Maternal Iron and Vitamin D Status During and After Pregnancy and Their Relationships With Maternal and Child Health Outcomes: Evidence From the APrON Cohort Study

by

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ABSTRACT

Background: Investigations that assess how maternal iron or vitamin D status biomarkers change across pregnancy and postpartum, and their relationships with maternal and offspring outcomes, in cohorts of healthy pregnant individuals from high-income countries are limited. Recent reports suggest that the prevalence of iron or vitamin D insufficiencies among pregnant people are underestimated. Further, evidence supporting associations between maternal iron and vitamin D status and prospective maternal and child health outcomes is mixed, and the possible combined impact of both micronutrients is poorly described.

Objectives: The first objective was to determine how maternal concentrations of iron and vitamin D biomarkers changed across pregnancy into postpartum and to assess the combined maternal status of iron and vitamin D during the 2nd trimester (mid-pregnancy). Relationships between maternal iron biomarkers and birth weights (BWs) and birth head circumferences (BHCs) were examined. Lastly, relationships between maternal iron and vitamin D biomarkers and maternal depression symptoms along with internalizing and externalizing behaviours in children at age 5 were determined.

Methods: The Alberta Pregnancy Outcomes and Nutrition (APrON) prospective cohort study recruited 2,189 generally healthy pregnant individuals from Calgary and Edmonton, Alberta. Maternal blood was drawn at each trimester of pregnancy and at 3 months postpartum, and maternal hemoglobin (Hb) concentrations were immediately quantified. Concentrations of maternal serum ferritin (SF) were measured using chemiluminescent microparticle immunoassays, and erythropoietin (EPO), hepcidin and soluble transferrin receptor (sTfR) using enzyme-linked immunosorbent assays. Maternal 25-hydroxyvitamin D3 (25(OH)D3) and 3-epi-

25-hydroxyvitamin D3 (3-epi-25(OH)D3) concentrations were quantified by liquid chromatography with tandem mass spectroscopy. Ratios of sTfR:SF and hepcidin:EPO were calculated, and four categories of maternal iron and vitamin D status conceptualized. Neonatal BWs (n=2,022) and BHCs (n=1,873) were obtained. Maternal Edinburgh Postnatal Depression Scale (EPDS) scores, collected during the 3rd trimester (n=1,920) and at 3 months postpartum (n=1,822), and the Behavior Assessment System for Children 2nd Edition internalizing and externalizing T-scores from children at age 5 (n=662) were also assessed. Child outcome models were stratified by offspring sex. Directed acyclic graphs and change-in-estimate rules determined multivariate linear regression model adjustment.

Results: Concentrations of maternal Hb, hepcidin and SF decreased, and sTfR and sTfR:SF increased as pregnancy progressed, with Hb and SF rebounding by 3 months postpartum (all $p<0.001$). Maternal 25(OH)D3 and 3-epi-5(OH)D3 concentrations decreased between the 2nd trimester and 3 months postpartum ($p<0.01$). In 627 pregnant participants with 2nd trimester SF and 25-hydroxyvitamin D data, 63% were replete in both micronutrients, 15% were iron replete but low in vitamin D, 18% were vitamin D replete but low in iron and 4% were low in both. Higher maternal 3rd trimester SF and hepcidin:EPO concentrations were linearly associated with lower BWs in male (SF: $p<0.01$; hepcidin:EPO: $p=0.03$) and female newborns (SF: $p=0.02$; hepcidin:EPO: $p=0.02$). There were inverse associations between BWs and 3rd trimester maternal hepcidin ($p=0.03$) and Hb ($p<0.01$), and BHCs and maternal 2nd trimester SF ($p<0.05$) and 3rd trimester Hb ($p=0.02$), but only in the male models. Higher 2nd trimester maternal SF ($p<0.05$), hepcidin ($p<0.01$) and 25(OH)D3 ($p<0.01$) concentrations predicted lower 3rd trimester maternal EPDS scores. Pregnant individuals that were vitamin D replete but low in iron ($p=0.04$) or low in iron and vitamin D ($p=0.02$) during mid-pregnancy had higher 3rd trimester EPDS scores

compared to those that were replete in both micronutrients. An inverse association between 3rd trimester maternal sTfR:SF and externalizing T-scores was observed in female children ($p=0.04$), whereas in male children a negative relationship was observed between maternal 3-epi-25(OH)D3 concentrations at 3 months postpartum and externalizing T-scores ($p=0.02$). Child externalizing T-scores differed depending on the combined maternal iron and vitamin D status during mid-pregnancy (all $p<0.04$).

Conclusions: Iron and vitamin D biomarker concentrations were dynamic across gestation and into the postpartum period in generally healthy pregnant individuals. Maternal SF concentrations were inversely related to BWs, BHCs, prenatal maternal depression symptoms and child externalizing behaviours at age 5, but these associations may be time and offspring sex dependent. Negative relationships were also observed between maternal vitamin D biomarkers and maternal prenatal depression symptoms as well as child externalizing behaviours at age 5. The potential impact of concurrent micronutrient deficiencies on maternal and offspring health outcomes should be explored in future research.

PREFACE

The Alberta Pregnancy Outcomes and Nutrition (APrON) study, the investigation that generated data that was analyzed in the thesis research, was granted ethical approval by the Conjoint Health Research Ethics Board (CHREB) at the University of Calgary, Project Name: "Alberta Pregnancy Outcomes and Nutrition (APrON): The Impact of Maternal Nutrient Status During Pregnancy on Maternal Mental Health and Child Development", No. REB14-1702_REN9, January 15, 2023.

Chapter 4 of this thesis is a manuscript that has been submitted to The Journal of Nutrition and was co-authored by the candidate and Anita Kozyrskyj, Natalie Hanas, Susan Goruk, Elnaz Vaghef-Mehrabani, Carolina M. Archundia-Herrera, Kimberly O. O'Brien, Nicole L. Letourneau, Gerald F. Giesbrecht, Rhonda C. Bell, and Catherine J. Field, titled "Maternal iron status is dynamic throughout pregnancy and might predict birth outcomes in a sex-dependent manner: Results from the APrON cohort study." The candidate was responsible for the conceptualization of project objectives, quantification of biomarker concentrations, statistical analyses of the data, manuscript writing and the application of revisions. A. Kozyrskyj was involved with the conceptualization of project objectives, statistical analyses and manuscript writing. N. Hanas was involved with the quantification of biomarker concentrations and manuscript writing. S. Goruk was involved with the quantification of biomarker concentrations and statistical analyses. E. Vaghef-Mehrabani was involved with manuscript writing. C.M Archundia-Herrera was involved with the statistical analyses and manuscript writing. K.O. O'Brien was involved with the conceptualization of project objectives and manuscript writing. N.L. Letourneau was involved with the conceptualization of project objectives. G.F. Giesbrecht was involved with the conceptualization of project objectives. R.C. Bell was involved with the conceptualization of project objectives, statistical analyses and manuscript writing. C.J. Field was the supervisory and corresponding author, and was involved with the conceptualization of project objectives, statistical analyses and manuscript writing.

Chapter 5 of this thesis is a manuscript that has been submitted to The American Journal of Clinical Nutrition and was co-authored by the candidate and Anita Kozyrskyj, Elnaz Vaghef-Mehrabani, Yvonne Lamers, Gerald F. Giesbrecht, Nicole L. Letourneau, Fariba Aghajafari, Deborah Dewey, Brenda Leung, Rhonda C. Bell, and Catherine J. Field, titled "Maternal iron and vitamin D status

during pregnancy and the postpartum period is associated with maternal prenatal depression and child externalizing behaviours in the APrON cohort." The candidate was responsible for the conceptualization of project objectives, quantification of iron biomarker concentrations, statistical analyses of the data, manuscript writing and the application of revisions. A. Kozyrskyj was involved with the conceptualization of project objectives, statistical analyses and manuscript writing. E. Vaghef-Mehrabani was involved with statistical analyses and manuscript writing. Y. Lamers was responsible for the quantification of vitamin D biomarker concentrations and was involved in manuscript writing. G.F. Giesbrecht was involved with the conceptualization of project objectives and manuscript writing. N.L. Letourneau was involved with the conceptualization of project objectives and manuscript writing. F. Aghajafari was involved with manuscript writing. D. Dewey was involved with the conceptualization of project objectives and manuscript writing. B. Leung was involved with the conceptualization of project objectives and manuscript writing. R.C. Bell was involved with the conceptualization of project objectives, statistical analyses and manuscript writing. C.J. Field was the supervisory and corresponding author, and was involved with the conceptualization of project objectives, statistical analyses and manuscript writing.

DEDICATION

I want to thank my family, partner and friends for their continuous encouragement and love. My involvement in this program would not have been possible without all of you.

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APrON, Alberta Pregnancy Outcomes and Nutrition
BASC-2, Behavior Assessment System for Children 2nd Edition
BBB, blood brain barrier
BHC, birth head circumference
BMI, body mass index
BW, birth weight
C3, carbon 3
CFG, Canadian Food Guide
CRP, C-reactive protein
CSF, cerebral spinal fluid
DAG, directed acyclic graph
DBP, vitamin D binding protein
ELISA, enzyme-linked immunosorbent assay
EM, effect modifier
EPDS, Edinburgh Postnatal Depression Scale
EPO, erythropoietin
FPN, ferroportin
Hb, hemoglobin
Hepcidin:EPO, hepcidin-EPO ratio
ID, iron deficiency
IDA, iron deficiency anemia
IL, interleukin
IRE, iron responsive element
IRP, iron regulatory protein
IU, International Unit
LC-MS/MS, liquid chromatography with tandem mass spectroscopy
RCT, randomized controlled trial
RBC, red blood cell
RDA, Recommended Dietary Allowance

SES, socioeconomic status

SF, serum ferritin

STB, syncytiotrophoblast

sTfR, soluble transferrin receptor

sTfR:SF, sTfR-SF index

Tf, transferrin

TfR, transferrin receptor

UVB, ultraviolet B

VDR, vitamin D receptor

$1,25(OH)_2D$, 1,25-dihydroxyvitamin D

25(OH)D, 25-hydroxyvitamin D

25(OH)D2, 25-hydroxyvitamin D2

25(OH)D3, 25-hydroxyvitamin D3

3-epi-25(OH)D3, 3-epi-25-hydroxyvitamin D3

CHAPTER 1: INTRODUCTION

1.1 Iron

Iron is an essential trace element that is crucial for optimal health (Dutt et al., 2022; Lieu et al., 2001). Dietary and body iron can be found in either heme or non-heme forms (Monsen, 1988). Heme iron is covalently bound to four pyrrole rings (a tetrapyrrole), whereas non-heme iron is not associated with this structure (Pauling & Coryell, 1936; Perutz, 1978). Iron is constantly shuttled through a series of ionic states by oxidoreductases, producing Fe^{3+} (ferric iron) and Fe^{2+} (ferrous iron) (Roman et al., 1993).

1.1.1 Iron metabolism and homeostasis

1.1.1.1 Dietary sources, digestion and absorption

The absorption of iron from dietary sources involves an orchestrated series of steps involving gastrointestinal enzymes, transporters and cells (Miret et al., 2003; Muñoz et al., 2009). Foods that contain non-heme iron are primarily plant-based and include legumes (lentils, lima beans and chickpeas), seeds (flax and pumpkin), vegetables (broccoli, spinach and kale) and grains (oats, quinoa and fortified flours) (National Institutes of Health [NIH], 2022a). The non-heme iron in foods is less bioavailable compared to heme iron; in fact, it is estimated that only 2% to 20% of non-heme iron present in the gastrointestinal tract is absorbed (Kalpalathika et al., 1991; Mayer Labba et al., 2022). Heme iron is absorbed with greater ease (between 15% to 35%) (Uzel & Conrad, 1998), and is mainly supplied from the hemoglobin (Hb) and myoglobin contained in the tissues of shellfish (oysters, mussels), fish (tuna), meat (beef, pork), and poultry (chicken, turkey) (Kongkachuichai et al., 2002; NIH, 2022a). Upon entry into the stomach, acidic degradation by hydrochloric acid can unbind iron from food matrices (Schubert, 2017). The simultaneous consumption of foods that are rich in vitamin C is recommended to improve the absorption of non-heme iron because ascorbic acid can bind to the ferric form to increase its solubility in the duodenum (Lynch & Cook, 1980). Liberated non-heme iron is then apically transferred into enterocytes by divalent metal transporter 1, which has a specificity for divalent metal ions (Au et al., 2008). The mode of heme iron import from the gut lumen into enterocytes is a point of debate, but there is evidence to support receptor-mediated endocytosis (West & Oates, 2008). Next, iron

is either utilized or stored in enterocytes or expelled into the circulation by ferroportin (FPN) (Pan et al., 2020), an iron exporter (Montosi et al., 2001).

1.1.1.2 Circulatory transport and cellular uptake

The transport of systemic iron is primarily conducted by transferrin (Tf) (Morgan & Laurell, 1963). After non-heme iron is transported across basolateral membranes of enterocytes, two ferric ions can bind to a Tf molecule (diferric Tf) for circulatory transport (Dutt et al., 2022; Yang et al. 2000). Other carriers are hemoplexin or albumin, which bind heme, and haptoglobin, which binds Hb (Smith & McColluh, 2015). Without sequestration, free systemic iron can promote macromolecular, such as lipid and nucleic acid, oxidative damage (Meneghini, 1997), and it may also function as a metabolic source for pathogens (Otto et al., 1992). In states of iron excess, non-transferrin-bound iron has been detected (Cogswell et al., 1998). Interestingly, immune, hepatic and pancreatic cells are known to have membrane proteins that are able to import unbound iron (Mleczko-Sanecka & Silvestri, 2021; Pinto et al., 2014).

The cellular uptake of iron is mainly facilitated by the binding of diferric Tf to transferrin receptor (TfR) 1 on cell membranes, triggering receptor-mediated endocytosis (Yang et al., 2000). After internalization, endosomal reductases reduce the iron to its ferrous form for liberation from Tf and subsequent exportation into the cytosol (Ohgami et al., 2005). The regulation of cellular uptake, through the expression of proteins like TfR1, is translationally controlled (Theil, 1994). Specifically, iron-responsive elements (IREs) are positioned at both ends of messenger ribonucleic acids (mRNAs) which bind to iron regulatory proteins (IRPs) (Anderson et al., 2012). If IRPs bind the 3' or 5' IREs on mRNAs, translation will either be promoted or prevented, respectively. The events that dictate binding of IRPs to a given mRNA position reflect iron abundance in the cell (Corral et al., 2021). Therefore, the translocation of iron into cells is generally dictated by its intracellular availability. For descriptions of other aspects of iron homeostasis, including intracellular trafficking, recycling and elimination, see Appendix 1.1.

1.1.2 Important functions of iron in physiology

1.1.2.1 Cell metabolism and regulation

Iron has a vital role in cell metabolism and regulation (Gao et al., 2021). The mitochondrion is arguably the most important organelle in cellular iron balance, being not only the location of

heme and Hb biosynthesis (Pauling & Coryell, 1936), but also where iron-sulfur clusters are synthesized (Beinert et al., 1997; Wiedemann et al., 2006). These clusters are cofactors that are required by enzymes that perform critical steps in cell metabolism (Johnson et al., 2005). For example, they are found in complexes I, II and III as well as the cytochromes of electron transport chains within inner mitochondrial membranes (Dutt et al., 2022; Trumpower & Edwards, 1979). Iron is also needed for cell function beyond metabolism (Muñoz et al., 2009). Iron-sulfur clusters are necessary in polymerase and helicase function in the synthesis of deoxyribonucleic acid (DNA) (Rudolf et al., 2006; Netz et al., 2012). Heme molecules are essential parts of hemoproteins, a group of metalloproteins with a variety of functions, including nitric oxide synthase and guanylyl cyclase (Dutt et al., 2022; Kanner at al., 1992). Another area of interest is the impact of cellular iron availability in the progression of the cell cycle (Mueller et al., 2006). Notably, iron sequestration studies have suggested that the gap 1 to synthesis cell cycle checkpoint may be related to intracellular iron status (Pop et al., 2010). Ultimately, iron is recognized to be an important regulator of metabolism and energy balance, implying its relevance for cell and tissue growth and development.

1.1.2.2 Neurological function

Iron can be transported across the blood brain barrier (BBB) (Aschner & Aschner, 1990; Fishman et al., 1987). Although the exact mechanism is yet to be confirmed, the majority of iron uptake into the brain is thought to be mediated by the binding of diferric Tf to TfR1 on the BBB endothelium or glial cells (Marell et al., 2019; Roberts et al., 1993). However, other carrier molecules, like lactoferrin and ferritin, may facilitate iron import into the cerebral spinal fluid (CSF) (McCarthy & Kosman, 2015; Rothenberger et al., 1996). After crossing the BBB, iron may be transported through the CSF to neurons or other cells via transporters like citrate (Mills et al., 2009). Another possible paradigm of iron delivery is an endothelial cell-astrocyte-neuron axis where a central glial cell regulates iron release to adjacent neurons (McCarthy & Kosman, 2015).

In the brain, iron supports neuronal myelination and neurotransmitter synthesis (Lozoff, 2000). Iron, bound to Tf or ferritin, can be delivered to oligodendrocytes, the myelin sheath producing cells of the central nervous system (Brand et al., 1993). These glial cells are extremely metabolically active, requiring efficient oxidative phosphorylation. As iron is a required cofactor in various metabolic pathways (Trumpower & Edwards, 1979), iron deficiency (ID) could impair

the production of energy needed for myelination. Iron is also a cofactor of enzymes that synthesize precursors of myelin, like 3-hydroxy-3-methylglutaryl coenzyme-A (Connor & Menzies, 1996). Indeed, in several animal model studies, iron deprivation during key periods of early neurological development led to inadequate myelination (Beard et al., 2003; Jorgenson et al., 2003). Moreover, iron is also required for neurotransmitter production and regulation, particularly dopamine and serotonin (Chen et al., 1995; Youdim et al., 1980). Tyrosine hydroxylase, an enzyme that contributes to the synthesis of dopamine, requires iron (Goodwill et al., 1997), and changes in dopamine receptors and transporters have been detected in states of ID (Chen et al. 1995). The impairment of serotonin producing enzymes, like tryptophan hydroxylase, and transporters have also been reported during iron deprivation (Kuhn et al., 1980).

1.1.3 Iron demands during pregnancy

Considering its critical role in a multitude of biological processes (Muñoz et al., 2009), the requirements for iron significantly increase during pregnancy (Fisher & Nemeth, 2017; Chandra et al., 2012). There are three compartments that have increased iron demands during this life stage, the pregnant person, placenta and offspring (O'Brien, 2022), but the adaptations of iron biology in gestating individuals is relevant to the thesis research and will be a focus of this section. Details of iron acquisition and utilization in the placenta and fetus are also described below.

1.1.3.1 Maternal demands

Many physiological changes occur within pregnant people, some of which necessitate increased iron availability (Fisher & Nemeth, 2017). The maternal demands for this micronutrient can be estimated by summating the amount of iron needed for processes that deplete it coupled by those that preserve it (Hallberg & Rossander-Hulten, 1991). Pregnancy results in the cessation of menstruation, leading to the retainment of iron that would otherwise be dispelled (Theobald, 1936). However, other normal avenues of iron loss, including gut enterocyte sloughing, still occur and may amount to >200 mg across gestation (Fisher & Nemeth, 2017; Hallberg & Rossander-Hulten, 1991). There are also increases in the plasma and red blood cell (RBC) volumes of pregnant individuals, starting around the middle of the 1st trimester (~6 weeks gestation) through to the early to mid 3rd trimester (~30 to 34 weeks) (de Haas et al., 2017; Lurie & Mamet, 2000). These adaptations improve blood flow to the placenta to facilitate an efficient transfer of nutrients to the

growing embryo or fetus (O'Brien, 2022). Importantly, the volumetric growth of maternal blood and RBCs do not occur to the same extent; plasma increases by ~50% of its original volume, exceeding the 15% to 20% rise in RBCs from pre-pregnancy values (Costantine, 2014; Koller, 1982). The result is hemodilution, a drop in systemic Hb concentrations of pregnant females, which is typically the most apparent during the later stages of the 2nd trimester (Fisher & Nemeth, 2017). Accordingly, as an attempt to adjust for hemodilution, several obstetric agencies lower the Hb-based threshold for maternal anemia during the 2nd trimester to <105 g/L, a decrease from <110 g/L during the 1st and 3rd trimesters (American College of Obstetricians and Gynecologists [ACOG], 2021; O'Connor et al., 2016). The additional iron required to produce extra Hb molecules needed for RBC expansion can total ~450 mg (Chiabrando et al., 2014), but this can vary depending on maternal anthropometrics (Hallberg & Rossander-Hulten, 1991).

A compensatory adaptation for the enhanced maternal demand for iron is that the proportion of absorbed non-heme iron progressively rises across pregnancy (Barrett et al., 1994). Previous evidence suggests that the latter change is highly correlated with declines in both the hepatic expression and systemic concentrations of maternal hepcidin (see 1.1.5.3) (Young et al., 2012), but the downregulatory mechanism is still poorly understood. Moreover, the iron status of people who recently gave birth may be decreased because of blood loss during parturition (Quinlivan et al., 1970; Wilcox et al., 1959). However, the normalization of maternal RBCs to pre-pregnancy values typically recycles enough iron to replenish hemorrhage-related losses (de Haas et al. 2017; Lurie & Mamet, 2000). With all the above considered, ~700 mg of iron is required across gestation to sustain the needs of the pregnant person, which does not account for increased placental or fetal demands (Fisher & Nemeth, 2017; O'Brien, 2022).

There have been recent calls to update the recommendations for maternal iron supplementation during gestation (Fairweather-Tait, 2022; Flores et al., 2017). Pregnancy-related iron guidelines vary by country or district. For example, the United Kingdom does not universally recommend additional iron supplementation in pregnancy compared to pre-pregnancy (~15 mg of iron per day) (Pavord et al., 2020), whereas an increased intake of 27 mg of iron a day is recommended for pregnant people in Australia (Queensland Government, 2020), Canada (Health Canada, 2009) and the United States (Institute of Medicine [IOM], 2006). Despite this, recent investigations that assessed the risk of maternal ID suggest that the prevalence of pregnant people with an insufficient status of iron may be underestimated (Beckert et al., 2019; Delaney et al.,

2020). This idea held even among a healthy pregnant cohort that was consuming the Canadian recommended dietary allowance (RDA) for iron as 80% were estimated to have depleted iron stores (serum ferritin <30 ug/L) (Cochrane et al., 2022). Still, the latter types of studies are sparse in the literature, which may stem from the limited iron screening guidelines or protocols in many obstetric practices where clinicians solely quantify Hb concentrations to determine the presence of anemia and rarely explore underlying ID (ACOG, 2021; O'Connor et al., 2016). Therefore, the knowledge gap of whether current iron recommendations are enough to sustain maternal iron adequacy remains to be addressed (Cochrane et al., 2022; Delaney et al., 2021a). Conversely, other researchers have raised concerns whether universal maternal iron supplementation should be required at all, and if the risk for iron overload is increased through these practices (Georgieff et al., 2019; Pavord et al., 2020).

Another aspect of maternal iron adequacy that is beginning to receive more attention is the pre-pregnancy period (Aranda et al., 2011). The status of iron prior to conception may be associated with obstetric outcomes (Davies et al., 2021; Viteri & Berger, 2005). The latter is especially important considering that people of reproductive age who may become pregnant are a population that appear to be vulnerable to ID and iron deficiency anemia (IDA) (Goonewardene et al., 2012); in fact, globally, an estimated 30% are anemic (Stevens et al., 2013).

1.1.3.2 Placental demands

The placenta is the main avenue by which iron from the pregnant individual is supplied to the growing embryo or fetus (King, 1976; O'Brien, 2022). Placental development during the early stages of a pregnancy is a dynamic process involving the reorganization of maternal and offspring tissues (Brosens et al., 2011). For the majority of the 1st trimester, the maternal spiral arteries are not adequately formed and can be anatomically blocked from supplying nutrients to the placental syncytiotrophoblast (STB) by early trophoblasts (Burton et al., 2002; Huppertz et al., 2014). As a result, the limited endowment of embryonic and early fetal nutrients is obtained from uterine secretions, a process referred to as histiotrophic nutrition. From ~13 gestational weeks onward, the spiral arteries are fully formed, optimizing access to nutrients like iron (Huppertz et al., 2014; O'Brien, 2022). This stage is known as hemotrophic nutrition. The majority of non-heme iron destined for the developing offspring approaches the maternal-placental interface as diferric Tf (King, 1976; Okuyama et al., 1985). The apical surface of the STB contains TfRs that bind diferric

Tf to trigger receptor-mediated endocytosis (Dutt et al., 2022). After uptake, ferrireductases reduce ferric iron causing its release from Tf and subsequent transport into the STB cytosol (Ohgami et al., 2005).

The placenta itself requires iron to sustain its high metabolic and functional demands (Delaney et al., 2020; Gao et al., 2021). In fact, it has been estimated that ~40-90 mg of iron is acquired by the placenta over the course of a normal term pregnancy (Barad et al., 2022). Therefore, this organ is thought to independently express its own iron regulatory hormones and may uniquely respond to the iron status of the pregnant individual throughout gestation (O'Brien, 2022). The expression of hepcidin (Yang et al., 2016), erythropoietin (EPO) (Conrad et al., 1996) and erythroferrone (ERFE) have been detected in placental tissues (Srole & Ganz et al., 2021). While hepcidin and EPO are described elsewhere (Chapter 1; Appendix 1.2), ERFE is a peptide that supports erythropoietic efficiency (Kautz et al., 2014; Srole & Ganz et al., 2021). Interestingly, when these iron-regulatory hormones are expressed by the pregnant individual they do not appear to impact placental iron homeostasis and in a comparable manner, no associations between fetal iron status and placental versions of these regulators have been reported (O'Brien, 2022). Therefore, the actions of EPO, ERFE and hepcidin may be limited to wherever they were synthesized. Conversely, other reports suggest that the status of maternal iron may lead to a modulation in placental transcription (Sangkhae et al., 2020). For example, TfR1 expression has been shown to increase during states of maternal ID (Li et al., 2008). Moreover, placental FPN may be downregulated to prevent the escape of intracellular iron during maternal ID (Cao et al., 2021). These observations could imply that the placenta prioritizes its own iron needs over the demands of the developing offspring (Keegan et al., 2022; O'Brien, 2022). Potential changes in iron-related gene expression or homeostasis may be further mediated by apparent differences in placental programming and function depending on the biological sex of the fetus (Tarrade et al., 2015; Rosenfield, 2015).

1.1.3.3 Fetal demands

Some of the regulatory mechanisms of fetal iron status have been described, but important questions remain. Estimates suggest that the offspring must endow ~270 mg of iron from the maternal compartment during gestation (Fisher & Nemeth, 2017). Once iron has been pumped from the placenta into the stromal space, its subsequent mode of entry into the circulation of the

fetus has not been confirmed (Cao & Fleming, 2022). Diferric Tf or unbound iron may pass through fetal endothelial cells into the circulation (Okuyama et al., 1985; Sweet et al., 2001), but more research is warranted. Nonetheless, erythropoiesis is a crucial iron-related process in the developing embryo and later fetus and has been reported to begin at or just beyond the first week of growth during the yolk sac stage (Palis et al., 1999). The anatomical location of erythropoiesis shifts as organogenesis progresses; the fetal liver gains erythropoietic control during the 1st trimester, which dominates for the rest of gestation (Popescu et al., 2019). To regulate RBC synthesis and iron availability, the fetus has been shown to produce its own supply of hepcidin, EPO and ERFE, which has been identified through the quantification of these biomarkers in umbilical cord blood (Bahr et al., 2021; O'Brien, 2022). The impacts of these hormones on fetal iron homeostasis appear to be similar to their role after birth (Delaney et al., 2021b). However, it is still unclear whether fetal hepcidin, EPO and ERFE modulates iron status in the pregnant person or placenta. While correlations between iron sufficiency in the pregnant person and fetal iron regulatory biomarkers, like EPO, have been reported (Macphail et al., 1981), other evidence suggests that these fetal mediators may not exert control over the expression of placental TfR1 or FPN (Sangkhae et al., 2020).

1.1.4 A low or high maternal iron status during pregnancy: implicated health outcomes

1.1.4.1 Maternal outcomes

Iron status in pregnant individuals may contribute to several adverse maternal symptoms or events during or after pregnancy. It is well-established that IDA can result in tiredness, reduced strength as well as poor mental concentration and thermoregulation (Knizley & Noyes, 1972), and individuals may experience similar or exacerbated versions of these symptoms during pregnancy or postpartum (Chandra et al., 2012; Goonewardene et al., 2012). Given the importance of iron in neurological function (Chen et al., 1995; Connor & Menzies, 1996), maternal iron depletion before, during or after pregnancy could lead to poor maternal mental health outcomes (Thirupathi & Chang, 2019, pp. 6–10). Indeed, associations between several iron status biomarkers, such as serum ferritin, or the presence of IDA during pregnancy and more maternal depressive symptoms, often in the postpartum period, have been reported (Dama et al., 2018; Hameed et al., 2022). A newer analytic approach is to assess the trajectory of maternal stress or mental health symptoms

across time (Chow et al., 2019). Such analyses have identified risk factors that may contribute to maternal depression during or after pregnancy (Korja et al., 2018; Vanwetswinkel et al., 2022).

There are other medical conditions that may be influenced by gestational iron overload (Ng et al., 2019; Zein et al., 2014). Gestational diabetes mellitus was positively associated with maternal iron status in several meta-analyses (Fu et al., 2016; Iqbal & Ekmekcioglu, 2019). Further, in several investigations that analyzed hematological differences in pregnant individuals with preeclampsia compared to those without preeclampsia, significantly higher maternal concentrations of several iron biomarkers, like ferritin, were identified in those with the condition (Mannaerts et al., 2018; Siddiqui et al., 2011). Health Canada recommends that pregnant people should not consume >45 mg/day of iron to prevent toxicity (Government of Canada, 2006).

1.1.4.2 Offspring outcomes

Embryonic or fetal exposures to maternal ID may modify offspring growth and development (Hales & Barker, 1992; Silveira et al., 2007). In Taeuburt et al. (2022), maternal ferritin concentrations were significantly related to cord blood epigenetic modifications that persisted in children up to 10 years of age. Relationships between maternal ID or IDA and a higher risk of poor birth outcomes, including preterm deliveries and low birth weights (BWs) have been reported (Aranda et al., 2011). An increased risk of offspring mortality has also been linked with maternal IDA (Stephansson et al., 2000). Several previous meta-analyses suggested that maternal iron or a multi-micronutrient (generally with ≥30 mg of iron) during gestation could reduce the risk of low BWs or small for gestational age (Devakumar et al., 2016; Keats et al., 2022). In contrast, other studies have reported associations between high maternal iron status and poor birth outcomes (Dewey & Oaks, 2017). The impact that maternal iron status during or after pregnancy may have on behavioural outcomes from infancy to adolescence has also been explored (Cortes-Albornoz et al., 2021; Iglesias et al., 2018), and while there is some evidence, the overall body of literature remains mixed.

1.1.5 Detecting iron deficiency or excess during pregnancy: the utility of biomarkers

The consumption of dietary and supplemental iron are major contributors to its biological status (Björn-Rasmussen & Hallberg, 1979). Unfortunately, foods that are rich in iron, particularly bioavailable heme sources, are financially or geographically inaccessible for many populations

(Kongkachuichai et al., 2002; Monsen, 1988). For this reason and others, iron continues to be one of the most deficient micronutrients worldwide, particularly among groups with increased iron demands like pregnant people (Cappellini et al., 2020).

Iron deficiency has been clinically conceptualized into distinct stages starting with the depletion of ferritin stores eventually leading to IDA (Choi, 2005; Lynch et al., 2018). The earliest indication of a low iron status is a decline in intracellular ferritin stores, known as the 1st or pre-latent stage (Plays et al., 2021). The 2nd (latent) stage is marked by distinct changes in circulatory iron biomarkers, which will be further described below. At this point heme synthesis for erythropoiesis may become dysregulated (Galmozzi et al., 2019; Zheng et al., 2008), which has been identified as part of stage 2 or as its own distinct period termed iron-deficient erythropoiesis (Brugnara, 2003). The most severe period of ID, the 3rd or direct stage, is IDA, indicated by decreases in systemic Hb concentrations (Knizley & Noyes, 1972). IDA occurs when the Hb synthesis in bone marrow erythroblasts is hindered by the depletion of iron containing heme groups (Schneider-Yin et al., 2000).

An important consideration of several iron biomarkers is the potential compounding impact of inflammation and acute phase responses on their systemic concentrations (Mei et al., 2017). Innate immune cells, like macrophages and neutrophils, can increase their expression of cytokines, including interleukin (IL)-1, IL-6, and interferon gamma, following the detection of pathogens or other non-host materials (Pradhan et al., 2020; Tran et al., 1997). These mediators can cause an induction of the hepatic acute phase response, marked by elevated circulatory concentrations of acute phase reactants like C-reactive protein (CRP), α-1-glycoprotein, hepcidin and ferritin (Dutt et al., 2022; Feelders et al., 1998). Resultingly, concentrations of certain iron biomarkers might appear normal or elevated when in reality, ID could be present (Mei et al., 2017). The latter may contribute to the anemia of chronic disease (Nemeth & Ganz, 2014). The reported changes of iron biomarkers of interest in the presence of inflammation will be further described below.

1.1.5.1 Serum ferritin (SF)

Iron that exceeds intracellular demands can be stored conjugated to ferritin until functional needs within the cell or elsewhere triggers its release (Cook et al., 1974). Ferritin molecules are multiprotein complexes comprised of 24 subunits of either light chains (L-chains) or heavy chains (H-chains), and the specific ratio of these subunit types depends on the tissue (**Figure 1**) (Wang et

al., 2010). Ferritin is often secreted into the circulation by hepatic or splenic macrophages during RBC recycling or by iron storing hepatocytes (Wang et al., 2010). Concentrations of ferritin molecules quantified from serum samples are clinically known as serum ferritin (SF) (Cook et al., 1974). This biomarker is highly correlated with cytosolic ferritin within various tissues and its systemic concentration is often utilized for the prediction of body iron stores (Daru et al., 2017). The quantification of SF concentrations is particularly useful because they can detect the earliest pre-latent stage of ID (Lynch et al., 2018), providing a window of opportunity for early intervention. Other details about ferritin and SF biology are provided in Appendix 1.2.1.

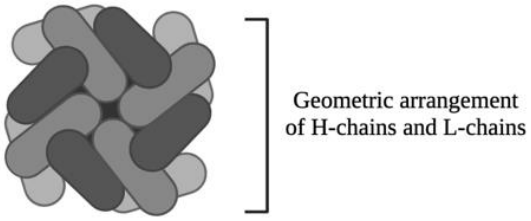

Figure 1. A representation of the globular structure of ferritin. The heavy chain (H-chain) and light chain (L-chain) subunits can be conceptualized by the different coloured units (Wang et al., 2010). Figure created using BioRender.com.

Serum ferritin concentrations may be increased during states of inflammation (Fisher & Nemeth, 2017; Bertoli et al., 2019). A concurrent state of inflammation may overestimate iron stores because concentrations may be more reflective of an ensuing acute phase response rather than iron sufficiency. Furthermore, ferritin itself has also been reported to exacerbate inflammatory cascades (Ng et al., 2019). Particularly, SF may initiate intracellular cascades which have the potential to increase nuclear factor kappa B expression, a transcription factor involved in diverse cellular outcomes including inflammation (Chen et al., 2005; Wang et al., 2010). Several techniques to adjust SF for the influence of inflammation have been proposed, including an equation that accounts for CRP and α-1-acid glycoprotein (Mei et al., 2017).

Investigations that report on SF concentrations in large cohorts of pregnant individuals from high-income countries, including Canada, are limited. In the few studies that have recruited from these populations, SF is rarely measured during each trimester and the postpartum period. Nonetheless, previous investigations suggest that SF concentrations generally decline as pregnancy progresses (**Table 1**) (Akesson et al., 1998; Bencaiova et al., 2012; Berglund et al.,

2016; Berglund et al., 2017; Bowers et al., 2016; Cochrane et al., 2022; Khambalia et al., 2015; Gernand et al., 2019; Lee et al., 2014). Concentrations of either <12 μg/L or more commonly <15 μg/L are cut-offs for iron storage depletion during pregnancy (Walsh et al., 2011; Lee et al., 2014). The World Health Organization recently published guidelines that recommended the use of SF <15 μg/mL during the 1st trimester but did not list cut-offs for the 2nd or 3rd trimester citing the unknown impacts of hemodilution and inflammation on SF in mid to late pregnancy (WHO, 2020). However, a high threshold of <30 μg/L has been proposed by others (Nelson et al. 1978; O'Connor et al., 2016). From several investigations, the proportion of pregnant people with iron storage depletion increased across gestation (Table 1) (Hernández-Martinez et al., 2011; Walsh et al., 2011; Lee at al., 2014; Cochrane et al., 2022).

Table 1. Ranges of mean or median maternal SF concentrations and the prevalence of iron storage depletion among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
SF (μg/L)	25.4 – 95.3 [1-3]	19.9 – 34.0 [4-6]	10.0 – 19.2 [2,5-9]
Low iron stores (%)	10 – 20 [10,11]	22 – 57 [6,10,11]	35 – 71 [6,9-11]

Superscripts indicate citations for the data included in Table 1: [1]Bowers et al. 2016; [2]Gernand et al. 2019; [3]Khambalia et al. 2015; [4]Bencaiova et al. 2012; [5]Berglund et al. 2016; [6]Lee et al. 2014; [7]Akesson et al. 1998; [8]Berglund et al. 2017; [9]Cochrane et al. 2022; [10]Hernández-Martinez et al. 2011; [11]Walsh et al. 2011. Abbreviations: serum ferritin (SF).

1.1.5.2 Soluble transferrin receptor (sTfR)

Systemic concentrations of soluble transferrin receptor (sTfR) are derived from the cleavage of TfRs from the exosomal compartments of cells (Ahn et al., 1993; Cao & O'Brien, 2013). After the binding of diferric or empty Tf to TfRs, a fraction of the empty Tf-TfR complexes are not recycled back to the plasma membrane and is instead rerouted to endosomes (Hopkins & Trowbridge, 1983). In these spaces, the extracellular domains of TfR can be cleaved creating two monomers, known as sTfRs, each with a weight of ~320 kDa (**Figure 2**) (Harms & Kaiser, 2015). In a state of intracellular ID, the expression of TfR1 is upregulated by IRP/IRE translational control mechanisms (Anderson et al., 2012; Dutt et al., 2022). While the proportion of extracellular TfR cleavage in endosomes is thought to be constant, a higher expression of TfR increases the

production and release of sTfR (Beguin, 2003). Therefore, as sTfR concentrations increase so does the extent of poor iron status. Many sTfRs are derived from iron deficient erythroid precursors, so its quantification can give insight into erythropoietic efficiency (Cao & O'Brien, 2013; Fisher & Nemeth, 2017), and higher sTfR concentrations are considered to be indicative of the 2nd stage of ID (Lynch et al., 2018).

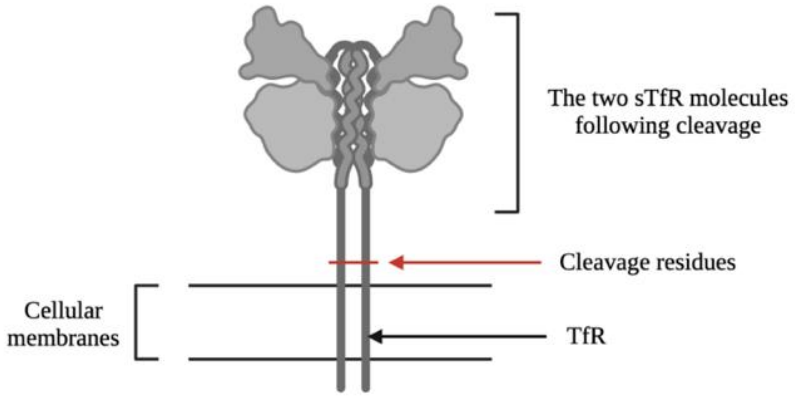

Figure 2. A representation of a transferrin receptor (TfR) with its extracellular domains that become two soluble transferrin receptors (sTfRs) after cleavage (Hopkins & Trowbridge, 1983). Figure created using BioRender.com.

The quantification of maternal sTfR concentrations at multiple timepoints in cohorts of pregnant women from high-income nations are limited, especially among healthy participants. However, while the mean (or median) maternal sTfR concentrations were variable between studies, they generally suggest that this biomarker increases across pregnancy (**Table 2**) (Akesson et al., 1998; Bowers et al., 2016; Lee et al., 2014; Cochrane et al., 2022). A limitation with the use of this iron biomarker is that there is currently no consensus on the most clinically accurate sTfR cut-off representative of ID in pregnant people (WHO, 2014). Concentrations of >2.4 mg/L, >4.4 mg/L and >8.5 mg/L have been proposed (Akesson et al., 1998; Walsh et al., 2011; Lee et al., 2014; Næss-Andresen et al., 2019).

Table 2. Ranges of mean or median maternal sTfR concentrations among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
sTfR (mg/L)	1.4 – 4.1 [1,2]	4.2 (3.8-4.6) [3]	4.1 – 5.4 [2-4]

95% confidence intervals are shown in parenthesis and are only shown when there was only one study with a sTfR measurement for that timepoint. Superscripts indicate citations for the data included in Table 2: [1]Akesson et al. 1998; [2]Bowers et al. 2016; [3]Lee et al 2014; [4]Cochrane et al. 2022. Abbreviations: soluble transferrin receptor (sTfR).

1.1.5.3 Hepcidin

Hepcidin is a peptide involved in the regulation of iron mobilization from cellular stores and non-heme iron absorption in the small intestine (Park et al., 2001; Jordan et al., 2009). Both of these actions are critical for the maintenance of iron homeostasis, particularly the prevention of overload (Vela, 2018). When intracellular iron is high, a majority of FPN molecules are loaded with ferrous iron (Dutt et al., 2022). Hepcidin binds to an extracellular pocket of FPN, and it does so with a higher affinity when iron or other metals are present because of the resulting covalent interactions (**Figure 3**) (Billesbelle et al., 2020). In this configuration, hepcidin obstructs the FPN ion channel preventing iron export from cells into the circulation (O'Brien, 2022). As for its negative control on iron absorption, hepcidin is known to downregulate the expression of divalent transporter 1 (Mastrogiannaki et al., 2013), which hinders the apical transport of non-heme iron from the gut lumen into enterocytes. Decreases in the expression or systemic concentrations of hepcidin signals low iron status or iron-deficient erythropoiesis (Park et al., 2001; Jordan et al., 2009). Other details about hepcidin are provided in Appendix 1.2.2.

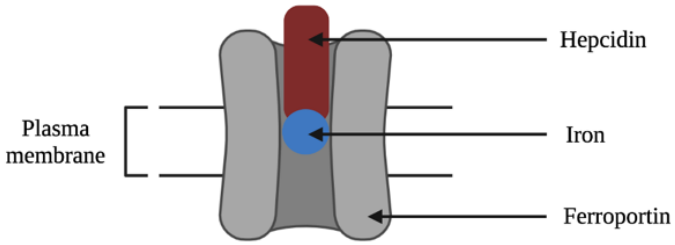

Figure 3. A representation of hepcidin (red) preventing the release of iron (blue) into the circulation through ferroportin (FPN; grey iron channel) (Billesbelle et al., 2020). Figure created using BioRender.com.

Beyond its role in iron regulation, systemic hepcidin concentrations may be reflective of inflammation (Tabbah et al., 2018). Hepatocytes are responsive to pro-inflammatory cytokines produced by innate immune cells when foreign materials are detected (Feelders et al., 1998). When IL-6 concentrations rise, hepcidin expression may proportionally increase as IL-6 receptors trigger pathways that can activate *HAMP,* the hepcidin gene (Pietrangelo et al., 2007). Therefore, inflammation may increase hepcidin concentrations, which could overestimate iron status adequacy and contribute to less iron availability (Nemeth & Ganz, 2014). During pregnancy, a downregulation of hepcidin may be essential to fulfill the increasing iron demands of the fetus, placenta and pregnant person (O'Brien, 2022). If elevated hepcidin is more reflective of maternal infections or chronic conditions that raise the expression of IL-6, there may be an increased risk of ID or even IDA (Anelli et al., 2018; Jones et al., 2021).

As with SF and sTfR, the quantification of maternal concentrations of hepcidin at different periods of gestation among generally healthy pregnant people is sparse. Clinical cut-offs of hepcidin concentrations that are suggestive of ID are not well-established (Fisher & Nemeth, 2017; O'Brien, 2022). In the studies that have reported maternal hepcidin concentrations in pregnancy, this biomarker is often shown to decrease throughout gestation, but variability in the quantified values is apparent among the investigations (**Table 3**) (Braithwaite, et al. 2019; Delaney et al., 2021a; Hedengran et al., 2016; Lee et al., 2014; Lee et al., 2016). Discrepancies in the hepcidin concentrations may reflect differences in several factors, including the risk of inflammation or malnutrition (Cappellini et al., 2020; Pietrangelo et al., 2007), between pregnant cohorts.

Table 3. Ranges of mean or median maternal hepcidin concentrations among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
Hepcidin (ng/mL)	5.8 – 7.3 [1,2]	1.2 – 24.0 [2-5]	0.8 – 21.3 [1,2,4]

Superscripts indicate the citations for the data included in Table 3: [1]Braithwaite et al. 2019; [2]Hedengran et al. 2016; [3]Delaney et al. 2021a; [4]Lee et al. 2014; [5]Lee et al. 2016.

1.1.5.4 Erythropoietin (EPO)

Erythropoietin is a sugar-conjugated peptide that regulates iron homeostasis by enhancing erythropoiesis during hypoxia (**Figure 4**) (Goldberg et al., 1988). The majority of systemic EPO is derived from peritubular interstitial fibroblasts in the kidneys (O'Brien, 2022; Peng et al., 2020). When oxygenation is adequate, the biosynthesis of EPO is either absent or very low, but its circulatory presence significantly increases during the deprivation of circulatory oxygen (Goldberg et al., 1988; Schuster et al., 1987). Upon arrival in the bone marrow, EPO increases the efficiency and success of erythropoiesis by triggering signaling cascades in erythrocyte precursors that reinforce cell division, differentiation and survival (Lacombe & Mayeux, 1999). Ultimately, increased EPO concentrations are reflective of a low iron status. Other details about EPO biology are provided in Appendix 1.2.3.

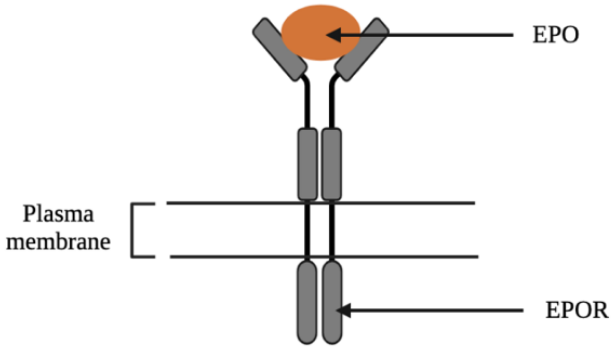

Figure 4. A representation of erythropoietin (EPO; orange) binding to the EPO receptor (EPOR; grey) on a cell surface. Binding of EPO to EPOR on erythrocyte precursors generate signal cascades that support the division, differentiation and survival of the cell (Lacombe & Mayeux, 1999). Figure created using BioRender.com.

Previous investigations suggest that maternal EPO concentrations increase throughout pregnancy (**Table 4**) (Delaney et al., 2021a; Kosiński et al., 2016; Lee at al., 2014), but these reports are limited. The quantification of maternal EPO concentrations may more reliably reveal the biological status of iron in the presence of inflammation; in fact, EPO may be involved in enhancing anti-inflammatory cascades (Peng et al., 2020). However, because this hormone is upregulated in response to hypoxia (Goldberg et al., 1988; Schuster et al., 1987), changes in maternal EPO concentrations are likely to occur further along in the ID continuum, closer to the onset of IDA (Delaney et al., 2021a). Nonetheless, maternal EPO concentrations may provide an

important indication of the extent of ID or erythropoietic demands during pregnancy (Nemeth & Ganz, 2014; Peng et al., 2020).

Table 4. Ranges of mean or median maternal EPO concentrations among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
EPO (mIU/mL)	11.2 ± 5.2 [1]	28.3 – 29.7 [2,3]	30.6 (26.3, 35.9) [3]

Standard deviations (mean ± SD) or 95% confidence intervals (shown in parenthesis) are only shown when there was only one study with a EPO measurement for that timepoint. Superscripts indicate the citations for the data included in Table 4: [1]Kosiński et al. 2016; [2]Delaney et al. 2021a; [3]Lee et al. 2014. Abbreviations: erythropoietin (EPO).

1.1.5.5 Combined biomarker measurements

The use of two iron biomarkers may be more informative about iron status compared to the utilization of either one alone (Delaney et al., 2021a; Nadeem et al., 2011). More research is required to determine their physiological and statistical relevance (Curran-Everett, 2013).

1.1.5.5.1 sTfR-SF index (sTfR:SF)

During states of low iron status or ID, concentrations of SF and sTfR decrease and increase, respectively (Allen et al., 1998; Nelson et al., 1978). It has been proposed that combining these biomarkers may provide a more accurate insight into the extent of ID or iron-deficient erythropoiesis (Castel et al., 2012; Nadeem et al. 2011). As SF concentrations may be increased in the presence of inflammation, factoring in sTfR concentrations, which are thought to be less influenced by these environments, could be used to partially control for this confounding factor (Mei et al. 2017; Pradhan et al. 2020). sTfR-SF index (sTfR:SF) measurements are calculated by dividing sTfR concentrations by the logarithm of SF concentrations [sTfR/log(SF)] (Castel et al., 2012). As iron status decreases and accordingly, sTfR (the numerator) increases and SF (the denominator) decreases, and the sTfR:SF ratio increases. Investigations that report this index at more than one pregnancy timepoint in generally healthy females are limited, but van Santen et al. (2013) observed significant increases (<1 mg/μg in the 1st and 2nd trimesters; ~1.5 mg/μg in the 3rd trimester).

1.1.5.5.2 Hepcidin-EPO ratio (hepcidin:EPO)

The ratio of hepcidin to EPO (hepcidin:EPO) was recently found to be highly indicative of both maternal and fetal iron status (Delaney et al. 2021a; Delaney et al. 2021b). The integration of hepcidin and EPO into one measure could be especially useful because it can reflect the extent of ID and oxygenation, respectively (Goldberg et al., 1988; Jordan et al., 2009). Like sTfR:SF, adding another biomarker that may not be influenced by pro-inflammatory states, like EPO (Peng et al. 2020), could provide a more accurate measure of hepcidin (O'Brien 2022). Therefore, this indicator is thought to be less impacted in the presence of inflammation. When the status of iron is low, hepcidin (the numerator) decreases and EPO (the denominator) increases, leading to lower hepcidin:EPO values. There may be only one study to date that has reported on maternal hepcidin:EPO concentrations during pregnancy and in this investigation, there was no difference between 2nd trimester and delivery measurements (Delaney et al. 2021a).

1.2 Vitamin D

Vitamin D has a multifaceted role in physiology (Bikle & Christakos, 2020). Humans can obtain vitamin D endogenously or exogenously (Webb et al., 1988), which will be further explained below. Broadly, there are two types of vitamin D, cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2). These forms and their derivatives retain the same secosteroid backbone but retain unique functional groups (Lips, 2006), however, their biological impacts are thought to be similar (Bikle & Christakos, 2020; Tripkovic et al., 2012).

1.2.1 Vitamin D metabolism and homeostasis

1.2.1.1 Endogenous synthesis

Endogenous production of vitamin D3 is localized in the epidermis and begins in the presence of an adequate dose of ultraviolet B (UVB) radiation (Clemens et al., 1982; Webb et al. 1988). The sunlight penetrates into the skin to promote the conversion of 7-dehydrocholesterol to pre-vitamin D3 (Bikle & Christakos, 2020). Pre-vitamin D3 is sterically unstable and quickly undergoes a reversible rearrangement into its epimer, vitamin D3. After vitamin D3 is formed, it can enter into the systemic pool by diffusion (Haddad et al., 1993).

The extent of cutaneous vitamin D production is dependent on several factors or personal characteristics (Dominguez et al., 2021). Vitamin D3 synthesis is positively correlated with the

surface area of the non-clothed skin that is exposed to UVB and the duration of time spent in sunlight (Webb et al., 1988; Holick et al., 2002). Endogenous production is also reliant on the intensity of UVB radiation which can be modified by weather events, climate patterns, season and latitude of residence (Lehmann, 2009). In fact, very little to no vitamin D3 is endogenously produced at or past latitudes of ~35 degrees north or south of the equator during non-summer months (Cannell et al., 2008), a phenomenon coined as the 'vitamin D winter' (Webb et al., 1988). Another consideration is skin pigmentation (Clemens et al., 1982). The darkness of skin is related to the expression of epidermal melanin (Zonios et al., 2001). Overtime, melanin molecules migrate up the epidermis to the skin surface where they can absorb UVB rays. Resultingly, only a fraction of radiation, proportional to the concentration of epidermal melanin, is accessible for the light-dependent biosynthesis of vitamin D3 (Lehmann, 2009; Osmancevic et al., 2015).

1.2.1.2 Exogenous sources, digestion and absorption

The consumption of vitamin D in food or supplements can be a significant source of this micronutrient (Dominguez et al., 2021; Vieth, 1999). In fact, the vitamin D dietary reference intakes outlined by Health Canada were derived under the assumption that endogenous production is minimal (Government of Canada, 2020). Solar radiation increases the risk of oncogenic phenotypes (Armstrong et al. 1997), so these guidelines favour sun avoidance to protect against skin cancer rather than recommending UVB exposure to ensure vitamin D repletion (Government of Canada, 2020). Food sources that contain vitamin D, either as vitamin D2 or vitamin D3, are generally limited (Dominguez et al., 2021). However, fatty fish (cod, salmon and sardines) (Barnett, 1982), organ meats and egg yolks contain vitamin D3, whereas uncooked mushrooms (shiitake or maitake) are the highest natural sources of vitamin D2 (NIH, 2022b). The addition of vitamin D to milk derived from cows and margarine through fortification is mandated in Canada (Health Canada, 2020; Langlois et al. 2010). Despite these food sources, the highest exogenous source of vitamin D is thought to be from supplements (Vieth, 1999). Vitamin D3 or D2 or other derivatives of these compounds, with or without additional nutrients, are commercially available in a variety of doses from different brands (Pludowski et al., 2018).

As a lipid-soluble micronutrient, the digestion and absorption of vitamin D forms are highly related to other dietary fats (Maurya & Aggarawal, 2017). The stomach is thought to be an important mediator of vitamin D liberalization. If vitamin D is bound to vitamin D binding protein

(DBP), gastric pepsin or duodenal trypsin can degrade this carrier (Dominguez et al., 2021; Tso & Fujimoto, 1991). Entry of lipids and proteins into the small intestine signals the release of cholecystokinin triggering the secretion of bile acids into the duodenum (Dockray, 2012; Polak et al., 1975). Bile acids further emulsify and solubilize vitamin D into amphipathic micelles that diffuse into enterocytes (Maurya & Aggarawal, 2017). Inside enterocytes, vitamin D and other lipids are integrated to form chylomicrons (Ghosh et al., 2022, pp. 65–67), and these transport vesicles are secreted into lymphatic circulation destined for the liver (Reboul, 2015).

1.2.1.3 Hormonal activation

Regardless of endogenous or exogenous origin, vitamin D undergoes two regulated hydroxylation reactions to yield its hormonally active form, 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Ponchon et al., 1969; Norman et al., 1971). The first modification occurs at the 25^{th} carbon by the addition of a hydroxyl group via mitochondrial 25-hydroxylases in the liver (Jenkinson, 2019); the resulting product is calcidiol or 25-hydroxyvitamin D (25(OH)D) (Blunt et al., 1968), the most abundant vitamin D metabolite in the circulation (Galior et al., 2018). Hepatic cytochrome P450 2R1 is the major enzyme that contributes to 25(OH)D formation (Cheng et al., 2004). In the kidneys, a second hydroxylation occurs at the 1^{st} carbon of 25(OH)D yielding $1,25(OH)_2D$, a reaction mediated by 1-α-hydroxylases (Xu et al., 2015; Zhu et al., 2013).

1.2.1.4 Circulatory transport and intracellular binding proteins

The majority of systemic vitamin D metabolites are bound to DBP, or the vitamin D binding protein (Daiger et al. 1975; Imawari et al., 1976). Indeed, previous studies suggest that over 80% of 25(OH)D and $1,25(OH)_2D$ are bound to DBP when present in the blood stream (Bikle & Christakos, 2020; Galior et al., 2018). Lower affinity chelation to albumin makes up the majority of the remaining non-DBP vitamin D transport (Swamy, 2008), but a small proportion (<1%) is thought to be present in a non-bound, free form (Bikle & Christakos, 2020).

Vitamin D receptors (VDRs) are structurally nuanced receptors with a plethora of transcriptional and non-transcriptional related activities (Brumbaugh & Haussler, 1974). These receptors contain a ligand binding domain, that binds $1,25(OH)_2D$, and a DNA binding domain (Freedman & Towers, 1991; Vanhooke et al., 2004). Interestingly, the particular way in which a ligand binds to VDR can modify the overall conformation and function of the complex. In the

nucleus, 1,25(OH)$_2$D-VDR complexes bind retinoid X receptor-α to form its fully functional state as a transcription factor (Orlov et al., 2012). Recruitment of other mediating molecules, including steroid receptor co-activators 1, 2 and 3, dictate the specificity of genomic actions, usually via chromatin-dependent or other epigenetic changes (Bikle & Christakos, 2020; Oda et al., 2009). The target genes of VDR action are often specific to the particular cell environment.

For additional details regarding the regulation of vitamin D activation, cellular uptake and storage, see Appendix 2.1.

1.2.2 An important physiological role of vitamin D: neurological function

There is evidence that suggests the presence of vitamin D in brain tissues (Eyles et al., 2005; Landel et al., 2018). Molecules of 25(OH)D can traverse the BBB (Christensen & Birn, 2002), and they are likely converted to 1,25(OH)$_2$D as one of the major 1-α-hydroxylases, hepatic cytochrome P450 27B1, is expressed in the brain (Eyles et al., 2005; Landel et al., 2018). Other vitamin D metabolites have been located in the CSF (Zelzer et al., 2021), but whether they can cross the BBB like 25(OH)D is unclear. Neuronal and glial cells are known to express VDRs (Eyles et al., 2005), further implicating a demand for vitamin D in this environment.

Previous studies propose a role for vitamin D in the development and ongoing maintenance of the brain (Groves et al., 2014). VDR-related changes in protein expression, like glial or T cell cytokine concentrations, have been shown to be anti-inflammatory or anti-oxidative (Dulla et al., 2016; Won et al., 2015; Smolders et al., 2009). Moreover, during early life, the coordinated actions of 1,25(OH)$_2$D may program the development of neurons through the expression of nerve growth factor (Brown et al., 2003) and glial derived neurotrophic factor (Sanchez et al., 2009). Most of the above evidence was derived from in vitro and pre-clinical studies, although research in humans is ongoing (Eyles et al. 2013). The role of vitamin D in immunomodulation is discussed in Appendix 2.1.

1.2.3 Vitamin D demands during pregnancy

There is compiling evidence suggesting the importance of vitamin D in the regulation of maternal, placental and fetal health during pregnancy (Karras et al., 2018). Although the specific requirements of each compartment are less defined compared to iron, the proposed gestational

adaptations of vitamin D homeostasis in pregnant people will be discussed below (Cyprian et al., 2019). Placental and fetal vitamin D dynamics and regulation are also detailed next.

1.2.3.1 Maternal demands

The availability of maternal $1,25(OH)_2D$ and more recently, DBP, may increase as pregnancy progresses (Bikle et al., 1984; Steichen et al., 1980). It has also been proposed that the regulation of maternal vitamin D metabolism is unique to the gestational period. Notably, the renal expression of 1-α-hydroxylases are reported to significantly increase across pregnancy (Kirby et al., 2013). Pregnancy-specific mediators, like estradiol, and their physiological cascades may contribute to the upregulation of the latter enzymes and subsequently, $1,25(OH)_2D$ (Baksi & Kenny, 1978). Increases in maternal DBP concentrations across pregnancy may function to improve systemic vitamin D binding capacity (Zhang et al., 2014). In contrast, others have concluded that DBP affinities for vitamin D metabolites decrease across gestation to facilitate easier offloading for maternal use or fetal mobilization (Bikle et al., 1984; Steichen et al., 1980).

An adequate status of vitamin D is thought to promote the ongoing health of pregnant individuals (Kirby et al., 2013). As in non-pregnant people, $1,25(OH)_2D$ is osteoprotective (Karras et al., 2018). During pregnancy, vitamin D dependent upregulation of calcium transporters and receptors in gut enterocytes and renal tubular cells is crucial to ensure enough calcium is accrued to maintain maternal calcium homeostasis in the presence of fetal endowment (Morrison et al., 1994; Priemel et al., 2010). Indeed, it has been reported that by late pregnancy, the amount of absorbed calcium is significantly increased compared to the pre-pregnancy period (Ritchie et al., 1998). However, there may be other factors that further increase maternal calcium status during gestation, such as estradiol (Karras et al., 2018). Vitamin D may also promote maternal immunosuppression towards microchimeric fetal cells bearing paternal antigens (Tamblyn et al., 2015; Lee et al., 2020). It has been postulated that without vitamin D and other contributors to immunotolerance, inflammation could lead to adverse health outcomes in the developing offspring and pregnant individual (Lapillonne, 2010).

Despite these findings, global surveillance of vitamin D status among different populations, including pregnant individuals, is limited (Bodnar et al., 2007). As a result, the true prevalence of vitamin D insufficiency among pregnant people is unclear (Özdemir et al., 2018). Health agencies from Canada and the United States currently recommend that pregnant people should consume

600 (International Units) IU/day of vitamin D (ACOG, 2021; Government of Canada, 2020; NIH, 2022b), whereas the Australian and United Kingdom recommendations are 400 IU/day (Australian Government, 2019; National Health Service, 2020). In a policy statement from the World Health Organization, various experts stated there was insufficient evidence to warrant the universal supplementation of vitamin D during pregnancy (WHO, 2012). This is in spite of other observational and clinical studies that concluded doses beyond current recommendations, including 1000 IU or 2000 IU per day, significantly reduced maternal vitamin D deficiency compared to lower amounts (400 IU/day) (Hollis et al., 2011; Pérez-López et al., 2015). In another investigation, healthy pregnant females living in a Canadian city reported that the RDA of 600 IU/day throughout gestation did not appear to protect against vitamin D insufficiency in many participants (Aghajafari et al., 2016). Moreover, maternal vitamin D status screening during pregnancy is not recommended because an evidence-based consensus about the risks and benefits of the practice has not been established (ACOG, 2011). Still, there are apparent caveats to this guideline as there is an acknowledgement of risk factors that may make pregnant individuals more vulnerable to a vitamin D deficiency (Pearce et al., 2010), as described above. Overall, the decision to screen for vitamin D status or recommend a supplement of this micronutrient during pregnancy seems to rely on the discretion of healthcare providers (Rockwell et al., 2018).

1.2.3.2 Placental demands

The placenta may house a unique environment for vitamin D metabolism (Shin et al., 2010). Previous studies have confirmed the presence of important mediators of vitamin D homeostasis, including VDR and DBP, and enzymes, like 1-α-hydroxylases, in trophoblast samples (Díaz et al., 2000; Shahbazi et al., 2011). Despite this, the regulation of vitamin D may be different within the placenta compared to the kidneys (Karras et al., 2018). Notably, the placental biosynthesis of $1,25(OH)_2D$ seems to outweigh its degradation so that more of it is readily available (Zehnder et al., 2002). A possible mechanism for this observation could relate to observed increases in the expression and efficiency of 1-α-hydroxylases (Díaz et al. 2000; Evans et al. 2004). To support the increased biosynthesis of $1,25(OH)_2D$, a higher amount of 25(OH)D molecules must be acquired from the maternal circulation. Previous evidence suggests that 25(OH)D-DBP complexes can enter the STB via megalin-cubilin transporters, providing an avenue of placental importation (Burke et al., 2013; Christensen & Birn, 2002; Shin et al., 2010). Along this line,

positive correlations between maternal 25(OH)D concentrations and placental 1,25$(OH)_2$D abundances have been reported (Park et al. 2017).

Vitamin D may be responsible for various placental functions (Evans et al., 2004; Shin et al., 2010). The decidual and extravillous trophoblast remodelling that occurs during placental development could be partially modulated by VDR signaling cascades (Emerson et al., 1985; Zehnder et al., 2002). Moreover, when the placenta is developed, vitamin D may also aid in the transfer of essential nutrients from the maternal to the fetal circulation (Ganguly et al., 2018). Specifically, VDR mediated changes in gene expression and DBP dynamics support calcium and amino acid transfer by targeting calcium absorption proteins and amino acid transporters, respectively (Cleal et al., 2015; Young et al., 2014). An adequate placental status of vitamin D could promote the establishment and maintenance of immune tolerance at the STB interface (Tamblyn et al., 2015). In fact, 1,25$(OH)_2$D has been shown to program placental natural killer and T cells towards immunosuppressive phenotypes, and it may also be involved in the upregulation of antigenic sensors, like toll-like receptors (Evans et al., 2006; Shin et al., 2010).

1.2.3.3 Fetal demands

The fetus is reliant on maternal vitamin D availability to endow its own supply of the vitamin, which is critical for their development and growth (Eyles et al., 2013). As quantified in umbilical cord blood, a major contributing metabolite in the fetal blood stream is 25(OH)D, primarily 25(OH)D3 (Markestad et al., 1984), which is reasonable considering this compound has been found to cross the placenta from the maternal circulation (Bouillon et al., 1981). Positive relationships between maternal vitamin D concentrations and cord blood vitamin D concentrations were deemed significant in a recent meta-analysis (Wong et al., 2022). Further evidence of the importance of fetal vitamin D acquisition is that the offspring may express modulators of its metabolism in utero. Notably, the fetal parathyroid gland can synthesize parathyroid hormone-related peptide (Moniz et al., 1990), which may enhance the activity of renal 1-α-hydroxylases to produce 1,25$(OH)_2$D (Ardawi et al., 1997; Kovacs, 2014).

1.2.4 A low or high maternal vitamin D status during pregnancy: implicated health outcomes

1.2.4.1 Maternal outcomes

Maternal vitamin D deficiency or toxicity may increase the risk for certain medical conditions (Cannell et al., 2008), but evidence from human investigations is limited. If a pregnant individual has a low vitamin D status, they may not absorb and retain calcium as optimally, potentially leading to maternal hypocalcemia (Hashemipour et al., 2013; Lapillonne, 2010). This could lead to dental and skeletal mineralization impairments and possibly a future predisposition to low bone mineral densities, osteoporosis or osteomalacia (Lips, 2006). A low maternal vitamin D status has also been linked to adverse mental health outcomes, like more self-reported symptoms of depression (Aghajafari et al., 2018b; Brandenberg et al., 2012; Robinson et al., 2014). However, there are seemingly more studies that examined the risk of postpartum depression and less that considered the impact of gestational vitamin D status on maternal mental health during pregnancy, and even fewer have assessed outcomes at both timepoints in the same investigation. An excessively high maternal vitamin D status may lead to toxicities and other adverse conditions, including hypercalcemia, calcium-related kidney precipitations or changes in mental processing or attention (Dinour et al., 2015; Inzucchi et al., 2004; Schoenmakers et al., 2020). Accordingly, pregnant people in Canada are recommended to not consume over 4000 IU/day of vitamin D (Government of Canada, 2020; Tripkovic et al., 2012).

1.2.4.2 Offspring outcomes

An insufficient maternal vitamin D status throughout gestation has also been associated with poor fetal outcomes (Cyprian et al., 2019; Wong et al., 2022). Without an appropriate amount of vitamin D available in the maternal circulation, the adequacy of fetal $1,25(OH)_2D$ may be compromised, which has been reported to increase the risk of improper fetal skeletal development (Mahon et al., 2010) and adverse birth outcomes (Rodriguez et al., 2015; Wei et al., 2013). Furthermore, maternal vitamin D insufficiency and abnormal DBP dynamics have been implicated in preterm birth (Amegah et al., 2017; Kook et al., 2018). As labour is thought to be at least partially induced by a series of inflammatory cascades, it has been postulated that vitamin D could be protective by suppressing the premature activation of these signals (Evans et al., 2006; Ota et al., 2015). A lower fetal status of vitamin D has also been associated with cortical modifications in the brain along with other behavioural outcomes in postnatal life (Gooch et al., 2019;

Whitehouse et al., 2012), including attention-deficit hyperactivity disorders (García-Serna & Morales, 2020). Still, whether inadequacies of maternal vitamin D during pregnancy contribute to permanent neurodevelopmental outcomes is yet to be determined in human studies.

1.2.5 Detecting vitamin D deficiencies or excess during pregnancy: the utility of biomarkers

A vitamin D deficiency can be conceptualized into different steps or benchmarks (Heaney, 2004). The first indication is typically low serum calcium, or hypocalcemia, which can be followed by several bone-related or other pathologies that may worsen overtime if the deficiency is not corrected. Specifically, osteoporosis, or the loss of bone mass, could manifest independently or together with osteomalacia, a state of insufficient mineralization by unchecked bone resorption (Elbossaty, 2017; Golounina et al., 2020). Rickets is a form of osteomalacia that impacts skeletal development during growth periods, making it potentially relevant during adolescent or early adulthood pregnancies before bone plate fusion (Wharton & Bishop, 2003; Lerner, 1980). Although toxicity is thought to be uncommon (Galior et al., 2018), the popularization of vitamin D as a universal health promotor, especially in the treatment of SARS-CoV-2, may lead to an increased prevalence of toxic exposures in the future (Mohan et al., 2020).

The contribution of endogenous and exogenous sources to the status of vitamin D make biomarkers of this micronutrient descriptive and clinically informative tools (Clemens et al., 1982; Norman, 1998). As metabolomic research has been proliferating over recent years, a new understanding about the complexities of biological vitamin D metabolism has emerged (Jenkinson, 2019). For example, vitamin D compounds with diverse chemical modifications, such as those conjugated with glucuronide groups, have been identified (Jenkinson et al., 2022; Slominski et al., 2015). Carbon 3 (C3)-epimerization is another pathway that will be further described below (Jenkinson, 2019).

1.2.5.1 25-hydroxyvitamin D (25(OH)D)

Clinically, the most common method of determining vitamin D status is through the quantification of systemic 25(OH)D concentrations (Heaney, 2004; Ponchon et al., 1969), but there is contention about the appropriate cut-offs to define vitamin D deficiency or insufficiency when using this biomarker. Health Canada defines vitamin D deficiency as a systemic 25(OH)D concentrations <30 nmol/L (<12 ng/mL) in most people (Health Canada, 2020). However, among

other researchers and agencies, the general consensus is that 25(OH)D concentrations <50 nmol/L (or <20 ng/mL) indicate a deficiency (Munns et al., 2016), whereas concentrations between 50 and <75 nmol/L (or 20 to <30 ng/mL) represent insufficiency (Holick et al., 2011). In one investigation, skeletal pathologies were present even if 25(OH)D concentrations were between 50 to <75 nmol/L (Priemel et al., 2010), supporting the use of the more sensitive cut-off of <75 nmol/L to indicate a low vitamin D status (Hanley et al., 2010). In the context of pregnancy, maternal 25(OH)D concentrations typically fall between 50 to 100 nmol/L, but these figures can vary depending on the population (Karras et al., 2018; Zhang et al., 2014).

The term 25(OH)D actually refers to multiple metabolites (Ponchon et al., 1969). Many of these compounds can now be individually quantified through liquid chromatography with tandem mass spectroscopy (LC-MS/MS) (Jenkinson et al., 2022), which is presently considered to be the gold standard for the measurement of vitamin D metabolites (Erdman et al., 2019; Galior et al., 2018). The biomarkers below fall into the group of 25(OH)D compounds (Jenkinson, 2019).

1.2.5.1.1 25-hydoxyvitamin D3 (25(OH)D3)

Vitamin D3 can be hepatically hydroxylated to form 25-hydroxyvitamin D3 (25(OH)D3) (**Figure 5**) (Blunt et al., 1968; Zhu et al., 2013). As 25(OH)D3 comprises a large fraction of total 25(OH)D, its systemic concentrations are clinical proxies of vitamin D status when measured in human blood (Heaney, 2004).

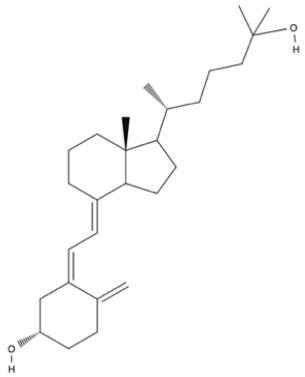

Figure 5. The chemical structure of 25-hydroxyvitamin D3 (25(OH)D3) (Jenkinson, 2019). Figure drawn using MolView.org.

The quantification of maternal 25(OH)D3 concentrations at multiple gestational timepoints among pregnant individuals from high-income countries is limited. Nonetheless, previously

reported mean or median maternal concentrations of 25(OH)D3 are quite variable between studies (**Table 5**) (Fernández-Alonso et al., 2012; Karras et al., 2013; Mao et al., 2022; Markestad et al., 1984; Morales et al., 2012; Rodriguez et al., 2015). As with total 25(OH)D, there is presently no cut-off concentration of 25(OH)D3 to define vitamin D insufficiency or deficiency when this biomarker is independently quantified. However, it appears that the proposed total 25(OH)D thresholds have been extrapolated for use with 25(OH)D3; for example, 25(OH)D3 concentrations <50 nmol/L to define a vitamin D deficiency (Fernández-Alonso et al., 2012). Still, this practice may not have the highest accuracy considering other metabolites comprise total 25(OH)D, such as 25-hydroxyvitamin D2 (25(OH)D2) the status of which could vary depending on the dietary and supplemental practices of individuals (Jenkinson, 2019; Wiebe & Binkley, 2014).

Table 5. Ranges of mean or median maternal 25(OH)D3 concentrations among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
25(OH)D3 (nmol/L)	69.0 – 73.5 [1,2]	53.0 – 74.0 [3,4]	34.6 – 90.8 [1,5,6]

Superscripts indicate the citations for data included in Table 5: [1]Fernández-Alonso et al. 2012; [2]Rodriguez et al. 2015; [3]Mao et al. 2022; [4]Morales et al. 2012; [5]Karras et al. 2013; [6]Markestad et al. 1984. Abbreviations: 25-hydroxyvitamin D3 (25(OH)D3).

1.2.5.1.2 3-epi-hydroxyvitamin D3 (3-epi-25(OH)D3)

Although epimers of vitamin D metabolites have been detected in humans (Bailey et al., 2013), their metabolic pathways, biological activities and implications in clinical measurements remain largely undescribed. An epimer is a stereoisomer that contains a different configuration at only one stereogenic centre (Rosanoff, 1906). In the case of the 3C epimer of 25(OH)D3, 3-epi-hydroxyvitamin D3 (3-epi-25(OH)D3), the stereogenic centre at the 3rd carbon is enzymatically converted from the α to the β configuration (**Figure 6**) (Al-Zohily et al., 2020; Bailey et al. 2013). Many vitamin D metabolites may be susceptible to epimerization, but 3-epi-25(OH)D3 has been of particular interest because it is the isomer of the highly abundant 25(OH)D3 (Jenkinson, 2019). Other epimers, including 3-epi-hydroxyvitamin D2, are also known to exist but their contribution to human vitamin D status is even less understood (Wiebe & Binkley, 2014). Although the

mechanistic details of C3-epimerization are not well-described, its enzymatic synthesis is achieved by 3-epimerases (Al-Zohily et al., 2020). Regarding biological activity, some in vitro investigations suggest that fully active C3-epimers retain less functional capacity possibly from different interactions with the VDR (Al-Zohily et al., 2020; Kamao et al., 2004).

Concentrations of 3-epi-25(OH)D3 can be reliably quantified from human blood samples via LC-MS/MS (Bailey et al., 2013). In previous studies, the proportion of 3-epi-25(OH)D3 to 25(OH)D3 concentrations were estimated to be between 4% to 10% (Hanson et al., 2016; Mao et al., 2022). Some researchers have expressed a concern whether the inclusion of these epimers within total 25(OH)D concentrations lead to the overestimation of vitamin D adequacy if they are indeed less biologically active (Al-Zohily et al., 2020; Cooke et al., 2015; Kamao et al., 2004).

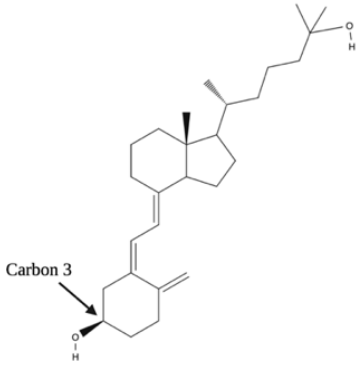

Figure 6. The chemical structure of 3-epi-25-hydroxyvitamin D3 (3-epi-25(OH)D3) (Jenkinson, 2019). The 3rd carbon where epimerization occurs is indicated. Figure drawn using MolView.org.

The quantification of this biomarker among pregnant individuals is rare. Notably, compared to adults, 3-epi-25(OH)D3 concentrations in children under 24 months of age appear to be significantly higher (Singh et al., 2006; Yazdanpanah et al., 2013). However, similarities between maternal and infant 3-epi-25(OH)D3 concentrations were reported at delivery in Zhang et al., (2013), suggesting that gestation may be a time of increased vitamin D epimerization. Maternal 3-epi-25(OH)D3 concentrations in generally healthy pregnancies have been reported (Hanson et al., 2016; Karras et al., 2013; Mao et al., 2022), but not in all trimesters (**Table 6**).

Table 6. Ranges of mean or median maternal 3-epi-25(OH)D3 concentrations among pregnant participants from high-income countries

	1st Trimester	2nd Trimester	3rd Trimester
3-epi-25(OH)D3 (nmol/L)	NA	1.9 (1.5) [1]	4.3 – 7.1 [2,3]

The interquartile range (IQR), as shown in parenthesis, is given in the 2nd trimester column because there was only one study with a 3-epi-25(OH)D3 measurement at that timepoint. Superscripts indicate the citations for data included in Table 6: [1]Mao et al. 2022; [2]Hanson et al. 2016; [3]Karras et al. 2013. Abbreviations: not available (NA); 3-epi-hydroxyvitamin D3 (3-epi-25(OH)D3).

1.3 Evidence for relationships between iron and vitamin D

Relationships among iron and vitamin D biomarker concentrations or clinically defined deficiencies have been identified in human populations, including children and older adults (Kang et al., 2015; Masoud et al., 2020; Yoo & Cho, 2015). Results from studies in human pregnancies as well as proposed pre-clinical mechanisms will be discussed below.

1.3.1 Observational evidence during human pregnancies

Significant positive relationships between maternal iron and vitamin D biomarkers during different timepoints of pregnancy were reported in some studies. An observational study that included pregnant participants in their 3rd trimester found that the presence of anemia and ID were significantly related to a low maternal vitamin D status, defined by maternal 25(OH)D concentrations <50 nmol/L (Bener et al., 2013). Similarly, low maternal 25(OH)D concentrations across mid to late gestation or delivery were significantly associated with low maternal Hb concentrations in other investigations (Braithwaite et al., 2019; Finkelstein et al., 2012; Park, 2017; Thomas et al., 2015). However, only two of the five studies that found a positive relationship between low maternal 25(OH)D and Hb concentrations also measured at least one other iron biomarker during gestation (Braithwaite et al., 2019; Thomas et al., 2015). The sole assessment of maternal Hb without simultaneously measuring other iron biomarkers, like SF or sTfR, is not the most accurate proxy of maternal iron status as other factors can contribute to anemia during pregnancy (Fisher & Nemeth, 2017). Indeed, many of these investigations were cohort studies that included pregnant people with particular characteristics that may significantly increase their risk

for non-iron related anemia, including infections (Finkelstein et al., 2012; Thomas et al., 2015). This idea may be exemplified in a study where low 25(OH)D concentrations were associated with an increased risk of maternal anemia at delivery, but there were no significant differences in maternal SF concentrations among vitamin D deficient (<50 nmol/L) or sufficient (≥50 nmol/L) groups at the same timepoint (Park, 2017). Moreover, the positive associations between 25(OH)D and Hb concentrations could reflect the high prevalence of maternal low status or deficiencies in iron and vitamin D independently (Kiely et al., 2021; Parisi et al., 2014), and not necessarily a metabolic interaction between these micronutrients.

In contrast, other evidence suggests that maternal 25(OH)D concentrations were inversely associated with iron biomarkers. In one study, after pregnant individuals with ID (SF <12 μg/L) received a 1 g injection of ferric carboxymaltose in either their 2nd or 3rd trimester, maternal concentrations of SF and hepcidin increased and 1,25$(OH)_2$D concentrations decreased (Huang et al., 2018). In two other studies, maternal SF concentrations significantly decreased and 25(OH)D concentrations increased across mid to late gestation compared to concentrations in the 1st trimester after the consumption of either a multiple micronutrient supplement, containing iron (27 mg) and vitamin D (200 IU) (Schulze et al., 2019), or constantly supplemented iron (8 mg/day) and vitamin D (280 IU/day) (Looman et al., 2019).

Still, there is other evidence that iron and vitamin D status associations are only specific to certain gestational timepoints or biomarkers. For example, no relationships were reported between maternal SF, hepcidin, or serum iron concentrations and 25(OH)D concentrations during early pregnancy or at delivery, but relationships were identified during mid-gestation (Braithwaite et al., 2019; Thomas et al., 2015). In another study, variations in maternal 25(OH)D, SF and hepcidin occurred simultaneously, but there were no significant changes in 1,25$(OH)_2$D concentrations across the same time period (Braithwaite et al., 2021). A particular strength of Braithwaite et al., (2021) is that multiple vitamin D status biomarkers, 25(OH)D and 1,25$(OH)_2$D, were quantified from maternal blood samples. However, the measurement of multiple iron or vitamin D biomarkers in the same investigation has rarely been conducted.

1.3.2 Proposed metabolic interactions

Metabolic pathway interactions between various metabolites and regulators of iron and vitamin D have been explored but not fully elucidated (**Figure 7**). Alon et al. (2002) found that

the application of 1,25(OH)$_2$D to a cell line of erythroid precursors increased the expression of their EPO receptors, and that in the presence of EPO, the division of these cells were enhanced. Moreover, positive associations between iron depletion, EPO and the enhanced expression of fibroblast growth factor 23 have been reported (Braithwaite et al., 2021; Clinkenbeard et al., 2014). The latter growth factor has been negatively correlated with 1,25(OH)$_2$D expression (Icardi et al., 2013). Together these findings suggest that the abundance of 1,25(OH)$_2$D may modulate EPO function, such as erythropoiesis, but that EPO might also negatively regulate this metabolite.

Another group of studies reported associations between vitamin D biomarkers and hepcidin (Thomas et al. 2015; Zughaiger et al. 2014). Specifically, hepcidin expression may be suppressed via VDR-dependent downregulation of its gene, *HAMP* (Bacchetta et al. 2014; Zughaiger et al. 2014). Bacchetta et al. (2014) also showed that the expression of ferritin was significantly reduced when high doses of either 25(OH)D or 1,25(OH)$_2$D were applied to hepatocytes in vitro. These findings are consistent with the reported inverse relationships among both 1,25(OH)$_2$D and 25(OH)D with hepcidin and SF concentrations in pregnant women (Braithwaite et al. 2021; Huang et al. 2018; Looman et al. 2019; Schulze et al. 2019). However, given the relationship between SF and hepcidin with inflammation (Lynch et al. 2018; Mei et al. 2017), it is not clear whether these relationships reflect an interaction between vitamin D and iron pathways, or between vitamin D and immunomodulation (Meza-Meza et al., 2022). Still, potential changes in systemic SF and hepcidin concentrations in the presence of vitamin D, immune-related or not, may still have physiological or clinical implications. Ultimately, these interactions provide mechanistic frameworks that should be explored in future experiments to determine if they are relevant to maternal iron and vitamin D status during pregnancy.

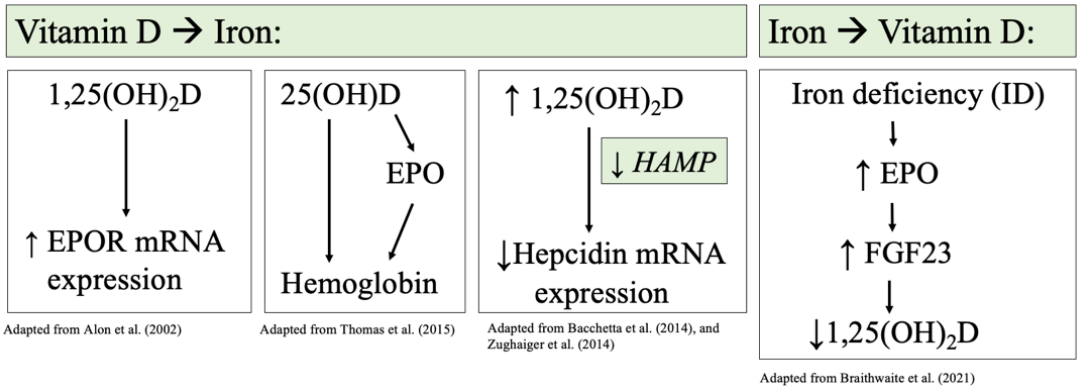

Figure 7. Several proposed interactions between vitamin D and iron metabolites or regulators. The availability of 1,25(OH)$_2$D may upregulate the expression of EPOR on erythroid precursors (Alon

et al., 2002). Systemic concentrations of EPO may mediate the correlation between 25(OH)D and hemoglobin concentrations (Thomas et al., 2015). The hormonal form of vitamin D, $1,25(OH)_2D$, may suppress *HAMP* gene expression through interactions with the VDR (Bacchetta et al., 2014; Zughaiger et al., 2014). Finally, prolonged ID can lead to iron-deficient erythropoiesis and an enhanced expression of EPO (Braithwaite et al., 2021). Increases in EPO may upregulate FGF23, a down-regulator of $1,25(OH)_2D$ biosynthesis. Abbreviations: erythropoietin (EPO); fibroblast growth factor 23 (FGF23); *human antimicrobial peptide* (*HAMP*); 25-hydroxyvitamin D (25(OH)D); 1,25-dihydroxyvitamin D ($1,25(OH)_2D$).

1.3.3 Methodological inconsistencies and research gaps

Beyond certain critiques specific to individual studies, there are other general inconsistencies in this area of the literature. Crucial characteristics of pregnant individuals that may influence nutrient status were not regularly reported in many studies (Dominguez et al., 2021). Particularly, the self-reported ethnicity of participants was only identified in a subset of studies (Braithwaite et al., 2021; Looman et al., 2019; Thomas et al., 2015). The range of maternal ages in many of the studies were variable, including some where younger females made up the majority of the included participants (Braithwaite et al., 2021; Finkelstein et al., 2012; Thomas et al., 2015). Although the important question of whether there is a relationship between iron and vitamin D should be explored in all expectant people, results from studies that include adolescent participants may not be generalizable to pregnant adults because younger females are still growing, and their requirements are different (Partridge, 2020).

There were also differences in the methodological approaches between investigations. Inconsistencies in the techniques used to measure the same biomarkers might have contributed to discrepancies in results. For example, 25(OH)D concentrations were measured by either chemiluminescent immunoassays (Finkelstein et al., 2012; Huang et al., 2018; Schulze et al., 2019), radioimmunoassays (Bener et al., 2013; Braithwaite et al., 2019; Park, 2017) or mass spectroscopy (Looman et al., 2019). Finally, different thresholds were used to define vitamin D status between studies. Maternal vitamin D deficiency was defined by 25(OH)D concentrations of <25 (Braithwaite et al., 2019), <30 (Braithwaite et al., 2021; Thomas et al., 2015), <50 (Finkelstein et al., 2012; Park, 2017; Schulze et al., 2019) or <72.5 nmol/L (Bener et al., 2013).

1.3.4 Conclusions about the current state of research

Ultimately, interactions between iron and vitamin D status during pregnancy are still poorly understood. The number of mechanistic investigations exploring this topic are increasing, but more research, especially in pregnancy-specific settings, is warranted. In future studies there should be an emphasis on adjusting for relevant maternal characteristics, and examining whether maternal iron and vitamin D status adequacy influences maternal and offspring health outcomes.

1.4 The Alberta Pregnancy Outcomes and Nutrition (APrON) study

1.4.1 Rationale, aim and objectives

The Alberta Pregnancy Outcomes and Nutrition (APrON) longitudinal cohort study was conceptualized to explore nutritional contributions to important maternal and child health outcomes (Kaplan et al., 2014; Letourneau et al., 2022). The investigators of APrON aimed to assess relationships between pre-pregnancy, pregnancy or postpartum nutrition and key maternal and offspring health outcomes, including obstetric conditions and offspring neurodevelopment.

A strong rationale for the development of the APrON study was the increasing prevalence of maternal and youth mental illness in Alberta and Canada (Kaplan et al., 2014; Letourneau et al., 2022). Estimates from the Canadian government in 2019 suggested that nearly a quarter of pregnant individuals across the country may experience postpartum depression or anxiety; in Alberta, this figure was 22% (Statistics Canada, 2019). An even higher percentage of pregnant females (33%) identified mental health to be a worrisome factor in their lives. Since the onset of the COVID-19 pandemic, investigations have reported that the prevalence of poor maternal mental health has significantly increased across Canada (Lebel et al., 2020) and globally (Tomfohr-Madsen et al., 2021). In addition, the proportion of youth that experience mental illness or neurodevelopmental disorders has been progressively rising (Fombonne, 2009), but the recent pandemic may have substantially amplified this health concern. According to Statistics Canada, approximately 60% of surveyed Canadian children reported having suboptimal mental health in the spring of 2020, coinciding with the beginning of the pandemic, whereas in 2018 this figure was lower at ~38% (Statistics Canada, 2022). The rate of medical visits related to mental health disturbances among youth also climbed in recent years, further suggesting an increased severity of mental illness in this age group (Canadian Institute for Health Information, 2022).

The rising prevalence of poor mental health among pregnant individuals and children is coupled with the inadequate nutrition of a considerable proportion of Canadians (Statistics Canada, 2018), despite dietary quality being implicated in brain health (Gómez-Pinilla, 2008). Recent population-based surveys, including the Canadian Community Health Survey, reported that on average, the intake of fruits and vegetables, which are rich sources of a variety of important minerals and vitamins, did not meet Canadian Food Guide recommendations during the time of the assessment (Statistics Canada, 2018). Previous evidence has suggested the essentiality of certain nutrients, including lipids like long-chain omega-3 fatty acids and micronutrients, in the development and maintenance of mental functions (Gómez-Pinilla, 2008). In pregnancy, proper nutrition supports maternal physiological adaptations along with embryonic and fetal requirements for proper development and growth (Fisher & Nemeth, 2017; Koletzko et al., 2019). A poor pre-pregnancy dietary pattern could lead to nutrient inadequacies that may be exacerbated across gestation, possibly leading to adverse outcomes for the offspring and pregnant individual.

Therefore, the aim of the APrON study was to determine how maternal nutrition before, during and after pregnancy may influence maternal moods and mental health along with child neurodevelopment to ultimately improve the prospective health of Albertans, from mothers to children (Kaplan et al., 2014; Letourneau et al., 2022). The establishment of this comprehensive investigation has and will continue to allow novel research questions to be explored overtime across many disciplines, including nutrition, psychology, medicine, genetics and epidemiology.

The *main objectives* of the APrON study are to:

Determine if associations exist between maternal nutrient intake and status, during the pre-pregnancy, gestational or postpartum periods, and:

1. Maternal mental and obstetric health, along with;
2. Offspring birth outcomes, neurodevelopment and subsequent mental health

1.4.2 Components of the APrON study that are relevant to the thesis research

1.4.2.1 Ethical approval, inclusion criteria and recruitment

There were only a few key pieces of criteria that prospective participants needed to retain to be eligible for entry into APrON cohort (Kaplan et al., 2014). Specifically, the pregnant person

needed to be at less than 27 gestational weeks, within their 1st or 2nd trimester of pregnancy. Individuals also needed to be 17 years of age or older with language literacy in English (Kaplan et al., 2014; Letourneau et al., 2022). Finally, residency in either Edmonton or Calgary during pregnancy was another important inclusion criteria. If any of the above scenarios could not be met, then that person would be excluded from study enrollment. After lead investigators conceptualized the study aims, objectives and design (as described above), both the University of Alberta Health Research Ethics Panel (Pro00002954) and the University of Calgary Health Research Ethics Board (REB14-1702) approved the APrON study in 2008 (Kaplan et al. 2014; Letourneau et al., 2022).

The recruitment of APrON study participants commenced in May 2009 and lasted until July 2012 in Calgary and Edmonton (Letourneau et al., 2022). Approaches to increase enrollment were tailored to the specific pregnancy care services in the two cities (Kaplan et al., 2014). In Calgary, maternity and obstetric medical practices are more centralized in a few common sites, and it was convenient for study promotors to station themselves at these locations. Whereas in Edmonton, expectant individuals could be attending a variety of clinics or practices dispersed across different sites. For the latter, the APrON investigators partnered with local organizations, including the Women and Children's Health Research Institute, to aid in a widespread disbursement of information. Societal awareness about the APrON study was further promoted by community advertisements and media or event appearances by investigators (Kaplan et al., 2014). If potential participants met the inclusion criteria and voluntarily expressed their interest in joining the study, a research assistant would follow up to provide a more detailed explanation of the study. If a willingness to enroll remained, an initial study visit was scheduled. All participants provided consent before any data collection commenced, and they were assured that consent could be retracted at any time (Letourneau et al., 2022). In total, over twenty-one hundred (n=2,187) pregnant participants were enrolled.

1.4.2.2 Collection of maternal information and biological samples

Maternal data were collected at study timepoints ranging from the earliest pregnancy visit to over a decade after delivery (Kaplan et al., 2014). At present, the goal of the APrON study team is to further expand this follow up window so that intergenerational influences may be captured within the cohort (Letourneau et al. 2022). The latter is a strong study design for research questions related to the developmental origins of adult disease (Silveira et al., 2007; Tarrade et al., 2015).

Although maternal data was collected at additional timepoints, the thesis research utilized the pregnancy and 3 months postpartum study visit data (**Figure 8**). The details of these 4 timepoints are as follows:

1st trimester timepoint: biological samples and other data were collected from pregnant participants at <14 gestational weeks. The mean ± standard deviation weeks of gestation at this timepoint was 10.8 ± 2.4 weeks.

2nd trimester timepoint: biological samples and other data were collected from pregnant participants between 14 to 26 gestational weeks. The mean ± standard deviation weeks of gestation at this timepoint was 19.0 ± 3.4 weeks.

3rd trimester timepoint: biological samples and other data were collected from pregnant participants between 27 to 40 gestational weeks. The mean ± standard deviation weeks of gestation at this timepoint was 32.5 ± 1.3 weeks.

3 months postpartum timepoint: biological samples and other data were collected from participants at approximately 12 weeks post-delivery.

Data collected at the first study visit, during the 1st or 2nd trimester depending on the participant, was more extensive, whereas subsequent questionnaires were used to gather information that could change with the progression of time (Letourneau et al., 2022). Pregnant participants could either complete these surveys at home or in-person at study visits (Kaplan et al., 2014). Details of maternal data that relevant to this thesis research are described below.

1.4.2.2.1 Sociodemographic information

Participant characteristics or demographics, including age, ethnicity and country of birth, were self-reported during the first study visit (Kaplan et al., 2014; Letourneau et al., 2022). Other sociodemographic factors, including the highest level of educational attainment, annual household income and marital status, were also collected.

1.4.2.2.2 Medical information

Self-reporting of historical or current medical diagnoses were collected through the administration of other questionnaires (Letourneau et al., 2022). Pre-existing (anxiety, asthma, celiac disease, Crohn's disease, depression, type 1 or type 2 diabetes, epilepsy, heart disease, hyperthyroidism, hypothyroidism, irritable bowel syndrome, polycystic ovarian syndrome, primary hypertension, ulcerative colitis) and pregnancy-specific (gestational diabetes mellitus, gestational hypertension, preeclampsia) conditions were included. Questionnaires were provided at each prenatal study visit and 3 months postpartum in case a participant had a new diagnosis or symptom(s) (Kaplan et al., 2014).

Maternal mental health was further assessed through validated questionnaires and surveys, such as the Edinburgh Postnatal Depression Scale (EPDS) (Cox et al., 1987) and Symptom Checklist-90-Revised (Derogatis, 1994). The EPDS is a validated and widely used 10-item tool to measure depressive symptoms (Cox et al., 1987), and was utilized to assess maternal depression in this thesis research. Maternal EPDS scores were collected at each trimester of pregnancy and at 3 months postpartum (Letourneau et al., 2022). During each administration, participants were asked to self-report if they had specific moods or symptoms in the last week that are reflective of depression (Bergink et al., 2011; Pop et al., 1992). The severity or frequency of symptoms is reflected by the magnitude of the score from each of the 10 questions, ranging from 0 (no presence of the symptom) to 3 (a high frequency or severity of the symptom) (Cox et al., 1987). Accordingly, a total score of 30 is reflective of the highest risk of depression, whereas a lower score suggests a lower risk of depression. A EPDS score ≥13 was the cut-off for probable depression (Cox et al., 1987; Levis et al., 2020).

1.4.2.2.3 Nutritional information

Several assessments were used to estimate maternal dietary and supplemental intakes (Kaplan et al., 2014; Letourneau et al., 2022). Twenty-four hour dietary recalls assessed maternal food and drink intake during the day prior to a study visit. There was also an APrON-adapted supplemental intake questionnaire (SIQ) that estimated maternal supplements and other non-food product intakes across the study (Csizmadi et al., 2007). Maternal 24-hour dietary recalls and SIQs were collected at each prenatal study visit and at ~3 months postpartum. After the collection of maternal 24-hour dietary recall data during pregnancy, a method to estimate overall dietary quality

using criteria from the 2007 Canadian Food Guide (CFG) was developed (Jarman et al., 2017; Health Canada, 2007). A score ranging from 0 to 9 was assigned to dietary intake of each participant, with a higher score indicating a closer adherence to the CFG.

1.4.2.2.4 Anthropometric measurements

The height, weight and various skinfold measurements of pregnant individuals were assessed at each study visit (Kaplan et al., 2014; Letourneau et al., 2022). Established protocols for the calculation of other anthropometric measurements, including pre-pregnancy body mass index (BMI) and gestational weight gain, were also utilized (Begum et al., 2012).

1.4.2.2.5 Blood draws and sample processing

Venous blood samples were drawn from participants at each study visit by a certified phlebotomist (Kaplan et al., 2014). Assessment of maternal concentrations of Hb were immediately performed using whole blood samples via HemoCue 201® analyzers (HemoCue, Cypress, CA, USA). The remaining blood was promptly processed into different components: RBCs, buffy coat, plasma and serum. Each fraction was transferred into vials and stored at -80°C until other analyses, including iron and vitamin D biomarker quantification (see Chapter 3).

1.4.2.3 Collection of offspring information and outcomes

A child cohort of the pregnant females that enrolled in the APrON study have and will continue to be followed (Kaplan et al., 2014; Letourneau et al., 2022). Although there are other timepoints when data was collected from children, the thesis research focussed on two time periods: (1) birth and (2) 5 years of age. Relevant offspring data is detailed next.

1.4.2.3.1 Birth outcomes

Delivery records were accessed for the collection of birth outcomes (Kaplan et al., 2014). A non-exhaustive list of these variables includes BW, size for gestational age (small- or large-for-gestational age) and birth head circumference (BHC).

1.4.2.3.2 Child behaviour outcomes

Child neurodevelopment and behaviours were estimated through a variety of questionnaires, like the Quantitative Checklist of Autism in Toddlers (Magiati et al., 2015) and Behavior Assessment System for Children (Reynolds, 2010). The Behavior Assessment System for Children 2nd Edition (BASC-2) Parent Rating Scale is a validated and widely used questionnaire that estimates varied aspects of behaviours and emotions in youth (Kamphaus, 2014; Reynolds, 2010). BASC-2 internalizing and externalizing composite scores, that were collected from children at approximately 5 years of age, were used in this thesis research. Internalizing scores estimate behaviours related to anxiety, depression and somatization, and externalizing scores examine conduct problems, hyperactivity and aggression (Kamphaus, 2014; Reynolds, 2010). After collection, raw BASC-2 child internalizing and externalizing scores were converted to T-scores using normative tables. Higher internalizing and externalizing T-scores are reflective of a higher occurrence of internalizing or externalizing behaviours among children, respectively.

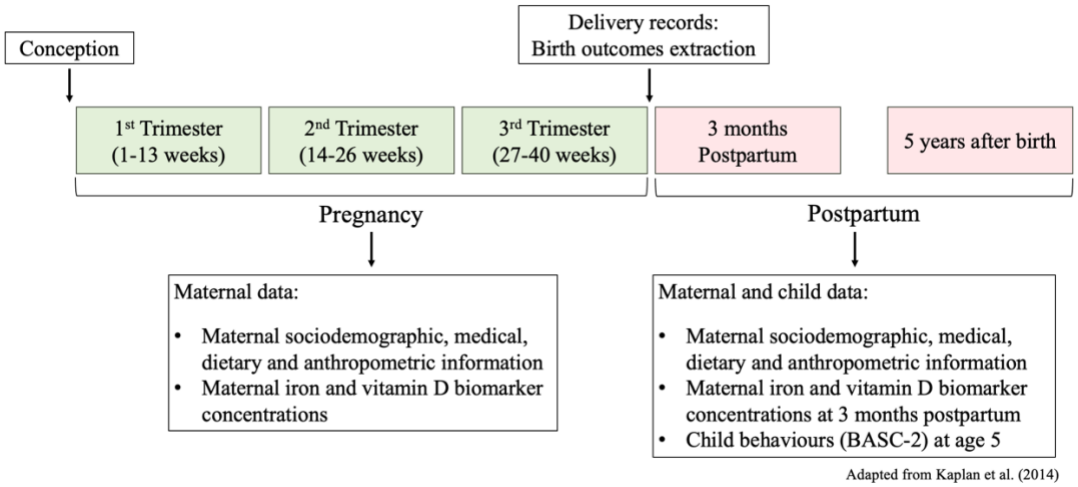

Figure 8. Overview of APrON study timelines and variables relevant to the thesis research. Data, including maternal iron and vitamin D biomarker concentrations (see Chapter 3), were collected from pregnant participants across gestation and at 3 months postpartum. Birth outcome data were obtained from the extraction of delivery records. Child behaviours were estimated through different questionnaires, such as the BASC-2, at various timepoints in early life, including at 5 years of age. This figure was adapted from Kaplan et al. (2014).

1.4.3 Previous research relating to maternal iron and vitamin D status in the APrON cohort

There is published data describing maternal vitamin D status in a sub-cohort of the APrON study (Letourneau et al. 2022; Aghajarfari et al., 2016; Aghajafari et al., 2017; Aghajafari et al., 2018a). Maternal concentrations of 25(OH)D3 and 3-epi-25(OH)D3 were previously quantified in 2nd trimester (n=537) and delivery (n=92) maternal plasma samples. During the 2nd trimester, 20% of the included pregnant participants had 25(OH)D concentrations that were <75 nmol/L, even though many were meeting the RDA for vitamin D (600 IU/day) through supplements alone (Aghajarfari et al., 2016). These results call into question whether the current Canadian recommendations for vitamin D are enough to achieve maternal vitamin D status sufficiency during mid-pregnancy. In a subsequent investigation a significant positive relationship was detected between maternal vitamin D supplementation during lactation and vitamin D status in infants (Aghajarfari et al., 2018a), suggesting the importance of maternal vitamin D status during the postpartum period. Results related to maternal iron status in the APrON cohort, including the dynamics of biomarkers and their relationships with health outcomes, have not been published.

1.5 Offspring sex: a potential modulator of development

Although discrepancies in offspring health outcomes depending on biological sex have been reported for decades (Gualtieri & Hicks, 1985), there has been a resurgence of interest in the role of fetal sex during critical periods of development (Al-Qaraghouli & Fang, 2017). A significant body of evidence suggests that male fetuses may be more susceptible to adverse gestational conditions, including poor maternal nutrient status, during pregnancy (DiPietro & Voegtline, 2017). For example, males newborns often have a higher risk of preterm birth (Cooperstock & Campbell, 1996; McGregor et al., 1992) and delivery complications (Sheiner et al., 2004). In recent months alone, investigations detailing the possible sex-dependency of maternal stress (Vrijkotte et al., 2023), fetal responses to acute SARS-CoV-2 infections (Shook et al., 2023) and brain-related inflammation (Emezienna et al., 2023) have been published.

There are several mechanisms that may underlie observations of male vulnerability. Sex-specific differences in growth patterns, gene expression, immunotolerance and endocrinological regulation during development have all been proposed as potential mediators (DiPietro & Voegtline, 2017; Nugent et al., 2018). As many of the latter pathways likely involve the placenta, an important consideration is assessing its function in the context of fetal sex. Placental efficiency

is typically defined as fetal growth as compared to placenta growth, or the ratio of BW to placental weight (Wilson & Ford, 2001). Previous evidence suggests male fetuses have higher placental efficiencies; they optimize physical growth during gestation while being supported by a smaller placenta (Richardson et al., 2022; Roland et al., 2014). However, male fetuses may be more negatively impacted by inadequate nutrient endowment from maternal reserves, whereas females may have a higher ability to tolerate these conditions. Indeed, females have been shown to allocate more energy and nutrients to ensure the proper development of vital organs in a period of vulnerability (Cogollos et al., 2016). Relevant to nutrition, previous findings suggest that the amount and timing of fetal requirements for specific nutrients may differ depending on their biological sex (Roland et al., 2014).

Despite an accumulating body of evidence, deliberate steps are needed to further elucidate the role of offspring sex in development as well as prospective health outcomes. Retrospectively, the use of fetal sex as a confounder may obscure relationships. Instead, the treatment of fetal sex as an effect modifier may unveil sex-specific associations and should be prioritized in future research (Al-Qaraghouli & Fang, 2017; DiPietro & Voegtline, 2017).

CHAPTER 2: RATIONALE, OBJECTIVES AND HYPOTHESES

2.1 Rationale

Despite the essentiality of iron and vitamin D for optimal pregnancy outcomes (Goonewardene et al., 2012; Özdemir et al., 2018), evidence describing how the maternal status of these two micronutrients impact maternal and offspring health in cohorts of healthy pregnant individuals from North America is rare (O'Brien & Ru, 2017). Limitations of nutritional studies can stem from the estimation of nutrient intake from self-reported surveys and questionnaires, which may be influenced by a number of biasing factors like subjective reporting and differences in bioavailability (Kirkpatrick et al., 2018). The quantification of validated biomarkers can provide an objective indication of nutrients status (Combs Jr. et al., 2013). The measurement of several types of biomarkers for a given nutrient may provide additional insights into the extent of nutritional adequacy, and their quantification across various timepoints can reveal changes overtime.

During pregnancy, micronutrient status may be important for not only fetal growth, but also the gestational, postpartum and lifelong health of people who give birth (Silveira et al., 2007). Even though the progressive depletion of maternal nutrient reserves throughout the course of pregnancy has been considered a physiologically normal phenomenon (Fisher & Nemeth, 2017; Karras et al., 2018), the possible implications of these changes for maternal well-being and quality of life should not be overlooked. Excitingly, maternal concentrations of multiple iron and vitamin D biomarkers were quantified during several study timepoints in the APrON study (Letourneau et al., 2022), allowing for a comprehensive analysis of iron and vitamin D status among generally healthy pregnant people from Alberta.

Birth outcomes, early child behaviours and maternal mental health are the health outcomes of interest because they have been independently associated with maternal iron or vitamin D status during pregnancy in previous studies (Aghajafari et al., 2018b; Dewey & Oaks, 2017; Iglesias et al., 2018; Quezada-Pinedo et al., 2021; Wassef et al., 2019; Whitehouse et al., 2012). Birth outcomes are critical as they have been correlated with prospective health outcomes, from growth during early childhood to the onset of metabolic conditions later in life (Belbasis et al., 2016). At present, there appears to be more evidence supporting relationships between maternal iron status during pregnancy and birth outcomes (Dewey & Oaks, 2017), which informed the second objective

below. Furthermore, although behaviours are likely a product of many influential mediators, the sufficiency of neurodevelopment during pregnancy, especially the 3rd trimester, may be an important contributing factor (Gong et al., 1998). Previous evidence suggests children that are approximately 5 years of age may exhibit behaviours that are related to the brain development that occurs during the perinatal period (Acosta et al., 2019; Trønnes et al., 2020). The latter makes this age group an important population to study for evidence of nutritional programming during pregnancy. Indeed, previous investigations have reported that the maternal status of iron or vitamin D during gestation were significantly associated with behaviours in children around the age of 5 (Whitehouse et al., 2012; Quezada-Pinedo et al., 2021). Finally, the determination of how the nutritional adequacy of pregnant individuals may impact their own well-being is critical. Although research involving pregnant people, and more broadly, women, was neglected for a long time, over the past few decades there has been an emergence of research dedicated to these populations (Chen et al., 2022; Lapillonne et al., 2010). Nonetheless, the rising prevalence of maternal mental illness, not just during the postpartum period but across pregnancy, must be addressed, especially in the wake of the COVID-19 pandemic (Lebel et al., 2020; Tomfohr-Madsen et al., 2021).

It is unclear whether concurrent iron and vitamin D deficiencies in pregnant individuals influence pregnancy outcomes. This is in spite of previous reports of potential associations between systemic biomarkers of these micronutrients during gestation (Braithwaite et al., 2021; Braithwaite et al., 2019; Thomas et al., 2015) as well as pre-clinical evidence of metabolic interactions between iron and vitamin D (Alon et al., 2002; Bacchetta et al., 2014; Zughaiger et al., 2014), as described above. Provided that the maternal status of iron or vitamin D during pregnancy may be independently associated with maternal mental health and offspring neurodevelopment (Aghajafari et al., 2018b; Iglesias et al., 2018; Quezada-Pinedo et al., 2021; Wassef et al., 2019; Whitehouse et al., 2012), studies that assess whether these health outcomes differ depending on the maternal status of both micronutrients are needed.

Ultimately, the design of the APrON study provides an excellent opportunity to explore many of the aforementioned knowledge gaps and important topics that require investigation (Kaplan et al., 2014; Letourneau et al., 2022).

2.2 Research question, objectives and hypotheses

Given the current state of relevant literature, the main research question, objectives and hypotheses of this thesis were conceptualized.

Main research question:

How does maternal iron and vitamin D status change during and after pregnancy and what are their relationships with maternal depression and child birth and neurodevelopmental outcomes in the APrON cohort?

Research objectives and hypotheses:

1. Determine how maternal concentrations of systemic iron and vitamin D biomarkers change across time, and the combined maternal status of both micronutrients.
    a. Determine how maternal Hb, hepcidin, SF, sTfR and sTfR:SF concentrations change between different trimesters of pregnancy and at 3 months postpartum.

        I hypothesized that maternal Hb, hepcidin and SF would decrease, and maternal sTfR and sTfR:SF would increase as pregnancy progressed.
    b. Determine how maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations change between the 2nd trimester and 3 months postpartum. Prior to the thesis research, concentrations of maternal 25(OH)D3 and 3-epi-25(OH)D3 were quantified in 644 pregnant participants during the 2nd trimester because this study visit had the largest prenatal sample size in the APrON study (Aghajarfari et al., 2016). We quantified these two metabolites during the 2nd trimester in an additional 1,249 participants in the current research to improve the statistical power at this timepoint, and in 1,251 participants at 3 months postpartum to assess postnatal vitamin D status.

        I hypothesized that maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be lower during the 2nd trimester compared to 3 months postpartum.
    c. Describe the combined status of maternal iron and vitamin D during the 2nd trimester.

        I hypothesized that the majority of pregnant participants would have a replete status of iron and vitamin D during the 2nd trimester.

2. Determine the relationships between maternal concentrations of systemic iron biomarkers and birth outcomes.
    a. Assess relationships between 1st, 2nd or 3rd trimester maternal EPO, Hb, hepcidin, hepcidin:EPO, SF, sTfR and sTfR:SF concentrations and BWs.

        I hypothesized that lower 1st, 2nd or 3rd trimester maternal Hb, hepcidin, hepcidin:EPO and SF, and higher maternal EPO, sTfR and sTfR:SF, would be associated with lower BWs.

    b. Assess relationships between 1st, 2nd or 3rd trimester maternal EPO, Hb, hepcidin, hepcidin:EPO, SF, sTfR and sTfR:SF concentrations and BHCs.

        I hypothesized that lower 1st, 2nd or 3rd trimester maternal Hb, hepcidin, hepcidin:EPO and SF, and higher maternal EPO, sTfR and sTfR:SF, would be associated with lower BHCs.

3. Determine the relationships between maternal concentrations of systemic iron and vitamin D biomarkers and maternal antenatal and postpartum depression symptoms.
    a. Assess relationships between 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin, SF, sTfR and sTfR:SF concentrations and maternal EPDS scores during the 3rd trimester and at 3 months postpartum.

        I hypothesized that lower 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin and SF, and higher maternal sTfR and sTfR:SF, would be associated with higher maternal EPDS scores at both timepoints.

    b. Assess relationships between 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations and maternal EPDS scores collected during the 3rd trimester and at 3 months postpartum.

        I hypothesized that lower 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be associated with higher maternal EPDS scores at both timepoints.

    c. Determine if maternal EPDS scores during the 3rd trimester or at 3 months postpartum differ depending on the combined status of maternal iron and vitamin D during the 2nd trimester.

I hypothesized that compared to pregnant participants that were replete in iron and vitamin D during mid-pregnancy, maternal EPDS scores at both timepoints would be higher if participants had a low status in one or both micronutrients during mid-pregnancy.

4. Determine the relationships between maternal concentrations of systemic iron and vitamin D biomarkers and child internalizing and externalizing behaviours at age 5.
    a. Assess relationships between 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin, SF, sTfR and sTfR:SF concentrations and BASC-2 child internalizing and externalizing T-scores at age 5.

        I hypothesized that lower 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin and SF, and higher maternal sTfR and sTfR:SF, would be associated with higher child internalizing and externalizing T-scores at age 5.

    b. Assess relationships between 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations and BASC-2 child internalizing and externalizing T-scores at age 5.

        I hypothesized that lower 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be associated with higher child internalizing and externalizing T-scores at age 5.

    c. Determine if BASC-2 child internalizing or externalizing T-scores at age 5 differ depending on the combined status of maternal iron and vitamin D during the 2nd trimester.

        I hypothesized that compared to pregnant participants that were replete in iron and vitamin D during mid-pregnancy, child internalizing and externalizing T-scores at age 5 would be higher if participants had a low status in one or both micronutrients during mid-pregnancy.

CHAPTER 3: METHODS

3.1 Quantification of maternal iron and vitamin D biomarkers

Circulatory concentrations of maternal biomarkers that are indicative of iron status were quantified in serum samples from pregnant participants in the APrON cohort (Kaplan et al., 2014). These biomarkers include SF, sTfR, hepcidin and EPO, which were measured at different gestational or postpartum timepoints (**Table 7**). Maternal SF concentrations were quantified through the use of an i2000sr Architect Plus machine (Abbott, Chicago, IL, USA) with chemiluminescent microparticle immunoassay (CMIA) functionality (**Figure 9**a). Preparation for SF quantification involved complete thawing of maternal samples before aliquoting ~150 µL of serum into separate i2000sr Architect-specific loading cups followed by the addition of assay buffer (1:2 serum-buffer dilution). The accuracy of SF quantification was ensured by the use of SF calibrators and the daily running of SF controls, as specified by i2000sr Architect+ protocols. If resulting maternal SF concentrations were lower or higher than the reference range, they were concentrated or diluted, respectively, before re-analysis. The inter-day CMIA performance was high; the mean coefficient of variation (CV) between same samples whose SF concentrations were re-analyzed daily was <5%.

Serum concentrations of maternal sTfR, hepcidin and EPO were quantified through sandwich enzyme-linked immunosorbent assays (ELISAs) protocols (R&D Systems®, Minneapolis, MN, USA) (Figure 9b). Concentrations of the biomarker of interest were estimated from a standard curve depending on the extent of absorption in each sample well as determined by a spectrophotometer. A new standard curve was generated with each group of ELISA plates through serial dilutions of kit specific standards. Reference ranges for each iron biomarker are given in Table 7. Prior to serum addition, maternal samples were completely thawed and diluted with reagent diluent. The reliability of ELISA quantification was assessed by running each maternal sample in duplicate. If a resulting CV was >15%, the sample was re-analyzed. If a resulting concentration was outside of the kit specific reference range (an 'outlier'), then the sample would also be re-analyzed. Note that maternal CRP concentrations, which were utilized for the adjustment of several statistical models (described below), were measured in 3rd trimester maternal serum samples using similar sandwich ELISA techniques by the candidate (Jenna).

Quantification of maternal vitamin D metabolites were performed at the Nutritional Biomarker Laboratory at the University of British Columbia (UBC). Before shipment to this site, maternal samples from the 2nd trimester and 3 months postpartum were extracted from storage containers and organized into new boxes. This procedure was conducted entirely on dry ice to prevent sample thawing. Once received by the appropriate facility at UBC, the samples were kept frozen until analyses. LC-MS/MS was utilized for the determination of maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations, which is the current gold standard procedure for vitamin D metabolite quantification (Alexandridou et al., 2021) (Figure 9c; Table 7). Protocols were adapted to the available equipment of the laboratory but were highly comparable to the methods of others (Abu Kassim et al., 2018; van den Ouweland et al., 2011; van den Ouweland et al., 2010; Shah et al., 2011). Hexane and ethyl acetate were employed for hydrophobic separation prior to reverse-phase LC (Agilent 1290) and an Agilent 6495B MS/MS was utilized in its positive-ion setting. Standards of 25(OH)D3 and 3-epi-25(OH)D3 (Cayman Chemicals, Sigma Supelco, Inc, Belleftone, PA, USA) provided a quantitative comparison, but concentrations lower than 0.78 ng/mL (or 1.95 nmol/L) could not be reliably detected and measured. LC-MS/MS validation and reliability was ensured by comparing the mean concentrations of both metabolites to internal and external (Standard Reference Material 2970 from the National Institute for Standards and Technologies) standards, which gave inter-assay CVs of <10% and <7%, respectively.

Table 7. Methodological details for the quantification of maternal iron and vitamin D biomarkers

Biomarker	Study timepoint	Technique	Reference range	Clinical cut-offs
EPO	3rd Tri.	ELISA	1.95 to 125 mIU/mL	ND
Hepcidin	2nd & 3rd Tri.	ELISA	3.13 to 800 pg/mL	ND
SF	All Tri. & 3 months postpartum	CMIA	Determined using daily controls	<15 µg/L for iron storage depletion[1]
sTfR	1st & 3rd Tri.	ELISA	5 to 80 nmol/L	ND
25(OH)D3	2nd Tri. & 3 months postpartum	LC-MS/MS	≥1.95 nmol/L	ND
3-epi-25(OH)D3	2nd Tri. & 3 months postpartum	LC-MS/MS	≥1.95 nmol/L	ND

[1]WHO, 2020. Abbreviations: chemiluminescent microparticle immunoassay (CMIA); enzyme-linked immunosorbent assays (ELISA); erythropoietin (EPO); liquid chromatography with tandem mass spectroscopy (LC-MS/MS); not determined (ND); serum ferritin (SF); soluble transferrin receptor (sTfR); trimester (Tri.); 25-hydroxyvitamin D3 (25(OH)D3); 3-epi-25-hydroxyvitamin D3 (3-epi-25(OH)D3).

The candidate (Jenna) conducted a majority of the quantification assays to determine maternal EPO, hepcidin, SF and sTfR concentrations. She quantified maternal SF concentrations in ~2,500 serum samples collected at prenatal and 3 months postpartum visits via CMIA. Jenna also conducted sandwich ELISAs to determine serum hepcidin concentrations in nearly 3,000 samples (collected at 2nd and 3rd trimester visits; estimated ~40 ELISA plates), serum EPO concentrations in >1,350 samples (3rd trimester; ~18 plates) and serum sTfR concentrations in >300 samples (1st and 3rd trimesters; ~5 plates).

The measurement of maternal nutrient biomarkers would not have been possible without additional technical assistance and other facilities. Susan Goruk, the senior manager and technician of the Field lab, assisted with the quantification of SF concentrations. Lauren Brown, who was a visiting summer research student from the United Kingdom, assisted with the quantification of sTfR and EPO concentrations. Natalie Hanas, who was an undergraduate volunteer and later research assistant, assisted with the measurement of maternal hepcidin and sample organization prior to UBC shipment. Dr. Yvonne Lamers, a Principal Investigator and Associate Professor of Food, Nutrition and Health at UBC, coordinated and ensured the reliability and validity of maternal vitamin D biomarker measurements. Shujun Lin, a senior research technician in the Lamers lab, conducted LC-MS/MS processing at the UBC Nutritional Biomarker Laboratory.

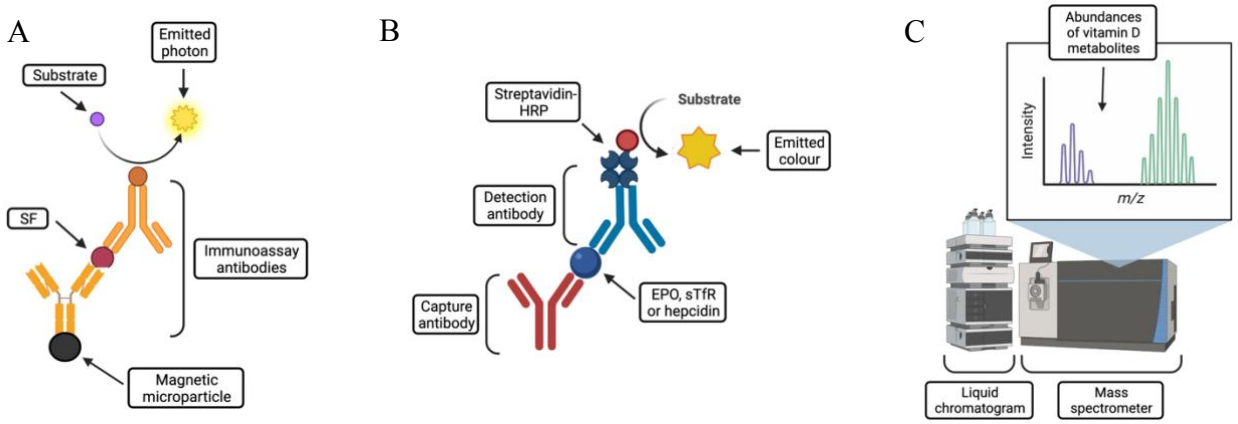

Figure 9. Details of CMIA, sandwich ELISA and LC-MS/MS protocols. (A) CMIA involves a SF-specific antibody tethered to a magnetic particle that binds available SF in maternal serum. Photon emission directly related to the amount of SF in the sample occurs following the enzymatic conversion of added substrates. (B) The general procedure of sandwich ELISAs, using 96-well plates, is pre-incubation with capture antibodies, blocking non-specific sites with reagent diluent, sample addition and incubation, detection antibody incubation, streptavidin-horseradish peroxidase (HRP) incubation and finally, colorimetric conversion through the addition of substrates. (C) In LC-MS/MS, the LC phase is critical for the extraction of vitamin D fractions from maternal samples, whereas subsequent MS cycles involve ionization and electromagnetic acceleration to quantify the abundance of particular metabolites based on their unique combination of mass and charge, or m/z ratios. Abbreviations: erythropoietin (EPO); serum ferritin (SF); soluble Transferrin Receptor (sTfR). All images were generated using BioRender.com.

3.1.1 Calculation of additional biomarker variables

3.1.1.1 sTfR-SF index

$$\mathrm{sTfR{:}\,SF} = \frac{[\mathrm{sTfR}]}{\log([\mathrm{SF}])}$$

where concentrations of sTfR and SF are in units of mg/L and µg/L, respectively; therefore, sTfR:SF have units of mg/µg (Nadeem et al., 2011). This variable was calculated during the 1st or 3rd trimester if a participant had sTfR and SF data available at one or both timepoints. The brackets represent concentrations.

3.1.1.2 Hepcidin-EPO ratio

$$\mathrm{Hepcidin{:}\,EPO} = \frac{[\mathrm{Hepcidin}]}{[\mathrm{EPO}]}$$

where concentrations of hepcidin and EPO are in units of ng/mL and mIU/mL, respectively; therefore, hepcidin:EPO have units of ng/mIU (Delaney et al., 2021a). This variable was calculated during the 3rd trimester if a participant had hepcidin and EPO data available. The brackets represent concentrations.

3.1.1.3 Changes in maternal SF concentrations

Change in maternal SF between time A and time B = $[SF]_{time\ B} - [SF]_{time\ A}$

where the change in maternal SF concentrations is in units of μg/L. Time A is an earlier timepoint and time B is a later timepoint. This variable was calculated between the (1) 1st and 2nd trimesters, (2) 2nd and 3rd trimesters, and (3) 1st and 3rd trimesters. The brackets represent concentrations.

3.1.1.4 Changes in maternal body iron stores

Change in maternal body iron stores between time A and time B = $[SF_{Time\ B - Time\ A}] * 8$

as 1 μg/L of SF is thought to represent ~8 mg of body iron stores (Walters et al., 1973), the body iron storage variables were calculated by multiplying the change in SF by a factor of 8, and therefore, are in units of mg. Time A is an earlier timepoint and time B is a later timepoint. This variable was calculated between the (1) the 1st and 2nd trimesters, (2) 2nd and 3rd trimesters, and (3) 1st and 3rd trimesters. The brackets represent concentrations.

3.2 The maternal iron and vitamin D adequacy variable

A new categorical variable was generated to assess the combined adequacy of maternal iron and vitamin D status during the 2nd trimester. Four categories were conceptualized:

1. A replete maternal status of both micronutrients:
    a. Maternal SF concentrations ≥15 μg/L, and
    b. Maternal 25(OH)D* concentrations ≥75 nmol/L

2. Replete maternal iron and low vitamin D:
    a. Maternal SF concentrations ≥15 μg/L, and
    b. Maternal 25(OH)D concentrations <75 nmol/L

3. Replete maternal vitamin D and low iron:
    a. Maternal SF concentrations <15 μg/L, and
    b. Maternal 25(OH)D concentrations ≥75 nmol/L

4. A low maternal status of both micronutrients:
    a. Maternal SF concentrations <15 μg/L, and
    b. Maternal 25(OH)D concentrations<75 nmol/L

*Maternal 25(OH)D concentrations were determined by summing maternal 25(OH)D3 and 25-hudroxyvitamin D2, or 25(OH)D2, concentrations in the 2nd trimester (n=644)

3.3 Statistical analyses

The determination of maternal vitamin D and iron biomarker dynamics across different study visits (objective 1) involved several statistical procedures. Concentrations of maternal biomarkers, with the exception of maternal Hb and 25(OH)D3, were right skewed and required log transformation prior to tests that required data normality. Skewed biomarker concentrations are presented as medians with interquartile ranges (IQR), whereas means and standard deviations (SD) are given for normally distributed data. Maternal SF<15 μg/L indicated iron storage depletion (WHO, 2020). Paired t-tests were used to determine if maternal hepcidin, sTfR, sTfR:SF, 25(OH)D3 or 3-epi-25(OH)D3 concentrations changed between two study timepoints (Table 7). Repeated measures analysis of variance was used to assess changes in Hb and SF concentrations across each trimester and 3 months postpartum. Kruskal-Wallis tests were used to assess if there were differences between median changes in maternal SF or body iron stores across time.

The determination of relationships between maternal iron and vitamin D biomarker concentrations during and after pregnancy and child and maternal health outcomes (objectives 2, 3 and 4) were primarily conducted via multivariate linear regression models. Univariate (crude) models included the exposure as the only independent variable. When birth anthropometrics (BWs and BHCs) and child behaviours (BASC-2 externalizing and internalizing T-scores) were the outcomes, regression models were stratified by offspring sex. Although this was not part of the initial statistical plan, variability in maternal iron biomarker concentrations depending on fetal sex was observed. Using Student's t-tests, it was determined these differences were significant during

the 3rd trimester (Table 10). As described in Chapter 1 (section 1.5), there is also accumulating evidence to suggest that susceptibility to adverse maternal conditions, like poor nutritional status, during fetal development may be sex-dependent (Gualtieri & Hicks, 1985; Nugent et al., 2018). It was decided a posteriori to treat offspring sex as an effect modifier (EM) for neonatal and child outcome models.

Adjustment of regression models were dictated by a two-step process (Weng et al., 2009). Firstly, the identification of potential confounders was achieved by the construction of directed acyclic graphs (DAGs) for each statistical question of interest (**Figures 10-12**) (Williams et al., 2018). To be included as a confounder in a DAG, there needed to be existing evidence, primarily in the form of meta-analyses, of associations between the given variable with both the exposure and outcome of interest. Next, change-in-estimate rules were applied (Weng et al., 2009). All potential confounders and the exposure of interest were added into models as independent variables, and if the exposure coefficient changed by ≤10% after the removal of a potential confounder, then the variable would be removed. Resultingly, each multivariate (adjusted) model was adjusted for a minimum set of confounders specific to the dataset, which reduced the risk of overadjustment while ensuring that important confounding variables were controlled. To assess the combined impact of maternal iron and vitamin D status on child BASC and maternal EPDS scores, generalized linear models were used.

Statistical analyses were primarily conducted using SPSS (V28.0, IBM Corporation, Armonk, NY, USA), with support from SAS (V9.3, IBM Corporation , Cary, NC, USA). Statistical significance was defined by a p-value (two-sided) of <0.05. Figures were created using BioRender.com, Microsoft PowerPoint, MolView.org, Prism or SPSS.

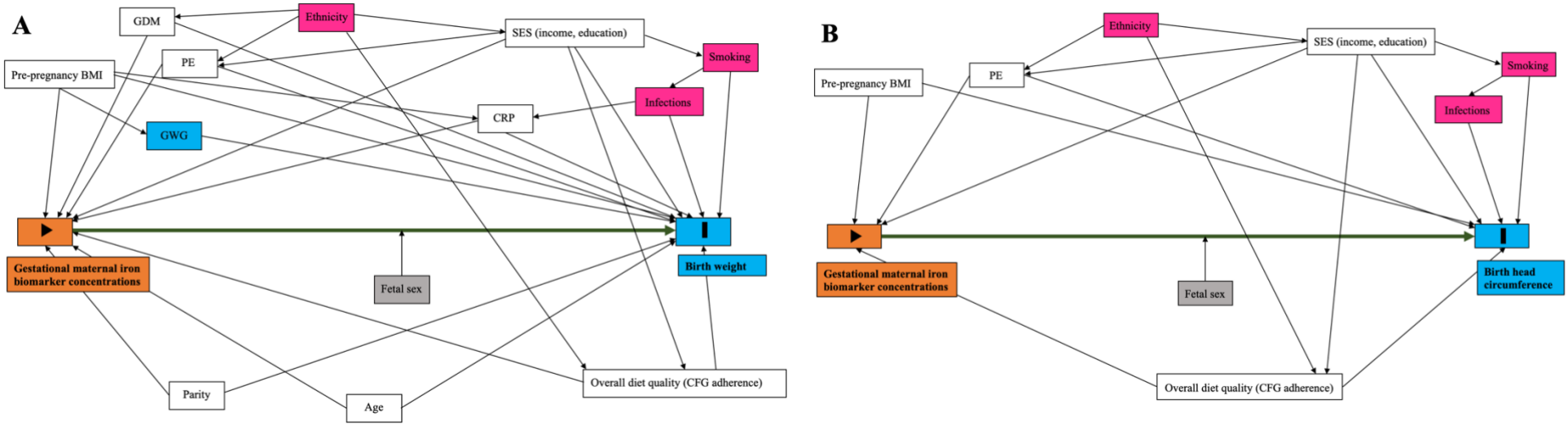

Figure 10. Directed acyclic graphs (DAGs) depicting the relationships between maternal iron status biomarkers during pregnancy and birth anthropometrics of interest with potential confounders of the pathway. (A) is specific to birth weight (BW), whereas (B) relates to birth head circumference (BHC). The green line represents the causal-like association pathway between the exposure (orange; triangle) and outcome (blue; "I") variables (Williams et al., 2018). The variables in white boxes represent potential confounders. Variables in blue and pink are not confounders, but ancestors of either the exposure and outcome (pink boxes) or just the outcome (blue boxes). Fetal sex was treated as an effect modifier (EM). Abbreviations: body mass index (BMI); Canadian Food Guide (CFG); C-reactive protein (CRP); gestational diabetes mellitus (GDM); preeclampsia (PE); socioeconomic status (SES). The DAGs were originally constructed using DAGitty.net and remodelled using PowerPoint.

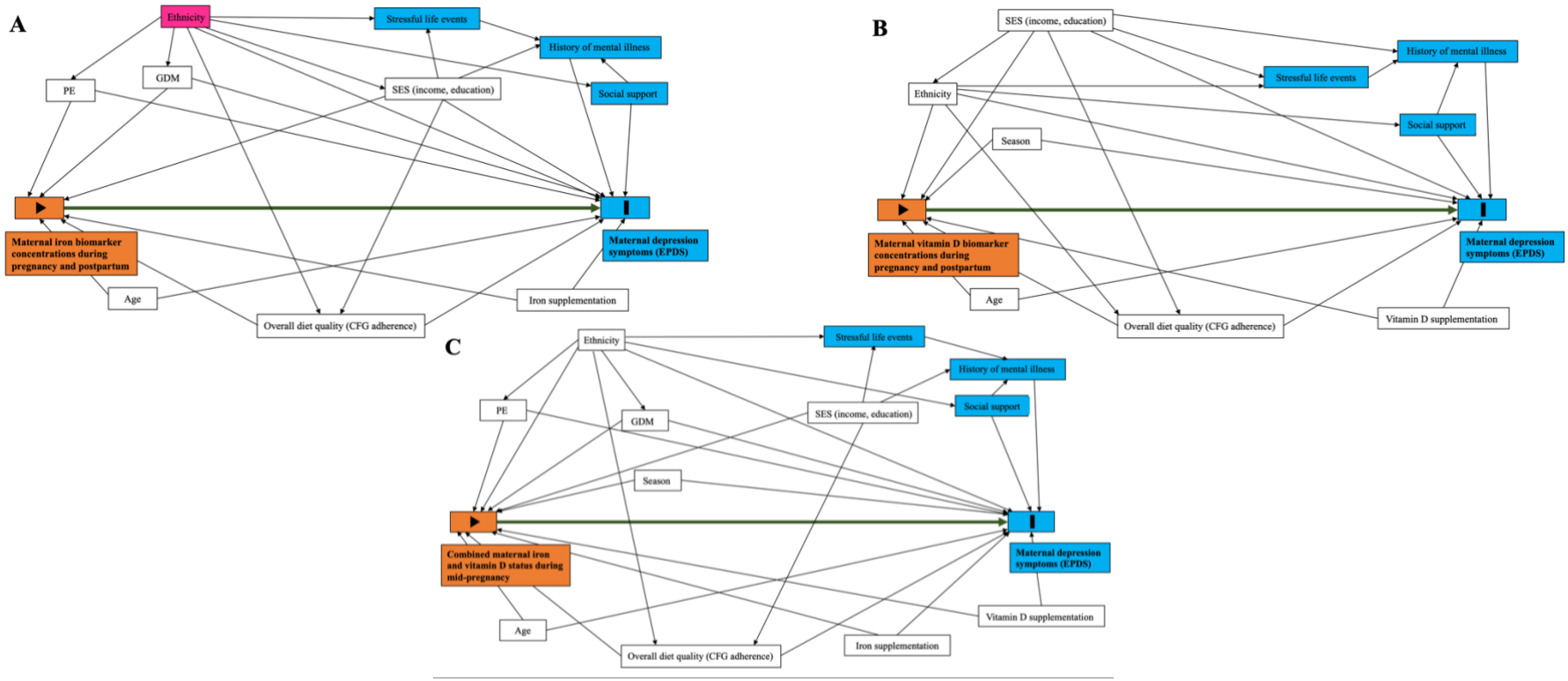

Figure 11. Directed acyclic graphs (DAGs) depicting the relationships between maternal iron and vitamin D status biomarkers and maternal EPDS scores with potential confounders of the pathway. (A) is specific to maternal iron biomarker exposures, (B) to vitamin D biomarker exposures and (C) has combined maternal iron and vitamin D status as exposures. The green line represents the causal-like association pathway between the exposures (orange; triangle) and outcome (blue; "I") variables (Williams et al., 2018). The variables in white represent potential confounders. Variables in blue and pink are ancestors of either the exposure and outcome (pink) or just the outcome (blue). Abbreviations: body-mass-index (BMI), Canadian Food Guide (CFG), Edinburgh Depression Scale (EDS), gestational diabetes mellitus (GDM), pre-eclampsia (PE), socioeconomic status (SES). DAGs were originally constructed using DAGitty.net and remodelled using PowerPoint.

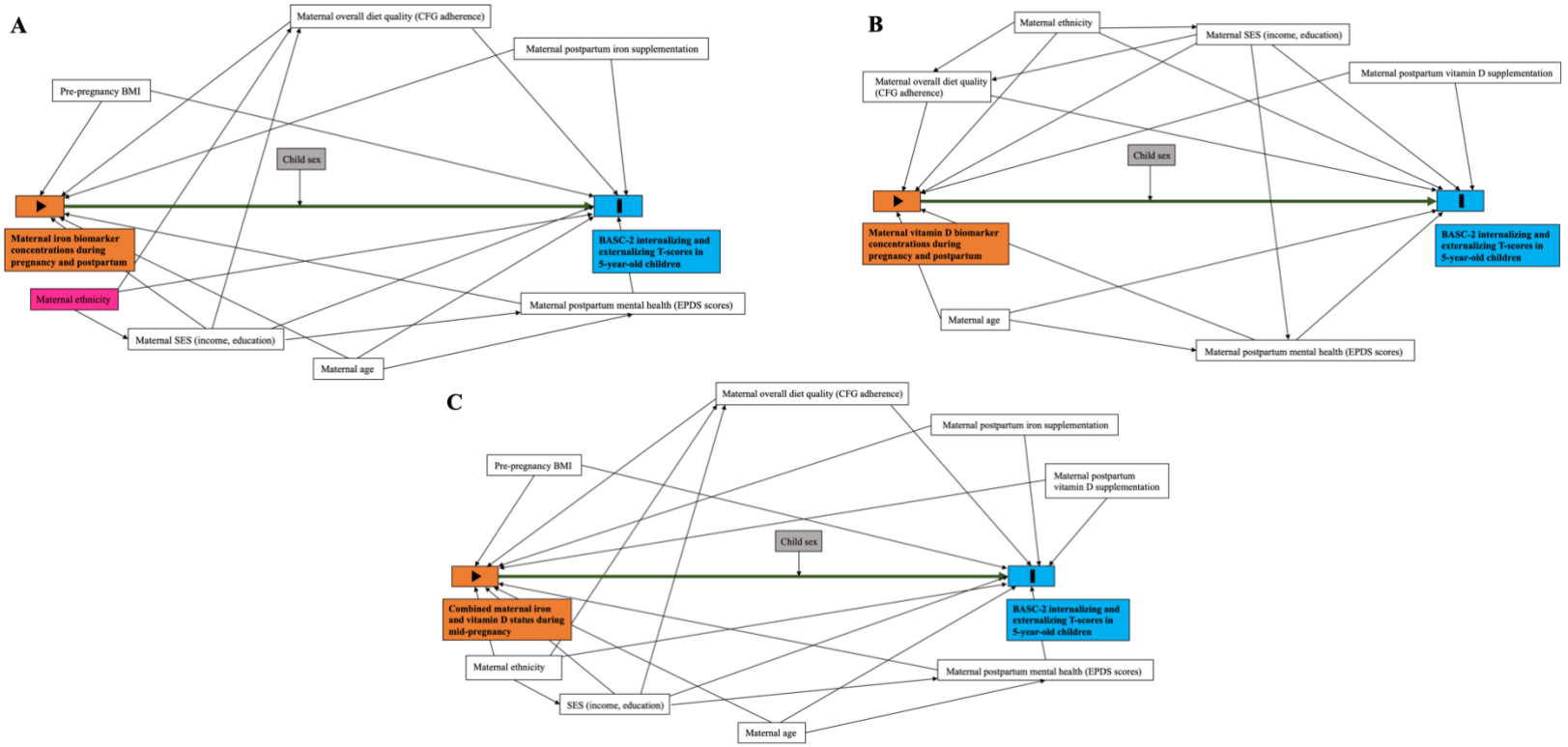

Figure 12. Directed acyclic graphs (DAGs) depicting the relationships between maternal iron and vitamin D status biomarkers and BASC-2 internalizing and externalizing T-scores in 5-year-old children with potential confounders of the pathway. (A) is specific to maternal iron biomarker exposures, (B) to vitamin D biomarker exposures and (C) has combined maternal iron and vitamin D status as exposures. The green line represents the causal-like association pathway between the exposures (orange; triangle) and outcome (blue; "I") variables (Williams et al., 2018). The variables in white represent potential confounders. Variables in blue and pink are ancestors of either the exposure and outcome (pink) or just the outcome (blue). Child sex was treated as an effect modifier (EM). Abbreviations: body-mass-index (BMI), Behaviour Assessment System for Children (BASC), Canadian Food Guide (CFG), Edinburgh Depression Scale (EDS), socioeconomic status (SES). The DAGs were originally constructed using DAGitty.net and remodelled using PowerPoint.

CHAPTER 4: MANUSCRIPT 1

Maternal iron status is dynamic throughout pregnancy and might predict birth outcomes in a sex-dependent manner: Results from the APrON cohort study

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4.1 Introduction

Despite the well-established importance of sufficient maternal iron status for optimal gestational outcomes (Allen, 2000; Klebanoff et al., 1991; Murphy et al., 1986; Steer, 2000), the prevalence of inadequacies of this micronutrient remains high among both pregnant individuals (Garzon et al., 2020; Tang et al., 2019) and people of reproductive age (Mawani et al., 2016). Those with excessive menstrual bleeding can also be additionally predisposed (Mirza et al., 2018). Recent estimates suggest approximately 40% of pregnant people may be at risk for anemia (Goonewardene et al., 2012; Stevens et al., 2013). Half of these cases of anemia may be attributable to the lack of systemic iron available for maternal, placental and fetal demands, resulting in iron deficiency anemia (IDA) (Stevens et al., 2013). Beyond iron, other micronutrient deficiencies, certain pathogens and inflammatory conditions can contribute to gestational anemia. Although there are continued attempts to estimate the global prevalence of IDA, diagnoses of deficiency as well as the prevalence of iron overload is underreported because of a lack of research in this area and limited iron screening practices (Mirza et al., 2018; Zhao et al., 2022). The incidence of iron deficiency (ID) within high-income countries are suggested to impact about a quarter of all

pregnancies (Stevens et al., 2013), but some recent studies caution that this figure is an underestimation (Beckert et al., 2019; Cochrane et al., 2022; Milman et al., 2017).

Iron is a critical micronutrient that facilitates systemic respiration, mitochondrial activity in essential cellular networks, such as those in the heart, kidney and thyroid gland, and the proper development and maintenance of the immune and nervous systems (Dutt et al., 2022; Lynch et al., 2018; Ward & Coonon, 2019). Its role as a cofactor in key proteins that regulate DNA replication and cellular checkpoints have also been elucidated (Khan et al., 2020). ID is a continuum where iron is preferentially shunted to accommodate sustained erythropoiesis while certain biomarkers of status change differentially depending on the extent of depletion (Cappellini et al., 2020; Lynch et al., 2018). Cellular stores, primarily in the liver, spleen and skeletal muscles, are the first to decline during ID, which can be assessed by concentrations of serum ferritin (SF) (Georgieff, 2020; Harrison & Arosio, 1996). If iron is not replenished, circulatory iron concentrations may change, orchestrated by the master regulator of iron absorption and distribution, hepcidin (Park et al., 2001; Nicolas et al., 2001). Decreased concentrations of this hepatic peptide negatively regulate non-heme iron absorption in the gut and release from cellular stores. Increases in soluble transferrin receptor (sTfR), a peptide that is cleaved from transferrin receptors during a state of intracellular iron depletion (Huebers et al., 1990), may also be observed during an extended period of ID. An indication of the most extreme manifestation of ID is IDA, leading to increasingly hypoxic conditions that upregulates the renal production of erythropoietin (EPO), a hormone that protects RBCs and promotes erythropoiesis (McMullin et al., 2003). Although some of these biomarkers are validated indicators of iron sufficiency or regulation, their precision may be modulated if inflammation is probable (Delaney et al., 2021a; Lynch et al., 2018).

Pregnancy is a period of significant physiological change coupled with tissue growth, which substantially increases iron requirements (Fisher & Nemeth, 2017). Accordingly, the proper allocation, storage and utilization of iron for not only the pregnant individual, but also the placenta and growing fetus is crucial (McArdle et al., 2011; Sangkhae et al., 2020). There is evidence that each component independently regulates these processes through the secretion of their own supply of hormones, like hepcidin, EPO and erythroferrone (O'Brien, 2022). Iron has been implicated in fetal development and growth (Allen, 2000; Scholl, 2011), but the literature substantiating connections between the maternal status of iron and birth outcomes remains surprisingly mixed with an array of either no associations as well as positive or negative relationships with birth

weights (BWs) or birth head circumferences (BHCs) (Haider et al., 2013; Iqbal & Ekmekcioglu, 2019). This is in spite of the importance of neonatal anthropometrics, including BWs, in the health outcomes of individuals, from future weight status to chronic disease predisposition (Belbasis et al., 2016). In addition, there may be sex-specific differences in fetal growth and development (DiPietro & Voegtline, 2017), a finding that could be relevant to iron biology.

The aim of this study was to determine which maternal iron biomarkers at different gestational timepoints predicted two key birth outcomes and if these relationships varied depending on fetal sex in a cohort of generally healthy pregnant females. Our first objective was to determine the prevalence of maternal ID and whether concentrations of various iron biomarkers changed across different pregnancy timepoints. Our second objective was to explore potential associations between maternal biomarker concentrations at different trimesters of pregnancy and BWs and BHCs while treating the sex of the fetus as an effect modifier (EM).

4.2 Methods

As part of the Alberta Pregnancy Outcomes and Nutrition (APrON) prospective cohort study (Kaplan et al., 2014; Letourneau et al., 2022), the dietary intakes and biological status of iron among pregnant females were assessed in a sub-study called APrON-Iron (APrON-Fe). The APrON study was conducted in Calgary and Edmonton, Alberta, Canada, with family-medicine and obstetric clinics as the primary recruitment locations. Throughout the intake period of APrON (May 2009 – July 2012), 2,189 pregnant individuals either in their 1st or 2nd trimester were recruited into the study (Letourneau et al., 2022). They were excluded if they were 16 years old or younger, could not read and write in English or if they were planning to move out of the Edmonton or Calgary area before giving birth (Kaplan et al., 2014). Maternal and neonatal data was also extracted from labour and delivery records. Other information and details about the design of the APrON study are described extensively elsewhere (Kaplan et al., 2014; Letourneau et al., 2022). Informed consent about the collection and use of all data was obtained from each APrON participant. Ethical approval was granted by both the Calgary Health Research Ethics Board (REB14-1702) and the University of Alberta Health Research Biomedical Panel (Pro00002954) (Letourneau et al., 2022).

4.2.1 Demographic, health and dietary data

The ethnicity of pregnant participants was self-reported. Other aspects of maternal socioeconomic status (SES), including information about household income and educational attainment, were collected at the first study visit (Kaplan et al., 2014). Pre-pregnancy body mass index (BMI) and gestational weight gain were calculated from anthropometric data collected at study visits (Begum et al., 2012). History of a medical diagnosis with pre-existing conditions, including type 1 or type 2 diabetes, depression and anxiety, or gestational-related complications, like gestational diabetes, gestational hypertension and preeclampsia, were obtained by self-report from participants at each trimester of pregnancy and at ~3 months postpartum. Through the use of 24-hour dietary recall data, which was collected at each prenatal timepoint, maternal adherence to Canadian Food Guide (CFG) recommendations were quantified into scores (CFG Scores) as previously described (Jarman et al., 2017). Delivery records were consulted for offspring sex, BWs and BHCs (Kaplan et al., 2014).

4.2.2 Blood collection and biomarker measurements

At each trimester of pregnancy and at approximately 3 months postpartum, maternal venous blood samples were drawn at clinics by certified phlebotomists (Kaplan et al., 2014). The mean (range) gestational age of blood sample collection across pregnancy was 10.8 (3.1-13.9) weeks in the 1st, 19.0 (14.0 – 26.9) weeks in the 2nd and 32.5 (27.0 – 39.0) weeks in the 3rd trimester. Hemoglobin (Hb) concentrations were immediately measured in whole blood samples using HemoCue 201® analyzers (HemoCue, Cypress, CA, USA) with the utilization of appropriate controls. After, the maternal blood was processed into fractions (serum, plasma, buffy coat and red blood cells) that were aliquoted into independent 1.5 mL microcentrifuge tubes and stored in -80 °C freezers until other analyses. SF concentrations were measured by transferring ~150 µL of thawed maternal serum into buffer (1:2 serum:buffer) before loading into a i2000sr Architect Plus blood analyzer (Abbott, Chicago, IL, USA) that utilizes chemiluminescent microparticle immunoassays. This machine was continuously calibrated and control samples were run daily. Random serum aliquots were re-analyzed each day to determine any variation in quantified SF concentrations, which was found to be <5%. Serum hepcidin, sTfR and EPO concentrations were measured using enzyme-linked immunosorbent assays (R&D Systems®, Minneapolis, MN, USA). The serum samples were appropriately diluted in reagent diluent then pipetted into 96-well plates

in duplicate. Co-efficient of variations, or CVs, between replicates were determined. A CV of <10% was considered reproducible and samples were re-analyzed if CVs were larger than this threshold. The hepcidin kits had a detection range of 3.13 to 800 pg/mL whereas this range was 5 to 80 nmol/L and 1.95 to 125 mIU/mL for the sTfR and EPO kits, respectively. If samples were outside of the detectable range, they were diluted or concentrated and re-analyzed.

Pregnant participants were considered to have anemia if Hb concentrations were <110 g/L during the 1st or 3rd trimesters or <105 g/L during the 2nd trimester to adjust for the unequal balance between plasma and red blood cell expansion during mid-gestation, termed hemodilution (Costantine, 2014; Koller et al., 1980). Hb concentrations <120 g/L were considered indicative of anemia at 3 months postpartum (World Health Organization [WHO], 2011). Deficient iron stores were defined as SF <15 μg/L, although the accuracy of this threshold is poorly understood beyond early pregnancy (WHO, 2020). If pregnant participants had Hb <110 g/L (1st and 3rd trimesters), <105 g/L (2nd trimester) or <120 g/L (3 months postpartum) and SF <15 μg/L, they were considered to have IDA (Costantine, 2014; Koller et al., 1980; WHO, 2011; WHO, 2020). A cut-off for maternal sTfR concentrations has not been well-defined, so the percentage of participants during the 3rd trimester with sTfR above the 75th and 90th percentiles from the 1st trimester were used to describe gestational dynamics of this biomarker. During the 1st and 3rd trimesters, a sTfR-SF index (sTfR:SF) was calculated by dividing maternal sTfR concentrations by the logarithm of maternal SF concentrations [sTfR/$\log_{10}$(SF)] (Zimmermann, 2008) in 246 individuals with both measurements available. The ratio between maternal hepcidin and EPO concentrations (hepcidin:EPO) in the 3rd trimester was quantified by dividing hepcidin by EPO concentrations (n=1291) (Delaney et al., 2021a).

4.2.3 Statistical analysis

Maternal concentrations of SF, hepcidin, sTfR, sTfR:SF, EPO and hepcidin:EPO at each study timepoint were positively skewed. Therefore, we logarithmically transformed the data to ensure their normality before conducting applicable statistical tests. Descriptive estimates of normal data are presented as mean ± standard deviation (SD) and skewed data are given as median (interquartile range, IQR). Paired t-tests were used to compare any differences between maternal hepcidin, sTfR and sTfR:SF concentrations at two timepoints and repeated measures general linear models were used to compare Hb and SF concentrations at each trimester and 3 months

postpartum. Independent t-tests were conducted to assess any differences in maternal biomarker concentrations by fetal sex, and Kruskal-Wallis tests examined differences in median SF and body iron storage changes.

To explore relationships between maternal iron biomarker concentrations at different gestational timepoints and neonatal BWs and BHCs, multivariate linear models were built. Pregnant participants were identified for inclusion into each type of model depending on the availability of associated fetal sex and birth outcome **data (Supplementary Figure 1).** Univariate (crude) models included only the exposure and outcome of interest, and for model adjustment, a two-step method was performed (Weng et al., 2009). Firstly, multivariate (adjusted) models were initially adjusted for confounders as indicated by directed acyclic graphs (DAGs), which were constructed using evidence from relevant literature (Williams et al., 2018). The potential confounding variables included in the DAGs differed depending on the particular birth outcome (see **Figure 10** in Chapter 3), and included maternal age (years), ethnicity (White or other ethnicity), income (≥$70,000 or <$70,000), education (some type of post-secondary education or not), parity (≥1 or 0), CFG scores, maternal C-reactive protein concentrations during the 3rd trimester, gestational diabetes mellitus (yes or no) and preeclampsia (yes or no). Fetal sex was treated as an EM as males may be more vulnerable to poor in utero conditions (Gualtieri & Hicks, 1985; Nugent et al., 2018). Next, using backward removal, a change in estimate rule was applied such that if the elimination of a covariate changed the ß-coefficient for the exposure variable by >10% of its original adjusted value, it would be identified as a confounder and kept in the model (Weng et al., 2009). If the change was ≤10%, then that variable was removed from the model to reduce the risk of overadjustment. A detailed list of the confounders that were included in each model is provided in **Supplementary Table 1**. All statistics were performed using SPSS (V28.0, IBM Corporation, Armonk, NY, USA) and two-sided *p*-values of <0.05 were considered statistically significant.

4.3 Results

4.3.1 Maternal and newborn characteristics

At enrollment into the APrON study, pregnant participants had an average age of 31.5 years (SD: 4.5) and a median pre-pregnancy BMI of 23.0 (IQR: 5.4) (**Table 8**). The majority of participants self-identified as White, were born in Canada, were either married or cohabiting with

a partner, had completed some type of post-secondary education and had a household income of $70,000 or more. Just over half of the participants were primiparous. The prevalence of self-reported maternal pre-pregnancy and prenatal health conditions were generally low, with many affecting less than 1% of the pregnant individuals. Among newborns, 52.7% (n=1066) were male (Table 8). Mean BHCs and BWs were 34.5 ± 0.1 cm and 3341.9 ± 547.1 g, respectively, and 6.6% of neonates were born preterm.

Table 8. Pregnant participant and newborn characteristics in the APrON cohort

Characteristic	Count (n)	Average or proportion (%)
Pregnant participants		
Age (years)	2,134	31.5 ± 4.5[1]
Pre-pregnancy BMI (kg/m^2)	1,947	23.0 (5.4)[2]
Gestational weight gain (kg)	1,541	15.2 ± 5.9
Marital status, %		
Married	1,772	86.7
Common-law	236	11.3
Divorced	8	0.4
Separated	7	0.3
Single	69	3.3
Education, %		
Completed post-grad	470	22.7
Completed university	943	45.5
Completed trade/tech	401	19.4
Completed high school	200	9.6
Less than high school	58	2.8
Household income, %		
$100 000 or more	1,143	55.2
$70 000–$99 999	463	22.4
$40 000–$69 999	276	13.3
$20 000–$39 999	122	5.9
Less than $20 000	65	3.1
Primiparous, %		
Yes	1,119	53.5
No	971	46.5
Born in Canada, %		
Yes	1,612	77.1
No	479	22.9
Ethnicity (%)		
White	1,674	80.2
Other	412	19.7
Maternal conditions, %		
Anxiety	121	5.5
Depression	122	5.6
Diabetes		
Type 1	4	0.2

Type 2	2	0.1
Gestational	65	3.0
Hypertension		
Primary	12	0.5
Gestational	15	0.7
Preeclampsia	7	0.3
Polycystic Ovarian Syndrome	61	2.8
Newborns		
Sex		
Male	1066	52.7
Female	956	47.3
Preterm, %	134	6.6
Birthweight (g)	2022	3341.9 ± 547.1
Birth length (cm)	1751	51.0 (3.5)
Birth head circumference (cm)	1873	34.5 ± 0.1

[1]Mean ± SD; [2]Median (IQR). This table was adapted from Leung et al. (2016). Abbreviations: body mass index (BMI).

4.3.2 Changes in maternal iron biomarkers during and after pregnancy

First trimester mean maternal Hb concentrations were significantly higher compared to the 2nd and 3rd trimesters ($p<0.001$), however there was no difference between Hb concentrations in the 2nd and 3rd trimesters (**Table 9; Supplementary Figure 2**). The mean maternal Hb concentration at 3 months postpartum was higher than the means at any of the three pregnancy timepoints ($p<0.001$). Although the prevalence of maternal anemia during gestation and the postpartum period was generally low in this cohort, the proportion of participants with this condition increased as pregnancy progressed.

Maternal SF concentrations declined significantly with each successive trimester of pregnancy (all $p<0.001$) (Table 9; Supplementary Figure 2), but there was no difference between the mean maternal SF concentration in the 1st trimester compared to the mean at 3 months postpartum ($p>0.05$). Between the 1st and 3rd trimester, the median changes in maternal SF concentrations and body iron stores were respectively -28.1 μg/L (IQR: 36.9) and -224.8 mg (IQR: 294.9) (**Figure 13)**, given that 1 μg/L of SF has been estimated to reflect approximately 8 mg of body iron stores (Walters et al., 1973). Accordingly, the prevalence of maternal iron storage depletion increased across gestation, with nearly 61% of pregnant individuals falling below the threshold of SF <15 μg/L by the 3rd trimester study visit (Table 9).

As shown in Table 2, maternal concentrations of sTfR and the sTfR-SF index both significantly increased from the 1st to the 3rd trimester (both $p<0.001$) (Supplementary Figure 2).

There was an increase in the proportion of participants with 3rd trimester sTfR concentrations that fell over the 1st trimester 75th (25% to 65.3%) and 90th percentiles (10% to 34.1%). Mean maternal hepcidin concentrations significantly dropped between the 2nd and 3rd trimesters (p<0.001) (Table 9; Supplementary Figure 2).

Table 9. Changes in maternal iron biomarker concentrations and the prevalence of insufficient status across different study timepoints

Biomarker	1st Trimester	2nd Trimester	3rd Trimester	3 months Postpartum
Hb (g/L)				
Sample size (n)	542	1895	1676	1765
Mean ± SD[1]	127.9 ± 9.1	122.4 ± 8.8*	122.4 ± 9.1*	133.8 ± 9.2*
Anemia,[2] %	0.9	1.4	4.2	4.2
IDA,[3] %	--	17.6	65.9	10.3
SF (µg/L)				
Sample size (n)	379	1639	1466	1435
Median (IQR)	43.7 (42.4)	29.8 (29.7)*	12.8 (9.8)*	40.9 (43.0)
Depleted iron stores[3]				
SF <15 µg /L,[4] %	7.4	15.6	60.7	10.5
SF <12 µg /L,[4] %	2.9	10.9	43.9	6.3
sTfR (mg/L)				
Sample size (n)	313	--	352	--
Median (IQR)	3.5 (1.0)		4.3 (1.8)*	
Above 1st trimester 75thile, %	25.0		65.3	
Above 1st trimester 90thile, %	10.0		34.1	
sTfR:SF (mg/µg)				
Sample size (n)	246	--	246	
Median (IQR)	2.0 (0.8)		3.8 (1.8)*	
Hepcidin (ng/mL)				--
Sample size (n)	--	1491	1504	
Median (IQR)		10.7 (17.2)	2.8 (5.8)*	
EPO (mIU/mL)				
Sample size (n)	--	--	1385	--
Median (IQR)			12.9 (9.1)	
Hepcidin:EPO (ng/mIU)				
Sample size (n)	--	--	1291	--
Median (IQR)			0.2 (0.5)	

[1]Mean ± SD or Median (IQR) depending on data normality; [2]Anemia: Hb <110 (1st or 3rd trimesters) or <105 g/L (2nd trimester); [3]IDA: Hb <110 g/L (1st and 3rd trimesters) or Hb <105 g/L (2nd trimesters) and SF <15 µg/L (WHO, 2011). Proposed thresholds for low maternal iron status: [4]low iron stores, SF <15 µg/L or SF <12 µg/L (WHO, 2020). Abbreviations: erythropoietin (EPO); hemoglobin (Hb); hepcidin-EPO ratio (hepcidin:EPO); iron deficiency anemia (IDA); interquartile range (IQR); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF);

data not available (--). Paired t-tests and repeated measures generalized linear models were conducted to determine if there was a statistical difference between mean maternal concentrations (after log transformation for all biomarkers except Hb) at that timepoint compared to the 1st (Hb, SF, sTfR, sTfR-SF index) or 2nd (hepcidin) trimester concentration. *Indicates a significant difference at p<0.01.

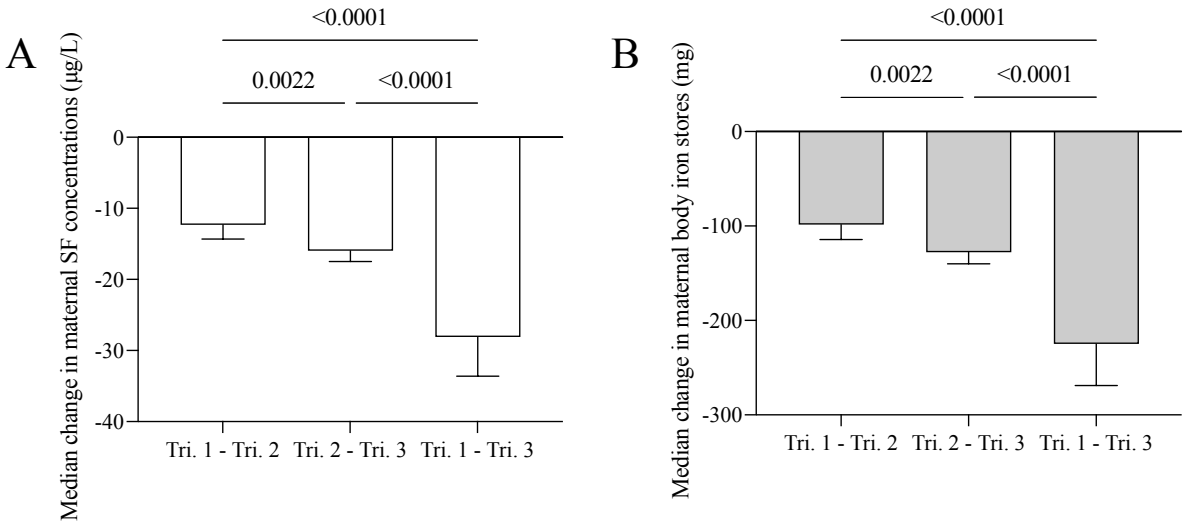


Figure 13. Median changes in maternal (A) SF concentrations and (B) body iron stores across pregnancy. Changes between three different gestational time intervals (1st to 2nd trimester, 2nd to 3rd trimester and 1st to 3rd trimester) are given for both measurements. Previous estimates suggest that 1 µg/L of SF may represent ~8 mg of body iron stores (Walters et al., 1973). Bars and error bars represent the median and 95% CI, respectively. Non-parametric Kruskal-Wallis tests were used to determine statistical differences between the medians. Abbreviations: trimester (Tri.).

4.3.3 Differences in maternal iron biomarker concentrations depending on offspring sex

Table 10 shows statistical estimates of differences between maternal concentrations of several iron biomarkers during the 3rd trimester when stratified by fetal sex. If participants were pregnant with a male fetus, they had significantly higher concentrations of SF, hepcidin and hepcidin:EPO during the 3rd trimester compared to those that were having a female fetus (medians: 13.6 µg/L vs 12.2 µg /L for SF; 3.1 ng/mL vs 2.4 ng/mL for hepcidin; 0.3 vs 0.2 ng/mIU for hepcidin:EPO) (Table 10). Maternal concentrations of sTfR, sTfR:SF and EPO in the 3rd trimester were lower if the pregnant individuals were carrying a male compared to a female (sTfR: 4.2 mg/L vs 4.5 mg/L; sTfR:SF: 3.5 vs 4.1 mg/µg; EPO: 12.5 mIU/mL vs 13.3 mIU/mL). No additional

differences in maternal biomarker concentrations were found at different study timepoints after sex stratification.

Table 10. Differences in 3rd trimester maternal iron biomarker concentrations by fetal sex

Biomarker	Sample size (n)	Mean difference (M-F) [95% CI]	*p*-value
SF			
Males	731	1.1 [1.1, 1.2]	<0.001
Females	680		
sTfR			
Males	177	-1.0 [-1.0, -0.9]	0.037
Females	158		
sTfR:SF			
Males	134	-0.9 [-1.0, -0.8]	0.024
Females	134		
Hepcidin			
Males	744	1.2 [1.1, 1.4]	0.005
Females	690		
EPO			
Males	704	-0.9 [-1.0, -0.9]	0.025
Females	646		
Hepcidin:EPO			
Males	663	1.3 [1.1, 1.6]	0.005
Females	595		

The maternal iron biomarker concentrations in this table were log transformed before conducting independent t-tests. Mean differences and 95% CIs of male minus female (M-F) values were back transformed [biomarker concentration units: EPO (mIU/mL); hepcidin (ng/mL); hepcidin: EPO (ng/mIU); SF (µg/L); sTfR (mg/L); sTfR:SF (mg/µg)]. The sample sizes in this table reflect the number of pregnant participants with biomarker measurements during the 3rd trimester if the sex of their infant was also defined. There were no significant differences in maternal biomarker concentrations by fetal sex at any other study timepoint (p>0.05). Abbreviations: confidence interval (CI); erythropoietin (EPO); hepcidin-EPO ratio (hepcidin:EPO); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF).

4.3.4 Relationships between maternal iron biomarkers and birth outcomes

After model adjustment, only maternal iron biomarker concentrations from the 3rd trimester remained significantly associated with offspring BWs (**Table 11**). Specifically, linear increases in SF and hepcidin:EPO during the 3rd trimester in both male (SF: p=0.006; hepcidin:EPO: p=0.033) and female (SF: p=0.020; hepcidin:EPO: p=0.019) fetal models were associated with lower BWs. In addition, maternal hepcidin and Hb concentrations from the 3rd trimester were inversely associated with BWs in those having males (p=0.033 for hepcidin; p=0.004 for Hb), but this was

not observed for females (both p>0.05) (Table 11). The significant BW relationships are visualized via scatter plots in **Supplementary Figure 3**.

Statistical outputs for relationships between maternal iron biomarker concentrations and BHCs are shown in **Table 12**. After adjustment, a negative association between mid-pregnancy maternal concentrations of SF and BHCs were only present in male offspring (p=0.048). Maternal Hb concentrations during the 3rd trimester were inversely related with BHCs in male (p=0.017), but not female models. The significant BHC relationships are visualized via scatter plots in **Supplementary Figure 4**.

Table 11. Relationships between maternal iron biomarker concentrations and birth weights (BWs) stratified by offspring sex

	Biomarker	Univariate β [95% CI]		Multivariate β [95% CI]	
		Male	Female	Male	Female
1st Tri.	SF	-107.3 [-395.0, 180.5]	-150.8[-425.2, 123.6]	-3.8 [-347.4, 339.8]	-196.7 [-482.8, 89.4]
	sTfR	442.5 [-356.9, 1241.9]	**-845.2 [-1666.9, -23.5]**	408.9 [-461.4, 1279.3]	-807.2 [-1817.5, 203.1]
	sTfR:SF	259.0 [-371.5, 889.5]	-287.7 [-922.2, 346.9]	79.6 [-665.6, 824.7]	-165.5 [-986.9, 655.8]
	Hb	-5.4 [-14.7, 3.8]	6.3 [-0.5, 13.0]	-7.6 [-17.5, 2.3]	5.1 [-1.9, 12.1]
2nd Tri.	SF	-59.8 [-170.2, 50.6]	**-151.9 [-259.7, -44.1]**	67.5 [-188.0, 322.9]	-226.9 [-503.7, 49.9]
	Hepcidin	-30.2 [-95.2, 34.8]	-20.7 [-78.4, 37.0]	-42.9 [-171.4, 85.7]	-74.0 [-215.6, 67.6]
	Hb	0.02 [-4.2, 4.2]	-1.7 [-5.5, 2.1]	-1.5 [-12.9, 9.9]	-0.4 [-10.6, 9.8]
3rd Tri.	SF	**-276.1 [-417.4, -134.7]**	**-215.2 [-356.1, -74.3]**	**-228.8 [-392.3, -65.3]**	**-203.1 [-372.5, -33.7]**
	sTfR	**906.282 [237.8, 1574.7]**	-341.2 [-1153.5, 471.0]	714.6 [-358.1, 1787.3]	-274.6 [-1599.9, 1050.8]
	sTfR:SF	**587.7 [29.1, 1146.3]**	100.6 [-475.4, 676.6]	299.1 [-466.6, 1064.8]	-81.7 [-949.6, 786.2]
	Hepcidin	-52.4 [-107.4, 2.5]	-50.3 [-102.6, 1.9]	**-83.2 [-146.0, -20.4]**	-49.2 [-149.7, 24.0]
	EPO	111.2 [-27.9, 250.3]	127.5 [-9.7, 264.7]	80.0 [-101.0, 260.9]	130.5 [-32.8, 293.7]
	Hep:EPO	-40.4 [-90.7, 9.9]	**-53.4 [-102.0, -4.7]**	**-64.1 [-123.1, -5.1]**	**-68.1 [-224.8, -11.4]**
	Hb	**-7.8 [-11.4, -4.2]**	-3.0 [-6.5, 0.6]	**-7.1 [-11.9, -2.2]**	-2.6 [-7.2, 1.9]

All maternal biomarker concentrations were logarithmically transformed for normality (except for Hb) prior to regression modelling. The study timepoint when a given group of biomarkers were quantified is indicated by the vertical labels on the left side of the table. Multivariate models were informed by the birth weight (BW) DAG (Figure 10a) followed by the identification of minimum dataset covariates based on change-in-estimate rules (see Supplementary Table 1). Bolded results are statistically significant at p<0.05. Abbreviations: confidence interval (CI); erythropoietin (EPO); hemoglobin (Hb); hepcidin:EPO (hep:EPO); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF); 1st trimester (1st Tri.); 2nd trimester (2nd Tri.); 3rd trimester (3rd Tri.).

Table 12. Relationships between maternal iron biomarker concentrations and birth head circumferences (BHCs) stratified by offspring sex

	Biomarker	Univariate β [95% CI]		Multivariate β [95% CI]	
		Male	Female	Male	Female
1st Tri.	SF	-0.3 [-1.3, 0.7]	-0.4 [-1.3, 0.5]	-0.7 [-1.8, 0.4]	-0.9 [-1.9, 0.2]
	sTfR	2.1 [-0.6, 4.8]	-0.7 [-4.9, 3.5]	2.7 [-0.1, 5.5]	-0.5 [-3.9, 2.9]
	sTfR:SF	2.1 [-0.6, 4.8]	-0.5 [-2.7, 1.6]	2.7 [-2.0, 7.3]	0.7 [-1.7, 3.2]
	Hb	-0.02 [-0.06, 0.02]	0.03 [-0.01, 0.06]	-0.01 [-0.05, 0.03]	0.02 [-0.01, 0.04]
2nd Tri.	SF	-0.4 [-0.08, 0.07]	-0.4 [-0.8, 0.02]	**-1.1 [-2.1, -0.01]**	-0.9 [-1.9, 0.1]
	Hepcidin	-0.1 [-0.5, 0.2]	-0.06 [-0.3, 0.2]	-0.2 [-0.6, 0.2]	-0.06 [-0.5, 0.4]
	Hb	0.01 [-0.03, 0.01]	-0.01 [-0.02, 0.01]	-0.02 [-0.07, 0.03]	-0.01 [-0.04, 0.03]
3rd Tri.	SF	-0.5 [-1.1, 0.01]	-0.3 [-0.8, 0.2]	-0.5 [-1.1, 0.2]	-0.1 [-0.7, 0.4]
	sTfR	1.8 [-0.4, 4.0]	2.8 [-1.1, 6.7]	2.2 [-0.5, 5.0]	2.4 [-0.9, 5.7]
	sTfR:SF	1.8 [-0.05, 3.7]	1.7 [-0.1, 3.5]	2.0 [-0.0.8, 4.2]	1.7 [-0.7, 4.1]
	Hepcidin	-0.06 [-0.3, 0.2]	-0.1 [-0.3, 0.1]	-0.1 [-0.4, 0.1]	-0.1 [-0.3, 0.1]
	EPO	**0.6 [0.1, 1.1]**	0.2 [-0.3, 0.7]	0.1 [-0.5, 0.7]	0.2 [-0.3, 0.8]
	Hep:EPO	-0.05 [-0.2, 0.1]	-0.09 [-0.3, 0.1]	0.07 [-0.3, 0.1]	-0.1 [-0.3, 0.08]
	Hb	**-0.02 [-0.04, -0.01]**	0.002 [-0.01, 0.02]	**-0.02 [-0.03, -0.003]**	-0.003 [-0.02, 0.01]

All maternal biomarker concentrations were logarithmically transformed for normality (except for Hb) prior to modelling. The study timepoint when a given group of biomarkers were quantified is indicated by the vertical labels on the left side of the table. Multivariate models were informed by the birth weight DAG (Figure 10b) followed by the identification of minimum dataset covariates (Supplementary Table 1). Bolded *p*-values are statistically significant at $p<0.05$. Abbreviations: confidence interval (CI); erythropoietin (EPO); hemoglobin (Hb); hepcidin:EPO (hep:EPO); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF); 1st trimester (1st Tri.); 2nd trimester (2nd Tri.); 3rd trimester (3rd Tri.).

Table 6. A summary of the important findings from manuscript 1

1. Changes in maternal iron biomarker concentrations from an earlier pregnancy timepoint
▪ ↓ Hb in 2nd trimester, no change in 3rd trimester, rebound at 3 months postpartum
▪ ↓ SF in 2nd and 3rd trimester, rebound at 3 months postpartum
▪ ↓ Hepcidin in the 3rd trimester
▪ ↑ sTfR and sTfR:SF in the 3rd trimester
2. Differences in maternal iron biomarker concentrations depending on fetal sex
▪ ↑ SF, hepcidin and hepcidin:EPO in participants having a male fetus during the 3rd trimester
▪ ↑ sTfR, sTfR:SF and EPO in participants having a female fetus during the 3rd trimester
3. Associations between maternal iron biomarkers and birth outcomes by fetal sex
▪ Lower BWs were associated with (after adjustment):
o ↑ SF during the 3rd trimester in participants having males or females
o ↑ Hepcidin:EPO during the 3rd trimester in participants having males or females
o ↑ Hepcidin during the 3rd trimester in participants having males only
o ↑ Hb during the 3rd trimester in participants having males only

- Lower BHCs were associated with (after adjustment):
  - ↑ SF during the 2nd trimester in participants having males only
  - ↑ Hb during the 3rd trimester in participants having males only

↑ or ↓ indicates a significant change at $p<0.05$. Abbreviations: erythropoietin (EPO); hemoglobin (Hb); hepcidin-EPO ratio (hepcidin:EPO); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF); 1st (1st trimester); 2nd (2nd trimester); 3rd (3rd trimester); 3 months postpartum (pp.); ↑ (higher); ↓ (lower).

4.4 Discussion

The prevalence of pregnant individuals at risk for ID, as showcased by declines in the systemic concentrations of several biomarkers across gestation, was high in the APrON cohort. This finding was especially apparent during the 3rd trimester, when over 60% had depleted iron stores. Maternal anemia remained low across pregnancy, with a range of about 1% to 4%, despite the underlying risk of maternal ID. Interestingly, a higher maternal iron status was consistently detected in the 3rd trimester among participants carrying male fetuses compared to those having a female (**Table 13**). Inverse associations between maternal SF and hepcidin:EPO concentrations during the 3rd trimester and BWs were identified in both offspring sexes. Additionally, 3rd trimester hepcidin and Hb concentrations were associated with BWs in males but not females. Similarly, BHCs were only associated with maternal iron biomarkers (SF and Hb) in males.

While there are a limited number of studies that have investigated similar maternal iron status dynamics in healthy adult pregnant populations (O'Brien & Ru, 2017), there are a few that reported comparable results. In a group of >1,000 pregnant individuals recruited as part of the National Health and Nutrition Examination Survey in the United States, the prevalence of low maternal iron status, as estimated by low SF and high sTfR concentrations, increased across pregnancy and was relatively high (a range of 28% to 39%) in the 3rd trimester (Mei et al., 2011). These estimates were lower compared to those in the APrON cohort, but the threshold utilized to define ID using SF was more conservative at <12 µg/L. Recently, Cochrane et al. (2022) conducted a retrospective analysis of 60 pregnant individuals who received the recommended dietary allowance for iron during gestation, 27 mg/day (Health Canada, 2009). The ethnic composition of their cohort and ours are different, but many other demographic details were similar. At the first blood collection, which spanned 8 to 21 gestational weeks, nearly 30% had SF concentrations <30 µg/L whereas 81% had SF that fell under this threshold during mid to late pregnancy (Cochrane et al., 2022). The prevalence of ID was higher than the 61% observed in our study, but they employed

the use of a Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia, or BRINDA, algorithm that attempts to adjust for SF increases if inflammation is probable (Namaste et al., 2017). High incidences of poor maternal iron status in generally healthy individuals have also been reported during early pregnancy (Auerbach et al., 2021) and Teichman et al. (2021) recently found that nearly 53% of >40,000 pregnant females in Ontario, Canada were ID (SF <30 μg/L) during the course of their study.

A considerable proportion of these maternal iron insufficiencies would be otherwise undetected without the measurement of other biomarkers of this nutrient beyond Hb. Although clinical practices vary by location or the particular physician, many practitioners measure maternal Hb concentrations early and later on in gestation, generally during the 1st and 3rd trimesters, respectively (American College of Obstetricians and Gynecologists [ACOG], 2021; Pavord et al., 2020; O'Connor et al., 2016). If anemia is not detected, additional screening with other iron biomarkers is rarely conducted (Abdulrehman et al., 2019). This is despite the risk of other iron-related deficiencies that do not modify erythropoiesis, as suggested by the results of our study. Therefore, opportunities to intervene earlier in a pregnancy may be missed (Flores et al., 2017; Sharma et al., 2021), even among healthy populations. Additionally, the percentage of individuals with anemia at 3 months postpartum remained the same as those with this condition during the 3rd trimester, even though the prevalence of maternal IDA seemingly decreased from ~66% to 10% between these timepoints. This could imply that there are other contributing causes to maternal anemia beyond iron in the postpartum period, such as inflammation or other micronutrient deficiencies (O'Brien & Ru, 2017), which should be explored.

It is widely understood that the increased iron demands of pregnancy are critical to sustained growth and homeostatic mechanisms during this life stage (Fisher & Nemeth, 2017; Sangkhae et al., 2020). However, the normal or reference concentration ranges of iron or hypoxic biomarkers beyond Hb, including SF, sTfR, hepcidin and EPO, at different gestational periods during healthy pregnancies remain to be fully elucidated. Measurements of sTfR:SF or hepcidin:EPO, which may detect iron repletion with improved accuracy in the physiologically complex environment of pregnancy (Mor & Cardenas, 2010; Delaney et al., 2021a), were quantified in the 3rd trimester in over a thousand APrON participants. The use of these concentration ratios may be more predictive of iron status compared to the use of any one biomarker independently, warranting the need for future investigations that analyze these

measurements to determine what they mean and how they relate to outcomes during and after pregnancy.

The inverse associations detected between maternal iron status during gestation and birth outcomes is consistent with the findings of some studies but contrasts others. In pregnant individuals from Turkey, a positive relationship between maternal Hb concentrations in late pregnancy and BWs was reported, but the prevalence of maternal anemia was high at ~20% (Yildiz et al., 2014). Dewey and Oaks (2017) highlighted non-linear (U-shaped) relationships between maternal Hb and other iron biomarker concentrations and the risk for adverse birth outcomes. Their analyses included data from over twenty-five investigations and included an extensive range of maternal iron biomarker concentrations as well as neonatal health outcomes. The results from our study may depict only a portion of this relationship (Dewey & Oaks, 2017), linking higher maternal iron status with lower BWs and BHCs. Indeed, infants in the APrON study generally did not have poor birth outcomes; in fact, 95% had a BW between the normal range of 2500-4500 g (Belbasis et al., 2016). Conversely, perhaps newborns with adverse birth anthropometrics, including those that are either small- or large-for-gestational-age, are born to gestating people with IDA, the prevalence of which was low in our cohort. Still, other studies have reported comparable findings to ours (Puerto et al., 2021; von Tempelhoff et al., 2008; Yuan et al., 2019); lower maternal iron status during the 3rd trimester (lower concentrations of SF and higher sTfR and sTfR:SF) were associated with higher BWs among >10,000 pregnant individuals from China (Yuan et al., 2019). In another prospective investigation that recruited nearly 5000 participants from Germany, mid-gestation maternal Hb concentrations were elevated in those with several adverse pregnancy outcomes, including low BWs (von Tempelhoff et al., 2008). Similar negative relationships between maternal ID and poor birth outcomes were reported in other research (Puerto et al., 2021; Symington et al., 2019). Importantly, in the current investigation fetal sex was not adjusted as a confounder, which has been a common procedure in previous studies that explored comparable questions (DiPietro & Voegtline, 2017). Instead, this variable was treated as an EM which could be an important statistical consideration to unveil sex-specific influences of birth outcomes and may account for the discrepancies in findings between this study and others.

All associations between maternal iron and BWs were observed when the biomarkers were quantified during the 3rd trimester, suggesting a possible time-dependency of these relationships. This may coincide with previous studies that analyzed the temporality of maternal-placental iron

mobilization throughout gestation (Bothwell et al., 1958; Glasser et al., 1968). These and more recent investigations (Bradley et al., 2004) have shown that the majority of iron that is reserved for fetal growth (>70%) is transported during the 3rd trimester (O'Brien, 2022). Therefore, the availability of maternal iron during later pregnancy is critical for fetal growth, which is supported by our results. Further, the inverse connection between iron status during later pregnancy and birth outcomes could be related to evidence that poor iron mobilization may be influenced by systemic or placental complications (Lao et al., 2000; Stoffel et al., 2022). A higher absolute amount of circulating metabolites, including RBCs, SF or other carriers like transferrin, could increase the viscosity of blood, decreasing the speed of its flow to the placental interface for transcellular transport across the syncytiotrophoblast (Chernyavsky et al., 2010; Viteri, 2011; Zondervan et al., 1988). The latter phenomenon may result from inadequate maternal plasma expansion (Costantine, 2014; Garn et al., 1981; Koller et al., 1982).

Although the investigation of sex differences between the relationships of interest was not an initial objective, preliminary explorations of the data highlighted potential discrepancies that dictated the remainder of the analyses. Indeed, different associations depending on fetal sex were revealed in this APrON-Fe study. There is evidence supporting disparities in the vulnerabilities of fetal programming and development depending on biological sex (DiPietro & Voegtline, 2017; Gualtieri & Hicks, 1985; Zarén et al., 2000). Recently, Nugent et al. (2018) suggested that placental enzyme O-linked N-acetylglucosamine transferase may be implicated in discrepant stress tolerances possibly through differences in epigenetic processes between sexes. Interestingly, when maternal iron biomarker concentrations were stratified by fetal sex, those with female fetuses had consistently lower iron statuses, or a higher risk of ID, as indicated by six biomarkers at nearly 33 gestational weeks compared to those having males. These sex-related results may display distinctions in the timing of nutrient transport, which perhaps was not captured by the APrON study timepoints, or in the regulation of nutrient movement depending on the sex chromosomal composition of the fetus.

The APrON cohort study enabled the collection of an extensive set of demographic, lifestyle, nutritional, medical and health information from over two thousand pregnant individuals and their offspring in Alberta, Canada (Kaplan et al., 2014; Letourneau et al., 2022). Another strength is that seven maternal bioindicators of iron status, many of which are not routinely measured during typical, healthy pregnancies (ACOG, 2021; Pavord et al., 2020; Teichman et al.,

2021), were quantified at multiple timepoints across gestation. Analyzing concentrations at different prenatal periods may provide insights into time-specific interactions when interventions may yield the greatest benefit or protection. APrON-Fe study limitations are that systemic concentrations of several iron biomarkers, including SF, can be impacted by inflammation and may overestimate iron status adequacy (Lynch et al., 2018; Delaney et al., 2021a). There are proposed strategies to correct for such discrepancies if inflammation is probable, including a regression algorithm (Namaste et al., 2017). Although maternal C-reactive protein concentrations were measured during the last trimester in the APrON study, a required metabolite for the adjustment (alpha-1-acid glycoprotein) was not quantified, preventing the use of this adjustment technique. However, given that the vast majority of participants had a pre-pregnancy BMI in the normal range and did not have pre-existing or pregnancy-related medical conditions, the risk of inflammation is low in this cohort. In fact, the occurrence of chronic or low-grade inflammation over the course of a healthy pregnancy is still unconfirmed (Challis et al., 2009; WHO, 2020). Still, 3rd trimester maternal C-reactive protein concentrations were included as covariates in regression models if there was convincing evidence supporting its inclusion (Figure 10). Therefore, we are confident that potential inflammatory impacts, if any, are controlled. Finally, because both the pregnant participants and newborns were generally healthy, the generalizability of our findings to people with different demographics, lower SES, obstetric complications or those with infants that had poor birth outcomes are unknown.

In conclusion, maternal SF was the only iron biomarker associated with both BWs and BHCs, but at different gestational timepoints and only in those having males. Maternal iron appears to consistently decline across pregnancy in the absence of anemia, revealing the high prevalence of low status especially in the 3rd trimester. BWs and BHCs may be reflective of mid to late pregnancy maternal iron status in a fetal sex-dependent manner, which was supported by differences in iron biomarker concentrations depending on the sex of the offspring. Particularly high iron biomarker concentrations during the 3rd trimester could signal a dysfunction in iron mobilization with the potential for low fetal endowment of this critical micronutrient.

These results reveal the complexity of maternal iron biomarker dynamics and their implications for offspring health during and after pregnancy. Investigating generally high SES participants provides a rare insight into the status of maternal iron in healthy populations, who are not typically monitored in this way (ACOG, 2021; O'Connor et al., 2016; Teichman et al., 2021).

As the calls for updated iron screening protocols in obstetric care continue (Cappellini et al., 2020; Fairweather-Tait, 2022), the relatively inexpensive practice of screening for iron biomarkers beyond Hb should be considered. Reproducibility in other pregnant populations is required.

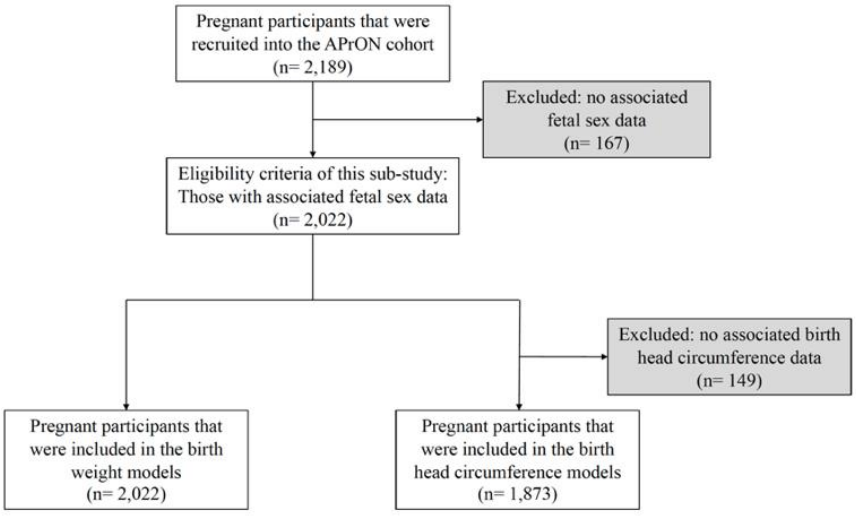
4.5 Supplementary materials

Supplementary Table 1. Lists of variables included in multivariate regression models

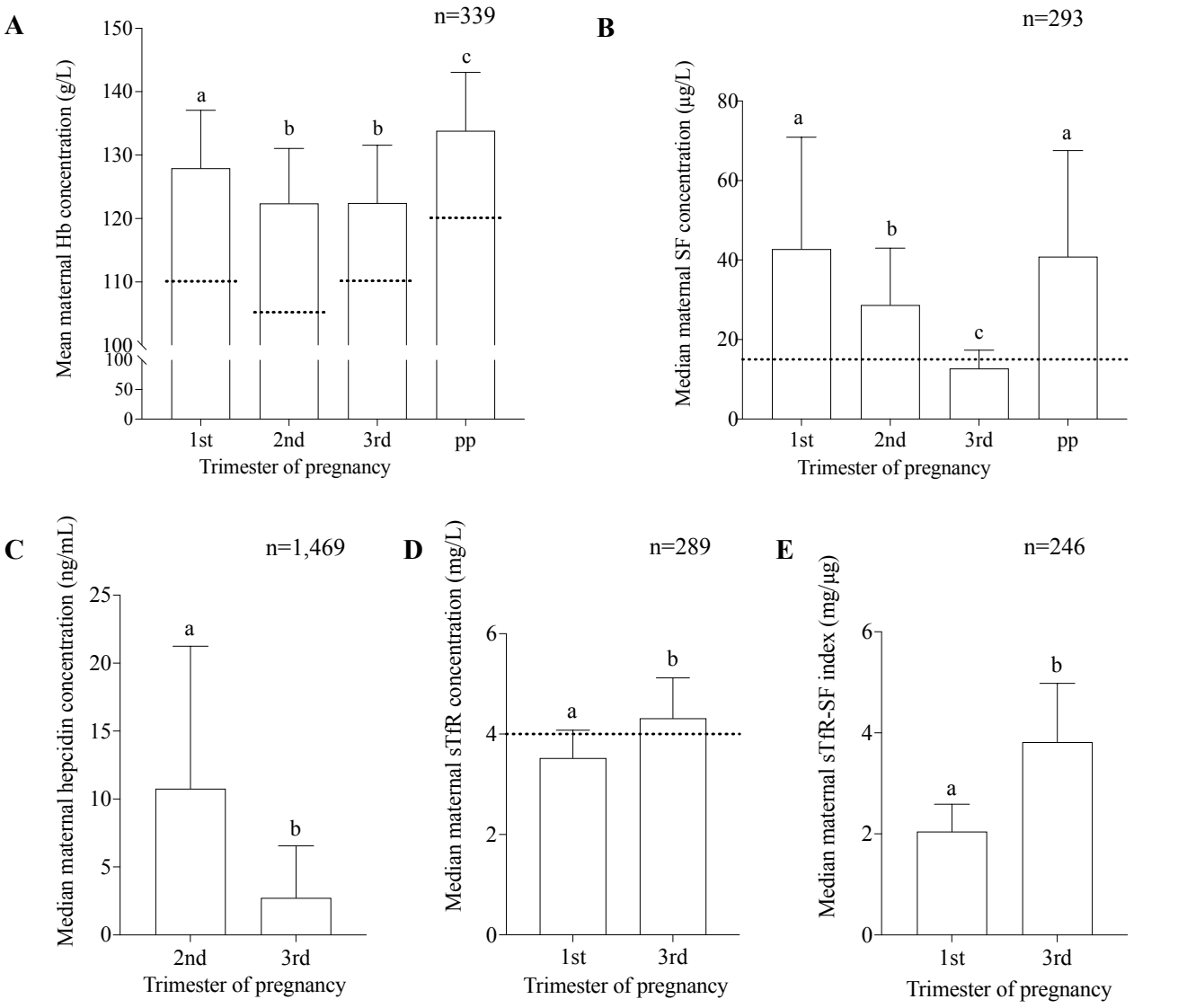
Exposure	Outcome	Minimum covariates
Hepcidin 2ndTri	BW	Males: ppBMI, parity, GDM, income, CFG scores Females: ppBMI, parity, GDM
Hepcidin 3rdTri	BW	Males: parity, CRP, CFG scores, age Females: parity, CRP, CFG scores
SF 1stTri	BW	Males: ppBMI, parity, GDM, education, income, CFG scores, age Females: CFG scores, age
SF 2ndTri	BW	Males: parity, GDM, education, income, CFG scores, age Females: ppBMI, GDM, CFG scores
SF 3rdTri	BW	Males: ppBMI, parity, GDM, age Females: ppBMI, CRP, parity, CFG scores
sTfR 1stTri	BW	Males: ppBMI, parity, GDM, education, age Females: ppBMI, income, CFG scores, age
sTfR 3rdTri	BW	Males: CRP, parity, PE, income, CFG scores Females: ppBMI, CRP, parity, GDM, education, income, CFG scores, age
sTfR:SF 1stTri	BW	Males: ppBMI, parity, GDM, education, income, CFG scores, age Females: ppBMI, parity, GDM, education, income, CFG scores, age
sTfR:SF 3rdTri	BW	Males: ppBMI, CRP, parity, education, CFG scores, age Females: ppBMI, CRP, parity, GDM, education, income, CFG scores, age
EPO 3rdTri	BW	Males: ppBMI, CRP, parity, GDM, income, CFG scores, age Females: ppBMI, parity, GDM 3rdTri, income, CFG scores, age
Hepcidin:EPO 3rdTri	BW	Males: ppBMI, CRP, parity, education, income, CFG scores, age Females: ppBMI, parity, GDM
Hb 1stTri	BW	Males: ppBMI, GDM, income, age Females: GDM, CFG scores, age
Hb 2ndTri	BW	Males: ppBMI, parity, GDM, education, income, CFG scores, age Females: ppBMI, parity, GDM, education, income, CFG scores, age
Hb 3rdTri	BW	Males: ppBMI, CRP, GDM, CFG scores, age Females: ppBMI, CRP, parity, income, CFG scores, age
Hepcidin 2ndTri	BHC	Males: ppBMI, PE, income Females: ppBMI, PE, income, CFG scores
Hepcidin 3rdTri	BHC	Males: ppBMI, PE, income, CFG scores Females: ppBMI, PE
SF 1stTri	BHC	Males: ppBMI, income, CFG scores Females: ppBMI, income, CFG scores
SF 2ndTri	BHC	Males: ppBMI, PE, income Females: ppBMI, PE, CFG scores
SF 3rdTri	BHC	Males: ppBMI, PE, income

		Females: ppBMI, education
sTfR 1stTri	BHC	Males: education, CFG scores Females: ppBMI, education, income, CFG scores
sTfR 3rdTri	BHC	Males: PE, CFG scores Females: ppBMI, education, income, CFG scores
sTfR:SF 1stTri	BHC	Males: PE, education, income, CFG scores Females: ppBMI, income, CFG scores
sTfR:SF 3rdTri	BHC	Males: ppBMI, PE, education Females: ppBMI, PE, CFG scores
EPO 3rdTri	BHC	Males: ppBMI, PE, education, income Females: ppBMI, income, education, CFG scores
Hepcidin:EPO 3rdTri	BHC	Males: PE, education, income, CFG scores Females: ppBMI, PE
Hb 1stTri	BHC	Males: ppBMI, PE, education, income Females: ppBMI, PE, CFG scores
Hb 2ndTri	BHC	Males: PE, education, CFG scores Females: ppBMI, PE, education, CFG scores
Hb 3rdTri	BHC	Males: PE Females: ppBMI, education, income

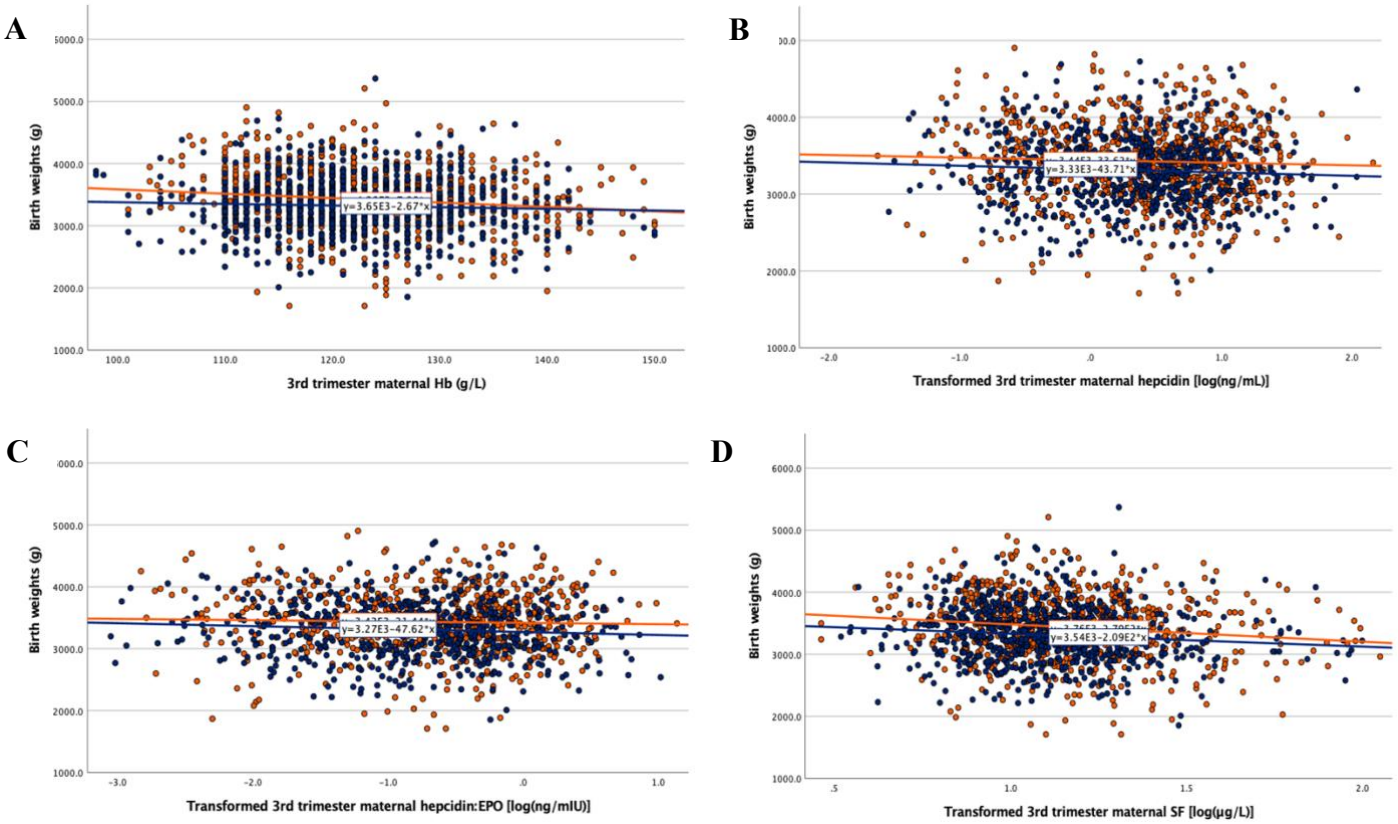
A two-step method, including the use of directed acyclic graphs (DAGs) and change in estimate rules, was utilized to determine the minimum set of covariates that may confound relationships between the exposure and outcome (Williams et al., 2018). The minimum covariates used in the final models are presented for male and female fetuses for each exposure and outcome variable. Abbreviations: birth head circumference (BHC); birth weight (BW); Canadian Food Guide (CFG); C-reactive protein (CRP); erythropoietin (EPO); gestational diabetes mellitus (GDM); hemoglobin (Hb); preeclampsia (PE); pre-pregnancy body mass index (ppBMI); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF index (sTfR:SF); 2nd trimester (2ndTri); 3rd trimester (3rdTri).



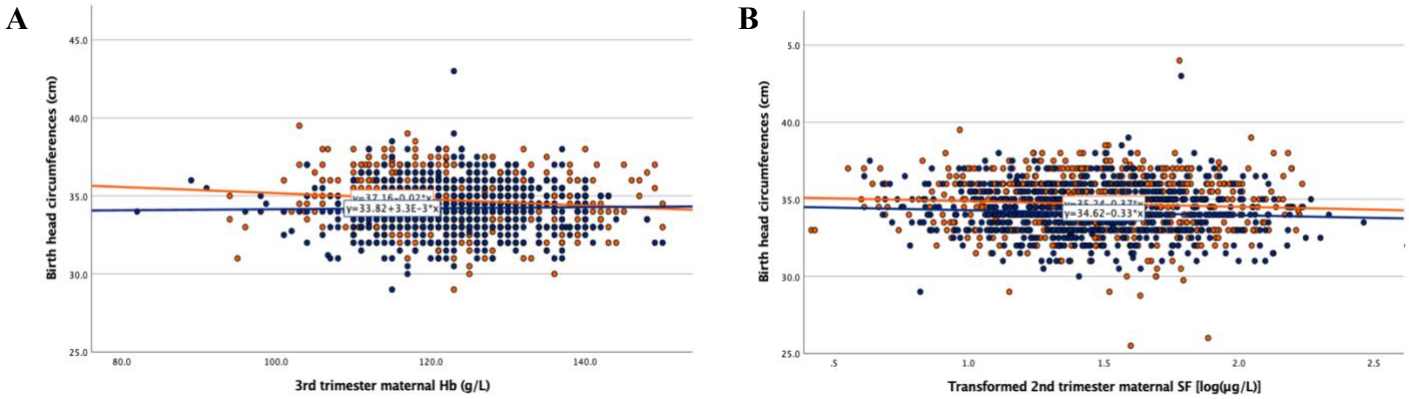
Supplementary Figure 1. Flow chart of pregnant participants from the APrON cohort that were included in birth weight (BW) or birth head circumference (BHC) regression models



Supplementary Figure 2. Median maternal (A) hemoglobin (Hb), (B) serum ferritin (SF), (C) hepcidin, (D) soluble transferrin receptor (sTfR) and (E) sTfR-SF index measurements by APrON study timepoint. The number of pregnant participants with paired data at each study timepoint is given in the top right of each panel, and bars and error bars represent the median and IQR, respectively (except for maternal Hb where means and SEMs are provided). The horizontal dotted lines in (A), (B) and (D) represent the proposed concentration thresholds for anemia or a low maternal iron status, respectively, Hb: <110 g/L in the 1st and 3rd trimesters, <105 g/L in the 2nd trimester and <120 g/L in the postpsrtum period (WHO, 2011; WHO, 2020); SF: <15 µg/L (WHO, 2020); sTfR >4.0 mg/L (1st trimester 75thile). Bars with different letters are significantly different ($p<0.001$) following paired t-tests.



Supplementary Figure 3. Scatter plots and best-fit lines of birth weights (BWs) against maternal (A) hemoglobin (Hb), (B) hepcidin, (C) hepcidin:erythropoietin (hepcidin:EPO) and (D) serum ferritin (SF) concentrations during the 3rd trimester. Data points and regression lines are stratified by child sex where males are represented by orange and females are represented by blue.



Supplementary Figure 4. Scatter plots and best-fit lines of birth head circumferences (BHCs) against maternal (A) 3rd trimester hemoglobin (Hb) and (B) 2nd trimester serum ferritin (SF) concentrations. Data points and regression lines are stratified by child sex where males are represented by orange and females are represented by blue.

CHAPTER 5: MANUSCRIPT 2

Maternal iron and vitamin D status during pregnancy and the postpartum period is associated with maternal prenatal depression and child externalizing behaviours in the APrON cohort

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5.1 Introduction

An adequate status of iron and vitamin D can be difficult to achieve for many reasons, including limited access to food sources, low bioavailability of nutrient forms in consumed foods, the observance of specific dietary patterns, location or seasonality of residence, certain medical conditions and demographic factors, such as ethnicity and age (Kulkarni et al., 2022; Parva et al., 2018; Pearce & Cheetham, 2010). Pregnant people have a higher risk of low status or deficiency in one or both of these micronutrients due to the increased requirements necessitated for optimal fetal neurodevelopment and maternal health (Allen, 2000; Mulligan et al., 2010; Palacios & Gonzalez, 2014; Pet & Brouwer-Brolsma, 2016; Mehrpouya et al., 2015). Iron deficiency anemia is estimated to affect 20% of global pregnancies (Stevens et al., 2013). There are limited data, however, on the global prevalence of vitamin D deficiency, typically defined as 25-hydroxyvitamin D (25(OH)D) concentrations <50 nmol/L (Munns et al., 2016), during pregnancy

(Palacios & Gonzalez, 2014; van der Meer et al., 2006). Despite investigations that recently attempted to estimate the risk of iron or vitamin D deficiency during gestation (Cochrane et al., 2022; Özdemir et al., 2018; Stevens et al., 2022), very few have assessed the maternal status of both nutrients in healthy pregnant cohorts.

Even though iron and vitamin D are essential for health during gestation and childhood (Özdemir et al., 2018; Stevens et al., 2013), they are often not monitored among pregnant people from high-income countries (American College of Obstetricians and Gynecologists [ACOG], 2011; Fairweather-Tait, 2022; O'Connor et al., 2016). Health Canada recommends iron supplementation for pregnant individuals to meet their increased iron needs (Health Canada, 2009; Institute of Medicine, 2006). Still, recent investigations have questioned whether the current RDA of 27 mg of iron each day during pregnancy is sufficient to sustain the increased maternal, placental and fetal iron demands (Cochrane et al., 2022; O'Brien, 2022). The recommendations for vitamin D do not increase during the reproductive years, pregnancy or lactation relative to the dietary vitamin D requirements for children and non-pregnant adults aged <50 years in Canada (Health Canada, 2010). Moreover, pregnant individuals living at higher latitudes are less likely to be vitamin D status replete when consuming the current Canadian RDA of 600 International Units/day (Aghajafari et al., 2016). The use of iron and vitamin D biomarkers could aid in a more objective assessment of iron and vitamin D adequacy among pregnant people, compared to dietary assessments (Kirkpatrick et al., 2018), if they are measured in a variety of populations. Fortunately, biomarkers that estimate the status of these nutrients, including hepcidin, serum ferritin (SF), soluble transferrin receptor (sTfR) for iron, and 25(OH)D for vitamin D, are becoming more clinically available (Fisher & Nemeth, 2017; Jenkinson, 2019; O'Brien, 2022).

Evidence related to the potential combined impact of maternal iron and vitamin D status on pregnancy-related health outcomes are limited, despite an emergence of studies reporting mechanistic interactions between their biological pathways (Alon et al., 2002; Bacchetta et al., 2014; Braithwaite et al., 2021; Braithwaite et al., 2019; Thomas et al., 2015; Zughaiger et al., 2014). For example, Braithwaite et al. (2021) reported associations between erythropoietin concentrations, indicative of hypoxia and iron deficiency (Goldberg et al., 1988), and an increased expression of fibroblast growth factor 23, a negative regulator of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) (Fukumoto, 2014). Relationships between $1,25(OH)_2D$ and erythropoietin receptors have also been reported (Alon et al., 2002). Further, elevated $1,25(OH)_2D$ has been shown to

reduce the expression of hepcidin (Bacchetta et al., 2014; Zughaiger et al., 2014), a negative regulator of non-heme iron absorption (Jordan et al., 2009). Positive associations between biomarkers of iron and vitamin D have been described in pregnant individuals (Kang et al., 2015), adolescents (Masoud et al., 2020), athletes (Malczewska-Lenczowska et al., 2018) and older adults (Yoo & Cho, 2015). The findings from these studies suggest that a history of anemia (Kang et al., 2015) or other measures of iron depletion, such as hemoglobin, SF and total iron binding capacity (Malczewska-Lenczowska et al., 2018; Masoud et al., 2020; Yoo & Cho, 2015), are related to a lower vitamin D status in humans. Whether there is a combined effect of concurrent maternal vitamin D and iron deficiency on health outcomes is unknown.

Neurobiological evidence links iron status to energy and neurotransmitter balance in the brain (Aschner & Aschner, 1990; Chen et al., 1995; Lozoff, 2000), and vitamin D to neuronal messaging and immunomodulation (Brown et al., 2003; Dulla et al., 2016), suggesting their importance for the maintenance of mental health. Previous observational studies have reported associations between higher iron and vitamin D status, independently, and a reduced risk of maternal depression (Aghajafari et al., 2018b; Gowtham et al., 2022; Lamb et al., 2018; Lin et al., 2022), but others did not (Armony-Sivan et al., 2012; Nielsen et al., 2014). Nonetheless, most of the available evidence suggests that a higher maternal iron or vitamin D status predicts less prenatal or postpartum depression symptoms (Hameed et al., 2022; Jani et al., 2020; Lamb et al., 2018). Few studies have assessed nutrient risk factors for maternal depression at both timepoints in the same investigation (Gowtham et al., 2022; Ribamar et al., 2020). A recent study by Lin et al. (2022) may be one of the only investigations that reported on the maternal status of iron and vitamin D (as well as folic acid), and their associations with gestational depression symptoms, but the contribution of each micronutrient was assessed separately.

Mechanistic and pre-clinical investigations also suggest the independent impact of iron and vitamin D on early brain development (Becker et al., 2005; Jorgenson et al., 2003), but there is mixed evidence from pregnancy cohort studies in humans (Quezada-Pinedo et al., 2021). Maternal vitamin D and iron biomarker concentrations in the perinatal period have been associated with hyperactivity (Daraki et al., 2018) or inattention (Santa-Marina et al., 2020) in children at age 4, but others did not observe significant relationships (Lewis et al., 2014; Strom et al., 2014). The possible adverse effects of an excessively high maternal iron status on child neurodevelopment is another important consideration (Quezada-Pinedo et al., 2021; Zhou et al., 2006).

There are notable differences in study designs, particularly in the type and timing of maternal or child outcome assessments and biochemical quantification of biomarkers, that complicate the synthesis of the body of evidence linking maternal iron or vitamin D status to maternal mental health and early child behaviours. In addition, very few studies to date have recruited large numbers of generally healthy pregnant individuals and their children to explore how both outcomes may be impacted by maternal iron or vitamin D status. Considering previous evidence independently linking both micronutrients to maternal depression and childhood behaviours (Aghajafari et al., 2018b; Daraki et al., 2018; Gowtham et al., 2022; Lamb et al., 2018; Lin et al., 2022; Santa-Marina et al., 2020), an investigation that examines potential differences in these outcomes depending on the combined adequacy of maternal iron and vitamin D is warranted.

One of the primary aims of the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort study is to examine if maternal nutrient status in pregnancy and postpartum are related to maternal mental health and child neurodevelopmental outcomes (Kaplan et al., 2014). The goal of this sub-study was to examine the individual and combined associations of maternal iron and vitamin D status during and after pregnancy on mothers' depression symptoms and children's internalizing and externalizing behaviours at age 5.

5.2 Methods

The Alberta Pregnancy Outcome and Nutrition (APrON) study recruited 2,189 pregnant people from Calgary and Edmonton, Alberta, Canada between 2009 and 2012 (Kaplan et al., 2014). Detailed methods have been published elsewhere (Kaplan et al., 2014; Letourneau et al., 2022). Briefly, pregnant people were eligible for this prospective cohort if they were over 16 years of age, could read and write in English and intended to reside in Calgary or Edmonton throughout gestation and to 3 months postpartum. These participants visited the clinic up to three times during pregnancy (weeks of gestation (range): 10.8 (3.1-13.9) in the 1st, 19.0 (14.0- 26.9) in the 2nd and 32.5 (27.0-39.0) in the 3rd trimester) and at multiple timepoints during the postpartum period (Kaplan et al., 2014; Letourneau et al., 2022). We employed data for this secondary analysis from the pregnancy and the 3 and 60 month (5 year) visits. Collection of data on the health outcomes of the female participants and their children in this cohort is ongoing. The Calgary Health Research Ethics Board (REB14-1702) and the University of Alberta Health Research Biomedical Panel

(Pro00002954) provided ethical approval, and informed consent was obtained from all participants (Kaplan et al., 2014; Letourneau et al., 2022).

5.2.1 Maternal and child demographic information

An extensive list of self-reported demographic characteristics, including maternal ethnicity, age, highest level of education and average family income, were collected at the first study visit (Kaplan et al., 2014; Letourneau et al., 2022). The occurrence of gestational medical conditions was determined through self-report questionnaires at several pregnancy visits. Heights of the pregnant participants were measured at the first visit, and weight was measured at all prenatal visits to estimate pre-pregnancy body-mass-index and weight gain in pregnancy as previously reported (Begum et al., 2012). Birth outcomes were accessed from delivery records.

5.2.2 Predictors

5.2.2.1 Maternal nutrition information

Maternal intakes of iron and vitamin D from supplements was estimated by Supplemental Intake Questionnaires (SIQs) (Kaplan et al., 2014; Letourneau et al., 2022). Maternal 24-hour dietary recalls and SIQs were collected at each prenatal study visit and at ~3 months postpartum. A maternal healthy eating score was calculated based on Canada's Food Guide (CFG) to estimate overall diet quality (Jarman et al., 2017; Health Canada, 2007).

5.2.2.2 Iron and vitamin D biomarker quantification

Maternal venous blood samples were drawn at each study visit by a trained phlebotomist (Kaplan et al., 2014). Whole blood was immediately fractioned into red blood cell, serum and plasma portions, and were stored separately at -80 °C. Serum ferritin was quantified in maternal serum at all three trimesters and at ~3 months postpartum using a i2000sr Architect Plus blood analyzer machine (Abbott, Chicago, IL, USA) that used chemiluminescent microparticle immunoassay techniques. The daily variation in the SF concentrations of random samples that were run daily was <5%. Iron storage depletion was defined as SF <15 µg/L (O'Connor et al., 2016; WHO, 2020). Enzyme-linked immunosorbent assays (R&D Systems®, Minneapolis, MN, USA) were used to measure hepcidin (detection range: 3.13-800 pg/mL) and soluble transferrin receptor (sTfR) (5-80 nmol/L) concentrations in serum samples in duplicate. Maternal hepcidin

was measured in 2nd and 3rd trimester samples, and maternal sTfR quantified in 1st and 3rd trimester samples. A co-efficient of variation (CV) of <10% between the absorbances of duplicate samples was considered reproducible. If a particular sample had a CV ≥10% or had a concentration that fell outside of the detection range, the sample was re-quantified after dilution or concentration. If maternal SF and sTfR concentrations were quantified during the 1st (n=267) or 3rd trimester (n=280), the ratio of sTfR to the logarithmic of SF [sTfR/$\log_{10}$(SF)] was utilized to calculate the sTfR-SF ratio (sTfR:SF) (Zimmermann, 2008).

Second trimester plasma concentrations of 25-hydroxyvitamin D (25(OH)D) and the prevalence of maternal vitamin D deficiency (25(OH)D <50 nmol/L) (Holick et al., 2011; Malabanan & Veronikis, 1998) and insufficiency (25(OH)D <75 nmol/L) (Dawson-Hughes et al., 2005) among 537 pregnant participants are published elsewhere (Aghajafari et al., 2016). For the present investigation, vitamin D metabolites were quantified in an additional 2500 maternal samples during the 2nd trimester (n=1,249) and at ~3 months postpartum (n=1,251). In brief, vitamin D metabolites were extracted from human plasma by liquid-liquid extraction using hexane and ethyl acetate (Abu Kassim et al., 2018; Shah et al., 2011; van den Ouweland et al., 2010). The analytes, including 25-hydroxyvitamin D2 (25(OH)D2), 1,25-dihydroxyvitamin D2 ($1,25(OH)_2D2$), 25-hydroxyvitamin D3 (25(OH)D3), 3-epi-25hydroxyvitamin D3 (3-epi-25(OH)D3), and 1,25-hydroxyvitamin D3, were separated by liquid chromatography (LC) (Agilent 1290) using reverse-phase conditions and detected by tandem mass spectrometry (MS/MS) in positive-ion mode (Agilent 6495B MS/MS). Quantitation was achieved by an isotope dilution method using stable-isotope-labelled standards (Cayman Chemicals, Sigma Supelco, Inc, Belleftone, PA, USA). The lowest limit of quantitation (LLOQ) for each of the vitamin D metabolites was 0.78 ng/mL. The assay was validated using pooled plasma samples and the Standard Reference Material 2970 (National Institute for Standards and Technologies, NIST). The assay showed high accuracy (compared to NIST values) and precision reflected in the <6% inter-assay CV for mean ± SD of 9.65 ± 0.58 ng/mL 25(OH)D3 (certified value: 9.63 ± 0.31 ng/mL), 23.19 ± 1.45 ng/mL 25(OH)D2 (certified value: 23.5 ± 0.3 ng/mL) and 1.98 ± 0.11 ng/mL 3-epi-25(OH)D3. The concentrations of the forms $1,25(OH)_2D2$, $1,25(OH)_2D2$ and 1,25-hydroxyvitamin D3 were below the LLOQ in all maternal samples in the most recent LC-MS/MS analysis. Maternal 25(OH)D concentrations are the sum of maternal 25(OH)D3 and 25(OH)D2 concentrations during the 2nd trimester if both forms were quantified (n=644).

To examine relationships between different levels of maternal iron and vitamin D status adequacy on the outcomes of interest, a categorical variable was created. Four categories were defined: (1) iron and vitamin D replete (SF ≥15 µg/L and 25(OH)D ≥ 75nmol/L), (2) iron replete and low vitamin D (SF ≥ 15 µg/L and 25(OH)D <75nmol/L), (3) low iron and vitamin D replete (SF <15 µg/L and 25(OH)D ≥ 75nmol/L), and (4) low iron and vitamin D (SF <15 µg/L and 25(OH)D <75nmol/L). Participants with SF and 25(OH)D quantified in the 2nd trimester (n=627) were categorized.

5.2.3 Outcomes

5.2.3.1 Maternal depression

The 10-item Edinburgh Postnatal Depression Scale (EPDS) was used to assess maternal depression symptoms during the 3rd trimester and at 3 months postpartum (Cox et al., 1987). This questionnaire is a widely researched tool to measure depressive symptoms in pregnant individuals, and it asks participants about varied aspects of their feelings in the past week. Scores could range from 0, indicating the absence of depressive symptoms, to 30, suggesting a high frequency or severity of depressive symptoms (Bergink et al., 2011; Cox et al., 1987). An EPDS score of ≥13, estimated to have a sensitivity of 66% and specificity of 95% in a recent meta-analysis (Levis et al., 2020), was used as a threshold to indicate probable depression.

5.2.3.2 Child behaviour

To operationalize child behaviour outcomes, the Behavior Assessment System for Children 2nd Edition (BASC-2) was administered when participating children were approximately 5 years of age (n=662) (Kamphaus, 2014; Kaplan et al., 2014; Reynolds, 2010). The BASC-2 Parent Rating Scale was utilized, a reliable and validated questionnaire that assesses behavioural and emotional characteristics of children that can be quantified into internalizing and externalizing composite scores (Community-University Partnership for the Study of Children, Youth and Families, 2011; Kamphaus, 2014; Reynolds, 2010). BASC-2 internalizing scales assess symptoms of depression, anxiety or somatization, whereas externalizing scales examine symptoms of aggression, hyperactivity and conduct problems (Reynolds, 2010). After collection, both sets of raw scores were converted to T-scores using normative tables. The latter standardizes participant scores against a mean T-score of 50 for classification in either the normal (T-score <60), at-risk

(T-score between 60 and 69) and clinically significant (T-score >70) categories (Leclerc et al., 2016). Higher BASC-2 internalizing and externalizing T-scores are reflective of more internalizing or externalizing behaviours in children, respectively (Kamphaus, 2014; Reynolds, 2010).

5.2.4 Statistical analyses

Variables that are normally distributed data are presented as mean ± standard deviation (SD); skewed data are presented as medians (interquartile range, IQR) and were log transformed as needed for statistical tests. Maternal vitamin D biomarker concentrations during the 2nd trimester and 3 months postpartum were compared using paired t-tests.

Multivariate regression models were utilized to explore relationships between maternal iron or vitamin D biomarkers at different study timepoints and EPDS scores during the 3rd trimester and 3 months postpartum, and child internalizing and externalizing T-scores at age 5. The availability of EPDS or BASC-2 data dictated the number of pregnant participants that were eligible for inclusion into the statistical models (see **Supplementary Figure 5**). Directed acyclic graphs (DAGs) were constructed based on pre-existing evidence to identify variables that could be associated with both the exposures and outcomes of interest (**Figures 11 & 12** in Chapter 3) (Williams et al., 2018). This information was used to guide multivariate adjustment. Change-in-estimate rules were applied to each model as a means of adjusting for the minimum number of confounders for each relationship to reduce the risk of overadjustment (Weng et al., 2009). A threshold of 10% was used because of the large sample sizes in the APrON cohort. The biological sex of the children was treated as an effect modifier (EM) (Gualtieri & Hicks, 1985), and analyses were stratified by this variable in both univariate (crude) and multivariate (adjusted) models. Refer to **Supplementary Table 2** for a detailed description of variables that were included in each model. Generalized linear models, adjusted for the covariates identified by relevant DAGs (Figures 11c & 12c), were used to assess relationships between the iron and vitamin D status adequacy categorical variable and maternal depressive symptoms or child behaviours. SPSS (V28.0, IBM Corporation, Armonk, NY, USA) was used to conduct all statistical analyses and two-tailed *p*-values <0.05 were considered statistically significant.

5.3 Results

Characteristics of the cohort is shown in **Table 14**. Generally, pregnant participants were married or co-habiting with a partner, highly educated and had a household income ≥$70,000. The majority self-identified as White, were born in Canada and 53% were pregnant with their first child. The prevalence of pregnancy-related medical conditions was low. Most of the pregnant individuals consumed iron and vitamin D supplements at each trimester of pregnancy (**Table 15**).

Table 14. Maternal and child characteristics in the APrON cohort

Variables	Participants with data (n)	Mean, median or proportion	Participants with missing data (n)
Pregnant participants			
Age (years)	2,134	31.5 ± 4.5[1]	55
Pre-pregnancy BMI (kg/m^2)	1,947	23.0 (5.4)[2]	242
Marital status[3]			97
Married	1,772	86.7	
Common-law	236	11.3	
Separated or divorced	15	0.7	
Single	69	3.3	
Education[3]			117
Completed post-grad	470	22.7	
Completed university	943	45.5	
Completed trade/tech	401	19.4	
Completed high school	200	9.6	
Less than high school	58	2.8	
Household income[3]			120
$100 000 or more	1,143	55.2	
$70 000–$99 999	463	22.4	
$40 000–$69 999	276	13.3	
$20 000–$39 999	122	5.9	
Less than $20 000	65	3.1	
Primiparous[3]			99
Yes	1,119	53.5	
No	971	46.5	
Immigrated to Canada[3]			98
No	1,612	77.1	
Yes	479	22.9	
Ethnicity[3]			103
White	1,674	80.2	
Other	412	19.7	
Maternal pregnancy conditions[3,4]			
Gestational diabetes mellitus	1,695	3.0	494
Preeclampsia	1,695	0.3	494
Edinburgh depression scale (EPDS)[5]			
3rd trimester	1,920	4 (0 – 23)	269
	107	5.6	

Probable depression (EPDS ≥13), %	1,822 80	4 (0 – 22) 4.4	367
3 months postpartum			
Probable depression (EPDS ≥13), %	661	3393.0 ± 503.1 4.1	1
Children		1.4	
Birth weight (g)	662		0
<2500 g[3]		48.9	
>4500 g[3]		51.1	
Biological sex[3]			
Female	662	49 (27 – 78)	--
Male	662	47 (31 – 85)	--
BASC-2 (age 5)[6]			
Internalizing (T-scores)			
Externalizing (T-scores)			

[1]Mean ± SD; [2]Median (IQR); [3]Percentage; [4]Information collected during the last gestational visit (3rd trimester). The percentage of participants with the condition is given in the third column for these variables; [5]Median scores (range of scores). [6]The children of 662 pregnant females in the APrON cohort had BASC-2 data available at age 5. Median BASC-2 T-scores are displayed along with the range of T-scores in the child cohort. Table adapted from Leung et al. (2016). Not applicable (--).

Table 15. Maternal iron and vitamin D supplementation and status adequacy during pregnancy

	1st Trimester[1]	2nd Trimester	3rd Trimester
Supplement intake			
Iron			
Proportion taking, % (n)	93.0 (1,769)	95.4 (2,008)	93.7 (1,299)
Median intake (mg/day)[2]	22.9 (13.5)	27.0 (7.4)	27.0 (19.3)
Vitamin D			
Proportion taking, % (n)	65.2 (1,241)	70.2 (1,478)	68.2 (1,059)
Median intake (IU/day)[2]	250.0 (461.5)	342.9 (857.1)	338.5 (1000.0)
Iron and vitamin D status adequacy, % (n)[3]	--		--
(1) Iron and vitamin D replete		63.3 (397)	
(2) Iron replete, low vitamin D		14.8 (93)	
(3) Low iron, vitamin D replete		18.4 (115)	
(4) Low iron and vitamin D		3.5 (22)	

[1]Timepoints: 1st [mean (range) 10.8 (3.1-13.9) weeks], 2nd [19.0 (14.0- 26.9) weeks] and 3rd [32.5 (27.0-39.0) weeks]; [2]Supplement intakes given as median (IQR); [3]Determined in pregnant participants with SF and 25(OH)D quantified during the 2nd trimester (n=627). Abbreviations: International Units (IU).

5.3.1 Maternal vitamin D biomarkers and the combined adequacy of iron and vitamin D

Mean maternal concentrations of 25(OH)D3 were significantly lower at 3 months postpartum (83.8 ± 24.8 nmol/L) compared to the 2nd trimester of pregnancy (90.7 ± 25.8 nmol/L) (**Figure 14**a). The same relationship was detected between 2nd trimester [median (IQR): 6.7 (3.2)] and postpartum timepoint [5.4 (2.1)] for 3-epi-25(OH)D3 (Figure 14b) and the ratio of 3-epi-25(OH)D3 to 25(OH)D3 [median (IQR): 7.1 (0.04) during the 2nd trimester and 6.7 (0.03) at 3 months postpartum] (Figure 14c). When participants were categorized based on their iron and vitamin D status during the 2nd trimester, about 65% were replete for both micronutrients; however, a third had a low status in either iron or vitamin D and less than 5% had a low status for both (Table 15). A bar graph depicting the proportion of participants that fell into each category is given in **Supplementary Figure 6.**

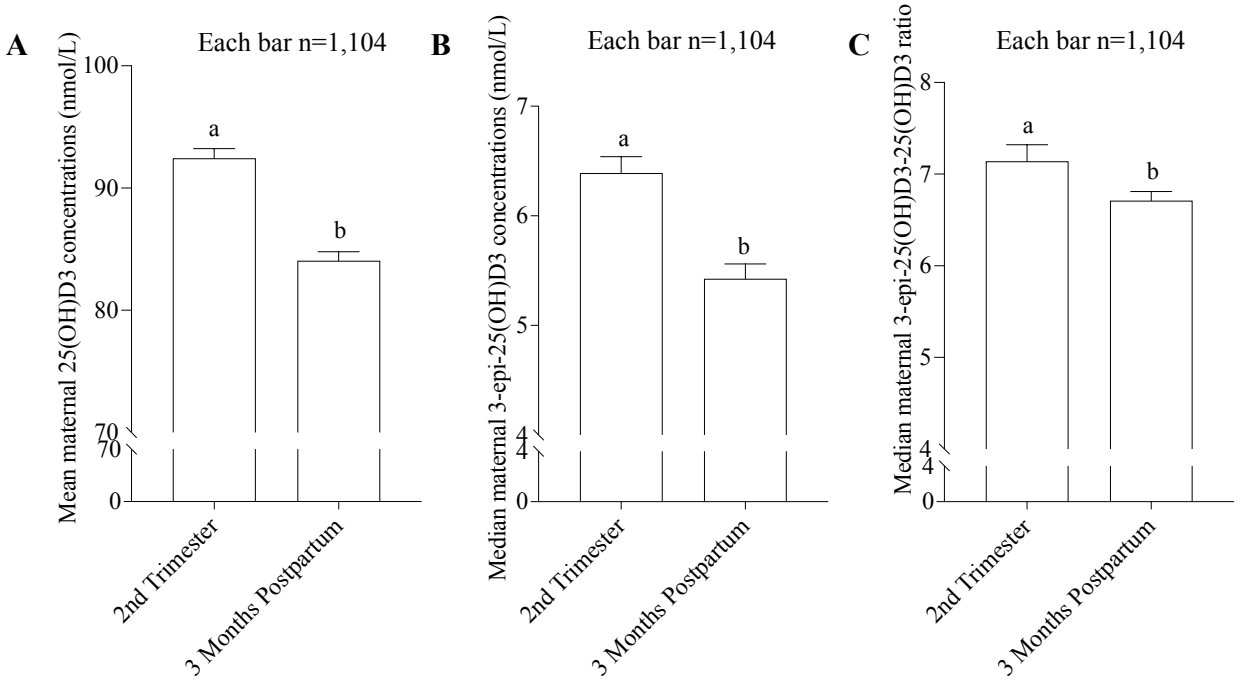


Figure 14. Changes in maternal (A) 25(OH)D3, (B) 3-epi-25(OH)D3 and (C) the 3-epi-25(OH)D3-25(OH)D3 ratio between the 2nd trimester and 3 months postpartum. Concentrations of 3-epi-25(OH)D3 and the 3-epi-25(OH)D3-25(OH)D3 ratio were skewed, and accordingly were log transformed before conducting paired t-tests. Bars and error bars respectively represent the mean and SD in panel (A) and the median and 95% confidence intervals in panels (B) and (C). Bars with different letters are significantly different ($p<0.05$) following paired t-tests.

5.3.2 Associations between biomarkers of maternal iron and vitamin D and maternal EPDS scores

Associations between maternal iron and vitamin D biomarker concentrations and 3rd trimester maternal EPDS scores were observed after adjustment (**Table 16**). Higher maternal hepcidin (p=0.005), SF (p=0.048) and 25(OH)D3 (p=0.001) concentrations during the 2nd trimester were significantly associated with lower EPDS scores during the 3rd trimester. There were no relationships between maternal sTfR, sTfR:SF or 3-epi-25(OH)D3 and 3rd trimester EPDS, or between maternal biomarkers and EPDS scores at 3 months postpartum. Refer to **Supplementary Table 3** for statistical outputs between other maternal iron and vitamin D biomarkers and maternal EPDS scores during the 2nd trimester or at 3 months postpartum.

Table 16. Relationships between 2nd trimester maternal hepcidin, SF and 25(OH)D3 concentrations and 3rd trimester maternal EPDS scores

Biomarker models	Univariate ß [95% CI]	Multivariate ß [95% CI]
Hepcidin[1]	-0.5 [-0.9, -0.2]	-0.5 [-0.9, -0.2]
SF[2]	-0.5 [-1.1, 0.1]	-0.8 [-1.5, -0.01]
25(OH)D3[3]	-0.01 [-0.02, -0.004]	-0.01 [-0.02, -0.004]

This table only includes variables that were significant predictors of maternal EPDS scores during the 3rd trimester (p<0.05). Hepcidin and SF concentrations were log transformed before regression analysis. The use of directed acyclic graphs (DAGs) identified potential confounding variables which were maternal age (years), ethnicity (White, other), income (≥$70,000, <$70,000), educational history (some type of post-secondary education, no post-secondary education), diet quality (CFG scores), gestational diabetes mellitus (GDM) (yes, no), preeclampsia (PE) (yes, no), season (summer, winter) and current iron or vitamin D supplementation (mg/day) (Figure 11a & b). Minimum covariates each multivariate model was adjusted for: [1]Maternal age; [2]3rd trimester maternal PE and 3rd trimester maternal iron supplementation; [3]no covariates (Supplementary Table 2).

5.3.3 Associations between biomarkers of maternal iron and vitamin D and child internalizing and externalizing T-scores

Maternal concentrations of iron and vitamin D biomarkers during the 3rd trimester and at 3 months postpartum were inversely related to externalizing T-scores at age 5 (**Table 17**). Higher maternal sTfR:SF indices during the 3rd trimester were associated with lower externalizing T-scores in female children (p=0.038), but not in males (p=0.860). Furthermore, there was an association between higher maternal 3-epi-25(OH)D3 concentrations at 3 months postpartum and

lower externalizing T-scores in 5-year-old male children (p=0.019). **Figure 15** shows scatter plots with trend lines between maternal concentrations of sTfR:SF (3rd trimester) and 3-epi-25(OH)D3 (3 months postpartum) and externalizing T-scores stratified by child sex. There is variability in the magnitude (Figure 15a & b) and direction (Figure 15a) of trend line slopes depending on child sex. Maternal iron or vitamin D biomarker concentrations were not related to internalizing T-scores at age 5 (**Supplementary Table 4**).

Table 17. Relationships between individual maternal iron and vitamin D biomarker concentrations during pregnancy and postpartum and child externalizing T-scores at age 5

	Biomarker	Univariate β [95% CI]		Multivariate β [95% CI]	
		Male[1]	Female[2]	Male	Female
1st Tri.	SF	2.6 [-6.6, 11.8]	-2.8 [-9.7, 4.2]	4.8 [-7.5, 17.1]	-4.6 [-16.8, 7.6]
	sTfR	15.7 [-6.6, 38.1]	-12.8 [-36.3, 10.6]	21.1 [-11.1, 53.3]	-21.0 [-50.3, 8.4]
	sTfR:SF	10.5 [-9.4, 30.4]	-6.9 [-26.3, 12.5]	11.3 [-18.3, 40.9]	-7.0 [-35.9, 20.9]
2nd Tri.	SF	-1.3 [-4.2, 1.6]	-1.7 [-4.4, 0.9]	-2.1 [-5.5, 1.2]	-1.2 [-4.2, 1.7]
	Hepcidin	0.05 [-1.9, 2.0	-0.7 [-2.2, -0.9]	-0.2 [-2.8, 2.4]	-0.3 [-2.0, 1.4]
	25(OH)D3	0.008 [-0.03, 0.05]	0.01 [-0.03, 0.05]	0.005 [-0.04, 0.05	0.02 [-0.02, 0.05]
	3-epi-25(OH)D3	3.2 [-2.2, 8.5]	2.1 [-3.0, 7.2]	3.3 [-3.8, 10.0]	2.6 [-2.6, 7.9]
3rd Tri.	SF	0.5 [-3.7, 4.8]	2.1 [-1.2, 5.5]	-1.1 [-6.7, 4.4]	2.2 [-2.2, 6.7]
	sTfR	10.8 [-10.8, 32.4	-14.4 [-35.1, 6.3]	6.4 [-20.4, 33.2]	-17.7 [-43.8, 8.4]
	sTfR:SF	0.2 [-17.6, 18.0]	-11.6 [-24.3, 1.1]	2.2 [-23.1, 27.5]	**-15.9 [-30.9, -0.9]**
	Hepcidin	0.5 [-1.2, 2.2]	-0.03 [-1.3, 1.3]	-0.4 [-2.6, 1.8]	-0.3 [-2.0, 1.4]
3-mo. Pp.	SF	1.0 [-2.2, 4.1]	0.7 [-2.1, 3.4]	-0.7 [-4.7, 3.2]	-0.4 [-3.9, 3.1]
	25(OH)D3	-0.04 [-0.08, 0.02]	-0.01 [-0.05, 0.02]	-0.03 [-0.08, 0.03]	0.02 [-0.02, 0.05]
	3-epi-25(OH)D3	**-9.3 [-18.0, -0.7]**	-3.3 [-10.0, 3.4]	**-11.9 [-21.8, -1.9]**	-0.1 [-9.6, 7.8]

[1]The male child sample size was n=338; [2]The female child sample size was n=324. Maternal biomarker concentrations were transformed if they were not normally distributed before regression analysis. The study timepoint when a given group of biomarkers were quantified is indicated by the vertical labels on the left side of the table. The following covariates were included in multivariate regression models related to iron status: maternal age (years), income (≥$70,000, <$70,000), education (some type of post-secondary education, no pos-secondary education), pre-pregnancy BMI, dietary quality (CFG scores), postpartum iron supplementation (mg/day) and postpartum EPDS scores (Figure 12a). Maternal age (years), ethnicity (White, other), income (≥$70,000, <$70,000), education (some type of post-secondary education, no post-secondary education), dietary quality (CFG scores), 3 months postpartum vitamin D supplementation (mg/day) and 3 months postpartum EPDS scores were included in the vitamin D models (Figure 12b). Through change-in-estimate rules, only the minimum set of covariates (Supplementary Table 2) were used to generate the statistical estimates shown in this table. Abbreviations: 1st trimester (1st Tri.); 2nd trimester (2nd Tri.); 3rd trimester (3rd Tri.); 3 months postpartum (3-mo. Pp.).

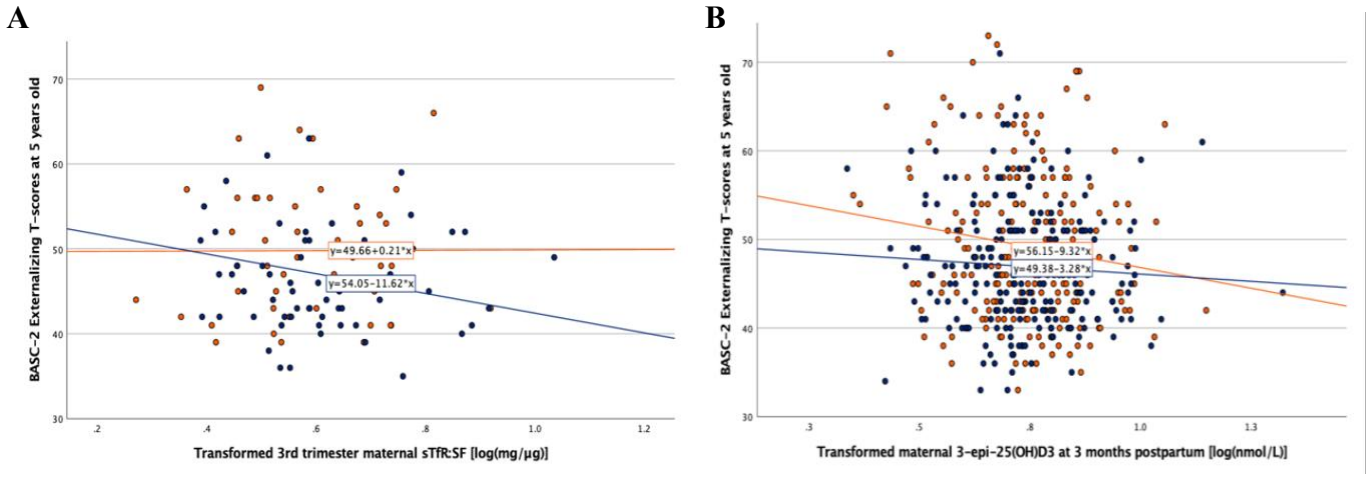


Figure 15. Scatter plots and trend lines between child externalizing T-scores against log transformed maternal (A) sTfR:SF and (B) 3-epi-25(OH)D3 concentrations in the 2nd trimester and 3 months postpartum, respectively. Data points and regression lines are stratified by child sex; males are represented by orange and females are represented by blue.

5.3.4 Differences in maternal depression symptoms and child behaviours depending on the combined adequacy of maternal iron and vitamin D status

There were differences in EPDS scores during the 3rd trimester and externalizing T-scores at age 5 depending on the combined status of maternal iron and vitamin D during mid-pregnancy (**Table 18**). Compared to pregnant participants who were replete in both micronutrients (category 1), EPDS scores were significantly higher when iron status was low and vitamin D was replete (category 3; p=0.044) or when iron and vitamin D status were both low (category 4; p=0.024). Relationships between the combined maternal adequacy of iron and vitamin D during the 2nd trimester and externalizing behaviour T-scores differed by child sex (Table 18); compared to the maternal replete group (category 1), externalizing T-scores in female children were significantly higher when iron was replete and vitamin D was low (category 2; p=0.031), but in male children externalizing T-scores were significantly higher when iron was low and vitamin D was replete (category 3; p=0.007). The adequacy of maternal iron and low vitamin D status was not related to either EPDS scores at 3 months postpartum or child internalizing T-scores (Table 18).

Table 18. Differences in maternal EPDS scores and child internalizing and externalizing T-scores depending on the adequacy of maternal iron and vitamin D status during mid-pregnancy

	Maternal iron and vitamin D status adequacy (2nd Trimester)[1]							
	Both replete		Replete iron, low vitamin D		Low iron, replete vitamin D		Both low	
	β[2]	[95% C]	β	[95% CI]	β	[95% CI]	β	[95% CI]
Maternal depression symptoms[3]								
3rd trimester	Ref.		0.5	[-0.6, 1.7]	**1.1**	**[0.03, 2.1]**	**2.2**	**[0.3, 4.2]**
3 months postpartum	Ref.		0.1	[-1.0, 1.3]	0.2	[-0.9, 1.2]	1.0	[-1.1, 0.9]
Child behaviours[4]								
Internalizing								
Females	Ref.		0.5	[-4.3, 5.3]	2.2	[-2.9, 7.3]	--[5]	
Males	Ref.		2.6	[-2.8, 8.1]	0.3	[-4.9, 5.6]	--	
Externalizing								
Females	Ref.		**7.3**	**[2.0, 12.6]**	-0.2	[-5.1, 4.8]	--	
Males	Ref.		2.2	[-1.6, 5.9]	**4.3**	**[0.4, 8.1]**	--	

[1]Categories (left to right) of maternal iron and vitamin D adequacy: (1) iron and vitamin D replete, (2) iron replete, low vitamin D, (3) low iron, vitamin D replete, (4) low iron and vitamin D. [2]Reference group (Ref.) for Generalized Linear Model (GLM) assessment. The β and 95% CI for the remainder of the categories represent the mean difference in the outcome variable between that category and the reference category (Ref.). [3]Maternal GLM models were adjusted for maternal age (years), ethnicity (White, other), income (≥$70,000, <$70,000), education (some type of post-secondary education, no post-secondary education), diet quality (CFG scores), GDM (yes, no), PE (yes, no), iron and vitamin D supplementation (3rd trimester or 3 months postpartum; mg/day) and season (summer, winter) (Figure 1c). [4]Child BASC-2 models were adjusted for maternal age (years), ethnicity (White, other), income (≥$70,000, <$70,000), education (some type of post-secondary education, no post-secondary education), diet quality (CFG scores), pre-pregnancy BMI, iron and vitamin D supplementation (3 months postpartum; mg/day) and EPDS scores (3 months postpartum). [5]Sex stratified sample sizes were too small to determine a reliable statistical estimate.

Table 19. A summary of the important findings from manuscript 2

1. Associations between individual maternal iron and vitamin D biomarkers and maternal depression
▪ Inverse association: maternal SF concentrations (2nd Tri.) and EPDS scores (3rd Tri.)
▪ Inverse association: maternal hepcidin concentrations (2nd Tri.) and EPDS scores (3rd Tri.)
▪ Inverse association: maternal 25(OH)D3 concentrations (2nd Tri.) and EPDS scores (3rd Tri.)
2. Associations between individual maternal iron and vitamin D biomarkers and child behaviours
▪ Inverse association: maternal sTfR:SF (3rd Tri.) and externalizing T-scores (age 5) for female children
▪ Inverse association: maternal 3-epi-25(OH)D3 (3 mo. pp.) and externalizing T-scores (age 5) for male children

3. Differences in the outcomes depending on the combined adequacy of maternal iron and vitamin D
▪ Compared to those that were iron and vitamin D replete (category 1): • Maternal EPDS scores (3rd Tri.) were higher in those with either low in iron but replete in vitamin D (category 3) or low in iron and vitamin D (category 4) during the 2nd trimester • Child externalizing T-scores (at age 5) were higher when pregnant participants had replete iron but low vitamin D (category 2) during the 2nd trimester for female children • Child externalizing T-scores (at age 5) were higher when pregnant participants had low iron but replete vitamin D (category 3) during the 2nd trimester for male children

Only the significant relationships associated with each of the three objectives (after appropriate covariate adjustment) are provided in this table. Abbreviations: months (mo.); postpartum (pp.); trimester (Tri.).

5.4 Discussion

A low maternal iron status in the 2nd trimester was associated with higher maternal EPDS scores during the 3rd trimester (**Table 19**). Maternal iron depletion, defined as a higher sTfR:SF index, was associated with lower externalizing T-scores in 5-year-old female children. We also observed an inverse association between maternal 3-epi-25(OH)D3 concentrations at 3 months postpartum and externalizing T-scores at age 5 in male offspring, potentially for the first time. Maternal depression scores and externalizing scores in 5-year-old children differed depending on the combined adequacy of maternal iron and vitamin D during mid-pregnancy (Table 19). This study provides evidence that maternal iron and vitamin D status are important predictors of maternal mental health during pregnancy, and that they also contribute to the neurodevelopmental characteristics of children at least until 5 years of age.

Results of other work examining the effects of maternal iron or vitamin D status in pregnancy on maternal depression outcomes are difficult to interpret, and some have found contradictory results. Similar to the present study, Lin et al. (2022) did not find a relationship between 1st trimester maternal SF concentrations and 3rd trimester EPDS scores. Gowtham et al. (2022) observed that concentrations of SF in pregnant people during early gestation were not related to maternal EPDS scores in the 2nd trimester of pregnancy. Other studies reported that a low maternal iron or vitamin D status were simultaneously related to higher maternal EPDS scores (Dama et al., 2018; Jani et al., 2020; Lamb et al., 2018); for example, in Jani et al. (2020), pregnant participants with 1st trimester EPDS scores ≥13 were more likely to have a lower vitamin D status during the 1st trimester. In contrast, we did not observe a significant association between any of the 3rd trimester maternal iron biomarker concentrations and 3rd trimester EPDS scores. However, in the present study, maternal vitamin D metabolites were not quantified during the 3rd trimester

preventing us from comparing 3rd trimester maternal depression symptoms to concurrent maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations. Considering this body of evidence, reported associations between iron or vitamin D biomarkers and symptoms of maternal depression appear to be temporally nuanced among different study populations, including ours. While other vitamin D metabolites, including 25(OH)D2, 25(OH)D3 and $1,25(OH)_2D$ were measured in a few investigations (Cunha Figueiredo et al., 2017; Gur er al 2014), maternal concentrations of total 25(OH)D were measured in many cases (Aghajafari et al., 2018b; Jani et al., 2022; Lamb et al., 2018). The analysis of a more diverse set of nutrient biomarkers across an array of timepoints has the potential to inform the association between maternal nutrient status and maternal depression during or after pregnancy, which should be a focus of future research.

A particularly novel finding in this investigation was that there appeared to be differences in maternal EPDS scores during the 3rd trimester depending on the combined adequacy of maternal iron and vitamin D status during the 2nd trimester. For example, the mean difference in EPDS scores when pregnant individuals had a low status of both micronutrients was more than 2 points above the reference group, suggesting that individuals with a concurrent depletion of iron and vitamin D during mid-pregnancy may experience more symptoms associated with depression during the last trimester (Cox et al., 1987). At this point, it is difficult to discuss these findings in relation to other comparable evidence as it is either extremely limited or non-existent.

Others have postulated bidirectional influences of inadequate nutrient status and poor mental health (Reesor-Oyer et al., 2021). Micronutrient availability may impact the development of mental health issues, but on the other hand, people with low moods or depression may consume food or supplements in an inconsistent or disordered manner which could have consequences on nutrient status (Maynard et al., 2018). Still, based on previous data suggesting a mechanistic role for poor nutrition in the development of maternal mental illness (Gregorio et al., 2022; Zhao et al., 2020), we deemed there was enough evidence to justify DAGs and statistical analyses that assumed one direction, with maternal micronutrient status as predictors and EPDS scores as the outcome (Williams et al., 2018). While our results suggest that a low maternal status of iron or vitamin D may be related to more symptoms of maternal depression at a later pregnancy timepoint, bidirectional associations should be considered (Maynard et al., 2018; Reesor-Oyer et al., 2021).

Recent evidence also suggests that the unique trajectories of maternal stress or mental illness may be influenced by different risk factors depending on whether they exist during

pregnancy or postpartum (Chow et al., 2019; Korja et al., 2018; Vanwetswinkel et al., 2022). Indeed, in our investigation, concentrations of iron or vitamin D biomarkers, even those that were significantly related to 3rd trimester depressive symptoms, were not associated with maternal EPDS scores at 3 months postpartum, consistent with the findings of Armony-Sivan et al. (2012). Still, others have reported that maternal iron or vitamin D biomarkers measured during pregnancy were related to postpartum depression symptoms among people who recently gave birth (Gowtham et al., 2022; Gur et al., 2014). Considering this mixed evidence, the temporal trajectory of maternal depression is an important variable to consider (Vanwetswinkel et al., 2022).

Previous literature relating maternal iron or vitamin D status to child neurodevelopment in humans remains mixed. The Cork BASELINE Birth Study showed no associations between 2nd trimester maternal 25(OH)D concentrations and either internalizing or externalizing behaviours in 4-year-old children (McCarthy et al., 2018), which is similar to our observation of no relationships between 2nd trimester maternal 25(OH)D3 or 3-epi-25(OH)D3 and child externalizing or internalizing behaviours. In contrast, Daraki et al. (2018) found an association between higher maternal 25(OH)D in the 1st trimester and lower externalizing and overall problematic behaviour scores in children at 4 years of age. These investigations and ours suggest that relationships between maternal nutrient status and childhood behaviours may be gestationally time-dependent. Furthermore, about a decade ago it was determined that 3-epi-25(OH)D3, a stereoisomer of 25(OH)D3, contributes to human adult 25(OH)D status (Lensmeyer et al., 2012). Despite it being reported to have an increased abundance during the first year of human life (Singh et al., 2006; Yazdanpanah et al., 2013), there is limited evidence about the potential role of 3-epi-25(OH)D3 in early behaviours. In this investigation we observed an inverse association between postnatal maternal 3-epi-25(OH)D3 concentrations and externalizing T-scores at age 5 in male children, suggesting that this vitamin D metabolite might be reflective of prospective behaviours in this sex and age group. Although there were no associations among individual 2nd trimester vitamin D biomarkers and externalizing T-scores in female children, analysis of the combined maternal adequacy of both micronutrients during mid-pregnancy suggested that externalizing T-scores in females were significantly higher when the maternal status of vitamin D was low (category 2) compared to the replete group (category 1). Together this evidence suggests that vitamin D status in pregnant individuals may be related to offspring behaviours at 5 years of age and provides

evidence for the possible long-term impacts of maternal nutrient availability in pregnancy (Pet & Brouwer-Brolsma, 2016).

While several previous studies observed relationships between maternal iron status during pregnancy and childhood behaviours (McCarthy et al., 2021; Quezada-Pinedo et al., 2021), some of the details of these associations differed from our findings. Santa-Marina et al. (2020) found an inverse association between maternal plasma ferritin during the 1st trimester and inattentive behaviour scores in male children at 4 years old, but we observed a relationship between concentrations of a maternal iron biomarker (sTfR:SF) during the 3rd trimester and externalizing behaviours at age 5 in female children. There are notable differences in the biomarker types, relationship temporalities and sex-specificities between their results (Santa-Marina et al., 2020) and ours. However, the coexistence of rapid placental iron mobilization along with the growth and development of the neocortex during the 3rd trimester (Bothwell et al., 1958; Bradley et al., 2004; Gong et al., 1998) may rationalize the relationship between maternal iron depletion at this time and externalizing behaviours. It is not clear why the sTfR:SF index was the only iron biomarker independently associated with child externalizing T-scores in the present investigation, but this measurement may reflect the biological adequacy of this nutrient more accurately because it integrates a proxy of both iron stores (Walters et al., 1973) and iron-deficient erythropoiesis (Allen et al., 1998). The analysis of the combined mid-pregnancy status of both micronutrients suggested that externalizing T-scores in male children were higher when maternal iron status was low (category 3) compared to the replete reference group (category 1). Overall, antenatal maternal iron status may be related to externalizing behaviours in male and female children at 5 years of age.

It is unclear why the relationships between maternal iron and vitamin D biomarkers and externalizing behaviours differed between the sexes. Although evidence related to the possible sex-dependency of gestational programming has been described for years (Gualtieri & Hicks, 1985; Zarén et al., 2000), it has become of more interest recently (DiPietro & Voegtline, 2017). It is possible that this is a general effect given that several authors have suggested that male and female fetuses may differ in their reaction to developmental insults, including sub-optimal nutrient conditions, in pregnancy (Nugent et al., 2018). More research is urgently needed to delineate potential interactions of fetal biological sex and maternal nutrient status in pregnancy and their impact on child neurodevelopment.

Despite evidence supporting associations between maternal iron and vitamin D status and maternal depression and child externalizing behaviours, there was not a particular biomarker that was related to both outcomes. However, the inclusion of 3 months postpartum EPDS scores as a covariate in many of the child externalizing T-score models (Supplementary Table 1) highlighted significant positive associations between the two variables in many instances (not reported). While mediation analysis was not conducted in this investigation, it is possible that maternal depression may be a mediator in the potential pathway between maternal iron or vitamin D status, either independently or together, and externalizing behaviours at age 5. This idea has also been proposed by others (Giallo et al., 2015; Glover, 2014), and further research should prioritize the exploration of this hypothesis.

Finally, further to previous estimates of vitamin D status during the 2nd trimester among a sub-cohort of pregnant participants (Aghajafari et al., 2016), this investigation reported significant changes in maternal 25(OH)D3, 3-epi-25(OH)D3 and 3-epi-25(OH)D3:25(OH)D3 ratios between mid-pregnancy to approximately 3 months postpartum for the first time in APrON. Other studies that describe maternal vitamin D status after birth are sparse, but Narchi et al. (2010) found that the proportion of pregnant individuals at risk for deficiency increased across gestation through to 6 months postpartum. As maternal vitamin D biomarker concentrations were lower ~3 months after birth in our investigation, the postnatal maternal status of this micronutrient should be explored in different pregnant populations.

Multiple biomarkers for each micronutrient were quantified at different antenatal and postnatal timepoints. Sex-specific differences in the associations between maternal nutrient status and offspring externalizing behaviours highlight the benefit of treating child sex as an EM to provide insights into the possible fetal sex-specificity of in utero programming (DiPietro & Voegtline, 2017). Further, the inclusion of prenatal and postpartum maternal depression scores provided evidence of its potential risk factors during both periods (Vanwetswinkel et al., 2022). A limitation is that gestational concentrations of maternal vitamin D metabolites have only been measured during mid-pregnancy, but we are hopeful that they will be quantified during the 1st and 3rd trimesters in the future. Maternal 25(OH)D3 and 3-epi-25(OH)D3 are precursors to their activated derivatives, 1,25$(OH)_2$D3 and 3-epi-1α,25-dihydroxyvitamin D3, respectively, which retain many of the functional capabilities of vitamin D (Kamao et al., 2004; Jenkinson, 2019). Still, maternal 25(OH)D metabolites, including 25(OH)D3, cross the placenta (Christensen & Birn,

2002) and are known to significantly contribute to the fetal status of vitamin D (Cyprian et al., 2019; Wierzejska et al., 2017). Moreover, pregnant participants in the APrON cohort are generally of a high SES, which likely limited the group of individuals that had a low status of both iron and vitamin D (category 4) in this investigation. Resultingly, the sample size of the low iron and vitamin D group (category 4) was too small to determine reliable statistical estimates after offspring sex stratification, impacting our ability to estimate the mean internalizing and externalizing T-scores in this category. In this line, we also recognize that the maternal and child participants in our study are of a generally high SES and would caution against the generalizability of the findings to populations at a higher risk of obstetric complications or malnutrition.

To conclude, maternal iron and vitamin D biomarkers, measured during mid to late pregnancy or at 3 months postpartum, were independently associated with 3rd trimester maternal depression symptoms and child externalizing behaviours at age 5 in the APrON cohort. There were sex-specific differences in the child-related findings; a biomarker of maternal iron depletion (sTfR:SF) was inversely associated with externalizing T-scores in female children, whereas maternal 3-epi-25(OH)D3, a novel vitamin D metabolite (Lensmeyer et al., 2012; Yazdanpanah et al., 2013), was inversely related to externalizing behaviours in males. A replete maternal status of iron and vitamin D during mid-pregnancy was related to less maternal depression symptoms during the 3rd trimester and lower externalizing scores in male and female children at 5 years of age.

This investigation is one of the first to report on the combined adequacy of maternal iron and vitamin D status during pregnancy and its impact on maternal depression and child behaviour. The novelty of this work reinforces the need to ask similar questions in other pregnant populations. Future investigations should report on the status of multiple micronutrients and explore their independent and combined impact on the health outcomes of children and pregnant individuals in an offspring sex-stratified manner.

5.5 Supplementary materials

Supplementary Table 2. Lists of variables included in multivariate regression models

Exposure	Outcome	Minimum covariates
Hepcidin 2ndTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., CFG scores, Fe suppl 3 mo. pp. Females: Maternal EPDS 3 mo pp., ppBMI, CFG scores, Fe Suppl 3 mo. pp., education, income, age

Hepcidin 3rdTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp, income, age Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores
SF 1stTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., Fe suppl 3 mo. pp., income, age Females: Maternal EPDS 3 mo. pp., ppBMI, Fe suppl 3 mo. pp.
SF 2ndTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, Fe Suppl 3 mo. pp., income Females: Maternal EPDS 3 mo pp, ppBMI, CFG scores, Fe suppl 3 mo pp, education
SF 3rdTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., CFG scores, Fe suppl 3 mo. pp., income Females: Maternal EPDS 3 mo. pp., ppBMI, education
SF 3mo. pp	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., CFG scores, Fe suppl 3 mo. pp., education, income, age Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, income, age
sTfR 1stTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, age Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, income
sTfR 3rdTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., CFG scores, Fe suppl 3 mo. pp., education, income Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., income
Index 1stTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, age Females: Maternal EPDS 3 mo. pp., ppBMI, Fe suppl 3 mo. pp., education
Index 3rdTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, income, age EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education
25(OH)D3 2ndTri	Internalizing T-scores	Males: Maternal VD suppl 3 mo. pp., income, age Females: Maternal EPDS 3 mo. pp., CFG scores, education, income, age
25(OH)D3 3 mo. pp	Internalizing T-scores	Males: Maternal CFG scores, VD suppl 3 mo. pp., age Females: Maternal EPDS 3 mo pp., ethnicity, CFG scores, VD suppl 3 mo. pp., education, income
3-epi-25(OH)D3 2ndTri	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., VD suppl 3 mo. pp., income, age Females: Maternal EPDS 3 mo. pp., ethnicity, CFG scores, VD suppl 3 mo pp, education, income, age
3-epi-25(OH)D3 3 mo. pp	Internalizing T-scores	Males: Maternal EPDS 3 mo. pp., CFG scores, VD suppl 3 mo. pp., education, income Females: Maternal EPDS 3 mo. pp., CFG scores
Hepcidin 2ndTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., income, age

		Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, income, age
Hepcidin 3rdTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., income, age Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, age
SF 1stTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, age Females: Maternal EPDS 3 mo. pp., ppBMI, Fe suppl 3 mo. pp., income
SF 2ndTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, Fe suppl 3 mo. pp., education, income Females: Maternal EDS 3 mo. pp., ppBMI, CFG scores, income
SF 3rdTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., income, age Females: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, age
SF 3mo. Pp	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education Females: Maternal CFG scores, Fe suppl 3 mo. pp., education, income, age
sTfR 1stTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, age Females: Maternal EPDS 3 mo. pp., ppBMI, Fe suppl 3 mo. pp., income, age
sTfR 3rdTri	Externalizing T-scores	Males: Maternal ppBMI, Fe suppl 3 mo. pp., education, income Females: Maternal EPDS 3 mo. pp., CFG scores, Fe suppl 3 mo. pp.
Index 1stTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., age Females: EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp.
Index 3rdTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ppBMI, CFG scores, Fe suppl 3 mo. pp., education, income Females: Maternal EPDS 3 mo. pp., CFG scores
25(OH)D3 2ndTri	Externalizing T-scores	Males: Maternal ethnicity, CFG scores, VD suppl 3 mo. pp., education, income, age Females: Maternal EPDS 3 mo. pp., VD suppl 3 mo. pp., education, income, age
25(OH)D3 3 mo. pp	Externalizing T-scores	Males: Maternal CFG scores, income Females: Maternal EPDS 3 mo. pp., VD suppl 3 mo. pp., education, income, age
3-epi-25(OH)D3 2ndTri	Externalizing T-scores	Males: Maternal EPDS 3 mo. pp., ethnicity, CFG scores, VD suppl 3 mo. pp., education, income, age Females: Maternal EPDS 3 mo. pp., education, income, age
3-epi-25(OH)D3 3 mo. pp	Externalizing T-scores	Males: Maternal VD suppl 3 mo. pp., education, age

		Females: Maternal EODS 3 mo. pp., CFG scores, VD suppl 3 mo. pp., income, age
Hepcidin 2ndTri	EPDS scores 3rdTri	Maternal age
Hepcidin 3rdTri	EPDS scores 3rdTri	Maternal PE, CFG scores, Fe suppl 3rdTri, education, income, age
SF 1stTri	EPDS scores 3rdTri	Maternal PE, Fe suppl 3rdTri, income, age
SF 2ndTri	EPDS scores 3rdTri	Maternal PE, Fe suppl 3rdTri
SF 3rdTri	EPDS scores 3rdTri	Maternal PE, CFG scores, Fe suppl 3rdTri, income
sTfR 1stTri	EPDS scores 3rdTri	Maternal PE, Fe suppl 3rdTri, education
sTfR 3rdTri	EPDS scores 3rdTri	Maternal PE, CFG scores, Fe suppl 3rdTri
Index 1stTri	EPDS scores 3rdTri	Maternal PE, Fe suppl 3rdTri, age
Index 3rdTri	EPDS scores 3rdTri	Maternal CFG scores, Fe suppl 3rdTri, age
25(OH)D3 2ndTri	EPDS scores 3rdTri	[no covariates]
3-epi-25(OH)D3 2ndTri	EPDS scores 3rdTri	Season, maternal age
Hepcidin 2ndTri	EPDS scores 3 mo. pp.	Maternal PE, CFG scores, Fe suppl 3 mo. pp, age
Hepcidin 3rdTri	EPDS scores 3 mo. pp.	Maternal PE, CFG scores, Fe suppl 3 mo. pp., education, age
SF 1stTri	EPDS scores 3 mo. pp.	Maternal GDM, PE, CFG scores, Fe suppl 3 mo. pp., education, income, age
SF 2ndTri	EPDS scores 3 mo. pp.	Maternal GDM, Fe suppl 3 mo. pp., education
SF 3rdTri	EPDS scores 3 mo. pp.	Maternal PE, CFG scores, Fe suppl 3 mo. pp., education, age, income
SF 3mo. Pp	EPDS scores 3 mo. pp.	Maternal PE, age
sTfR 1stTri	EPDS scores 3 mo. pp.	Maternal GDM, CFG scores, Fe suppl 3 mo. pp., education, income
sTfR 3rdTri	EPDS scores 3 mo. pp.	Maternal PE, education, income
Index 1stTri	EPDS scores 3 mo. pp.	Maternal GDM, CFG scores, Fe suppl 3 mo. pp., education, income, age
Index 3rdTri	EPDS scores 3 mo. pp.	Maternal Fe suppl 3 mo. pp., education
25(OH)D3 2ndTri	EPDS scores 3 mo. pp.	Season, maternal CFG scores, VD supp 3 mo. pp., age
25(OH)D3 3 mo. pp	EPDS scores 3 mo. pp.	Season, maternal CFG scores, VD supp 3 mo. pp., education, age
3-epi-25(OH)D3 2ndTri	EPDS scores 3 mo. pp.	Season, maternal CFG scores, VD suppl 3 mo. pp.
3-epi-25(OH)D3 3 mo. pp	EPDS scores 3 mo. pp.	Season, maternal CFG scores, VD suppl 3 mo. pp., income

A two-step method, including the use of directed acyclic graphs (DAGs) and change-in-estimate rules, was utilized to determine the minimum set of covariates that may confound relationships between the exposure and outcome variables (Weng et al., 2009). The child behaviour models were stratified by child sex. Abbreviations: Canadian Food Guide (CFG); Edinburgh Postnatal Depression Scale (EPDS); gestational diabetes mellitus (GDM); iron (Fe); preeclampsia (PE); pre-pregnancy body mass index (ppBMI); serum ferritin (SF); soluble transferrin receptor (sTfR); sTfR-SF

index (sTfR:SF); supplement (suppl); vitamin D (VD); 1st trimester (1stTri); 2nd trimester (2ndTri); 3rd trimester (3rdTri).

Supplementary Table 3. Relationships between individual maternal iron and vitamin D biomarker concentrations during pregnancy and postpartum and maternal EPDS scores

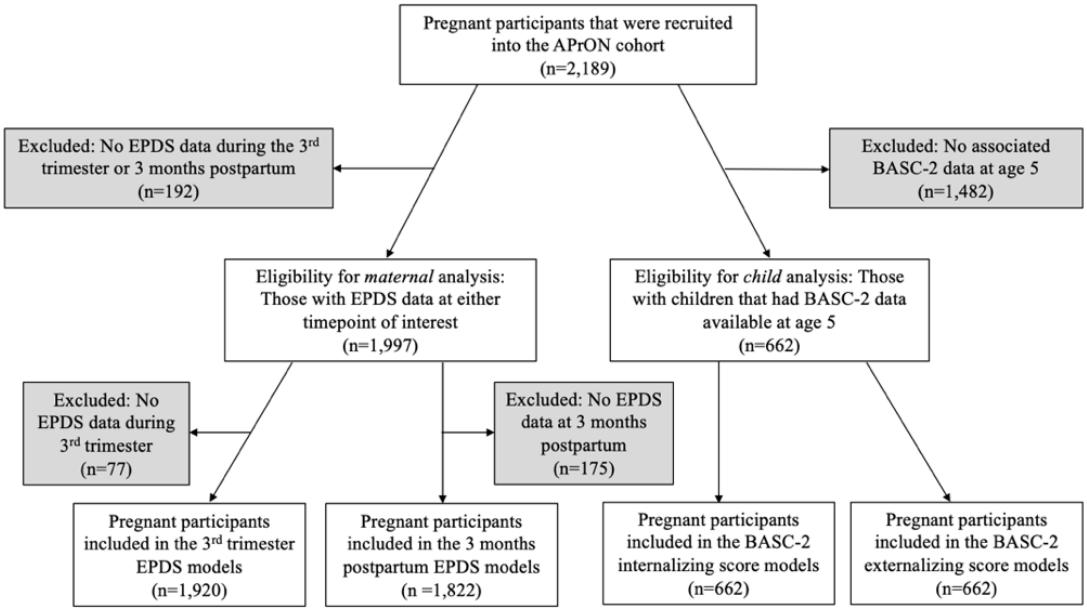
	Biomarker	3rd trimester EPDS scores		EPDS scores 3 months postpartum	
		Univariate	Multivariate	Univariate	Multivariate
1st Tri.	SF	-1.1 [-2.6, 0.5]	-0.4 [-2.4, 1.7]	-0.4 [-2.0, 1.1]	-0.01 [-2.4, 2.4]
	sTfR	2.3 [-2.4, 6.9]	4.3 [-1.3, 9.9]	2.9 [-1.8, 7.8]	2.9 [-4.1, 10.0]
	sTfR:SF	2.3 [-1.1, 5.6]	3.9 [-0.2, 8.0]	2.0 [-1.7, 5.6]	1.6 [-4.1, 7.4]
2nd Tri.	SF	-0.5 [-1.1, 0.1]	**-0.8 [-1.5, -0.01]**	-0.6 [-1.2, 0.08]	-0.2 [-1.0, 0.6]
	Hepcidin	**-0.5 [-0.9, -0.2]**	**-0.5 [-0.9, -0.2]**	-0.2 [-0.6, 0.2]	-0.3 [-0.7, 0.2]
	25(OH)D3	**-0.01 [-0.02, -0.004**]	**-0.01 [-0.02, -0.004]**	0.002 [-0.01, 0.01]	2.3 [-0.6, 5.1]
	3-epi-25(OH)D3	0.1 [-1.0, 1.2]	-0.7 [-2.5, 2.4]	0.7 [-0.4, 1.8]	0.9 [-2.3, 4.0]
3rd Tri.	SF	-0.4 [-1.2, 0.5]	-0.7 [-1.7, 0.2]	-0.4 [-1.2, 0.5]	0.02 [-1.0. 1.1]
	sTfR	3.2 [-1.1, 7.4]	2.3 [-2.9, 7.6]	3.6 [-0.7, 7.9]	3.4 [-1.8, 8.5]
	sTfR:SF	0.5 [-2.6, 3.6]	1.0 [-2.6, 4.5]	2.3 [-0.9, 5.6]	3.2 [-0.7, 7.1]
	Hepcidin	-0.09 [-0.4, 0.2]	-0.05 [-0.4, 0.3]	-0.1 [-0.4, 0.2]	-0.1 [-0.5, 0.3]
3-mo. Pp.	SF	--	--	-0.2 [-0.8, 0.4]	-0.5 [-1.2, 0.2]
	25(OH)D3	--	--	0.001 [-0.01, 0.01]	0.01 [-0.01, 0.02]
	3-epi-25(OH)D3	--	--	-0.07 [-1.7, 1.6]	-0.8 [-1.2, 2.9]

Maternal biomarker concentrations were transformed if they were not normally distributed before regression analysis. The study timepoint when a given group of biomarkers were quantified is indicated by the vertical labels on the left side of the table. The use of directed acyclic graphs (DAGs) identified potential confounding variables which were maternal age (years), ethnicity (White, other), income (≥$70,000, <$70,000), educational history (some type of post-secondary education, no post-secondary education), diet quality (CFG scores), gestational diabetes mellitus (GDM) (yes, no), preeclampsia (PE) (yes, no), season (summer, winter) and current iron or vitamin D supplementation (mg/day) (Figure 11a & b). Through change-in-estimate rules, only the minimum set of covariates (Supplementary Table 1) were used to generate the statistical estimates shown in this table. Abbreviations: 1st trimester (1st Tri.); 2nd trimester (2nd Tri.); 3rd trimester (3rd Tri.); 3 months postpartum (3-mo. Pp.).

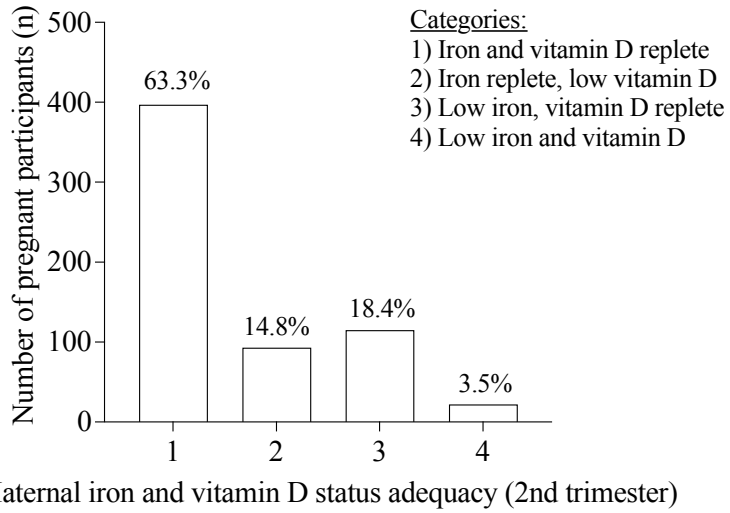
Supplementary Table 4. Relationships between individual maternal iron and vitamin D biomarker concentrations during pregnancy and postpartum and child internalizing T-scores at age 5

	Biomarker	Univariate β [95% CI]		Multivariate β (95% CI)	
		Male[1]	Female[2]	Male	Female
1st Tri.	SF	-2.7 [-11.1, 5.7]	-3.3 [-10.7, 41.4]	-4.2 [-13.2, 4.7]	-4.7 [-15.6, 6.2]
	sTfR	14.4 [-5.7, 34.6]	-15.6 [-40.5, 9.4]	12.7 [-14.1, 39.5]	-19.2 [-50.7, 12.3]
	sTfR:SF	11.6 [-6.5, 29.6]	-9.0 [-30.3, 12.3]	8.2 [-15.6, 32.1]	-7.0 [-32.2, 18.2]
2nd Tri.	SF	-3.0 [-6.1, 0.01]	-1.6 [-4.7, 1.6]	-1.9 [-5.3, 1.5]	-1.0 [-5.1, 3.2]
	Hepcidin	-1.2[(-3.1, 0.7]	-0.6 [-2.5, 1.4]	-1.3 [-3.3, 0.8]	0.4 [-2.4, 3.2]
	25(OH)D3	0.01 [-0.02, 0.05]	0.001 [-0.04, 0.04]	0.03 [-0.02, 0.07]	0.02 [-0.03, 0.06]
	3-epi-25(OH)D3	-0.5 [-5.9, 4.9]	1.5 [-4.6, 7.8]	3.6 [-2.6, 9.7]	0.5 [-7.4, 8.3]
3rd Tri.	SF	-2.3 [-6.7, 1.0]	0.3 [-4.0, 4.6]	-2.8 [-7.8, 2.2]	2.7 [-1.9, 7.3]
	sTfR	0.0 [-20.1, 20.1]	-17.8 [-39.6, 3.9]	-0.02 [-22.7, 22.4]	-17.0 [-42.1, 8.1]
	sTfR:SF	0.03 [-16.5, 16.5]	-11.0 [-25.0, 3.0]	-0.5 [-22.1, 21.0]	-7.7 [-26.9, 11.5]
	Hepcidin	-0.014 [-1.7, 1.7]	-1.1 [-2.8, 0.6]	-1.8 [-0.9, 0.6]	-1.0 [-3.0, 0.9]
3-mo. Pp.	SF	0.6 [-2.6, 3.8]	1.1 [-2.3, 4.4]	1.2 [-2.8, 5.2]	0.1 [-4.5, 4.8]
	25(OH)D3	-0.002 [-0.05, 0.05]	-0.01 [-0.05, 0.03]	-0.02 [-0.08, 0.04]	-0.002 [-0.07, 0.06]
	3-epi-25(OH)D3	-5.9 [-14.6, 2.7]	-5.0 [-13.2, 3.3]	-3.3 [-13.6, 7.0]	-8.6 [-18.3, 1.1]

[1]The male child sample size was n=338; [2]The female child sample size was n=324. Maternal biomarker concentrations were transformed if they were not normally distributed before regression analysis. The study timepoint when a given group of biomarkers were quantified is indicated by the vertical labels on the left side of the table. The following covariates were included in multivariate regression models related to iron status: maternal age (years), income (≥$70,000, <$70,000), education (some type of post-secondary education, no post-secondary education), PE (yes, no), GDM (yes, no), diet quality (CFG scores), 3 months postpartum iron supplementation (mg/day) (Figure 12a). Maternal age (years), income (≥$70,000, <$70,000), education (some type of post-secondary education, no post-secondary education), diet quality (CFG scores), 3 months postpartum vitamin D supplementation (mg/day) and season (summer, winter) were included in the vitamin D models (Figure 12b). Through change-in-estimate rules, only the minimum set of covariates (see Supplementary Table 2) were used to generate the statistical estimates shown in this table. Abbreviations: 1st trimester (1st Tri.); 2nd trimester (2nd Tri.); 3rd trimester (3rd Tri.); 3 months postpartum (3-mo. Pp.).



Supplementary Figure 5. Sample size flow chart of the pregnant participants from the APrON cohort that were eligible for inclusion into the maternal EPDS (left side) or child BASC-2 internalizing or externalizing (right side) regression models.



Supplementary Figure 6. The number of pregnant participants in each category of the maternal iron and vitamin D status adequacy variable during the 2nd trimester.

CHAPTER 6: CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

6.1 Summary of the major findings and main conclusions by thesis objective

Objective 1: Determine how maternal concentrations of systemic iron and vitamin D biomarkers change across time, and the combined maternal status of both micronutrients.

1. Determine how maternal Hb, hepcidin, SF, sTfR and sTfR:SF concentrations change between different trimesters of pregnancy and at 3 months postpartum.

   I hypothesized that maternal Hb, hepcidin and SF would decrease, and maternal sTfR and sTfR:SF would increase as pregnancy progressed. Similarly, through this thesis research it was determined that concentrations of maternal Hb, hepcidin and SF decreased whereas sTfR and sTfR:SF increased throughout pregnancy. Maternal Hb and SF concentrations rebounded by 3 months postpartum. These changes suggest that there is a decline in maternal iron status across gestation. Although the prevalence of anemia was relatively low, there was a high risk of maternal iron storage depletion during the 3rd trimester. If a pregnant person were to experience another complication during this time, such as an infection, depleted iron stores could put them and their offspring at a higher risk for adverse outcomes as this micronutrient has been implicated in immunomodulatory processes (Dutt et al., 2022; Pradhan et al., 2020).

2. Determine how maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations change between the 2nd trimester and 3 months postpartum.

   I hypothesized that maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be lower during the 2nd trimester compared to 3 months postpartum. In contrast, maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations were lower at 3 months postpartum compared to the 2nd trimester. These results suggest that maternal vitamin D status is lower at 3 months postpartum compared to mid-pregnancy, supporting the need for more studies that assess the postnatal status of maternal vitamin D.

3. Describe the combined status of maternal iron and vitamin D during the 2nd trimester.

I hypothesized that the majority of pregnant participants would have a replete status of iron and vitamin D during the 2nd trimester. It was determined that among participants with SF and 25(OH)D data available in the 2nd trimester, ~63% were replete in both micronutrients (category 1), ~15% were iron replete but low in vitamin D (category 2), 18% were vitamin D replete but low in iron (category 3) and ~4% were low in both (category 4). Although the majority were replete for iron and vitamin D during mid-pregnancy, ~37% had a low status in one micronutrient or both, even in this generally healthy cohort. This variable was only calculated during the 2nd trimester because of data availability, but it could be hypothesized the proportion of participants that fall into the vitamin D replete but low in iron (category 3) or the low status for both (category 4) groups might increase during the 3rd trimester considering the high prevalence of maternal iron storage depletion during this time. Similar analyses should be replicated in other pregnant cohorts.

Objective 2: Determine the relationships between maternal concentrations of systemic iron biomarkers and birth outcomes.

1. Assess relationships between 1st, 2nd or 3rd trimester maternal EPO, Hb, hepcidin, hepcidin:EPO, SF, sTfR and sTfR:SF concentrations and BWs.

   I hypothesized that lower 1st, 2nd or 3rd trimester maternal Hb, hepcidin, hepcidin:EPO and SF, and higher maternal EPO, sTfR and sTfR:SF, would be associated with lower BWs. In contrast, inverse associations between concentrations of several maternal iron biomarkers during the 3rd trimester and BWs and BHCs were observed, which were often offspring sex-specific. Higher 3rd trimester maternal concentrations of hepcidin:EPO and SF were associated with lower BWs in male and female neonates. Higher maternal Hb and hepcidin during the 3rd trimester were also associated with lower BWs, but only in males. The negative direction of relationships between 3rd trimester maternal iron biomarker concentrations and BWs were consistent among male and female neonates. This could suggest that a particularly high maternal iron status during the 3rd trimester, when the fetal endowment of maternal iron is the highest (Bradley et al., 2004; Glasser et al., 1968), might predict lower BWs in both sexes. However, more maternal iron biomarkers (Hb, hepcidin,

hepcidin:EPO and SF) were related to BWs in male newborns, compared to females (hepcidin:EPO and SF).

2. Assess relationships between 1st, 2nd or 3rd trimester maternal EPO, Hb, hepcidin, hepcidin:EPO, SF, sTfR and sTfR:SF concentrations and BHCs.

   I hypothesized that lower 1st, 2nd or 3rd trimester maternal Hb, hepcidin, hepcidin:EPO and SF, and higher maternal EPO, sTfR and sTfR:SF, would be associated with lower BHCs. In contrast, higher maternal SF concentrations during the 2nd trimester and higher maternal Hb during the 3rd trimester were independently associated with smaller BHCs among male newborns but not females.

   Together, the findings from objective 2 might imply that maternal iron biomarkers are more predictive of BW and BHC outcomes if the fetus is male. Significant differences in 3rd trimester concentrations of six maternal iron biomarkers depending on fetal sex were also detected; pregnant participants carrying a female fetus consistently had a lower iron status during the last trimester compared to those with males. There could be differences in the timing or regulation of maternal iron transport depending on the biological sex of the fetus.

Objective 3: Determine the relationships between maternal concentrations of systemic iron and vitamin D biomarkers and maternal antenatal and postpartum depression symptoms.

1. Assess relationships between 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin, SF, sTfR and sTfR:SF concentrations and maternal EPDS scores during the 3rd trimester and at 3 months postpartum.

   I hypothesized that lower 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin and SF, and higher maternal sTfR and sTfR:SF, would be associated with higher maternal EPDS scores at both timepoints. Similarly, inverse linear relationships between maternal iron status biomarkers during mid-pregnancy and maternal EPDS scores during the 3rd trimester were observed. Specifically, higher maternal hepcidin and SF during 2nd trimester were independently predicted lower 3rd trimester maternal EPDS scores. However, there

were no associations between maternal iron biomarkers at any timepoint and maternal EPDS scores at 3 months postpartum.

2. Assess relationships between 2nd trimester or 3 month postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations and maternal EPDS scores collected during the 3rd trimester and at 3 months postpartum.

   I hypothesized that lower 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be associated with higher maternal EPDS scores at both timepoints. Indeed, higher 2nd trimester 25(OH)D3 concentrations linearly predicted lower EPDS scores during the 3rd trimester. Like iron, there were no associations between maternal vitamin D biomarkers at any timepoint and maternal EPDS scores at 3 months postpartum.

3. Determine if maternal EPDS scores during the 3rd trimester or at 3 months postpartum differ depending on the combined status of maternal iron and vitamin D during the 2nd trimester.

   I hypothesized that compared to pregnant participants that were replete in iron and vitamin D during mid-pregnancy, maternal EPDS scores at both timepoints would be higher if participants had a low status in one or both micronutrients. Similarly, compared to pregnant participants that were replete for both micronutrients during the 2nd trimester (category 1), those that were low in iron and vitamin D replete (category 3) or low in iron and vitamin D (category 4) had significantly higher 3rd trimester EPDS scores.

   The results from the objective 2 suggest that a lower maternal iron and vitamin D status during the 2nd trimester, independently and together, significantly predicted more symptoms of maternal depression during the 3rd trimester. These findings imply that maternal nutrient status during an earlier pregnancy timepoint is related to maternal mental health outcomes at a later pregnancy timepoint. Therefore, ensuring that pregnant individuals are replete for iron and vitamin D during mid-pregnancy, before the majority of iron is mobilized to the fetus (Bradley et al., 2004; Glasser et al., 1968), may be

associated with less depressive symptoms in the last trimester. As there were no associations between maternal iron or vitamin D status biomarkers with maternal depression symptoms at 3 months postpartum, the nutritional risk factors for antenatal and postpartum depression symptoms may differ.

Objective 4: Determine the relationships between maternal concentrations of systemic iron and vitamin D biomarkers and child internalizing and externalizing behaviours at age 5.

1. Assess relationships between 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin, SF, sTfR and sTfR:SF concentrations and BASC-2 child internalizing and externalizing T-scores at age 5.

   I hypothesized that lower 1st, 2nd or 3rd trimester or 3 months postpartum maternal hepcidin and SF, and higher maternal sTfR and sTfR:SF, would be associated with higher child internalizing and externalizing T-scores at age 5. In contrast, higher maternal sTfR:SF during the 3rd trimester was associated with lower externalizing T-scores in female but not male children at age 5. There were no significant relationships between maternal iron biomarker concentrations at any timepoint and child internalizing scores at age 5. Therefore, the depletion of maternal iron status during the 3rd trimester, as suggested by higher maternal sTfR:SF, may be related to less externalizing behaviours in 5-year-old female children. This result along with the findings from objective 2 are similar in that maternal iron biomarkers during the 3rd trimester were significantly related to birth outcomes and behaviours in children at age 5, suggesting that the surveillance of maternal iron status during this timepoint may be predictive of multiple offspring outcomes.

2. Assess relationships between 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations and BASC-2 child internalizing and externalizing T-scores at age 5.

   I hypothesized that lower 2nd trimester or 3 months postpartum maternal 25(OH)D3 and 3-epi-25(OH)D3 concentrations would be associated with higher child internalizing and externalizing T-scores at age 5. Similarly, it was observed that there was an inverse relationship between maternal 3-epi-25(OH)D3 concentrations at 3 months postpartum and

externalizing T-scores in male but not female children. There were no significant relationships between maternal vitamin D biomarker concentrations at any timepoint and child internalizing scores at age 5. Given these findings, a higher maternal status of 3-epi-25(OH)D3 may be predictive of prospective externalizing behaviours in males at age 5.

3. Determine if BASC-2 child internalizing or externalizing T-scores at age 5 differ depending on the combined status of maternal iron and vitamin D during the 2nd trimester.

   I hypothesized that compared to pregnant participants that were replete in iron and vitamin D during mid-pregnancy, child internalizing and externalizing T-scores at age 5 would be higher if participants had a low status in one or both micronutrients. Compared to those with a mother that was replete for both micronutrients during the 2nd trimester (category 1), female children had significantly higher externalizing T-scores if maternal iron was replete and vitamin D was low (category 2), and male child had higher externalizing T-scores if maternal iron was low and vitamin D was replete (category 3).

   The results from objective 4 suggest that more externalizing behaviours at age 5 are associated with a lower maternal vitamin D status during the 2nd trimester and a higher maternal iron status during the 3rd trimester in female children, but a lower maternal iron status during the 2nd trimester and a lower maternal vitamin D status at 3 months postpartum in males. It is unclear why the micronutrient and timing of these relationships differ between female and male children, but it could imply that the timing of fetal demands for iron and vitamin D during neurodevelopment change depending on fetal sex. Indeed, maternal SF concentrations during the 2nd trimester were also related to BHCs only in males, further supporting the potential importance of maternal iron availability during mid-pregnancy for growth and neurodevelopment in this sex. There is also evidence that child internalizing behaviours at age 5 do not appear to be associated with maternal iron or vitamin D status, independently or together, during gestation or at 3 months postpartum.

6.2 Strengths and limitations of the thesis research

The meticulous design of the APrON study enabled the collection of an extensive set of sociodemographic, nutritional and medical data from pregnant participants and their children (see

Chapter 1.4) (Kaplan et al., 2014; Letourneau et al., 2022). Access to this data was crucial to enable the adjustment of statistical models for potential confounders to isolate associations between the exposure and outcome variables of interest (Williams et al., 2018). Moreover, a review of existing literature highlighted that there has been a limited number of investigations that quantified iron and vitamin D biomarkers during gestation in large cohorts of pregnant people, especially those from North America (O'Brien & Ru, 2017). In the APrON study, maternal concentrations of multiple types of maternal iron and vitamin D biomarkers, including many that are not routinely measured in healthy populations (O'Connor et al., 2016; Teichman et al., 2021), were quantified at multiple timepoints (Kaplan et al., 2014; Letourneau et al., 2022). This design not only addressed identified knowledge gaps (O'Brien & Ru, 2017), but it also allowed for the determination of time-specific relationships between maternal biomarkers and key health outcomes. For example, inverse relationships between 3rd trimester maternal concentrations of several iron biomarkers and BWs were observed because these metabolites were quantified at this timepoint. If maternal iron biomarkers were only measured during the 1st or 2nd trimesters, these relationships would not have been examined or detected in this cohort. Therefore, the APrON study design provided an opportunity to generate evidence about when the clinical screening of particular biomarkers, during or after pregnancy, may be the most informative of prospective health outcomes. Further, the inclusion maternal EPDS scores collected during the 3rd trimester and at 3 months postpartum facilitated the determination of whether maternal iron and vitamin D status were risk factors for antenatal and postpartum maternal depression symptoms. Presently, only a limited number of studies have assessed whether maternal nutrition is associated with maternal depression at both time periods in the same investigation (Gowtham et al., 2022; Ribamar et al., 2022). Finally, during analyses of relationships between maternal iron and vitamin D status and child outcomes, offspring sex was treated as an EM, necessitating the stratification of male and female statistical models. Given the compiling evidence that there might be differences in in utero developmental programming depending on the sex of the fetus (DiPeitro & Voegtline, 2017; O'Brien, 2022), stratification enabled potential sex-specific relationships to be examined.

This thesis research was also subject to several limitations that are important to acknowledge. The observational design of the APrON study prevents the determination of causation, but deliberate steps were taken to adjust for the minimum set of confounders through the construction of DAGs and the application of change-in-estimate rules (Weng et al. 2009;

Williams et al., 2018). As there is evidence that SF concentrations may be elevated during states of inflammation (Feelders et al., 1998; Mei et al., 2017), the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia, or BRINDA, working group suggest a corrective equation (Namaste et al., 2017). This model requires the input of two inflammatory biomarkers, including alpha-1-acid glycoprotein, that was not quantified in the APrON study. However, whether there is significant inflammation, aside from acute infections, over the course of a healthy pregnancy remains to be confirmed (Challis et al., 2009; WHO, 2020), despite limited evidence (Palm et al., 2013). Still, if it was warranted by the appropriate DAG (Figures 10-12), 3rd trimester CRP concentrations were included as potential confounders if indicated after the two- step method (Weng et al., 2009). Moreover, more pregnant people were recruited into the APrON study during the 2nd trimester compared to the 1st trimester (Kaplan et al., 2014; Letourneau et al., 2022). Resultingly, there was less maternal ion biomarker data available during the 1st trimester, which may have influenced the significance of relationships between these exposures at this study visit and the outcomes of interest. Maternal concentrations of 25(OH)D3 and 3-epi-25(OH)D have only been quantified during the 2nd trimester and 3 months postpartum timepoints, which prevented the assessment of relationships between 1st and 3rd trimester maternal concentrations of these biomarkers and the outcomes of interest. Nonetheless, the APrON study team is hopeful that the measurement of these metabolites at the remaining trimesters will be possible in the coming years.

Another limitation is that the categorization of maternal iron and vitamin D status could only be performed during the 2nd trimester, and only in pregnant participants with SF and 25(OH)D data available at this study visit. Therefore, this analysis was only conducted in a sub-cohort of 627 pregnant participants. This difference might explain why a negative association between 2nd trimester maternal SF concentrations and externalizing T-scores in male children at age 5 was not observed in regression models, when significantly higher externalizing T-scores were detected in male children from the low iron and vitamin D replete group (category 3) compared to the reference group (category 1) during the 2nd trimester. In addition, given that APrON participants were generally of a high SES (Kaplan et al., 2014; Letourneau et al., 2022), the number of participants in the low iron and vitamin D group (category 4) was low. In the child models, these sample sizes were too small for the determination of reliable mean difference estimates in the generalized linear models following stratification for offspring sex. Therefore, a comparison of externalizing and internalizing T-scores in male and female children between the replete (category

1) and low iron and vitamin D group (category 4) was not possible. Assessments of the combined maternal adequacy of iron and vitamin D among maternal-child dyads that are at a higher risk of malnutrition or deficiency is needed. Ultimately, provided that the majority of pregnant participants in the APrON cohort are healthy with a high SES, the generalizability of the thesis findings to low SES populations or those at a higher risk of poor nutrition or obstetric complications is unknown.

6.3 Future directions

6.3.1 Short-term objectives

Beyond the assessment of iron and vitamin D status dynamics during and after pregnancy in other populations for comparability to the thesis findings, the determination of reference ranges for maternal biomarkers of both micronutrients is needed. The use of receiver operating characteristic curves or other tests that estimate the sensitivity and specificity of concentration cut-offs may help to define or validate biomarker thresholds that effectively predict nutrient deficiency or overload (Hoo et al., 2017; Søreide, 2009). A consensus on the most reliable cut-off for SF (<12, <15 or <30 μg/L) (Lee et al., 2014; Walsh et al., 2011; WHO, 2020) and 25(OH)D (<50 or <75 nmol/L) (Munns et al., 2016; Holick et al., 2011) during pregnancy is imperative because the current use of different thresholds makes comparing evidence between investigations difficult. The assessment of biomarker reference ranges in diverse cohorts of pregnant people is required as there are important characteristics that could influence inter-individual concentrations (Kant & Graubard, 2008). As many of the biomarkers measured in this research do not have a well-established quantification protocol, reported concentrations could also be biased by the specific biochemical assay used or other research-related factors. In the context of iron, there are questions as to whether high adiposity or metabolic dysfunction in pregnant people, or broadly in females of reproductive age, impacts iron availability because systemic hepcidin concentrations can be increased by the chronic inflammation that often accompany these conditions (Anelli et al., 2018; Jones et al., 2021; Nemeth & Ganz, 2014). Although the pregnant females in the APrON study are generally healthy, significant positive (univariate) associations were detected between maternal pre-pregnancy BMI and maternal concentrations of hepcidin, in the 2nd and 3rd trimesters, and CRP in the 3rd trimester prior to the thesis research. The latter results are comparable to other evidence (Dao et al., 2013; Garcia-Valdes et al., 2015; Mayasari et al., 2021). Overall, future research should

aim to understand and account for the sources of variability in maternal iron biomarker concentrations and to assess how these differences may impact the availability of this critical micronutrient before, during and after pregnancy. Given that 3rd trimester maternal concentrations of sTfR:SF and hepcidin:EPO were associated with offspring health outcomes, the quantification of these measurements across gestation, which are underreported (Delaney et al., 2021a; van Stanten et al., 2013), should be prioritized in other studies involving pregnant people. Furthermore, although the mean proportion of participants with anemia and IDA was low, a peculiar finding was that the prevalence of maternal anemia was the same during the 3rd trimester and at 3 months postpartum. This was in spite of a decrease in the prevalence of IDA and a significant rebound of SF concentrations in pregnant individuals between the two timepoints. These results suggest that there may be other contributors to maternal anemia during the postpartum period beyond iron (Breymann, 2015; Lops et al., 1995), which should be further explored. Finally, as was determined for maternal iron and vitamin D during the 2nd trimester, more studies should examine the prevalence of concurrent micronutrient deficiencies across pregnancy and postpartum.

Relationships between maternal iron and vitamin D status and child health outcomes were often moderated by offspring sex, and if similar findings are observed in future studies, prospective investigations should aim to elucidate their biological mechanisms. Evidence from the thesis research suggests that the maternal status of iron during the 3rd trimester was lower if pregnant people were carrying a female compared to a male fetus. These sex-dependent findings allude to the question of whether the regulation of fetal iron acquisition differs depending on the biological sex of the fetus, and more particularly, whether female fetuses are more efficient at endowing maternal iron by the 3rd trimester in healthy pregnancies? These questions could be addressed by examining if there are differences in the expression of important mediators of iron transport, including TfR or FPN, at the level of the placenta depending on fetal sex. The latter type of investigation could be carried out in pre-clinical models. While animal studies enable researchers to control experimental conditions to determine causality, one of the major trade-offs is the generalizability of findings to human populations (Carter, 2007), who in themselves are variable (Kant & Graubard, 2008). Still, important questions about the key biological processes that regulate iron and vitamin D dynamics and the fetal endowment of these nutrients during pregnancy remain, which necessitates pre-clinical model investigations alongside studies with human participants (Fisher & Nemeth, 2017; O'Brien et al., 2022). That is, animals model studies can

generate hypotheses that can be examined in prospective research involving humans. For example, Cao et al. (2021) reported that iron homeostatic processes in placentas were modified to a greater extent in response to maternal ID when dams gave birth to male pups compared to those that had female pups. Animal models with knockouts of genes thought to be involved in critical iron-related processes could also be employed. The outcomes of studies involving genetic modification may not only provide evidence relating to iron and vitamin D metabolism (Rees & Alcolado, 2005), but also the potential sex-specificity of mechanisms (Cao et al., 2017). Another approach could be to quantify concentrations of different iron biomarkers, like SF, sTfR or sTfR:SF, in umbilical cord blood from fraternal twins, each with a different biological sex. Although previous reports have suggested that males may be at a higher risk for ID during infancy (Campbell et al., 2020; Domellöf et al., 2002), differences in the maternal environment during pregnancy and potential postnatal contributors to iron status adequacy in infants would be eliminated in twin investigations. Given the findings in this thesis, it could be hypothesized that the iron status of cord blood would be higher in female compared to male twins at delivery.

A crucial next step in the consistency of the reported associations between maternal iron and vitamin D status and depressive symptoms is to determine whether these relationships are observed in other groups of pregnant people, especially in those at a higher risk of mental illness. In the APrON cohort, the prevalence of maternal probable depression was low at approximately 6% and 4% during the 3rd trimester and 3 months postpartum, respectively (Table 14). A cohort with a higher incidence of maternal depression during or after pregnancy may improve the statistical power for detecting associations between maternal depression and iron or vitamin D biomarker concentrations. Moreover, considering that maternal iron and vitamin D biomarkers only appeared to predict antenatal but not postpartum depression symptoms, the determination of maternal mental health trajectories among the APrON cohort could be conducted (Chow et al., 2019; Korja et al., 2018; Vanwetswinkel et al., 2022). Trajectory analyses enable an assessment of how mental health symptoms change overtime, providing a retrospective and prospective context. An objective of future research could be to examine whether certain maternal iron and vitamin D biomarkers significantly predict a certain trajectory of maternal depression symptoms. Based on the thesis findings, it could be hypothesized that a lower maternal iron and vitamin D status during the 2nd trimester, either independently or in combination, could be associated with a trajectory containing higher 3rd trimester EPDS scores.

As maternal concentrations of several biomarkers were significantly related to externalizing T-scores in children at age 5, it would be insightful to examine whether these metabolites are associated with a particular type of behaviour that is assessed within the BASC-2 externalizing composite score (Kamphaus, 2014; Reynolds, 2010). In other words, the determination of relationships between maternal iron and vitamin D biomarker concentrations during and after pregnancy with BASC-2 hyperactivity, conduct problems and aggression scores in 5-year-old children. In addition, previous evidence suggests that when a pregnant person experiences more depressive symptoms during pregnancy, their offspring may subsequently have a higher risk of behaviour problems during childhood (Giallo et al., 2015; Glover, 2014). Considering the latter and that maternal EPDS scores and child externalizing T-scores were both independently related maternal iron and vitamin D biomarker concentrations, maternal mental health may a mediator in the potential relationships between maternal nutrient status and child externalizing behaviours at age 5. Therefore, it might be prudent to examine whether maternal iron and vitamin D biomarkers are related to child externalizing T-scores in cohorts that are at a higher risk of maternal stress or depression. A mediation analysis between maternal iron or vitamin D status, depression symptoms and child behavioural outcomes could also be conducted if statistically indicated (Lockword et al., 2010; Maric et al., 2012). Regardless of the study design, the sex-dependency of findings in the thesis provides a rationale for stratification by offspring sex if fetal, infant or child outcomes are assessed in the future.

Finally, as briefly introduced in Chapter 1, there is mounting evidence supporting the influence of pre-pregnancy nutrient status on pregnancy outcomes (Aranda et al., 2011; Davies et al., 2021; Viteri & Berger, 2005). Although pre-conception concentrations of iron and vitamin D biomarkers were not quantified in the APrON study (Kaplan et al., 2014; Letourneau et al., 2022), examining each thesis objective with pre-pregnancy iron and vitamin D biomarkers as predictors could help to determine whether pre-pregnancy screening of maternal iron, vitamin D or both may be informative of prospective maternal and offspring health outcomes.

6.3.2 Long-term objectives

In the long-term, experimental or clinical trials followed by meta-analyses to synthesize a rigorous body of evidence should be prioritized. Despite previous randomized controlled trials (RCTs) (Iglesias-Vázquez et al., 2022; Jayasinghe et al., 2018; Pérez-López et al., 2015),

investigations the recruit healthy pregnant participants are limited in this area. It is critical to determine whether the current Canadian RDAs for iron and vitamin D (Health Canada, 2009; Health Canada, 2010; IOM, 2006) are enough to sustain maternal nutrient adequacy in diverse populations, especially among those that may be at a higher risk of a low status or deficiency in one or both micronutrients (Kulkarni et al., 2022; Pearce & Cheetham, 2010). Considering the findings from in thesis, forthcoming RCTs could also examine whether a lower maternal iron and vitamin D status during mid-pregnancy might predict more depressive symptoms in the 3rd trimester, provided that this health outcome is specifically assessed.

If there is more observational evidence suggesting that concurrent maternal deficiencies of multiple micronutrients might interact during pregnancy to influence health outcomes, the employment of RCTs may serve to confirm these findings. In the context of iron and vitamin D, an experimental study design recruiting individuals before or during pregnancy could contain 4 groups: those receiving: 1) a placebo (iron and vitamin D RDAs; standard-of-care), 2) an additional iron supplement only (total iron dose below the iron upper limit), 3) an additional vitamin D supplement only (total vitamin D dose below the vitamin D upper limit) and 4) an additional iron and vitamin D supplement (total doses below upper limits of both micronutrients). After taking steps to ensure the randomization of group placement and homogeneity of potential confounding variables among participants, differences in pregnancy outcomes, such as antenatal maternal depression or child neurodevelopment, depending on the supplemental regimen could be examined.

Ideally, the hope is that reliable reference ranges for maternal nutrient biomarkers during, and possibly before, pregnancy will be established and universally validated in a variety of clinical settings. These ranges should consistently predict the most optimal maternal and offspring health outcomes. Considering the potential risks of deficiency and overload of iron and vitamin D (Ng et al., 2019; Dinour et al., 2015; Schoenmakers et al., 2020), the cut-off concentrations that define not just the low, but also the high ends of biomarker reference ranges are needed for both micronutrients. If these ranges are validated across high-quality observational studies, RCTs and meta-analyses, the evidence may be used to inform updates to nutritional recommendations or clinical screening practices during pregnancy.

6.4 Final conclusions

Overall, the only maternal iron biomarker that was significantly related to BWs, BHCs, maternal depression symptoms and child externalizing behaviours was SF. When measured in the 2nd trimester, higher maternal SF concentrations predicted lower BHCs in male newborns, less maternal depressive symptoms during the last trimester and less externalizing behaviours in 5-male children at age 5. In the 3rd trimester, higher maternal SF concentrations predicted lower BWs in male and female newborns. Although the replication of these results in other populations of gestating people are warranted, the findings from this thesis research suggest that maternal SF concentrations during the 2nd and 3rd trimester could be informative of future maternal and offspring health outcomes in generally healthy pregnant individuals and their children. Significant inverse relationships between the maternal status of vitamin D and maternal depression symptoms and child externalizing behaviours at age 5 were reported, but evidence supporting the potential predictive ability of a specific vitamin D biomarker is less convincing. There should be an emphasis on assessing the impact or interaction of concurrent micronutrient deficiencies on maternal and offspring health outcomes in future research.

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APPENDIX 1: IRON

1.1 Additional details of iron homeostasis and biology

1.1.1 Intracellular trafficking

To avoid the oxidative toxicity of excess iron (Meneghini, 1997), its movement and transport is tightly controlled in cells (Ganz, 2013). The mitochondria have the highest demands for iron within the cell (Gao et al., 2021; Levi & Rovida, 2009), but the way the iron is acquired into this organelle is unconfirmed (Lange et al. 1999; Mastroberardino et al., 2009). Still, there is evidence for direct fusion between endosomal and mitochondrial membranes allowing for ferrous iron entry into the mitochondria (Dutt et al., 2022). There may also be a pools of cytosolic iron that enter the mitochondria through endocytosis (Gao et al., 2021).

The transport of iron-containing macromolecules, such as enzymes, around the cell is even less explored (Leung et al., 2021). Given that the majority of heme is produced in the mitochondria (Gao et al. 2021; Levi & Rovida 2009), this organelle is thought to be the first location is a series of transportation steps (Dutt et al., 2022). Movement may vary depending on the final destination or function of a hemoprotein. For example, some molecules may be transferred directly into the cytosol, or trafficked out of the cell (Yang et al., 2010). Others suggest that inter-membrane systems, like mitochondrial associated membranes, facilitate the movement of iron-related proteins (Chambers et al., 2021; Neuspiel et al., 2008).

1.1.2 Recycling and elimination

The iron released during the breakdown of red blood cells (RBCs) is a major biological source of the mineral (Sukhbaatar & Weichhart, 2018). Resident macrophages present in the spleen and liver, respectively known as red pulp macrophages and Kupffer cells, are largely responsible for scavenging old RBCs for iron recycling (Fang et al., 2009; Pradhan et al., 2020; Saba, 1970). After engulfment, the merging of RBCs with lysosomal compartments leads to the degradation of heme and hemoglobin, which liberates iron (Medina et al., 2020; Rajagopal et al., 2008).

Apart from specific infections, hemorrhages and menstruation (Chiabrando et al., 2014), regulated iron excretion was historically thought to be non-existent (Dubach et al., 1955; McCance & Widdowson, 1937). Despite this, Prajapati et al. (2021) recently observed that an unbound form of systemic iron, often referred to as non-transferrin bound iron (Brissot et al., 2012), may be stored

in the liver and subsequently excreted through the biliary system. There is also evidence in animal models that iron is excreted by gastrointestinal enterocytes (Mercadante et al., 2019).

1.2 Biological details of the iron biomarkers of interest

1.2.1 Serum ferritin (SF)

After iron is transported into a cell, it can have multiple fates (Cook et al., 1974; Dutt et al., 2022). If it is not required for incorporation into key enzymes or macromolecules, poly(rC)-binding protein may traffic excess ferric iron to ferritin for storage (Patel et al., 2021). A single cytosolic ferritin molecule has been reported to carry up to 4500 Fe^{3+} ions (Watt, 2011). If the cellular demand for iron increases, ferritin-iron complexes can be shuttled to lysosomal compartments for breakdown (Fujimaki et al., 2019). Occasionally, lysosomal and ferritin fragments can be combined into hemosiderin, another form of stored iron typically encountered in macrophages (Leftin et al., 2017). The translation of ferritin is upregulated when intracellular iron concentrations increase (Kawabata, 2019; Muckenthaler et al., 1998).

In the circulation, serum ferritin (SF) contributes to iron chelation and transport (Plays et al., 2021). Interestingly, TfR1 has a SF-specific binding site, facilitating another method of cellular iron uptake, which has been reported in glial cells and neurons in the brain (Aschner & Aschner, 1990). In the context of pregnancy, maternal SF has been reported to facilitate iron delivery to the placenta by binding receptors on the syncytiotrophoblast (STB) (Li et al., 2009). Moreover, previous structural investigations of SF have revealed the presence of several iron binding pockets, which could aid in the in-vivo delivery of compounds or molecules beyond iron for a variety of clinical applications (Chen et al., 2020; Jian et al., 2016).

1.2.2 Hepcidin

The vast majority of hepcidin, which is encoded by the *human antimicrobial peptide* (*HAMP)* gene, is derived from hepatocytes (Park et al., 2001). The biosynthesis of this iron regulator is controlled through transduction pathways in response to an array of extracellular mediators (Jordan et al., 2009). One of the most significant pathways is the bone morphogenic protein (BMP) cascade involving the binding of BMP2 and BMP6 on hepatocytes (Babitt et al., 2006), which signals the upregulation of *HAMP* expression (Wang et al., 2019). When the demand for iron is high during periods of increased erythropoiesis, increased ERFE concentrations

downregulate hepcidin by interrupting BMP signalling (Kautz et al., 2014). Other ligands, like diferric Tf, or membrane proteins, such as TfR2 and hemojuvelin, may also regulate the expression of hepcidin (Dutt et al., 2022).

1.2.3 Erythropoietin (EPO)

The synthesis of EPO is dependent on the nuclear binding of hypoxia inducible factor (HIF) with other transcription factors to promote the expression of *EPO* gene transcripts (Kuhrt & Wojchowski, 2015; Peng et al., 2020). The latter phenomenon occurs during hypoxia. During normoxia, HIF, which is constitutively expressed, is chemically modified with hydroxyl groups and is digested via ubiquitin-lysosomal pathways. The enzyme that catalyzes this hydroxylation is called HIF prolyl hydroxylase domain and requires diatomic oxygen to function (Haase, 2017). Therefore, when oxygen is present in EPO-producing cells, EPO is not produced. Interestingly, other oxygen containing biomolecules, like succinate, may be able to inhibit the HIF prolyl hydroxylase domain enzyme to increase EPO expression (Haase, 2017).

The functionality of EPO may extend beyond iron homeostasis and RBC erythropoiesis (Nairz et al., 2012). In fact, EPO mRNA transcripts have been discovered in different tissues, including in bone, heart, lungs and immune cells (Lacombe & Mayeux, 1999). Protective phenotypes may be promoted by EPO through heterodimeric tissue-protective receptors (TPRs), which are comprised of one EPO receptor and one β common receptor (Peng et al., 2020). TRPs have been localized on the plasma membranes of innate and adaptive immune cells, and there is increasing evidence to support its role in anti-inflammatory cascades. For example, under the influence of EPO, macrophages may secrete less pro-inflammatory cytokines and phagocytose damaging apoptotic fragments with enhanced efficiency via TRP-dependent signalling (Nairz et al., 2012). A similar transduction pathway has also been shown to shift T helper cells to regulatory T cells (Tregs) (Peng et al., 2020). Finally, EPO may promote neuronal and glial cell survival function (Noguchi et al. 2007), which is reasonable considering it has been observed to traverse the blood brain barrier (Brines et al., 2000).

APPENDIX 2: VITAMIN D

2.1 Additional details of vitamin D homeostasis, biology and function

2.1.1 Hormonal activation

The synthesis of 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is controlled by several regulators (Dominguez et al., 2021). Parathyroid hormone and estradiol have been shown to stimulate $1,25(OH)_2D$ production (Bergwitz & Jüppner, 2010; Hagenfeldt et al., 1991). In contrast, fibroblast growth factor 23 and high serum calcium downregulate $1,25(OH)_2D$ (Shimada et al., 2004). When the abundance of $1,25(OH)_2D$ increases, deactivating hydroxylases chemically degrade $1,25(OH)_2D$ and 25-hydroxyvitamin D (25(OH)D) (Zierold et al., 1994).

2.1.2 Cellular uptake

Mechanisms of vitamin D uptake into cells may be more tissue-specific than originally recognized (Bikle et al., 2018; Bikle & Christakos, 2020). Along with other lipid-based molecules, 25(OH)D and its derivatives can passively diffuse through the plasma membrane of cells (Mendel, 1989). This transport can occur after vitamin D metabolites have been dislodged from vitamin D binding protein (DBP) or albumin carriers (Bhan et al., 2012; Chun et al., 2014; Imawari et al., 1976). However, in some tissues the cellular uptake of 25(OH)D may depend on membrane proteins megalin and cubilin (Bikle & Christakos, 2020; Nykjaer et al., 1999). Renal tubular, parathyroid gland and placental STB cells may express megalin-cubilin (Burke et al., 2013), which have been shown to bind and transport DBP-25(OH)D complexes (Christensen & Birn, 2002).

2.1.3 Storage and excretion

As steroids, most vitamin D metabolites can diffuse into adipose tissues and to a lesser extent, skeletal muscle, for long-term storage (Dominguez et al., 2021). 25(OH)D is the most abundant vitamin D metabolite detected in subcutaneous or organ-related adipocytes (Pramyothin et al., 2011). Conversely, with an additional hydroxyl functional group, $1,25(OH)_2D$ is less likely to aggregate in fat stores (Jenkinson, 2019). When there is a large influx of vitamin D into the blood stream, regardless of its origin, storage occurs relatively quickly (Ghosh et al., 2022; Speeckaert et al., 2006). Accordingly, previous studies have reported strong positive associations

between systemic and fat deposited vitamin D (Didriksen et al., 2015). The release of vitamin D from adipose stores is gradual, a possible protection against toxicity (Rosenstreich et al., 1971).

Alternatively, if the status of vitamin D is exceeding its physiological demands, certain compounds can be enzymatically catabolized for excretion (Jenkinson, 2019). High $1,25(OH)_2D$ or fibroblast growth factor 23 concentrations may lead to the chemical deactivation of $1,25(OH)_2D$ by 24-hydroxylases, predominantly in the kidney (Zierold et al., 1994). Elevated 25(OH)D can also be catabolized by 24-hydroxylases (Jenkinson, 2019). Subsequently, a cascade of hydroxylation reactions eventually generates calcitroic acid and other waste metabolites that can either be secreted through the biliary system or concentrated in urine (Reddy & Tserng, 1989).

2.1.4 Immunomodulation

Vitamin D has been shown to activate certain innate cells while inhibiting others, leading to a generally anti-inflammatory first line of defense (Chen et al., 2007; Chen et al., 2017; Meza-Meza et al., 2022). This micronutrient may enhance the maturation and actions of natural killer cells, monocytes and macrophages while hampering the differentiation of dendritic cells (Balogh et al., 1999; Cyprian et al., 2019; Penna & Adorini, 2000). This could lead to the clearance or destruction of foreign materials by several innate cells, while preventing a widespread activation of adaptive cells and subsequent inflammation. In addition, vitamin D receptor (VDR) signaling has been implicated in the upregulation of cathelicidin and defensins, two types of antimicrobial peptides (Wang et al., 2004), as well as several lysosomal enzymes (Lerner, 1980). Moreover, vitamin D has been observed to promote the adoption of an M2 phenotype by macrophages, a state that prioritizes anti-inflammatory pathways and the repair of damaged tissues (Liang et al., 2019). Inflammatory conditions may also be resolved by anti-inflammatory cytokines, like interleukin 4 and 10, which were increased by $1,25(OH)_2D$ in several studies (Bakdash et al., 2014; Cantorna et al., 2015).

The programming of B and T cells towards tolerogenic phenotypes may also be promoted by vitamin D (Chen et al., 2007; Liang et al., 2019; Meza-Meza et al., 2022). A body of evidence suggests that vitamin D plays a crucial role in the production of a subset of T cells called Tregs (Jeffery et al., 2009); in fact, $1,25(OH)_2D$-VDR activities have been documented to increase fully activated populations of Tregs (Cyprian et al., 2019). As their name implies, Tregs are known for their regulatory, anti-inflammatory or tolerogenic properties (Roncarolo et al., 2001). Finally,

1,25$(OH)_2$D might impair humoral immune function by downregulating key aspects of B cell development and function, including cell division, survival and antibody production (Morgan et al., 2000; Chen et al., 2007).

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