.g. seizures [eclampsia], platelet count < $50x10^9$/L).[29]

Hypertensive disorders, which include preeclampsia, are the number one cause of maternal death in Latin America and the Caribbean (26%), and a leading cause of maternal death in Africa and Asia (9%) and developed countries (16%).[31] The Preeclampsia Foundation estimates the costs attributed to hypertensive disorders of pregnancy are $7 billion per year in the USA alone.[32] These costs are due to longer hospitalization, increased use of labor induction, more caesarean births, and an increased need for intensive care in newborns of affected mothers.[33] In the Montreal Prematurity Study, the study on which the thesis projects are based, 40% of newborns born to mothers who developed preeclampsia in pregnancy received intensive care after birth versus 19% among babies born to control mothers.[34]

Although substantial progress has been made in understanding the pathogenesis of preeclampsia in recent decades, the cause or causes of preeclampsia remain unknown.[35-37] Because of this limited knowledge, effective strategies for prevention of preeclampsia are lacking. While some studies have reported small to moderate protective effects of some interventions, such as administration of aspirin,[38] calcium,[39] or nitric oxide donors or precursors (e.g. L-arginine),[40] results have been inconsistent. As a consequence, these interventions are variably endorsed in guidelines for preeclampsia prevention.[3,41,42] Further, the only definitive cure for preeclampsia is delivery, as the syndrome usually resolves promptly after delivery of the placenta,[43] though in rare instances it can occur after delivery.[44] As a consequence of early interventions aimed at minimizing harms to the mother and fetus, preeclampsia is responsible for the largest proportion of medically-indicated preterm births.[45,46]

The most well established risk factors for preeclampsia are nulliparity (first pregnancy), family history of preeclampsia, multifetal gestations, and obesity.[47] Furthermore, some preexisting maternal conditions such as chronic hypertension, diabetes, kidney disease, antiphospholipid syndrome, and autoimmune disease confer higher risk.[48] Extremes of maternal age[47] and low socioeconomic status are also risk factors for preeclampsia.[49] Ethnic groups of African origin have consistently been found to be at increased risk of preeclampsia.[50-52] One of the strongest predictors is preeclampsia in a previous pregnancy, which is consistently found to be associated with an approximately 7-fold increased risk of preeclampsia in a subsequent pregnancy.[47] Smoking, on the other hand, has been consistently shown to be associated with a reduced risk.[53,54] As risk factors for preeclampsia, including obesity and diabetes, are increasingly prevalent in the population, and the incidence of preeclampsia has risen in the USA in recent years,[55,56] understanding what causes preeclampsia and how to prevent it is even more pressing.

Preeclampsia is often conceptualized as a two-stage disorder. The first stage is characterized by inadequate placental perfusion; the second stage is the maternal syndrome.[36] The link between these stages is still unresolved. During normal placentation, the spiral arteries of the uteroplacental interface undergo significant changes that result in wide, flaccid vessels lined by trophoblast cells. In preeclampsia, this process often fails, resulting in incompletely transformed spiral arteries with increased resistance to flow, as evidenced by Doppler

velocimetry[57] and documented by placental bed biopsies collected after caesarean delivery.[58] On the other hand, abnormal placentation may not be a prerequisite for the condition, in the presence of strong predisposing maternal constitutional factors, or increased fetal demand in the case of multiple pregnancy.[36]

No single disease model proposed can account for the heterogeneity in preeclampsia presentation and outcomes. This heterogeneity has led some to suggest that the syndrome should be stratified into distinct subgroups.[7] For example, preeclampsia cases with early versus later onset and associated with reduced fetal growth (generally measured with SGA) versus those without may not arise from the same underlying processes as term preeclampsia without SGA.[8] The link between the placental and maternal stages of the disorder may vary, as may the contribution of placental and maternal factors to the pathophysiology.

Key features of the maternal syndrome, hypertension and proteinuria, are generally believed to result from endothelial dysfunction.[59] Indeed, one author has suggested that the heterogeneous causes of preeclampsia resemble a flight map with flights converging at a single airport because, despite the various etiologic pathways to preeclampsia, endothelial cell dysfunction is a consistent feature of the disorder.[60] Endothelial cell activation involves a loss of vascular integrity, increased expression of immune cell adhesion molecules, a shift towards a prothrombotic environment, increased production of inflammatory signaling molecules (cytokines), and increased expression of HLA molecules used by immune system to distinguish self from foreign cells.[61] Of interest, plasma removed from women at high risk of preeclampsia at 22 and 26 weeks gestation (before clinical manifestation of preeclampsia) alters endothelial cell function in vitro.[62]

2.2 Small for Gestational Age

Compromised fetal growth is a related complication of pregnancy. An infant is defined as being SGA if his/her birth weight corresponds to less than the 10th percentile of gestational-age- and sex-specific-birth weight, according to an appropriate population reference. SGA is often the result of intrauterine growth restriction (IUGR), also known as fetal growth restriction (FGR), but may also include some constitutionally small babies. IUGR is defined as the failure to reach one's

growth potential, which is difficult to predict.[63] The study of IUGR is hence often operationalized by studying SGA in epidemiologic research. The prevalence of SGA in a population should be, by definition, approximately 10% among live births, provided that the reference used to classify birth weight for gestational age is based on the same population, or at least one very similar to it. However, because of the recent trend toward increasing size at birth and the absence of regular updating of the references, the observed prevalence of SGA birth is often lower.

Nulliparous women are at increased risk of giving birth to SGA babies, with odds ratios ranging between 1.3 and 2.1, according to the literature.[64] Some ethnic groups are reported to be at increased risk for SGA, including African-American, Indian, and Asian (vs. Caucasian) women in developed countries, but some of this may be due to the use of an inappropriate standard. Maternal low birth weight, low pre-pregnancy BMI, and short stature are also risk factors for SGA birth. Low socioeconomic status (SES) has been linked to SGA, but the association is likely mediated by factors such as poor diet[65] and tobacco use.[64] Cigarette smoking is the single most important risk factor for SGA birth in developed countries; however, smoking cessation in early pregnancy can reduce the risk to that of non-smokers.[66] Cocaine use in pregnancy is also associated with birth weight reduction and increased risk of SGA birth.[67]

Chronic hypertension is associated with an increased risk of SGA birth, which is even greater among women who develop superimposed preeclampsia.[68] Diabetes is typically associated with large babies; however, vascular complications of severe, longstanding diabetes have been associated with increased SGA birth. Renal disease, auto-immune conditions including antiphospholipid syndrome (APS), systemic lupus erythematosus (SLE), and asthma (small effect) are also risk factors for SGA birth. Malaria is also an important risk factor for SGA birth in endemic areas,[64] although maternal malnutrition is probably a more important risk factor in these areas.

Short interpregnancy interval (<18 months) and long interpregnancy interval (>59 months) are associated with an increased risk of SGA birth, compared to an optimum interval of 18-23 months, according to a meta-analysis of 67 studies.[69] Previous pregnancy complicated by SGA birth or stillbirth, and abnormal uterine artery Doppler wave forms are a predictors of SGA birth in a current pregnancy.[70,71]

2.3 Links Between Preeclampsia and SGA

The underlying pathophysiology of preeclampsia and intrauterine growth restriction may be intimately related. Ananth *et al* coined the term "ischemic placental disease," asserting that preeclampsia, SGA birth (a proxy for IUGR), and placental abruption all result from a failure to achieve normal placentation. One study found that, among women who delivered at or before 37 completed weeks of gestation, birth weights were significantly lower among women with preeclampsia vs. normotensive controls; this trend was similar for nulliparous and multiparous women.[10] Another study noted a strongly significant ($p<0.0001$) inverse relationship between gestational age at delivery and prevalence of SGA birth among women with preeclampsia. In that study, the median birth weight centile for pregnancies unaffected by preeclampsia was the expected 50, whereas among women with preeclampsia, the median birth weight centile was 13.[71] Further, in a Chinese cohort of singleton deliveries between 37 and 42 completed weeks of gestation, the odds ratio for SGA birth was 2.55 (95% CI 1.84-3.55) for pregnancies complicated by preeclampsia/eclampsia compared to normotensive controls,[72] In the Montreal Preeclampsia Study population, approximately 25% of pregnancies complicated by preeclampsia resulted in an SGA birth.[73]

A number of risk factors and biomarkers are shared between preeclampsia and SGA. Low SES,[74] maternal age ≥35, infertility, chronic hypertension, and black race have been associated with SGA birth as well as preeclampsia.[75] Biomarkers found to be predictive of both preeclampsia and SGA birth include decreased placental growth factor (PlGF) and elevated soluble endoglin (s-Eng), two proteins that are important for blood vessel formation.[75,76]

Preeclampsia and SGA birth are both associated with an increased risk of later cardiovascular disease in the affected women themselves. Women who have had an SGA infant or a pregnancy complicated by preeclampsia are at increased risk for cardiovascular disease later in life.[48,77,78] Women who have had preeclampsia experience an even greater risk of cardiovascular disease if the pregnancy was further complicated by poor fetal growth.[79]

However, preeclampsia and SGA diverge with respect to other important risk factors and biomarkers. In particular, smoking is a risk factor for SGA birth but is associated with a lower risk of preeclampsia, and low BMI is associated with SGA birth, whereas obesity or high BMI is

associated with an increased risk of preeclampsia.[75] Elevated sFlt-1 (or sVEGFR-1), which has been consistently found to be predictive of preeclampsia and is elevated in women with preeclampsia, is not elevated in pregnancies complicated by SGA birth alone.[76]

Endothelial dysfunction may be common to both preeclampsia and SGA birth. Hypertension, renal disease, SLE, and advanced age are associated with endothelial dysfunction and are risk factors for preeclampsia and SGA.[64,77] Ness and Sibai, leading experts in this field, have hypothesized in a highly-cited narrative review that both SGA birth and preeclampsia arise due to a predisposition to endothelial dysfunction, which results in abnormal placentation.[77] Low levels of the angiogenic placenta derived growth factor (PlGF) has been described in both of these conditions at the end of the first trimester, which corresponds to the timing of the second phase of placentation.[77]

2.3.1 Oxidative Stress

Inadequate placental perfusion is thought to cause intermittent hypoxia leading to oxidative stress. Hypoxia and reperfusion injury may cause increased generation of oxygen-free radicals in the placenta.[80] Insufficient antioxidant defenses can be deleterious to the placenta and the maternal vascular system.[81] The presence of increased levels of oxygen free radicals initiates oxidative damage to lipids, nucleic acids, and proteins, causing cellular dysfunction and resulting in vascular endothelial dysfunction and leukocyte activation, recognized features of preeclampsia.[82] Oxidative stress may also contribute to the risk of SGA birth by provoking endothelial dysfunction. Endothelial dysfunction has also been documented in SGA birth, although to a lesser extent than in preeclampsia.[77] Bretelle et al compared levels of soluble E-selectin, intracellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM1) from blood samples taken from all groups at a mean gestational age of 31 weeks between women with SGA birth, preeclampsia, and healthy controls. They reported that soluble E-selectin was significantly increased in both SGA birth and preeclampsia compared to controls, whereas ICAM-1 and VCAM1 were significantly increased only in women with preeclampsia.[83] The mechanism whereby oxidative stress is involved in the pathophysiology of IUGR/SGA birth may therefore be shared in preeclampsia and SGA.

Animal studies have provided additional evidence for a role of oxidative stress in preeclampsia. While there is no ideal animal model of preeclampsia that is able to replicate all features of the human syndrome, one of the best models is induced by clamping the abdominal aorta and ovarian arteries in the rat.[84] This reduced utero-placental perfusion pressure (RUPP) model is characterized by hypertension, proteinuria, and growth restriction,[85] and elevated markers of oxidative stress.[86] Animal studies have also suggested potential benefits of antioxidant-rich diet and supplementation in reducing blood pressure and proteinuria as well as improving fetal growth.[87,88]

A number of case-control studies have reported elevated levels of biomarkers of oxidative stress in women with preeclampsia and SGA birth. A systematic review of studies assessing lipid peroxidation and/or antioxidant status in pregnancy found that markers of lipid peroxidation, including malondialdehyde and thiobarbituric acid reactive substances (TBARS), were, on average, higher around the time of delivery among women with preeclampsia.[21] Antioxidants provide defenses against oxidative stress, and their levels are thought to reflect the extent of oxidative stress in the body.[89] The same systematic review observed significantly lower mean concentrations of vitamins C and E in plasma taken from preeclampsia cases as compared to normotensive pregnant controls. However, it is unclear if those lower levels contribute to the development of preeclampsia or instead reflect consequences of the disorder, since blood samples among cases were taken from women whose preeclampsia had already been clinically manifested.

Data are far more limited regarding the status of antioxidant levels in early- and mid-pregnancy among women eventually diagnosed with the disorder in comparison to those whose pregnancies progress without developing preeclampsia. At least two randomized controlled trials (RCTs) have taken longitudinal measurements of levels of antioxidant vitamins C and E (the vitamins given to women in the supplementation group) in the placebo group and noted lower vitamin C levels from mid-pregnancy in women who eventually developed preeclampsia compared with women who did not develop preeclampsia.[90,91] Although both of these trials had limited statistical power due to the small number of subjects in whom serial measurements were taken, they suggest that differences in antioxidant levels may exist before preeclampsia is diagnosed, though the process leading to preeclampsia may have already been underway.

Another study assessed total antioxidant power (which provides an overall measure of the antioxidant potential from a variety of antioxidant defenses) in 307 young pregnant women at 20-28 weeks gestation. The authors found that lower total antioxidant power at cohort entry was associated with later preeclampsia. Being in the highest tertile of total antioxidant power was associated with a 3-fold reduced risk of subsequent preeclampsia compared with the lowest tertile.[92]

While the above observational studies provide some evidence that antioxidant levels in pregnancy may be important for the development of preeclampsia, the associations may have been confounded by diabetes, hypertension, obesity, and smoking status, since those factors, which are risk factors for preeclampsia and may be related to antioxidant levels, were not adjusted for. Despite the limitations of previous work, the link between antioxidants and preeclampsia is mechanistically plausible and worth pursuing further, as oxidative stress is a known cause of endothelial dysfunction and leukocyte activation.[93,94]

A number of studies have investigated markers of oxidative stress and antioxidant levels in women who have recently delivered an SGA infant or in women whose fetus is judged to be growth-restricted by ultrasound. Gupta *et al.* reported higher levels of malondialdehyde in cord blood of SGA infants born to undernourished mothers, compared with appropriate-for-gestational age (AGA) infants born to healthy mothers.[22] Saker *et al.* noted increased markers of oxidative stress, hydroperoxides and carbonyl proteins in both newborns and mothers, although more pronounced in maternal blood.[23] Gveric-Ahmetasevic *et al.* observed increased markers of lipid peroxidation in SGA newborns and their mothers immediately after delivery.[19] Gupta *et al.* and Saker *et al.* found that, in a cohort of recent deliveries, SGA newborns and their mothers had lower levels of total antioxidant activity and lower levels of vitamins C and E within 48 hours of birth among a cohort of recent deliveries, compared with AGA newborns and mothers.

As is the case with preeclampsia, fewer studies have analyzed blood samples taken before labor and delivery. In one study by Mert *et al.*, women diagnosed by ultrasound with a growth-restricted fetus between 28 and 41 weeks gestation were included. Total oxidant status and total antioxidant status were both increased.[20] However, most assays of total antioxidant status are strongly influenced by uric acid levels, which are significantly higher among cases as compared to controls.[95] Stephan *et al.* recruited women with Doppler evidence of poor uterine perfusion at

18-23 weeks gestation and found lower total antioxidant capacity among women with poor uterine profusion in the second trimester,[96] which is a predictor of both IUGR and preeclampsia.[70,97] Each of the above-mentioned studies excluded smokers, since smoking is potentially an important confounder of the relation between oxidative stress and SGA.

There is thus some evidence to suggest that oxidative stress may link preeclampsia and SGA. A more complete consideration of the antioxidant literature is described in the next chapter. It is possible that oxidative stress contributes to both of these via different mechanisms, resulting in the co-occurrence of preeclampsia and SGA. Alternatively, oxidative stress may contribute to both of these conditions via similar mechanisms involving endothelial dysfunction.

2.4 Dietary Antioxidants for Prevention

The Institute of Medicine has defined a dietary antioxidant as "...a substance in food that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans."[98] The body contains both enzymatic and non-enzymatic antioxidants. Synthesis of antioxidant enzymes protects cells from oxidative damage. These include superoxide dismutase, catalase, and glutathione. Antioxidant enzymes require small quantities of minerals derived from the diet, including selenium, zinc, copper, and manganese, which act as cofactors for their reactions with oxidants. Dietary antioxidant micronutrients that are not synthesized by the body must be obtained through diet or dietary supplements. Vitamins C and E are major antioxidants in the body; vitamin A and various carotenoids are also believed to serve as antioxidants in the body. Prenatal supplements typically contain vitamins A, C, E and some carotenoids but not others. For example, lutein is not commonly included in prenatal supplement formulations.

Vitamin C (ascorbic acid, ascorbate) is a water-soluble antioxidant which is also required for collagen synthesis.[99] Vitamin C is derived from fruits and vegetables--in particular, citrus fruits, tomato, broccoli, peppers, and potatoes. It acts as a reversible reducing agent by donating an electron to an unstable reactive oxygen species. It is also believed to be important for recycling vitamin E to its protective form. It may also have a beneficial effect on vasodilatation by preventing inactivation of nitric oxide (a potent vasodilator) by superoxide radicals.[98]

Vitamin E (tocopherol) is a fat-soluble vitamin that exists in eight different forms; the most common dietary forms are α- and γ-tocopherol. In European diets, α-tocopherol is the most common form; it is highly concentrated in sunflower and olive oils. In North America, γ-tocopherol is more common in the diet, highly concentrated in corn and soybean oils.[100] In the body, however, α-tocopherol is preferentially secreted by the liver and circulated,[98] so that even in North America, blood concentration of α-tocopherol is higher than γ-tocopherol. Vitamin E protects cell membranes from oxidative damage by preventing propagation of lipid peroxidation. Frank vitamin E deficiency is uncommon.

Vitamin A is vital for reproduction, vision, and a host of other biological processes. Preformed vitamin A (retinols) are derived only from animal sources.[101] High intake of retinol in pregnancy is associated with increased risk of birth defects; therefore, supplementation with pre-formed vitamin A in pregnancy may be risky, depending on the timing of supplementation. Supplementation of vitamin A is not recommended in countries where intake is generally adequate.[101]

A large number of carotenoids are found in nature, but only some are present in measureable concentrations in human plasma. Carotenoids include carotenes and oxy-carotenoids. Carotenes include α-carotene, β-carotene, and lycopene. Oxy-carotenoids include lutein, α-cryptoxanthin, and β-cryptoxanthin. Some carotenoids (including α-carotene, β-carotene and β-cryptoxanthin) are forms of pro-vitamin A that are processed in the body to form vitamin A. β-carotene is the most well-studied carotenoid. Carotenoids are ingested in fruits and vegetables, eggs, as well as derived from dietary supplements.

Observational studies reporting differences in antioxidant levels between cases and controls have provided the rationale for randomized controlled trials (RCTs) to assess the effect of antioxidant supplements to prevent preeclampsia and associated sequelae, and most included SGA birth as a secondary outcome.[24,91,102-105] Disappointingly, a 2008 Cochrane systematic review of 10 RCTs involving 6533 women concluded that antioxidant interventions (mostly vitamins C and E) were not significantly associated with a reduction in the risk of preeclampsia (relative risk (RR)=0.73 [95% confidence interval (CI): 0.51, 1.06]) or SGA (RR=0.83 [95% CI: 0.62, 1.11]) and may be associated with increased risks of preterm birth (RR=1.10 [95% CI:

0.99, 1.22]) and miscarriage (RR=1.32 [95% CI: 0.92, 1.90]).[25] Two other published systematic reviews (2007, 2012) reached similar conclusions (see details and comparison of the 3 reviews, Appendix Table 1).[26,27] However, the RCTs conducted to date studied a small number of antioxidant interventions, and have mainly targeted women at high risk for preeclampsia (see details of individual trials, Appendix Tables 2, 3, 4). The largest trials uniformly studied an intervention of 1000 mg vitamin C and 400 international units (IU) vitamin E daily. Some small trials with multifaceted interventions had some promising results, but it is impossible to determine which component of the intervention was responsible for a protective effect.[106-108] Examples of multifaceted interventions included providing medical food bars containing L-arginine and antioxidant vitamins,[107] or multivitamins containing antioxidants[106] (also see Appendix Tables 2, 4). Many studies were under-powered to detect a modest, but clinically relevant effect.

In summary, preeclampsia and SGA birth are common and serious pregnancy-related disorders that have shared risk factors. Observational data suggest that oxidative stress may play a role in each but intervention studies of vitamins C and E have so far failed to show benefit. We believe it is important to carefully re-examine the observational and clinical trial data to ascertain if other antioxidant interventions could be beneficial if applied to relevant subpopulations of women at an appropriate time in pregnancy.

Chapter 3. Objectives

The overall objective of my thesis is to evaluate the potential role of antioxidants in reducing the risks of preeclampsia and SGA birth. Specific objectives are:

1. To conduct a systematic literature review to summarize the published evidence on the association between antioxidant levels in pregnancy and risk of preeclampsia and SGA birth

2. Using data from an existing multicentre pregnancy cohort, to assess differences in the risk of SGA birth according to antioxidant levels in mid-pregnancy by comparing term SGA cases to appropriate for gestational age (AGA) controls

3. Using the same data source (as in #2), to assess differences in the risk of preeclampsia according to antioxidant levels in mid-pregnancy by comparing preeclampsia cases to normotensive controls

4. Again using the same data source, to assess the impact of antioxidant levels on the timing of progression to clinically manifest preeclampsia

Chapter 4. [Manuscript 1] Maternal antioxidant levels in pregnancy and risk of preeclampsia and SGA birth: A systematic review and meta-analysis

4.1 Preface to Manuscript 1

Studies of the pathophysiology of preeclampsia and SGA suggest that oxidative stress may be causally related to these conditions and that antioxidant supplementation in pregnancy may (at least theoretically) prevent them. In the Background chapter, we discussed the many clinical trials of antioxidant supplements for preventing preeclampsia and associated sequelae, including SGA birth. We also cited several systematic reviews and meta-analyses, which concluded that antioxidant interventions are not effective for preeclampsia prevention. However, there is inconclusive evidence on antioxidants overall as one review of any antioxidant supplementation reported a pooled RR of 0.73 (95% CI: 0.51, 1.06),[25] which does not rule out a protective effect. We were interested in reviewing the observational literature that forms the empirical basis for RCTs to understand whether these studies suffered from specific shortcomings, and to try to understand the heterogeneity in their findings. We also sought to determine whether certain antioxidants showed a stronger association with these outcomes than others, and may represent good candidates for future interventions.

We felt that prospective studies deserved greater attention in the search for an intervention to prevent preeclampsia. However, we were also interested in reviewing the traditional case-control studies and cross-sectional studies that were likely used in providing justification for the trials and to examine specific subgroups of the population in which the differences in antioxidant levels between preeclampsia cases and controls may have been more pronounced.

We identified a single systematic review on this topic, which also covered markers of oxidative stress.[21] That review had several limitations. Many studies published at the time of the search were not included or described. Some may have been excluded because of concern regarding confounding, however, no explanation of the criteria for assessing potential confounding were provided. No prospective studies were identified, despite that some had been

published. There were inconsistencies between the tables and figures (author names, biomarkers included, patient numbers), which make it difficult to interpret these results adequately. While the review acknowledged that there was heterogeneity between the study findings, it was not meaningfully investigated. Finally, the review did not include SGA as an outcome. We therefore wanted to conduct a thorough systematic review of the topic to better explore additional markers, time periods (first, second trimester studies), and reasons for heterogeneity.

Finally, we also sought to identify knowledge gaps that we could fill in subsequent analyses of two case-control studies nested within a large cohort of pregnant women in Montreal. The manuscript has been prepared according to the specifications of the journal *Obstetrics & Gynecology*.

4.2 Title Page

Maternal antioxidant levels in pregnancy and risk of preeclampsia and small-for-gestational age birth: a systematic review and meta-analysis

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Short Title: Antioxidants in preeclampsia and SGA

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Précis: Maternal antioxidant levels may be lower in preeclampsia or small for gestational age birth but publication bias and confounding may contribute to this association.

4.3 Abstract

OBJECTIVE: To conduct a systematic review of the association between maternal antioxidant levels during pregnancy and preeclampsia and/or SGA.

DATA SOURCES: We searched PubMed, MEDLINE, Embase, CAB Abstracts, BIOSIS Previews, ProQuest Dissertations & Theses, CINAHL, FSTA Direct, and POPLINE databases from 1970-2013.

METHODS OF STUDY SELECTION: We selected observational studies published in English or French that measured maternal blood levels of non-enzymatic antioxidants (vitamins A, C, E, and carotenoids) during pregnancy or within 72 hours of delivery. We reviewed 1,882 unique citations. Title and abstract screen, assessment of eligibility, and data extraction were carried out in duplicate. Study quality was assessed using the Newcastle-Ottawa Scale, supplemented by additional questions. We pooled the standardized mean difference (SMD) across studies, stratified by outcome and pregnancy trimester, and investigated heterogeneity due to study design, quality, and other factors using meta-regression.

TABLULATION, INTEGRATION, AND RESULTS: Sixty-four studies were included. Among 58 that addressed preeclampsia, 9 studies recruited subjects prior to diagnosis. Among 9 that addressed SGA, 6 recruited subjects prior to recognition of SGA. Most studies were small with high risk of bias. Significant heterogeneity was observed in all meta-analyses. The SMD for vitamins A, C, and E in preeclampsia were significantly lower than controls for overall preeclampsia, but not in mild and severe preeclampsia subtypes. Measurement methods and fasting status were identified as sources of heterogeneity for vitamin E. Evidence for carotenoid antioxidants was limited and inconclusive. Publication bias appears likely.

CONCLUSION: Observational studies do not consistently show that vitamins C and E or other antioxidants are lower in women who develop preeclampsia or SGA. Reverse causality remains a possible explanation for associations observed. Additional rigorous studies measuring antioxidant

levels before clinical detection of preeclampsia and SGA, ideally in early pregnancy, will help to better understand whether levels are altered at a causally-relevant time of pregnancy.

4.4 Introduction

Preeclampsia is a hypertensive disorder that affects 2-8% of pregnancies worldwide, and is most common in first pregnancies.[109] Despite remarkable progress in the understanding of the pathophysiology of preeclampsia in the last few decades, the etiology of this disorder remains unclear; a problem compounded by its heterogeneity.[36] Preeclampsia is typically diagnosed by the presence of high blood pressure and protein in the urine or adverse conditions including low platelet count, pulmonary edema, elevated liver enzymes, etc.[2-4] It is a multisystem disorder, which can result in severe complications including seizures (eclampsia), multi-organ failure, stroke, and death.[5] Fetuses are at increased risk of preterm birth, growth restriction, and stillbirth.[6] Effective strategies for preventing preeclampsia are lacking; delivery is the only definitive cure.

Small for gestational age (SGA) is often used as a proxy for fetal growth restriction. It is defined as the birth of an infant below the 10th percentile for birth weight according to a population reference, and is more common in preterm pregnancies complicated by preeclampsia.[9,10] Infants born SGA are at increased risk of neonatal morbidity and mortality.[11,12] SGA is adversely associated with future growth,[16] neurodevelopment, including intelligence and risk of cerebral palsy,[13-15] and adult chronic disease.[17]

The pathophysiology of preeclampsia and SGA are incompletely understood, but endothelial dysfunction may play a role in both conditions. Oxidative stress, caused by increased production of free radicals and insufficient antioxidant defenses, is a known cause of endothelial dysfunction, and may thus be causally related to preeclampsia and SGA.[93] Hypertension, renal disease, lupus, and older age are associated with endothelial dysfunction and are risk factors for both conditions.[64,77] Indeed, several studies to date have shown that markers of oxidative damage are elevated and antioxidant vitamin levels are lower, in women with preeclampsia[110-114] and SGA.[19,22,23] Therefore, it has been hypothesized that low antioxidant levels in pregnancy may increase the risk of preeclampsia and SGA.

One systematic review and meta-analysis of observational studies has assessed the association between lipid peroxidation and/or antioxidant status in pregnancy and preeclampsia.[21] That review showed that markers of lipid peroxidation were, on average, higher in preeclampsia cases, and vitamins C and E were significantly lower. A number of important limitations of this

review justify further evaluation. Substantial heterogeneity was observed but not investigated. Many studies published at the time were not included or described, including at least one prospective study.[90] Further, the review did not include SGA as an outcome which is of interest as SGA and preeclampsia often appear together.[10]

The findings of early observational studies and the success of a small pilot intervention study,[103] inspired a number of large randomized controlled trials (RCTs) to assess the use of antioxidant supplements to prevent preeclampsia and associated sequelae, including SGA.[24,91,115,116] Disappointingly, these trials mostly showed null results. Our goal was to summarize studies that formed the empirical basis for trials, investigate sources of heterogeneity, and investigate whether observational studies suffered from specific shortcomings. We conducted a systematic review of studies of the maternal levels of non-enzymatic antioxidants that could be given as dietary supplements to pregnant women. Ultimately, this review will enable us to understand whether additional observational studies or trials are warranted to comprehensively assess the suitability of antioxidant supplements for prevention of preeclampsia and SGA.

4.5 Methods

We followed MOOSE guidelines in planning and carrying out the systematic review and meta-analysis.[117] Before undertaking the review, we registered our systematic review protocol in the PROSPERO database (www.crd.york.ac.uk/PROSPERO; registration no. CRD42013003519).

We searched for studies published in English or French from 1970-January 2013 that measured maternal blood levels of non-enzymatic antioxidants in any population of pregnant women. Such antioxidants included tocopherols (vitamin E), carotenes (α-carotene, β-carotene, lycopene), oxy-carotenoids (lutein, zeaxanthin, cryptoxanthin), retinol (vitamin A), and ascorbic acid (vitamin C). We included studies in which samples were obtained during pregnancy or within 72 hours of delivery. Pregnancy outcomes of interest included any preeclampsia, mild preeclampsia, severe preeclampsia (including eclampsia), early- and late-onset preeclampsia, and SGA. We included prospective cohort studies, nested case-control studies, RCTs (analyzed as cohorts), case-control studies and cross-sectional studies in our search.

Sources

We searched the following databases without language restrictions: Pubmed; Medline, Embase, CAB Abstracts, and BIOSIS Previews via OvidSP; ProQuest Dissertations & Theses, CINAHL, FSTA Direct, and POPLINE. The search strategy was developed in consultation with an experienced health sciences librarian. We pilot tested the ability of our search strategy to identify records of known studies and further refined it after preliminary searches to include all relevant controlled vocabulary (subject headings that included MeSH terms in Medline and Emtree terms in Embase) and free-text words in the title or abstract.

Search terms for exposures included: antioxidant, tocopherol, vitamin E, beta-carotene, carotene, carotenoid, lycopene, cryptoxanthin, lutein, vitamin A, retinol, vitamin C, ascorbic acid, and ascorbate. Search terms for outcomes were: preeclampsia, pre-eclampsia, toxemia, small for gestational age, SGA, fetal growth retardation, fetal growth restriction, FGR, intrauterine growth retardation, intrauterine growth restriction, and IUGR. The complete Pubmed search is shown in **Appendix 1**, available online.

In addition to the above database searches, we also hand-searched the reference lists of relevant retrieved studies and of narrative and systematic reviews to find additional studies. Unpublished literature was sought by searching the ProQuest Dissertations & Theses database.

Study Selection

Two trained reviewers independently screened all titles and abstracts of records retrieved from database searches. Records considered potentially relevant by one or both reviewers proceeded to the secondary screen of full-text articles. Two reviewers independently assessed each of these studies using a structured study eligibility form. Where there were disagreements, the reviewers discussed and came to a consensus, sought additional information from authors to make a decision, or discussed with a third reviewer.

We excluded studies that did not provide diagnostic criteria for the outcome of interest and those that did not provide gestational age (GA) at time of blood sampling. We were interested in whether differences in diagnostic criteria and timing of biomarker measurement could explain heterogeneity of results; hence we required that these be reported. We excluded studies that obtained samples >72 hours after delivery, as antioxidant levels obtained more than a few days after delivery may not be representative of levels during pregnancy.[118] We excluded studies published in a language other than English or French, owing to resource constraints that limited our ability to obtain translations. Finally, we excluded studies published prior to 1970, when high-performance liquid chromatography technology was developed used for precise measurement of many antioxidant biomarker levels.[119,120] No review articles, letters to the editor, or duplicate publications were included; however, published abstracts were considered eligible. In the case of abstracts, definition of the outcome was not required due to acknowledged space constraints.

Data Extraction

For studies deemed eligible for inclusion based on the secondary full-text screen, data were independently extracted by two reviewers using a data extraction form developed and pilot tested for this literature. The following data were extracted from each study:

- Study characteristics: study design, setting (country), time period
- Population characteristics: inclusion/exclusion criteria, covariates measured, prevalence of risk factors, by study group
- Description of cases and controls: definition of preeclampsia and/or SGA, gestational age distribution at recruitment (first blood draw), characteristics of study controls (and matching criteria, if applicable)
- Exposure characteristics: timing of antioxidant measurement, antioxidants assessed, biomarker assay methods, adjustment for blood lipids (yes/no, method)
- Outcome: mean and SD of each antioxidant measured, by group; measures of association for preeclampsia and SGA, covariates included in multivariable models

Where data for meta-analysis were available only in figures, we extracted the data using WebPlotDigitizer Version 3.4, a free online program (Rohatgi A. WebPlotDigitizer. 3.4 ed. http://arohatgi.info/WebPlotDigitizer; 2014).

Methodological quality and potential biases were also evaluated for each study. Data were entered into two separate Access 2010 databases (Microsoft, Redmond, WA) and compared for agreement. Disagreements were resolved by coming to consensus or discussing with a third reviewer.

Risk of Bias Assessment

Two reviewers independently assessed the quality of each study using the Newcastle-Ottawa Scales (NOS) for cohort and case-control studies (http://www.ohri.ca/programs/clinical_epidemiology/oxford.asp). This scale uses multiple-choice questions to address the areas of selection, comparability, and exposure/outcome assessment. Points (stars) are given for high-quality rating in each of these areas and studies earned a maximum of 9 points. We tailored the NOS to this literature by specifying the key confounders *a priori*: gestational age at blood sampling, smoking, parity, and BMI. We also rated studies on overall quality of reporting and confounding control using "risk of bias" additional questions. We prepared a manual to guide our assessments and make the judgements as consistently as possible (**Appendix 2**, available online).

Statistical Analysis

We conducted meta-analyses for four distinct outcomes: all preeclampsia, mild preeclampsia, severe preeclampsia or eclampsia, and SGA. Since the antioxidants were reported in different units, we pooled the standardized mean difference (SMDs) across three or more studies for each antioxidant, stratified by trimester of measurement. The SMD provides an estimate of the average difference, in standard deviations, between cases and controls in the levels of a continuous exposure, when different units are reported across studies. We converted standard errors to standard deviations; where the median and range of values were reported, and

we estimated the mean and SD using the method of Hozo *et al.*[121] We pooled subgroup means and standard deviations (e.g. combined mild and severe for all preeclampsia analysis) using formulae described in the Cochrane Handbook (details in **Appendix 3**, available online).[122] Meta-analysis was carried out using Dersimonian and Laird random effects models, with I^2 used to quantify heterogeneity.

For our primary analysis of vitamin E status, we constructed a composite variable which refers to α-tocopherol status where available, or vitamin E provided as total tocopherol or unspecified. We conducted influence analyses to assess the robustness of our findings by assessing whether any individual studies were highly influential on the pooled result, sequentially excluding one study at a time from the analysis.

We investigated heterogeneity due to study design, quality, population, setting, exposure characteristics, variation in definition of severe preeclampsia, and possible confounding by gestational age, maternal age, and BMI via univariate and multivariate random-effects meta-regression. We classified study designs as prospective if they measured biomarker levels before preeclampsia or SGA became clinically apparent. Study quality was assessed by (1) whether authors addressed confounding in the design or analysis (yes or unclear versus no), (2) NOS score at or above versus below the median. We stratified by study population in three categories: general, high-risk only, or other. We stratified studies carried out exclusively in low- and middle-income countries (LMIC), where nutritional deficiencies may be more common, vs. high-income countries (HIC). For exposure characteristics, we assessed fasting status, assay method (high-performance liquid chromatography, HPLC vs. other), and timing of sample collection (before versus during or after labor). For vitamin E, we assessed whether the magnitude of association differed for total tocopherol or unspecified vs. α-tocopherol. Finally, we calculated the mean difference in gestational age, maternal age, and BMI between cases and controls in each study and used this variable in meta-regression analyses to assess potential confounding by these characteristics. As a sensitivity analysis for vitamin E studies, we restricted to studies measuring α-tocopherol to see if meta-regression findings were consistent.

Publication bias occurs when studies with null findings are less likely to be published than those finding a significant association.[123] As recommended by Sterne, Egger and Davey Smith, we examined publication bias by using funnel plots.[123] We also restricted to higher-

quality studies (those that addressed confounding in design or analysis) to assess whether publication bias was less evident among them.

Stata 11 (StataCorp, College Station, Texas) was used for statistical analyses and for generating figures.

4.6 Results

Database searches returned 3,939 total records and search of reference lists of relevant studies and literature reviews identified an additional 24 records (**Figure 1**). Reviewers completed the title and abstract screen on 1,882 unique records and selected 135 for full-text review and eligibility assessment. Sixty-four studies published from 1973 to 2012 were selected for inclusion. The complete list of included studies is provided in **Appendix 4**, available online. Fifty-five studies addressed the outcome preeclampsia, six addressed SGA, and three addressed both outcomes (**Table 1**, available online). Sample sizes ranged from 20 to 1231 subjects. Forty-two studies (66%) were conducted in general population samples; others were carried out in selected groups, including women with diabetes, HIV, high-risk for preeclampsia, first pregnancy, elective cesarean, etc. The most common antioxidant assessed was vitamin E (unspecified, total tocopherol, or α-tocopherol), followed by vitamins C and A.

The 58 preeclampsia studies comprised 48 traditional case-control studies, 1 cross-sectional study, and 9 prospective designs (nested case-control, cohort, RCT). Thirty-three were conducted exclusively in LMICs; most commonly Turkey (n=11) and India (n=8). Fourteen studies stratified cases as mild and severe for their primary analysis. Only one stratified early- (<34 weeks) and late-onset preeclampsia. Biomarker measurement spanned all three trimesters across the reviewed literature (**Table 2**, available online). Three studies reported serial measures of biomarker levels.

Among the 9 SGA studies, 2 were case-control studies, 1 was cross-sectional, and 6 used prospective designs. Three studies were conducted in LMICs. These studies measured biomarker levels in the 2nd and 3rd trimesters; hence there were no SGA studies reporting first-trimester levels of any of the exposures of interest. Two studies reported serial measures of biomarker levels.

Risk of Bias

The results of the risk of bias assessment are shown in **Table 3**. With "No" being the rating associated with the highest risk of bias, the studies were weakest with respect to accounting for confounding in the design or analysis. Twenty-eight studies (44%) did not account for confounding and another 22 (34%) were rated as unclear because of a concern of important residual confounding.

The median score for the Newcastle-Ottawa Scale was 4.5/9 (shown in Table 1) which is sub-optimal. Although there is no established cut-off for high-quality, several studies have used 6/9 to denote moderate or high-quality.[124,125] Representativeness of cases was a concern in many of the case-control studies. Only 23% reported recruiting consecutive eligible cases or an otherwise obviously representative sample. Confounding was also an issue identified with the Newcastle-Ottawa scale. Thirty-eight percent of studies controlled for confounding by gestational age, 25% controlled for parity, 16% for smoking, and 11% for pre-pregnancy BMI.

Data for Meta-Analysis

We included 53 studies in the quantitative synthesis of results. We extracted the mean and standard deviation or standard error from the figures of five studies.[90,126-129] Description of the data which prevented statistical pooling are detailed in **Appendix 5**, available online. We were unable to pool ORs across studies since ORs were reported for various heterogeneous comparisons: across quantiles (tertiles, quartiles, quintiles), above or below a specified cut-off value or percentile, and per unit SD (z-score). Nine studies provided ORs for an association between one of the antioxidant biomarkers of interest and preeclampsia and/or SGA. Eight of these studies provided adjusted ORs.

Vitamin E

Vitamin E reported as α-tocopherol, total tocopherol, or unspecified was measured in the first trimester in one study, second trimester in five studies, and third trimester in 41 studies in relation to preeclampsia. Meta-analysis of three studies estimated no difference in second

trimester vitamin E levels between preeclampsia cases and controls; pooled SMD=0.32 (-0.12, 0.76), I^2=56% (0, 88%). The meta-analysis of 34 third trimester studies showed a significantly negative SMD but substantial heterogeneity (**Table 4, Figure 2**). For both mild and severe preeclampsia, however, the pooled SMDs were null with substantial heterogeneity (**Table 4, Figure 2**). Additional studies we were unable to pool had mixed results, consistent with the meta-analyses (**Appendix 6**, available online).

Many of the studies corrected each individual's vitamin E for a measure of their blood lipid concentration (total cholesterol, total lipid, or other). None of the studies reported any significant difference in lipid-corrected vitamin E in the second trimester. Meta-analyses of third trimester levels did not suggest systematic differences between preeclampsia cases and controls for vitamin E, once differences in blood lipid levels were accounted for (**Table 4**). Four additional studies had consistent results (**Appendix 6**, available online).

Vitamin E in the form of γ-tocopherol was measured in the first trimester in one study, in the second trimester in three studies, and in the third trimester in five studies. Most studies found no significant difference but results suggested that higher levels may be associated with a modest increase in risk of preeclampsia. We were unable to perform meta-analyses. Additional details on the results of individual studies in **Appendix 6**, available online. ORs for vitamin E and preeclampsia are presented in the reviewed studies are detailed in **Appendix 7**, available online.

In a total of eight studies, vitamin E (reported as α-tocopherol, total tocopherol, or unspecified) was measured in the second trimester in five studies, and third trimester in five studies in relation to SGA. No meta-analyses were undertaken. Two studies with serial measures across both trimesters showed that lipid-adjusted α-tocopherol was not significantly different during the second trimester but became significantly lower in SGA cases versus controls at the start of the third trimester (28 weeks).[90,130] Additional details in **Appendix 6**.

All three studies that measured γ-tocopherol in the second trimester adjusted for total cholesterol, and each reported no difference between SGA cases and controls. [130-132] Two studies measured γ-tocopherol levels in the third trimester. One reported non-significantly higher γ-tocopherol in late third trimester[133] and the other study found no difference in γ-tocopherol adjusted for total cholesterol at 28 weeks.[130] Scholl *et al.* took serial measures at 16 and 28 weeks and reported no difference at either time point.[130]

Vitamin C

Vitamin C was measured in the first trimester in one study, second trimester in two studies, and third trimester in 30 studies in relation to preeclampsia. The meta-analysis of 29 third trimester studies showed a significantly negative SMD but substantial heterogeneity (**Table 4, Figure 3**). For both mild and severe preeclampsia, the pooled SMDs were not significant with substantial heterogeneity (**Table 4, Figure 3**); however, the influence analysis revealed that exclusion of just one study in either case would have resulted in a significantly negative pooled SMD (**Appendix 8**). Additional studies we were unable to pool had mixed results, consistent with the meta-analyses (**Appendix 6**).

Vitamin C was measured in the second trimester in two studies, and third trimester in two studies in relation to SGA. The results showed consistently lower vitamin C levels in SGA cases compared to controls.

Retinol/Vitamin A

Retinol was measured in the first trimester in one study, second trimester in two studies, and third trimester in thirteen studies in relation to preeclampsia. The meta-analysis of twelve third trimester studies showed a significantly negative SMD but substantial heterogeneity (**Table 4**). One additional study reported that retinol was non-significantly lower for preeclampsia cases versus controls.[134] For mild and severe preeclampsia where there were fewer studies, the pooled SMDs were null with substantial heterogeneity (**Table 4**).

Three studies found that retinol levels measured in the second trimester were similar among pregnancies resulting in SGA versus appropriate for gestational age (AGA) birth.[131,132,135] Three other studies measured retinol in the third trimester, all of which provided raw data for meta-analysis. Results were very heterogeneous (**Table 4**). Only one study measured levels before delivery and found significantly higher retinol in mothers who delivered SGA babies.[133] Two of the studies measured retinol levels shortly after delivery and found no significant differences for mothers who delivered SGA compared to AGA babies.[23,136]

Carotenoids

Carotenes assessed in relation to preeclampsia included total carotene in five studies,[111,137-140] β-carotene in nine studies,[112-114,129,141-145] α-carotene in four studies,[114,129,142,143] and lycopene in five studies.[114,129,142,143,146] Our meta-analyses estimated significantly negative pooled SMDs for total carotene, β-carotene, and lycopene with substantial heterogeneity (**Table 4**). For α-carotene, the SMD was not significant and the I^2 was 0%.

Other carotenoids assessed in relation to preeclampsia in the reviewed studies included lutein,[114,129,143] zeaxanthin,[114,143] β-cryptoxanthin,[114,143] and canthaxanthin.[142] Meta-analysis of three studies of lutein levels showed no significant difference between cases and controls (**Table 4**). Two large case-control studies did not report any ORs significantly different from 1 but subjects in the lowest quartiles of lutein, zeaxanthin, and β-cryptoxanthin had the highest odds of preeclampsia.[114,143] Palan *et al.* (2001) reported that levels of canthazanthin were non-significantly lower in preeclampsia cases in the third trimester.

Kerver *et al.* (2012) reported an adjusted OR for SGA of 0.2 (95% CI 0.1, 0.8) for subjects above the 75th percentile for total carotenoids measured in the second trimester. It was unclear what factors were adjusted for since the study was a published abstract with limited information.

Investigation of Heterogeneity

There were sufficient numbers of studies for a thorough investigation of heterogeneity among studies of vitamin E and C and preeclampsia. For Vitamin E and preeclampsia studies, we examined each factor in univariate models (**Table 5**). Since there were only three prospective studies in the meta-analysis, we restricted the multivariable model to studies measuring biomarker levels after diagnosis. Each factor that explained >1% of the variation, according to the adjusted R^2, was included in the initial multivariable model. The final model described in the table included factors with $p<0.1$. Collectively, these factors explained some of the variation but the residual I^2 remained above the threshold of 50% that is considered moderate heterogeneity. The model suggested that the negative SMD was driven by studies that used spectrometric methods as opposed to HPLC. More strongly negative SMDs were also observed among studies in which subjects were fasting. Among the studies of α-tocopherol, associations with HPLC and fasting were similar, and each explained some variation in the pooled SMD. A more negative

SMD for studies with fasting samples and a less negative SMD for studies with HPLC measurement were also identified in the meta-regressions for mild and severe preeclampsia. However, univariable and multivariable models did not explain a large proportion of the variation amongst studies included in these models (**Appendix 9**, available online).

For vitamin C and all preeclampsia, we were unable to explain heterogeneity in univariable meta-regressions. Although the studies by Howlader *et al.*[127] and Nilar *et al.*[147] were somewhat influential, based on the influence analysis, exclusion of neither study resulted in a change in the overall conclusion (**Appendix 8** available online).

For vitamin C and mild and severe preeclampsia, less heterogeneity was observed than for any preeclampsia. Matched studies had a pooled SMD closer to the null for severe preeclampsia. Whether or not the definition of severe PE included consideration of adverse conditions was important; for the definitions that more strictly relied on presence of severe hypertension and proteinuria, the pooled SMD was not significantly different from zero (**Appendix 9**, available online).

Publication Bias

Publication bias was evident among studies of vitamin E and C and all preeclampsia; many of the point estimates fell outside of the pseudo 95% confidence limits (i.e. limits assuming the pooled point estimate is the true association) in both directions. However, among higher-quality studies that addressed confounding (and the subset that measured α-tocopherol, not shown), publication bias appears to be less problematic. Publication bias was less evident among studies of mild preeclampsia, but could not be excluded in studies of severe preeclampsia or eclampsia, since very few of the studies fell on the right side of the funnels (**Figures 4, 5**). The funnel plot for retinol suggested publication bias but there were few data points (not shown).

4.7 Conclusion

Our systematic review identified a large number of studies that assessed levels of various antioxidants during pregnancy in relation to preeclampsia, and fewer that assessed an association

with SGA. With some exceptions, the studies were generally small (less than 50 cases) with high risk of bias. The majority of included studies compared antioxidant levels in women with clinically manifest preeclampsia to third trimester controls. Meta-analyses revealed heterogeneous evidence for reduced third trimester levels of vitamins A, C, E, and carotenoids in preeclampsia. However, confounding was inherent in the crude comparisons that were drawn. Publication bias was also apparent. There was little evidence for differences in antioxidant levels that predated preeclampsia diagnosis. In SGA, there was some limited evidence for reduced levels of vitamin C from the second trimester and lower carotenoid levels in the second trimester.

Several systematic reviews of RCTs have concluded that antioxidant supplementation is not associated with a significant reduction in the risk of preeclampsia or SGA, and may be associated with an increased risk of preterm birth and miscarriage.[25-27] However, the RCTs conducted to date studied a small set of antioxidant interventions (mostly 1000 mg vitamin C and 400 international units vitamin E daily), mainly in women at high risk of preeclampsia. The results of this systematic review may undermine the premise that vitamins C and E are consistently lower in women who develop preeclampsia (and perhaps SGA), on which clinical trials of vitamin C and E for prevention were predicated. Our results suggest that publication bias and confounding may have played a role in forming this premise. We identified significantly negative SMDs for vitamins C and E and preeclampsia, but the findings were very heterogeneous, null for the subset of studies that stratified mild and severe PE, and null for lipid-corrected vitamin E. There was also limited evidence that vitamin C and E levels are reduced in the first or second trimester in pregnancies resulting in preeclampsia or SGA. Among studies that measured other antioxidant levels prospectively, some suggest that differences in vitamins E and A may arise later in pregnancy. Hence, we must consider whether these results, in combination with evidence of a lack of a protective effect of vitamins C and E in RCTs, suggest that lower antioxidant levels may be a consequence, rather than a cause, of these conditions.

While the meta-analysis of any vitamin E showed a negative SMD in preeclampsia cases vs. controls, the meta-analysis of lipid-corrected vitamin E showed no difference. The literature has suggested that correction for cholesterol levels in analyses of tocopherols is appropriate as this may better reflect the potential impact of these antioxidants on reducing oxidative damage.[148] However, women with preeclampsia have been shown to have significantly altered blood lipid measurements.[149] This correction could therefore introduce bias; conversely, failure

to adjust may also be problematic. More research on prospectively measured vitamin E levels is needed; preferably with both crude and lipid-corrected levels presented so that is the impact of lipid differences can be better understood.

Our review follows a systematic review published in 2009 that focused on markers of lipid peroxidation and antioxidant levels.[21] Compared to those findings, our pooled SMDs were closer to the null. We believe our approach was more comprehensive and inclusive, since the previous review did not include any prospective studies, and focused more narrowly on vitamins C and E. Further, many new studies have been published since the previous review. We made an effort to explain the heterogeneity observed, even though much of the heterogeneity remained unexplained.

RCTs to date have mainly focused on vitamins C and E. The evidence presented here does not show that vitamins C or E are consistently low in preeclampsia. Other interventions might be beneficial. Our results suggest that carotenes may be an alternative intervention; however, unless future studies show that levels are truly lower before the onset of clinical disease, the evidence is insufficient to recommend carotene supplementation to prevent preeclampsia. We also found evidence that total carotenoids are lower in the second trimester in SGA. Few studies measured one or more carotenoids in preeclampsia, and more data are needed to assess whether supplementation with carotenoid antioxidants could be an effective intervention to prevent preeclampsia or SGA.

Few studies included in our review addressed confounding at the design or analysis stage. Most studies reported only means and standard deviations and a t-test for differences. Thus, confounding likely explains at least part of the crude associations observed. Some studies used regression models to adjust for confounding. Studies that reported both crude and adjusted ORs allow a more in-depth consideration of the extent of confounding. When the data were not presented in a way amenable to statistical pooling of adjusted effect estimates, we tried to summarize the results qualitatively. We also assessed whether excluding studies that did not address confounding affected the point estimate and our assessment of publication bias. We found that the SMD was shifted toward the null, with less evidence of publication bias.

Inclusion and exclusion criteria of individual studies differed substantially. Some studies included only women at high risk for preeclampsia, while others excluded women who had risk factors such as pre-existing hypertension, diabetes, or multiple pregnancies. We investigated

heterogeneity in the study results due to differences in study populations, but we were limited in number of comparisons we could make. Nevertheless, the population studied was not significantly associated with the pooled SMD.

Most of the preeclampsia studies measured antioxidants in late pregnancy, in most cases, after the clinical diagnosis. Hence, we are unable to infer whether differences observed predated clinical signs and participated in a causal mechanism. Only one study stratified the cases by the timing of preeclampsia, early (<34 weeks) vs. late, which is a widely accepted subgrouping that may represent distinct conditions with different causal mechanisms.[7]

Due to limited resources available, we could not translate studies in languages other than English and French. However, we searched many databases without language restrictions. Only 5% (7 of 135) of the studies we judged as potentially eligible based on a translated title and abstract were published in other languages. However, we acknowledge that at least a few relevant studies could have been missed by the language restrictions we applied.

Very few studies provided adjusted ORs for the association in which we are interested: the risk/odds of preeclampsia or SGA according to level of antioxidant. Given the data available to us, we calculated the pooled SMD, which provides a measure of the average difference (in terms of SD units) between study groups. Our interpretation is hence limited, because each of the pooled SMD estimates is likely confounded to some degree. Studies that accounted for confounding in the design by matching or by restriction are less likely to be confounded.

We did not consider studies of other micronutrients that act as co-factors to antioxidant enzymes (e.g. selenium, zinc) and that may also modulate oxidative stress in preeclampsia and SGA. Several studies have looked at whether levels of these micronutrients are differential in preeclamptic pregnancies,[150,151] and clinical trials of selenium supplementation have been reported.[152] While these were outside of the scope of this review, they may be relevant to explore in future systematic reviews.

We did not exclude studies on the basis of research design or population. Hence, we could investigate sources of heterogeneity in the literature and seek to understand if differences in these factors led to different conclusions. We carried out an exhaustive search strategy developed in consultation with an experienced librarian that covered a wide time range and included many databases. We also carefully reviewed the references of included studies and review articles to identify any studies we may have missed. The entire review process was

carried out in duplicate. The reviewers also met early in each stage to review whether they were interpreting eligibility criteria in the same way and whether the data extraction process was consistent.

Our review assessed many relevant markers and included prospective studies so that differences in early- to mid-pregnancy could be investigated, in addition to evaluating whether consistent differences were apparent in late pregnancy. We were able to determine that there was limited high-quality evidence for systematic differences in antioxidant levels before clinical detection of preeclampsia or recognition of SGA birth. We believe that such evidence would be needed to justify future trials of any antioxidant supplementation intervention.

A large number of studies have measured non-enzymatic antioxidant levels in maternal blood during pregnancy in relation to preeclampsia and several have assessed them in relation to SGA. The results for crude differences between cases and controls, as assessed by the pooled SMD showed a tendency toward lower vitamin E and C levels in cases of preeclampsia and SGA, and lower vitamin A in preeclampsia, but substantial heterogeneity of results suggests the need for cautious interpretation. Some of this heterogeneity was explained by assay methods, including HPLC and fasting status. Many of the studies were of low quality, and confounding and publication bias are potentially problematic. Future observational studies with a sufficiently large sample size and high methodologic quality are needed to determine if antioxidant levels are low before clinical manifestation of preeclampsia or SGA. Studies with serial measures are also of interest to more clearly identify when differences may arise. The results of our review do not suggest an imminent need for new clinical trials. Substantial unexplained heterogeneity in findings among the studies we reviewed limits our ability to know which populations might benefit from future interventions. However, it may be beneficial to focus future interventions on populations that are known to have higher baseline levels of oxidative stress, for example, smokers, diabetic, and obese women.

Authors' roles

JMC, SRK, RWP, OB, and MSK participated in the design of the study. JMC and MB screened studies, selected studies for inclusion, performed data extraction, quality assessment,

and data cleaning. JMC conducted data analyses (advised by RWP) and wrote the first draft of the manuscript. All authors provided feedback on the manuscript and approved the final version.

4.7 Tables

Table 1: Characteristics of included studies

PREECLAMPSIA STUDIES

Study	Design	Country	Population	Study Groups	Group Ns	NOS	RB7*
Akyol et al. (2000)	Case-control	Turkey	General population	Controls / Mild / Severe PE	15 / 17 / 16	5	Unclear
Azar et al. (2011)	Nested case-control	Norway, Australia, USA	Women with diabetes	Controls / PE Cases	24 / 23	7.5	Yes
Bakheit et al. (2010)	Case-control	Sudan	General population	Controls / PE Cases	38 / 37	5	Yes
Basu and Arulanantham (1973)	Cross-sectional	India	Low SES women	Controls / PE Cases	56 / 50	1	No
Ben-Haroush et al. (2002)	Nested case-control	Israel	High-risk for PE	Controls / PE Cases	36 / 8	4	Unclear
Bowen et al. (1998)	Case-control	S. Africa	Low SES, black African	Controls / Mild / Severe PE	32 / 15 / 31	5	Unclear
Bowen et al. (2001)	Case-control	S. Africa	First pregnancy	Controls / PE / Eclampsia	29 / 21 / 6	3.5	No
Dehghan, Daryani, and Dehghanan (2007)	Case-control	Iran	General population	Controls / PE Cases	100 / 50	5.5	Unclear
Dirican et al. (2008)	Case-control	Turkey	General population	Term Controls / Mild / Severe PE	20 / 21 / 21	4	No
Elsen et al. (2012)	RCT sub-study	Venezuela, Tanzania	High-risk for PE	Controls / Mild / Severe PE	394 / 20 / 17	5	No
Gratacos et al. (1999)	Case-control	Spain	General population	Term Controls / PE / Superimposed	36 / 34 / 11	6	No
Harma and Erel (2005)	Case-control	Turkey	General population	Control / PE Cases	18 / 24	4.5	Unclear
Harsem, Braekke, and Staff (2006)	Case-control	Norway	Elective caesarean	Control / PE Cases	38 / 21	4	No
Harsem et al. (2008)	Case-control	Norway	Elective caesarean	Control / PE Cases	38 / 36	3	No
Howlader et al. (2007)	Case-control	Bangladesh	General population	Control / PE Cases	22 / 25	3.5	Unclear
Hubel et al. (1997)	Case-control	USA	General population	Control / PE Cases	17 / 14	4.5	No
Ikpen et al. (2012)	Case-control	Nigeria	General population	Control / PE Cases	80 / 80	7	Yes
Islam et al. (2004)	Case-control	Bangladesh	General population	Control / PE Cases / Eclampsia	35 / 44 / 50	4	Unclear
Jendryczko and Drozdz (1989)	Prospective cohort	NR (Poland)	General population	Term Controls / PE Cases	11 / 9	2	No
Kaur et al. (2008)	Case-control	India	General population	Controls / Mild / Severe PE	56 / 47 / 77	1	Unclear
Kharb (2000)	Case-control	India	First pregnancy	Controls / PE Cases	30 / 30	5	Yes
Kiondo et al. (2012)	Case-control	Uganda	General population	Controls / PE Cases	400 / 215	6.5	Unclear
Kolusari et al. (2008)	Case-control	Turkey	General population	Controls / PE Cases	48 / 47	4	No
Llurba et al. (2004)	Case-control	Spain	General population	Controls / PE Cases	30 / 53	5.5	Yes
Madazli et al. (1999)	Case-control	Turkey	General population	Controls / PE Cases	21 / 22	4	No
Mehendale et al. (2008)	Case-control	India	General population	Controls / PE Cases	55 / 60	4.5	Unclear
Mikhail et al. (1994)	Case-control	USA	General population	Controls / Mild / Severe PE	44 / 22 / 8	5	No
Mohanty et al. (2006)	Case-control	India	First pregnancy, low SES	Controls / Mild / Severe PE	20 / 38 / 12	3.5	Unclear
Mohindra et al. (2002)	Case-control	India	Primiparous women	Controls / PE Cases	54 / 33	2.5	No
Morris et al. (1998)	Case-control	UK	General population	Controls / PE Cases	19 / 19	4	Yes
Mutlu-Turkoglu et al. (1998)	Case-control	Turkey	General population	Controls / PE Cases	10 / 17	4.5	Unclear

Nilar et al. (2009)	Case-control	Myanmar	General population	Controls / PE Cases	48 / 25	5	No
Noyan et al. (2006)	Case-control	Turkey	General population	Controls / PE / Eclampsia	19 / 21 / 11	3	No
Ozan et al. (1997)	Case-control	Turkey	General population	Controls / Mild / Severe PE / Eclampsia	20 / 14 / 12 / 13	4	No
Palan, Mikhail, and Romney (2001)	Case-control	USA	General population	Controls / PE Cases	22 / 19	5	Yes
Palan et al. (2004)	Case-control	USA	General population	Controls / Mild / Severe PE	42 / 25 / 28	5	No
Panburana, Phuapradit, and Puchaiwatananon (2000)	Case-control	Thailand	General population	Controls / Mild / Severe PE	60 / 30 / 20	3	No
Roland et al. (2010)	Case-control	Canada	General population	Controls / PE Cases	30 / 29	3	Unclear
Sagol, Ozkinay, and Ozsener (1999)	Case-control	Turkey	General population	Term Controls / Mild / Severe PE	33 / 8 / 16	5.5	Unclear
Serdar et al. (2003)	Case-control	Turkey	Elective caesarean	Term Controls / Mild / Severe PE	50 / 30 / 30	4	No
Serdar et al. (2002)	Case-control	Turkey	Elective caesarean	Term Controls / PE Cases	72 / 70	3	No
Sharma et al. (2006)	Case-control	India	General population	Controls / PE Cases	50 / 50	2.5	Unclear
Sharma et al. (1984)	Case-control	NR	General population	Controls / Moderate / Severe PE	86 / 12 / 41	3	No
Suhail and Faizul-Suhail (2009)	Case-control	India	General population	Controls / Severe PE Cases	21 / 21	4	No
Uotila et al. (1994)	Case-control	Finland	Elective caesarean	Controls / PE Cases	9 / 11	4	No
Uotila et al. (1993)	Case-control	Finland	General population	Controls / Mild / Severe PE	20 / 20 / 23	3	No
Wei et al. (2012)	Nested case-control	Canada, Mexico	High-risk & low-risk RCT participants (INTAPP trial)	Controls / PE Cases	229 / 115	5.5	Yes
Wikstrom et al. (2009)	Case-control	Sweden	General population	Early Controls / Early PE / Late Controls / Late PE	22 / 18 / 18 / 20	3.5	Unclear
Williams et al. (2003)	Case-control	Zimbabwe	General population	Controls / PE	186 / 173	7.5	Yes
Xu (2011)	Nested case-control	Canada, Mexico	High-risk & low-risk RCT participants (INTAPP trial)	Controls / PE Cases	229 / 115	7.5	Yes
Yanik et al. (1999)	Case-control	Turkey	General population	Controls / PE / Eclampsia	25 / 18 / 15	5	No
Zhang, Luthy, et al. (2001)	Nested case-control	NR	General population	Controls / PE Cases	241 / 24	7	Yes
Zhang, Williams, et al. (2001)	Case-control	Peru	General population	Controls / PE Cases	179 / 125	7.5	Yes
Ziari et al. (1996)	Case-control	Nigeria	1st or 2nd pregnancy	Controls / PE / Eclampsia	16 / 9 / 7	4	Unclear
Zusterzeel et al. (2002)	Case-control	Netherlands	General population	Controls / Mild PE	14 / 14	5	No
PREECLAMPSIA & SGA STUDIES							
Study	**Design**	**Country**	**Population**	**Study Groups**	**Group Ns**	**NOS**	**RB7***
Chappell et al. (2002)	Semi-nested case-control	UK	High-risk cases; low-risk controls	Controls / PE / SGA Cases	27 / 21 / 17	5.5	Unclear
Rajasingam et al. (2009)	RCT sub-study	UK, Netherlands	High-risk for PE (obese, nulliparous)	Total Cohort	385	7	Unclear
Schiff et al. (1996)	Case-control	USA	General population	Controls / PE Cases / SGA+PE (subgroup of PE)	90 / 48 / 14	5	No
SGA STUDIES							
Study	**Design**	**Country**	**Population**	**Study Groups**	**Group Ns**	**NOS**	**RB7***

Agarwal et al. (2008)	Cross-sectional	India	Healthy term mother-newborn pairs	Control / SGA Cases	30 / 30	4	No
Dreyfuss et al. (2001)	RCT sub-study	Tanzania	HIV-positive women	Total Cohort	822	6	Yes
Kerver et al. (2012)	Nested case-control	USA	General population (POUCH study)	Total Cohort	547	4	Unclear
Ortega-Senovilla et al. (2010)	Case-control	Italy	General population	Controls / SGA Cases	25 / 24	5	Unclear
Saker et al. (2008)	Prospective cohort	Algeria	Term births	Controls / SGA Cases	56 / 45	6	Unclear
Scholl et al. (2006)	Prospective cohort	USA	General population (Camden study)	Total Cohort	1231	6.5	Yes

*RB7, Risk of Bias question #7 ("Was confounding accounted for in the design and/or analysis?"); PE, preeclampsia; SGA, small for gestational age; NOS, Newcastle-Ottawa Scale [score out of 9 maximum]; SES, socioeconomic status; INTAPP, An international trial of antioxidants in the prevention of preeclampsia, POUCH, Pregnancy outcomes and community health

Table 2: Details of antioxidant biomarker measurements

Study	Outcome	Markers measured	Sample Timing	GA Range (wk)	Fasting	Units
Akyol et al. (2000)	PE	Vitamin E**	Before delivery	28-40	Unclear	mg/dL
Azar et al. (2011)	PE	Retinol , α-, γ-tocopherol, α-, β-carotene, lycopene, lutein	3 samples; once per trimester		Yes	umol/L
Bakheit et al. (2010)	PE	Retinol, α-tocopherol	Before delivery	Third trimester	Unclear	ug/dL
Basu and Arulanantham (1973)	PE	Retinol*	Before delivery	25-40	Unclear	ug/dL
Ben-Haroush et al. (2002)	PE	Vitamin E*	Before labor	7-32	Yes	mg/dL
Bowen et al. (1998)	PE	Vitamin C*, vitamin E	Before labor	Third trimester	Unclear	umol/L
Bowen et al. (2001)	PE	Vitamin C, vitamin E	Before delivery	Third trimester	Unclear	umol/L
Dehghan, Daryani, and Dehghanan (2007)	PE	Vitamin C*	Before delivery	28-42	Unclear	mg/dL
Dirican et al. (2008)	PE	Vitamin C*, vitamin E*, total carotene*	Before delivery	Third trimester	Unclear	umol/L
Elsen et al. (2012)	PE	Retinol, vitamin E	7 samples; every 4 weeks from week 12		Unclear	ug/L
Gratacos et al. (1999)	PE	α-tocopherol	Before labor	Third trimester	Yes	mg/mL
Harma and Erel (2005)	PE	Vitamin C*	Before labor	Third trimester	Unclear	umol/L
Harsem, Braekke, and Staff (2006)	PE	α-tocopherol	Before labor	25-40	Yes	umol/L
Harsem et al. (2008)	PE	Vitamin E	Before labor	24-40	Yes	umol/L
Howlader et al. (2007)	PE	Vitamin C*	Before delivery	Third trimester	Unclear	mg/dL
Hubel et al. (1997)	PE	Vitamin C*, α-tocopherol	Before delivery	Third trimester	Unclear	nmol/mL
Ikpen et al. (2012)	PE	Vitamin C*, vitamin E*	Before delivery	Third trimester	Unclear	mg/dL
Islam et al. (2004)	PE	Retinol, vitamin C*, α-tocopherol	Before delivery	28-42	Unclear	umol/L
Jendryczko and Drozdz (1989)	PE	Retinol, vitamin E, β-carotene	5 samples; Every 2 weeks from week 28		Unclear	ug/L
Kaur et al. (2008)	PE	Retinol, vitamin C*, vitamin E	Before delivery	Third trimester	Unclear	ug/mL, mg% (vitamin C)
Kharb (2000)	PE	Vitamin C*, Vitamin E*	Before labor	28-40	Yes	umol/L
Kiondo et al. (2012)	PE	Vitamin C*	Before delivery	20-	Unclear	mg/L
Kolusari et al. (2008)	PE	Retinol, α-tocopherol	Before delivery	29-38	Yes	mmol/L
Llurba et al. (2004)	PE	Vitamin C, α-tocopherol	Before labor	24-40	Yes	umol/L
Madazli et al. (1999)	PE	Vitamin C*, α-tocopherol*	Before delivery	Third trimester	Yes	mg/dL
Mehendale et al. (2008)	PE	Vitamin C*, α-tocopherol*	Before labor	36-	Unclear	mg/dL (mg%)
Mikhail et al. (1994)	PE	Retinol, α-tocopherol, β-carotene	Before labor	28-42	Yes	mg/dL
Mohanty et al. (2006)	PE	Vitamin C*, vitamin E*	Before delivery	28-42	Yes	mg/L, mol/L (vitamin E)

Mohindra et al. (2002)	PE	Total carotene (NR)	Before delivery	Third trimester	Yes	ug/dL
Morris et al. (1998)	PE	Vitamin E	Before delivery	Third trimester	Unclear	ug/mL
Mutlu-Turkoglu et al. (1998)	PE	Vitamin C*	Before labor or after delivery	Third trimester	Unclear	umol/L
Nilar et al. (2009)	PE	Vitamin C*, vitamin E*	Before labor	28-40	Unclear	mg/dL
Noyan et al. (2006)	PE	Vitamin C*	Before delivery	23-40	Yes	mg/dL
Ozan et al. (1997)	PE	Vitamin C*	Before delivery	Third trimester	Yes	mg/dL
Palan, Mikhail, and Romney (2001)	PE	α-, β-carotene, lycopene, canthaxanthin	After delivery	30-42	Unclear	ug/dL
Palan et al. (2004)	PE	α-, γ-tocopherol	Before delivery	30-41	No	umol/L
Panburana, Phuapradit, and Puchaiwatananon (2000)	PE	Retinol, vitamin C*, α-tocopherol	Before delivery	Third trimester	Yes	umol/L
Roland et al. (2010)	PE	α-, γ-tocopherol, total tocopherol, β-carotene	Before delivery	Third trimester	Unclear	umol/L
Sagol, Ozkinay, and Ozsener (1999)	PE	Vitamin C, α-tocopherol	Before delivery	20-40	Unclear	ug/L
Serdar et al. (2003)	PE	Vitamin E*, total carotene*	Before labor	31-38	Yes	umol/L
Serdar et al. (2002)	PE	Vitamin E*, total carotene*	Before labor	31-38	Yes	umol/L
Sharma et al. (2006)	PE	Vitamin C*, lycopene	Before delivery	26-42	Unclear	mg/dL
Sharma et al. (1984)	PE	Vitamin C*	Before delivery	28-40	Unclear	mg/dL
Suhail and Faizul-Suhail (2009)	PE	Retinol*, vitamin C*, vitamin E*	Before delivery	Third trimester	Unclear	umol/L
Uotila et al. (1994)	PE	Vitamin C, vitamin E	Before labor	Third trimester	Yes	umol/L
Uotila et al. (1993)	PE	Vitamin E	Before delivery	31-38	Unclear	umol/L
Wei et al. (2012)	PE	β-carotene	3 samples; 12-18 (pre-intervention), 24-26, 32-34 weeks		Unclear	ug/mL
Wikstrom et al. (2009)	PE	Vitamin C*, α-tocopherol	Before delivery	Third trimester	Unclear	umol/L
Williams et al. (2003)	PE	Retinol , α-, γ-tocopherol, α-, β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin	After delivery (12-72 hours)	Third trimester	Unclear	ug/mL
Xu (2011)	PE	α-, γ-tocopherol, total tocopherol	3 samples; 12-18 (pre-intervention), 24-26, 32-34 weeks		Unclear	NR
Yanik et al. (1999)	PE	Vitamin E*	Before delivery	28-41	Unclear	mg/mL
Zhang, Luthy, et al. (2001)	PE	Vitamin C	Before labor	First trimester	Unclear	umol/L
Zhang, Williams, et al. (2001)	PE	Retinol , α-, γ-tocopherol, α-, β-carotene, lycopene, lutein, zeaxanthin, β-cryptoxanthin	Before delivery	Third trimester	Unclear	umol/L
Ziari et al. (1996)	PE	Retinol, α-tocopherol, β-carotene	Before labor	Third trimester	Yes	ug/dL
Zusterzeel et al. (2002)	PE	Vitamin C, α-tocopherol	Before labor	Third trimester	Yes	umol/L
Chappell et al. (2002)	PE & SGA	Vitamin C, α-tocopherol	5 samples; every 4		Unclear	umol/L

			weeks from week 20			
Rajasingam et al. (2009)	PE & SGA	Retinol, vitamin C, α-, γ-tocopherol	Before labor	14-22	No	umol/L
Schiff et al. (1996)	PE & SGA	Vitamin E	Before delivery	22-41	Unclear	mg/dL
Agarwal et al. (2008)	SGA	Retinol*	After delivery	37-42	Unclear	ug/dL
Dreyfuss et al. (2001)	SGA	Retinol, vitamin E (NR)	Before labor	12-27	Unclear	umol/L
Kerver et al. (2012)	SGA	Retinol, α-, γ-tocopherol, total carotenoids	Before labor	16-27	Unclear	not reported
Ortega-Senovilla et al. (2010)	SGA	Retinol, α-, γ-tocopherol	Before labor	Third trimester	Yes	umol/L
Saker et al. (2008)	SGA	Retinol, vitamin C*, α-tocopherol	After delivery	37-42	Yes	umol/L
Scholl et al. (2006)	SGA	α-, γ-tocopherol	2 samples	16 & 28 weeks	Unclear	ug/mL

NR, not reported; PE, preeclampsia; SGA, small for gestational age; *=Spectrophotometric method, **=Enzymatic Assay (method is HPLC unless otherwise indicated)

Table 3: Study quality items assessed in addition to Newcastle-Ottawa Scale

Quality Assessment Item	Yes - n (%)	Unclear - n (%)	No - n (%)
RB1. Was the study clinical setting well-described?	28 (44)	25 (39)	11 (17)
RB2. Were incomplete data (i.e. missing data) adequately described?	13 (20)	47 (73)	4 (6)
RB3. Were statistical analyses described adequately?	41 (64)	15 (23)	8 (13)
RB4. Were analyses appropriate?	39 (61)	24 (38)	1 (2)
RB5. Did analysis provide sufficient presentation of data?	52 (81)	2 (3)	10 (16)
RB6. Is the study report free of the suggestion of selective reporting?	55 (86)	5 (8)	4 (6)
RB7. Was confounding accounted for in the design and/or analysis?	14 (22)	22 (34)	28 (44)

RB, "Risk of Bias"; Manual for assigning "yes", "unclear," and "no" available in Appendix 2. Percents may not sum to exactly 100 due to rounding

Table 4: Meta-analyses of third trimester antioxidant levels

		Random Effects Model			Heterogeneity	
Marker, Outcome	**No. of Studies**	**SMD**	**95% CI**	**p-value**	**I^2, %**	**95% CI**
Retinol						
SGA	3	0.47	-0.56, 1.49	0.37	92	79, 97
All preeclampsia	12	-0.51	-0.88, -0.15	0.01	91	85, 94
Mild preeclampsia	3	-0.08	-0.58, 0.42	0.76	76	21, 93
Severe preeclampsia	6	-0.62	-1.58, 0.34	0.21	95	91, 97
Vitamin C						
All preeclampsia	29*	-0.56	-0.83, -0.28	<0.01	91	88, 93
Mild preeclampsia	11	-0.32	-0.67, 0.03	0.07	77	59, 87
Severe preeclampsia	15	-0.35	-0.72, 0.01	0.06	84	74, 90
Vitamin E						
All preeclampsia	34*	-0.42	-0.72, -0.13	0.01	93	91, 94
Mild preeclampsia	12	0.08	-0.55, 0.72	0.79	93	90, 95
Severe preeclampsia	17	-0.13	-0.57, 0.30	0.55	90	85, 93
Lipid-adj. Vitamin E						
All preeclampsia	10*	-0.04	-0.41, 0.33	0.82	86	76, 92
Mild preeclampsia	4	0.16	-0.48, 0.80	0.63	83	56, 93
Severe preeclampsia	5	-0.30	-1.17, 0.58	0.51	91	82, 95
α-tocopherol						
All preeclampsia	15*	-0.35	-0.66, -0.03	0.03	88	83, 92
Mild preeclampsia	4	-0.20	-0.47, 0.07	0.15	0	0, 85
Severe preeclampsia	6	-0.56	-0.99, -0.14	0.01	66	17, 86
Lipid-adj. α-tocopherol						
All preeclampsia	7*	0.02	-0.37, 0.41	0.91	82	65, 91
Others - All preeclampsia						
α-carotene	3	-0.01	-0.16, 0.15	0.94	0	0, 90
β-carotene	7	-0.40	-0.72, -0.08	0.01	74	45, 88
Total carotene	4	-1.06	-1.65, -0.47	<0.01	86	66, 94
Lycopene	4	-1.05	-2.09, -0.00	0.05	97	96, 99
Lutein	3	-0.07	-0.34, 0.20	0.61	61	0, 89

*Wikstrom *et al.* (2009) early-onset cases and controls and late-onset cases and controls were treated as two separate studies. SGA, small for gestational age; SMD, standardized mean difference; CI, confidence interval

Table 5: Meta-regression for vitamin E and any preeclampsia

Covariate	No.	β-coef.	95% CI	P-value	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	3	-0.60	-1.92, 0.73	0.37	93.3	-3.85
Study Quality						
Addressed Confounding	18	0.46	-0.24, 1.16	0.19	92.6	2.24
NOS>4.5 (above median)	15	-0.16	-0.88, 0.56	0.65	93.3	-2.71
Matched	9	0.31	-0.50. 1.11	0.45	93.1	-1.84
Population / Setting						
General Population	25	0.27	-0.54, 1.09	0.50	92.8	-1.12
High-risk for preeclampsia	6	-0.36	-1.29, 0.58	0.45	93.2	-1.07
LMIC	21	-0.46	-1.18, 0.27	0.21	93.2	4.51
Exposure Characteristics						
Fasting	12	-0.63	-1.34, 0.09	0.09	91.6	10.96
HPLC	23	1.07	0.42, 1.72	<0.01	88.6	35.71
Pre-labor sample	14	-0.57	-1.27, 0.13	0.11	92.2	5.77
α-tocopherol	16	0.23	-0.49, 0.94	0.53	93.1	-2.80
MD gestational age (per wk)	29	-0.09	-0.31, 0.13	0.41	92.8	-2.08
MD maternal age (per year)	28	0.00	-0.22, 0.23	0.96	91.0	-4.36
MD BMI (per unit kg/m2)	10	0.07	-0.24, 0.39	0.62	91.1	-9.04
Multivariable model intercept*	31	-0.85	-1.27, -0.43	<0.01	58.6	17.76
HPLC		1.15	0.67, 1.63	<0.01		
Fasting		-0.78	-1.26, -0.29	<0.01		

N overall=34; SMD= - 0.42, 95% CI -0.72,-0.13, I^2=93%

*Restricted to prospective studies. CI, confidence interval; HPLC, high-performance liquid chromatography; LMIC, low- or middle-income country; MD, mean difference; NOS, Newcastle-Ottawa Scale [score]

4.8 Figures

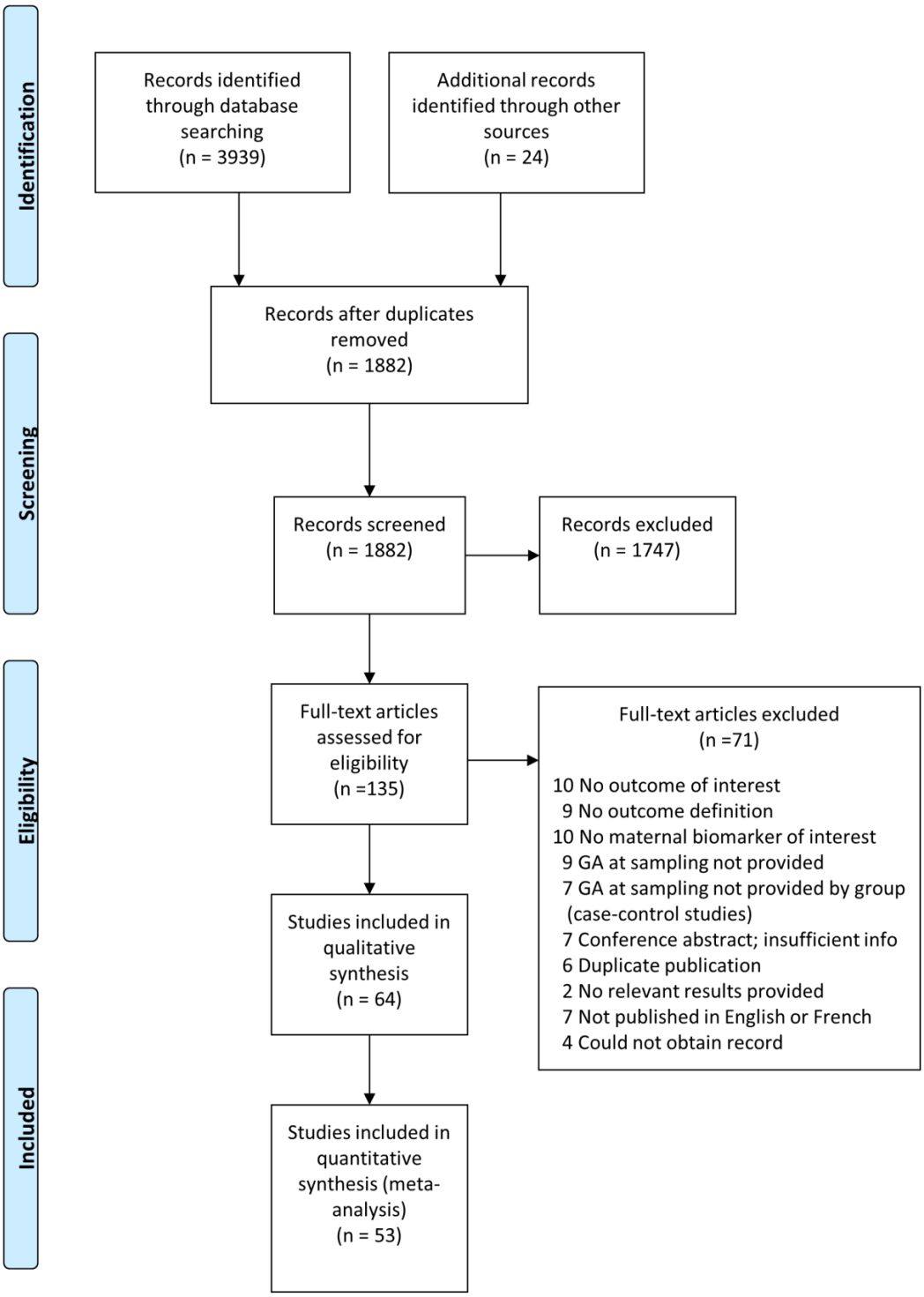

Figure 1: Flow diagram of study selection process. Figure adapted from*:* Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

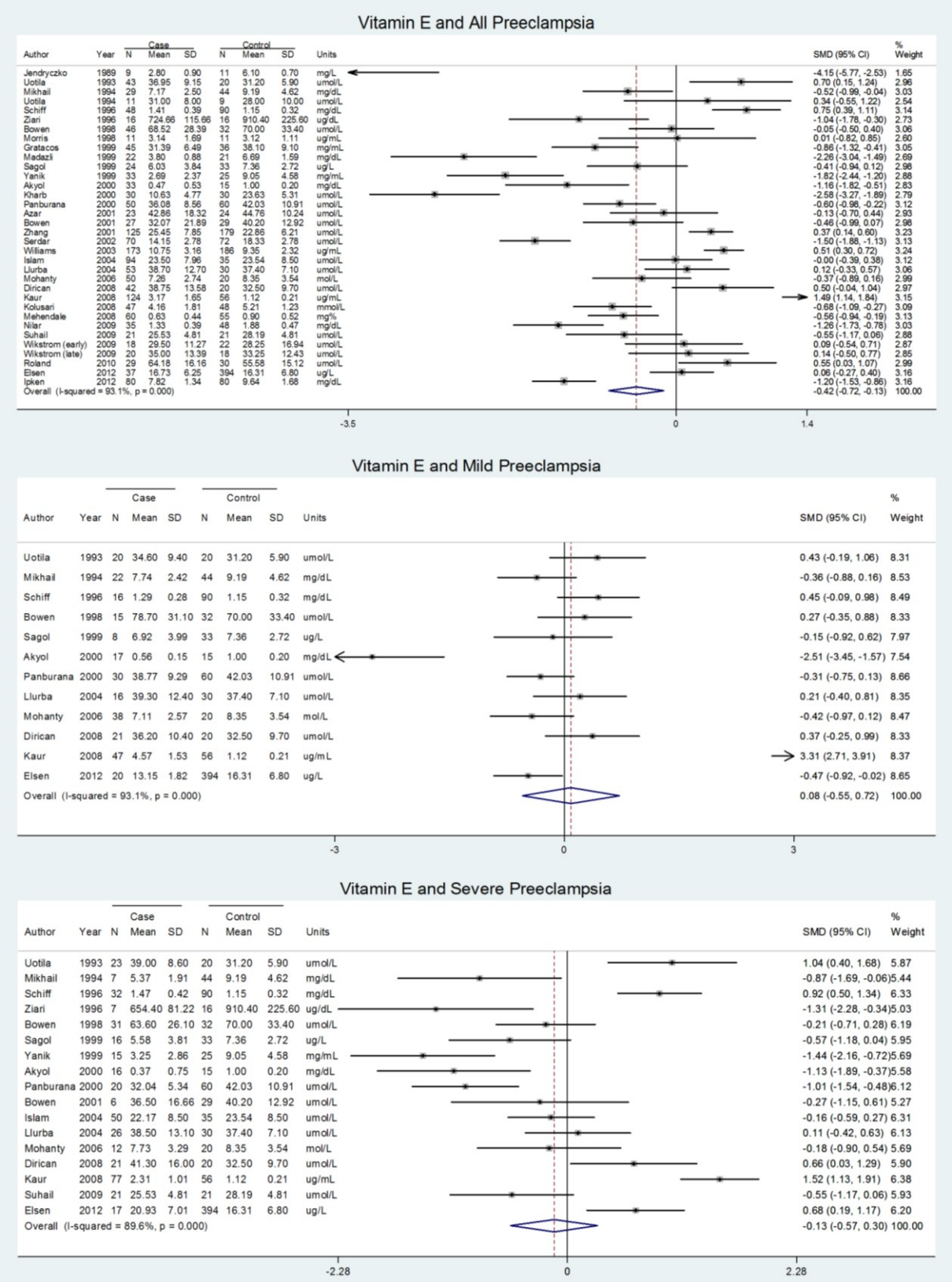

Figure 2: Forest plots for vitamin E and preeclampsia containing raw data

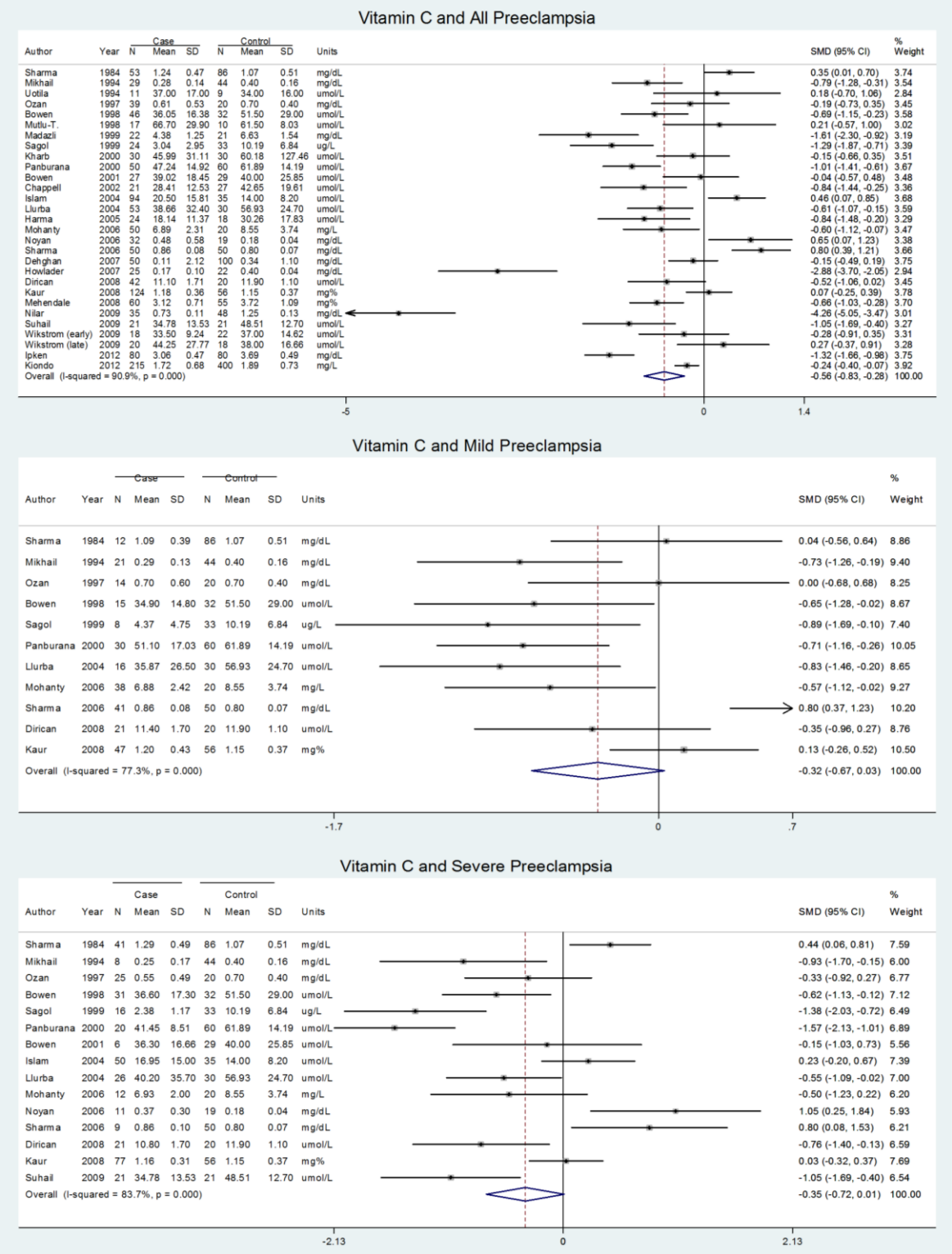

Figure 3: Forest plots for vitamin C and preeclampsia containing raw data

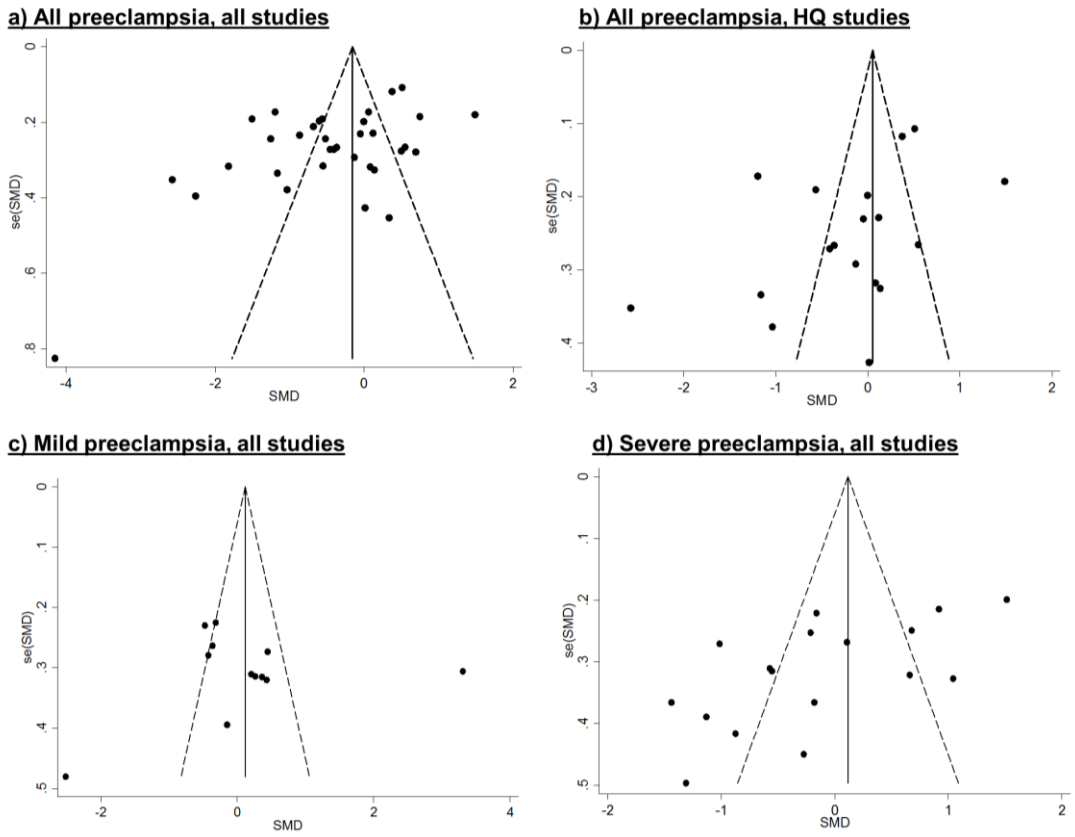

Figure 4: Funnel plots with pseudo-95% confidence intervals for vitamin E studies. High-quality (HQ) studies refer to those that addressed confounding in the design or analysis

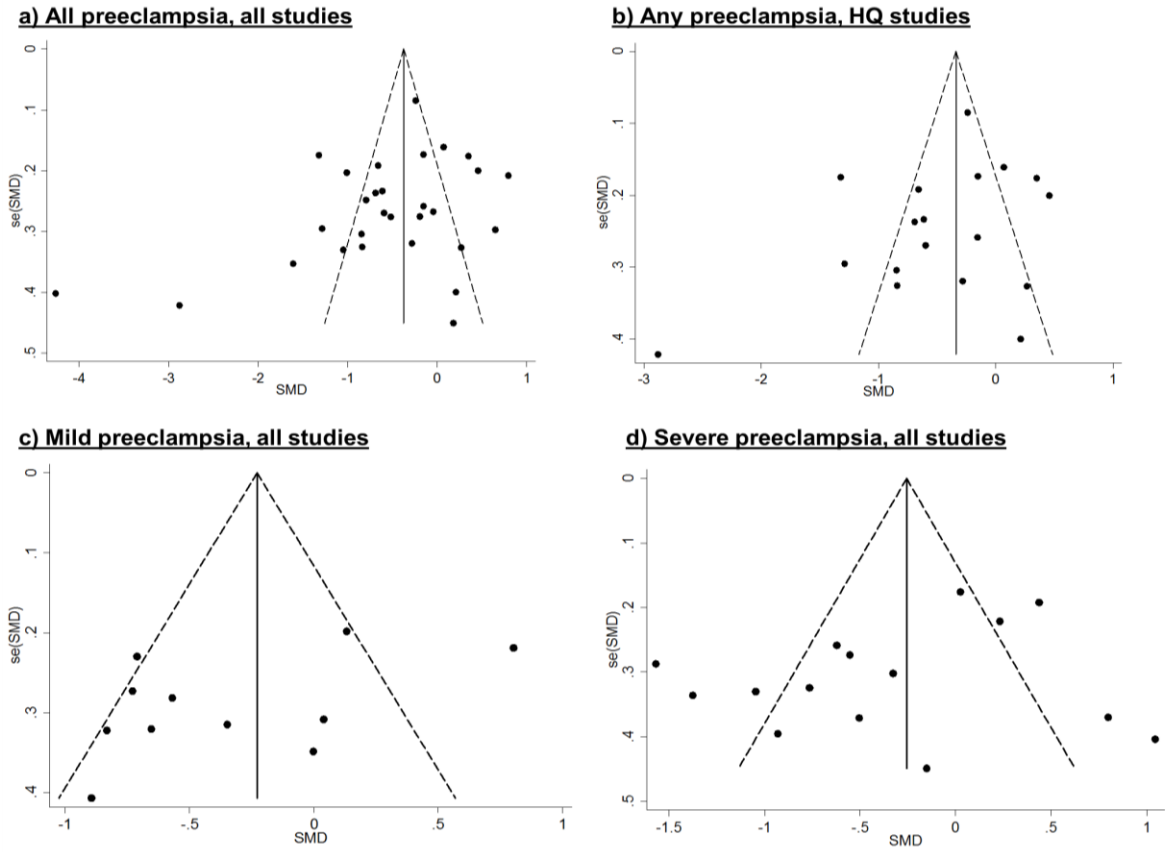

Figure 5: Funnel plots with pseudo-95% confidence intervals for vitamin C studies. High-quality (HQ) studies refer to those that addressed confounding in the design or analysis

4.9 Supplemental Digital Content

Appendix 1: Complete PubMed Search

Appendix 2: Manual for risk of bias assessment

Appendix 3: Details of methods used to combine study groups and description of pooling

Appendix 4: Complete list of included studies

Appendix 5: Description of data available that was not amenable to statistical pooling

Appendix 6: Additional details of study results not meta-analyzed

Appendix 7: Odds ratios reported in the reviewed studies for vitamin E and preeclampsia

Appendix 8: Results of influence analyses (for meta-analyses with 10 or more observations)

Appendix 9: Tables from additional meta-regression analyses

Appendix 1: Complete PubMed Search

Pubmed search conducted January 16, 2013 which was adapted to other databases:
(("Antioxidants"[Mesh] OR "Carotenoids"[Mesh] OR "Vitamin A"[Mesh] OR "Ascorbic Acid"[Mesh] OR antioxidant*[tiab] OR anti-oxidant* OR tocopherol*[tiab] OR "vitamin e"[tiab] OR carotenoid*[tiab] OR betacarotene*[tiab] OR beta-carotene*[tiab] OR carotene*[tiab] OR lycopene*[tiab] OR cryptoxanthin*[tiab] OR lutein*[tiab] OR "vitamin a"[tiab] OR retinol[tiab] OR "vitamin c"[tiab] OR ascorbate*[tiab] OR ascorbic acid[tiab]) AND ("Pre-Eclampsia"[Mesh] OR pre-eclampsia[tiab] OR preeclampsia[tiab] OR (toxemia[tiab] AND pregnancy[tiab]) OR "Infant, Small for Gestational Age"[Mesh] OR "small for gestational age"[tiab] OR "small-for-gestational-age"[tiab] OR SGA[tiab] OR "Fetal Growth Retardation"[Mesh] OR "fetal growth retardation"[tiab] OR "fetal growth restriction"[tiab] OR FGR[tiab] OR "intrauterine growth retardation"[tiab] OR "intra-uterine growth retardation"[tiab] OR "intrauterine growth restriction"[tiab] OR "intra-uterine growth restriction"[tiab] OR IUGR[tiab])) NOT animals[Mesh:noexp] AND ("1970/01/01"[PDAT] : "2013/12/31"[PDAT])

Appendix 2: Manual for risk of bias assessment

RB1. Was the study clinical setting well-described?
Yes = described clinical setting (teaching vs. non / tertiary care, location)
No = did not describe any
Unclear = missing one key descriptor

RB2. Were incomplete data (i.e. missing data) adequately described?
Yes = described proportion of missing data or described there was no missing data
No = no description of missing data or incomplete information on missing data
Unclear = no mention of missing data

RB3. Were statistical analyses described adequately?
Yes = described all analyses presented in methods section
No = missing important information on analyses
Unclear = some unclear portions of results

RB4. Were analyses appropriate?
Yes = appropriate
No = did not account for matching [if they did individual matching, they should use statistical methods that treat subjects as pairs, e.g. paired t-test, or conditional logistic regression], described distributions as skewed but used a t-test (which assumes normality), etc.
Unclear = not well described or missing key information

RB5. Did analysis provide sufficient presentation of data?
Yes = means and SD or median and IQR in figures or tables or text; AND presented patient characteristics by group (same groups as presented in analysis)
No = did not present characteristics of patients by groups stratified in analyses
Unclear = not well described or missing key covariates

RB6. Is the study report free of the suggestion of selective reporting?
Yes = all exposure-outcome relationships described
No = indication of selective outcome reporting, only select time points / markers
Unclear = judgement (data not shown)

RB7. Was confounding accounted for in the design and/or analysis?
Yes = used restriction or matching [design], stratification or adjustment [analysis] to control for confounding
No = did not account for confounding (only presented crude results)
Unclear = concern of residual confounding or unclear description of methods used

Appendix 3: Details of methods used to combine study groups and description of pooling

eTable 1: Description of case groups included and pooled by meta-analysis

Meta-analysis	Case groups pooled
All preeclampsia Includes any, mild, severe, superimposed, early-onset, late-onset preeclampsia, eclampsia	Mild & severe Preeclampsia & eclampsia Preeclampsia & superimposed preeclampsia Mild & severe & eclampsia
Mild preeclampsia	None
Severe preeclampsia Includes eclampsia	Severe & eclampsia

eTable 2: Equations used to pool means and standard deviations across study case groups*

	Group 1 (e.g. mild)	Group 2 (e.g. severe)	Combined groups
Sample size	N_1	N_2	$N_1 + N_2$
Mean	M_1	M_2	$\frac{N_1 M_1 + N_2 M_2}{N_1 + N_2}$
SD	SD_1	SD_2	$\sqrt{\frac{(N_1 - 1)SD_1^2 + (N_2 - 1)SD_2^2 + \frac{N_1 N_2}{N_1 + N_2}(M_1^2 + M_2^2 - 2M_1 M_2)}{N_1 + N_2 - 1}}$

*Adapted with permission from Table7.7.a in Higgins JPT, Deeks JJ (editors). Chapter 7: Selecting studies and collecting data. In: Higgins JPT, Green S (editors), Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 (updated March 2011). The Cochrane Collaboration, 2011. Available from http://handbook.cochrane.org/.

Stata code for program for combining groups

```
program combgroups, rclass
        version 11
        args n1 n2 m1 m2 sd1 sd2
        scalar n_comb=`n1'+`n2'
        scalar mean_comb=( [(`n1'*`m1') + (`n2'*`m2')]  / (`n1'+`n2') )
        scalar sd_comb= sqrt( ( [(`n1'-1)*`sd1'^2 ] + [(`n2'-1)*`sd2'^2 ] +
        [(`n1'*`n2')/(`n1'+`n2')] * [`m1'^2+`m2'^2-(2*`m1'*`m2')] ) / [`n1'+`n2'-1] )

        display "Combined N = " `n1'+`n2'
        display "Combined Mean = " ( [(`n1'*`m1') + (`n2'*`m2')]  / (`n1'+`n2') )
        display "Combined SD = " sqrt( ( [(`n1'-1)*`sd1'^2 ] + [(`n2'-1)*`sd2'^2 ] +
        [(`n1'*`n2')/(`n1'+`n2')] * [`m1'^2+`m2'^2-(2*`m1'*`m2')] ) / [`n1'+`n2'-1] )

        return scalar n=n_comb
        return scalar mean=mean_comb
        return scalar sd=sd_comb

end
```

Appendix 4: Complete list of included studies

Agarwal K, Dabke AT, Phuljhele NL, and Khandwal OP. Factors affecting serum vitamin A levels in matched maternal-cord pairs. *Indian journal of pediatrics* 2008: **75**; 443-446.

Akyol D, Mungan T, Gorkemli H, and Nuhoglu G. Maternal levels of vitamin E in normal and preeclamptic pregnancy. *Arch Gynecol Obstet* 2000: **263**; 151-155.

Azar M, Basu A, Jenkins AJ, Nankervis AJ, Hanssen KF, Scholz H, Henriksen T, Garg SK, Hammad SM, Scardo JA*, et al.* Serum carotenoids and fat-soluble vitamins in women with type 1 diabetes and preeclampsia: a longitudinal study. *Diabetes care* 2011: **34**; 1258-1264.

Bakheit KH, Ghebremeskel K, Zaiger G, Elbashir MI, and Adam I. Erythrocyte antioxidant enzymes and plasma antioxidant vitamins in Sudanese women with pre-eclampsia. *Journal of obstetrics and gynaecology* 2010: **30**; 147-150.

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Appendix 5: Description of data available that was not amenable to statistical pooling

eTable 3: Description of data available that was not amenable to statistical pooling

Study	Antioxidant	Data provided that prevented statistical pooling
Azar et al. (2011)	Lipid-corrected vitamin E α-carotene β-carotene lycopene	Geometric means (in figures)*
Bakheit et al. (2010)	Vitamin E Retinol	Median and interquartile range
Chappell et al. (2002)	Lipid-corrected vitamin E	Geometric means (in figures)
Dirican et al. (2008)	Lipid-corrected vitamin E	None, result described in text
Harsem, Braekke, and Staff (2006)	Vitamin E	Median and 95% confidence interval
Harsem et al. (2008)	Vitamin E	Median and 95% confidence interval
Hubel et al. (1997)	Vitamin E Lipid-corrected vitamin E Vitamin C	Median and interquartile range
Palan et al. (2004)	Vitamin E	Median and 10^{th} to 90^{th} percentiles
Rajasingam et al. (2009)	Lipid-corrected vitamin E	None, result described in text
Serdar et al. (2003)	Vitamin E Total carotene	ORs across tertiles ; only mean (SD) for lipid-corrected form
Wei et al. (2012)	β-carotene	OR for lowest versus highest quartile
Zusterzeel et al. (2002)	Vitamin E Vitamin C	Median and interquartile range

*The authors provided arithmetic means for retinol, α-tocopherol, and lutein; hence we could meta-analyzed the data extracted from figures for these markers. We were unable to pool the geometric means for γ-tocopherol, α-carotene, β-carotene, lycopene, α-tocopherol/total lipids, γ-tocopherol/total lipids

Appendix 6: Additional details of study results not meta-analyzed

Vitamin E

Vitamin E (α-tocopherol, total tocopherol, or unspecified) and preeclampsia

First/Second Trimester (5 studies)

One study measured vitamin E in the first trimester among women with diabetes and found no significant difference for vitamin E or lipid-corrected vitamin E between subjects who subsequently developed preeclampsia and controls (Azar et al. 2011). Five studies measured vitamin E in the second trimester (Azar et al. 2011; Ben-Haroush et al. 2002; Chappell et al. 2002; Rajasingam et al. 2009; Xu 2011). Four of the five studies reported no significant difference between cases and controls in vitamin E (Ben-Haroush et al. 2002; Xu 2011), or lipid-corrected vitamin E (Chappell et al. 2002; Rajasingam et al. 2009). Only the study by Azar et al. (2011) reported finding a significantly higher level of vitamin E among preeclampsia cases compared to controls; however, no difference was observed for lipid-corrected vitamin E.

Third Trimester (41 studies)

Among four other studies not included in this meta-analysis for reasons provided in Appendix 5, two reported similar levels in cases and controls (Harsem, Braekke, and Staff 2006; Harsem et al. 2008), and two reported higher levels of vitamin E in cases (Bakheit et al. 2010; Hubel et al. 1997); and in one, the difference was statistically significant (Bakheit et al. 2010). Two studies we were unable to pool reported significantly lower vitamin E in the third trimester for both mild and severe preeclampsia (Palan et al. 2004; Serdar et al. 2003), and one study found nonsignificantly higher levels in mild preeclampsia (Zusterzeel et al. 2002). Among four studies of preeclampsia that we were unable to pool, none reported an association between lipid-adjusted vitamin E measured in the third trimester and any preeclampsia (Hubel et al. 1997; Chappell et al. 2002; Azar et al. 2011) or mild/severe preeclampsia (Dirican et al. 2008).

γ-Tocopherol and preeclampsia

First/Second Trimester (3 studies)

Azar et al. (2011) found γ-tocopherol to be non-significantly higher in all three trimesters among women who developed preeclampsia, and correction for total lipids did not alter the findings. Rajasingam et al. (2009) reported that γ-tocopherol corrected for total cholesterol was similar at 14-22 weeks between women who would and would not later develop preeclampsia in a cohort of 385 high-risk (nulliparous, obese) women. The larger study by Xu (2011) found significantly elevated uncorrected γ-tocopherol at 12-18 weeks; the adjusted OR per 1 unit SD was 1.35 (1.02, 1.78).

Third Trimester (5 studies)

Among five studies that measured γ-tocopherol in the third trimester, none identified significant differences between cases of mild or severe preeclampsia and controls (adjusted for apoB) (Palan et al. 2004), or of any preeclampsia and controls (Zhang, Williams, et al. 2001; Williams et al. 2003; Roland et al. 2010; Azar et al. 2011). One of two large case-control studies observed a trend toward higher odds of preeclampsia among those in higher quartiles of uncorrected γ-tocopherol, even after adjustment for confounding (Williams et al. 2003).

Vitamin E (α-tocopherol, total tocopherol, or unspecified) and SGA (8 studies)

Two studies reported that vitamin E was significantly lower in the second trimester for pregnancies that resulted in an SGA birth vs. controls (Dreyfuss et al. 2001; Kerver et al. 2012); however, one reported that after adjusting for total cholesterol, the levels of lipid-adjusted vitamin E were similar between the groups (Kerver et al. 2012). This is consistent with the findings of three other studies which found no significant difference in the second trimester for lipid-adjusted vitamin E (total cholesterol) (Rajasingam et al. 2009; Scholl et al. 2006; Chappell et al. 2002).

Five studies measured vitamin E in the third trimester. One study reported it was significantly lower (Saker et al. 2008), one reported it was nonsignificantly higher (Ortega-Senovilla et al. 2010), and one study reported it was significantly higher for SGA vs. appropriate for gestational age (AGA) births (Schiff et al. 1996). Interestingly, the two studies with serial measures showed that lipid-adjusted α-tocopherol was not significantly different during the second trimester but became significantly lower in SGA vs. AGA at the start of the third trimester (28 weeks) (Scholl et al. 2006; Chappell et al. 2002).

γ-Tocopherol and SGA (4 studies)

No additional details

Vitamin C

Vitamin C and preeclampsia

First/Second Trimester (3 studies)

One study measured levels of vitamin C in the first trimester and found lower levels in women who subsequently developed preeclampsia (Zhang, Luthy, et al. 2001); the adjusted OR was 3.1 (95% CI: 1.1-9.4) for those below the 10^{th} percentile for vitamin C. Two studies measured vitamin C in the second trimester. One found similar levels for cases and controls at 14-22 weeks (Rajasingam et al. 2009), and the other, which assessed vitamin C every four weeks from 20 weeks, found significantly lower vitamin C at every time point except 24 weeks (Chappell et al. 2002).

Third Trimester (30 studies)

One additional study reported that vitamin C levels were significantly lower among preeclampsia cases (Hubel et al. 1997). Another study reported that the levels were nonsignficantly lower among mild preeclampsia cases (Zusterzeel et al. 2002).

Vitamin C and SGA (3 studies)

Three studies examined the association between vitamin C levels in pregnancy and birth of an SGA infant. Two studies took samples during the second trimester and reported

significantly lower levels among pregnant women with subsequent SGA birth (Rajasingam et al. 2009; Chappell et al. 2002). Two of these studies measured levels in the third trimester and also found lower vitamin C levels among cases (Chappell et al. 2002; Saker et al. 2008). In the one study with serial measurements, taken every 4 weeks from 20 weeks, women with SGA birth had significantly lower vitamin C levels than controls at every assessment (Chappell et al. 2002). It should be noted, however, that cases were women with SGA birth who were also at high risk for preeclampsia, and controls were women with AGA births and at low-risk for preeclampsia, so there may well have been confounding by baseline risk.

Vitamin A/Retinol

Retinol and preeclampsia

First/Second Trimester (2 studies)

One study measured retinol in each trimester of pregnancy among women with diabetes and similar found levels at each timepoint between women who developed preeclampsia and those who did not (Azar et al. 2011). Another study measured retinol levels in the second trimester and also found that levels were similar between PE cases and controls (Rajasingam et al. 2009).

Third Trimester (13 studies)

No additional details

Retinol and SGA (6 studies)

No additional details

Carotenoids

Total carotene and preeclampsia (5 studies)

Additionally, Dirican et al. (2008) found total carotene to be significantly lower in the third trimester for both mild and severe preeclampsia cases vs. controls. Serdar et al. (2003) reported a statistically significant OR of 7 for severe cases in the lowest versus highest tertile of total carotene in the third trimester; and all other ORs were above 1 but not significantly so.

β-carotene and preeclampsia (9 studies)

Among those we were unable to pool, Azar et al. (2001) found β-carotene was lower in preeclampsia cases in the first and third trimesters; the difference was not statistically significant in the second trimester. After adjusting for confounding (BMI, HDL cholesterol, prandial status), the difference was significant only in the third trimester, and results were consistent for lipid-corrected β-carotene (total lipids). A published abstract by Wei et al. (2011) reported an adjusted OR for the lowest vs. the highest quartile of 6.0 (3.1, 11.9) at 24-26 weeks (adjustment variables

unclear from abstract). However, in two large case-control studies, neither reported any crude or adjusted ORs across quartiles significantly different from 1, nor did they suggest any consistent trend across quartiles (Zhang, Williams, et al. 2001; Williams et al. 2003).

α-carotene and preeclampsia (4 studies)

Azar et al. (2001) could not be pooled and found significantly lower levels in the third trimester, even after values were corrected for total lipids; however, no association was observed in the first or second trimester. Crude and adjusted ORs were not significantly different from 1 in either study that reported ORs (Zhang, Williams, et al. 2001; Williams et al. 2003).

Lycopene and preeclampsia (5 studies)

Azar et al. (2001) found that lycopene was higher in the second, but not in the first or third trimester, for cases of preeclampsia vs. controls, even after adjusting for confounding. Zhang, Williams et al. (2001) reported ORs by quartile; crude ORs were not significantly different from 1 but all were below 1 (reference is lowest level) suggesting that higher levels could be protective; however, the adjusted ORs suggested the opposite. Adjustment variables included total cholesterol.

Other carotenoids and preeclampsia (4 studies)

No additional details

Carotenoids and SGA (1 study)

No additional details

Appendix 7: Odds ratios reported in the reviewed studies for vitamin E and preeclampsia

eTable 4: Odds ratios reported in the reviewed studies for vitamin E and preeclampsia

Xu 2011 12-18 weeks	Zhang 2001 36.8 ± 3.8 (cases and controls combined)	Williams 2003 37.4 ± 3.4 (cases and controls combined)	Serdar 2003 31-38 weeks
By quartile, lowest=reference	By quartile, lowest=reference	By quartile, lowest=reference	By tertile, highest=reference
Total tocopherol Q2 OR = 1.30 (0.67, 2.52) Q3 OR = 1.13 (0.54, 2.37) Q4 OR = 1.45 (0.65, 3.23) z-score OR = 1.11 (0.85, 1.46) Q2 AOR = 1.60 (0.78, 3.27)[1] Q3 AOR = 1.17 (0.54, 2.53) Q4 AOR = 1.34 (0.58, 3.08) z-score AOR = 1.11 (0.83, 1.49) a-tocopherol Q2 OR = 0.96 (0.50, 1.83) Q3 OR = 0.90 (0.44, 1.85) Q4 OR = 1.06 (0.49, 2.32) z-score OR = 1.05 (0.81, 1.37) Q2 AOR = 1.07 (0.54, 2.11)[1] Q3 AOR = 0.10 (0.48, 2.14) Q4 AOR = 1.00 (0.44, 2.24) z-score AOR = 1.06 (0.79, 1.42) g-tocopherol Q2 OR = 1.34 (0.65, 2.76) Q3 OR = 1.02 (0.49, 2.11) Q4 OR = 2.00 (0.95, 4.23) z-score OR = 1.48 (1.13, 1.92) Q2 AOR = 1.24 (0.58, 2.64)[1] Q3 AOR = 1.00 (0.46, 2.10) Q4 AOR = 1.63 (0.75, 3.37) z-score AOR = 1.35 (1.02, 1.78) g-/a-tocopherol ratio Q2 OR = 1.10 (0.58, 2.09) Q3 OR = 0.80 (0.41, 1.59) Q4 OR = 1.88 (0.94, 3.76) z-score OR = 1.52 (1.16, 2.00) Q2 AOR = 1.08 (0.56, 2.10) Q3 AOR = 0.80 (0.39, 1.67) Q4 AOR = 1.39 (0.71, 3.10) z-score AOR = 1.43 (1.08, 1.90)	g-tocopherol Q2 OR = 0.75 (0.38, 1.49) Q3 OR = 1.13 (0.59, 2.16) Q4 OR = 1.30 (0.69, 2.45) Q2 AOR = 0.47 (0.20, 1.08)[2] Q3 AOR = 1.30 (0.61, 2.77) Q4 AOR = 1.27 (0.59, 2.71) a-tocopherol Q2 OR = 1.24 (0.61, 2.52) Q3 OR = 1.26 (0.62, 2.54) Q4 OR = 2.38 (1.23, 4.60) Q2 AOR = 1.71 (0.75, 3.93)[2] Q3 AOR = 1.83 (0.70, 4.75) Q4 AOR = 4.98 (1.77, 13.98) Q2 AOR = 1.43 (0.61, 3.34)[3] Q3 AOR = 1.22 (0.45, 3.32) Q4 AOR = 3.13 (1.06, 9.23) a-tocopherol/total cholesterol Q2 OR = 1.69 (0.82, 3.38) Q3 OR = 1.44 (0.68, 3.02) Q4 OR = 2.88 (1.22, 5.57) Q2 AOR = 1.73 (0.76, 3.92)[4] Q3 AOR = 1.85 (0.81, 4.24) Q4 AOR = 3.47 (1.60, 7.57) a-tocopherol/total lipids Q2 OR = 1.29 (0.66, 2.54) Q3 OR = 1.28 (0.65, 2.52) Q4 OR = 1.63 (0.84, 3.13) Q2 AOR = 1.49 (0.69, 3.19)[4] Q3 AOR = 1.57 (0.73, 3.35) Q4 AOR = 2.16 (1.03, 4.52)	g-tocopherol Q2 OR = 1.68 (0.92, 3.12) Q3 OR = 1.32 (0.70, 2.50) Q4 OR = 2.26 (1.24, 4.14) Q2 AOR = 1.43 (0.68, 2.98)[5] Q3 AOR = 0.97 (0.45, 2.08) Q4 AOR = 1.44 (0.68, 3.06) a-tocopherol Q2 OR = 1.16 (0.58, 2.31) Q3 OR = 1.78 (0.93, 3.42) Q4 OR = 3.69 (1.99, 6.82) Q2 AOR = 1.10 (0.50, 2.46)[6] Q3 AOR = 1.15 (0.52, 2.56) Q4 AOR = 1.65 (0.75, 3.60)	T1 OR mild = 3.6 (0.9, 14.9) T2 OR mild = 1.9 (0.6, 6.1) T1 OR severe = 6.6 (1.6, 27.7) T2 OR severe = 2.0 (0.6, 7.4)

1. Adjustment variables: smoking, the presence of pre-selected clinical risk condition (i.e. chronic hypertension, history of preeclampsia, diabetes), prenatal regular using of vitamins or mineral supplementation, intervention status (vitamins supplementation vs. placebo), gestational age and baseline BMI

2. Adjustment variables: maternal age, nulliparity, prepregnancy body mass index (quartile), use of prenatal vitamins, gestational age at blood collection, education, planned pregnancy, and total cholesterol concentration
3. Adjustment variables: maternal age, nulliparity, prepregnancy body mass index (quartile), use of prenatal vitamins, gestational age at blood collection, education, planned pregnancy, and total lipid concentration (2 x cholesterol + triglycerides)
4. Adjusted for maternal age, nulliparity, prepregnancy body mass index (quartile), use of prenatal vitamins, gestational age at blood collection, education, and planned pregnancy
5. Adjustment variables: maternal age (<19; 19–34; and ≥35 years), nulliparity (yes/no), maternal adiposity, midarm circumference (continuous), gestational age (continuous), and prenatal vitamin use (yes/no)
6. Adjustment variables: maternal age (<19; 19–34; and ≥35 years), nulliparity (yes/no), maternal adiposity, midarm circumference (continuous), gestational age (continuous), prenatal vitamin use (yes/no), and plasma total triglycerides (quartile)

Appendix 8: Results of influence analyses (for meta-analyses with 10 or more observations)

eTable 5: Results of influence analyses (for meta-analyses with 10 or more observations)

Meta-Analysis	Results of Influence Analysis
Vitamin A, All PE	None of these studies were highly influential on the overall result; however, the Jendryczko 1989 study was an outlier and the confidence interval did not overlap with any of the others; SMD -4.35 (-6.02, -2.68)
Vitamin C, All PE	Howlader 2007 and Nilar 2009 were somewhat influential on the overall result; however, exclusion of neither study changed the overall conclusion
Vitamin C, Mild PE	Sharma 2006 study was influential on the overall result. It is the only study that found significantly higher levels of vitamin C in cases but mistakenly reported in the text that they found the levels were lower. When we omitted this study from the meta-analysis, we obtained a significantly negative pooled SMD; -0.43 (-0.69, -0.18).
Vitamin C, Severe PE	None of these studies were very highly influential on the overall result. Exclusion of Sharma 1984, Islam 2004, Noyan 2006, or Sharma 2006 studies would have resulted in a significantly negative pooled SMD.
Vitamin E, All PE	None of these studies were highly influential on the overall result
Vitamin E, Mild PE	Kaur 2008 study was influential on the overall result. Omitting this study from the meta-analysis resulted in a narrower confidence interval and a pooled SMD -0.18 (-0.54, 0.18). However, exclusion of this study would not change the overall conclusion of no significant difference. The Akyol 2000 study was also somewhat influential in the opposite direction, but did not impact the conclusion of no difference.
Vitamin E, Severe PE	None of these studies were highly influential on the overall result
Lipid-Adjusted Vitamin E, All PE	None of these studies were highly influential on the overall result

Appendix 9: Tables from additional meta-regression analyses

eTable 6: Meta-regression for α-tocopherol & all preeclampsia (N=15; SMD= -0.35, 95% CI -0.66,-0.03, I^2=88%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	1					
Study Quality						
Addressed Confounding	10	0.85	0.23, 1.46	0.01	78.7	48.34
NOS>4.5 (above median)	7	0.47	-0.24, 1.18	0.18	84.3	10.05
Matched	4	0.28	-0.57, 1.14	0.48	88.5	-3.41
Population / Setting						
General Population	13	0.23	-0.95, 1.40	0.68	89.0	-7.30
High-risk for preeclampsia	2	-0.23	-1.40, 0.95	0.68	89.0	-7.30
LMIC	8	-0.22	-0.98, 0.54	0.55	88.7	-9.15
Exposure Characteristics						
Fasting	8	-0.72	-1.34, -0.10	0.03	80.3	36.91
HPLC	13	1.06	0.10, 2.01	0.03	86.5	25.56
Pre-labor sample	6	-0.22	-1.00, 0.55	0.55	86.9	-4.45
MD gestational age (per wk)	13	-0.22	-0.49, 0.06	0.12	84.1	21.94
MD maternal age (per year)	15	-0.14	-0.42, 0.13	0.27	89.2	-2.47
MD BMI (per unit kg/m2)	7	-0.09	-0.51, 0.33	0.62	81.7	-23.11
Multivariable model intercept*	14	-0.85	-1.51, -0.20	0.02	63.4	76.81
HPLC		1.07	0.39, 1.76	0.01		
Fasting		-0.84	-1.30, -0.39	<0.01		

eTable 7: Meta-regression for vitamin E & mild preeclampsia (N=12; SMD= 0.09, 95% CI -0.55, 0.72, I^2=93%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	1					
Study Quality						
Addressed Confounding	6	0.14	-1.59, 1.86	0.86	93.4	-10.57
NOS>4.5 (above median)	7	-1.00	-2.61, 0.59	0.19	93.0	8.40
Matched	3	0.14	-1.86, 2.15	0.88	93.3	-10.42
Population / Setting						
General Population	9	0.39	-1.58, 2.36	0.67	93.4	-8.52
High-risk for PE	2	-0.64	-2.89, 1.62	0.54	93.2	-6.22
LMIC	7	-0.06	-1.81, 1.69	0.94	93.8	-11.13
Exposure Characteristics						
Fasting	4	-0.46	-2.26, 1.34	0.58	93.2	-6.83
HPLC	9	1.18	-0.65, 3.01	0.18	93.3	8.33
Pre-labor sample	4	-0.26	-2.08, 1.56	0.75	93.5	-9.68
α-tocopherol	4	-0.35	-2.17, 1.46	0.67	93.4	-8.55
Definition includes adverse events	8	-0.13	-1.96, 1.70	0.88	93.8	-10.98
MD gestational age (per wk)	8	0.05	-0.51, 0.60	0.84	81.8	-22.92
MD maternal age (per year)	7	0.15	-0.40, 0.71	0.50	82.2	-12.60
MD BMI (per unit kg/m2)	0					
Multivariable model intercept*	11	-0.36	-1.89, 1.17	0.60	92.8	35.4
NOS>4.5 (above median)		-1.40	-2.96, 0.17	0.07		
HPLC		1.71	-0.05, 3.48	0.06		

eTable 8: Meta-regression for vitamin E & severe preeclampsia (N=17; SMD= -0.13 95% CI -0.57, 0.30, I^2=90%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	1					
Study Quality						
Addressed Confounding	8	-0.13	-1.07, 0.82	0.78	90.2	-7.15
NOS>4.5 (above median)	8	-0.28	-1.22, 0.65	0.52	90.1	-4.43
Matched	5	-0.07	-1.11, 0.97	0.89	90.0	-7.61
Population / Setting						
General Population	12	0.12	-0.93, 1.16	0.81	90.2	-7.04
High-risk for PE	4	-0.11	-1.25, 1.02	0.84	90.2	-7.08
LMIC	12	-0.40	-1.41, 0.61	0.41	90.0	-2.39
Exposure Characteristics						
Fasting	5	-0.70	-1.67, 0.28	0.15	88.4	8.83
HPLC	12	0.54	-0.45, 1.54	0.26	89.0	3.35
Pre-labor sample	5	-0.19	-1.23, 0.84	0.70	90.1	-6.59
A-tocopherol	6	-0.74	-1.64, 0.17	0.10	87.3	13.13
Definition includes adverse events	10	-0.26	-1.21, 0.69	0.57	90.2	-5.27
MD gestational age (per wk)	13	0.05	-0.16, 0.25	0.62	83.1	-5.33
MD maternal age (per year)	12	0.13	-0.04, 0.31	0.12	78.2	17.16
MD BMI (per unit kg/m2)	4	0.27	-0.76, 1.30	0.38	78.5	15.44
Multivariable model intercept	12	-0.18	-0.60, 0.24	0.35	63.4	56.50
WMD maternal age (per year)		0.17	0.02, 0.31	0.03		
Fasting		-1.01	-1.90, -0.11	0.03		

eTable 9: Meta-regression for vitamin C & all preeclampsia (N=29; SMD= - 0.56, 95% CI -0.83,-0.28, I^2=91%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	1					
Study Quality						
Addressed Confounding	18	0.19	-0.62, 1.00	0.49	91.2	-3.39
NOS>4.5 (above median)	10	-0.66	-1.44, 0.12	0.09	90.5	7.94
Matched	9	0.21	-0.63, 1.05	0.62	90.8	-3.08
Population / Setting						
General Population	23	-0.25	-1.22, 0.72	0.60	91.2	-3.42
High-risk for PE	4	0.19	-0.95, 1.33	0.74	91.2	-4.00
LMIC	21	-0.26	-1.13, 0.62	0.55	91.2	-3.15
Exposure Characteristics						
Fasting	9	0.15	-0.70, 1.00	0.72	91.1	-4.11
HPLC	6	-0.01	-0.99, 0.96	0.98	91.1	-4.38
Pre-labor sample	9	-0.54	-1.37, 0.28	0.19	90.4	3.73
MD gestational age (per wk)	23	0.00	-0.25, 0.25	0.99	92.3	-5.47
MD maternal age (per year)	24	0.01	-0.26, 0.27	0.96	91.1	-5.36
MD BMI (per unit kg/m2)	7	0.09	-0.16, 0.34	0.40	64.5	3.65

eTable 10: Meta-regression for vitamin C & mild preeclampsia (N=11; SMD= -0.32, 95% CI -0.67, 0.03, I^2=77%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	0					
Study Quality						
Addressed Confounding	6	-0.24	-1.02, 0.54	0.5	79.0	-8.05
NOS>4.5 (above median)	4	-0.69	-1.39, 0.00	0.05	68.9	35.51
Matched	3	0.27	-0.59, 1.13	0.49	78.7	-7.26
Population / Setting						
General Population	9	0.36	-0.64, 1.36	0.44	77.6	-3.03
High-risk for PE	1					
LMIC	7	0.42	-0.37, 1.20	0.26	76.0	6.07
Exposure Characteristics						
Fasting	5	-0.51	-1.19, 0.18	0.13	67.5	26.59
HPLC	3	-0.67	-1.46, 0.13	0.09	72.1	25.42
Pre-labor sample	3	-0.58	-1.37, 0.21	0.13	72.8	20.58
Definition includes adverse events	8	0.18	-0.70, 1.07	0.65	78.51	-9.15
MD gestational age (per wk)	6	0.13	-0.05, 0.31	0.11	0	
MD maternal age (per year)	6	0.06	-0.10, 0.23	0.36	0	
MD BMI (per unit kg/m2)	0					
Multivariable model intercept	11	0.12	-0.30, 0.54	0.55	55.1	58.10
NOS>4.5 (above median)		-0.63	-1.26, 0.00	0.05		
Fasting		-0.46	-1.05, 0.13	0.11		

eTable 11: Meta-regression for vitamin C & severe preeclampsia (N=15; SMD= -0.35, 95% CI -0.72, 0.01, I^2=84%)

Covariate	No.	β-coef	95% CI	P	I^2, %	Adj. R^2, %
Univariate models						
Prospective design	0					
Study Quality						
Addressed Confounding	7	0.08	-0.78, 0.95	0.84	83.6	-8.63
NOS>4.5 (above median)	4	-0.69	-1.57, 0.21	0.12	81.2	13.3
Matched	4	0.60	-0.29, 1.49	0.17	77.7	13.55
Population / Setting						
General Population	12	0.11	-1.00, 1.22	0.83	84.5	-8.66
High-risk for PE	2	0.02	-1.32, 1.36	0.98	84.8	-8.73
LMIC	11	0.29	-0.68, 1.25	0.53	84.9	-7.15
Exposure Characteristics						
Fasting	6	-0.24	-1.12, 0.65	0.57	83.1	-5.07
HPLC	4	-0.55	-1.50, 0.39	0.23	82.6	5.56
Pre-labor sample	3	-0.42	-1.48, 0.63	0.40	83.6	-2.52
Definition includes adverse events	9	-0.73	-1.50, 0.05	0.06	79.8	23.15
MD gestational age (per wk)	10	0.09	-0.14, 0.32	0.41	83.9	-3.64
MD maternal age (per year)	10	0.02	-0.32, 0.36	0.89	84.2	-16.26
MD BMI (per unit kg/m2)	3	0.11	-1.71, 1.92	0.59	56.6	-48.8
Multivariable model intercept	15	0.19	-0.41, 0.79	0.51	76.8	32.17
NOS>4.5 (above median)		-0.57	-1.40, 0.27	0.16		
Definition includes adverse events		-0.64	-1.40, 0.11	0.09		

For all tables in Appendix 9: *Restricted to prospective studies. CI, confidence interval; HPLC, high-performance liquid chromatography; LMIC, low- or middle-income country; MD, mean difference; NOS, Newcastle-Ottawa Scale [score]

Chapter 5. [Manuscript 2] Small-for-gestational-age birth and maternal plasma antioxidant levels in midgestation: a nested case-control study

5.1 Preface to Manuscript 2

In our systematic review, we identified nine studies which assessed the association between antioxidant levels in pregnancy and SGA. Six of those studies measured antioxidants before recognition of SGA in the context of nested case-control or cohort study or an RCT sub-study. Limitations of previous studies suggested the need for additional rigorous prospective studies of the association between maternal antioxidant levels in pregnancy and SGA birth. Existing studies were mostly small, with a wide gestational age range within which antioxidant levels were measured. While it would be of interest to look at different time windows of gestational age, most studies were small and grouped together all cases and non-cases, regardless of the timing of exposure measurement. Most prior studies measured few biomarkers. Only one unpublished study measured carotenoids, and its findings therefore required confirmation. In addition, confounding may have been responsible for some of the reported associations.

In this study, we examined the independent association between antioxidant levels and SGA birth by restricting to non-preeclamptic term births. The systematic review demonstrated some suggestive associations between antioxidant levels and preeclampsia. Furthermore, a previous study by Kramer *et al.* based on the same pregnancy cohort found associations between antioxidant levels and preterm birth.[153] By restricting to term births, we remove the potential confounding by preterm birth. Hutcheon and Platt also showed that SGA birth is underestimated at preterm ages when defined by birth weights of live-born preterm infants.[154] We avoid this problem by restricting our study to term births.

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5.2 Title Page

Small for gestational age birth and maternal plasma antioxidant levels in midgestation: a nested case-control study

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Shortened running title: Midgestation antioxidant levels and SGA birth

5.3 Abstract

Objective: To assess whether maternal plasma antioxidant levels in mid-pregnancy are associated with small for gestational age (SGA) birth.

Design: Case-control study nested within a population-based cohort study.

Setting: Four hospitals in Montreal, Canada.

Population: Pregnant women recruited before 24 weeks gestation, whose pregnancies were not complicated by preeclampsia or pre-term delivery.

Methods: Blood samples were obtained at 24-26 weeks and assayed for nutritionally-derived antioxidant levels in SGA cases (n=342) and randomly selected controls with birthweights between the 25th and 75th percentiles (n=672). We performed logistic regression analyses using the standardized z-score of each antioxidant as the main independent variable, after summing highly correlated antioxidants or combining via principal component analysis. We adjusted for risk factors for SGA that were associated with antioxidant levels.

Main Outcome Measures: SGA, birthweight <10th percentile for gestational age and sex

Results: Retinol was positively associated with risk of SGA [adjusted OR=1.41 (95% CI 1.22 – 1.63) per SD increase]. Carotenoids (log of the sum of β-carotene, lutein/zeaxanthin, α- and β-cryptoxanthin) were negatively associated with SGA [adjusted OR=0.64 (0.54 – 0.78) per SD increase]. We found no significant associations between SGA and lycopene or any of the forms of vitamin E assessed including α-tocopherol, corrected α-tocopherol (per nmol/L of LDL particles), or γ-tocopherol.

Conclusions: Elevated retinol may be associated with an increased risk of SGA, whereas elevated carotenoid levels may reduce the risk. A better understanding of the nature of these associations is required, however, before recommending specific nutritional interventions in an attempt to prevent SGA birth.

Keywords: antioxidants, carotenoids; retinol; small for gestational age; vitamin E

Abbreviations: SGA, small for gestational age; IUGR, intrauterine growth restriction; BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; OR, odds ratio

5.4 Introduction

Small for gestational age (SGA) is usually defined as an infant below the 10th percentile of gestational age- and sex-specific birthweight, according to an appropriate population reference.[155] Being SGA is a strong predictor of stillbirth, [156,157] neonatal morbidity and mortality, [11,12] though whether disrupted growth is simply a marker or has any causal impact is unclear.[158] Fetal growth restriction and resultant SGA predict later growth,[16] neurodevelopment (including cognitive ability and risk of cerebral palsy),[13-15] and adult chronic disease.[17]

Well-established risk factors for SGA include primiparity, low pre-pregnancy body mass index (BMI), short stature, smoking, and preeclampsia.[9,64,159,160] Cigarette smoking is the single-most important risk factor for SGA in developed countries; however, smoking cessation in early pregnancy can reduce the risk to that of non-smokers.[66]

Oxidative stress, which results from an imbalance between generation of potentially harmful oxygen free radicals and antioxidant defenses, may be involved in the etiology of fetal growth restriction and subsequent SGA delivery. A number of studies reporting markers of oxidative stress and antioxidant levels in women who have recently delivered an SGA infant, or in women whose fetus is estimated to be at increased risk of SGA by ultrasound, suggest that this mechanism may play a role.[19,20,22,23,96] Previous studies have repeatedly shown that markers of oxidative stress are also increased in cord blood among SGA births compared to non-SGA births.[19,22,23] Furthermore, there is some evidence to suggest elevated levels of oxidative stress exist earlier in pregnancy and hence may be of causal importance, rather than being a consequence of SGA.[161]

In contrast to many of the risk factors for SGA that have been identified, nutritionally-derived antioxidants represent potentially modifiable exposures. Antioxidants are derived in large part from fruits and vegetables in the diet. There is limited evidence, however, as to whether maternal antioxidant levels in early to mid-pregnancy, a causally relevant time period, are systematically different among pregnancies resulting in SGA birth compared to those resulting in an appropriate for gestational age (AGA) birth. We hypothesized that higher levels of antioxidants in maternal blood may provide some protection from SGA birth. Large, prospective studies assessing the association between antioxidant levels in pregnancy and risk of SGA, while controlling for potential confounding factors are lacking. Hence, the aim of this

study was to prospectively measure a broad panel of plasma antioxidant biomarker levels in relation to the risk of SGA.

5.5 Methods

We carried out secondary data analyses from a case-control study nested within a population-based cohort study of over 5,000 pregnant women recruited from four Montreal hospitals in 1999-2004. The McGill Faculty of Medicine Institutional Review Board approved the current study in November 2012. Details of the study have been described elsewhere.[162] Briefly, pregnant women at least 18 years of age, fluent in English or French, and planning to deliver at one of the study hospitals were eligible to participate. Consecutive pregnant women were approached for recruitment at each of four participating hospitals up to 24 weeks gestation. With the exception of diabetes, hypertension, and asthma, women with pre-existing chronic conditions were excluded. In addition, pregnancies with a diagnosed fetal abnormality, multifetal gestation, or a history of incompetent cervix were excluded. Subjects provided written informed consent at the time of recruitment.

Cases of SGA were identified retrospectively based on recorded birthweight and gestational age in medical records. Cases were mothers of live-born term (≥37 completed weeks) singleton infants whose birthweight fell below the 10^{th} percentile of weight for gestational age and sex based on a Canadian national reference.[163] For some of the analyses, cases were subdivided into severe SGA (<5^{th} percentile) and mild SGA (5^{th} to <10^{th} percentile). Control subjects were randomly selected at an approximate 2:1 ratio among the mothers of healthy, term live births with a birthweight between the 25^{th} and 75^{th} percentiles of birthweight for gestational age. While appropriate for gestational age (AGA) birth is most often defined as a birthweight in the 10^{th} to 90^{th} percentile for gestational age and sex, we chose to focus on birth weights in a more narrow range to avoid misclassification and allow greater contrast between cases and controls. Pregnancies complicated by preeclampsia or resulting in preterm birth were ineligible to be cases or controls, as the blood samples for those subjects had previously been analyzed for other biomarker studies,[34,164] leaving insufficient sample for the current study. However, these

exclusions may improve the internal validity of the current study by eliminating potential confounding by preterm birth and preeclampsia.

All cohort participants attended a study visit at 24-26 weeks and responded to a prenatal questionnaire which addressed demographic, medical, obstetrical, and family history. At the same visit, a research nurse obtained a non-fasting blood sample in two 7-ml EDTA-containing tubes by venipuncture. The tubes were placed on ice and brought immediately to the hospital's biochemistry laboratory, where plasma was separated by centrifugation. Five aliquots of 1.0 mL plasma were stored in 2-mL cryovials. These samples were stored for up to one week at -20°C. We were not concerned that short term storage at -20°C would affect antioxidant concentrations since a review of the literature concluded that retinol, α-tocopherol, and carotenoids are stable for up to 5 months at -20°C.[29] Each week, samples were transported on dry ice to a -80°C freezer for later analysis of case and control samples. Samples were stored until 2012, when they were analyzed for antioxidant levels for the current study. Samples were handled under subdued lighting to prevent photo-oxidative damage.

Plasma α- and γ-tocopherol, β-carotene, retinol, lycopene, lutein/zeaxanthin, and α- and β-cryptoxanthin concentrations were analyzed in the Heinz Nutrition Laboratory (Pittsburgh, PA). These compounds have been shown to be stable in storage at -70^{o}C for at least 15 years.[119,165] Antioxidants were measured using a modification of an isocratic high-performance liquid chromatography (HPLC) procedure developed by Chromsystems Diagnostics (Munich, Germany). Retinol acetate and tocopherol acetate were added as internal standards prior to extraction. Ascorbic acid and butylhydroxytoluene (BHT) were added to prevent carotenoid losses during extraction and evaporation. The HPLC consisted of Water Corp. (Milford, MA) modules: two 515 pumps, a 717 Plus auto-sampler, and a 2996 photodiode array detector. The tocopherols, retinol, and carotenoids were monitored at 290 nm, 325 nm, and 453 nm, respectively. Blanks, calibrators, and controls were analyzed with each set of samples.

The samples were assayed blind to case versus control status. The intra-assay coefficients of variation were: β-carotene, 4.1%; retinol, 5.0%; α-tocopherol, 3.2%; γ-tocopherol, 6.9%; α-cryptoxanthin, 5.7%; β-cryptoxanthin, 2.9%; lutein/zeaxanthin, 2.2%; lycopene 2.6%. The inter-assay CV% for the control pools were: β-carotene, 8.4%; retinol, 9.1%; α-tocopherol, 4.2%; γ-

tocopherol, 8.4%; lycopene 8.4%. In the control pools, the concentrations of xanthophylls were much lower than in the samples hence we do not report a CV%.

Lipoprotein profiles were determined at Liposcience (Raleigh, NC) by nuclear magnetic resonance (NMR) spectroscopy using the LipoProfile-3 algorithm, as detailed in a previous publication.[162]

Statistical Analysis

Antioxidant levels were assessed as continuous variables in all analyses. Since the distributions of most markers were positively skewed, we examined medians and interquartile ranges (IQR) for each of the antioxidants measured and assessed crude differences by the non-parametric Wilcoxon rank-sum test. If distributions were skewed, we log-transformed raw values before standardizing based on the mean and SD among study controls. Given our sample size, we determined that we had 90% power to detect a difference of 0.22 units in the z-score between groups, based on the two-sided t-test with $\alpha=0.05$.

The ratio of α-tocopherol to cholesterol has been reported to be the most useful measurement of vitamin E status in plasma.[148] Since tocopherols are carried in the plasma by lipoproteins, primarily LDL cholesterol,[166] we calculated corrected α-tocopherol levels adjusted for LDL lipoprotein particle concentration in maternal plasma (nmol/L), as total cholesterol was not measured.

Since some of the antioxidants we studied are highly related in terms of dietary origins and characteristics, we expected that some would be highly correlated. We wished to avoid statistically adjusting models of one marker for other correlated markers, owing to concerns about multicollinearity. If correlation between the biomarkers among controls exceeded the pre-specified cut-off ($r^2>0.3$, chosen based on discussion amongst the co-authors), the sum was used as the predictor variable in further analyses. As a sensitivity analysis, we also performed a principal component analysis (PCA) on the control sample to identify a set of uncorrelated components or factors that account for the majority of the variation within the dataset. Variables included in the PCA were the z-scores of each antioxidant (including the adjusted α-tocopherol concentration, not the unadjusted form).

We used logistic regression models to assess whether antioxidant levels were associated with the risk of SGA, after accounting for important confounders. We used the z-score of each antioxidant as the main independent variable, after pooling highly correlated and structurally similar antioxidants. We assessed linear, quadratic, and cubic models for each antioxidant. We also carried out multinomial logistic regression analyses to simultaneously estimate adjusted odds ratios across the three categories of birthweight among cases and controls.

Variables considered as potential confounders included maternal age, height, pre-pregnancy BMI, primiparity, alcohol use, smoking, geographic region of birth, language spoken at home, maternal education, family income, living arrangement, asthma, and pre-existing hypertension. Confounders selected *a priori* included maternal age, pre-pregnancy BMI, height, and smoking. We assessed whether to include other potential confounders by examining the relation between the variable and the outcome using logistic regression, and each antioxidant z-score in the study controls using linear regression. If the variable or at least one indicator variable was associated with SGA, and with one or more of the antioxidant biomarkers among the controls with $p<0.2$, it was included in all adjusted models. Since we had a large number of cases and controls, we were not very limited in terms of the number of degrees of freedom in our models.[167] We used the opportunity to more accurately capture the association between age and BMI with SGA. We compared linear, quadratic, and cubic models for the relation between each of the continuous covariates (maternal age, BMI, height) and SGA using the likelihood ratio test (LRT).

Some data were missing for confounders, particularly income and BMI. We assumed that these were missing at random (MAR),[168] i.e. that these values did not depend on unobserved variables conditional on observed data, and that we could therefore impute these values. We imputed height and weight separately and recalculated BMI. We used multiple imputation with chained equation according to the procedures described by White *et al.*[169]

A recent publication suggested that associations between elevated lipophilic biomarkers and birthweight reductions may be attributable to confounding by gestational weight gain. Gestational weight gain is associated with increases in the volume of distribution for lipophilic molecules, and is also positively associated with birthweight. Therefore, in sensitivity analyses, we assessed the associations between each of the antioxidant biomarkers and SGA risk, with

adjustment for gestational weight gain up to 24-26 weeks, calculated by subtracting the weight measured at that visit from self-reported pre-pregnancy weight.

5.6 Results

Among 5,337 women recruited in early pregnancy who attended the 24-26 week study visit, 176 were lost to follow-up (**Figure 1**). The 4,885 term deliveries included 324 cases of isolated SGA. We randomly selected 672 controls between the 25th and 75th percentile of birthweight for gestational age and sex. The mean gestational age in both groups was 39 weeks.

Baseline characteristics of cases and controls are described in **Table 1**. As expected, the SGA group included a higher proportion of underweight women, primiparae, and smokers. Cases tended to have fewer years of education and a lower annual family income.

Table 2 compares medians and interquartile ranges of the eight antioxidant biomarkers in cases and controls. The crude levels of retinol, β-carotene, lutein/zeaxanthin, and α- and β-cryptoxanthin were significantly different between cases and controls. However, when we assessed correlations amongst the biomarkers, we found that β-carotene, lutein/zeaxanthin, and α- and β-cryptoxanthin were highly correlated (**Tables S1, S2**). Hence, we chose to pool these markers together by summing the four into a single variable called "carotenoids," which we log-transformed before calculating the z-score for further analysis.

A quadratic model for maternal age, cubic for BMI, and a linear model for height provided the best-fitting models for the relationship with SGA. In all cases except for retinol, neither the quadratic nor cubic models fit the data significantly better than the linear model. For retinol, the quadratic model fit significantly better than the linear model, but since the relation was close to linear and the confidence intervals for the linear and quadratic models overlapped for most of the range of the observed data, we chose to model it linearly in further analyses (**Figure 2**).

Slight differences were observed between the crude models including only the z-score for the antioxidant in question and the multivariable adjusted model including confounders (**Table 3**); however, the adjusted models did not change our conclusions. Higher levels of retinol were associated with an increased risk of SGA, whereas higher levels of carotenoids were associated

with a reduced risk of SGA; adjusted ORs were 1.41 (95% CI 1.22, 1.63) and 0.64 (0.54, 0.78) per SD increase, respectively. The tocopherols were non-significantly associated with an increased risk, and lycopene with a reduced risk of SGA. A sensitivity analysis excluding subjects with diabetes, chronic hypertension, or asthma resulted in similar ORs and did not alter any conclusions (results not shown). The principal components analysis (**Tables S3, S4**) did not change our conclusions with respect to the negative association between carotenoid levels and risk of SGA and positive association between retinol and risk of SGA described above. Further, PCA emphasized that the effects of the individual carotenoids measured, with the exception of lycopene, could not be reliably estimated due to a high degree of correlation amongst these markers.

Consistent with the primary analysis, increased plasma levels of retinol were associated with a significantly increased risk of both mild and severe SGA, and increased carotenoid levels were associated with a significantly reduced risk of both mild and severe SGA in the multinomial logistic regression analyses (**Table 4**). Among the tocopherol biomarkers, point estimates were in the positive direction.

Sensitivity analyses did not demonstrate substantial confounding of the observed relationships by early gestational weight gain (data not shown). As expected, weight gain was negatively associated with the risk of SGA (OR = 0.85 per 5 lbs). Based on the model by Verner et al, we expected higher weight gain to be associated with a reduction in plasma biomarker levels. With the exception of γ-tocopherol and corrected α-tocopherol, however, this was not observed. Weight gain was positively associated with the z-score of retinol, suggesting some confounding in the direction opposite to that observed by Verner *et al.*

5.7 Discussion

Main findings

In this case-control study nested within a general obstetrical population cohort, we found an increased risk of SGA associated with elevated retinol and decreased risk associated with elevated carotenoids in maternal plasma in mid-pregnancy. Tocopherol biomarkers were positively, and lycopene negatively, but non-significantly associated with the risk of SGA.

Strengths and Limitations

One strength of our study is the large, multi-centre cohort of pregnant women from whom we derived our cases and controls. Unlike traditional case-control studies, recall bias with respect to confounders is not an issue here, owing to the prospective nature of data collection. The nested case-control design enabled prospective biomarker measurement. Blood samples were taken within a short time window of pregnancy (24-26 weeks gestation), thus ensuring that the samples could be compared in a valid fashion between cases and controls. Since blood antioxidant levels may naturally change over the course of pregnancy, it is important that samples be obtained at a similar gestational age for all study subjects. Other strengths include that we measured a broad range of antioxidants, which were selected by reviewing the literature and expert advice.

A limitation of our study was that antioxidant levels were measured only at a single time point in pregnancy. However, we believe that a single blood sample measured at 24-26 weeks will reliably represent the time period in the weeks surrounding this measurement; furthermore, errors would be non-differential and likely result in bias toward a null effect. Samples were obtained in the non-fasting state; however, there is limited diurnal variation in the lipid-soluble markers measured.[170,171] We also did not collect dietary information from study subjects and hence, we cannot consider whether differences in plasma levels resulted from differences in dietary intake; however, plasma levels reflect concentration closer to the active site than food intake.

Since we measured antioxidant levels only in the second trimester, we cannot rule out the possibility that associations we observed may be a result of a pathologic process that had already been initiated, rather than a cause of SGA. It is possible that for some cases of SGA, the process is already underway by the second trimester or even earlier.[172,173]

Another limitation is that we measured levels of antioxidants only. Markers of oxidative stress levels of antioxidant enzymes may also be of interest to more fully understand the balance of factors that contribute to a state of oxidative stress in SGA. However, nutritionally derived antioxidants are potentially modifiable, and were hence the focus of our study.

Interpretation

Our study is one of only a few prospective studies to assess antioxidant levels during pregnancy and the risk of SGA. To our knowledge, this study includes the largest number of cases and most comprehensive set of antioxidants measured in studies published to date. One prospective study that included 88 cases of SGA and measured antioxidant levels at 28 weeks gestation found that maternal plasma α-tocopherol was positively associated with birthweight.[130] This conflicts with our results; however, the previous study did not observe a consistent dose-response effect. Most prospective studies have not measured biomarkers within a narrow time window of pregnancy, as we have done. One study (published only as a conference abstract at the time of this writing), reported results consistent with ours, with carotenoids levels at 16-27 weeks negatively associated with SGA risk.[174]

Several mechanisms have been proposed to describe how oxidative stress may lead to SGA. Oxidative stress may lead to placental infarction or placental calcification, resulting in suboptimal placental function and restricted fetal growth.[175] Oxidative stress may also contribute to the risk of SGA by provoking endothelial dysfunction, which is known to occur in isolated SGA, although to a lesser extent than has been demonstrated in preeclampsia.[77,83] Resulting endothelial dysfunction could lead to fibrinogen synthesis increasing the blood viscosity and limiting placental perfusion. Alternatively, the carotenoids may stimulate growth directly at the cellular level.[176] Hence, the mechanisms whereby antioxidants may be related to risk of SGA may or may not be shared with preeclampsia. Therefore, we still expected that we may find an association between antioxidants and SGA, despite the fact that clinical trials have not demonstrated a benefit of antioxidant supplements for prevention of preeclampsia, a condition with similar pathophysiology. Further, virtually all large trials have used a combination of vitamin C and vitamin E, whereas, we investigated other antioxidants including carotenoids in this study.

Alternatively, SGA may be the result of placental dysfunction that leads to defective transport of micronutrients, specifically retinol, across the placenta. One study found that amniotic fluid levels of retinol were higher among normal pregnancies than those with complications such as diabetes and preeclampsia. The authors interpreted their results to suggest

that complications may affect transport of retinol into the amniotic fluid, possibly through a mechanism involving poor uteroplacental perfusion.[177] This is consistent with studies by Neel *et al.* that found cord serum levels of retinol were positively associated with birthweight,[178] and Ortega-Senovilla *et al.* that plasma retinol at delivery was higher in IUGR mothers and that the ratio of umbilical vein plasma levels to maternal levels of retinol were lower in IUGR than AGA controls.[133] Hence, higher average levels of retinol in maternal blood, as we observed among SGA pregnancy, may be only a marker of poor placental function and risk of SGA or could signify lower levels of retinol reaching the fetus, and being of causal importance for fetal growth.

Further, retinol has many functions in addition to its antioxidant properties including roles in transcription regulation and gene expression, vision, immunity, reproduction, growth, and development.[179] Therefore, the contribution of retinol to the etiology of SGA may have to do with its function as a regulator of gene expression, or another function, as opposed to solely acting as an antioxidant. Findings recently published by our group also suggest reduced transport of apo-A1 and HDL particles in SGA pregnancies, providing further evidence to support the impaired transport hypothesis.[162]

A future study aiming to identify whether the relationships observed are causal would ideally be a prospective cohort study that measures levels of individual antioxidant biomarkers from the first trimester, or even before pregnancy, and onward at regular intervals. This would better characterize the levels across pregnancy and reveal when differences between pregnancies resulting in SGA versus AGA infants become apparent. Further, if serial ultrasounds are also incorporated, when growth restriction arises could be more precisely timed and antioxidant levels before and after could be compared to see whether differences observed are more likely to be a cause or an effect of the disrupted growth process which can lead to an SGA birth.

Our findings with respect to carotenoids should not influence clinical practice but support current recommendations for pregnant women to eat a variety of fruits and vegetables;[180,181] however the antioxidant content of individual fruits and vegetables varies greatly.[182] If antioxidants are of particular nutritional value to pregnant women, specific food choices may be advised. While the cost of antioxidant rich foods is sometimes prohibitively expensive, government programs such as The Special Supplemental Nutrition Program for Women, Infants, and Children (WIC) in the United States, and Healthy Start in the United Kingdom, increase

accessibly for low-income women. Hence, our research provides additional support for the value of these programs.

While our study was conducted in North America, the results may not apply to resource-poor settings. Our study population included women across a wide socioeconomic spectrum, and although low intake of certain dietary nutrients may be similar to low intakes observed in resource-poor settings, supplementary multivitamin use was very common. We may have therefore missed associations between vitamin deficiency and SGA that might occur in low-income, developing country settings.

Conclusions

In conclusion, elevated retinol may be associated with an increased risk of being small for gestational age, whereas elevated carotenoid levels may be associated with a reduced risk. Elevated retinol may indicate problems with transport mechanisms; hence, the elevated levels may be an effect of the mechanism leading to SGA, or they may form a part of the causal pathway. On the other hand, the protective effect of elevated carotenoids observed is consistent with our initial hypotheses about antioxidant defenses. If differences in carotenoid levels are corroborated in future studies, nutritional intervention trials may be warranted to assess whether intervening on antioxidant levels would improve other outcomes, beyond SGA. Further, this research provides some evidence to support recommendations and public health programs that aim to promote adequate intake of fruits and vegetables.

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Disclosure of Interests

The authors report no competing interests

Contribution of Authorship

All authors participated in the design of the present study and interpretation of the results. MK and SK designed the cohort study and oversaw data collection. RE oversaw the biomarker analyses. RP provided statistical guidance. JC undertook statistical analyses, and provided the first draft of the manuscript. All authors critically reviewed drafts of the manuscript and approved the final version.

Details of Ethics Approval

The current study was approved by the McGill Faculty of Medicine Institutional Review Board on November 22, 2012 and renewed in 2013 (IRB Study Number A11-M120-12B).

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5.8 Tables

Table 1. Baseline characteristics, mean ± SD or n (%), among SGA cases and AGA controls

Maternal Characteristic*	SGA Cases (n=324)	AGA Controls (n=672)
Maternal age	29.01 ± 5.74	29.07 ± 5.16
BMI (kg/m^2)	22.93 ± 5.03	23.32 ± 4.90
Underweight (BMI <18.5)	46 (14.9)	52 (8.1)
Normal (BMI 18.5 - <25)	188 (60.8)	430 (67.3)
Overweight (BMI 25 - <30)	46 (14.9)	102 (16.0)
Obese (BMI 30+)	29 (9.4)	55 (8.6)
Height (cm)	162 ± 6.4	164 ± 6.7
Primiparous (first birth)	220 (67.9)	395 (58.8)
Current smoker (24-26 weeks)	87 (26.9)	102 (15.3)
Maternal education		
High school or less	64 (19.8)	104 (15.5)
Partial college	63 (19.4)	120 (17.9)
Completed college or some university	82 (25.3)	192 (28.6)
University graduate or more	115 (35.5)	256 (38.1)
Annual family income ($/year)		
<15,000	44 (13.6)	73 (10.9)
15,000 to <30,000	56 (17.3)	88 (13.1)
30,000 to <50,000	60 (18.5)	121 (18.0)
50,000 to <80,000	70 (21.6)	177 (26.3)
≥80,000	58 (17.9)	112 (16.7)
Missing	36 (11.1)	101 (15.0)
Region of Birth		
North America/Europe	249 (76.9)	536 (79.9)
Asia	10 (3.1)	29 (4.3)
Sub-Saharan Africa/Caribbean	32 (9.9)	54 (8.1)
Middle East	13 (4.0)	33 (4.9)
Latin America	20 (6.2)	19 (2.8)
Language Spoken at Home		
French	186 (57.4)	382 (56.9)
English	49 (15.1)	114 (17.0)
Other	89 (27.5)	175 (26.1)
Living arrangements		
Legally married	130 (40.1)	321 (47.9)
Cohabiting	157 (48.5)	275 (41.0)
Neither	35 (10.8)	74 (11.0)
Missing	2 (0.6)	2 (0. 0)

Hypertension	13 (4.0)	28 (4.2)
Asthma	35 (10.8)	50 (7.5)

*Missing data: 33 BMI values for controls, 15 values for cases; 12 height values for controls, 6 values for cases; 7 smoking values for controls, 4 for cases; one region of birth value for a control; one language value for a control; 19 hypertension responses for controls, 18 responses for cases; 3 asthma responses for controls, 1 response for cases; region of birth missing for a control; language spoken at home for a control. These missing data were imputed by multiple imputation.

Table 2. Distributions of antioxidants among case (n=324) and control (n=672) subjects

Biomarker (units) *	Median (IQR) in SGA cases	Median (IQR) in AGA controls	Wilcoxon p-value
α-tocopherol (ug/mL)	9.11 (8.09 – 10.94)	9.10 (7.88 – 10.69)	0.3174
γ-tocopherol (ug/mL)	0.84 (0.58 – 1.19)	0.80 (0.53 – 1.20)	0.2755
retinol (ug/mL)	0.28 (0.22 – 0.32)	0.25 (0.21 – 0.30)	<0.0001
lycopene (ng/mL)	363 (266 – 470)	365 (273 – 488)	0.2163
β-carotene (ng/mL)	234 (135 – 380)	260 (151 – 412)	0.0406
lutein/zeaxanthin (ng/mL)	236 (162 – 351)	274 (177 – 389)	0.0057
α-cryptoxanthin (ng/mL)	26.9 (18.2 – 39.7)	39.7 (27.5 – 55.0)	<0.0001
β-cryptoxanthin(ng/mL)	127 (70 – 218)	166 (98 – 281)	<0.0001

*We excluded 1 outlier for each of a-tocopherol (a case), retinol (a control), and lycopene (a control).

Table 3. Odds ratios (ORs) associated with 1 SD increase in z-score according to logistic regression models

Biomarker	OR_{crude} [95% CI]	$OR_{adjusted}$* [95% CI]
α-tocopherol	1.05 [0.95, 1.24]	1.06 [0.92, 1.23]
ln(γ-tocopherol)	1.08 [0.94, 1.24]	1.14 [0.98, 1.33]
retinol	1.38 [1.21, 1.58]	1.41 [1.22, 1.63]
lycopene	0.90 [0.79, 1.02]	0.89 [0.77, 1.03]
ln(carotenoids)**	0.75 [0.67, 0.86]	0.64 [0.54, 0.78]
α-tocopherol/LDL	1.06 [0.93, 1.21]	1.06 [0.92, 1.23]

*Adjusted for maternal age, BMI, height, primiparity, smoking, geographic region of birth (North America, Asia, Africa/Caribbean, Middle East, Latin America; as proxy for ethnicity), maternal education, family income (as proxies for socioeconomic status), and asthma

** Sum of β-carotene, lutein/zeaxanthin, α- and β-cryptoxanthin

Table 4. Odds ratios for 1-SD increase in z-score in multinomial logistic regression models

	OR_{crude} [95% CI]	$OR_{adjusted}$ [95% CI]*
α-tocopherol		
Mild SGA**	1.15 [0.97, 1.35]	1.11 [0.93 – 1.32]
Severe SGA	0.99 [0.82, 1.19]	1.00 [0.82, 1.23]
γ-tocopherol		
Mild SGA	1.09 [0.92, 1.29]	1.13 [0.94, 1.37]
Severe SGA	1.07 [0.89, 1.29]	1.15 [0.93, 1.41]
retinol		
Mild SGA	1.37 [1.16, 1.62]	1.39 [1.17, 1.66]
Severe SGA	1.40 [1.17, 1.68]	1.45 [1.18, 1.75]
lycopene		
Mild SGA	0.89 [0.76, 1.04]	0.89 [0.75, 1.06]
Severe SGA	0.92 [0.77, 1.09]	0.88 [0.72, 1.07]
carotenoids		
Mild SGA	0.73 [0.63, 0.86]	0.61 [0.49, 0.77]
Severe SGA	0.79 [0.67, 0.94]	0.70 [0.54, 0.89]
α-tocopherol/LDL		
Mild SGA	1.06 [0.90, 1.25]	1.05 [0.88, 1.25]
Severe SGA	1.07 [0.89, 1.27]	1.08 [0.89, 1.31]

*Adjusted for maternal age, BMI, height, primiparity, smoking, geographic region of birth, maternal education, family income, and asthma

**Mild (5^{th}-$<10^{th}$ percentile), Severe ($<5^{th}$ percentile)

5.9 Figures

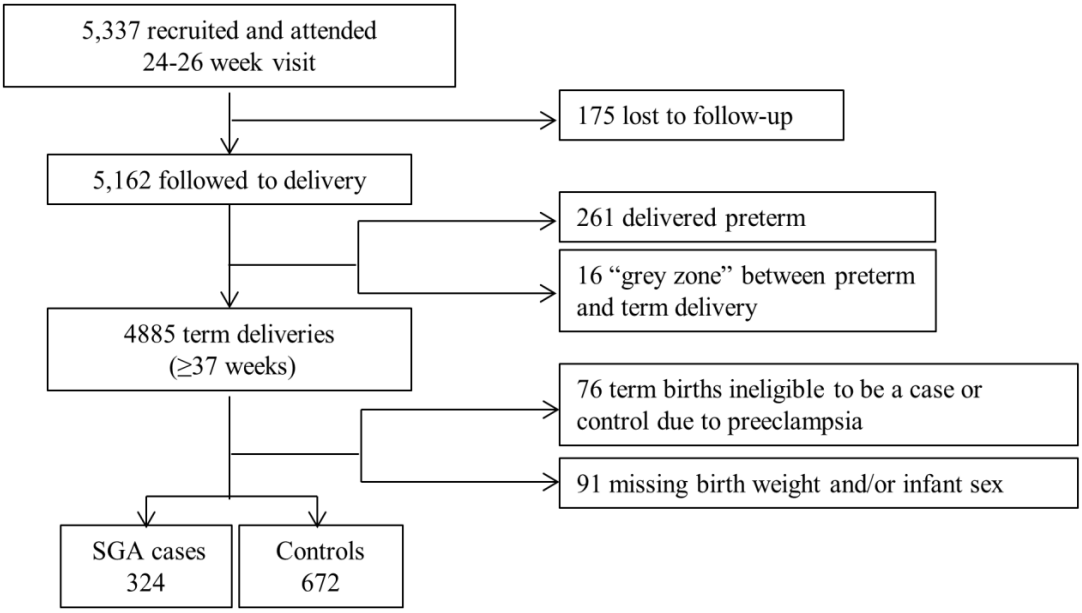

Figure 1. Flow diagram of study sample selection

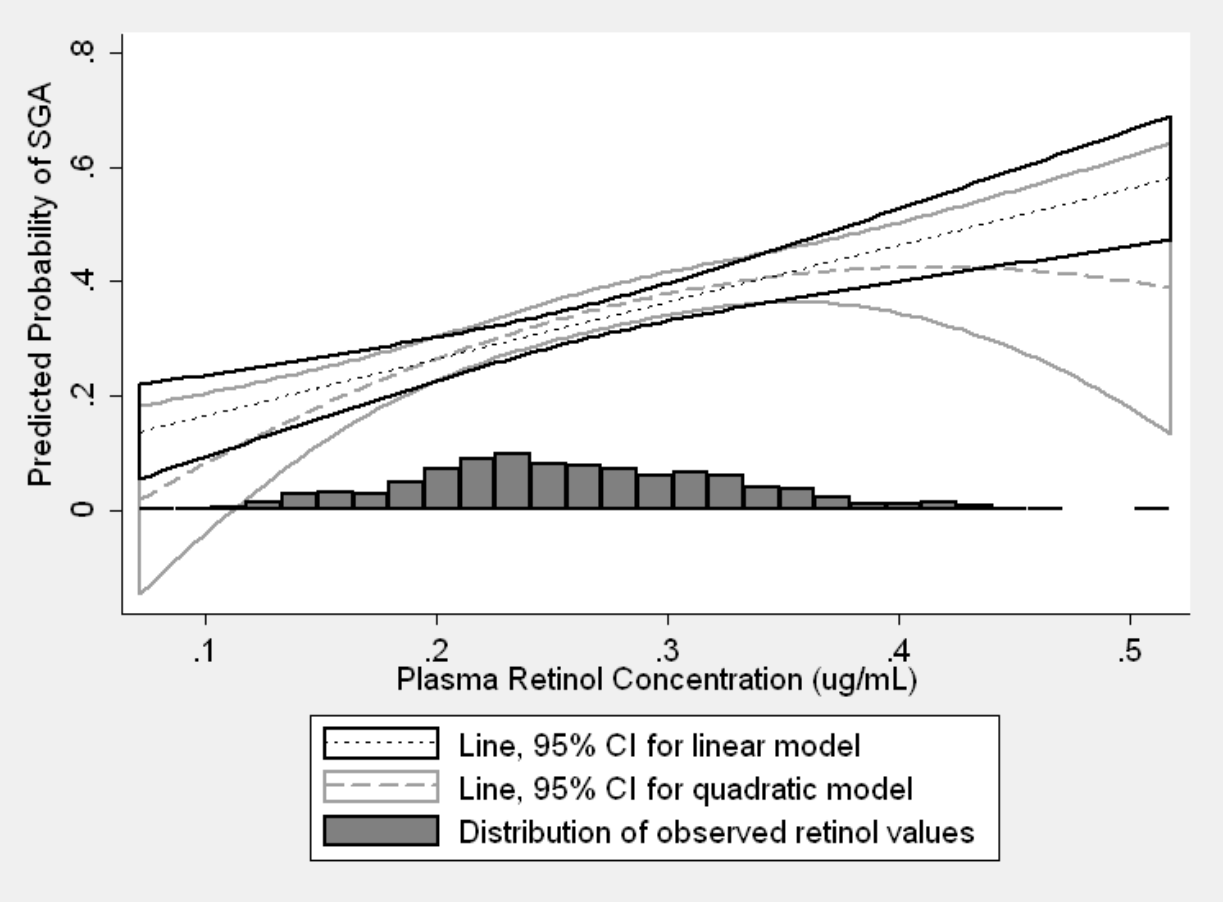

Figure 2. Comparison of linear and quadratic models for retinol

Best-fitting lines and confidence intervals for the predicted probabilities of SGA according to the linear and quadratic models with distribution of observed data over the range of retinol values, shown in a histogram.

5.10 Supporting Information (BJOG online only)

Table S1. Pearson correlations between individuals for antioxidant levels measured at 24-26 weeks gestation

	α-tocopherol	γ-tocopherol	retinol	lycopene	β-carotene	lutein/ zeaxanthin	α-crypto-xanthin	β-crypto-xanthin
γ-tocopherol*	0.12							
retinol	0.09	-0.14						
lycopene	0.29	0.02	0.01					
β-carotene*	0.23	-0.40	0.18	0.22				
lutein/zeaxanthin*	0.21	-0.17	0.29	0.12	0.52			
α-cryptoxanthin*	0.18	-0.22	0.15	0.21	0.45	0.49		
β-cryptoxanthin*	0.23	-0.26	0.18	0.18	0.56	0.48	0.65	
α-tocopherol/LDL	0.33	-0.13	-0.07	-0.07	0.10	0.10	0.07	0.19

*Log transformed (natural log)

Table S2. Pearson correlations between individuals for antioxidant levels measured at 24-26 weeks gestation, with carotenoids (b-carotene, lutein/zeaxanthin, a- and b-cryptoxanthin) pooled

	α-tocopherol	γ-tocopherol	retinol	lycopene	α-tocopherol/LDL
carotenoids*	0.26	-0.35	0.26	0.21	0.15

*Log transformed (natural log)

Table S3. Description of principal components derived from the eight biomarkers of antioxidant level

	Eigenvalue [95% CI]	Cumulative Proportion of Variance Explained	Adjusted OR for SGA [95% CI]*
Component 1	2.93 [2.61, 3.24]	0.366	0.71 [0.63, 0.79]
Component 2	1.15 [1.03, 1.27]	0.510	0.75 [0.65, 0.85]
Component 3	0.95 [0.87, 1.03]	0.629	1.52 [1.31, 1.76]
Component 4	0.89 [0.82, 0.96]	0.740	
Component 5	0.79 [0.71, 0.86]	0.838	
Component 6	0.57 [0.51, 0.63]	0.909	
Component 7	0.43 [0.38, 0.47]	0.963	
Component 8	0.30 [0.26, 0.32]	1.000	

*Adjusted for maternal age, BMI, height, primiparity, smoking, geographic region of birth, maternal education, family income, and asthma

Table S4. Principal components analysis eigenvectors and unexplained variance when three components are utilized for analysis

	Component 1	Component 2	Component 3	Proportion of Variance Unexplained
α-tocopherol/LDL	0.13	-0.57	-0.44	0.389
ln(γ-tocopherol)	-0.27	0.37	0.18	0.597
retinol	0.21	-0.26	0.82	0.155
lycopene	0.17	0.66	0.12	0.399
ln(β-carotene)	0.46	0.03	-0.08	0.363
ln(lutein/zeaxanthin)	0.43	0.00	0.23	0.398
ln(α-cryptoxanthin)	0.45	0.18	-0.08	0.365
ln(β-cryptoxanthin)	0.48	0.04	-0.14	0.303

5.11 Supplemental Material for Manuscript 2

Selection of potential confounders

Table 5.11-1: p-values from investigation of the association between potential confounders and outcome (logistic model for SGA) and exposures (linear regression for each antioxidant biomarker among controls); shaded cells contain p<0.2

	Outcome	**Exposures / Biomarkers**					
	SGA	**α-tocopherol**	**γ-tocopherol**	**retinol**	**lycopene**	**carotenoids**	**α-tocopherol/LDL**
Primiparity	p=0.006	0.049	0.273	0.076	0.231	<0.001	0.040
Alcohol	p=0.993	0.063	0.536	0.765	0.166	0.216	0.012
Education	p=0.475 p=0.077 p=0.106	0.027 <0.001 <0.001	0.735 0.713 0.001	0.943 0.154 <0.001	0.241 0.638 0.138	<0.001 <0.001 <0.001	0.276 0.006 <0.001
Income	p=0.832 p=0.431 p=0.076 p=0.544	0.684 0.034 0.034 <0.001	0.204 0.900 0.935 0.569	0.554 0.324 0.031 <0.001	0.565 0.149 0.327 0.004	0.267 0.079 0.025 <0.001	0.900 0.960 0.504 0.037
Region	p=0.426 p=0.302 p=0.624 p=0.013	0.014 <0.001 0.574 0.374	0.810 <0.001 0.711 0.169	0.048 0.054 0.216 0.086	<0.001 0.710 0.288 0.081	0.015 0.006 0.025 0.113	0.958 0.040 0.560 0.156
Language	p=0.518 p=0.783	0.752 <0.001	0.617 0.010	<0.001 0.276	0.001 <0.001	<0.001 <0.001	0.571 0.003
Partner*	P=0.934	.002	0.290	0.001	0.683	<0.001	0.117
Hypertension	p=0.977	0.403	0.041	0.964	0.799	0.004	0.940
Asthma	p=0.078	0.985	0.011	0.432	0.670	0.013	0.391

*(1 = married or cohabiting)

Alcohol, language, partner and hypertension were not associated with SGA risk, hence should not be included as confounders in the adjusted models. All other potential confounders were associated with the outcome and the exposure (for at least one category in the categorical variables) at p<0.2.

Details of multiple imputation procedure and diagnostics

We used multiple imputation to account for missing data and conducted diagnostics on the resulting data (particularly BMI and income, where we had the most missing data, 5% and 14%, respectively) to assess the behavior of the imputation models.

We imputed all covariates using chained equations. We dropped an implausible value for height (>2 meters) before imputation. A linear model was used for height and weight and BMI was later calculated within the imputed datasets. A logistic model was used for smoking, primiparity, hypertension, diabetes, and asthma; ordinal regression was used for income; multinomial regression was used for living arrangement, birthplace, language, and global region of birth. Other variables used to inform the imputation models were maternal education (no missing data), SGA status, z-scores of each antioxidant or combination [variables shown in Table 4-5], total HDL, total IDL (intermediate density lipoprotein), total LDL, maternal age, multivitamin use, medication use, use of food supplement program available for low-income women in Quebec, and previous infertility. We imputed 50 cycles, each containing n=995 observations. We used the Stata –ice– command (Stata 11).

Across each of the imputation cycles, we observed a consistent median and IQR for BMI **(Figure 5.11-1)**. Only two low outliers (<1.5*IQR) were imputed. The imputation models rarely resulted in high outliers (>1.5*IQR). High values were mostly observed, i.e. not imputed. Therefore, it appears that the imputation models produced reasonable estimates of missing BMI values.

In the imputed datasets, we observed a consistently larger proportion of subjects in the lowest two income categories, and a smaller proportion in the two highest categories, compared with the observed data **(Figure 5.11-2)**. This is consistent with the expected pattern of missing income data.

When we fit the multivariable logistic models within each of the imputed data sets, we observed minimal variation in the point estimate for the odds ratio associated with a 1-SD change in each biomarker **(Table 5.11-2)**.

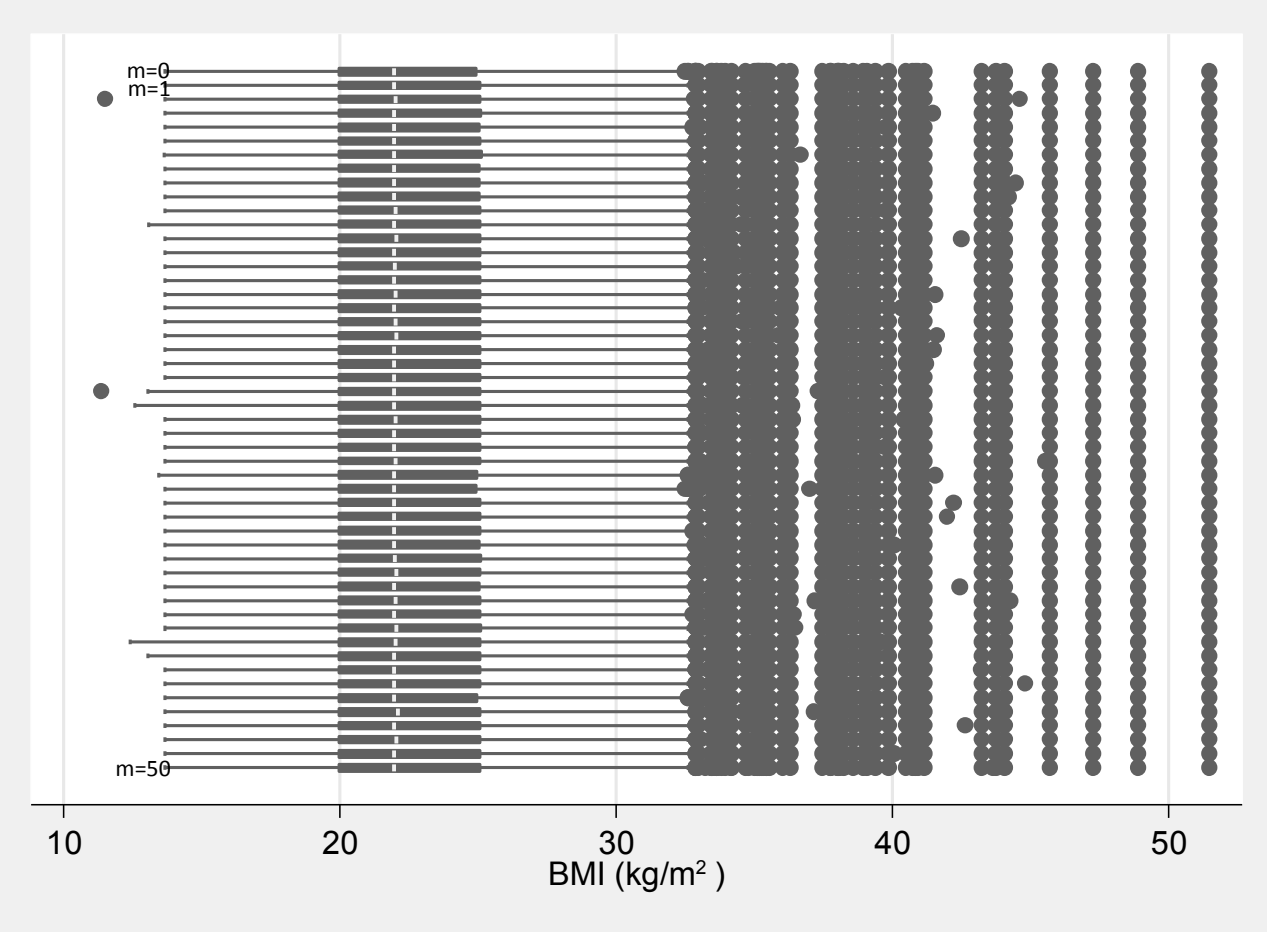

Figure 5.11-1. Boxplots for the distributions of BMI in the observed data (top bar) and in each of the m=50 imputed datasets (n=996 in each)

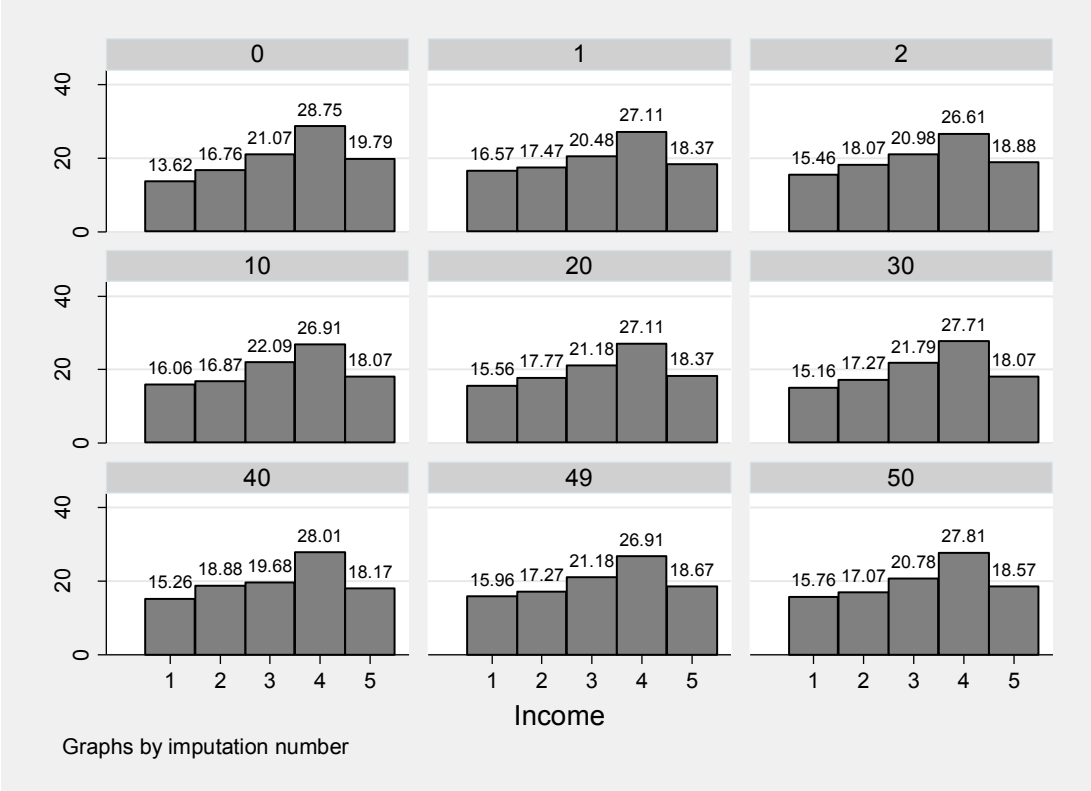

Figure 5.11-2. Histogram of each income category in the observed data (imputation number =0) and in several other imputed datasets

Table 5.11-2: Variation in adjusted point estimates across the 50 imputation cycles

Biomarker	Mean OR	SD	Min	Max
α-tocopherol	1.063	0.004	1.055	1.073
ln(γ-tocopherol)	1.139	0.005	1.128	1.147
retinol	1.411	0.005	1.398	1.423
lycopene	0.889	0.005	0.875	0.899
ln(carotenoids)	0.646	0.004	0.638	0.657
α-tocopherol/LDL	1.064	0.006	1.056	1.080

Effect modification by obesity and smoking assessed by an interaction term in the model

Table 5.11-3: Exploration of effect modification by pre-pregnancy obesity and smoking

	OR_{obese} [95% CI]	$OR_{non\text{-}obese}$ [95% CI]	$OR_{smokers}$ [95% CI]	$OR_{non\text{-}smokers}$ [95% CI]
α-tocopherol	1.11 [0.73 -1.67]	1.08 [0.94 -1.24]	1.05 [0.79 – 1.39]	1.11 [0.95 – 1.29]
γ-tocopherol	0.84 [0.56 – 1.27]	1.10 [0.95 -1.28]	1.11 [0.82 – 1.49]	1.05 [0.90 – 1.23]
retinol	1.70 [1.05 – 2.77]	1.36 [1.18 – 1.57]	1.77 [1.27 – 2.45]	1.33 [1.14 – 1.55]
lycopene	0.66 [0.45 – 0.98]	0.94 [0.82 – 1.08]	0.68 [0.52 – 0.90]	1.01 [0.87 – 1.18]
carotenoids	0.75 [0.49 – 1.13]	0.75 [0.65 -0.87]	0.70 [0.53 – 0.94]	0.87 [0.74 – 1.03]
α-tocopherol/LDL	1.46 [0.95 – 2.25]	1.02 [0.88 – 1.17]	1.08 [0.82 – 1.43]	1.06 [0.91 – 1.24]

We assessed effect modification between each of the antioxidant biomarkers and pre-pregnancy obesity and smoking, as a hypothesis-generating exercise. There were no significant interactions identified between any of the antioxidants and obesity or smoking. There was a marginally non-significant interaction between lycopene and smoking. Higher levels of lycopene were associated with a significantly reduced risk of SGA among smokers, only. We also found that lycopene was associated with a significantly reduced risk of SGA in women with pre-pregnancy obesity; however, this was not significantly different from the effect identified in non-obese women.

Chapter 6. [Manuscript 3] The association between maternal antioxidant levels in mid-pregnancy and preeclampsia

6.1 Preface to Manuscript 3

In our systematic review, we identified 58 studies that assessed one or more antioxidants in relation to preeclampsia. Only nine of these measured biomarkers prospectively, i.e., before preeclampsia was diagnosed. Most studies were small and suffered from substantial risk of confounding and other sources of bias, which suggested the need for larger and more rigorous studies with adequate control for confounding. Many of the studies had a wide range of sampling times, and only assessed a few antioxidants. We sought to contribute high-quality evidence to address the association between antioxidant levels in pregnancy and preeclampsia in a second nested case-control study within the Montreal Prematurity Study cohort. Ideally, we would want to know about their status in early pregnancy, but lipid-soluble antioxidant levels change slowly over time, and we therefore believe that our measurement of biomarkers at 24-26 weeks may represent levels from several weeks or months earlier.

Only one study in our systematic review treated early- and late-onset preeclampsia subtypes as separate outcomes. However, that study measured antioxidant levels only among women with diagnosed preeclampsia. In our study, we were interested in assessing whether the timing of onset of preeclampsia was associated with antioxidant levels. We were especially interested to test the hypothesis that reduced antioxidant levels in the second trimester were associated with the early-onset subtype.

We assessed a large panel of antioxidants, with measurement within the narrowest time window reported to date. Ours is also the first prospective study in a general population sample. Most prior prospective studies of antioxidants were conducted in samples of women at high risk of preeclampsia. We have the advantage of drawing cases and controls from a population-based cohort. Since most women in our population take prenatal supplements, our study explores whether antioxidant levels are related to preeclampsia risk in a population at low risk for vitamin deficiency. Therefore, any associations observed in this population would suggest that antioxidant supplementation, in addition to standard prenatal supplements, could be beneficial for prevention.

In our SGA birth study, we found that carotenoids and retinol were associated with the outcome. In our preeclampsia study, we assess similar associations, which inform our understanding of potential pathophysiologic links between these two conditions.

6.2 Title Page

Original Article [*Prepared for submission to Epidemiology*]

The association between maternal antioxidant levels in mid-pregnancy and preeclampsia

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Suggested running head: Mid-pregnancy antioxidant levels and preeclampsia

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6.3 Abstract

Background: Oxidative stress and insufficient antioxidant defenses may play a causal role in the development of preeclampsia. However, most studies to date have examined antioxidant vitamin levels after the clinical occurrence of preeclampsia.

Methods: We carried out a case-control study, nested within an existing cohort of 5,337 pregnant women in Montreal, Canada. Blood samples obtained at 24-26 weeks were assayed for non-enzymatic antioxidant levels among cases of preeclampsia (n=111) and unaffected controls (n=441). We excluded women diagnosed with gestational hypertension only. We used logistic regression with the z-score of each antioxidant level as the main predictor variable for preeclampsia risk. We further stratified early-onset (<34 weeks) and late-onset preeclampsia and carried out multinomial logistic regression. Finally, we assessed associations between antioxidant biomarkers and timing of onset (in weeks) by Cox regression, with appropriate selection weights. We summed levels of correlated biomarkers (r-squared>0.3) and log-transformed positively skewed distributions. We adjusted for body mass index, nulliparity, pre-existing diabetes, hypertension, smoking, and proxies for ethnicity and socioeconomic status.

Results: The ORs for α-tocopherol, α-tocopherol:cholesterol, lycopene, lutein and carotenoids (sum of α-carotene, β-carotene, anhydrolutein, α-cryptoxanthin, and β-cryptoxanthin) suggested an inverse association between antioxidant levels and overall preeclampsia risk; however, only lutein was significantly associated with overall preeclampsia in adjusted models, OR=0.60 (95% CI 0.46 – 0.77) per SD. In multinomial logistic models, the relative risk ratios (RRR) estimates for the early-onset subgroup were farther from the null than those for the late-onset subgroup. The ratio of α-tocopherol to cholesterol and retinol were significantly associated with early- but not late-onset preeclampsia; RRRs for early-onset preeclampsia 0.67 (0.46-0.99) and 1.61 (1.12-2.33), respectively. Lutein was significantly associated with both early- and late-onset subtypes in adjusted models; RRRs 0.53 (0.35 – 0.80) and 0.62 (0.47-0.82), respectively. Survival analyses confirmed these trends.

Conclusion: Most of the examined antioxidants were more strongly associated with early-onset preeclampsia, suggesting that the oxidative stress pathway may be more relevant for this subtype, although reverse causality could also explain this pattern. Lutein was associated with both early- and late-onset preeclampsia and may be a promising nutrient to consider in preeclampsia prevention trials, if this finding is corroborated by future studies.

6.4 Introduction

Preeclampsia affects 2-8% of pregnancies and is diagnosed by the development of new, sustained hypertension and proteinuria or other adverse conditions in the second half of pregnancy.[2,6] Preeclampsia is a leading cause of iatrogenic and spontaneous preterm delivery and a major cause of maternal morbidity and mortality worldwide.[6,46,183] Despite substantial progress in understanding the pathophysiology of preeclampsia, many knowledge gaps limit the ability to identify effective prevention strategies.[184]

Preeclampsia is often conceptualized as a two stage disorder.[80,185] The first stage is a failure of normal placentation which results in a hypoxic placenta. In the second stage, the maternal syndrome develops and is characterized by endothelial dysfunction and clinical signs including hypertension.[59] The link between these stages is unclear. Oxidative stress is an imbalance between generation of reactive oxygen species and antioxidant defenses. In preeclampsia, oxidative stress may result from reperfusion injury to a chronically hypoxic placenta.[80] Since oxidative stress is a known cause of endothelial dysfunction, it represents a putative link between the stages of preeclampsia.[80,186] We hypothesized that insufficient antioxidant defenses may therefore predispose women to preeclampsia, or may play a causal role by bringing about the syndrome at an earlier stage of pregnancy than in the presence of adequate antioxidant defenses.

Several studies have reported lower levels of antioxidant vitamins in preeclampsia. However, most studies measured antioxidant levels in late pregnancy and had heterogeneous findings. Determining the status of antioxidants earlier in pregnancy should help understand if antioxidant deficit predates clinical symptoms. The few prospective studies were generally small and did not adequately control for confounding.[90,129] Since most prior studies focused on a limited set of antioxidants, with some exceptions,[114,129,143] our study may suggest other modifiable nutrients that could be considered in future intervention trials. Our study objective was to assess whether antioxidant levels in the second trimester of pregnancy are associated with preeclampsia occurrence; and, to assess whether any associations differ by presentation (early- vs. late-onset).

6.5 Methods

Study Population

The Montreal Preeclampsia Study was nested within the established Montreal Prematurity Study, a population-based cohort of over 5,000 women. Consecutive pregnant women were recruited at one of four participating hospitals in Montreal, Canada up to 24 weeks of gestation. Recruitment sites included (1) first trimester blood draw (usually 8-12 weeks), (2) routine ultrasound examination (16-18 weeks), and (3) first- or second-trimester obstetric clinic visit (until 24 weeks). At the time of recruitment to the Montreal Prematurity Study, participants were asked to provide separate consent for the Montreal Preeclampsia Study.

Inclusion criteria were age ≥18 years of age, fluency in English or French, and plan to deliver at one of the study hospitals. Women were excluded if they had any pre-existing chronic conditions, except for diabetes, hypertension, or asthma. Women diagnosed with fetal abnormality, multifetal gestation, or history of incompetent cervix were also excluded.

All participants attended a study visit at 24-26 weeks gestation. A baseline questionnaire that assessed demographic, medical, obstetrical, and family history was administered by a research nurse, and a non-fasting blood sample was obtained for later analysis of case and control samples.

Case and Control Selection

Preeclampsia cases and controls were identified at the time of delivery. The delivery wards of the study hospitals were monitored on a daily basis for deliveries of study subjects. Each woman identified by the case room obstetrician, nurse, or by self-report as having had hypertension or preeclampsia during pregnancy had her chart reviewed in detail to assess whether she met the criteria for preeclampsia. Medical charts were assessed independently by two physicians using a standardized checklist to ensure that the definition of preeclampsia was consistent with the Canadian Hypertension Society Consensus Conference definition.[187] The current version of these guidelines is largely consistent with the previous version employed in

this study,[29] as well as with the recently updated American College of Obstetrics and Gynecology (ACOG) guidelines.[28]

Preeclampsia was defined in one of four ways: (1) gestational hypertension without proteinuria but with adverse conditions; (2) gestational hypertension with proteinuria but without adverse conditions; (3) gestational hypertension with both proteinuria and adverse conditions; (4) preexisting hypertension and superimposed gestational hypertension with proteinuria.

Gestational hypertension was defined as diastolic hypertension ≥90 mmHg measured on two occasions at least 4-6 hours apart after 20 weeks gestation. Proteinuria was defined as 24-hour urine protein excretion of ≥0.3 g/day or positive dipstick result ≥2. Adverse conditions included convulsions, diastolic BP >110 mmHg, low platelet count (<100,000 X 10^9/L), oliguria, protein excretion >3g/day, pulmonary edema, elevated liver enzymes, severe nausea and vomiting, frontal headache, visual disturbances, persistent abdominal pain in upper right quadrant, chest pain or shortness of breath, suspected abruptio placentae, HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count), intrauterine growth restriction (IUGR), oligohydramnios, and absent or reverse umbilical artery end diastolic flow (detected by Doppler).

Preeclampsia was considered "severe" when it was associated with one or more of the following adverse conditions: seizure (i.e. eclampsia), HELLP syndrome, proteinuria >3g/day, or diastolic BP>110mmHg.[34,187] Early-onset preeclampsia was defined as preeclampsia diagnosed before 34 completed weeks of gestation.[7,187,188]

Controls were selected from study women who delivered closest in time to the case at the same hospital. Our controls included some women who delivered preterm, in order to represent the population that gave rise to the cases. Whenever possible, three or four controls per case were identified among women who did not develop preeclampsia or gestational hypertension in the course of pregnancy.

Exposure measurement

We measured mid-pregnancy levels of non-enzymatic antioxidant micronutrients, capable of scavenging free radicals and inhibiting reactive oxygen species.[18] At the 24-26 week study visit, the research nurse drew blood into two 7ml EDTA-containing tubes by venipuncture.

Tubes were placed on ice and brought immediately to each hospital's biochemistry laboratory where plasma was separated by centrifugation. Five aliquots of 1.0 mL plasma were stored in 2 mL cryovials. These samples were stored for up to one week at -20°C. Each week samples were transported on dry ice to a central -80°C freezer for later analysis.

Plasma α- and γ-tocopherol, α- and β- carotene, retinol, lycopene, lutein, anhydrolutein, and α- and β-cryptoxanthin concentrations were analyzed in the Heinz Nutrition Laboratory according to the procedures by Talwar *et al*. using high performance liquid chromatography (HPLC).[119] The intra-assay coefficients of variation were: retinol, 2.60%; α-tocopherol, 3.84%; γ-tocopherol, 4.43%; lycopene, 8.71%; α-carotene, 7.48%; β-carotene, 5.23%. We adjusted α-tocopherol for cholesterol, since the ratio of α-tocopherol to cholesterol has been reported to be the most useful measurement of Vitamin E status in plasma.[148] Total cholesterol was determined using the enzymatic method of Allain *et al*.[189] Laboratory technicians conducting these assays were blind to the case vs. control status of the participants.

Statistical Analysis

Antioxidant levels were examined as continuous variables in all analyses. We assessed the distribution of each antioxidant for extreme outliers, which were excluded only from models including the antioxidant in question. As some of the antioxidants had a skewed distribution, we report the median and interquartile range and compare cases and controls using the non-parametric Wilcoxon test.

We then transformed each biomarker value to a z-score, based on the control distribution of the marker or its log-transformation, if positively skewed. We expected levels of these biomarkers to be correlated, owing to their derivation from common dietary sources. We assessed the correlations among the antioxidant biomarkers to rule out potential collinearity. If antioxidant levels were moderately correlated (r^2>0.3, based on *a priori* discussion among co-authors), the sum was used as the main predictor variable in further analyses. We expected the most highly correlated variables to be biologically similar and therefore decided to sum their concentrations. We also performed a principal components analysis (PCA) based on the control sample to identify a set of uncorrelated factors to assess for association with preeclampsia.

We assessed the overall association between each antioxidant z-score as the exposure of interest and preeclampsia (yes/no) through (unconditional) logistic regression models. We estimated the odds ratio for a 1-SD increment in the biomarker. In a secondary analysis, we examined early- and late-onset preeclampsia separately through multinomial logistic regression which estimates the relative risk ratio (RRR) for each outcome associated with a 1-SD increase in each antioxidant biomarker; e.g. RRR= $(\Pr(Y = 2 \mid x = 1)/\Pr(Y = 0 \mid x = 1))/(\Pr(Y = 2 \mid x = 0)/\Pr(Y = 0 \mid x = 0))$.

We additionally used survival analysis to assess the association between antioxidant levels and the timing of onset of preeclampsia, based on the onset of hypertension, proteinuria, and time of delivery. We most often used the gestational week in which hypertension first arose to assign the week of preeclampsia onset. Details on the timing of onset algorithm are available in **eAppendix 1**. To represent the cohort from which the cases and controls were sampled, we used inverse probability of selection weights. All cases received a weight of 1 and all controls 11.18, which accounts for exclusion of subjects ineligible to be a control, i.e., those who were lost to follow-up or developed gestational hypertension or preeclampsia.

We used extended Cox proportional hazards models for the survival analysis.[190] Pregnancies were censored at the time of delivery if the pregnancy did not result in preeclampsia. Preeclampsia after delivery is a rare occurrence,[44] and no such cases were found after reviewing the medical records of study participants. We used the Schoenfeld residuals test to assess the non-proportionality of hazards and plotted the scaled Schoenfeld residuals, which represent a smoothed function of the time-dependent log hazard ratio for a 1-SD increase in the antioxidant level. [190]

We considered the following known risk factors for preeclampsia to be potential confounders: maternal age, body mass index (BMI), parity (nulliparous yes/no), smoking status, pre-existing medical conditions (diabetes, hypertension), ethnicity, and socioeconomic status (maternal education, income, and marital status). Variables for geographic region of birth and language spoken at home served as proxies for maternal ethnicity. Data on potential confounding variables were obtained from the prenatal questionnaire.

We had some missing data for covariates, especially income (10%) and BMI (6%). Based on the assumption that, conditional on other covariates, these data were missing at random (i.e., their values did not depend on unobserved variables), we imputed values using the other data

available. Multiple imputation using chained equations was used to estimate 50 complete data sets, according to the procedures described by White *et al.*[169]

Analyses were carried out in Stata 11.2 (StataCorp, College Station, TX) and R version 3.0.2. This study was approved by the McGill Faculty of Medicine Institutional Review Board.

6.6 Results

Among the cohort participants, 113 cases of preeclampsia arose and 443 controls were selected. Two cases and two controls were missing antioxidant biomarker data and were excluded from the present analyses (**Figure 1**).

As expected, cases had higher mean BMI, were more likely to be nulliparous and nonsmokers, and to have diabetes and chronic hypertension compare to controls. Cases also had lower educational attainment, on average, than controls (**Table 1**).

Cases had significantly lower levels of α-carotene, β-carotene, lutein, and anhydrolutein than controls (**Table 2**). However, several of the biomarkers were highly correlated (**eTable 1**). Hence, we pooled α-carotene, β-carotene, anhydrolutein, α-cryptoxanthin, and β-cryptoxanthin. After summing the concentrations of these carotenoids, some residual correlation with lutein remained (**eTable 2**). Therefore, we also carried out sensitivity analyses in which lutein was pooled with the other carotenoids, and where lutein and carotenoids were included in the same regression model.

According to the crude logistic regression analyses using the standardized z-score as the main predictor variable, corrected α-tocopherol (per mmol cholesterol), lutein, and carotenoids were all negatively associated with risk of preeclampsia (**Table 3**). However, after adjusting for key confounders, only lutein remained statistically significantly associated with a reduced risk of preeclampsia. In our sensitivity analysis where we pooled lutein with the other carotenoids, we found a marginally non-significant reduced risk of preeclampsia associated with higher carotenoid levels in maternal blood at 24-26 weeks. When we included both lutein and carotenoids in the same model, the association between carotenoids and preeclampsia disappeared, while the association with lutein remained unchanged.

Our PCA analysis confirmed that, with the exception of lycopene, most of the carotenoids measured in our study were highly correlated (**eTable 3**). The component dominated by the carotenoids was significantly associated with a reduced risk of preeclampsia (**eTable 4**). When we separated early-onset (<34 weeks) and late-onset preeclampsia, we observed stronger associations between each antioxidant and early-onset preeclampsia, with the exception of lycopene (**Table 4**). Both positive (RRR>1; γ-tocopherol, retinol) and negative (RRR<1; α-tocopherol, lutein, carotenoids) associations arose. After adjustment for confounding, significant associations were observed between both the ratio of α-tocopherol to cholesterol and retinol with early- but not late-onset preeclampsia. A significant association was seen between lutein and both early- and late-onset preeclampsia. Adjustment for confounding had a stronger impact on risk estimates in the early-onset subgroups.

In survival analysis, the crude and adjusted HRs were almost identical to the ORs obtained in the primary analysis; however, tests of the proportional hazards assumption suggested non-proportionality for many of the markers (**Table 5**). The plots of the scaled Schoenfeld residuals versus time of preeclampsia onset showed that the hazard ratio was farthest from the null for earlier onset cases and gradually returned to the null for the later onset cases (**eFigure 1**). This analysis reinforced our observations from the multinomial logistic regression analysis in which we dichotomized the timing of preeclampsia onset.

6.7 Discussion

Main Findings

In this population-based nested case-control study, we found that most of the studied antioxidants biomarkers assessed at 24-26 weeks, with the exception of lutein, were not significantly associated with preeclampsia overall. However, when we separately examined early- (<34 weeks) and late-onset subtypes, we observed stronger associations between most antioxidants and early-onset preeclampsia. Higher levels of lutein were significantly associated with a reduced risk of both early- and late-onset subtypes, whereas the ratio of α-tocopherol to cholesterol and retinol were significantly associated with early- but not late-onset preeclampsia.

Higher levels of retinol and γ-tocopherol appeared to be associated with an elevated risk of early-onset preeclampsia.

Strengths and Limitations

Our nested case-control design provided an efficient strategy with much lower cost and almost identical precision as analyzing antioxidant levels for the full cohort. Among previous studies that measured antioxidants prior to the diagnosis of preeclampsia, only one other, nested within the INTAPP trial, included a similar number of cases;[191] most had substantially fewer cases.[90,129,141]

We measured a broad panel of individual antioxidants, whereas several previous studies captured only total antioxidant capacity. While two large retrospective studies have measured many of the same markers,[114,143] most have measured fewer markers. Furthermore, we also considered the correlated nature of these markers, which has not been taken into account previously.

Our study employed a number of procedures to minimize bias in exposure and outcome ascertainment. Exposures were assessed in a blinded manner. To reduce the variability due to procedures and instruments, all samples were analyzed according to a standardized process in a single laboratory using the same equipment. Our study employed a highly specific case definition for preeclampsia based on published guidelines and used a standardized diagnostic checklist to minimize inter-rater variability. Moreover, two physicians independently verified the accuracy of the diagnosis for each case, and disagreements were resolved by consensus.

One limitation of our study is that exposure was based on a single, nonfasting blood sample. However, diurnal variation is limited for the markers examined here.[170] We chose not to measure vitamin C since levels of this water-soluble antioxidant are highly variable in response to dietary intake. We believe that the single blood sample measured at 24-26 weeks reflects the time period in the weeks surrounding the measurement. Our samples were taken later in pregnancy than is ideal for assessing a causal relation with preeclampsia; pre-pregnancy or first trimester samples would have been preferable. However, to ensure that the blood samples could be reliably compared among the study subjects, blood samples were taken within a narrow (3-

week) time window during pregnancy—in fact, the narrowest time window in prospective studies to date.

We had no information on dietary intake; therefore, we cannot identify the source of variation for the differences in antioxidant levels (dietary, metabolic, prenatal vitamin use). However, plasma levels may be more informative than dietary intake data, since they have less measurement error, account for absorption, and reflect levels closer to the biological site of action.

We cannot rule out unmeasured or residual confounding. We did not collect information on ethnicity; maternal language and place of birth were used as a proxy. Nor did we collect information on physical activity, which may be related to both preeclampsia risk[192] and antioxidant status,[193] or on dietary fiber, which has also been linked to preeclampsia and may correlate with fruit and vegetable intake.[194]

Interpretation

Our findings suggest that either the oxidative stress pathway may be more strongly implicated in early-onset preeclampsia, or that depleted antioxidant stores may accelerate the progression from pre-clinical to clinically detectable preeclampsia. An alternative explanation for this pattern of findings is reverse causation. If oxidative stress is not a cause but an effect of the underlying pathophysiology, oxidative stress and consequent depletion of antioxidants would be more pronounced at 24-26 weeks, closer to the time of diagnosis of preeclampsia. However, accumulating evidence suggests that the early and late forms of preeclampsia may have different etiologic pathways. Differences between the early and late subgroups are also reinforced by our observation that the impact of adjustment for confounding was stronger in the early-onset subgroups, consistent with another study suggesting different risk factors for these subtypes.[195]

Absence of association between α-tocopherol in the second trimester and overall preeclampsia may help explain why previous antioxidant supplementation trials have not been successful for preeclampsia prevention.[25] Moreover, most trials focused on a single intervention (1000 mg vitamin C and 400 IU vitamin E daily), and other nutrients could potentially have a

beneficial effect. Few observational studies have examined lutein in relation to preeclampsia. Ours is the first to report a significant association.

Acute atherosis, also known as decidual vasculopathy, which is sometimes observed in preeclampsia, resembles the early stages of atherosclerosis.[37] Kramer *et al.* found that among the same antioxidants assessed in our study, only lutein was associated with a reduced risk of decidual vasculopathy in placentas of spontaneous preterm births (OR=0.5, 95% CI 0.3–0.9).[153] The Los Angeles Atherosclerosis Study reported that in a cohort of middle-aged men and women without atherosclerosis followed for 18 months, plasma lutein was inversely related to intima-media thickness (IMT) progression, a proxy for early atherosclerosis. Further, *in vitro* studies showed that lutein was effective in reducing attraction of monocytes to artery wall, a feature of acute atherosis.[196] The Beijing Atherosclerosis Study found a cross-sectional relationship between serum lutein and common carotid IMT, but no effect for other carotenoids.[197] Additionally, in experiments in mice, dietary supplementation with lutein enhanced antioxidant enzyme production. [198]

We found higher levels of retinol to be associated with an increased risk of early-onset preeclampsia. In another study in the same study population, retinol levels were higher in mothers who gave birth to small-for-gestational-age (SGA) babies at term (BJOG, *in press*). Early-onset preeclampsia or preeclampsia associated with preterm delivery have been more strongly associated with SGA than the late-onset form.[10] We also noted a trend toward a higher risk of preeclampsia associated with γ-tocopherol, as has been reported for preterm birth and SGA in this population.[153] Although our results for retinol and γ-tocopherol were contrary to our study hypothesis, the consistency with our previous findings suggests the need for further study.

Conclusions

Most of the antioxidants examined in this study were more strongly associated with early- than with late-onset preeclampsia, raising the possibility that the oxidative stress pathway is more relevant for early-onset preeclampsia. Future studies with serial antioxidant measures taken pre-pregnancy or in the first trimester should help to assess whether the associations observed in our study are causal or, alternatively, due to reverse causation. Lutein was inversely associated with both early- and late-onset preeclampsia. If this finding is corroborated in future

studies, lutein may be a promising nutrient to consider in preeclampsia prevention trials. However, the issue of reverse causation must also be carefully considered in future studies.

6.8 Tables

Table 1. Baseline characteristics of preeclampsia cases and controls

Maternal Characteristic[1]	Preeclampsia Cases (n=111) *mean ± SD or %*[2]	Preeclampsia Controls (n=441) *mean ± SD or n (%)*
Maternal age	29.3 ± 5.7	29.3 ± 5.4
BMI (kg/m^2)	25.7 ± 5.5	23.8 ± 5.1
Underweight (BMI <18.5)	2 (2)	37 (8)
Normal (BMI 18.5 - <25)	54 (49)	250 (57)
Overweight (BMI 25 - <30)	29 (26)	77 (17)
Obese (BMI 30+)	19 (17)	52 (12)
Missing	7 (6)	25 (6)
Nulliparous	86 (77)	253 (58)
Current smoker (24-26 weeks)	11 (10)	70 (16)
Language spoken at home		
French	52 (47)	280 (64)
English	20 (18)	72 (16)
Other	39 (35)	86 (20)
Region of birth of mother		
North America/Europe/Australia	84 (75)	362 (82)
Sub-Saharan Africa/Caribbean	15 (14)	31 (7)
Latin America	6 (5)	19 (4)
Asia	4 (4)	16 (4)
Middle East	3 (3)	11 (3)
Maternal education		
High school or less	20 (18)	65 (15)
Partial college	21 (19)	68 (15)
Completed college or some university	41 (37)	136 (31)
University graduate or more	29 (26)	172 (39)
Family Income ($/year)		
<15,000	5 (5)	50 (11)
15,000 to <30,000	13 (12)	66 (15)
30,000 to <50,000	26 (23)	88 (20)
50,000 to <80,000	26 (23)	114 (26)
≥80,000	23 (21)	85 (19)
Missing	18 (16)	38 (9)
Living arrangement		
Legally married	57 (52)	182 (42)
Cohabiting	45 (41)	208 (47)

Neither	7 (6)	48 (11)
Preexisting chronic conditions		
Hypertension	9 (9)	13 (3)
Diabetes	7 (7)	3 (1)
Asthma	9 (8)	45 (10)

[1]Missing information: parity for one control, smoking for three controls, language for three controls, geographic region of birth for two controls, living arrangement for two cases and three controls, hypertension for nine cases and eighteen controls, diabetes for eight cases and 34 controls, asthma for one control. [2]Percentages may not sum to exactly 100% due to rounding

Table 2. Description of the distribution of antioxidant levels in preeclampsia cases and controls

	Cases (n=111)	Controls (n=441)	
	Median (IQR)	Median (IQR)	P-value[a]
α-tocopherol[b]	17.8 (14.7 – 21.7)	18.9 (16.1 – 22.2)	0.07
γ-tocopherol	1.75 (1.18 – 2.72)	1.73 (1.21 – 2.46)	0.60
retinol[c]	0.38 (0.32 – 0.45)	0.38 (0.32 – 0.44)	0.57
lycopene	224 (102 – 317)	232 (135 – 357)	0.29
α-carotene	119 (72 – 205)	154 (84 – 250)	0.05
β-carotene	319 (180 – 571)	405 (250 – 667)	0.02
lutein	96 (67.1 – 133)	118 (87 – 158)	<0.01
anhydrolutein	63.9 (44.6 – 87.7)	73.4 (52.4 – 97.0)	0.01
α-cryptoxanthin	37.4 (29.2 – 56.9)	43.0 (31.5 – 60.0)	0.08
β-cryptoxanthin	92.3 (62.8 – 153)	108 (66.4 – 184)	0.08

[a]p-value for non-parametric Wilcoxon rank-sum test, [b]μg/mL for tocopherols and retinol and ng/mL for carotenoids, [c]n=110 cases; excluded one outlier for retinol in a case (retinol=0.92).

Table 3. Odds ratios for 1-SD difference in antioxidant biomarker in logistic regression models

	Crude OR	Adjusted OR[a]
α-tocopherol	0.82 (0.66 – 1.02)	0.92 (0.72 – 1.17)
α-tocopherol/cholesterol	0.75 (0.58 – 0.98)	0.82 (0.65 – 1.04)
Ln(γ-tocopherol)	1.05 (0.85 – 1.30)	1.01 (0.79 – 1.29)
Retinol	1.11 (0.90 – 1.35)	1.13 (0.90 – 1.43)
Ln(Lycopene)	0.89 (0.73 – 1.09)	0.93 (0.75 – 1.16)
Ln(Lutein)	0.59 (0.48 – 0.74)	0.60 (0.46 – 0.77)
Ln(Carotenoids)[b]	0.78 (0.64 – 0.97)	0.82 (0.62 – 1.07)

[a]Adjusted for diabetes, hypertension, BMI, nulliparity, smoking, maternal education, living arrangement, region of birth, and language spoken at home [b] Sum of α- and β-carotene, anhydrolutein, α- and β-cryptoxanthin

Table 4. Relative risk ratios for 1-SD difference in antioxidant biomarker in multinomial logistic regression models

		RRR_{crude} (95% CI)[a]	$RRR_{adjusted}$ (95% CI)[b]
α-tocopherol	Early Onset	0.59 (0.40 – 0.87)	0.74 (0.49 – 1.13)
	Late Onset	0.94 (0.73 – 1.20)	0.99 (0.75 – 1.30)
α-tocopherol/chol	Early Onset	0.55 (0.36 – 0.85)	0.67 (0.46 – 0.99)
	Late Onset	0.85 (0.64 – 1.13)	0.88 (0.69 – 1.14)
Ln(γ-tocopherol)	Early Onset	1.34 (0.94 – 1.92)	1.22 (0.81 – 1.83)
	Late Onset	0.94 (0.74 – 1.20)	0.94 (0.71 – 1.23)
retinol	Early Onset	1.45 (1.04 – 2.01)	1.61 (1.12 – 2.33)
	Late Onset	0.97 (0.76 – 1.24)	0.96 (0.74 – 1.26)
Ln(lycopene)	Early Onset	0.96 (0.68 – 1.36)	1.04 (0.72 – 1.50)
	Late Onset	0.84 (0.66 – 1.06)	0.90 (0.71 – 1.16)
Ln(lutein)	Early Onset	0.50 (0.35 – 0.72)	0.53 (0.35 – 0.80)
	Late Onset	0.64 (0.50 – 0.82)	0.62 (0.47 – 0.82)
Ln(carotenoids)	Early Onset	0.52 (0.37 – 0.73)	0.66 (0.42 – 1.02)
	Late Onset	0.94 (0.74 – 1.21)	0.89 (0.65 – 1.22)

[a] Relative risk ratio (RRR) = e.g. $(\Pr(Y = 1 \mid x = 1)/\Pr(Y = 0 \mid x = 1))/(\Pr(Y = 1 \mid x = 0)/\Pr(Y = 0 \mid x = 0))$

[b] Adjusted for diabetes, pre-existing hypertension, BMI, nulliparity, smoking, maternal education, marital status (living arrangement), geographic region (Africa/Caribbean vs. other), language

Table 5. Hazard ratios for 1-SD of each in z-score and p-value for proportional hazards test

	HR_{crude}	P-value	$HR_{adjusted}$	P-value[a]
α-tocopherol	0.83 (0.66 – 1.04)	0.001	0.88 (0.68 – 1.15)	0.001
α-tocopherol/cholesterol[b]	0.75 (0.57 – 0.99)	0.002	0.86 (0.70 – 1.05)	0.016
Ln(γ-tocopherol)	1.06 (0.86 – 1.30)	0.120	0.97 (0.75 – 1.27)	0.077
Retinol	1.12 (0.90 – 1.40)	0.208	1.13 (0.85 – 1.50)	0.033
Ln(Lycopene)	0.88 (0.72 – 1.08)	0.777	0.94 (0.75 – 1.16)	0.921
Ln(Lutein)	0.61 (0.50 – 0.75)	0.025	0.57 (0.44 – 0.72)	0.159
Ln(Carotenoids)[b]	0.79 (0.65 – 0.96)	0.004	0.79 (0.60 – 1.04)	0.093

[a]Test of proportional hazards in first complete dataset (m=1) from multiple imputation; [b]n=130 controls for corrected α-tocopherol; cholesterol only measured in 25% of controls

6.9 Figures

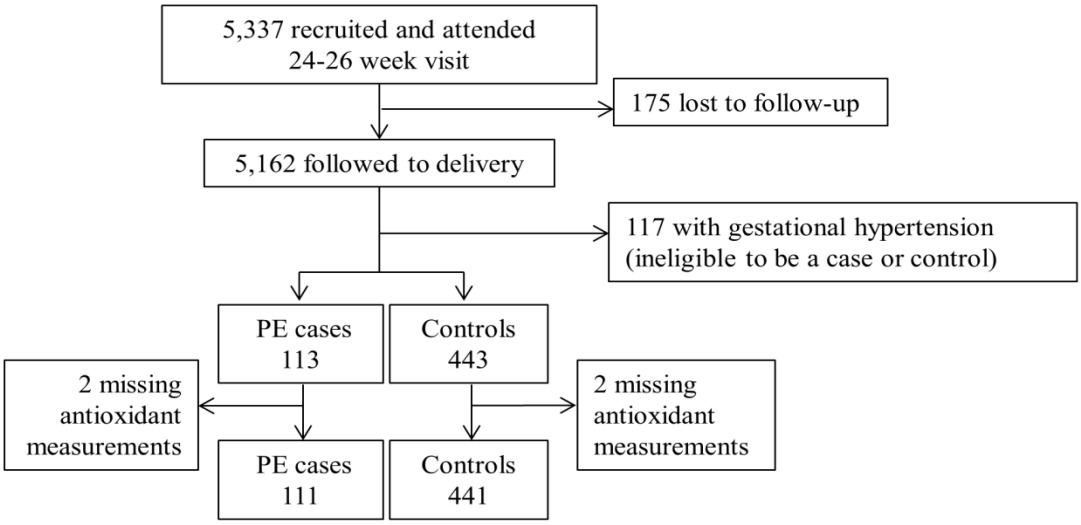


Figure 1. Flow diagram of study sample selection

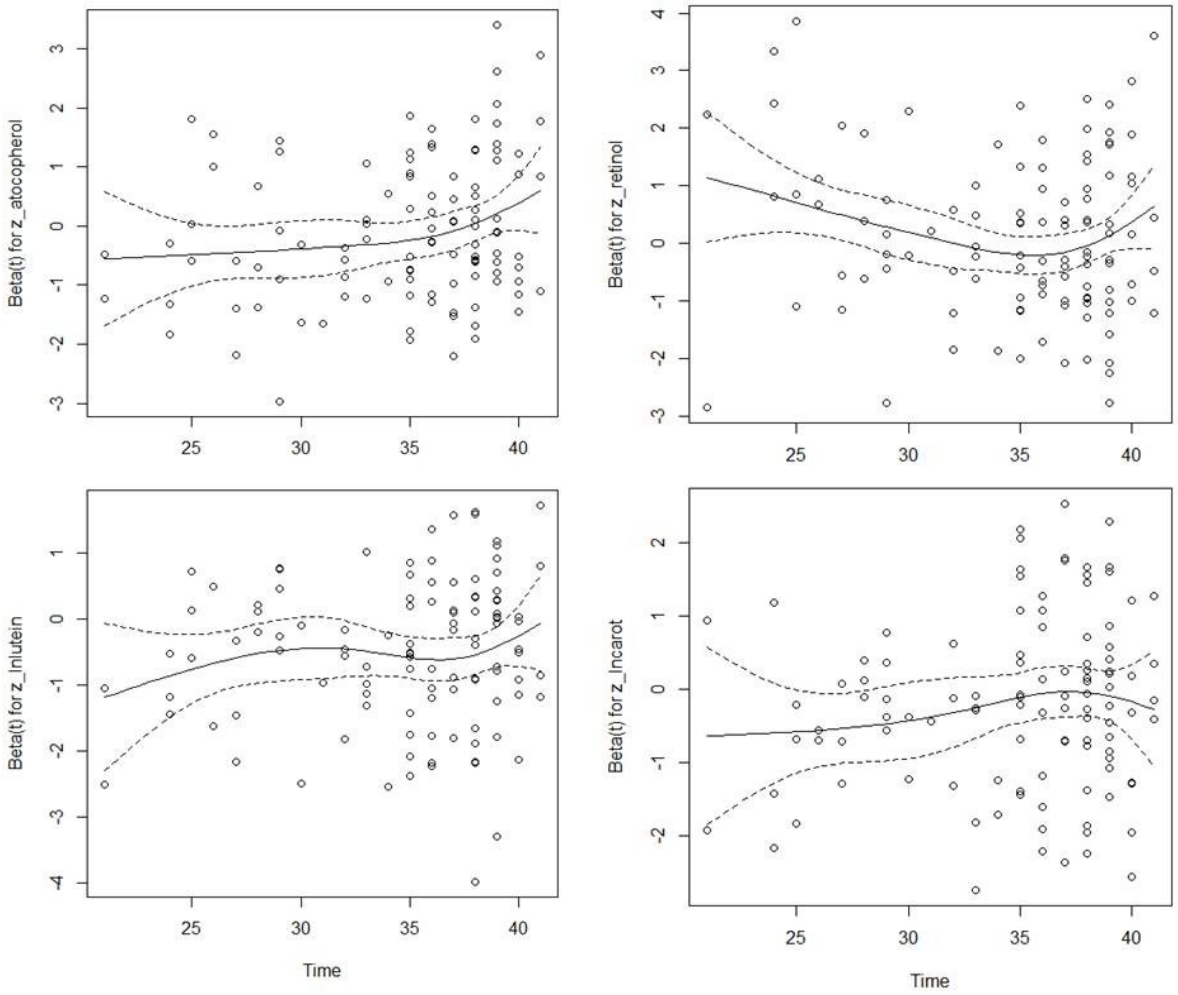


Figure 2. Selected scaled Shoenfeld residual plots from adjusted Cox models of a-tocopherol, retinol, lutein, and carotenoids

6.10 Online Supplemental Material

eAppendix 1: Timing of preeclampsia onset algorithm description, by Canadian Hypertension Society (CHS) class[1]

CHS class B1b – gestational hypertension without proteinuria with adverse conditions (n=49)

- Timing based on hypertension onset
- For the timing of adverse conditions, we only have before, during labor, and after delivery
- Missing timing of hypertension in four subjects – classify by GA at delivery

CHS class B2a – gestational hypertension with proteinuria without adverse conditions (n=12)

- Timing based on the earlier of hypertension or proteinuria
- We have no subjects missing hypertension or proteinuria timing

CHS class B2b – gestational hypertension with proteinuria with adverse conditions (n=49)

- Timing based on earlier of hypertension or proteinuria
- Missing timing of proteinuria in six subjects, none missing hypertension timing
    - Used timing of hypertension to assign timing of onset

CHS class C – Superimposed preeclampsia on preexisting hypertension (n=3)

- Based on timing of delivery

Note: Physicians reviewing patient charts with a standardized checklist extracted the earliest gestational week of sustained hypertension, defined as diastolic hypertension ≥90 mmHg measured on two occasions at least 4-6 hours apart, and the earliest gestational week of significant proteinuria, defined as 24-hour urine protein excretion of ≥0.3 g/day or positive dipstick result ≥2, in accordance with guideline recommendations. [1] We used the above algorithm with these weeks for our primary approach. In secondary analyses, we used an alternate approach of defining early-onset by week of delivery. The results were very similar, however, the average gestational age at "onset" was later for the second method based on delivery (37.1 versus 34.8 weeks) and there was a stronger suggestion of non-proportionality of the hazards for the primary timing of onset algorithm.

[1]Helewa ME, Burrows RF, Smith J, Williams K, Brain P, Rabkin SW. Report of the Canadian Hypertension Society Consensus Conference: 1. Definitions, evaluation and classification of hypertensive disorders in pregnancy. *CMAJ* 1997;**157**(6):715-725.

eTable 1. Pearson correlations between individuals for antioxidant levels measured at 24-26 weeks gestation among controls (n=441)

	α-tocopherol	γ-tocopherol	retinol	lycopene	α-carotene	β-carotene	lutein	anhydro-lutein	α-crypto-xanthin
γ-tocopherol*	0.29								
retinol	0.07	-0.08							
lycopene*	0.28	0.15	0.05						
α-carotene*	0.16	-0.25	0.02	0.22					
β-carotene*	0.25	-0.35	0.07	0.28	0.76				
lutein *	0.34	-0.04	-0.03	0.33	0.37	0.42			
anhydrolutein*	0.35	-0.17	0.11	0.48	0.56	0.62	0.59		
α-cryptoxanthin*	0.20	-0.19	0.07	0.33	0.49	0.55	0.38	0.56	
β-cryptoxanthin*	0.19	-0.20	0.08	0.25	0.43	0.52	0.34	0.48	0.88

*Log transformed (natural log)

eTable 2. Pearson correlations between individuals for antioxidant levels measured at 24-26 weeks gestation, with carotenoids (α-carotene, β-carotene, anhydrolutein, α-cryptoxanthin, β-cryptoxanthin) pooled

	α-tocopherol	γ-tocopherol*	retinol	lycopene*	lutein*
carotenoids*	0.25	-0.33	0.07	0.32	0.45

*Log transformed (natural log)

eTable 3. Principal components analysis eigenvectors and unexplained variance when three components are utilized for analysis

	Component 1 (carotenoid-dominated)	Component 2 (tocopherol-dominated)	Component 3 (retinol-dominated)	Proportion of Variance Unexplained
α-tocopherol	0.20	0.53	0.09	0.40
ln(γ-tocopherol)	-0.12	0.65	-0.03	0.40
retinol	0.05	-0.03	0.96	0.05
ln(lycopene)	0.25	0.38	0.05	0.52
ln(α-carotene)	0.37	-0.18	-0.14	0.38
ln(β-carotene)	0.41	-0.18	-0.06	0.27
ln(lutein)	0.32	0.22	-0.19	0.48
ln(anhydrolutein)	0.41	0.10	0.02	0.29
ln(α-cryptoxanthin)	0.40	-0.10	0.04	0.32
ln(β-cryptoxanthin)	0.38	0.14	0.07	0.38

eTable 4. Description of principal components derived from the ten antioxidant level biomarker z-scores

	Eigenvalue (95% CI)	Cumulative Proportion of Variance Explained	Adjusted OR for Preeclampsia (95% CI)*
Component 1	4.07 (3.53 – 4.61)	0.41	0.83 (0.73, 0.95)
Component 2	1.53 (1.33 – 1.73)	0.56	0.92 (0.75, 1.11)
Component 3	1.02 (0.90 – 1.15)	0.66	1.21 (0.96, 1.53)
Component 4	0.90 (0.80 – 1.01)	0.75	
Component 5	0.74 (0.65 – 0.82)	0.83	
Component 6	0.64 (0.57 – 0.72)	0.89	
Component 7	0.48 (0.42 – 0.54)	0.94	
Component 8	0.30 (0.18 – 0.23)	0.97	
Component 9	0.21 (0.18 – 0.23)	0.99	
Component 10	0.11 (0.10 – 0.13)	1.00	

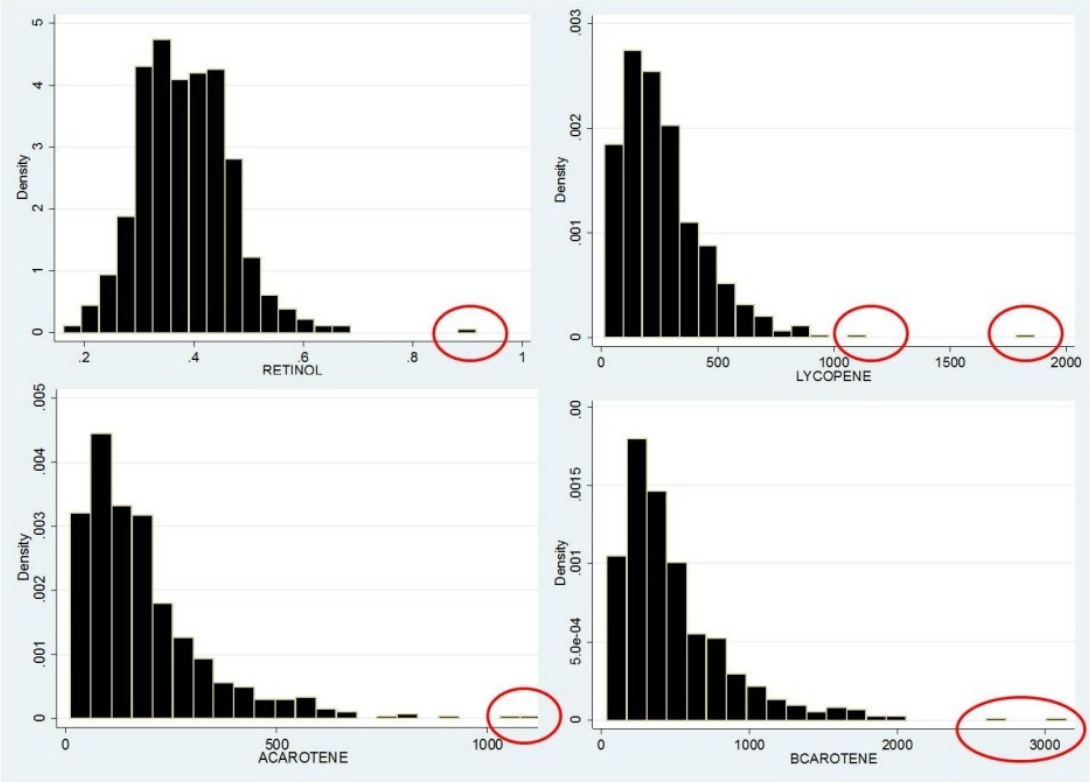
*Adjusted for diabetes, hypertension, BMI (continuous), primiparity, smoking, marital status, geographic region of birth (Africa/Caribbean vs. other) and language spoken at home (other vs. French/English)

6.11 Supplemental Material for Manuscript 3

Assessment of suspected outliers

The figure below shows the histograms for the untransformed antioxidant values for all subjects (cases and controls combined) where there were suspected outliers (**Figure 6.11-1**). In a sensitivity analysis with and without suspected outliers, inclusion of the outlier for retinol resulted in an OR further from the null; but it was still not statistically significant. ORs for lycopene and carotenes were very similar in models with and without suspected outliers (**Table 6.11-1**). Therefore, we decided to only exclude the one outlier value for retinol and retain other marker values to limit unnecessary reductions in sample size.

Figure 6.11-1: Histograms of markers with suspected outliers or influential values (circled in red)



- Retinol: one clear outlier in a case (>0.9)
- Lycopene: two outliers suspected (>1000) one in each group
- α-carotene: two outliers suspected (>1000) one in each group

- β-carotene: two outliers suspected (>2500) two in control group

Table 6.11-1. Effect of 1-SD difference in antioxidant level with and without potential outliers

	Crude OR	**Crude OR w/ outliers**
Retinol	1.11 (0.90 – 1.35)	1.15 (0.95 – 1.40)
Ln(Lycopene)	0.88 (0.71 – 1.07)	0.89 (0.73 – 1.09)
Ln(Carotenes)	0.80 (0.65 – 0.98)	0.80 (0.65 – 0.98)
Carotenoids	0.78 (0.64 – 0.96)	0.78 (0.64 – 0.97)

*carotenes = sum of α-carotene and β-carotene

Sensitivity Analysis: Adjustment for correlated markers

When we adjusted for the other carotenoid variables which have moderate residual correlation with each other, our conclusion that the protective effect of carotenoids in our primary logistic regression analysis is driven by correlation with lutein is reinforced (**Table 6.11-2**).

Table 6.11-2: Effect of 1-SD difference in antioxidant level with and without adjustment for correlated markers and covariates

	Crude OR	OR Adjusted for correlated markers	OR Adjusted for correlated markers & covariates
Carotenoid markers included in primary analysis			
Ln(Lutein)	0.59 (0.48 – 0.74)	0.59 (0.47 – 0.76)	0.60 (0.45 – 0.78)
Ln(Carotenoids)	0.78 (0.64 – 0.97)	1.00 (0.79 – 1.26)	1.01 (0.75 – 1.36)
Alternative set of carotenoid markers			
Ln(Carotenes)	0.80 (0.65 – 0.98)	1.04 (0.79 – 1.36)	1.10 (0.81 – 1.49)
Ln(Luteins)	0.62 (0.51 – 0.77)	0.59 (0.46 – 0.77)	0.61 (0.46 – 0.81)
Ln(Cryptos)	0.84 (0.68 – 1.04)	1.07 (0.82 – 1.39)	0.96 (0.71 – 1.29)

Carotenoids = sum of α-carotene, β-carotene, anhydrolutein, α-caryptoxanthin, β-cryptoxanthin; Carotenes = sum of α-carotene, β-carotene; Luteins = sum of lutein, anhydrolutein, Cryptos = sum of α-caryptoxanthin, β-cryptoxanthin

Correction for cholesterol

As expected, α-tocopherol was moderately correlated with cholesterol, and therefore we created another variable for cholesterol-corrected α-tocopherol. Lycopene and carotenoids also showed some moderate correlation with cholesterol (**Table 6.11-3)**. Therefore, we have also conducted sensitivity analyses where we correct those values for cholesterol (**Table 6.11-4**). We also excluded outliers for these corrected markers. The analysis showed that failure to adjust carotenoids for cholesterol may result in a bias away from the null.

Table 6.11-3: Correlation coefficients for markers and cholesterol (among controls, n=130)

	Total cholesterol	LDL cholesterol	HDL cholesterol
Markers included in primary analysis			
α-tocopherol	0.47	0.48	0.13
γ-tocopherol	0.08	0.12	-0.07
Retinol	0.11	0.03	0.22
Ln(Lycopene)	0.40	0.39	0.17
Ln(Lutein)	0.16	0.14	0.18
Ln(Carotenoids)	0.34	0.29	0.34
Alternative set of carotenoid markers			
Ln(Carotenes)	0.34	0.30	0.33
Ln(Luteins)	0.26	0.24	0.21
Ln(Cryptos)	0.21	0.14	0.24
All carotenoids pooled			
Ln(Carotenoids 2)	0.34	0.28	0.34

Carotenoids = sum of α-carotene, β-carotene, anhydrolutein, α-caryptoxanthin, β-cryptoxanthin; Carotenes = sum of α-carotene, β-carotene; Luteins = sum of lutein, anhydrolutein, Cryptos = sum of α-caryptoxanthin, β-cryptoxanthin; Carotenoids 2 = sum of α-carotene, β-carotene, lutein, anhydrolutein, α-caryptoxanthin, β-cryptoxanthin

Table 6.11-4: Comparison of uncorrected and cholesterol-corrected crude ORs

	Uncorrected OR_{crude}	Cholesterol-corrected OR_{crude}
α-tocopherol (n=241)	0.76 (0.58 – 1.01)	0.75 (0.58 – 0.98)
Ln(lycopene) (n=239)*	0.90 (0.70 – 1.15)	0.95 (0.73 – 1.24)
Ln(carotenoids) (n=240)*	0.66 (0.50 – 0.87)	0.86 (0.65 – 1.13)

*Excluded outliers: One z-score>4 for corrected lycopene in each group, one z-score>5 for corrected carotenoids in control group

Power Calculation

Since we had a set number of cases and controls, we determined the minimum detectable difference in antioxidant levels that could be ascertained from our study with varying power. Although we conducted multiple comparisons in this study, we did not think it was necessary to adjust our type 1 error rate because we did not want to dismiss potentially important findings.[199] Since we standardized our exposure variables to a mean=0 and a standard deviation (SD)=1 according to the distribution in the study controls, we calculated the study power according to this distribution of exposures. We calculated that for a t-test, we have 80% power to detect at difference of 0.296 SD and 90% power to detect a difference of 0.342 SD, at a fixed, two-sided type 1 error rate (α) of 5%. In reality, we also included several confounders in the models which reduce power.

Fractional Polynomial Assessment

To assess potential non-linear associations, we used fractional polynomials. Fractional polynomials were used to model the possibly non-linear effects of each individual antioxidant or the sum of highly correlated antioxidants, for those where specific hypotheses about nonlinearity have been formulated, based on the published literature. It has been previously shown that high levels of tocopherols in pregnancy are associated with preeclampsia and this is contrary to what we would have expected.[91,114] There is also conflicting evidence with respect to retinol. Therefore, we focused the fractional polynomial analysis on these markers.

We used fractional polynomials of degrees 1 and 2 to model the potential nonlinear effects of each prespecified antioxidants. These models allow for more flexible shapes than conventional polynomials; however, they do not add more than two parameters to the model, which is important because we have a relatively small number of cases and, therefore, we aimed to limit the number of additional parameters included in the models.

Fractional polynomials require positive values of the predictor variable. Therefore, we modeled the potential non-linear associations on the original scale of the antioxidant biomarker. According to analyses with the predictor variable on the original scale, no FP models significantly improved the fit beyond the linear model.

Assessment and Modeling of Confounders

We attempted to reduce the number of covariates by examining the association between each categorical variable and preeclampsia using logistic regression models. We then combined the groups with similar odds of preeclampsia to yield a new dichotomous variable. We assessed correlations among the potential confounders to rule out collinearity (**Table 6.11-5**).

Table 6.11-5: Correlation coefficients between potential confounders among controls; n=331 with complete information

	momage	bmi	primip	living~r	birthp~e	region	language	meduc4	income	smoker	alcohol	priorhtn	diabetes
bmi	0.0361	1.0000											
primip	-0.2010	-0.1441	1.0000										
livingarr	-0.2662	0.0059	0.1201	1.0000									
birthplace	0.1556	-0.0229	-0.1286	-0.3161	1.0000								
region	0.0161	0.0165	-0.1530	-0.1092	0.6386	1.0000							
language	0.1811	-0.0623	-0.1550	-0.3799	0.4289	0.3513	1.0000						
meduc4	0.4609	-0.1553	0.1183	-0.2700	0.0780	-0.0224	0.0369	1.0000					
income	0.4591	-0.0750	0.0778	-0.3497	-0.0484	-0.1579	-0.0213	0.4818	1.0000				
smoker	-0.1932	0.0607	0.0196	0.2463	-0.1570	-0.1109	-0.1308	-0.3207	-0.2779	1.0000			
alcohol	0.0722	-0.0213	0.0031	0.0317	-0.1284	-0.1866	-0.1687	0.1266	0.2048	0.0009	1.0000		
priorhtn	0.0294	0.0465	0.0439	0.0414	-0.0615	-0.0620	0.0174	-0.0129	-0.0073	0.1227	-0.0920	1.0000	
diabetes	-0.0102	0.0876	-0.1117	0.0021	-0.0563	-0.0336	0.0176	-0.0070	-0.0507	-0.0403	-0.0434	0.1694	1.0000
asthma	-0.0976	0.0362	0.0159	-0.0195	-0.0505	-0.0381	-0.0245	-0.0711	-0.0888	0.0626	-0.0405	-0.0603	0.0274

We (1) explored whether a change-in-estimate approach could be used to adjust for potential confounding; and (2) decided to adjust for *a priori* defined risk factors that were associated with the outcome in our study. BMI was the only confounder that effected a change of >10% in the estimate when controlled for. The effect of adjusting for BMI ranged from 1% for retinol to 14% for carotenoids and 17% for the ratio of α-tocopherol to cholesterol. Adjusting for other covariates had more modest effects (**Table 6.11-6**).

We used logistic regression to examine the association between each of the suspected confounders and preeclampsia. For those that were continuous or dichotomous and associated with preeclampsia ($p<0.2$), we included these factors. For multi-category variables we assessed the association between that factor and preeclampsia by a set of indicator variables. We aimed to collapse

categories to save degrees of freedom for our models. There was most often a single category that had association with preeclampsia whereas the other had no association. We therefore created dichotomous variables which compared the category of interest to the other categories for statistical adjustment. We collapsed maternal education from four categories to university graduate vs. others; we collapsed living arrangement from three categories to married or cohabiting vs. other; we collapsed geographic region of birth from five categories to born in Sub-Saharan Africa or Caribbean vs. other; we collapsed language spoken at home from three categories to Other vs. French or English. In this assessment, we found that income and maternal age were not associated with preeclampsia in our study and they were not included in adjusted models. Hence, we adjusted for *a priori* defined risk factors, after collapsing categories where possible, and excluding variables not found to be associated with PE in the dataset.

Table 6.11-6: Exploration of confounding via change in estimate approach

	Crude OR	Adjusted OR (one factor at a time)									
		diab	hyper	age	BMI	nullip	smoke	unigrad	partner	afric	olang
α-tocopherol	0.82 (0.66 – 1.02)	0.84	0.81	0.82	0.87	0.80	0.81	0.86	0.82	0.85	0.82
		2%	1%	0%	6%	2%	1%	5%	0%	4%	0%
α-tocopherol/chol.	0.75 (0.58 – 0.98)	1.11	1.04	1.05	0.93	1.11	1.05	1.01	1.05	1.08	1.05
		6%	1%	0%	17%	6%	0%	4%	0%	3%	0%
Ln(γ-tocopherol)	1.05 (0.85 – 1.30)	0.71	0.76	0.75	0.82	0.76	0.82	0.77	0.76	0.77	0.74
		5%	1%	0%	9%	1%	9%	3%	1%	3%	1%
Retinol	1.11 (0.90 – 1.35)	1.15	1.08	1.11	1.10	1.06	1.11	1.12	1.09	1.15	1.14
		4%	3%	0%	1%	5%	0%	1%	2%	4%	3%
Ln(lycopene)	0.89 (0.73 – 1.09)	0.93	0.87	0.87	0.89	0.89	0.88	0.86	0.86	0.88	0.87
		6%	1%	1%	1%	1%	0%	2%	2%	0%	1%
Ln(lutein)	0.59 (0.48 – 0.74)	0.60	0.58	0.59	0.64	0.56	0.58	0.61	0.59	0.59	0.59
		2%	2%	0%	8%	5%	2%	3%	0%	0%	0%
Ln(carotenoids)	0.78 (0.64 – 0.97)	0.75	0.80	0.78	0.89	0.72	0.74	0.83	0.78	0.77	0.76
		4%	3%	0%	14%	8%	5%	6%	0%	1%	3%

Diab=diabetes; hyper=hypertension; nullip=nulliparity; unigrad=university graduate; africar=geographic region of birth is Africa or Caribbean; olang=language spoken at home not French or English

Exploration of effect modification

We explored effect modification by diabetes, hypertension, obesity, and smoking as dichotomous variables with an interaction term in the regression model. The OR when adjusted for obesity versus continuous BMI had residual confounding; however, in these models, we included obesity instead of BMI just to look at effect modification.

Table 6.11-7: Exploratory analysis of interaction

	p-value for interaction with hypertension	p-value for interaction with smoking	p-value for interaction with obesity
α-tocopherol	0.331	0.422	0.176
α-tocopherol/cholesterol*	0.807	0.489	0.345
Ln(γ-tocopherol)	0.648	0.351	0.963
Retinol	**0.022**	0.540	0.176
Ln(lycopene)	0.728	0.614	0.112
Ln(lycopene)/cholesterol*	0.378	**0.029**	0.394
Ln(lutein)	0.353	0.477	0.179
Ln(carotenoids)	0.796	0.946	0.527
Ln(carotenoids)/cholesterol*	0.494	0.084	0.725

*Missing cholesterol data not imputed

Survival Analysis (additional methods details)

We wanted to determine if the rate of preeclampsia over the course of pregnancy is affected by levels of antioxidants measured in mid-pregnancy. If levels of these biomarkers do not change the overall risk of preeclampsia but do result in an earlier onset of preeclampsia, this would be important to know. Clinically, we may be more concerned if preeclampsia happens earlier as this usually means a more severe manifestation. Furthermore, if we are interested in examining the pathophysiologic process leading to the disease, this more nuanced understanding of the effect of antioxidant levels on the clinical manifestation of the disease is itself of interest.

We developed an algorithm for timing of onset and adjudicated complicated cases. As a sensitivity analysis, we used the gestational age at delivery at the indicator of preeclampsia timing of onset. Pregnancies were censored at the time of delivery if the pregnancy did not result in preeclampsia. Although preeclampsia can rarely occur after delivery,[44] research assistants scanned the records of study participants and none of these cases were identified in the cohort. While we were interested in accounting for potential informative censoring by preterm births, using inverse probability of censoring weights, preterm birth occurred in only 19 study controls. Therefore, it was impossible to model the birth process with precision. We used the Breslow method to deal with ties. While the Efron or exact methods would be preferred, the software would not allow us to use these in the presence of model weights and multiply-imputed data.[200] Exposures were the continuous z-score of antioxidants, as previously described.

Multiple Imputation Diagnostics

BMI (derived from imputed height and weight; **Figure 6.11-2**) and weight gain (**Figure 6.11-3**) seemed to be imputed successfully without many outliers generated. Imputation of cholesterol appears to have created more low value outliers and a wider spread of values (**Figure 6.11-4**). Since we only had cholesterol measurements for one in four controls, there was more missing data to impute, thus it is not surprising that there was more variation and outlier values in the imputed data (**Figure 6.11-5**). **Table 6.11-8** shows that values were more likely to be missing among subjects at the lower end of the income distribution than the upper end. This is consistent with what would be expected; especially since particularly high-income earners are not placed in a separate category.

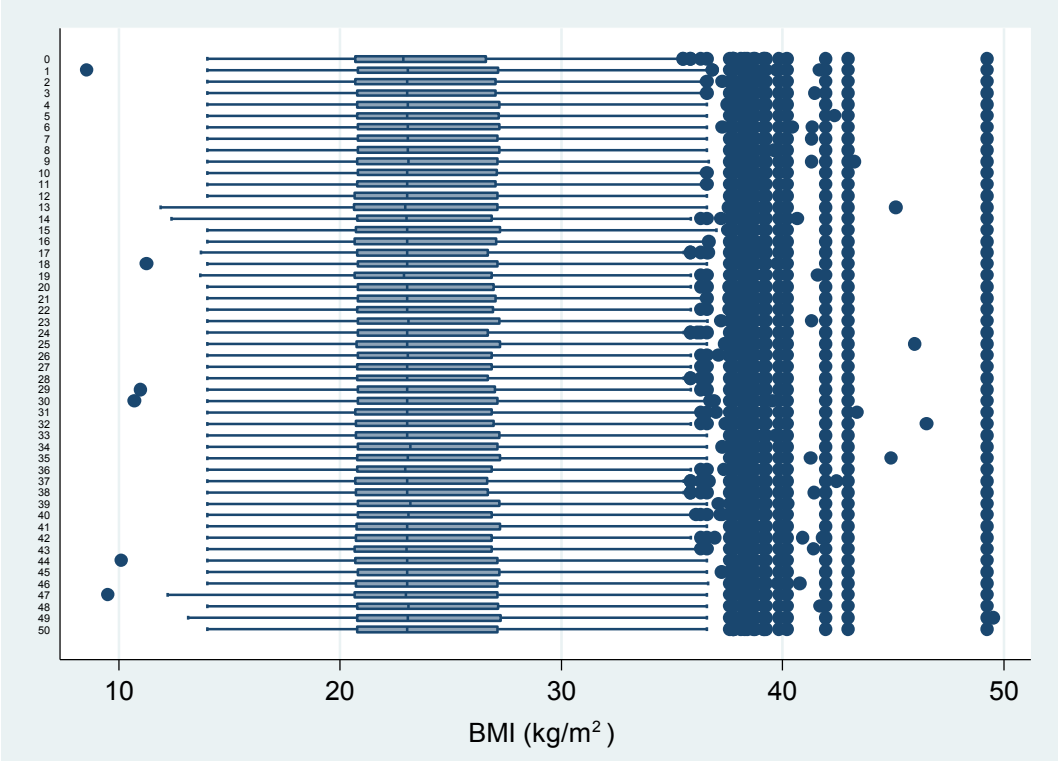


Figure 6.11-2: Distribution of BMI across imputation cycles; 0 = original data with missing values 1-50=MI datasets

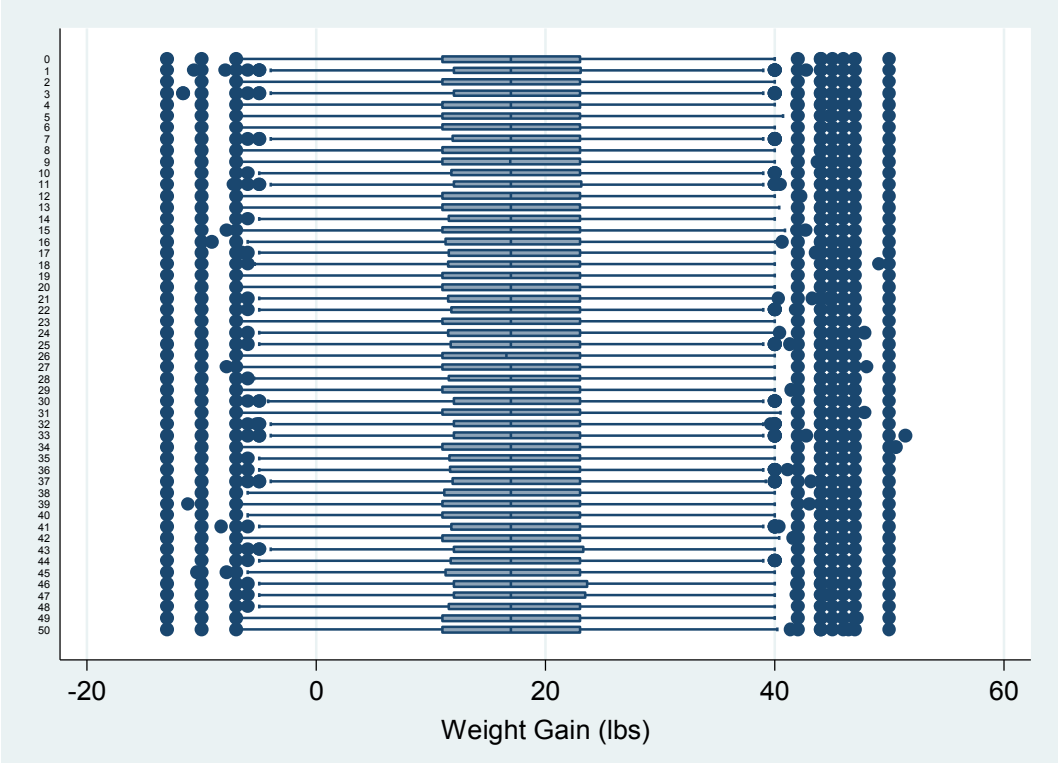


Figure 6.11-3: Distribution of weight gain across imputation cycles

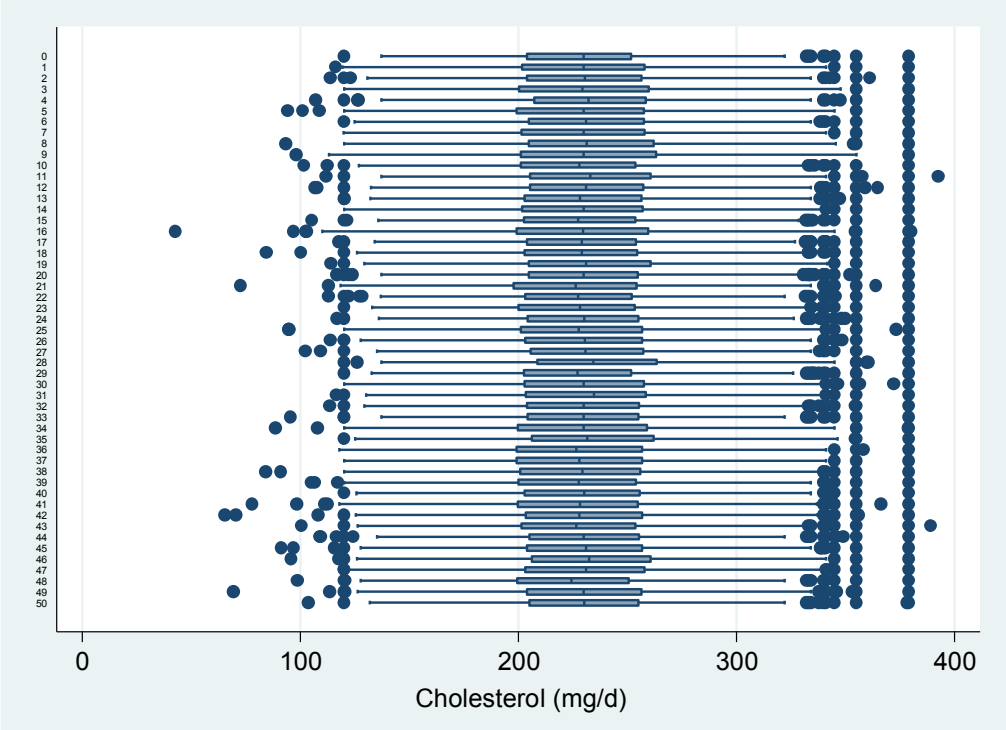


Figure 6.11-4: Distribution of cholesterol across imputation cycles

Table 6.11-8: Income distribution in original dataset with missing values compared to imputed datasets

Income category	Percent in original data with missing values (n=496)	Percent across all imputed datasets (m=50; n=27,600)
<15,000	11.1	12.6
15,000 to <30,000	15.9	16.7
30,000 to <50,000	23.0	23.3
50,000 to <80,000	28.2	27.0
≥80,000	21.8	20.5

m=number of imputation cycles; n=number of observations

Chapter 7. Discussion

This chapter summarizes the main findings of the thesis, strengths and limitations of our two original studies, and the overall contribution of this work and implications for future research.

7.1 Summary of Findings

In the first manuscript of this thesis, we wished to review the studies that formed the empirical basis for previous randomized controlled trials. Since RCTs to date have not demonstrated a significant benefit of antioxidant supplementation for preventing preeclampsia and associated sequelae, we wanted to assess whether observational studies suffered from specific shortcomings that may have led to misleading inferences about the effects of antioxidants and explore reasons for heterogeneous findings reported in the observational literature. Our systematic review and meta-analyses allowed us to identify several knowledge gaps. Moreover, the results did not suggest an imminent need for new RCTs, since many of the differences in antioxidant levels between preeclampsia cases and controls reported in observational studies could be attributable to confounding and publication biases. Our review, did suggest, however, that larger, high-quality observational studies were needed, particularly for carotenoids, for which findings appeared promising.

In our review, we noted that studies focusing on preeclampsia usually assessed antioxidant levels among women who had already been diagnosed with preeclampsia. Women were often recruited around the time of delivery, and controls typically had a more advanced gestational age than cases. With α-tocopherol in particular, such a difference is likely to induce confounding, which may have misled the investigators who designed the earliest RCTs.

We also noted that preeclampsia was usually considered as a single disorder, or stratified by severity (mild or severe preeclampsia or frank eclampsia). Only one study treated early- and late-onset preeclampsia separately; however, that study also recruited cases after the diagnosis of preeclampsia. Since observed magnitudes of association were rarely significantly different between cases of mild and severe preeclampsia, we were interested in filling this gap by stratifying cases into early- vs late-onset preeclampsia in our case-control study.

In the second manuscript, we addressed the hypothesis that lower antioxidant levels in pregnancy may be associated with an increased the risk of SGA birth. We found that carotenoid levels were inversely associated with SGA birth, whereas retinol levels were positively associated. To our knowledge, ours is the largest prospective study of antioxidant levels and SGA birth. We also stratified SGA cases by severity (degree), which had not been previously done in the literature reviewed in Manuscript 1. While we did not find differential associations between the antioxidants and the SGA subgroups, our findings were consistent across both the mild and severe subtypes. Our study isolated the association between antioxidants and SGA by excluding cases of preterm birth or preeclampsia, and demonstrated unconfounded associations with carotenoids and retinol. Our findings suggest that a carotenoid supplement might be useful for preventing SGA birth, but more confirmatory evidence is needed before a clinical trial would be justified. Our results are concordant with another study based in Michigan (POUCH study), which also identified that higher carotenoids levels in the second trimester were associated with a reduced risk of SGA birth.[132] On the other hand, the POUCH study did not observe an association with retinol.

In Manuscript 3, we focused on the continuing controversy surrounding the association between antioxidant levels in pregnancy and the risk of preeclampsia. We tested the hypothesis that antioxidant levels are lower before the onset of the clinically detectable syndrome and therefore may be part of the causal chain leading to preeclampsia. To our knowledge, ours is the first prospective study nested within a cohort from a general population of pregnant women. We also used data analysis techniques that provided greater statistical power to detect an association than previous studies by recruiting a larger sample size and analyzing biomarkers as continuous variables. We also assessed potential non-linear associations, among antioxidants for which the evidence on the association with preeclampsia from previous studies was mixed.

We found that lower levels of lutein were associated with an increased risk of preeclampsia and that higher levels of retinol were associated with an increased risk of early-onset preeclampsia. Decreased transfer of retinol across an abnormal placenta may be a potential explanation and mechanism shared by SGA birth and preeclampsia, and particularly by early-onset preeclampsia.

A unique feature of our study was attention to the timing of onset of preeclampsia signs and symptoms and assessment of whether associations differed for early- and late-onset disease. Our findings reinforced other evidence that preeclampsia is heterogeneous and that early- and late-onset subtypes may represent distinct conditions. We also applied survival analysis in a unique way (using time of onset as a continuous variable) to a nested case-control study, which reinforced our findings when we dichotomized the timing based on the well-established 34-week cut-off.

One unresolved issue that remains is whether the associations observed imply that low/high antioxidant levels are causes of preeclampsia, or whether they are due to reverse causation, i.e., differences that arose secondary to the pathologic changes that lead to preeclampsia and the clinically manifested hypertension, proteinuria, and other adverse conditions.

7.2 Strengths and Limitations

Strengths of the original studies in this thesis include the nested case-control study design, objective exposure measurement, use of general population controls, and statistical analysis designed to maximize the precision of effect estimates.

Preeclampsia is an uncommon condition. The high costs and large sample sizes required to conduct large cohort studies of preeclampsia have often proved prohibitive. Few previous observational studies obtained blood samples before the diagnosis of preeclampsia. A few clinical trials have conducted sub-studies in which antioxidant levels were assessed over time, but most were conducted in select populations at high risk for preeclampsia. However, these populations were not at homogenously increased risk of preeclampsia as the definitions of "high risk" varied across studies.

The original research studies described in this thesis leveraged previously collected data from a large cohort study that recruited a population-based sample. This design is superior to the traditional hospital-based case-control study, in which cases and controls are drawn from a selective clinical population. We also measured biomarker levels before the onset of clinical signs and symptoms of preeclampsia (as far as we know) and had prospectively collected data on

potential confounders and effect modifiers. Our study offers the benefits of a cohort design more efficiently, since costly exposure assessment was carried out only in cases and selected controls.

Plasma antioxidant levels were used to assess the associations between antioxidant vitamin and carotenoid levels and the preeclampsia and SGA birth. As opposed to survey methods that rely on self-reported diet and supplement use, with subsequent estimation of micronutrient intake, our measures reflect the balance of absorption, excretion, and metabolism, depletion due to oxidative stress, and availability closer to the site of biological action.

Controls were representative of the general population (Appendix Table 5). Therefore, our results may be more generalizable than those of many past studies, which focused on high-risk groups. Subjects from these studies were at increased risk for various reasons, and the prevalence of specific factors that may increase risk was not always stated.

We ensured that outcome measures were sensitive and specific, and thus we are unlikely to have missed many cases of preeclampsia or SGA birth. Our definition of preeclampsia included cases that presented without proteinuria but had other adverse conditions. Health care providers and study participants were asked whether they had experienced hypertension in pregnancy. Any suggestion of pregnancy-induced hypertension or preeclampsia was investigated, thus ensuring capture of all (or nearly all) cases that arose in the cohort. Our definition of SGA birth was based on birth weight, an objective outcome not subject to major measurement error, and thus we also have (near) complete ascertainment of SGA cases.

Our statistical analyses of biomarker levels avoided the common use of quantiles in favor of continuous z-scores. Some of the disadvantages of quantile-based analysis include concerns about lack of power, multiple testing, the assumption of homogeneous risk within categories, and the difficulty in comparing results across studies.[201] Since z-scores are based on the standard deviation in our specific population, and generalizability to other populations may therefore not be justified; however, they allowed us to estimate odds ratios that had a similar interpretation for all biomarkers studied. To assess non-linear associations, we used polynomials and fractional polynomials to allow flexible shapes without greatly increasing the number of parameters in the model, which would otherwise reduce our statistical power.

One of the limitations of our two original research studies was that blood samples were obtained at only a single time point. Maternal blood was stored from a single study visit and later analyzed for cases and selected controls for a series of plasma biomarkers, including levels of several antioxidants. However, we chose to measure lipid-soluble antioxidants whose concentrations are relatively stable over time, and change slowly over time in response to dietary changes, at least in non-pregnant adults. Midttun *et al.* analyzed samples collected 1-2 years apart from 40 healthy postmenopausal women in the Nurses' Health Study, and found the intraclass correlation coefficient (ICC) was 0.87 (0.77-0.93) for retinol and 0.86 (0.76-0.93) for α-tocopherol.[202] Cooney *et al.* collected plasma samples each month for one year from 21 healthy individuals and found that the ratio of within to between subject variability was <1 for all markers analyzed in our studies, except for retinol which showed greater within-individual variation in this study.[203] An RCT designed to assess the effect of increased daily consumption of fruits and vegetables on plasma antioxidant level reported that the mean plasma vitamin C, α- and β-carotene concentrations increased in parallel with increased dietary intake of fruit and vegetables in the intervention group at 8 weeks. However, concentrations of retinol, α-tocopherol, lipids, and lipoproteins remained unchanged.[204] Another RCT that encouraged consumption of fruits and vegetables as its intervention found that after 6 months, concentrations of α-and β-carotene, lutein, β-cryptoxanthin, and ascorbic acid increased by more in the intervention group than in controls. However, groups did not differ for changes in lycopene, retinol, α-tocopherol, γ-tocopherol, or total cholesterol concentrations.[205] Information is more limited about how these biomarkers change over the course of pregnancy. Several studies show that vitamin E levels rise in pregnancy, but this trend may be attenuated once cholesterol levels are taken into account.[90,91,206] Other antioxidants (retinol, vitamin C, carotenoids) may be more stable or slightly decreasing over pregnancy.[91,129,207] Since we were more interested in the short term levels that characterize early- to mid-pregnancy, our sample may provide a reliable representation of the levels for this time period. Although seasonal variation in antioxidant levels has been documented, we do not believe that this would have a major effect on our reliability because we were most concerned about a period of a few months which would be within one season or two.[203]

Reverse causation occurs when a study exposure is affected by the outcome, rather than the reverse. We attempted to address this bias by measuring antioxidant biomarker levels in mid-

pregnancy, when levels may still reflect the status of antioxidants in early pregnancy; however, the timing of measurement was likely later in pregnancy than the onset of pathologic processes leading to preeclampsia or SGA birth. Preeclampsia is thought to be initiated by inadequate placental blood supply and established in the first half of pregnancy. However, the timing of measurement of our antioxidant biomarkers should identify risk factors that link the first stage of inadequate placentation and the second stage (the overt maternal syndrome). Low levels of antioxidants in maternal blood may reflect depletion due to increased oxidative stress in the pre-clinical stage of preeclampsia. If oxidative stress is a key link between the stages, then even if levels reflect a pathophysiologic process already underway, reducing oxidative stress might be useful to prevent or delay preeclampsia. We conducted a survival analysis that demonstrated stronger associations with preeclampsia arising sooner after biomarker measurement. This finding provides some evidence of reverse causation (e.g., depleted antioxidant levels reflect oxidative stress that results from the pathologic process of preeclampsia), but it is also consistent with the hypothesis that antioxidant status may be a cause of early-onset preeclampsia (rather than late-onset) or may delay onset. While RCTs to date have not demonstrated a benefit of antioxidant supplementation, we have demonstrated that alternate supplementation strategies may be worth exploring. A survival analysis for time to preeclampsia onset may also be useful in supplementation trials to assess the potential for supplements to delay preeclampsia onset.

A related limitation is that detailed dietary information was not collected in the existing study cohort, which could have potentially avoided the issue of reverse causation. Measurement of diet with subsequent estimation of nutrient intake may suggest dietary interventions that could prevent preeclampsia and SGA. However, these measures have their own limitations due to poor recall, variation in nutrient content in food, and sometimes poor correlation with plasma levels. Rhee *et al.* assessed correlations between nutrient intake estimated by food frequency questionnaire (FFQ) and plasma carotenoids and retinol adjusted for serum cholesterol; they reported correlation coefficients of 0.15 for retinol and 0.19-0.29 for various carotenoids.[208] A similar study reported correlations of 0.03 for retinol and 0.21-0.40 for carotenoids.[209] A third study found a higher correlation between retinol not adjusted for cholesterol and a single FFQ ($r=0.17$) and the mean of four FFQs (0.32), with corresponding correlation coefficients for α-tocopherol/cholesterol of 0.19 and 0.06 and for β-carotene of 0.19 and 0.37.[210] Hence, correlation between estimated intake and blood levels were low to moderate for the biomarkers included in

our study. Measurement of blood biomarkers of nutritional status reflects our interest in better understanding the etiology of these conditions. We believe that this approach could also inform the design of future trials of interventions to prevent preeclampsia and SGA.

In our cohort, women had only a single study visit and otherwise received standard prenatal care. Therefore, exactly when hypertension and/or proteinuria arose was not assessed at regular intervals, except in the course of usual prenatal care. The timing of onset of preeclampsia was not ascertained systematically but based on an algorithm we developed. Some misclassification of time of onset is therefore likely to have occurred.

We studied SGA birth, rather than intrauterine growth restriction. While cases of SGA birth include some constitutionally small infants, definitions of IUGR vary widely. A recent survey in Ireland yielded a long list of definitions.[211] Some previous studies referred to their outcome as "IUGR" but defined it as SGA birth (<10^{th} percentile of birth weight). Studying SGA birth is valid to assess whether antioxidants are related to growth restriction, although we may have missed infants who failed to meet their ideal growth potential but were still above the 10^{th} percentile cut-off.

In our preeclampsia study, we had a relatively large sample size for this condition, but our modest absolute sample size limited our ability to conduct subgroup analyses and to investigate effect modification. The incidence of preeclampsia in our cohort was lower than expected. Estimates of the incidence of preeclampsia globally usually range from 2-8%; however, in our study population, the incidence was <2%. This may be due to our use of a strict definition that complied with the most up-to-date Canadian guidelines.

Residual or unmeasured confounding may have biased our results. We attempted to reduce residual confounding for BMI and maternal age by analyzing them as continuous variables. Although race/ethnicity has been associated with preeclampsia and SGA in previous research, we could only adjust for proxies (maternal birthplace, language), and residual confounding may have resulted. Since physical activity and diet are linked, a protective effect of physical activity against preeclampsia could also confound the association between antioxidant vitamin or carotenoid levels and preeclampsia.

Another limitation is that we did not consider how levels of antioxidant enzymes or their co-factor micronutrients/trace elements are potentially altered in preeclampsia and SGA. Since, for example, selenium, copper, iron, and zinc are required for the function of certain antioxidant enzymes, their intake and blood concentration may be causally related to preeclampsia and SGA birth. As micronutrient status is modifiable, these are worth considering in future studies. However, the relationship between these enzymes and co-factor is highly multifactorial and antioxidant vitamin levels may need to be considered concurrently.[212]

7.3 General Discussion and Implications for Future Research

7.3.1 Generalizability

In the population in which our studies were conducted, most women take multivitamin supplements, and serious vitamin deficiencies are uncommon. In resource-poor settings, however, vitamin deficiencies and much lower intakes are more common. We may thus have missed effects that only arise in the context of true vitamin deficiencies. We therefore believe that our results are likely to be generalizable to North America and other high-income countries, but may not be to low- and middle-income settings. Conversely, we did not find that setting or population was strongly related to the associations observed among studies included in our systematic review.

7.3.2 Causal inference in molecular epidemiologic studies of antioxidant levels

An assumption that is required for causal inference but difficult to assert in biomarker studies is consistency, i.e., a potential outcome at one exposure level is not strongly influenced by how a person arrived at that level.[213] In the context of antioxidant biomarkers, whether low levels are the result of increased oxidative stress or low dietary intake may be relevant to any casual effect of low antioxidant levels. Conversely, high levels may be due to high intake from diet, or perhaps lower utilization.

Temporality, one of Hill's causal criteria,[214] is difficult to establish in a biomarker study. Furthermore, the disease processes in preeclampsia and SGA birth are insidious and only revealed through screening or clinical manifestations. Although research suggests that lipid-soluble antioxidant levels change slowly over time, it is not clear that this would be true in

pregnancy, and particularly in abnormal pregnancy. Without a more complete understanding of the pathophysiology of these conditions, we cannot infer that our measurements truly predate the onset preeclampsia or growth restriction.

For these reasons, establishing that high or low antioxidant levels cause preeclampsia or SGA birth may be impossible in observational studies; perhaps only a randomized controlled trial may have potential to establish a causal link.

7.3.3. Results in the context of other prospective studies

Our systematic review was consistent in general with a prior systematic review, but our inclusion of many other studies resulted in pooled SMDs closer to the null for vitamins C and E and even more statistical heterogeneity. We investigated the sources of heterogeneity and observed that studies based on HPLC measurement methods did not find a significant association between vitamin E and preeclampsia, while those that used spectrometric methods did. Perhaps spectrometry has more cross-reactivity with other antioxidant species than HPLC. Alternatively, this discrepancy may be due to chance or to the fact that studies using HPLC also took greater care to reduce other sources of bias.

Retinol

We found that retinol was elevated in pregnancies with subsequent term SGA birth and early-onset preeclampsia. Previous prospective studies that measured retinol did not report a difference between SGA cases and controls.[131,132,135] In each of these prior studies, the focus was on all SGA, whereas we focused exclusively on term births. However, Kramer et al reported (in the same study cohort in a study of preterm birth cases and controls) that high levels of retinol were associated with an increased risk of decidual vasculopathy (crude OR 1.9 (95% CI 1.1-3.3), which may increase the risk of SGA birth.[153]

In preeclampsia, the evidence is mixed. Azar *et al.* and Rajasingam *et al.* reported that retinol levels were similar in cases and controls,[129,131] Jendryczko *et al.* reported significantly lower levels in nine cases than in 11 controls,[141] and Elsen *et al.* reported case levels similar to those in controls in mild cases but elevated at 28 weeks in severe cases.[126] Most previous preeclampsia studies have been quite small. Rajasingam *et al.* reported that 7/45 (15%) of

preeclampsia cases had an early onset. If it is true that the elevation in retinol is only observed in early-onset preeclampsia, an association may have been missed. On the other hand, in Elsen *et al.*, cases were stratified by severity and it is possible that more early-onset cases were included in the severe case group.[126] This case mix may have revealed a positive association that was consistent with ours.

α-tocopherol

In SGA birth, we found no association with α-tocopherol (corrected or uncorrected). Most prior studies reported either lower or similar levels in cases vs. controls. Our study associations were independent of (i.e., after excluding) preeclampsia. Since preterm or early-onset preeclampsia is associated with an increased risk of SGA birth, and since we found lipid-corrected α-tocopherol to be associated with the early-onset form, perhaps some previous reports of lower tocopherol levels may be explained by co-occurrence of preeclampsia. That was not the case for Chappell *et al.*, but additional confounding by baseline characteristics (e.g. prevalence of obesity in high risk vs. low risk women) in this study may have undermined the validity of the results.[90]

We found that lipid-adjusted α-tocopherol was associated with early-onset preeclampsia, whereas most previous prospective studies reported similar levels between cases and controls for lipid-adjusted α-tocopherol and more mixed results for α-tocopherol unadjusted. Again, it is possible that difference in case mix could (at least partly) explain these differences, or our increased power may have enabled us to detect a true association.

γ-tocopherol

We found that γ-tocopherol levels were non-significantly higher in preeclampsia, but similar in SGA birth, cases vs. controls, a finding consistent with the previous literature. Only Xu reported significantly higher γ-tocopherol levels in preeclampsia,[191] whereas Azar *et al.* reported non-significantly higher levels across all three pregnancy trimesters for preeclampsia cases vs. controls.[129]

Carotenoids

In preeclampsia and SGA, we found lower crude levels of lutein, carotenes, and other carotenoids. We observed similar levels of lycopene in both preeclampsia and SGA cases and controls. Only one other prospective study has reported on lycopene levels. Azar *et al.* found that lycopene levels were significantly higher in cases of preeclampsia, but only in the second trimester, which may have been a chance finding.[129] In SGA birth, we observed lower levels of pooled carotenoid in cases, which is consistent with the results of Kerver *et al*.[132]

Finally, we found lower levels of lutein to be associated with an increased risk of preeclampsia. Among the few prior studies that measured carotenoids prospectively to assess their association with preeclampsia, only Azar *et al.* measured lutein.[129] That study (of diabetic women) did not observe lower lutein levels but did find lower carotene levels in the third trimester. It was difficult to disentangle the effects of individual carotenoids in our study, owing to high correlations among them; therefore, this result may be roughly consistent with ours. Another observational study nested within an RCT with a similar sample size and timing of blood sampling as ours found that low β-carotene was associated with an increased risk of preeclampsia.[145] However, another study of only nine cases and 11 controls found no difference.[141] The latter study lacked sufficient details on the methods and study sample selection and it is therefore difficult to explain the discordant results.

7.3.4 Contribution

In the past decade, interest in using antioxidant vitamins for preventing preeclampsia and SGA birth has increased, but several clinical trials have yielded disappointing results. Those trials were justified by the finding that women with clinically manifest preeclampsia had lower blood levels of antioxidant vitamins compared to women without preeclampsia. Our systematic review aimed to provide a better understanding of how levels of antioxidants vary over the course of pregnancy and how those levels relate to the risks of preeclampsia and SGA birth. We found limited evidence comparing levels from the first and second trimester in women who went on to develop preeclampsia or SGA birth and those in women with normal pregnancies. Our results suggest that carotenoids may have the potential for prevention of preeclampsia and/or SGA birth, but more confirmatory evidence is needed. The results of our systematic review suggest that previous large-scale, multicenter trials assessing antioxidant vitamins C and E may have been unsuccessful in preventing preeclampsia because these vitamins are not consistently depleted in

women who later develop preeclampsia or SGA. High-quality prospective studies of preeclampsia were lacking, and most were small.

To help fill the evidence gaps concerning antioxidant levels and the risk of SGA birth and preeclampsia, we conducted two nested case-controls studies. These studies contributed more robust evidence for carotenoids, with improved consideration of confounding, and confirmed the lack of a strong association between vitamin E levels and either outcome. In the preeclampsia study, we conducted a novel analysis that suggests reverse causation, a phenomenon that should be carefully investigated before initiating future trials. Finally, we used a new application of survival analysis in perinatal research to nested case-control study data that provided results that nicely complemented our analysis of early- vs. late-onset subgroups.

7.3.5 Implications for prevention; rationale for trials

Our promising results for carotenoids should encourage a continuing interest in studying preventive antioxidant supplements. Such supplements, if proven effective, may help avoid the lengthy and costly process of developing new medications to treat preeclampsia, particularly as drug development for pregnant women is an area that is largely avoided by the pharmaceutical industry.[215]

While several previous systematic reviews of antioxidant interventions have concluded that these are ineffective, this thesis work suggests a potential role for carotenoid supplements. However, more research is clearly required to specifically address the possibility that low antioxidant levels may only be the result of preeclampsia or the processes leading to fetal growth restriction, rather than the reverse.

Preeclampsia is clearly a heterogeneous disorder, and antioxidant supplementation may benefit only certain subtypes or specific subgroups of pregnant women. Identification of these subtypes and subgroups needs greater attention and study.

7.3.6 Future research directions

An important future research priority is to investigate reverse causation more thoroughly. This would most likely be carried out in nested case-control studies using serial blood

measurements beginning early in pregnancy. Only one study, in diabetic women, has done this to date.[129]

Unresolved issues relating to biomarkers in pregnancy include whether changes in blood volume in pregnancy, alterations in lipid levels, and weight gain influence biomarker levels or measurement in pregnancy. In this thesis, we investigated confounding by gestational weight gain and could not confirm a strong confounding influence, but additional studies are needed.

Additional studies that measure carotenoids are needed for both preeclampsia and SGA birth, and supplements may be promising for future prevention trials.

Chapter 8. Conclusions

In this thesis, we have systematically reviewed the published literature and carried out two nested case-control studies that fill evidence gaps on the relationship between antioxidant levels in pregnancy and subsequent preeclampsia and SGA birth. In the systematic review, we found that the inverse association between vitamins C and E in late pregnancy and preeclampsia identified in previous reviews and meta-analyses were inconsistent across studies and could be the result of publication bias, confounding and reverse causation. The evidence was more limited on antioxidants and risk of SGA birth. Few studies have assessed the relationship between carotenoid antioxidants and preeclampsia or SGA birth.

In our two original case-control studies, carotenoids were associated with a reduced risk of SGA birth and preeclampsia, which is a novel finding. These studies supported our review's findings, which suggested that carotenoid levels may be lower in women who subsequently develop preeclampsia and SGA birth, and that vitamin E levels may be lower only in a subset of women who develop early-onset preeclampsia. Our two studies also observed significantly elevated retinol levels in women who delivered SGA infants and those who developed early-onset preeclampsia. The different results observed for early- and late-onset preeclampsia, and the consistency of our findings with respect to early-onset preeclampsia and SGA birth, provide further evidence of a relationship between preeclampsia and SGA birth, two components of what has been termed "ischemic placental disease." Further high-quality studies of the potentially protective role of carotenoid antioxidants are needed, before additional clinical trials of antioxidant supplements are undertaken to prevent preeclampsia and/or SGA birth. Understanding why retinol may be higher in ischemic placental disease may also further our understanding of the pathophysiology of the component conditions.

Appendix: Additional Tables

Appendix Table 1: Comparison of findings from three systematic reviews of antioxidant intervention trials

Review	Number of Studies	PE	SGA	PTB	Fetal loss/ neonatal death
Polyzos et al. (2007)[27]	4 RCTs	RR 0.97, 95% CI 0.82 to 1.13; four trials, 4680 women	RR 0.94, 95% CI 0.74 to 1.19; four trials	RR 1.07, 95% CI 0.96 to 1.20; four trials	RR 1.10, 95% CI 0.78 to 1.56; four trials
Rumbold et al. (2008; Cochrane Review)[25]	10 RCTs	RR 0.73, 95% CI 0.51 to 1.06; nine trials, 5446 women	RR 0.83, 95% CI 0.62 to 1.11; five trials, 5271 babies	RR 1.10, 95% CI 0.99 to 1.22; five trials, 5198 women	RR1.12, 95% CI 0.81 to 1.53; four trials, 5144 babies Miscarriage or stillbirth: RR 1.32, 95% CI 0.92 to 1.90; four trials, 5144 babies Neonatal death: RR 0.59, 95% CI 0.28 to 1.23; 3 trials, 4748 babies
Salles et al. (2012)[26]	15 RCTs	RR 0.92, 95% CI 0.82 to 1.04; fifteen trials, 21012 women	RR 0.92, 95% CI 0.80 to 1.05; eight trials, 9672 babies	RR 1.03, 95% CI 0.94 to 1.14; thirteen trials, 21166 babies	Miscarriage or stillbirth: RR 1.17, 95% CI 0.79 to 1.74; eight trials, 9209 babies Neonatal death: RR 0.79, 95% CI 0.54 to 1.17; eight trials, 19135 babies
Summary: All three systematic reviews of antioxidant intervention trials reached similar conclusions. Taken together, antioxidant interventions did not reduce the risk of preeclampsia or SGA and may be associated with an increased risk of PTB and miscarriage. However, these trials studied a limited set of interventions and patient populations varied. Therefore, we believe observational data which justified these trials should be comprehensively examined to assess whether there is evidence in support of future trials with improved rationale for intervention type, timing of initiation, and relevant sub-population.					

Abbreviations: CI=confidence interval, PE= preeclampsia, PTB=preterm birth, RCT=randomized controlled trial, RR=risk ratio, SGA=small for gestational age

Appendix Table 2: Description of antioxidant intervention trials from Cochrane systematic review by Rumbold, et al. (2008)[25] (Polyzos et al. 2007 review included the four trials of vitamin C and E interventions)[27]

Study (location)	Patient Population	Study N	Intervention	Outcomes Assessed
Beazley 2002 (USA)	14-20 weeks gestation Mix of high risk and moderate/low-risk[a]	N=109	1000 mg vitamin C and 400 IU vitamin E daily	PE (diagnostic criteria not defined) SGA (<10th centile)
Chappell 1999 (UK)	16-22 weeks gestation Mix of high risk and moderate/low-risk[b]	N=283	1000 mg vitamin C and 400 IU vitamin E daily	PE (ISSHP guidelines) SGA (<10th centile)
Han 1994 (China)	"High-risk factors of PIH," no further details; unclear, moderate/low-risk	N=100	100 µg selenium daily (6-8 weeks during late pregnancy)	PIH (not defined)
Mahdy 2004 (location unclear, published as abstract)	<16 weeks gestation Primigravidas; Moderate/low-risk	N=113	Red palm oil	PIH (not defined) PE (not defined)
Merchant 2005 (Tanzania)	12-27 weeks gestation HIV-1 infected women; Moderate/low-risk	N=1,078	Daily multivitamin[c] Daily multivitamin plus vitamin A (30 mg beta-carotene + 5000 IU preformed vitamin A) Vitamin A alone	PIH (systolic blood pressure >= 140 mmHg or diastolic blood pressure >= 90 mmHg at any time in pregnancy)
Poston 2006 (UK)	14-21 weeks gestation Mix of high risk and moderate/low-risk	N=2,395	1000 mg vitamin C and 400 IU vitamin E daily	PE (ISSHP guidelines) SGA (<5th, <10th centile)
Rivas 2000 (Venezuela)	<29 weeks gestation Mix of high risk and moderate/low-risk[d]	N=127	500 mg vitamin C and 400 IU vitamin E daily Plus 1g fish oil 3 times daily, 100 mg aspirin 3 times weekly	PE (not defined)
Rumbold 2006 (Australia)	14-21 weeks gestation Nulliparous women; Moderate/low-risk	N=1,877	1000 mg vitamin C and 400 IU vitamin E daily	PE (ASSHP criteria) SGA (<10th centile)
Sharma 2003 (India)	16-20 weeks gestation Primigravidas; Moderate/low-risk	N=251	2 mg lycopene daily until delivery	PE (ISSHP guidelines) IUGR (<10th centile)
Steyn 2002 (South Africa)	<26 weeks gestation Moderate/low risk	N=200	250 mg vitamin C twice daily until 34 weeks	PE
Overall meta-analysis results: No significant difference between antioxidant and control groups for the relative risk (RR) of **preeclampsia** (RR 0.73, 95% confidence interval (CI) 0.51 to 1.06; nine trials, 5446 women), **SGA** (RR 0.83, 95% CI 0.62 to 1.11; five trials, 5271 babies), or **preterm birth** (before 37 weeks) (RR 1.10, 95% CI 0.99 to 1.22; five trials, 5198 women).				

Abbreviations: ASSHP=Australian Society for the Study of Hypertension in Pregnancy, ISSHP=International Society for the Study of Hypertension in Pregnancy, PE= preeclampsia, PIH=pregnancy-induced hypertension, SGA=small for gestational age

Notes:

a) Previous preeclampsia, chronic hypertension, insulin dependent diabetes, multifetal gestation
b) Abnormal Doppler waveform (18-22 wks), history of preeclampsia requiring preterm delivery, eclampsia, or HELLP syndrome

c) Containing 500 mg C, 20 mg E, 20 mg thiamin, 20 mg riboflavin, 20 mg B-6, 50 µg B-12, 0.8mg folic acid
d) Nulliparity, previous PE, obesity, hypertension,<20 years of age, diabetes, nephropathy, positive roll over test, black race, family history of hypertension or PE, twin pregnancy, poor socioeconomic conditions

Appendix Table 3: Description of antioxidant intervention trials listed as ongoing studies in the Cochrane systematic review by Rumbold, et al. (2008);[25] Cochrane review update pending (results of individual trials did not show clear evidence of benefit to prevent PE or SGA)

Study (location)	Patient Population	Study N	Intervention	Outcomes Assessed
DAPIT trial McCance 2010[216] (UK)	8-22 weeks gestation Type 1 diabetes	762	1000 mg vitamin C and 400 IU vitamin E daily	PE (RR 0.81, 95% CI 0.59 to 1.12), rates of adverse perinatal outcomes did not differ significantly between the groups
INTAAP trial Xu 2010[115] (Canada)	Nulliparous women with and without PE risk factors and women with a history of PE[a]	2,647	1000 mg vitamin C and 400 IU vitamin E daily	GH and adverse conditions[b] (RR 0.99, 95% CI 0.78 to 1.26), increased risk of fetal loss or perinatal death, and preterm prelabor rupture of membranes (trial stopped early)
Spinnato 2007[217,218] (Brazil)	12-19 weeks gestation Chronic hypertension or past history of PE	739	1000 mg vitamin C and 400 IU vitamin E daily	PE (RR 0.87, 95% CI 0.61 to 1.25), increased risk of PROM (RR 1.89, 95% CI 1.11 to 3.23) and PPROM (RR 2.68, 95% CI 1.07-6.71
Roberts 2010[116] (USA)	9-16 weeks gestation Nulliparous, singleton pregnancy	10,154	1000 mg vitamin C and 400 IU vitamin E daily	PE (RR 1.07, 95% CI 0.93 to 1.24), Rates of adverse perinatal outcomes did not differ significantly between the groups
VIP WHO Trial Poston 2006[91] (India, Peru, Vietnam)	14-21 weeks gestation One or more risk factors[c]	2,410	1000 mg vitamin C and 400 IU vitamin E daily	PE (RR 0.97, 95% CI 0.80 to 1.17), SGA <5th centile (RR 1.12, 95% CI 0.96 to 1.31], low birth weight (<2500g) (RR 1.15, 95% CI 1.02 1.30)

Abbreviations: CI=confidence interval, GH = gestational hypertension, PE= preeclampsia, PROM=prelabor rupture of membranes, PPROM=preterm prelabor rupture of membranes, RR=risk ratio, SGA=small for gestational age

Notes:

a) Diabetes, chronic hypertension, obesity, multiple pregnancy, or multiparous women with history of PE
b) GH and ≥1 of the following: (1) diastolic pressure ≥110 mm Hg or systolic pressure ≥160 mm Hg; (2) proteinuria >300 mg/24-hour urine collection or dipstick ≥2+; (3) convulsion (eclampsia); (4) thrombocytopenia (platelet count <100,000 × 109/L); (5) elevated liver enzyme levels (AST or ALT >70 U/L); (6) hematocrit <24% and blood transfusion; (7) IUGR <3rd centile; and (8) perinatal death (fetal death >20 wk or neonatal death within 7 days)
c) Pre-eclampsia in the pregnancy preceding the index pregnancy, requiring delivery before 37 completed weeks' gestation, diagnosis of HELLP syndrome (haemolysis, elevated liver enzymes, and low platelets) in any previous pregnancy at any stage of gestation, eclampsia in any previous pregnancy at any stage of gestation; essential hypertension requiring medication, currently or previously; maternal diastolic blood pressure of 90 mm Hg or more before 20 weeks' gestation in the current pregnancy; type 1 or type 2 diabetes, requiring insulin or oral hypoglycaemic therapy before the pregnancy; antiphospholipid syndrome; chronic renal disease (creatinine ≥125 μmol/L pre-pregnancy or ≥100 μmol/L during pregnancy, or significant proteinuria [≥500 mg per 24 h]); multiple pregnancy; abnormal uterine artery doppler waveform (18–22 weeks' gestation, mean resistance index >0·67 or pulsatility

index >1·65 with or without the presence of unilateral or bilateral diastolic notches); primiparity with body-mass index (BMI) at first antenatal appointment of 30 kg/m^2or more

Appendix Table 4: Description of additional antioxidant intervention trials included in Salles et al. (2012)[26] but not included in Cochrane systematic review by Rumbold, et al. (2008)[25] or Polyzos et al. (2007)[27]

Study (location)	Patient Population	Study N	Intervention	Outcomes Assessed
Rumiris 2006 (Indonesia)	8-12 weeks gestation low antioxidant status	60	2.2 mg B-6 2.2 µg B-12 200 mg vitamin C 400 µg folic acid 200 mg n-acetylcysteine 2 mg copper 15 mg zinc 0.5 mg manganese 30 mg iron 800 mg calcium 100 µg selenium 1000 IU vitamin A 400 IU vitamin E (Control group received 30 mg iron and 400 µg folic acid)	PE (RR 0.24, 95% CI 0.06 to 1.01), PTB (RR 0.36, 95% CI 0.04 to 3.23), IUGR (RR 1.07, 95% CI 0.07 to 16.3)
Banerjee 2009 (India)	12-20 weeks gestation Nulliparous, singleton pregnancy	159	2 mg lycopene	PE (RR 0.99, 95% CI 0.51 to 1.92), PTB (RR 8.52, 95% CI 1.09 to 66.5), low birth weight (<2500g) (RR 2.26, 95% CI 1.04 to 4.94)
Vadillo-Ortega 2011 (Mexico)	14-32 weeks gestation personal or family history of preeclampsia in a first degree relative	444	Medical food bars containing 3.3 g L-arginine plus antioxidant vitamins: 250 mg vitamin C, 200 IU vitamin E, 25 mg niacin, 2 mg B-6 4.8 µg B-12, 200 µg folate, or antioxidant vitamins alone, or placebo	Vitamins alone vs. placebo: PE (RR 0.74, 95% CI 0.54 to 1.02), PTB (RR 1.18, 95% CI 0.82 to 1.68) L-arginine + vitamins vs. placebo: PE (RR 0.42, 95% CI 0.28 to 0.62), PTB (RR 0.53, 95% CI 0.33 to 0.84)

Abbreviations: CI=confidence interval, IUGR=intrauterine growth restriction, PE=preeclampsia, PTB=preterm birth, RR=risk ratio

Appendix Table 5: Maternal characteristics of the Montreal Prematurity Study cohort and controls from each nested case-control study; mean ± SD or %

	Total Cohort (n=5,337)	SGA Controls (n=672)	PE Controls (n=441)
Maternal age (years)	29.5 ± 5.4	29.1 ± 5.2	29.3 ± 5.4
<20	2.4	2.1	2.7
20-34	79.2	82.4	79.1
≥35	18.3	15.5	18.1
Missing	0.1	0.0	0.0
BMI (kg/m^2)[a]	23.8 ± 5.1	23.3 ± 4.9	23.8 ± 5.1
Underweight (BMI <18.5)	7.4	7.7	8.4
Normal (BMI 18.5 - <25)	60.1	64.0	56.7
Overweight (BMI 25 - <30)	17.3	15.2	17.5
Obese (BMI 30+)	10.6	8.2	11.8
Missing	4.6	4.9	5.7
Height (cm)[b]	164 ± 7	164 ± 7	164 ± 7
Parity			
0	58.4	58.8	57.4
1	29.6	29.9	32.0
≥2	11.8	11.3	10.4
Missing	0.2	0.0	0.2
Living arrangement			
Legally married	45.3	47.8	41.3
Cohabiting	43.8	40.9	47.2
Neither	10.5	11.0	10.9
Missing	0.4	0.3	0.7
Place of birth			
Quebec	67.5	68.3	69.4
Rest of Canada	4.3	3.3	5.0
Elsewhere	28.2	28.3	25.4
Missing	0.1	0.2	0.2
Region of Birth			
North America/Europe	79.9	79.8	82.1
Asia	3.3	4.3	3.6
Sub-Saharan Africa/Caribbean	8.0	8.0	7.0
Middle East	4.2	4.9	2.5
Latin America	4.5	2.8	4.3
Missing	0.2	0.2	0.5
Language spoken at home			
French	57.8	56.9	63.5
English	18.1	17.0	16.3
Other	24.0	26.0	19.5
Missing	0.2	0.2	0.7
Maternal education			
High school or less	15.6	15.5	14.7

Partial college	16.9	17.9	15.4
Completed college or some university	29.5	28.6	30.8
University graduate or more	37.9	38.1	39.0
Missing	0.0	0.0	0.0
Family Income ($/year)			
<15,000	10.5	10.9	11.3
15,000 to <30,000	13.8	13.1	15.0
30,000 to <50,000	20.3	18.0	20.0
50,000 to <80,000	24.1	26.3	25.9
≥80,000	18.7	16.7	19.3
Missing	12.7	15.0	8.6
Smoking			
Ever smoker	49.7	46.3	50.6
Smoked in first trimester	23.1	21.3	22.9
Smoked in second trimester	16.7	16.5	17.0
Current smoking (24-26 weeks)	15.7	15.2	15.9
Alcohol use during pregnancy			
None[c]	46.9	46.6	47.2
Less than once a month	63.9	62.7	64.4
Once or twice a month	23.9	25.6	24.9
Once or twice a week	10.4	10.3	9.0
Three or four times a week	1.4	1.1	1.3
Every day	0.1	0.0	0.4
Vitamin use during pregnancy			
Yes	92.3	91.2	91.8
Hypertension diagnosed when not pregnant			
No	92.8	93.0	93.0
Yes	3.8	4.2	3.0
Missing	3.4	2.8	4.0
Diabetes since the beginning of pregnancy			
No	89.5	88.4	91.6
Yes, resolved	0.1	0.0	0.0
Yes, current	1.9	1.9	0.7
Missing	8.6	9.7	7.7
Asthma since the beginning of pregnancy			
No	91.0	92.1	89.6
Yes, resolved	2.9	3.1	4.3
Yes, current	5.9	4.3	5.9
Missing	0.2	0.5	0.2

SGA, small for gestational age; PE, preeclampsia; BMI, body mass index

a) Missing BMI data: 244 cohort members, 33 SGA controls, 25 PE controls
b) Missing height data: 89 cohort members, 17 SGA controls, 10 PE controls
c) Missing alcohol data: 2 recorded as missing from entire cohort

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