# Risk of Iron Deficiency in Naturally Menstruating Women

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## Abstract

**Background:** Iron deficiency is a common nutrient deficiency that disproportionately affects women and is often attributed to menstrual blood loss in reproductive-aged women. Although iron biomarker fluctuations across the menstrual cycle have been reported in eumenorrheic women, the relationship between iron status and subclinical ovulatory disturbances (SOD) remains unclear. Studies are yet to explore iron biomarkers in women across multiple menstrual cycles, while characterising hormonal profiles for comparisons both between and within ovulatory status groups.

**Objectives:** To investigate variations in iron status between and within the menstrual cycle, considering ovulatory status and the influence of SOD in naturally menstruating women.

**Methods:** Ninety-seven healthy, naturally menstruating women participated in a prospective longitudinal cohort study across three to five menstrual cycles. Participants attended six laboratory sessions across the early-follicular, late-follicular and mid-luteal phases of the menstrual cycle. Serum ferritin (SF), haemoglobin (Hb), serum iron (SI), total iron-binding capacity (TIBC), transferrin saturation (TSAT) and C-reactive protein (CRP), were analysed alongside female sex steroid hormones (oestradiol and progesterone). Ovulatory status was determined using mid-luteal progesterone, with ≥10nmol/L classified as ovulatory and <10nmol/L or a luteal phase <10 days classified as SOD. A multiple linear regression analysis was conducted to identify predictors of SF change from baseline to study end.

**Results:** At baseline, 27.8% of participants were iron-insufficient (SF <30μg/L), while 70.1% and 18.6% of participants were classified as ovulatory or SOD, respectively. Iron biomarker concentrations were comparable between groups in all phases, except for higher TSAT in the late-follicular phase among ovulatory participants ($p$=0.049). All iron biomarkers, except Hb ($p$=0.942), significantly differed across menstrual cycle phases among ovulatory participants, whereas no such variation was observed among SOD participants. Change in CRP was the only significant predictor of SF change across the study (β=1.58 (95% CI 0.84, 2.32), $p$<0.001).

**Conclusion:** Menstrual cycle phase fluctuations in iron biomarkers were evident only in ovulatory women, suggesting altered iron regulation in those with SOD. Although the mechanisms underlying cyclical variations in iron biomarker concentrations among ovulatory women remain unclear, our study provides novel insights into iron status in women who present with SOD.

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# List of Abbreviations

| Abbreviation | Term |
|---|---|
| AGP | α-1 acid glycoprotein |
| ANOVA | Analysis of variance |
| BMI | Body mass index |
| BRINDA | Biomarkers Reflecting the Inflammation and Nutritional Determinants of Anaemia |
| CI | Confidence interval |
| CRP | C-reactive protein |
| DMT1 | Divalent metal transporter 1 |
| DNA | Deoxyribose nucleic acid |
| EAR | Estimated average requirement |
| Fe | Iron |
| $Fe^{2+}$ | Ferrous iron |
| $Fe^{3+}$ | Ferric iron |
| FeFFQ | Iron food frequency questionnaire |
| FFQ | Food frequency questionnaire |
| FSH | Follicle-stimulating hormone |
| Hb | Haemoglobin |
| HCP1 | Haem carrier protein 1 |
| HMB | Heavy menstrual bleeding |
| ID | Iron deficiency |
| IDA | Iron deficiency anaemia |
| IDMH | Iron deficiency with microcytosis and/ or hypochromia |
| IDWA | Iron deficiency without anaemia |
| IL-6 | Interleukin-6 |
| LH | Luteinising hormone |
| LPD | Luteal phase defect(s) |
| MC | Menstrual cycle(s) |
| MCH | Mean cellular haemoglobin |
| MCV | Mean cellular volume |
| NZ | New Zealand |
| PdG | Pregnanediol-3-glucuronide |
| RDI | Recommended dietary intake |
| RNI | Reference nutrient intake |
| SF | Serum ferritin |
| SH | Serum hepcidin |
| SI | Serum iron |
| SOD | Subclinical ovulatory disturbance(s) |
| sTfR | Soluble transferrin receptor |
| TIBC | Total iron-binding capacity |
| TSAT | Transferrin saturation |
| WHO | World Health Organisation |
| ZnPP | Zinc protoporphyrin |

# Chapter 1: Introduction

Nutrition plays a crucial role in promoting the optimal health and well-being of women throughout various life stages. Women have unique nutritional needs due to the physiological and hormonal changes that occur through adolescence, commencement of menstruation and the menstrual cycle, pregnancy, lactation, and menopause, leading to altered nutrient requirements during each of these phases of life (Bailey et al., 2022). Specifically, adequate nutrition is essential to achieve sufficient growth in adolescence, whilst in premenopausal years, adequate nutrition supports the maintenance of a regular menstrual cycle (Dunneram & Jeewon, 2015; Wohlgemuth et al., 2021). For main life events, sufficient dietary intake is required for a healthy pregnancy and baby (Dunneram & Jeewon, 2015). Finally, due to the protective role of oestrogen in bone mineral density and cardiovascular health, optimal nutrition in women throughout adolescence and adulthood is especially important in preparation for the decline in oestrogen post-menopause and for the prevention of chronic disease (Feskens et al., 2022; McPhee et al., 2022). A key component of women's nutrition is the consumption of adequate micronutrient intakes. Adverse health effects from micronutrient deficiencies in women such as iron, folate, zinc, iodine, vitamin D, calcium, and vitamin B12 are well documented within the literature (Dunneram & Jeewon, 2015; Wakeman et al., 2020). Despite this, few women manage to achieve micronutrient sufficiency, particularly during their premenopausal years, which has the potential to not only lead to adverse health consequences for the woman, but can impact the health of future offspring (Wakeman et al., 2020).

One particularly important micronutrient for women is iron. Iron plays an essential role in the body, supporting physiological functions such as erythropoiesis, oxygen transportation and storage, cognitive development, and energy production (Badenhorst et al., 2021; University of Otago & Ministry of Health, 2011). However, iron deficiency (ID) is the most common nutritional deficiency worldwide, affecting approximately 20-25% of the general population (Coad & Conlon, 2011). Iron deficiency is classified into three stages. In stage one, iron stores in the body begin to deplete, but haemoglobin (Hb) concentration and haematopoiesis are unaffected (Clénin et al., 2015). In stage two, known as iron deficiency without anaemia (IDWA), iron stores are depleted, circulating iron levels begin to decrease, and haematopoiesis is affected (Clénin et al., 2015). However, Hb concentrations remain normal (University of Otago & Ministry of Health, 2011). Within this thesis, unless otherwise stated, ID will collectively refer to both stage one and two. Finally, in stage three, known as iron deficiency anaemia (IDA), iron stores are severely depleted, with Hb concentrations falling below the normal reference range (Clénin et al., 2015). Iron deficiency is associated with a number of negative health consequences including deficits in cognitive function, reduced physical performance, and pregnancy complications, with further symptoms

including fatigue, lethargy and poor mood (Badenhorst et al., 2021; Beck et al., 2013; Coad & Conlon, 2011). Conversely, iron overload is also associated with a range of negative health consequences, emphasising the need for iron balance (Coad & Pedley, 2014).

Increased iron requirements, low dietary iron intakes, and increased blood loss are key causes of ID, all of which disproportionately impact women (Coad & Conlon, 2011). Subsequently, ID is especially prevalent in women of reproductive age who regularly menstruate (Coad & Conlon, 2011). Findings from the most recent New Zealand Adult Nutrition Survey (2008/09) revealed that 7.2% of New Zealand women were iron deficient (serum ferritin <12μg/L), with the highest prevalence of 12.1% among 31–50-year-olds (University of Otago & Ministry of Health, 2011). However, the serum ferritin (SF) threshold of <12μg/L for ID is noted as a relatively low cut-off, typically indicating depleted iron stores associated with IDWA and IDA and could potentially underestimate the prevalence of ID in New Zealand women (Clénin et al., 2015). For example, when the SF threshold was set at <20μg/L, Beck et al. (2013) found that 18.7% of female participants aged 18-44 years had suboptimal iron. Considering their heightened risk of ID, recommended dietary iron intakes for premenopausal women are more than twice that of their male counterparts and further increase by 50% during pregnancy (National Health and Medical Research Council et al., 2006). If dietary iron intake or absorption is low or exceeded by iron losses, a negative iron balance may occur, contributing to the development of ID (Badenhorst et al., 2022). In the current climate, the shift towards a more plant-based dietary pattern has seen a decline in women's red meat consumption, with 9.0% of New Zealand women in 2019/20 reporting never eating red meat compared to 6.6% in 2008/09 (Ministry of Health, 2022; University of Otago & Ministry of Health, 2011; Wakeman et al., 2020). This threatens to exacerbate ID, as non-haem iron from plant sources has lower bioavailability compared to haem iron from animal sources, leading to lower iron absorption (Wakeman et al., 2020). While iron absorption is upregulated in response to low iron stores, the body's capacity to increase iron uptake across the gastrointestinal tract is inherently limited (Coad & Conlon, 2011). Other factors contributing to ID as a result of increased iron loss in New Zealand women include blood donation, nose bleeds, exercise-related losses, and volume and duration of menstrual bleeding (Badenhorst et al., 2022; Beck, 2013; Heath et al., 2001).

Unsurprisingly, the literature often attributes ID in premenopausal women to menstrual blood loss; however, this fails to recognise that not all menstruating females are at risk of ID (Badenhorst et al., 2021). This often leads to biases in healthcare, as ID or IDA in women are assumed to be the result of menstruation without further investigation into other potential causes, such as gastrointestinal lesions (Miller, 2023). However, Miller (2023) suggests that iron homeostasis in women protects iron stores from normal iron loss during menstruation, concluding that menstruation alone should not be a risk factor for ID. The

exception to this proposal would be heavy menstrual bleeding, which is known to affect approximately 25% of women, significantly increasing susceptibility to ID and IDA (Bruinvels et al., 2016). Despite this, women who experience heavy menstrual bleeding often do not seek medical attention or are unaware that their menstrual bleeding is not normal (Bruinvels et al., 2016; Nelson & Ritchie, 2015). One study found that of women who did report heavy or very heavy menstrual bleeding, 35% were found to be anaemic (Bernardi et al., 2016). Moreover, Heath et al. (2001) reported that both the volume and duration of menstrual bleeding influence ID risk, with shorter bleeding durations associated with reduced odds of ID.

More recent literature is beginning to explore iron status changes within the menstrual cycle. Here, the research has sought to determine if there is a relationship between iron status and female sex steroid hormone fluctuations across the menstrual cycle (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016; Suzuki et al., 2018). The menstrual cycle can be divided into the follicular phase (occurring from the beginning of menstrual bleeding until ovulation) and the luteal phase (post-ovulation) (Rogan & Black, 2023). In eumenorrheic menstrual cycles, these can be broken down further into four distinct hormonal phases. In the early-follicular phase, both oestrogen and progesterone concentrations are low and menstrual bleeding is present (Rogan & Black, 2023). Oestrogen concentrations then increase with the maturation of a dominant follicle in the ovary until they peak in the late-follicular phase, while progesterone concentrations remain low (Mihm et al., 2011; Rogan & Black, 2023). The ovulatory phase is characterised by a surge in luteinising hormone (LH), rupture of the dominant follicle from the ovary (ovulation), a decline in oestrogen concentrations from their pre-ovulatory peak, and low progesterone concentrations (Mihm et al., 2011; Rogan & Black, 2023). Following ovulation and the formation of the corpus luteum, the mid-luteal phase consists of a secondary peak in oestrogen and high progesterone concentrations (Mihm et al., 2011; Rogan & Black, 2023). It is proposed that the fluctuations of these sex steroid hormones across the menstrual cycle influence various physiological processes, including nutritional status and iron regulation (Badenhorst et al., 2022; Rogan & Black, 2023). Prior research into biomarkers of iron status across the menstrual cycle reported Hb, SF, serum iron (SI), and transferrin saturation (TSAT) were highest in women during the luteal phase, and were at their lowest during menstruation, resulting in an increased prevalence of impaired iron status (Kim et al., 1993). These variations in iron status appear to coincide with hepcidin fluctuations, a hormone involved in the regulation of iron absorption, which is at its' lowest concentration during menstruation, increasing in the later phases of the cycle (Badenhorst et al., 2022). Due to the decline in hepcidin, iron uptake may be increased during the follicular phase of the menstrual cycle as a physiological response to help maintain iron balance (Badenhorst et al., 2021). However, in iron-deplete

women, there appears to be no correlation between iron biomarkers and menstrual phase (Badenhorst et al., 2022).

In healthy women, subclinical ovulatory disturbances (SOD), including anovulation and luteal phase defects (LPD), are a common occurrence and result in differing hormonal profiles from the previously described eumenorrheic cycle (De Souza, 2003). A three-month study into recreationally active females reported a 48% prevalence of combined LPD and anovulatory cycles, with a sample incidence of 79% (De Souza et al., 1998). However, in nonactive women, the prevalence of LPD is less understood but estimated to occur in 2-20% of nonactive women (De Souza, 2003). It is also recognised that it is not uncommon for women with regular menstrual bleeding to experience sporadic LPD or anovulatory cycles (De Souza, 2003; Rogan & Black, 2023). This could potentially lead to an underestimation of SOD prevalence in studies that are limited to a single cycle in duration or that only use calendar-based counting as a method of classifying menstrual cycle status (De Souza, 2003; Rogan & Black, 2023). Both under- and over-nutrition, including low energy availability and obesity, have been associated with menstrual disturbances such as SOD and amenorrhea (Kulsum & Astuti, 2019; Rogan & Black, 2023). It has been suggested that overweight or obese women are more likely to experience anovulation or menstrual irregularities due to excess fat cells leading to excess oestrogen production (Kulsum & Astuti, 2019). Opposingly, the limited quantities of fat cells present in underweight women may lead to insufficient oestrogen production required for ovulation, also resulting in possible menstrual disturbances (Kulsum & Astuti, 2019). While the influence of overall energy intake and body composition on menstrual disturbances has previously been investigated, our understanding of the association between micronutrient status and menstrual disturbances is still subject to investigation.

Existing research has investigated the association of iron status with hepcidin and female sex steroid hormones in a eumenorrheic cycle, however, this has not been conducted in a New Zealand population. Limited research to date has explored correlations between iron status and female sex steroid hormones longitudinally in women, and how this relationship is influenced by variations in female sex steroid hormones across menstrual cycles characterised by SOD. Furthermore, few studies have explored female-specific causes and risk factors of ID longitudinally or beyond menstruation (Badenhorst et al., 2022). Further research is therefore needed to address the gap in knowledge around the relationships between biomarkers of iron status and variations in female sex steroid hormones throughout the menstrual cycle and between different menstrual cycle types (eumenorrheic vs SOD). Overall, this study aims to bridge the gap in the literature, exploring iron status in New Zealand premenopausal women, the relationship to the menstrual cycle, and associated risk factors for ID.

## 1.1 Aim

To investigate variations in iron status between and within the menstrual cycle in naturally menstruating women.

### *1.1.1 Objectives*

1. To describe differences in biomarkers of iron status between menstrual bleeding, preovulation and the luteal phases of the menstrual cycle in naturally menstruating women.
2. To determine whether associations exist between female sex steroid hormones and biomarkers of iron status.
3. To determine whether differences in biomarkers of iron status exist between women with consistent ovulatory cycles (eumenorrheic) and those with an SOD (anovulation or luteal phase defect).
4. To investigate the change in iron status from baseline to end (over three to five menstrual cycles) and the factors associated with declining iron stores (e.g. menstrual bleeding duration, dietary intake, body composition, and physical activity).

### *1.1.2 Hypotheses*

1. Iron status, as reflected by serum ferritin, will be lowest during menstruation and will be highest in the mid-luteal phase of the menstrual cycle.
2. Iron deficiency in women will be associated with higher rates of luteal phase defects and/ or anovulation.
3. Increased menstrual bleeding duration and increased physical activity will be associated with reduced iron status and increased risk of ID.
4. Decreased meat intakes and/ or high BMI will be associated with a further increased risk of ID in women with prolonged menstrual bleeding.

## 1.2 Structure of Thesis

This thesis begins with an introduction to ID, and its' prevalence and known risk factors in premenopausal women. It discusses the impact of the menstrual cycle on iron status and the influence of female sex steroid hormones and SOD, concluding with the aims, objectives and hypotheses of the study. This is followed by chapter two which is an extended review of the current literature, exploring how ID is defined, prevalence and risk factors in premenopausal women, and an in-depth review of menstrual cycle physiology and relationships with iron status. Chapter three is a manuscript prepared for publication which includes an introduction, methodology, results, and discussion of the findings. Finally, chapter four provides a summary of the research, its' strengths and limitations, and how the findings contribute to the field within the New Zealand context. It concludes with final recommendations for future research in understanding ID in relation to the menstrual cycle in premenopausal women.

## 1.3 Researcher's Contributions

**Table 1.1** Summary of Researcher's Contributions to the Study

| **Researcher** | **Contribution to Thesis** |
|---|---|
| Alanah Prisk<br>MSc Nutrition and Dietetics Student | Primary author of the thesis<br>Assisted with participant recruitment, conducted statistical analysis |
| Dr Kathryn Beck<br>Primary Supervisor<br>Professor of Nutrition and Dietetics, School of Sport, Exercise and Nutrition | Co-investigator<br>Guidance on FFQ and statistical analysis |
| Dr Claire Badenhorst<br>Co-Supervisor<br>Associate Professor School of Sport, Exercise and Nutrition | Principal investigator<br>Designed research, applied for funding, applied for ethics, recruited participants, collected data, phlebotomist, processed blood samples |
| Rebecca Paul<br>Research Officer | Assisted with recruitment and data collection, compiled all data into spreadsheets |
| Dr Karen Mumme<br>Associate Professor Beatrix Jones | Cleaned data, assisted with statistical analysis |

# Chapter 2: Literature Review

## 2.0 Introduction

In New Zealand (NZ), the prevalence of iron deficiency (ID) is increasing among females aged 15 years and over from 2.9% in 1997 to 7.2% in 2008/09 (University of Otago & Ministry of Health, 2011). Iron deficiency anaemia (IDA) has recently been reported by the World Health Organisation (WHO) as affecting 29% of women aged 15-49 years globally (World Health Organisation, 2020). Given the scale and global prevalence of IDA in this population, the WHO aims to reduce anaemia in premenopausal women by 50% as part of the Global Nutrition Targets 2025 (World Health Organisation, 2024). However, to achieve this, the factors contributing to IDA must be understood. While many studies have found ID in premenopausal women to be associated with increased iron losses from menstruation, few studies have explored correlations between iron status, female sex steroid hormones, and variations in these hormones across different menstrual cycle presentations (e.g. subclinical ovulatory disturbances (SOD)), which can occur intermittently in healthy women over time (De Souza, 2003). This review aims to explore ID in premenopausal women aged 15-49 years, understanding iron regulation in the body, and examining the key factors that influence their risk of ID, with a particular focus on the role of the menstrual cycle and the hormonal fluctuations that occur.

## 2.1 Role of Iron in the Body

Iron is an essential micronutrient with a pivotal role in many physiological processes in the body. The transportation of oxygen around the body requires iron as a critical component of haemoglobin (Hb) in red blood cells known as erythrocytes (Gupta, 2014). Within Hb, iron acts as the site of oxygen binding, facilitating efficient oxygen delivery from the lungs to all tissues and organs throughout the body (Gupta, 2014). In muscle cells, oxygen is offloaded to myoglobin which requires iron similarly to Hb for the reversible storage and transport of oxygen within the muscle (Clénin et al., 2015; Gupta, 2014). Additionally, iron is also required in the production of erythrocytes, a process known as erythropoiesis (Gupta, 2014). Beyond its role in oxygen transport, iron is integral in energy production, DNA synthesis, immune function, electron transport, enzyme production, and as a component of some enzymes (Clénin et al., 2015; Coad & Pedley, 2014; Gupta, 2014). Coad and Pedley (2014) also recognised a clear relationship between iron levels and cognitive function. This can be attributed to the essential role of iron in the brain, with iron involved in the production of neurotransmitters, synaptogenesis and myelination of nerves (Clénin et al., 2015; Coad & Pedley, 2014).

### *2.1.1 Iron absorption, transport and storage*

Iron absorption primarily occurs in the duodenum and upper jejunum and is influenced by various physiological and dietary factors (Percy et al., 2017). Haem iron is efficiently absorbed and transported into enterocytes (Abbaspour et al., 2014). This is thought to occur via the intestinal haem iron transporter (HCP1), where it is then metabolised by haem oxygenase 1 to liberate ferrous ($Fe^{2+}$) iron (Abbaspour et al., 2014; Coad & Conlon, 2011; Percy et al., 2017). Non-haem iron, however, is predominantly found as insoluble ferric ($Fe^{3+}$) iron, which requires reduction to the $Fe^{2+}$ form by ferric reductases and/ or ingested ascorbic acid to optimise absorption (Coad & Pedley, 2014). Once in its ferrous form, divalent metal transporter 1 (DMT1) mediates the absorption of non-haem iron from the intestinal lumen into enterocytes (Percy et al., 2017). Iron absorbed into the enterocytes then enters a common labile iron pool where it is either stored intracellularly as ferritin or exported as $Fe^{2+}$ across the basolateral membrane into the bloodstream via export protein ferroportin (Abbaspour et al., 2014; Coad & Conlon, 2011). Subsequently, exported $Fe^{2+}$ is immediately oxidised to $Fe^{3+}$ by membrane-bound ferroxidase (Coad & Conlon, 2011). In this form, iron is then bound to transferrin, where it is transported through the bloodstream to the liver for storage as ferritin or transferred to required tissues (Clénin et al., 2015). Excess iron in the body is stored as ferritin, primarily in the reticuloendothelial cells of the liver, spleen and bone marrow as a reserve for future demand (Coad & Conlon, 2011; Percy et al., 2017).

### *2.1.2 Iron regulation and homeostasis*

Tight regulation of iron within the body is essential in ensuring iron requirements are met while preventing toxicity and reducing the risk of ID (Percy et al., 2017). While total body iron is regarded across the literature as ranging from 2.5-4.5g in adults (Angeli et al., 2016; Percy et al., 2017), Clénin et al. (2015) specified gender differences, with a total body iron content of approximately 2.5g in women. Of this, daily iron losses occur, with 1-2mg of iron typically lost daily through sweat, urine, and shedding of skin and gastrointestinal tract cells (Percy et al., 2017). Menstruating women are also cited as losing an additional 1mg of iron per day (Percy et al., 2017). This aligns with research by Hallberg et al. (1966), who calculated that menstrual blood losses of 40-60mL equate to ~1mg of daily iron loss when averaged across the menstrual cycle. However, a wider range of menstrual iron losses have also been reported, with Angeli et al. (2016) and Linder (2013) describing 10-40mg of iron (corresponding to 20-80mL of menstrual blood loss) and 4-37mg of iron lost total during menstrual bleeding respectively. Despite these passive losses, there is no regulated pathway for the active excretion of iron from the body (Angeli et al., 2016). Consequently, tight regulation of iron balance is also essential to prevent iron overload and its associated adverse health outcomes, including oxidative stress and cancer (Clénin et al., 2015).

Iron balance is mainly regulated through absorption of dietary iron intake (Abbaspour et al., 2014). Coordinated by the iron regulatory hormone hepcidin, dietary iron absorption primarily depends on an individual's physiological iron status (Badenhorst et al., 2022; Coad & Conlon, 2011). In general, 5-15% of dietary iron intake is absorbed which equates to approximately 1-2mg of iron per day (Coad & Pedley, 2014). However, intestinal iron absorption can increase up to four-fold in response to elevated iron losses or demand (Clénin et al., 2015). Hepcidin, synthesised in the liver, regulates iron absorption and recycling by binding to ferroportin which causes its internalisation and degradation, preventing iron export into circulation (Abbaspour et al., 2014). The resultant accumulation of intracellular iron inhibits DMT1 expression, thereby reducing dietary iron absorption (Coad & Pedley, 2014). Hepcidin production is increased in response to high concentrations of circulating iron and iron stores, reducing iron absorption, recycling, and release of stored iron to prevent iron toxicity (Angeli et al., 2016). Conversely, low concentrations of circulating iron and increased erythropoietic activity downregulate hepcidin production, thereby increasing iron absorption and release of stored iron to prevent ID (Angeli et al., 2016; Lim et al., 2016). Once iron is absorbed, it is retained and utilised by the body (Abbaspour et al., 2014). Recycled iron from the macrophage-mediated breakdown of erythrocytes contributes to the remainder of iron requirements, which is also regulated by hepcidin (Clénin et al., 2015; Percy et al., 2017). However, ID can occur if hepcidin-mediated regulatory mechanisms are impaired or if iron losses exceed 5mg/day (Briguglio et al., 2020; Percy et al., 2017).

## 2.2 Iron Deficiency

### *2.2.1 Key biomarkers of iron status*

Several biomarkers can be used to assess iron status and the presence and severity of ID within individuals, as summarised in table 2.1 (Clénin et al., 2015). However, the lack of consistency across the research in both the selection of biomarkers and the cutoff values used, makes comparison across studies challenging. While Badenhorst et al. (2021) states a minimum requirement of serum ferritin (SF), Hb and transferrin saturation (TSAT) to determine the progressiveness of ID, Clénin et al. (2015) also describes the addition of haematocrit, mean cellular volume (MCV) and mean cellular haemoglobin (MCH) as baseline parameters for ID diagnosis. Regardless, within the literature, SF is the most useful and widely used biomarker in the determination of iron status, and is commonly used in research as a marker of total body iron stores (Clénin et al., 2015; Northrop-Clewes & Thurnham, 2013; World Health Organisation, 2020). In general, 1µg/L of SF equates to approximately 8mg of stored iron (Rusch et al., 2023). However, there is much debate over the appropriate SF cutoffs to identify ID. The World Health Organisation (2020) recommends

a cutoff value of <15μg/L in adults, however, Rusch et al. (2023) note that while this threshold has high specificity for diagnosing ID, it lacks sensitivity in identifying those with mild ID. The WHO cutoff is challenged by Clénin et al. (2015) who proposes an SF cutoff of <30μg/L is more appropriate in identifying low iron stores, increasing sensitivity in detecting ID from 25% to 92%, with specificity remaining high at 98% (Percy et al., 2017; Rusch et al., 2023). Serum ferritin needs to be used in conjunction with Hb to determine if the severity of ID has progressed to IDA (Briguglio et al., 2020). While useful in the measure of anaemia, Hb should not be used alone due to its low sensitivity and specificity (Briguglio et al., 2020).

Transferrin saturation, and thereby serum iron (SI) and total iron-binding capacity (TIBC), are also useful in assessing ID, reflecting iron supply to the tissues (World Health Organisation, 2020). Transferrin saturation is calculated by dividing SI by TIBC multiplied by 100, which indicates the percentage of iron bound to transferrin (Rusch et al., 2023). Values below 20% are typically indicative of ID, with a TSAT <15% suggesting that there is insufficient iron to meet daily requirements for erythropoiesis (Clénin et al., 2015; World Health Organisation & Centers for Disease Control and Prevention, 2007). Serum iron has high daily fluctuations as it is influenced by circadian rhythm and dietary intake, and is therefore considered an unreliable and now obsolete standalone biomarker for ID (Clénin et al., 2015; Rusch et al., 2023). Similarly, TIBC has a normal concentration of 60μmol/L, which increases in ID but not until iron stores are depleted, so it is not used alone as a measure of iron status (Northrop-Clewes & Thurnham, 2013; World Health Organisation & Centers for Disease Control and Prevention, 2007). Soluble transferrin receptor (sTfR) is another iron biomarker used in research, which reflects erythropoietic activity and the demand for iron, making it useful in differentiating between IDA and anaemia of chronic disease (Northrop-Clewes & Thurnham, 2013; World Health Organisation & Centers for Disease Control and Prevention, 2007). However, there is no international reference range or standardised cutoffs for sTfR, leading to variations across studies (Northrop-Clewes & Thurnham, 2013).

Less common biomarkers that are emerging in research are zinc protoporphyrin (ZnPP) and serum hepcidin (SH). Zinc protoporphyrin reflects the severity of ID, rising in response to inadequate iron for Hb synthesis as zinc is inserted into the protoporphyrin molecule, replacing iron (Rusch et al., 2023). As an additional biomarker, ZnPP is re-entering routine testing for ID with a cutoff concentration of >60μmol/mol haem (Clénin et al., 2015; World Health Organisation & Centers for Disease Control and Prevention, 2007). Due to its role in iron regulation, SH is decreased in ID, often below the limit of detection in the assays used (Rusch et al., 2023). Lainé et al. (2016) emphasises the potential role of SH in determining the cause of ID and anaemia. However, the use of SH as a biomarker of iron status is still relatively novel and is not currently used in routine clinical testing (Clénin et al., 2015; Lim et al., 2016).

**Table 2.1** Summary of Key Biomarkers of Iron Status in Research

| Biomarker | Description | Proposed Cutoff in Premenopausal Women | Comments |
|---|---|---|---|
| Serum ferritin (SF) | An acute-phase protein reflective of the total amount of iron stored in the body in the absence of inflammation. | <12-20 µg/L<br><30 µg/L may be more sensitive in identifying low iron stores | The most widely used biomarker for ID but is increased during inflammation. |
| Haemoglobin (Hb) | A protein in erythrocytes that requires iron to transport oxygen around the body. | <120 g/L | Defines anaemia but must be used in conjunction with SF to determine IDA. |
| Transferrin saturation (TSAT) | Calculated by SI/TIBC x 100 and indicates the percentage of transferrin bound to iron. | <20% | Influenced by high daily fluctuations of serum iron. |
| Soluble transferrin receptor (sTfR) | Fragments of transferrin receptors shed into the blood, reflective of the body's demand for iron. | >0.75–1.5 mg/L | Not affected by inflammation and exercise but cutoff values vary. |
| Serum hepcidin (SH) | A hormone involved in the regulation of iron status. | Low in ID but has not yet entered routine testing | Can be elevated with inflammation. |
| Zinc protoporphyrin (ZnPP) | A protein that replaces iron in haem when iron is unavailable, reflecting a shortage of iron supply. | >60-100µmol/mol haem | Early marker of ID but should not be used alone. |

*ID = iron deficiency, IDA = iron deficiency anaemia, SI = serum iron, TIBC = total iron-binding capacity*
*Adapted from Clénin et al. (2015) and World Health Organisation and Centers for Disease Control and Prevention (2007)*

### *2.2.2 Classification of ID*

Iron deficiency can be classified into two distinct forms: absolute ID and functional ID (Dugan et al., 2024). Absolute ID occurs when inadequate iron stores are available to maintain normal physiological functions, with a compensatory decrease in SH concentrations observed to increase iron absorption (Northrop-Clewes & Thurnham, 2013; Zhang et al., 2024). In contrast, functional ID maintains adequate iron stores, but these cannot be mobilised or utilised for physiological needs, due to inappropriately elevated SH levels which may occur in chronic disease or inflammation (Dugan et al., 2024; Zhang et al., 2024). Absolute ID can be categorised into three stages. In stage one (iron depletion or mild ID), iron stores begin to deplete, resulting in a reduction of SF (Beck et al., 2014a; Clénin et al., 2015). However, in this stage Hb concentration and haematopoiesis are unaffected as erythropoiesis is prioritised (Briguglio et al., 2020; Clénin et al., 2015). If iron balance remains negative, ID progresses to stage two (Clénin et al., 2015). In stage two (iron deficiency without anaemia (IDWA) or iron-deficient erythropoiesis), iron stores are depleted, tissue iron stores and circulating iron levels decline and iron-dependent protein production decreases (Beck et al., 2014a; Coad & Conlon, 2011; University of Otago & Ministry of Health, 2011). Clénin et al. (2015) also refers to this stage as ID with microcytosis and/ or hypochromia (IDMH) as haematopoiesis is now affected. As the tissues are no longer provided with sufficient iron, ZnPP and sTfR concentrations increase (Beck et al., 2014a; Clénin et al., 2015). While Hb concentrations remain normal, SF continues to decline, TSAT decreases as a result of decreased SI and increased TIBC, and MCH and MCV decrease below the normal range (Beck et al., 2014a; Clénin et al., 2015). In stage three (IDA), iron stores are exhausted and circulating iron levels are low, resulting in reduced erythropoiesis and Hb concentrations decreasing below the normal reference range (Clénin et al., 2015; Dugan et al., 2024; University of Otago & Ministry of Health, 2011). For this review, unless otherwise stated, ID will collectively refer to both stage one and two.

While the literature tends to agree on the definition of each stage of ID, there is much debate around the appropriate biomarker cutoffs to distinguish each stage. The WHO specifies that adequate iron stores are available when SF is ≥15µg/L with an SF of <12-15µg/L reflecting depleted iron stores (World Health Organisation & Centers for Disease Control and Prevention, 2007). Conversely, Clénin et al. (2015) emphasises that SF <15µg/L is specific for empty iron stores while an SF of 15-30µg/L is still indicative of low iron stores which may benefit from treatment (Zhang et al., 2024). Dimas-Benedicto et al. (2024) acknowledges the usefulness of both cutoffs with an SF <30µg/L indicating mild ID and an SF <15µg/L indicating stage two ID. However, Galetti et al. (2021) proposed a functionally defined threshold for early ID, corresponding to the SF concentration at which the body begins to upregulate iron absorption. Their study suggested that an SF as high as <50µg/L

may be indicative of early ID in premenopausal women (Galetti et al., 2021). While much of the research uses the WHO cutoff to define ID and primarily differentiates between ID and IDA, Al-Naseem et al. (2021) highlights that an SF cutoff of 30µg/L is a common diagnostic for stage one ID in clinical practice. Briguglio et al. (2020) provides further elaboration on defining the stages of ID, with SF reducing to <20µg/L in stage two and <12µg/L in stage three. While these values are also used within research, the stage of ID they correspond to varies throughout the literature as evidenced by Heath et al. (2001) defining stage one ID as <20µg/L and stage two ID as <12µg/L. The WHO defines IDA as a Hb concentration of <120g/L in adult non-pregnant women in addition to the previously specified SF cutoff of <12-15µg/L (World Health Organisation, 2024). This appears to be universally accepted across the research; however, it is noted that the normal Hb range varies in different populations and 5% of healthy individuals will have a Hb value lower than the cutoff without anaemia (Percy et al., 2017; World Health Organisation, 2024). Table 2.2 summarises the main cutoffs used for each stage of ID.

**Table 2.2** Summary of Biomarker Cutoffs in Premenopausal Women for Each Stage of ID

| Biomarker | Stage 1 – Iron Depletion | Stage 2 – IDWA | Stage 3 – IDA | WHO Cutoff |
|---|---|---|---|---|
| Serum Ferritin (SF) | <30 µg/L | <20 µg/L | <12 µg/L | <12-15 µg/L |
| Haemoglobin (Hb) | >120 g/L | >120 g/L | <120 g/L | <120 g/L |
| Transferrin Saturation (TSAT) | >20% | <20% | <20% | <20% |
| Mean Cellular Volume (MCV) | Normal | <80 fL | <80 fL | <67-81 fL |
| Mean Cellular Haemoglobin (MCH) | Normal | <28 pg | <28 pg | Not defined |

*IDA = iron deficiency anaemia, IDWA = iron deficiency without anaemia*
*Adapted from Clénin et al. (2015) and World Health Organisation and Centers for Disease Control and Prevention (2007)*

### *2.2.3 Impact of inflammation on iron status and diagnosis of ID*

As an acute-phase protein, SF concentrations increase due to inflammatory cytokines when inflammation is present in the body, even in cases of low iron stores (Northrop-Clewes & Thurnham, 2013). This presents a challenge in the diagnosis of ID as high SF levels in these individuals can mask the presence of ID, indicating that SF is not always a reliable marker of iron status (Coad & Conlon, 2011). Additionally, inflammatory

states are also associated with an increased risk of ID (Coad & Conlon, 2011). During inflammation, hepcidin concentrations rise, primarily in response to the inflammatory marker interleukin-6 (IL-6) directly binding to the hepcidin (HAMP) gene, resulting in the upregulation of hepcidin transcription (Lim et al., 2020). The elevation of SH suppresses iron absorption and the release of stored iron, regardless of iron status (Lim et al., 2016). Along with chronic inflammatory conditions, obesity and intensive physical activity have been recognised as increasing inflammation in the body (Clénin et al., 2015; Jordaan et al., 2020). In studies of iron status among women with obesity, Jordaan et al. (2020) found a significant association between elevated SF and C-reactive protein (CRP) levels, concluding that the prevalence of ID could be underestimated in this cohort. Conversely, Cheng et al. (2013) reported no contribution of obesity to the prevalence of ID, evidenced by low median SH, and sTfR levels not detecting further cases of ID. Although, the authors did note that these findings differed from previous research and may be due to their population being otherwise healthy without any comorbidities often seen in individuals with obesity (Cheng et al., 2013).

With the increasing prevalence of overweight and obesity, there is a growing need to account for the limitations of iron biomarkers in the presence of inflammation (Coad & Conlon, 2011). It is suggested that SF should be assessed alongside markers of inflammation such as CRP and/ or α-1 acid glycoprotein (AGP), with CRP >5mg/L and AGP >1g/L indicating the presence of inflammation (Coad & Conlon, 2011; Northrop-Clewes & Thurnham, 2013). In these individuals, the WHO recommends that the SF cutoff to define ID should be increased to <70μg/L (World Health Organisation, 2020). This recommendation was based on analysis from the Biomarkers Reflecting the Inflammation and Nutritional Determinants of Anaemia (BRINDA) project, which also supported the use of regression correction of SF based on CRP and AGP to adjust for the confounding effects of inflammation (Namaste et al., 2017). Now known as the BRINDA inflammation adjustment method, regression correction accounts for the relationship between SF and acute-phase proteins; however, this method is based on inflammation levels in the context of the population, rather than adjusting for inflammation at an individual level (Luo et al., 2023; Namaste et al., 2017). Therefore, additional biomarkers such as sTfR may also be beneficial as, unlike ferritin, sTfR is not influenced by inflammation and exercise (Clénin et al., 2015).

### *2.2.4 Consequences of ID*

Iron deficiency is associated with negative health consequences, including deficits in cognitive function, reduced physical performance, and pregnancy complications such as low birthweight and prematurity, with further symptoms including fatigue, lethargy and poor mood (Badenhorst et al., 2021; Beck et al., 2013; Coad & Conlon, 2011). Emerging evidence suggests ID may impact neurocognitive function even before impairment of erythropoiesis

(Coad & Conlon, 2011). Neurophysiological consequences of ID in premenopausal women may include irritability, depression, and impaired concentration, reasoning, memory, and learning (Coad & Conlon, 2011; Coad & Pedley, 2014; Dimas-Benedicto et al., 2024). While improvements in cognitive function have been observed following iron supplementation (Dimas-Benedicto et al., 2024), Coad and Pedley (2014) also recognised that some of these neurological deficits appear to remain even after iron stores are replenished. Women with ID may also experience cold intolerance, headaches, dizziness and dyspnoea with exercise, progressing to possible tachycardia and cardiac failure in severe IDA (Percy et al., 2017).

## 2.3 Iron Deficiency in Premenopausal Women

### *2.3.1 Prevalence in New Zealand*

Iron deficiency is recognised as disproportionately affecting women, particularly those of reproductive age (Coad & Conlon, 2011). Several studies in NZ have investigated the prevalence of ID in NZ premenopausal women as summarised in table 2.3. The 2008/09 NZ Adult Nutrition Survey found females aged 31-50 years had the highest prevalence of ID (SF <12µg/L, ZnPP >60µmol/mol) at 12.1%, of which 6.3% had IDA (SF <12µg/L, ZnPP >60µmol/mol, Hb <120/L) (University of Otago & Ministry of Health, 2011). While Pacific women in this age group had a significantly greater prevalence of IDA at 11.1%, prevalence of both ID and IDA in women aged 19-30 years were much greater among Māori (10.8% ID, 5.1% IDA) and Pacific women (8.9% ID, 4.7% IDA), compared to NZ European/ Other women (3.8% ID, 0% IDA) (University of Otago & Ministry of Health, 2011). Other studies using SF <12µg/L to define ID have found a prevalence ranging from 4-7% in NZ premenopausal women (Ferguson et al., 2001; Heath et al., 2001). However, the prevalence of ID increases to 18.7-23% in studies using a higher SF of <20µg/L (Beck et al., 2014; Heath et al., 2001). Additionally, in a more recent study by Lim et al. (2020), 55.8% of participants were found to be iron-insufficient when using SF <30µg/L, or 43.0% when using SF <20µg/L. These figures were notably high compared to previous research, potentially due to the high proportion of vegetarian, vegan and South Asian participants. This result aligned with previous NZ research that found the likelihood of ID was increased almost fivefold in Asian females in comparison to European females (Beck et al., 2014). Further demonstrating the role of ethnicity, both ID and IDA were found by Schaaf et al. (2000) to be significantly higher among Māori, Pacific, and Asian females aged 14-21 years compared to NZ Europeans (see table 2.3). However, as this study was conducted among students from low socioeconomic backgrounds, these findings may reflect a combination of both ethnic and socioeconomic disparities. Low socioeconomic status has been shown to be significantly associated with lower iron status in young adult females compared to those of high

socioeconomic status (Chandra et al., 2017; Chandra et al., 2016). This is an important consideration as it is recognised that health disparities exist in NZ, with Māori having higher rates of poverty and are more likely to be obese than non-Māori, potentially further increasing their risk of ID (Grant  et al., 2010).

**Table 2.3** Summary of Studies Investigating the Prevalence of ID in New Zealand Premenopausal Women

| Study | Population | Prevalence | Definition of ID |
|---|---|---|---|
| Beck et al. (2014) | Auckland premenopausal females 18-44 years (*n*=375) | **ID** = 18.7%<br>**IDA** = 5.3% | **ID** = SF <20µg/L<br>**IDA** = SF <20µg/L, Hb <120g/L |
| Ferguson et al. (2001) | Premenopausal females 15-49 years (*n*=1751) | **ID** = 7% | **ID** = SF ≤12µg/L |
| Heath et al. (2001) | Dunedin premenopausal females 18-40 years (*n*=384) | **MID** = 23%<br>**ID** = 4%<br>**IDA** = 2% | **MID** = SF <20µg/L, Hb ≥120g/L<br>**ID** = SF <12µg/L<br>**IDA** = SF <12µg/L, Hb <120g/L |
| Lim et al. (2020) | Auckland premenopausal females 18-45 years (*n*=170) | **ID** = 55.8%<br>**IDA** = 8.5% | **ID** = SF <30µg/L<br>**IDA** = SF ≤12µg/L, Hb <120g/L |
| Schaaf et al. (2000) | Auckland females 14-21 years (*n*=896) | **ID** = 18.3% *(25.6% Māori, 20.9% Pacific, 15.4% Asian, 8.3% NZE)*<br>**IDA** = 8.7% *(10.4% Māori, 10% Pacific, 8.7% Asian, 3.5% NZE)* | **ID** = 2 of: SF <12µg/L, TSAT <14%, RDW >14.5%<br>**IDA** = ID + Hb <120g/L |
| University of Otago and Ministry of Health (2011) | Females 19-30 years (*n*=240) | **ID** = 5.2% *(10.8% Māori, 8.9% Pacific, 3.8% NZEO)*<br>**IDA** = 1.2% *(5.1% Māori, 4.7% Pacific, 0% NZEO)* | **ID** = SF <12µg/L, ZnPP >60µmol/mol<br>**IDA** = SF <12µg/L, ZnPP >60µmol/mol, Hb <120g/L |
| University of Otago and Ministry of Health (2011) | Females 31-50 years (*n*=508) | **ID** = 12.1% *(11.1% Māori, 12.6% Pacific, 12.1% NZEO)*<br>**IDA** = 6.3% *(4.9% Māori, 11.1% Pacific, 6.2% NZEO)* | **ID** = SF <12µg/L, ZnPP >60µmol/mol<br>**IDA** = SF <12µg/L, ZnPP >60µmol/mol, Hb <120g/L |

*Hb = haemoglobin, ID = iron deficiency, IDA = iron deficiency anaemia, MID = mild iron deficiency, NZE = New Zealand European, NZEO = New Zealand European/ Other, RDW = red cell distribution width, SF = serum ferritin, TSAT = transferrin saturation, ZnPP = zinc protoporphyrin*

### *2.3.2 Causes and risk factors of ID in premenopausal women*

Iron deficiency can generally be attributed to a negative iron balance that results from increased iron requirements, increased blood loss, inadequate dietary intake and/ or reduced iron absorption (Coad & Conlon, 2011; Percy et al., 2017). Within NZ premenopausal women, significant contributors to blood loss include blood donation, menstrual bleeding, and nose bleeds, with blood donation within the past 4-12 months described as the greatest risk factor for ID (Beck et al., 2014; Heath et al., 2001; Martin et al., 2023). This is further evidenced in an Australian study by Lim et al. (2016) who observed a decrease in SF by 8% for every 100mL of blood donated. However, menstrual blood loss is widely considered to be a causal factor of ID and IDA in premenopausal women (Percy et al., 2017). The extent and duration of menstrual bleeding significantly influence this risk, with abnormal uterine bleeding, specifically, heavy menstrual bleeding (HMB), contributing substantially to the depletion of iron stores and increasing the likelihood of deficiency (Dugan et al., 2024). However, it has also been recognised that many women with normal menstrual blood loss volumes have normal iron status, suggesting that ID in eumenorrheic women may not be directly caused by normal menstruation (Badenhorst et al., 2022).

Whitfield et al. (2003) found general genetic variations in dizygotic Australian female twins had a greater impact on iron stores than female-specific factors such as intensity and duration of menstrual blood loss. Another factor that has been debated in the literature is the impact of parity on iron status. While Looker et al. (1997) reported ID was more likely in multiparous North American women, Whitfield et al. (2003) found no association between parity and ID but noted this may be due to routine iron supplementation during pregnancy. As mentioned earlier in this literature review, reduced iron absorption can result from inflammatory states such as obesity (Zhang et al., 2024). Obesity is a prominent health condition in NZ, with one in three adults classed as obese (Ministry of Health, 2024). Lim et al. (2020) found that high body mass index (BMI) and body fat percentage in South Asian females contributed to their increased risk of ID. However, in non-obese premenopausal women, a higher body fat percentage appears to be protective against ID risk (Martin et al., 2023). Female athletes have also been found to be more at risk of ID due to increased iron losses and inflammation resulting in reduced iron absorption (Al-Naseem et al., 2021). Finally, with the increasing prevalence of vegetarian and vegan dietary patterns among premenopausal women, the lower bioavailability of iron in these diets could result in reduced absorption and inadequate intake, thereby increasing the risk of ID (Coad & Conlon, 2011).

## 2.4 Dietary Iron

Dietary iron occurs in two main forms known as haem iron and non-haem iron (Abbaspour et al., 2014). Haem iron is found in animal flesh foods as a component of Hb in red meats such as beef, lamb and venison, and myoglobin in white meats such as poultry and fish (Reeves et al., 2017). However, since haem iron concentrations are significantly greater in Hb than myoglobin, smaller portions of red meat are required to provide equivalent quantities of haem iron compared to white meat (Reeves et al., 2017). For example, approximately 60g of beef is required to provide 1mg of haem iron compared to approximately 140g of chicken (Reeves et al., 2017). Non-haem iron is primarily found in plant-based foods such as grains, cereals, legumes, fruits and vegetables, although small amounts are also present alongside haem iron in meat (Abbaspour et al., 2014; Beck et al., 2013; Jackson et al., 2016). Differences in bioavailability exist between haem and non-haem iron. While non-haem iron is more prevalent in the diet, haem iron has a higher bioavailability with 15-25% absorbed compared to 1-12% of non-haem iron (Abbaspour et al., 2014; Reeves et al., 2017). Iron bioavailability is further increased in the case of ID (Clénin et al., 2015); however, iron status and dietary factors have little impact on absorption of haem iron as opposed to their strong influence on non-haem iron (Abbaspour et al., 2014; Beck et al., 2013).

### *2.4.1 Dietary iron requirements for premenopausal women*

As iron balance is mainly regulated through dietary absorption, dietary iron intakes must ensure replenishment of daily losses (Briguglio et al., 2020). Premenopausal women subsequently have increased daily iron requirements to offset the additional losses from menstruation (Percy et al., 2017). In NZ, the estimated average requirement (EAR) for iron is 8mg/day to meet the requirements of 50% of premenopausal women (National Health and Medical Research Council et al., 2006). However, this indicates that 50% of women will not have adequate iron intake when set at this level, potentially resulting in ID. Therefore, the recommended dietary intake (RDI) for iron is 18mg/day, reflecting the average amount of iron sufficient to meet requirements of 97.5% of premenopausal women assuming 18% absorption (National Health and Medical Research Council et al., 2006). Comparatively, the United Kingdom has a reference nutrient intake (RNI) of 14.8mg/day while the WHO recommends a higher RNI of 29.4mg/day based on 10% iron absorption (Department of Health, 1991; World Health Organization & Food and Agricultural Organization of the United Nations, 2004). However, it is noted that the conservative approach to meet 97.5% of individuals needs is likely to exceed actual iron requirements for many women (Beck et al., 2014a). Conversely, premenopausal women consuming plant-based diets with lower

bioavailability are likely to have higher requirements and are thus recommended to increase iron intake to 32mg/day (Coad & Conlon, 2011).

### *2.4.2 Measuring dietary iron intake in research*

Throughout the research, three main methods are used to determine dietary iron intake: 24-hour diet recalls, food records, and food frequency questionnaires (FFQ's). Most national nutrition surveys employ a 24-hour diet recall due to their cost-effectiveness and low participant burden (University of Otago & Ministry of Health, 2011). Accordingly, this method was used in the 2008/09 NZ Adult Nutrition Survey and the NZ 1997 National Nutrition Survey which were interviewer administered using a standardised computer-prompted protocol (University of Otago & Ministry of Health, 2011). Dietary information from the previous day was collected in four stages: a quick list of all foods, drinks and supplements consumed; detailed descriptions of all items, cooking methods, and recipes; estimates of portion sizes of each item consumed; and a review of all items with any additions or changes made (University of Otago & Ministry of Health, 2011). However, as this method only assesses a single day, this data may not be representative of a person's usual intake and fails to capture daily variations in the diet. While approximately one-third of participants were asked to complete a second 24-hour diet recall to allow adjustment for intra-individual intake variability, it does not fully address this limitation (University of Otago & Ministry of Health, 2011). Other disadvantages of 24-hour diet recalls include high reliance on participants memory to recall all items consumed in the prior day and challenges in accurately estimating portion sizes.

While 24-hour recalls are done retrospectively, food records are assessed prospectively where participants record all foods and beverages consumed over a set period through either a weighed or estimated food record (Beck & Heath, 2013). This mitigates reliance on memory, providing an accurate record of all items consumed; however, this method requires participants to actively consider what they are consuming which may lead to adjustment of eating habits. Additionally, studies have shown that food records have high intra-participant variability in iron intake, requiring 11 days of data collection per participant to give an accurate measure of usual iron intake, resulting in significant participant burden (Heath et al., 2000). In contrast, FFQ's are designed to estimate usual intake, asking participants how often they have consumed particular foods from a predefined list over a set period of time (i.e. past month), minimising participant burden (Beck & Heath, 2013; Heath et al., 2000). However, the relative validity of an iron food frequency questionnaire (FeFFQ) should be assessed prior to use, usually by comparison against another dietary assessment method (typically a weighed food record) to ensure it provides an accurate estimate of iron intake (Beck & Heath, 2013). While FeFFQ's are relatively quick and easy to complete and

allow for estimation of iron inhibitors and enhancers, they often only use standard portion sizes which may not reflect individual intake variations, and accurate recall of dietary intake beyond a one-month period is unlikely (Heath et al., 2000).

Studies of iron intake in Australian premenopausal women have used the Dietary Questionnaire for Epidemiological Studies (DQES), which is a FFQ developed by the Cancer Council Victoria validated for use in this population (Lim et al., 2016; Reeves et al., 2017). In NZ research, Heath et al. (2001) administered an FeFFQ to estimate intakes of total dietary iron, haem and non-haem iron, and dietary factors affecting iron absorption. Similarly, Beck et al. (2012) developed and validated their own FeFFQ designed to assess intakes of food groupings containing iron and factors affecting iron absorption and to identify iron-related dietary patterns. This FeFFQ has subsequently been used in multiple studies (Beck et al., 2014; Beck et al., 2013; Martin et al., 2023). The use of dietary pattern analysis in the FeFFQ allows the whole diet to be considered as a combination of foods rather than as individual foods and nutrients, providing a more realistic insight into the impact of eating practices on iron status (Beck & Heath, 2013; Beck et al., 2012; Coad & Pedley, 2014).

### *2.4.3 Dietary iron intakes and contribution to iron status*

In NZ premenopausal women, mean dietary iron intakes of 10.3mg/day for those aged 19-30 years and 10.4mg/day for those aged 31-50 years were reported in the 2008/09 NZ Adult Nutrition Survey via 24-hour diet recall (University of Otago & Ministry of Health, 2011). These are similar to a previous NZ study which found average iron intakes of 10.4mg/day and 9.9mg/day in females aged 20-35 years and 36-49 years respectively (Ferguson et al., 2001). Although these findings met the EAR for dietary iron intake, full probability analysis estimated 6.0% of women aged 19-30 years and 15.4% of those aged 31-50 years had inadequate iron intake (University of Otago & Ministry of Health, 2011). In an Australian study by Lim et al. (2016), dietary iron intake assessed via FFQ was found to be a determinant of iron stores, with the authors reporting an associated SF increase of 5% for every 1mg/day increase in dietary iron. However, this finding directly opposes the majority of cross-sectional studies in premenopausal women which have failed to identify any association between total dietary iron intake and iron status (Beck et al., 2014a). Instead, it is more widely recognised that the type of iron consumed can have a greater influence on iron status, with a consensus among studies of a positive association with meat or haem iron intake on iron status (Beck et al., 2014a; Jackson et al., 2016). In NZ premenopausal women, approximately 18-20% of dietary iron is derived from haem sources (University of Otago & Ministry of Health, 2011). While Lim et al. (2016) noted a slightly lower contribution of total meat consumption to dietary iron intake at 14% among Australian women, their study reflected an SF increase of ~1% for each 10% increase in meat intake in

this population. Similarly, Reeves et al. (2017) identified Australian women with ID were consuming significantly less haem iron, noting that an increase of 1mg/day decreased the risk of developing ID within the next three years by 10%. However, other studies have noted that the impact of increasing haem iron intake is strongly predicted by baseline iron status and may demonstrate no association in women with sufficient stores (Jackson et al., 2016).

Various dietary factors are known to influence the absorption of non-haem iron and need to be considered in the determination of iron status (Coad & Pedley, 2014; Jackson et al., 2016). These include dietary enhancers of iron absorption, such as ascorbic acid (vitamin C) and an unidentified factor present in animal tissue known as the meat-fish-poultry factor, and dietary inhibitors of iron absorption, such as calcium, phytates found in grains, cereals and legumes, and polyphenols found in tea, coffee, and some vegetables (Coad & Pedley, 2014; Jackson et al., 2016). While ascorbic acid is recognised as the most effective enhancer of non-haem iron absorption, results on its relationship to iron status are mixed, with many studies finding no association (Beck et al., 2014a; Lim et al., 2016). However, a 16-week experimental study on women with low iron stores found that participants' SF concentrations significantly increased following daily consumption of an iron-fortified cereal when consumed with two gold kiwifruit, equivalent to 163mg of ascorbic acid (Beck et al., 2011). In comparison, no significant SF change was observed in participants consuming one banana in place of the kiwifruit, indicating the benefit of ascorbic acid in improving iron status (Beck et al., 2011). It is increasingly recognised that the combination of foods consumed may have a greater impact on iron status. When considering dietary patterns, only a 'meat and vegetable' dietary pattern and a 'milk and yoghurt' dietary pattern were observed to have an association with iron status, although these were noted to have less of an impact on iron status than non-dietary determinants (Beck et al., 2014a). High intakes of meat and vegetables were associated with decreased odds of ID, while high intakes of milk and yoghurt increased the odds of ID, likely due to calcium inhibiting iron absorption (Beck et al., 2014a). However, it has also been suggested that negative associations between calcium and iron status may be due to displacement of iron-containing foods from the diet in favour of dairy products (Heath et al., 2001). Similarly, while polyphenols are widely reported in the literature as inhibitors of non-haem iron absorption, the majority of studies have found no relationship between polyphenol intake and iron status in populations with adequate iron status (Beck et al., 2014a).

## 2.5 Natural Menstrual Cycle Physiology

In young women, menarche marks the onset of menstruation, usually occurring between 8-14 years of age (Mihm et al., 2011). Comparatively, analysis from the 2014/15 NZ

Health Survey revealed the average age of menarche in NZ was 13.2 years (Donovan & Telfar-Barnard, 2019). From menarche until menopause, women (who are not using a hormonal contraceptive or pregnant) should experience a roughly monthly menstrual cycle characterised by a period of menstrual bleeding, a short fertile phase leading up to ovulation, and a low fertile phase following ovulation (Mihm et al., 2011). While an average natural menstrual cycle is typically considered to be 28 days, high variability is observed with normal menstrual cycle lengths ranging between 21-35 days (Bull et al., 2019). Of this, menstrual bleeding typically occurs over 3-6 days in most women with a natural menstrual cycle but can range anywhere from 2-12 days (Mihm et al., 2011). During menses, normal blood loss varies between 20-80mL per cycle with an average of 35mL, although this may be lower in women on oral contraceptives (Coad & Conlon, 2011; Lainé et al., 2016). In a study of over 600,000 menstrual cycles, Bull et al. (2019) found mean cycle length in women aged 18-45 years was 29.3 days and the mean menstrual bleeding length was 4.0 days. However, the authors noted that menstrual cycle characteristics may be affected by individual factors such as BMI, ethnicity, and stress (Bull et al., 2019).

### *2.5.1 Female sex steroid hormones and phases of the menstrual cycle*

The menstrual cycle is tightly controlled by the hypothalamic-pituitary-ovarian (HPO) axis, with hormonal signals serving as chemical messengers that drive the physiological changes that occur throughout the ovarian and endometrial cycles (Badenhorst et al., 2022; Mihm et al., 2011). Commonly, the menstrual cycle is divided into two main phases, consisting of the follicular phase and the luteal phase (Rogan & Black, 2023). In a eumenorrheic cycle, these phases are divided by the timing of ovulation, with the follicular phase occurring from the beginning of menstrual bleeding until ovulation, and the luteal phase occurring post-ovulation, ending the day prior to the next menstrual bleed (Rogan & Black, 2023). In a textbook 28-day eumenorrheic cycle, ovulation is believed to occur on day 14, with both the follicular and luteal phases each lasting 14 days (Bull et al., 2019). However, wide ranges in follicular (10-23 days) and luteal (7-19 days) phase lengths have been reported, with variation in follicular phase lengths predominantly attributed to overall cycle length variations (Mihm et al., 2011). An analysis of more than 600,000 menstrual cycles found an overall mean follicular phase length of 16.9 days (Bull et al., 2019; Mihm et al., 2011). When grouped by menstrual cycle length, mean follicular phase lengths ranged from 12.4 days in those with 21-24 day cycles to 19.5 days in those with 31-35 day cycles (Bull et al., 2019). Comparatively, luteal phase lengths were found to have an overall mean of 12.4 days (Bull et al., 2019). When grouped by menstrual cycle length, mean luteal phase lengths ranged from 11.0 days in those with 21-24 day cycles to 12.9 days in those with 31-

35 day cycles (Bull et al., 2019). This indicates less variation in the luteal phase length in comparison to the greater variation observed in the follicular phase length (Bull et al., 2019).

The follicular and luteal phases in a eumenorrheic cycle can be broken down further into four distinct hormonal phases: the early-follicular phase, late-follicular phase, ovulatory phase, and the mid-luteal phase (Rogan & Black, 2023). Oestrogen and progesterone are the primary female sex hormones, and follicle-stimulating hormone (FSH) and luteinising hormone (LH) also play a crucial role in the reproductive axis, with fluctuations in each of these hormones occurring throughout the menstrual cycle (figure 2.1) (Aguree et al., 2023; Badenhorst et al., 2022). The early-follicular phase is defined by the presence of menstrual bleeding, lasting from day one to around day five of the menstrual cycle (Badenhorst et al., 2022; Rogan & Black, 2023). In this phase, oestrogen, progesterone, and LH concentrations are at their lowest baseline levels (Wohlgemuth et al., 2021). Following a rise in FSH concentrations beginning four days prior to menses and stimulating ovarian follicle development, FSH reaches maximum concentrations on the day of emergence of the dominant follicle (Badenhorst et al., 2022; Mihm et al., 2011). Oestrogen (predominantly oestradiol) concentrations increase with the maturation of the dominant follicle in the ovary, reaching its peak in the late-follicular phase as the dominant follicle reaches its final stages of maturation (Mihm et al., 2011; Rogan & Black, 2023). At the same time, progesterone concentrations remain low (Mihm et al., 2011; Rogan & Black, 2023). Simultaneously, negative feedback from the increasing levels of oestrogen and inhibin B produced by the developing and maturing follicle causes a gradual decline in FSH, with this hormone then reaching nadir concentrations in the late-follicular phase (Badenhorst et al., 2022; Mihm et al., 2011). The rise in LH initially begins in the late-follicular phase, approximately 28-48 hours before ovulation, due to positive feedback of oestrogen stimulating LH production in the pituitary gland (Badenhorst et al., 2022; Bull et al., 2019). The ovulatory phase consists of a surge in LH, which triggers ovulation of the dominant follicle, a drop in oestrogen as it is no longer being produced by a developing follicle, and low progesterone concentrations (Bull et al., 2019; Rogan & Black, 2023). Following ovulation, the remnants from the cellular wall of the ruptured follicle in the ovary form a temporary endocrine gland known as the corpus luteum (De Souza, 2003). The corpus luteum marks the beginning of the luteal phase and secretes progesterone and oestrogen in a pulsatile manner, with these hormones essential in preparing the endometrium for potential embryo implantation (Bull et al., 2019; De Souza, 2003). Progesterone concentrations peak in the mid-luteal phase, which coincides with a second medium oestrogen concentration peak (Aguree et al., 2023; Rogan & Black, 2023). In the absence of fertilisation, regression of the corpus luteum occurs, resulting in declining progesterone and oestrogen concentrations, and may be associated with the absence of the trophoblast and human chorionic gonadotropin (hCG) (Mihm et al., 2011). The withdrawal of progesterone triggers spontaneous decidualisation, the breakdown of the endometrium,

leading to the onset of menstruation to restart the cycle (Mihm et al., 2011). Concurrently, progesterone withdrawal induces an acute inflammatory response characterised by increased production of inflammatory cytokines and prostaglandins, with approximately a two-fold increase in IL-6 concentrations observed during menses (Badenhorst et al., 2022). In research, measurement of serum oestrogen and progesterone concentrations are considered the gold standard in verifying phases of the menstrual cycle, with urinary LH tests used to detect the day of ovulation (Rogan & Black, 2023). While the presence of menstrual bleeding indicates cyclicity, it does not confirm normal hormone fluctuations or ovulation (Mihm et al., 2011). Therefore, the measurement of sex steroid hormone concentrations is essential to accurately identify menstrual cycle phases and disturbances (Rogan & Black, 2023).

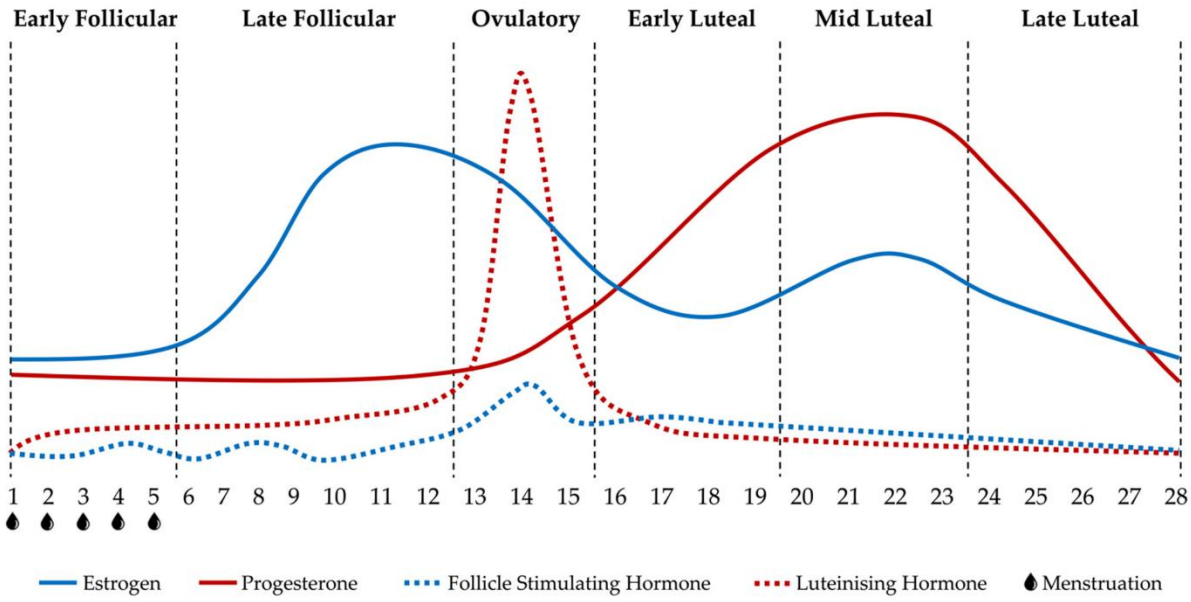


**Figure 2.1** Hormonal Fluctuations Across Phases of the Menstrual Cycle in a Typical 28-Day Eumenorrheic Cycle, reproduced under open access copyright (CC BY) from Carmichael et al. (2021)

### *2.5.2 Subclinical ovulatory disturbances*

In healthy premenopausal women, SOD, including luteal phase defects (LPD) and anovulation, typically present without any change in menstrual cycle length and therefore often remain undiagnosed (Badenhorst et al., 2022; De Souza, 2003). Luteal phase defects can present as either short luteal phases <10 days in length (clinical LPD) and/ or inadequate mid-luteal progesterone concentrations <10-16nmol/L (biochemical LPD), while anovulation is defined as a failure to ovulate with subsequent low progesterone

concentrations in the luteal phase (Badenhorst et al., 2022; Prior et al., 2015; Schliep et al., 2014; Shepard & Senturia, 1977). The literature suggests it is not uncommon for women with regular-appearing menstrual cycles to experience sporadic or intermittent LPD or anovulation, with inconsistent ovulatory status particularly observed in exercising women (46%) across two to three cycles (De Souza et al., 1998; Rogan & Black, 2023). Therefore, to accurately identify the true prevalence of SOD in women, studies should assess sex steroid hormone concentrations in each participant across multiple cycles (De Souza, 2003). A three-month study comparing the frequency of LPD and anovulation between sedentary and recreationally active women found significant differences between the groups (De Souza et al., 1998). Among sedentary women, 10% of menstrual cycles exhibited an LPD with no cases of anovulation, whereas in exercising women, 43% of cycles exhibited an LPD, while 12% were anovulatory (De Souza et al., 1998). However, the reliability of the results may be limited by the relatively small sample size, consisting of only 11 sedentary women (31 cycles) and 24 exercising women (66 cycles), which may not accurately reflect the true population (De Souza et al., 1998). To increase statistical power, this study was then combined with an additional dataset, resulting in a final sample of 20 sedentary women (48 cycles) and 48 physically active women (120 cycles), allowing for a more comprehensive analysis into the prevalence of SOD (De Souza et al., 2010). Comparatively, De Souza et al. (2010) found only 4.2% and 29.2% of all menstrual cycles for sedentary women and exercising women, respectively, exhibited an LPD, much lower than the previous study. However, while there remained no cases of anovulation among sedentary women, 20.8% of all menstrual cycles among exercising women were anovulatory, much higher than previously found (De Souza et al., 2010). Despite the variations in prevalence between the two studies, it is evident that physically active women are at increased risk of SOD.

Further studies among regularly menstruating women have found anovulation to be present in 2.7% and 2.8% of women in their respective cohorts (Dal et al., 2005; Schliep et al., 2014). However, these studies only evaluated anovulation in a single menstrual cycle, potentially underestimating its prevalence. While Malcolm and Cumming (2003) reported anovulation in 3.7% of women, errors were reported in both the results and the timing of testing for some women, and further analysis found that only 0.7% of women were consistently anovulatory. The reported prevalence of LPD varies across studies, with Schliep et al. (2014) identifying LPD in 13% of menstrual cycles ($n$=476) and Dal et al. (2005) reporting biochemical LPD in 25.3% of menstrual cycles ($n$=71). However, Dal et al. (2005) only evaluated a single menstrual cycle in each participant and did not report demographics or other participant characteristics that may explain the heightened prevalence. From the existing studies, it is evident that the prevalence of LPD tends to be highly variable, potentially due to a range of unknown individual and lifestyle factors. While no studies to date were found to explore associations between SOD and iron status, Gorczyca et al.

(2016) found no significant difference in iron intakes when comparing women with ovulatory cycles and women with anovulatory cycles.

## 2.6 Menstruation, Female Sex Steroid Hormones, and Iron Status

A small number of studies have previously investigated associations specifically between menstruation and biomarkers of iron status, as illustrated in table 2.4. While ID and IDA are widely attributed to menstrual blood loss, fluctuations in iron biomarkers are also reported to occur throughout the menstrual cycle (Badenhorst et al., 2021; Percy et al., 2017). In a study of regularly menstruating women, SI and TSAT were reported to be significantly lower in the early-follicular phase compared to both the mid-late-follicular phase and mid-luteal phase (Alfaro-Magallanes et al., 2023). These findings are congruent across the research with other studies reporting SI and TSAT to be lowest during menses and highest in the luteal phase (Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016). While Suzuki et al. (2018) reported no change in either SI or TSAT, the authors noted that this could be due to the limited sample size (*n*=4). Changes in Hb and SF are less consistent across studies. Both Angeli et al. (2016) and Lainé et al. (2016) described relatively stable SF and Hb levels across the menstrual cycle, despite notable inter-individual variability. Similarly, Alfaro-Magallanes et al. (2023) found that hormonal status in female eumenorrheic athletes did not influence SF, supporting its reliability as a measure of iron stores regardless of menstrual cycle phase. However, these findings contrast with those of Kim et al. (1993), who observed a higher prevalence of impaired iron status during menses compared to the luteal and late-luteal phases, suggesting that ID is more likely to be detected when assessments occur during menstruation. Further studies have reported significant associations between Hb and menstrual cycle phase, with Hb reported to be lowest during menses and highest in the late-luteal phase (Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Suzuki et al., 2018). While Kim et al. (1993) found a similar trend in SF to that of Hb, Aguree et al. (2023) reported the opposite, with SF concentrations highest in the early-follicular phase and lowest in the mid-luteal phase, although the mean of all iron biomarkers and prevalence of IDA were not significantly different across the menstrual cycle.

**Table 2.4** Summary of Studies Specifically Investigating Changes in Iron Status Biomarkers Across Phases of the Menstrual Cycle

| Author, Country | Study Aim/ Objectives | Population | Biomarkers Assessed | MC Time Points Sampled | Key Findings |
|---|---|---|---|---|---|
| Alfaro-Magallanes et al. (2023)<br>*Spain* | To identify potential differences in iron status biomarkers between naturally menstruating and oral contraceptive female athletes and changes in these biomarkers across the menstrual cycle. | Healthy females aged 18-40 years with regular menstrual cycles and participating in endurance training 3-12 hours per week (*n*=36). | • SI<br>• SF<br>• Transferrin<br>• TSAT | <u>1x cycle duration</u><br>Naturally menstruating women were tested in each of the following phases:<br>• EFP<br>• MLFP<br>• MLP | SI and TSAT were significantly lower in the EFP compared to the MLFP and MLP.<br><br>No association was found between menstrual cycle phase and SF or transferrin.<br><br>Menstrual cycle phase was associated with IDE. IDE was more likely in the EFP than the MLFP. |
| Suzuki et al. (2018)<br>*Japan* | To investigate iron status in healthy female cyclists across two menstrual cycles, one cycle without iron supplementation and one with iron supplementation. | Healthy female collegiate cyclists in their off season with normal menstrual cycles (*n*=4). | • Hb<br>• SI<br>• TSAT | <u>2x cycle duration</u><br>Women were tested in each of the following phases for two consecutive cycles:<br>• Menstrual phase<br>• Mid-follicular phase<br>• Luteal phase | Hb was significantly higher in the mid-follicular phase compared to the menstrual phase.<br><br>SF was significantly lower in the luteal phase compared to the mid-follicular and menstrual phases.<br><br>No association was found between menstrual cycle phase and SI or TSAT. |

| Author, Country | Study Aim/ Objectives | Population | Biomarkers Assessed | MC Time Points Sampled | Key Findings |
|---|---|---|---|---|---|
| Chandra et al. (2017)<br>*India* | To investigate iron status variables in young adult female students of high SES and whether the physiological changes that occur across the menstrual cycle impact iron status variables. | Females aged 18-25 years with regular menstrual cycles studying in their first year of medical school and not taking medication or hormonal supplements (*n*=50). | • Hb<br>• SI<br>• TSAT<br>• TIBC<br>• MCV | <u>1x cycle duration</u><br>Each participant was only tested once in either the:<br>• Menstrual, follicular, OR luteal phase | Hb, SI and TSAT were significantly associated with phase of the menstrual cycle being lowest in the menstrual phase and highest in the luteal phase.<br><br>TIBC was significantly associated with menstrual cycle phase being highest in the menstrual phase and lowest in the luteal phase.<br><br>MCV was significantly lower in the follicular phase compared to the menstrual phase. |
| Chandra et al. (2016)<br>*India* | To investigate iron status variables in young adult female students of low SES and whether the physiological changes that occur across the menstrual cycle impact iron status variables. | Females aged 18-25 years with regular menstrual cycles belonging to a low SES and not taking medication or hormonal supplements (*n*=50). | • Hb<br>• SI<br>• TSAT<br>• TIBC<br>• MCV | <u>1x cycle duration</u><br>Each participant was only tested once in either the:<br>• Menstrual, follicular, OR luteal phase | |

| Author, Country | Study Aim/ Objectives | Population | Biomarkers Assessed | MC Time Points Sampled | Key Findings |
|---|---|---|---|---|---|
| Angeli et al. (2016)<br>*France* | To investigate changes in iron status variables across the menstrual cycle in healthy menstruating women to determine when hepcidin should ideally be measured for diagnostic purposes. | Healthy females aged 19-44 years with regular menstrual cycles and menses lasting 3-5 days (*n*=90). | • SH<br>• SI<br>• TSAT<br>• Transferrin<br>• SF<br>• Hb | <u>1x cycle duration</u><br>Six samples per participant:<br>• 3x day 1-6<br>• 1x day 6-12<br>• 1x day 12-19<br>• 1x day 26-29 | Significant fluctuations in SI, SH and TSAT were observed across the menstrual cycle consisting of an initial decline during menses, a rebound above baseline mid-cycle, and stabilisation during the luteal phase.<br><br>No association was found between menstrual cycle phase and SF, Hb or transferrin. |
| Lainé et al. (2016)<br>*France* | To investigate changes in iron status variables across the menstrual cycle and whether menses induce significant variations in serum hepcidin concentrations. | Healthy females aged 18-45 years with normal iron status, regular menstrual cycles and menses lasting 3-5 days (*n*=90). | • TSAT<br>• SF<br>• Hb<br>• SI<br>• SH | <u>1x cycle duration</u><br>Six samples per participant:<br>• 3x day 1-6<br>• 1x day 6-12<br>• 1x day 12-19<br>• 1x day 26-29 | Significant fluctuations in SI, SH and TSAT were observed across the menstrual cycle consisting of an initial decline during menses, a rebound above baseline mid-cycle, and stabilisation during the luteal phase.<br><br>No association was found between menstrual cycle phase and SF or Hb. |

| Author, Country | Study Aim/ Objectives | Population | Biomarkers Assessed | MC Time Points Sampled | Key Findings |
|---|---|---|---|---|---|
| Kim et al. (1993)<br>*USA* | To investigate whether normal hormonal fluctuations across the menstrual cycle impact iron status variable concentrations and the prevalence of impaired iron status in different phases of the menstrual cycle. | Healthy naturally menstruating females aged 18-44 years with regular menstrual cycles from the NHANES II data (*n*=1712). | • Hb<br>• MCV<br>• SI<br>• TIBC<br>• TSAT<br>• SF | <u>1x cycle duration</u><br>Each participant was only tested once in either the:<br>• Menstrual, follicular, OR luteal phase | Hb, SI, TSAT, and SF were significantly associated with menstrual cycle phase being lowest in the menstrual phase and highest in the luteal and late luteal phases.<br><br>TIBC was highest in the menstrual phase and lowest in the luteal phase.<br><br>Prevalence of impaired iron status was highest during the menstrual phase and lowest in the luteal and late luteal phases. |

*EFP = early-follicular phase, Hb = haemoglobin, IDE = iron-deficient erythropoiesis, MC = menstrual cycle, MCV = mean corpuscular volume, MLFP = mid- late-follicular phase, MLP = mid-luteal phase, NHANES = National Health and Nutrition Examination Survey, SES = socioeconomic status, SF = serum ferritin, SH = serum hepcidin, SI = serum iron, TIBC = total iron-binding capacity, TSAT = transferrin saturation*

Overall, it is generally acknowledged within the literature that hormonal fluctuations across the menstrual cycle could influence the variation in iron status measures observed (Chandra et al., 2017). However, when considering these findings, it is important to note that Aguree et al. (2023) specifically excluded women classified as overweight or obese, while participants in the majority of other studies discussed were also predominantly within the normal BMI range. This imposes the limitation that these findings may not accurately reflect the relationship between iron status and the menstrual cycle in the broader female population. Chandra et al. (2017) also concluded that the changes in iron biomarkers observed across the menstrual cycle were due to hormonal fluctuations. However, the authors did not measure hormone concentrations, and their methodology was vague, leaving it unclear as to when in the menstrual, follicular, and luteal phases samples were taken and if this was consistent between participants (Chandra et al., 2017; Chandra et al., 2016). It is also worth noting that most studies investigating iron biomarkers across menstrual cycle phases have been limited to eumenorrheic cycles, thereby offering a limited understanding of the broader physiological impact of variations in female sex steroid hormones (e.g. SOD) on iron status.

To date, only two studies have investigated fluctuations in hepcidin throughout the menstrual cycle (Angeli et al., 2016; Lainé et al., 2016). Both studies reported an initial decline in SH during menses, a rebound above baseline concentrations mid-cycle, and stabilisation of hepcidin concentrations during the luteal phase (Angeli et al., 2016; Lainé et al., 2016). During the early-follicular phase, the decline in SH activity may compensate for menstrual iron losses by facilitating increased iron recycling and absorption of dietary iron (Badenhorst et al., 2022; Badenhorst et al., 2021). This is supported firstly by Angeli et al. (2016) who found that SI concentrations modified the release of SH and secondly, through TIBC being highest during menses (Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993). The influence of oestrogen on SH and iron regulation has also been described in the literature, with elevated oestrogen concentrations negatively correlated with SH (Badenhorst et al., 2022). Therefore, as oestrogen increases in the late-follicular phase, SH concentrations may remain low, sustaining enhanced iron absorption and release of stored iron following menstruation (Badenhorst et al., 2021). The mid-cycle rebound of SH is thought to coincide with the decline in oestrogen preceding ovulation, while the subsequent increase in progesterone may also increase SH expression, thereby reducing iron absorption (Badenhorst et al., 2021). However, Angeli et al. (2016) observed a minimal or absent SH rebound in approximately two-thirds of participants, despite a homogenous population of healthy iron-replete women. This may be explained by the positive correlation between menstrual blood loss intensity and the magnitude of SH variations, as a similar proportion of participants were reported using oral contraceptives, which are known to potentially reduce menstrual blood loss (Angeli et al., 2016; Lainé et al., 2016). While these findings highlight

the influence of menstruation on SH variations, there have been no prospective cohort studies to date in women with or without ID or across multiple menstrual cycles that have simultaneously tracked menstrual cycle features and characteristics such as menstrual bleeding duration and hormone concentrations with ovulation.

### *2.6.1 Heavy menstrual bleeding*

Abnormal uterine bleeding, specifically, heavy menstrual bleeding (HMB), has been identified as the primary cause of ID and IDA in premenopausal women, accounting for 20-30% of cases (Bruinvels et al., 2016; Percy et al., 2017). Heavy menstrual bleeding is defined as menstrual bleeding that regularly exceeds a total volume of 80mL per cycle (Badenhorst et al., 2022). This equates to iron losses five to six times greater in women with HMB, significantly increasing their risk of ID and IDA (Dugan et al., 2024). While HMB is thought to affect approximately 30% of premenopausal women, a much lower prevalence of only 3-5% is observed in healthcare, with evidence suggesting women with HMB often do not seek medical attention (Bernardi et al., 2016; Dugan et al., 2024; Munro et al., 2023; Percy et al., 2017). In a study of active females, Bruinvels et al. (2016) initially reported 54.1% of participants had experienced HMB, but acknowledged potential bias, as women with menstrual issues were more likely to have completed the online survey. Controlling for bias, further data was collected with HMB identified in 35.5% of female marathon runners (Bruinvels et al., 2016), while Dugan et al. (2024) reported a similar prevalence of 30.9% among recreationally active females. However, the prevalence of HMB could be as high as 50% in naturally menstruating women (Russo et al., 2024). This is of concern, as women reporting HMB have been shown to exhibit significantly lower Hb and SF levels, with the prevalence of ID and IDA reported as 80% and 35% respectively (Bernardi et al., 2016). However, this may not be an accurate reflection of women with HMB, as the high prevalence of ID observed even in women with normal menstrual blood loss (66.7%) suggests other factors could be contributing to ID in these participants (Bernardi et al., 2016). In contrast, the literature appears to be more accepting of the findings by Bruinvels et al. (2016), who reported that 38-43% of active females with HMB had a history of ID, while 32% had a history of IDA.

Identifying HMB is a known challenge, both in research and in practice (Dugan et al., 2024). Many women are unable to measure or accurately estimate their menstrual blood loss, with variations reported between subjective and objective measures of menstrual bleeding, and there is currently no validated survey to detect HMB (Badenhorst et al., 2022; Bernardi et al., 2016; Dugan et al., 2024). Additionally, many women appear to lack awareness of the difference between normal and heavy volumes of menstrual bleeding, with one study reporting 7.8% of women with HMB claimed their menses were normal

(Badenhorst et al., 2022; Bernardi et al., 2016). Typically, research has relied on indirect measures to determine menstrual blood loss such as self-perception, questionnaires, and pictograms, however, this can be subject to error as it relies on participant reporting accuracy (Badenhorst et al., 2022; Russo et al., 2024). Despite this, Russo et al. (2024) found no significant difference between women's self-perception and the use of pictograms in identifying normal versus heavy menstrual bleeding, concluding that self-perception alone could be used as a reliable method to classify HMB.

## 2.7 Conclusion

Iron deficiency is a common nutritional deficiency among NZ premenopausal women. However, the wide variation in ID categorisation and the use of biomarker cutoff values used across studies makes direct comparisons challenging. While ID can be attributed to increased iron requirements, increased blood loss, inadequate dietary iron intake and/ or reduced absorption, in premenopausal women, many studies attribute ID to increased iron losses from menstruation. Few studies to date explore the relationship between iron status, menstruation, and female sex steroid hormones in depth, and no studies have collected data prospectively. Of those investigating iron biomarker fluctuations across the menstrual cycle, conflicting results were observed between studies. No studies were also found to explore associations between iron status and SOD, which present with different sex steroid hormone concentrations throughout the cycle. Therefore, further research is required in this area to advance our understanding of the role of menstrual physiology in the determination of iron status.

# Chapter 3: Manuscript

## 3.0 Abstract

**Background:** Iron deficiency disproportionately affects women and is commonly attributed to menstrual blood loss in reproductive-aged women. Although iron biomarker fluctuations across the menstrual cycle have been reported in eumenorrheic women, the relationship between iron status and subclinical ovulatory disturbances (SOD) remains unclear. Few studies have investigated iron biomarkers across multiple menstrual cycles, with concurrent hormonal profiling for ovulatory status classification.

**Objectives:** To investigate variations in iron status between and within the menstrual cycle, considering ovulatory status and the influence of SOD in naturally menstruating women.

**Methods:** Ninety-seven healthy, naturally menstruating women participated in a prospective longitudinal cohort study over three to five menstrual cycles, attending six laboratory sessions across the early-follicular, late-follicular and mid-luteal phases. Iron biomarkers were analysed alongside female sex steroid hormones, and ovulatory status was determined using mid-luteal progesterone (≥10nmol/L = ovulatory; <10nmol/L or luteal phase <10 days = SOD). A multiple linear regression analysis identified predictors of serum ferritin (SF) change from baseline to study end.

**Results:** At baseline, 27.8% of participants were iron-insufficient (SF <30μg/L), while 70.1% and 18.6% of participants were classified as consistently ovulatory or SOD, respectively. Iron biomarker concentrations were comparable between groups in all phases, except for higher transferrin saturation in the late-follicular phase among ovulatory participants ($p$=0.049). All iron biomarkers, except Hb ($p$=0.942), significantly differed across menstrual phases among ovulatory participants, whereas no such variation was observed among SOD participants. Change in CRP was the only significant predictor of SF change from baseline to study end (β=1.58 (95% CI 0.84, 2.32), $p$<0.001).

**Conclusion:** Menstrual cycle phase fluctuations in iron biomarkers were evident only in ovulatory women, suggesting altered iron regulation in those with SOD. Although the mechanisms underlying cyclical variations in iron biomarker concentrations among ovulatory women remain unclear, our study provides novel insights into iron status in women who present with SOD.

## 3.1 Introduction

Iron deficiency (ID) is a common nutrient deficiency that disproportionately affects women, particularly those of reproductive age (Coad & Conlon, 2011). Findings from the most recent New Zealand Adult Nutrition Survey (2008/09) revealed that 7.2% of New Zealand (NZ) women were iron deficient (serum ferritin (SF) <12μg/L), with the highest prevalence of 12.1% among 31–50-year-olds (University of Otago & Ministry of Health, 2011). In contrast, when the SF threshold was increased and set at <20μg/L, a study by Beck et al. (2013) found that 18.7% of female participants aged 18-44 years had suboptimal iron status. Moreover, the prevalence of ID has been found to be further increased among ethnic cohorts within NZ, with Māori, Pacific and Asian women at increased risk compared to their NZ European counterparts (Beck et al., 2014; Schaaf et al., 2000; University of Otago & Ministry of Health, 2011). Women with ID may experience fatigue, cold intolerance, headaches, dizziness and dyspnoea with exercise, in addition to the negative health consequences associated with ID, including deficits in cognitive function, reduced physical performance, and pregnancy complications (Badenhorst et al., 2021; Beck et al., 2013; Coad & Conlon, 2011; Percy et al., 2017).

Identified contributors to ID include increased blood loss through blood donation or nose bleeds, exercise-related iron losses, and inadequate or low bioavailability of dietary iron intake (Badenhorst et al., 2022; Beck et al., 2014; Coad & Conlon, 2011). However, for women of reproductive age, ID is often attributed to menstrual blood loss and is widely recognised as the primary cause of ID in premenopausal women (Badenhorst et al., 2021; Percy et al., 2017). The extent and duration of menstrual bleeding significantly influences this risk, with heavy menstrual bleeding (HMB) contributing substantially to the depletion of iron stores and increasing the likelihood of ID (Dugan et al., 2024). However, it has also been recognised that many women with normal menstrual blood loss volumes have normal iron status, suggesting that ID in menstruating women may not be directly caused by non-heavy, regular menstruation (Badenhorst et al., 2022).

More recent literature is beginning to explore iron status changes within eumenorrheic menstrual cycles and the association between iron status and female sex steroid hormone fluctuations across the menstrual cycle (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016; Suzuki et al., 2018). A eumenorrheic menstrual cycle can be divided into four distinct hormonal phases: the early-follicular phase, late-follicular phase, ovulatory phase, and the mid-luteal phase (Rogan & Black, 2023). While oestrogen and progesterone are the primary female sex steroid hormones, follicle-stimulating hormone (FSH) and luteinising hormone (LH) also play a crucial role in the reproductive axis, with predictable fluctuations occurring in the concentrations of each of these hormones throughout the menstrual cycle (Aguree et al.,

2023; Badenhorst et al., 2022). It is proposed that the fluctuations of these sex hormones across the menstrual cycle could influence various physiological processes, including nutritional status and iron regulation (Badenhorst et al., 2022; Rogan & Black, 2023). Prior research into biomarkers of iron status across the menstrual cycle has reported that SF, haemoglobin (Hb), serum iron (SI), and transferrin saturation (TSAT) were highest in women during the luteal phase, and were at their lowest during menstruation, resulting in an increased prevalence of impaired iron status (Kim et al., 1993). However, findings are not consistent across studies, with several studies reporting relatively stable SF and Hb concentrations throughout the menstrual cycle (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Lainé et al., 2016). Among iron-deplete women, no clear association has been observed between iron biomarkers and menstrual cycle phase (Badenhorst et al., 2022).

Given the potential relationship between female sex steroid hormone fluctuations and iron status, it is also important to consider the role of subclinical ovulatory disturbances (SOD), including luteal phase defects (LPD) and anovulation. Luteal phase defects can present as either short luteal phases <10 days in length (clinical LPD) and/ or inadequate mid-luteal progesterone concentrations <10-16nmol/L (biochemical LPD), while anovulation is defined as a failure to ovulate with subsequently low progesterone concentrations in the luteal phase (Badenhorst et al., 2022; Prior et al., 2015; Schliep et al., 2014; Shepard & Senturia, 1977). In healthy premenopausal women, SOD typically present without any change in menstrual cycle length and therefore often remain undiagnosed (Badenhorst et al., 2022; De Souza, 2003). Additionally, it is not uncommon for women with regular menstrual bleeding to experience sporadic or intermittent LPD or anovulation (Rogan & Black, 2023). While previous research has established an increased prevalence of SOD in highly active women, limited research has explored SOD prevalence among a non-athlete recreationally active general population (De Souza et al., 1998; De Souza et al., 2010). Subsequently, the existing findings on SOD prevalence in previous research are highly variable. Previously reported SOD prevalence rates range from 0-4% of menstrual cycles for anovulation and 4-25% of menstrual cycles for LPD (Dal et al., 2005; De Souza et al., 1998; De Souza et al., 2010; Malcolm & Cumming, 2003; Schliep et al., 2014). To date, no studies have explored associations between SOD and iron status.

Few studies to date have examined longitudinal associations between iron status, menstrual cycle phase and female sex steroid hormones in women, with most previous research only investigating eumenorrheic cycles. No prospective cohort studies have simultaneously explored women with and without ID across multiple menstrual cycles while characterising menstrual cycle features, including menstruation duration, ovulation timing, and hormonal profiles. This study aims to explore iron status in relation to menstrual cycle

phase, female sex steroid hormone concentrations and SOD, as well as associated risk factors for ID in New Zealand premenopausal women.

## 3.2 Methodology

This study was part of a larger project investigating iron and menstrual cycle status in healthy naturally menstruating women within the Female Health Research Programme at Massey University. The project collected data over 19 months between July 2023 and March 2025. This study used a prospective longitudinal observational cohort design conducted in premenopausal NZ women. Data was collected for each participant over a period of three to five menstrual cycles and used to investigate changes in iron status both within and across menstrual cycles, and associations with menstrual cycle status and risk factors for ID.

### *3.2.1 Participants and recruitment*

A sample size calculation was used to determine the number of participants needed for results from the study to be reflective of the population. The formula $n = (Z^2 p(1-p)) / d^2$ was used (Cochran, 1977), where n = sample size, Z = 1.96 (5% type 1 error rate), p = 0.48 (48% expected prevalence of SOD), and d = 0.1 (10% absolute error). This gave a minimum sample size of 96 participants required for the study.

Participants were recruited through posters and flyers around Massey University, advertising on social media (on researchers' personal profiles and on community group pages) and on women's health community pages, and through email contacts, all of which contained a link or QR code to a pre-screening questionnaire. Participants were required to complete the pre-screening questionnaire to determine if they met the study's inclusion criteria. For inclusion in the study, participants were required to be biologically female between 18-40 years old and naturally menstruating without the use of hormonal contraception for at least six months prior to and throughout participation in the study. Participants were also required to be at least 12 months postpartum and no longer breastfeeding, as this could impact their menstrual cycle. To ensure an accurate overview of iron status, participants were excluded if they had taken iron supplements in the previous two months and were required to abstain for the duration of the study (multivitamins with very low dose iron were acceptable). Women who smoked or had a health condition impacting iron status, such as coeliac disease, inflammatory bowel disease, or a history of gastric ulcers, were excluded from the study. Figure 3.1 details the process of participants from screening to data collection. A total of 694 women were screened for the study, of which 385 were eligible for participation and followed up by email within 24 hours for registration in

the study and provided an information sheet. A total of 97 women completed more than two menstrual cycles' worth of data, meeting the sample size requirements. Five menstrual cycles' worth of data (two familiarisation cycles and three cycles of female sex steroid hormone measurements) was provided by 83 women, six women provided four cycles' worth of data (two familiarisation cycles and two cycles of female sex steroid hormone measurements), and eight women provided three cycles' worth of data (two familiarisation cycles and one cycle of female sex steroid hormone measurements). Eighteen women withdrew from the study after providing informed consent. Reasons for withdrawal included medical reasons (*n*=6), pregnancy (*n*=2) and other lifestyle factors (*n*=10). Ethics approval was obtained from Massey University Human Ethics Committee: Southern A, Application 22/56 and all participants provided written informed consent prior to participation.

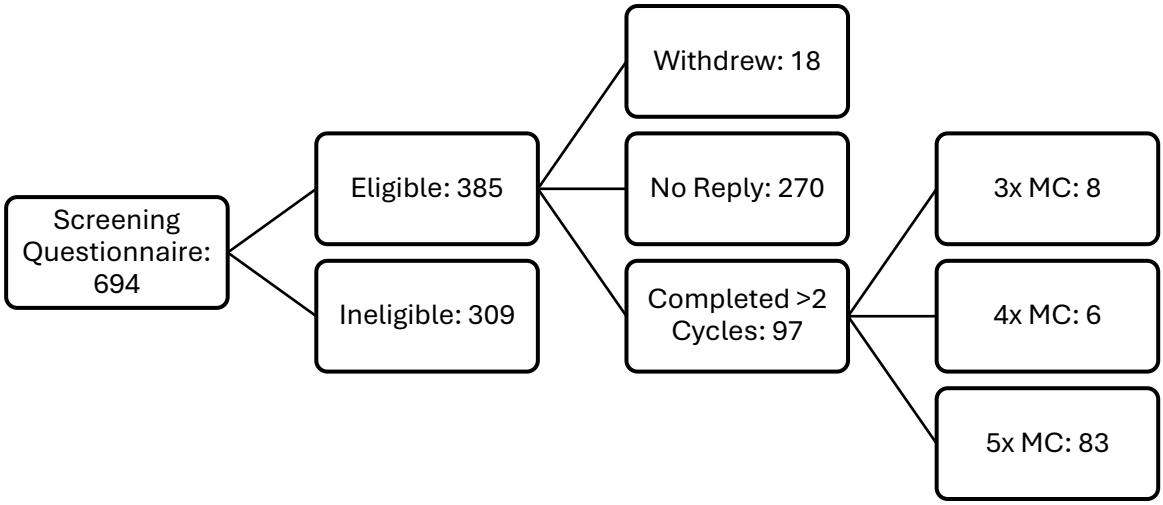


**Figure 3.1** Flow Chart of the Number of Participants from Screening to the Final Sample Size Used in the Study; MC = menstrual cycle

### *3.2.2 Procedures*

Following study registration, participants were scheduled for their initial in-person lab session for baseline data collection as soon as was convenient for them. In this session, informed consent was obtained before participants completed baseline questionnaires that collected information on the participants' health and demographics, reproductive status, and physical activity. To conclude, a food frequency questionnaire (FFQ) was completed. The questionnaires were followed by a body composition measurement and the collection of a blood sample to measure baseline iron status and inflammation. Participants were then

provided with urinary ovulation (Baby4You) tests for the next two menstrual cycles. After the baseline session, participants notified the research officer once their next menstrual bleed began. This was considered the start of cycle one and menstrual cycle data collection. The first two cycles collected menstrual cycle familiarisation data that was used to determine the timing of data collection in cycles three to five and required no in-person sessions. During these cycles, participants completed a daily survey during menstruation, reporting the presence of menstrual bleeding, and conducted urinary ovulation testing to help predict the timing of ovulation in each cycle. These procedures were also continued by each participant throughout all subsequent menstrual cycles of the study.

Table 3.1 outlines the data collected in each menstrual cycle. Participants attended their second laboratory session during cycle three, scheduled on days two to five of menstrual bleeding for the collection of early-follicular phase measurements. Late-follicular phase measurements were conducted in the fourth cycle, scheduled one to two days prior to predicted ovulation based on the timing of ovulation from the previous cycles. Mid-luteal phase measurements in cycles three to five were scheduled either seven days after a positive urinary ovulation test or five days before their next expected menstrual cycle. In each of these sessions, a blood sample was collected to assess iron biomarkers, inflammation, and female sex steroid hormones. The FFQ and body composition measurements were also repeated in cycle five mid-luteal phase testing, as this was the final testing session of the study.

**Table 3.1** Overview of Data Collected at Baseline and During Each Phase of Menstrual Cycles 1-5

| **MC Phase** | **Initial Session*** (Baseline/ Session 1) | **Cycles 1 & 2** (Familiarisation) | **Cycle 3** (Sessions 2 & 3) | **Cycle 4** (Sessions 4 & 5) | **Cycle 5** (Session 6) |
|---|---|---|---|---|---|
| **EFP** | Questionnaire: Demographics, ID history, MC status, PA<br>Food Frequency Questionnaire (FFQ)<br>Body composition (BIA)<br>Blood test: Fe | Daily menstrual survey | Blood test: Fe + FSSH*<br>Daily menstrual survey | Daily menstrual survey | Daily menstrual survey |
| **LFP** | | Urinary ovulation testing | Urinary ovulation testing | Blood test: Fe + FSSH*<br>Urinary ovulation testing | Urinary ovulation testing |
| **MLP** | | - | Blood test: FSSH* | Blood test: FSSH* | Blood test: Fe + FSSH*<br>FFQ + BIA* |

** = in-person session, BIA = Bioelectrical impedance analysis, EFP = Early-follicular phase, EFP blood test = day 2-4 of cycle, Fe = Iron biomarkers, FSSH = Female sex steroid hormones, ID = Iron deficiency, LFP = Late-follicular phase, LFP blood test = 1-2 days before expected ovulation, MC = menstrual cycle, MLP = Mid-luteal phase, MLP blood test = 5-7 days before expected period, PA = physical activity*

### *3.2.3 Body composition measurements*

Height was measured to the nearest centimetre using a stadiometer. Body composition was determined using bioelectrical impedance analysis (InBody 230). Data of interest included body mass, body fat mass, percentage body fat, and lean muscle mass. Body mass index (BMI) was calculated using participants' body weight in kilograms divided by their height in metres squared.

### *3.2.4 Blood analysis*

During each lab session, participants provided a venous blood sample while in a seated and rested position, collected by a trained phlebotomist from the antecubital vein. The blood samples were collected using a sterile 23-gauge butterfly needle into one to two plasma separator tubes (PST) and immediately inverted slowly eight times to ensure clotting did not occur prior to anticoagulation. Haemoglobin was measured on-site at Massey University, Auckland, using the HemoCue® Hb 201+ System, with a droplet of whole blood pipetted directly from the venous sample. The remainder of the blood sample was centrifuged within 30 minutes of collection at 2000rcf (4500rpm) for 10 minutes at 4°C. Once centrifuged, 2mL of plasma was aliquoted into an Eppendorf and stored in the freezer at -80°C until sent for analysis. Once a sufficient number of samples had been collected, plasma was sent to Auckland LabPlus in batches for analysis of iron biomarkers (SF, SI, TSAT, and total iron-binding capacity (TIBC)), inflammation (C-reactive protein (CRP)), and female sex steroid hormones (oestradiol and progesterone).

### *3.2.5 Menstrual cycle analysis*

Participants notified the research officer once menstrual bleeding began for each cycle. A survey was then emailed to participants to complete nightly for seven days or until menstrual bleeding ended. This survey asked participants to confirm the presence of menstrual bleeding and also allowed menstrual cycle length to be determined. Urinary ovulation (LH) testing to determine the timing of ovulation was conducted using ovulation urinary dip tests (Baby 4 You, 2009). Participants with cycle lengths <30 days began urinary ovulation testing on day eight of their cycle and continued testing daily until a positive test result or until day 21 of their cycle. Participants with cycle lengths ≥30 days commenced urinary ovulation testing on day 10 of their cycle and continued until a positive result or until day 24-25 of their cycle. A positive result was indicated by the presence of two dark lines 5-10 minutes after dipping the test strip into the urine sample. Images of the urinary ovulation

test results were sent to the research officer to monitor compliance and ensure correct interpretation of the test result.

### *3.2.6 Questionnaires*

Questionnaires were conducted online using Qualtrics software. Baseline questionnaires included a health and demographics questionnaire, a reproductive status questionnaire (Schmalenberger et al., 2021), and the New Zealand Physical Activity Questionnaire Short Form (NZPAQ-SF) (McLean et al., 2004). These collected self-reported information on the participants demographics (e.g. age, ethnicity), medical history (e.g. previous ID and/ or blood donation), menstrual history (e.g. age of menarche, typical cycle length), and physical activity (e.g. hours per week at different activity levels). Dietary intake of food groups was determined using a previously validated 109-item semi-quantitative FFQ, which asked participants to report how often they consumed various foods/ food groups in the previous month (Mumme et al., 2021).

### *3.2.7 Data handling and statistical analysis*

Data was initially cleaned by a statistician to ensure consistency of data across the multiple individual studies derived from the female health project. Cleaning of data involved compiling relevant participant data into spreadsheets, standardising variable names and coding across time points, and checking for implausible values or reporting errors. Iron insufficiency was defined as SF <30µg/L while iron sufficiency was defined as SF ≥30µg/L (Clénin et al., 2015). Subclinical ovulatory disturbances included anovulation, defined by a mid-luteal progesterone of <3nmol/L, and LPD, defined by either a mid-luteal progesterone of <10nmol/L or a short luteal phase <10 days (Prior et al., 2015; Schliep et al., 2014). Participants were characterised as consistently SOD if they had at least two cycles that presented with an SOD, while participants with at least two cycle measurements, of which no more than one was SOD, were characterised as consistently ovulatory (De Souza et al., 1998; De Souza et al., 2010). Physical activity was scored as per the International Physical Activity Questionnaire (IPAQ) in MET-minutes per week, and classified as low, moderate (≥3 days/ week vigorous activity (≥20 minutes/ day) or ≥5 days/ week moderate-intensity activity (≥30 minutes/ day) or ≥5 days/ week any activity (≥600 MET-minutes/ week)), or high (≥3 days/ week vigorous activity (≥1500 MET-minutes/ week) or ≥7 days/ week any activity (≥3000 MET-minutes/ week)) (Craig et al., 2003). Responses from the FFQ were converted into frequencies of intake per week for each participant, with one month considered equivalent to four weeks. The mid-point of each intake category was used, for example, a response of one to three times per month was converted to two times per month, equating to

0.5 servings per week. The resulting data were subsequently grouped into relevant food groups for analysis, as outlined in appendix A.

Statistical analysis was completed using IBM SPSS Statistics (version 30.0). Data was tested for normality using the Komogorov-Smirnov and Shapiro-Wilk tests, with $p>0.05$ reflecting no significant difference of the sample from the normal distribution, and thus, treated as normally distributed. Normally distributed data was reported as mean ± standard distribution, while non-normally distributed data was reported as median with $25^{th}$ and $75^{th}$ percentiles. However, where appropriate, data was reported in the same scale across groups for easy comparison. Categorical data were reported as frequencies with the number of participants and percentages for each group.

For comparison between groups, normally distributed data (with or without log transformation) was also tested for homogeneity using Levene's test, with $p>0.05$ reflecting equal variances between the groups, and thus, considered homogenous. Comparison between groups for parametric data (normally distributed and homogenous) was conducted through independent *t*-tests, while non-parametric data (non-normal and/ or non-homogenous) was compared through Mann-Whitney U tests and Friedman's analysis of variance (ANOVA). Categorical variables were compared using Pearson's Chi-square ($\chi^2$) test and Cochran's Q test. For the chi-square test, all groups were independent and <20% of the expected counts were <5, with none <1. Differences between groups were considered statistically significant with a *p*-value of <0.05 for all tests. For significant differences found using Friedman's ANOVA, Wilcoxon Signed-Rank tests were performed as post hoc analyses to determine between which groups the differences occurred. To adjust for type I error, a Bonferroni correction was applied to post hoc tests, with a new *p*-value of <0.0167 considered statistically significant. Correlations were conducted using Spearman's correlation coefficient as data for correlations violated parametric assumptions. A *p*-value (two-tailed) of <0.05 was considered statistically significant, with correlations of ±0.1 indicating a small effect, ±0.3 indicating a medium effect, and ±0.5 indicating a large effect (Field, 2024).

Multiple linear regression analysis was conducted to identify factors associated with the change in SF from baseline to study end. Continuous variables considered were gynaecologic age (chronological age minus age of menarche), change in CRP, change in body fat percentage, change in BMI, average menstrual cycle length, average menstrual bleed length, frequency of food group consumption (meat/ fish/ poultry, red meat, fruit and vegetables, tea and coffee servings per week), and number of days between baseline and study-end measurements. Ovulatory status (consistently ovulatory vs SOD) and iron aid supplementation (e.g. vitamin C and/ or multivitamins with very low dose iron) were also considered as categorical variables. The dependent variable, change in SF, was not

normally distributed, which was not improved with log transformation. However, the multiple linear regression analysis was deemed sufficiently robust to withstand the non-normality of change in SF as the residuals were normally distributed. The independence of residuals was confirmed by the Durbin-Watson test, with a test statistic of 1-3 indicating no correlation between residuals. Multicollinearity between independent variables was assessed, with variance inflation factor (VIF) values <10 and tolerance values >0.2 indicating no multicollinearity. Visual review of the residual's scatterplot indicated homoscedasticity, evidenced by an even horizontal spread and the absence of any distinct pattern.

## 3.3 Results

### *3.3.1 Participant characteristics*

Of the 115 participants recruited, 97 women provided at least three menstrual cycles' worth of data (two familiarisation cycles and at least one cycle of blood measurements) and were subsequently included in the final analysis. Of the 97 women included, 69 (71.1%) had sufficient iron stores (SF ≥30µg/L) at baseline, 27 (27.8%) had insufficient iron stores (SF <30µg/L), and one participant (1.1%) had no baseline SF so was excluded from baseline iron status classification. Four (4.1%) participants had a CRP >5mg/L so a higher SF cutoff of <70µg/L was used to define iron insufficiency in these participants in accordance with the World Health Organisation (2020) recommendations. However, using a higher SF cutoff did not reclassify iron status from sufficient to insufficient for these participants at baseline.

Table 3.2 summarises the characteristics, dietary intake and iron biomarkers of the participants. Previous blood donation included either whole blood or plasma. The average menstrual bleed and cycle lengths were calculated as the mean bleed and cycle lengths across the study (three to five cycles) for each participant. Participants had a median age of 31 years, with most participants identifying as European. For dietary intake, frequency of food group consumption per week did not significantly differ between iron-sufficient and iron-insufficient participants, except for white meat intake. White meat intake was significantly higher ($p$=0.005) in iron-sufficient participants than in iron-insufficient participants, despite the same medians for both groups. Significant differences in iron biomarkers were observed between iron-sufficient and insufficient groups. Participants classified as iron-insufficient at baseline had significantly lower SF ($p$<0.001), SI ($p$=0.042), and TSAT ($p$<0.001) compared to iron-sufficient participants. However, TIBC was significantly higher in iron-insufficient participants ($p$<0.001) than in iron-sufficient participants. There was no difference in Hb or CRP between iron-sufficient and insufficient participants. Other demographic, health, menstrual and physical activity characteristics did not significantly differ between groups.

**Table 3.2** Demographics, Dietary Intake and Iron Biomarkers of Participants at Baseline

| Characteristic | | Overall [a] n = 97 | Fe Sufficient [b] n = 69 (71.1%) | Fe Insufficient [c] n = 27 (27.8%) | P-value [d] |
|---|---|---|---|---|---|
| Age (years) † | | 31 (26, 35) | 31 (26, 35) | 30 (24, 38) | 0.639 |
| BMI (kg/m$^2$) † | | 24.4 (22.0, 26.3) | 24.5 (22.6, 26.3) | 24.4 (21.1, 28.7) | 0.690 |
| *Missing* | | *14* | *7* | *7* | |
| Body Fat (%) * | | 29.5 ± 8.7 | 28.8 ± 7.9 | 32.2 ± 10.7 | 0.126 |
| *Missing* | | *14* | *7* | 7 | |
| Ethnicity § | Māori/ Pacific | 8 (8.2) | 7 (10.1) | 1 (3.7) | 0.229 |
| | European | 70 (72.2) | 51 (73.9) | 18 (66.7) | |
| | Asian/ MELAA | 19 (19.6) | 11 (15.9) | 8 (29.6) | |
| Age of Menarche (years) † | | 13 (12,13) | 13 (12, 13) | 13 (11, 13) | 0.507 |
| *Missing* | | *1* | *0* | *1* | |
| Average MC Length (days) † | | 28 (27, 31) | 28 (27, 31) | 29 (27, 31) | 0.823 |
| Average MB Length (days) † | | 4.8 (4.2, 5.4) | 4.8 (4.0, 5.4) | 5.2 (4.5, 5.4) | 0.103 |
| *Missing* | | *3* | *2* | *1* | |
| Consistent Ovulatory Status § | Ovulatory | 68 (70.1) | 50 (79.4) | 18 (81.8) | 0.804 |
| | SOD | 18 (18.6) | 13 (20.6) | 4 (18.2) | |
| | *Missing* | *11* | *6* | *5* | |
| Having Children § | | 21 (21.6) | 13 (19.1) | 8 (29.6) | 0.265 |
| *Missing* | | *1* | *1* | *0* | |
| Previous ID § | | 44 (45.4) | 28 (40.6) | 15 (55.6) | 0.275 |
| Previous Blood Donation § | | 16 (16.5) | 10 (14.5) | 6 (22.2) | 0.361 |
| Use of Iron Aid Supplements [e] § | | 22 (22.7) | 14 (20.3) | 7 (25.9) | 0.548 |
| Physical Activity § | Low | 10 (10.3) | 7 (10.1) | 3 (11.1) | 0.244 |
| | Moderate | 32 (33.0) | 19 (27.5) | 12 (44.4) | |
| | High | 55 (56.7) | 43 (62.3 | 12 (44.4) | |
| Dietary Intake † (servings/week) | Fruits/ Vegetables | 44.5 (30.4, 56.1) | 48.1 (32.3, 60.5) | 40.6 (27.4, 50.6) | 0.151 |
| | Citrus Fruits | 1.0 (0.1, 2.5) | 0.5 (0.1, 2.5) | 1.0 (0.5, 2.5) | 0.074 |
| | Meat/ Fish/ Poultry | 8.8 (5.6, 12.8) | 9.3 (6.0, 13.1) | 7.8 (2.6, 10.9) | 0.080 |
| | Red Meat | 2.5 (1.0, 5.0) | 2.5 (1.0, 2.5) | 1.0 (0.5, 2.5) | 0.096 |
| | White Meat | 2.5 (1.0, 5.0) | 2.5 (2.5, 5.0) | 2.5 (0, 2.5) | **0.005** |
| | Dairy | 15.6 (8,4, 25.4) | 15.8 (7.3, 26.8) | 14.9 (9.0, 23.3) | 0.581 |
| | Cereals/ Grains | 14.1 (9.1, 21.0) | 13.3 (8.6, 19.4) | 15.5 (10.9, 23.4) | 0.453 |
| | Tea/ Coffee | 13.0 (7.5, 22.5) | 17.6 (8.0, 24.5) | 9.6 (2.6, 19.0) | 0.115 |
| | Alcohol | 1.3 (0.5, 2.6) | 1.4 (0.5, 2.8) | 0.9 (0.5, 2.3) | 0.412 |
| | *Missing* | *2* | *2* | *0* | |
| Fe Biomarkers † | SF (µg/L) | 41 (27, 58) | 51 (39, 75) | 19 (13, 26) | **<0.001** |
| | SI (µmol/L) | 15 (12, 19) | 16 (12, 20) | 13 (8, 18) | **0.042** |
| | TSAT (%) | 27 (20, 34) | 30 (22, 36) | 20 (14, 27) | **<0.001** |
| | TIBC (µmol/L) | 58 (52, 64) | 55 (51, 59) | 66 (61, 71) | **<0.001** |
| | *Missing* | *1* | *0* | *0* | |
| | Hb (g/L) | 133 (125, 141) | 136 (125, 141) | 132 (126, 139) | 0.374 |
| | *Missing* | *3* | *3* | *0* | |
| | CRP (mg/L) | 0.6 (0.6, 1.7) | 0.6 (0.6, 1.2) | 1.0 (0.6, 2.4) | 0.226 |
| | *Missing* | *2* | *0* | *1* | |

[a] One participant had no baseline SF for iron status classification

[b] Serum ferritin ≥ 30µg/L

[c] Serum ferritin < 30µg/L

[d] Significant differences between Fe sufficient and Fe insufficient groups (*p < 0.05)* as assessed by the Mann-Whitney U test (non-parametric data), independent *t*-test (parametric data), or Pearson's Chi-square ($\chi^2$) test (categorical data)

[e] E.g. vitamin C and/ or multivitamins with very low dose iron

§ Count (%)

* Mean ± SD

† Median (25$^{th}$, 75$^{th}$ percentiles)

*BMI = body mass index, CRP = C-reactive protein, Fe = iron, Hb = haemoglobin, ID =* iron deficiency, *MB = menstrual bleed, MC = menstrual cycle, MELAA = Middle Eastern, Latin American and African, SF = serum ferritin, SI = serum iron, SOD = subclinical ovulatory disturbance, TIBC = total iron-binding capacity, TSAT = transferrin saturation*

### *3.3.2 Between-group differences within menstrual cycle phases*

Table 3.3 compares iron biomarkers and female sex steroid hormone concentrations between consistently ovulatory participants and consistently SOD participants within the early-follicular, late-follicular and mid-luteal phases of the menstrual cycle. Of the 97 participants included in the study, 68 (70.1%) participants were considered consistently ovulatory (≥2 cycle measurements with <2 SOD) and 18 (18.6%) participants were considered consistently SOD (≥2 SOD cycles). One participant in the consistently SOD group had an iron infusion between the early-follicular and late-follicular phase measurements, so was excluded from analysis of iron status and biomarkers in the late-follicular phase. Eleven (11.3%) participants did not have sufficient data to determine consistent (≥2 cycles) ovulatory status classification. In participants with CRP concentrations >5mg/L, a higher SF cutoff of <70µg/L was applied to define iron insufficiency, as previously described. This impacted six (6.2%) participants in the early-follicular phase, one (1.0%) in the late-follicular phase, and two (2.1%) participants in the mid-luteal phase. All were reclassified as iron-insufficient under the higher cutoff, except for one participant who was already classified as such.

No significant differences in iron status (sufficient vs insufficient) were found between consistently ovulatory participants and consistently SOD participants within the early-follicular, late-follicular or mid-luteal phases of the menstrual cycle. In the early-follicular phase, there were also no significant differences in iron biomarkers (SF, Hb, SI, TIBC, TSAT and CRP) or female sex steroid hormones (oestradiol and progesterone) between consistently ovulatory participants and consistently SOD participants. In the late-follicular phase, consistently SOD participants had significantly lower TSAT ($p$=0.049) than consistently ovulatory participants. However, there were no other significant differences in iron biomarkers between consistently ovulatory and consistently SOD participants in either the late-follicular or mid-luteal phases. In terms of female sex steroid hormones, consistently SOD participants had significantly lower oestradiol ($p$<0.001) in the late-follicular phase while progesterone was significantly lower ($p$<0.001) in the mid-luteal phase compared to consistently ovulatory participants.

**Table 3.3** Comparison of Iron Biomarkers and Female Sex Steroid Hormones Across Menstrual Phases and Cycles Between and Within Consistently Ovulatory and Consistently SOD Participants

| | Early-Follicular Phase | | | Late-Follicular Phase | | | Mid-Luteal Phase | | | P-value[d] | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Ovulatory**[a] n = 68 (70.1%) | **SOD**[b] n = 18 (18.6%) | **P-value**[c] | **Ovulatory**[a] n = 68 (70.1%) | **SOD**[b] n = 18 (18.6%) | **P-value**[c] | **Ovulatory**[a] n = 68 (70.1%) | **SOD**[b] n = 18 (18.6%) | **P-value**[c] | **P-value Ovulatory** | **P-value SOD** |
| **Iron Status** | | | | | | | | | | | |
| Sufficient [§] | 44 (66.7) | 14 (82.4) | 0.209 | 47 (71.2) | 11 (73.3) | 0.869 | 44 (68.8) | 11 (68.8) | 1.000 | 0.779 | 0.368 |
| Insufficient [§] | 22 (33.3) | 3 (17.6) | | 19 (28.8) | 4 (26.7) | | 20 (31.3) | 5 (31.3) | | | |
| *Missing* | *2* | *1* | | *2* | *3* | | *4* | *2* | | | |
| **Iron Biomarkers** | | | | | | | | | | | |
| SF (µg/L) [†] | 52 (29, 70) | 44 (33, 57) | 0.361 | 42 (28, 61) | 46 (28, 58) | 0.516 | 42 (27, 59) | 38 (20, 54) | 0.361 | **0.007** | 0.640 |
| SI (µmol/L) [†] | 13 (10, 19) | 13 (10, 19) | 0.846 | 16 (13, 19) | 13 (10, 17) | 0.068 | 17 (13, 21) | 16 (10, 21) | 0.304 | **<0.001** | 0.276 |
| TSAT (%) [†] | 23 (17, 31) | 25 (18, 33) | 0.991 | 26 (21, 35) | 24 (16, 29) | **0.049** | 28 (21, 38) | 25 (17, 38) | 0.388 | **0.002** | 0.338 |
| TIBC (µmol/L) [†] | 58 (51, 63) | 58 (55, 67) | 0.419 | 58 (52, 62) | 59 (51, 66) | 0.291 | 59 (54, 66) | 60 (54, 72) | 0.567 | **0.001** | 0.943 |
| *Missing* | *2* | *1* | | *2* | *3* | | *4* | *2* | | | |
| Hb (g/L) [†] | 134 (122, 143) | 137 (125, 148) | 0.886 | 130 (125, 139) | 126 (100, 142) | 0.322 | 130 (124, 138) | 132 (123, 138) | 0.840 | 0.942 | 0.862 |
| *Missing* | *6* | *1* | | *4* | *4* | | *6* | *3* | | | |
| CRP (mg/L) [†] | 0.8 (0.6, 1.8) | 1.2 (0.6, 1.8) | 0.638 | 0.6 (0.6, 1.2) | 0.9 (0.6, 2.0) | 0.124 | 0.6 (0.6, 1.2) | 0.8 (0.6, 1.0) | 0.847 | **<0.001** | 0.318 |
| *Missing* | *5* | *1* | | *5* | *3* | | *4* | *2* | | | |
| **Female Sex Steroid Hormones** | | | | | | | | | | | |
| E2 (pmol/L) [†] | 123 (97, 162) | 119 (75, 162) | 0.399 | 705 (315, 1017) | 222 (167, 310) | **<0.001** | 436 (357, 641) | 333 (258, 613) | 0.152 | **<0.001** | **<0.001** |
| P4 (nmol/L) [†] | 0.7 (0.5, 1.0) | 0.6 (0.6, 1.0) | 0.875 | 0.9 (0.4, 2.5) | 0.5 (0.4, 0.6) | 0.144 | 33.4 (20.4, 55.1) | 1.7 (0.6, 14.5) | **<0.001** | **<0.001** | **0.013** |
| *Missing* | *1* | *2* | | *2* | *2* | | *4* | *2* | | | |

[a] Consistently ovulatory participants (≥2 cycle measurements and <2 SOD)

[b] Consistently SOD participants (≥2 SOD cycles)

[c] Significant differences ($p < 0.05$) between ovulatory and SOD groups within each cycle as assessed by the Mann-Whitney U test (non-parametric data), independent *t*-test (parametric data), or Pearson's Chi-square ($\chi^2$) test (categorical data)

[d] Significant differences ($p < 0.05$) within subjects (consistently ovulatory or consistently SOD) across each cycle as assessed by Friedman's ANOVA (non-parametric data), or Cochran's Q test (categorical data). For significant differences ($p < 0.05$), post hoc tests were conducted with Bonferroni correction ($p < 0.0167$) to determine between which groups the differences occurred

[§] Count (%)

[†] Median (25th, 75th percentiles)

*CRP = C-reactive protein, E2 = oestradiol, Hb = haemoglobin, P4 = progesterone, SF = serum ferritin, SI = serum iron, SOD = subclinical ovulatory disturbances, TIBC = total iron-binding capacity, TSAT = transferrin saturation*

### *3.3.3 Within-group differences between menstrual cycle phases*

Table 3.3 also compares differences in iron biomarkers and female sex steroid hormones across phases of the menstrual cycle within consistently ovulatory or consistently SOD participants. Iron status (sufficient vs insufficient) did not significantly differ between phases of the menstrual cycle in either consistently ovulatory or consistently SOD participants. In terms of iron biomarkers, significant differences across menstrual cycle phases were observed in consistently ovulatory participants but not in those who were consistently SOD. Significant differences between menstrual cycle phases were found in SF ($p$=0.007), SI ($p$<0.001), TIBC ($p$=0.001), TSAT ($p$=0.002) and CRP ($p$<0.001) for consistently ovulatory participants. Female sex steroid hormones also showed significant differences between menstrual cycle phases for both consistently ovulatory (oestradiol $p$<0.001; progesterone $p$<0.001) and consistently SOD participants (oestradiol $p$<0.001; progesterone $p$=0.013). However, no significant correlations between iron biomarkers (SF, Hb, SI, TSAT, and/ or TIBC) and female sex steroid hormones (oestradiol and/ or progesterone) were found in the early-follicular, late-follicular or mid-luteal phases of the menstrual cycle.

Following post hoc tests, serum ferritin was significantly lower in the late-follicular phase ($p$=0.007) than the early-follicular phase for consistently ovulatory participants. These participants also had significantly higher SI ($p$<0.001) and TSAT ($p$=0.002) in the mid-luteal phase compared to the early-follicular phase. Total iron-binding capacity was significantly higher in the late-follicular phase ($p$=0.015) and the mid-luteal phase ($p$=0.002) than the early-follicular phase in consistently ovulatory participants, despite no obvious differences in reported descriptive statistics. C-reactive protein was significantly higher in the early-follicular phase than both the late-follicular phase ($p$=0.003) and the mid-luteal phase ($p$=0.009) in consistently ovulatory participants. For oestradiol, each phase of the menstrual cycle in consistently ovulatory participants was found to be significantly different from the other phases (early-follicular vs late-follicular, $p$<0.001; late-follicular vs mid-luteal, $p$=0.001; early-follicular vs mid-luteal, $p$<0.001). As expected, oestradiol concentrations were lowest in the early-follicular phase and peaked in the late-follicular phase, with a decline in the mid-luteal phase. In consistently SOD participants, significant differences between oestradiol were only found between the early follicular and late-follicular phases ($p$=0.001) and the early-follicular and mid-luteal phases ($p$=0.002). Finally, in consistently ovulatory participants, progesterone was significantly higher in the mid-luteal phase compared to the early-follicular ($p$<0.001) and the late-follicular ($p$<0.001) phases. While progesterone was also significantly higher in the mid-luteal phase than the late-follicular phase ($p$=0.003) in consistently SOD participants, it was also significantly higher in the early-follicular phase than the late-follicular phase ($p$=0.014) in this group.

### *3.3.4 Overall predictors of change in SF*

Multiple linear regression analysis was conducted to identify significant predictors of the change in SF (0 (-13,7) µg/L) (median ($25^{th}$, $75^{th}$ percentiles)) from baseline to study end. One participant was excluded from the analysis as their baseline SF (137µg/L) was significantly higher than their following SF measurements (11µg/L, 74µg/L and 37µg/L respectively). Potential predictor variables were selected based on prior literature and included change in CRP (0 (-0.3, 0.1) mg/L), change in body fat percentage (0.1 (-1.0, 1.5)%), and change in meat/ fish/ poultry intake (0.4 (-1.4, 1.8) servings per week), as well as average menstrual bleed length (4.8 (4.2, 5.4) days) over the study period (median ($25^{th}$, $75^{th}$ percentiles)). Variables demonstrating correlations with the change in SF at $p<0.200$ were entered into the final model using the enter method. While additional variables such as change in red meat, fruit and vegetable, and tea and coffee intakes, change in BMI, and average menstrual cycle length were also tested for correlations with change in SF, only change in CRP met the significance threshold of $p<0.200$ to be entered into the final model. Gynaecologic age (18 (13, 23) years), ovulatory status (consistently ovulatory vs SOD), use of iron aid supplements, and the number of days between baseline and end measurements (178 (152, 211) days) were identified as potential confounding variables and were also included in the final model to account for their potential influence on the change in SF.

The final model indicated that a change in CRP was significantly associated with a change in SF ($p<0.001$), with the model explaining 24.7% of the variation in SF change after controlling for gynaecologic age, ovulatory status, iron aid supplementation and number of days between measurements (table 3.4). For every 1mg/L increase in the change in CRP, the change in SF increased by 1.58µg/L (95% CI 0.84, 2.32µg/L). A backward stepwise method was also trialled with all literature-based potential predictor variables previously mentioned initially entered, along with the potential confounding variables. This method also found that a change in CRP was the only significant variable associated with a change in SF.

**Table 3.4** Predictors of Overall Change in Serum Ferritin from Study Baseline to End

| Model | Coefficient *B* | Standard Error *B* | 95% CI for *B* | Standardised *B* | P-value |
|---|---|---|---|---|---|
| (Constant) | -15.9 | 15.6 | -47.1, 15.2 | | 0.312 |
| Change in CRP (mg/L) | 1.58 | 0.372 | 0.84, 2.32 | 0.480 | **<0.001** |
| Gynaecologic Age | 0.493 | 0.370 | -0.244, 1.231 | 0.142 | 0.187 |
| Ovulatory Status (Ovulatory) | 9.47 | 5.24 | -0.983, 19.91 | 0.197 | 0.075 |
| Supplement Use (Taking Iron Aid) | -7.05 | 5.09 | -17.21, 3.10 | -0.143 | 0.170 |
| Days Between Baseline and End | 0.036 | 0.051 | -0.066, 0.137 | 0.079 | 0.485 |

$n=78$; $F(5,77) = 4.73$; $p<0.001$; $R^2 = 0.247$; $R^2$ (adj) = 0.195

*B = beta, CI = confidence interval, CRP = C-reactive protein*

## 3.4 Discussion

To the best of our knowledge, this is the first longitudinal prospective cohort study that has simultaneously explored women with and without ID across multiple menstrual cycles, while characterising menstrual cycle features including menstruation duration, ovulation timing, and hormonal profiles with ovulatory status classification (ovulatory vs SOD). Our findings demonstrate that ovulatory status classification significantly influences fluctuations in iron biomarkers across menstrual cycle phases. While menstrual cycle phase was found to significantly influence iron biomarker concentrations, apart from Hb, in consistently ovulatory women, the same was not observed for consistently SOD women. Despite this, iron biomarkers did not significantly differ between ovulatory and SOD participants within any menstrual phase, and no direct correlations were found between iron biomarkers and female sex steroid hormones.

### *3.4.1 Prevalence of ID*

While many studies have explored the prevalence of ID in a population of NZ premenopausal women, there is considerable variability in the SF cutoff used to define ID. In the current study, an SF cutoff of <30µg/L was used to define iron insufficiency in accordance with Clénin et al. (2015), as a more sensitive threshold in detecting low iron stores associated with stage one ID. Prevalence of iron insufficiency at baseline within our study was relatively low (27.8%) compared to a previous NZ study using the same threshold (55.8%) (Lim et al., 2020). However, Lim et al. (2020) noted their rates of ID were unusually high, potentially due to the high proportion of vegetarian, vegan and/ or South Asian participants in their study, known to be at increased risk of ID (Beck et al., 2014). In contrast, Beck et al. (2014) and Heath et al. (2001) used a lower SF cutoff of <20µg/L to define ID in their studies, reporting a prevalence of 18.7% (*n*=375) and 23% (*n*=384), respectively. Comparatively, when our threshold for iron insufficiency was adjusted to this cutoff, 14.4% of participants were iron-insufficient at baseline. Older research among NZ premenopausal women used an even lower threshold of SF <12µg/L to define ID, with Ferguson et al. (2001) reporting a prevalence of 7%, and Schaaf et al. (2000) reporting a prevalence of 18.3%, while the 2008/09 NZ Adult Nutrition Survey reported a prevalence between 5.2% and 12.1% (University of Otago & Ministry of Health, 2011). However, an SF threshold of <12µg/L for ID is noted as a relatively low cut-off, typically indicating depleted iron stores associated with stage two and three ID, and could potentially underestimate the true prevalence of ID in NZ women. Therefore, future studies along with clinical practice should consider using a higher SF cutoff of <30µg/L to define ID and ensure participants with low iron stores are recognised.

In the current study, there were no significant differences in demographics, menstrual characteristics and dietary intake between iron-sufficient and iron-insufficient participants at baseline, with the exception of white meat intake. Lim et al. (2020) reported similar findings, with only meat intake significantly differing between iron status groups. In contrast, Beck et al. (2014) reported that being Asian ethnicity, having children, blood donation in the past year, increased duration of menstruation, and previous ID were all significantly associated with suboptimal iron status, while Heath et al. (2001) reported that low meat/ fish/ poultry intake, high menstrual blood loss, recent blood donation, nose bleeds, and low body mass index (BMI) were associated with an increased risk of ID. However, the use of a lower SF threshold of <20µg/L in these studies may have contributed to the greater differences observed between iron status groups, as iron-deficient participants would likely have more advanced ID than those in the present study. In the context of the literature, this suggests that risk factors for ID may be more associated with advanced stages of ID, rather than low iron stores.

### *3.4.2 Prevalence of SOD*

The prevalence of SOD, including anovulation and LPD, varies considerably across the literature, with research indicating that it is not uncommon for women with regular menstrual bleeding to experience intermittent SOD cycles (De Souza, 2003). In the current study, the prevalence of SOD ranged from 18.6 to 28.4% within each cycle, while 18.6% of participants (20.9% of those with sufficient data) were predominantly SOD (≥2 SOD cycles). In a large-scale population-based study (*n*=1545), Prior et al. (2015) found a 36.7% prevalence of SOD among naturally menstruating women, aged 20-49.9 years, with normal length menstrual cycles (21-35 days). However, the higher age range of their participants compared to our study (20-40 years) may have increased the prevalence of SOD, particularly in participants near the upper age range who may have been experiencing perimenopause (Mihm et al., 2011). Using the same classification criteria to define consistently SOD participants as used in the present study, De Souza et al. (1998) and De Souza et al. (2010) reported a higher prevalence of consistently SOD participants (33% and 31.9% respectively). However, this was reported among exercising women with less than 10% of sedentary women found to be consistently SOD (De Souza et al., 1998; De Souza et al., 2010). Additionally, De Souza et al. (1998) reported that up to 79% of women within the study experienced at least one SOD cycle compared to ~40% of women experiencing at least one SOD cycle in the present study. These statistics reinforce the idea that intermittent SOD cycles are common, even in women with regular-appearing menstrual cycles. Therefore, future research into iron biomarker fluctuations across the menstrual cycle should

consider measuring ovulatory function across multiple menstrual cycles to more accurately classify ovulatory status and account for changing hormonal profiles.

Across the literature, there is variability among research studies for SOD cycle classifications. In the current study, a progesterone cutoff of <10nmol/L in the mid-luteal phase was used to classify SOD (specifically biochemical LPD), based on research by Shepard and Senturia (1977) and Prior et al. (2015). A single random progesterone measurement of >3ng/mL (~9.54nmol/L) was previously found to be strongly associated with a secretory phase endometrium, thus, 10nmol/L was used as our cutoff for an ovulatory cycle to reduce the likelihood of false positives or negatives (Prior et al., 2015; Shepard & Senturia, 1977). Using this cutoff, our study found 24% of all cycles (*n*=254) were classified as SOD. However, De Souza et al. (1998) used peak pregnanediol-3-glucuronide (PdG) excretion of <3µg/mg creatinine, reporting an SOD cycle prevalence of 10% and 55% among sedentary (*n*=31) and exercising (*n*=66) women, respectively. Similarly, De Souza et al. (2010) used a peak PdG excretion value of <5µg/mL, reporting SOD cycle prevalence rates of 4% and 50% among sedentary (*n*=48) and exercising (*n*=120) women, respectively. These marked differences between sedentary and exercising women suggest that physical activity level may influence SOD prevalence, which may explain why the prevalence observed in the current study among a general recreationally active cohort falls between these values. Prevalence of SOD cycles in our study more closely aligns with findings by Schliep et al. (2014), who reported SOD in 15% of cycles (*n*=476) using a progesterone threshold of <5ng/mL (<16nmol/L). However, the varying thresholds used across the literature to classify SOD makes comparisons of SOD prevalence rates between populations challenging. Interpretation is further complicated by inconsistencies in whether studies report the prevalence of SOD among cycles or among women. Therefore, future research may consider standardising the measurement used to classify SOD and the way in which prevalence is reported to allow better comparisons across research.

### *3.4.3 Iron biomarkers: Menstrual cycle phase and status differences*

While our findings suggest that ovulatory status does not influence either iron status or iron biomarker concentrations within menstrual cycle phases, all iron biomarkers, except for Hb, appear to be associated with menstrual cycle phase among consistently ovulatory participants. Interestingly, the same was not observed for consistently SOD participants, with iron biomarkers not significantly differing between menstrual cycle phases in these participants. This is a novel finding, as most previous studies exploring iron status within the menstrual cycle have focused on eumenorrheic cycles or have not distinguished between ovulatory and SOD cycles. As the impact of SOD on iron biomarker fluctuations across menstrual phases has not previously been explored, these findings highlight the importance

of considering ovulatory status when interpreting iron biomarker changes across the menstrual cycle, as the hormonal patterns that characterise SOD cycles may alter expected fluctuations in iron metabolism. Failure to account for SOD cycles in future research may mask existing associations and contribute to inconsistent findings within the literature.

Among the consistently ovulatory women in our study, our findings on iron biomarker fluctuations across menstrual cycle phases are consistent with results from previous studies. Serum iron and TSAT were lowest in the early-follicular phase and highest in the mid-luteal phase, which is generally in agreement across the literature (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016). Serum ferritin was found to be highest in the early-follicular phase before stabilising between the late-follicular and mid-luteal phases, while Hb remained relatively stable across the menstrual cycle. However, reported changes in SF and Hb across the menstrual cycle are inconsistent across previous studies of eumenorrheic cycles. Both Angeli et al. (2016) and Lainé et al. (2016) described relatively stable SF and Hb concentrations across menstrual phases, despite notable inter-individual variability. In contrast, further studies have reported significant associations between Hb and menstrual cycle phase, with Hb reported to be lowest during menses and highest in the late-luteal phase (Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Suzuki et al., 2018). While Kim et al. (1993) found a similar trend in SF to that of Hb, Aguree et al. (2023) reported the opposite, with SF concentrations highest in the early-follicular phase and lowest in the mid-luteal phase.

A potential explanation for the decrease in SF following menstruation may be due to iron loss associated with menstrual bleeding (Nolte et al., 2025). Nolte et al. (2025) reported a significant association between menstrual blood loss and SF in eumenorrheic female athletes, thus, the decrease in SF observed in the late-follicular phase may reflect the delayed impact of menstrual blood loss on iron stores. Interestingly, CRP followed a similar cyclical pattern to that of SF in our study and was significantly higher in the early-follicular phase during menstruation than either the late-follicular or mid-luteal phases. This seemingly confirms a heightened inflammatory state during menstruation, coinciding with the observed progesterone withdrawal and its associated acute inflammatory response, marked by increased inflammatory cytokines and prostaglandins (Badenhorst et al., 2022). Given that SF is an acute-phase protein, the elevated CRP observed during the early-follicular phase may provide another physiological explanation for the concomitant increase in SF during menstruation (Namaste et al., 2017; World Health Organisation, 2020). However, despite the decrease in SF being statistically significant, it does not appear to significantly affect long-term iron status over consecutive cycles in consistently ovulatory women, supporting the claim by Alfaro-Magallanes et al. (2023) that SF is a reliable measure of iron status among premenopausal women regardless of menstrual cycle phase.

### *3.4.4 Iron biomarkers: Correlation with female sex steroid hormones*

It is generally acknowledged within the literature that hormonal fluctuations across the menstrual cycle could influence the variation in iron status measures observed (Chandra et al., 2017). However, the lack of correlations found between any of the iron biomarkers and female sex steroid hormones within each menstrual phase in our study suggests that the fluctuations in iron biomarkers that occur across the menstrual cycle in consistently ovulatory women are not directly associated with female sex steroid hormones themselves. This was an area of research that had not yet been investigated directly, so there were no previous studies to compare our findings to. Of the seven studies identified that investigated iron biomarkers across menstrual cycle phases, only Alfaro-Magallanes et al. (2023) measured oestradiol and progesterone concentrations to confirm menstrual phase. Although their study compared iron biomarker fluctuations across menstrual phases between naturally menstruating women and oral contraceptive users, they did not explore direct associations between iron biomarkers and female sex steroid hormones. Therefore, our findings present novel insights on the influence of the menstrual cycle, suggesting that variations in iron biomarkers across the menstrual cycle in consistently ovulatory women may be mediated by other physiological processes linked to menstrual cycle phase, rather than by direct effects of female sex steroid hormones. Future research may be necessary to provide more conclusive evidence, as well as investigate if intra-individual fluctuations in female sex steroid hormones across menstrual cycle phases may be associated with concurrent variation in iron biomarkers.

### *3.4.5 Overall predictors of SF*

Our final model showed a change in CRP was the only variable significantly associated with a change in SF from baseline to study end in our population of premenopausal women. Specifically, a 1mg/L decrease in CRP, and thus, inflammation, over the study period was associated with a 1.58µg/L (95% CI 0.84, 2.32µg/L) decrease in SF over the same period. However, because SF is an acute-phase protein, this model likely reflects the modulation of SF by inflammatory processes rather than an actual change in iron stores, and hence, may not be reflective of the risk of ID. While inflammatory states can be associated with an increased risk of ID due to elevated hepcidin concentrations suppressing iron absorption and the release of stored iron, this cannot be deduced from the model, due to SF being an inaccurate indicator of iron status under inflammatory conditions and the exclusion of hepcidin analysis in our study (Coad & Conlon, 2011; Lim et al., 2016).

Across the literature, a range of factors have been identified as potential predictors of SF, although findings are not always consistent across studies. In the current study, no other factors previously identified as predictors of SF were found to be associated with a change in

SF from study baseline to end. Beck et al. (2014) reported blood donation in the past year, being Asian ethnicity and previous ID to be the strongest predictors of SF among participants, with a milk and yogurt dietary pattern, duration of menstrual period, and a meat and vegetable dietary pattern in women with children to also be significant predictors. While Martin et al. (2023) reported low body mass index and blood donation in the past year as significant predictors of SF in healthy premenopausal women joining the NZ army, they reported no association between SF and a vegetarian dietary pattern or physical activity level. However, in studies with hepcidin measures, hepcidin was found to be the strongest predictor of SF (Lim et al., 2020; Lim et al., 2016). Despite these findings, our study differed by considering how variables known to be risk factors of ID may have changed over the course of the study and whether changes in these variables contributed to the changes in SF observed. However, this posed limitations in determining the overall impact of the variables tested on declining iron stores, as these variables may not have changed considerably over the course of the study. Future longitudinal research on changes in SF over time should therefore consider both the change in predictor variables over the course of the study, as well as the impact of the baseline and end values of these variables on SF change and the inclusion of serum hepcidin.

## 3.5 Conclusion

While ID in premenopausal women is widely attributed to menstrual blood loss, this fails to recognise that not all menstruating females are at risk of ID (Badenhorst et al., 2021). Such generalisations can contribute to biases in healthcare, where ID or IDA in women are often assumed to result from menstruation without further investigation into other potential causes (Miller, 2023). In the current study, SF was found to be partially influenced by menstrual cycle phase in consistently ovulatory participants. However, neither menstrual phase nor the duration of menstrual bleeding significantly affected iron status.

Our findings suggest that menstrual cycle phase significantly influences iron biomarker concentrations, except for Hb, in consistently ovulatory women but not for consistently SOD women. Despite this, iron biomarkers did not significantly differ between ovulatory and SOD participants within any menstrual phases. However, as only 18 participants were consistently SOD, we may not have had a large enough sample size for statistical power to detect differences in SOD participants. Although the mechanisms underlying cyclical variations in iron biomarker concentrations remain unclear, the inclusion of SOD cycles contributes to the growing body of female health research by broadening understanding of the physiological influence of female sex steroid hormones. This is particularly important given the high proportion of women with regular-appearing menstrual cycles who experience intermittent SOD, with approximately 40% of women in our study

exhibiting at least one SOD cycle. Therefore, future research should consider both eumenorrheic and SOD cycles to capture the full spectrum of hormonal and physiological variability in determining the risk of ID among women with regular menstrual cycles.

Longitudinal prospective studies assessing changes in iron biomarkers both across and within menstrual cycle phases over multiple cycles would further clarify the extent to which fluctuations are phase-specific versus attributable to external factors. However, this would entail considerable participant burden so feasibility must therefore be greatly considered. Overall, our study advances understanding of how menstrual cycle phases influence iron biomarker variations and highlights differences between women with consistently ovulatory and consistently SOD cycles, providing novel insight into the physiological influence of SOD, which has been largely overlooked in previous research.

# Chapter 4: Discussion

## 4.1 Achievement of Aims and Objectives

The overall aim of the research was to investigate variations in iron status between and within the menstrual cycle in naturally menstruating women. To achieve this, biomarkers of iron status were compared across menstrual cycle phases, assessed for associations with female sex steroid hormones, while considering ovulatory status and the influence of subclinical ovulatory disturbances (SOD) and factors associated with the change in serum ferritin (SF) over the study.

All iron biomarkers, except for haemoglobin (Hb), significantly differed across menstrual cycle phases in consistently ovulatory participants. Serum iron (SI) and transferrin saturation (TSAT) were lowest in the early-follicular phase and highest in the mid-luteal phase in consistently ovulatory participants which is generally in agreement across the literature (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016). While changes in SF across the menstrual cycle are inconsistent among previous research, we hypothesised that SF would be lowest during menstruation due to menstrual blood loss and highest in the mid-luteal phase of the menstrual cycle, similar to findings by Kim et al. (1993). However, our results differed, aligning more closely to those of Aguree et al. (2023), Angeli et al. (2016) and Lainé et al. (2016), with SF highest in the early-follicular phase before stabilising between the late-follicular and mid-luteal phases. This pattern may reflect the timing of early-follicular phase testing, as 61% of participants were tested on day two of their cycle, potentially before sufficient menstrual blood loss had occurred to affect iron stores. The subsequent decrease observed in the late-follicular phase may reflect the delayed impact of menstrual blood loss on iron stores. Alternatively, the same cyclical pattern as SF was observed between menstrual phases for C-reactive protein (CRP), suggesting increased inflammation during menstruation may contribute to the concomitant increase in SF, given SF is an acute-phase protein. Despite this, iron status (sufficient vs insufficient) did not significantly differ between phases of the menstrual cycle.

Interestingly, the same variations in iron biomarkers and CRP between menstrual cycle phases were not observed in consistently SOD participants, with no significant differences found. This was a novel finding within our research as previous studies investigating variations in iron status across the menstrual cycle primarily focused on eumenorrheic cycles or did not distinguish between ovulatory and SOD cycles. The absence of variation observed among consistently SOD participants could be influenced by the altered hormonal profiles observed, where lower oestradiol and progesterone peaks in these participants may have reduced the cyclical modulation of serum hepcidin (SH), leading to

more stable iron absorption and mobilisation throughout the menstrual cycle (Badenhorst et al., 2022). More research is therefore warranted in this area.

It is generally acknowledged within the literature that hormonal fluctuations across the menstrual cycle could influence the variation in iron biomarkers observed (Chandra et al., 2017). However, our study found no correlations between iron biomarkers and female sex steroid hormones within the early-follicular, late-follicular or mid-luteal phases of the menstrual cycle. No studies to date have previously explored direct correlations between these variables, and our findings suggest that variations in iron biomarkers across the menstrual cycle in consistently ovulatory women, may be mediated by other physiological processes linked to menstrual phase such as their influence on SH, rather than by direct effects of female sex steroid hormones. While these findings met our objective on a basic level, it remains unclear whether intra-individual fluctuations in female sex steroid hormones across the menstrual cycle are associated with concurrent changes in iron biomarkers.

Biomarkers of iron status did not significantly differ between consistently ovulatory and consistently SOD participants within the early-follicular, late-follicular or mid-luteal phases of the menstrual cycle in our study. The exception to this was TSAT in the late-follicular phase which was significantly higher in ovulatory participants compared to SOD participants. This was another area of research that had not yet been investigated so there were no previous studies to compare our findings to. We hypothesised that iron deficiency (ID) in women would be associated with a higher prevalence of SOD. However, as only a limited number of participants were identified as iron-insufficient, we did not further classify participants as ID or iron deficiency anaemia (IDA). Iron insufficiency was not significantly associated with consistently SOD participants, with a similar prevalence to that of consistently ovulatory participants within each menstrual phase, contradicting our hypothesis.

For our final objective, we used a multiple linear regression analysis to investigate factors associated with the change in SF from baseline to study end, rather than specifically addressing the change in iron status with declining stores. This approach was chosen as participants with baseline SF values near the threshold for iron sufficiency may have experienced minor decreases that shifted them towards iron insufficiency, even if not statistically significant. Given the inherent biological variation in SF concentrations along with analytical variation within laboratory testing, small fluctuations in SF over time may reflect physiological variability rather than true changes in iron status (Ozkanay et al., 2023). While a reference change value (RCV) was trialled to identify changes in SF that were statistically significant, very few participants showed a significant decrease in iron stores under this method.

We hypothesised that increased menstrual bleeding duration and increased physical activity would be associated with reduced iron status, while decreased meat intake and/ or high body mass index (BMI) would be associated with a further increased risk of ID in women with prolonged menstrual bleeding. However, physical activity levels were not reassessed at study end as participants verbally reported no change, and we did not assess the influence of prolonged menstrual bleeding as there was little variation in menstrual bleeding duration among participants. Our final regression model showed change in CRP as the only significant predictor of the change in SF from baseline to study end in our population of premenopausal women. While previously identified risk factors of ID such as heavy menstrual bleeding (HMB), increased menstrual bleeding duration, recent blood donation, obesity, and low meat intakes have been reported in the literature, none of these factors predicted SF change in our study (Beck et al., 2014; Dugan et al., 2024; Lim et al., 2020; Lim et al., 2016). In a similar New Zealand (NZ) cohort, Lim et al. (2020) found meat intake to be a significant predictor of SF in NZ Europeans, but not in South Asian or other ethnic groups. Hepcidin was also a strong predictor of SF across all ethnicities in their study, so further investigation into SH as a predictor of SF change over time could be warranted (Lim et al., 2020).

Overall, our study provides novel insights into the influence of SOD on biomarkers of iron status, which has been largely overlooked in previous research. To the best of our knowledge, this is the first longitudinal prospective cohort study that has simultaneously explored iron biomarkers across menstrual cycle phases over multiple cycles, while categorising participants by hormonal profile and comparing differences both between and within these ovulatory status groups. Although the mechanisms underlying cyclical variations in iron biomarker concentrations remain unclear, the inclusion of SOD cycles contributes to the growing body of female health research by enhancing understanding of the broader physiological influence of female sex steroid hormones on health outcomes. This is particularly important given the high proportion of women with regular-appearing menstrual cycles who experience intermittent SOD (De Souza et al., 1998).

## 4.2 Strengths

A key strength of our study was its longitudinal prospective cohort design, which allowed for the assessment of both inter- and intra-individual variations in iron biomarkers across multiple menstrual cycles, rather than the limitations imposed by cross-sectional analyses. This design provided a more comprehensive understanding of how iron biomarkers fluctuate in relation to menstrual cycle phase and ovulatory status over time. The inclusion of longitudinal data with measurements over three to five menstrual cycles per participant enabled a more accurate determination of overall ovulatory status by allowing

further analysis into the prevalence of SOD and the occurrence of sporadic SOD cycles. Comparatively, of other studies exploring variations in iron biomarkers across menstrual cycle phases, three studies were cross-sectional (Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993), three studies tested participants across one menstrual cycle (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Lainé et al., 2016), and one study tested participants across two menstrual cycles (Suzuki et al., 2018). Additionally, our study included a larger sample size (*n*=97) than the previous studies conducted across menstrual cycles, which ranged from four to 90 participants.

Our study included two familiarisation cycles with urinary luteinising hormone (LH) testing to determine timing of ovulation within each participant's cycles. This allowed us to more accurately estimate the timing of ovulation in the following cycles for late-follicular phase blood testing (1-2 days before expected ovulation), along with estimated cycle length to determine timing of mid-luteal phase blood testing (5-7 days before expected period), and to tentatively schedule early-follicular phase testing for day 2-4 of the menstrual cycle. The use of familiarisation cycles and urinary LH testing helped improve accuracy of menstrual phase identification and allowed timing of measurements within phases to be consistent across participants, strengthening the validity of our findings.

Another key strength of our study was the classification of ovulatory and SOD cycles through mid-luteal phase testing of female sex steroid hormones, and the distinction between consistently ovulatory and consistently SOD women. Previous studies exploring iron status and biomarker variations across the menstrual cycle have focused primarily on eumenorrheic cycles or have not distinguished between ovulatory and SOD cycles (Alfaro-Magallanes et al., 2023; Angeli et al., 2016; Chandra et al., 2017; Chandra et al., 2016; Kim et al., 1993; Lainé et al., 2016; Suzuki et al., 2018). This allowed our study to advance female health research, presenting novel findings on the physiological influence of SOD on iron biomarkers across the menstrual cycle that had not previously been explored.

A final key strength was our use of a higher SF cutoff (<30µg/L) to classify iron insufficiency. Previous NZ studies investigating the prevalence of ID in premenopausal women have typically used lower thresholds of <20µg/L or <12µg/L (Beck et al., 2014; Ferguson et al., 2001; Heath et al., 2001; Schaaf et al., 2000; University of Otago & Ministry of Health, 2011). However, an SF concentration <30µg/L is increasingly recognised as a more sensitive and physiologically appropriate indicator of low iron stores (Clénin et al., 2015), and has been adopted in more recent NZ literature (Lim et al., 2020). Therefore, our study was more likely to identify participants with stage one ID that may have remained undetected in studies using a lower threshold.

## 4.3 Limitations

A limitation of our study was its reliance on a volunteer population, which may not have been representative of the general population. This was particularly evident in the underrepresentation of Māori and Pacific participants within our study, who accounted for only 8.2% of our sample compared with 26.7% of the total NZ population (Stats NZ, 2024). Additionally, our study had a limited number of participants who were classified as consistently SOD. Only 18 participants were found to be consistently SOD, of which, only 13 had iron biomarker data over each of the three menstrual phases for analysis of between-phase differences. This compared to 68 participants classified as consistently ovulatory, of which 62 had sufficient data for between-phase iron analysis. While our study found no phase-related fluctuations in iron biomarkers among consistently SOD participants unlike those of consistently ovulatory participants, within each menstrual phase, iron biomarkers did not significantly differ between the two status groups. The small number of consistently SOD participants may have limited statistical power to detect subtle between-group or between-phase differences in iron biomarkers.

Our study also incorporated aspects of self-reporting within data collection, which may have introduced recall bias or reporting inaccuracies. Our use of a food frequency questionnaire to analyse dietary intake of food groups relied on participants' memory in estimating their frequency of intake of various foods over the previous month, which may have been prone to over- or under-estimation of intake. Similarly, self-reported physical activity would have been subject to the same limitations. While data was collected on self-perception of menstrual bleeding heaviness, it was considered highly subjective and subsequently excluded from analysis. However, this prevented a definitive conclusion on the full impact of the menstrual cycle on iron status.

Another limitation was how we accounted for participants with elevated CRP (>5mg/L) in the determination of iron status. For these participants, we increased the SF cutoff for iron insufficiency to <70µg/L for the affected cycle phase in accordance with World Health Organisation (WHO) guidelines, rather than adjusting SF for inflammation using a correction factor per the BRINDA method (Namaste et al., 2017; World Health Organisation, 2020). However, this meant that SF concentrations corresponding to elevated CRP, were likely increased due to the influence of inflammation and thus, not entirely reflective of iron stores. Despite this, there were insufficient participants with elevated CRP within our study to calculate a reliable correction factor and minimise the influence of inflammation. Additionally, we did not include a secondary inflammatory marker such as α-1 acid glycoprotein (AGP), which has a longer time-course than CRP and may have improved detection of acute and resolving inflammation, strengthening interpretation of SF concentrations that remain elevated after CRP has returned to baseline.

In our analysis of SF change over the study period, we considered previously identified risk factors of ID and how the change in these factors over the same time period influenced SF change. One key variable considered was the change in meat/ fish/ poultry intake from baseline to study end, but the limitation of this approach was that participants who experienced declining iron stores over the study may have already been consuming low meat intakes at baseline, which were not reflected by the change in meat intake. Therefore, meat intake may have contributed to the declining iron stores in line with findings by Lim et al. (2020), even without a change in meat intake over the study period. Another limitation with the analysis of SF change over the study was that baseline measurements were collected without regard to menstrual cycle phase. While final SF measurements were generally obtained during the mid-luteal phase, the menstrual phase of baseline measurements were unknown, and therefore, would likely vary between participants. This likely introduced additional phase-related variability of SF, potentially confounding the analysis of external factors influencing changes in SF over time.

### 4.4 Final Recommendations

Given that approximately 40% of participants in our study presented with at least one SOD cycle, it is evident that the occurrence of intermittent SOD cycles is relatively common in premenopausal women with regular-appearing menstrual cycles. Our findings highlight the importance of considering both menstrual cycle status and phase when interpreting iron biomarkers in this population. To extend upon these findings and address remaining gaps and limitations of our study, the following recommendations are proposed for future research and clinical practice:

- Studies on iron status and biomarkers in premenopausal women should continue to classify ovulatory and SOD cycles through mid-luteal phase testing of female sex steroid hormones. Classification of predominant ovulatory status as consistently ovulatory or consistently SOD should be maintained in future studies to capture the full spectrum of hormonal and physiological variability in determining the risk of ID among women with regular-appearing menstrual cycles.
- Further research should explore the role of hepcidin in regulating iron status across the menstrual cycle to better understand potential mechanisms underlying phase-related differences in iron biomarkers within both consistently ovulatory and consistently SOD participants.
- While our study found no correlation between iron biomarkers and female sex steroid hormones within each phase of the menstrual cycle, future research should

investigate how intra-individual fluctuations in hormones may relate to concurrent changes in iron biomarkers across the menstrual cycle.

- Longitudinal prospective studies with repeated testing of iron biomarkers in the early-follicular, late-follicular and mid-luteal phases of the menstrual cycle across multiple cycles per participant would further clarify the extent to which fluctuations are phase-specific versus attributable to external factors. However, this would entail considerable participant burden so feasibility must be greatly considered.
- Future research assessing external factors as predictors of the change in SF over time should control for menstrual cycle phase, with both start and end measurements timed to be collected in the same phase of the cycle. This will minimise potential confounding effects of phase-related changes in SF to ensure a more accurate interpretation of longitudinal changes.
- Heaviness of menstrual bleeding should be measured using objective and validated methods to improve the accuracy of estimating menstrual blood loss. Such data would further the understanding of the true impact of menstrual blood loss on iron status and biomarkers in healthy, naturally menstruating women.
- In clinical practice, testing of SF to determine iron status in premenopausal women should be conducted within the follicular phase of the menstrual cycle and interpreted alongside CRP to ensure identification of ID is not missed. Additionally, both research and clinical practice should consider measuring both CRP and AGP as measures of inflammation to more accurately interpret SF.

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# Appendices

**Appendix A:** Food Frequency Questionnaire Food Category Groupings

| Food Group | Foods Included |
|---|---|
| **Fruits and Vegetables** | Apples, pears, nashi pears, bananas, citrus fruits (e.g. oranges, tangelos, tangerines, mandarins, grapefruit, lemons, limes), stone fruit (e.g. apricots, nectarines, peaches, plums, lychees), avocado, olives, strawberries, blackberries, cherries, blueberries, boysenberries, loganberries, cranberries, gooseberries, raspberries, dried fruit (e.g. sultanas, raisins, currants, figs, apricots, prunes, dates), feijoa, persimmon, tamarillo, kiwifruit, grapes, mango, melon, watermelon, pawpaw, papaya, pineapple, rhubarb, potato, hot potato chips, French fries, wedges, kumara, taro, green banana, cassava, carrots, yams, parsnip, swedes, beetroot, turnips, peas, green beans, broad beans, runner beans, broccoli, cauliflower, brussels sprouts, cabbage, lettuce, cucumber, celery, sprouts, spinach, silver beet, Swiss chard watercress, puha, witloof, chicory, kale, chard, collards, Chinese kale, Bok choy, taro leaves, tomatoes, onions, leeks, garlic, corn, pumpkin, mushrooms, capsicum, peppers, courgette, zucchini, gherkins, marrow, squash, asparagus, radish, eggplant, artichoke |
| **Citrus Fruits** | Oranges, tangelos, tangerines, mandarins, grapefruit, lemons, limes |
| **Meat/ Fish/ Poultry** | Beef, lamb, hogget, mutton, pork, veal, chicken, turkey, duck, liver, kidney, offal, sausages, ham, bacon, luncheon, salami, pastrami, corn beef, boil up, pork bones, povi masima, meat pies, sausage rolls, fish, mussels, shellfish |
| **Red Meat** | Beef, lamb, hogget, mutton, pork, veal |
| **White Meat** | Chicken, turkey, duck |
| **Dairy** | Cheese, cottage cheese, ricotta cheese, sour cream, cream cheese, cream, cheese spreads, cow's milk, soy milk, coconut milk, rice milk, almond milk, oat milk, milk-based smoothies, milk shakes, milk-based puddings (e.g. rice pudding, custard, semolina, instant puddings, dairy food), yoghurt, ice cream |
| **Cereals and Grains** | Rolled oats, oat bran, oatmeal, All Bran, Sultana Bran, Weetbix, cornflakes, rice bubbles, Nutri grain, Fruit Loops, Honey Puffs, Frosties, Milo cereal, CocoPops, Special K, Light and Tasty, brown rice, white rice, white pasta, whole meal pasta, couscous, polenta, congee, Bulgur wheat, quinoa, pancakes, waffles, sweet buns, scones, sweet muffins, fruit bread, croissants, doughnuts, brioche, white bread and rolls, whole meal or wheat meal bread and rolls, crackers (e.g. crisp bread, water crackers, rice cakes, cream crackers, Cruskits, Meal mates, Vita wheat) |
| **Tea and Coffee** | Coffee, tea, herbal tea |
| **Alcohol** | Beer, lager, cider, red wine, white wine, port, sherry, liqueurs, spirits (e.g. gin, brandy, whiskey, vodka), RTD's |