An epidemiological investigation of iron deficiency in Indian women diagnosed with Peripartum Cardiomyopathy

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Impact of COVID-19

This DPhil project was an investigation of peripartum cardiomyopathy (PPCM), which is a rare cardiac complication of pregnancy. The project was primarily a secondary analysis of data that was being collected in hospitals across India between February 2019 and October 2021 as part of an ongoing study of heart failure in pregnancy.

In the first year of recruitment, the enrolment rate for the heart failure study was at its highest, at about 18 cases (3-4 cases of PPCM) per month. By March 2020, about 200 women with heart failure were enrolled in the study, and approximately 16% of these cases were diagnosed with PPCM. Thus, 100-110 women with PPCM were expected to be enrolled into the MaatHRI study by June 2021.

The initial wave of COVID-19 infections in India, which started around March 2020, significantly affected recruitment into the heart failure study in all participating hospitals until January 2021. By March 2021, the country was experiencing a devastating second wave of infections, which caused the number of births in the participating hospitals to decrease by more than 35%[1]. Overall, enrolment into the MaatHRI heart failure study decreased by 50% across all study hospitals over the course of the pandemic. Since then, enrolment rates continued to remain well below pre-pandemic levels until the end of 2021. Additionally, following the national restrictions that occurred during the first wave of the pandemic, access to national laboratories was restricted for several months, which directly impacted the ability to carry out the biological investigations needed for the project. The case fatality for heart failure among pregnant and postpartum women in these hospitals had also doubled to 58%, which further challenged data collection.

Overall, it is estimated that the COVID-19 pandemic resulted in a loss of 60% of the data expected for the DPhil project. The final dataset was deemed sufficient to carry out the main analyses. However the results of this study were limited by these events, and therefore must be interpreted with due considerations.

[1] Nair M. Reproductive health crisis during waves one and two of the COVID-19 pandemic in India: Incidence and deaths from severe maternal complications in more than 202,000 hospital births. EClinicalMedicine. 2021 Sep 1;39:101063.

Abstract

An epidemiological investigation of iron deficiency in Indian women diagnosed with Peripartum Cardiomyopathy

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Background: Peripartum cardiomyopathy (PPCM) is a serious cardiac complication affecting pregnant and postpartum women. The primary mechanism described for PPCM relates to prolactin signalling, but the determinants of the disease are known to vary across patient populations. In India, cardiac diseases account for up to 10% of maternal deaths. At the same time, maternal iron deficiency (ID) with or without anaemia is highly prevalent in this population. However, the epidemiology of PPCM in India is poorly studied, and it is unknown whether maternal ID contributes to the risk of PPCM among Indian women. The DPhil project sought to address this knowledge gap by 1) reviewing the literature on the biomolecular mechanisms suggested for PPCM, 2) profiling the epidemiology of PPCM in a population of Indian women, 3) investigating the association between maternal iron status and PPCM among Indian women, and 4) comparing serum concentrations of prolactin to maternal iron status as a determinant of PPCM in Indian women.

Method: The project involved a systematic review of the biological mechanisms involved in the development of PPCM, formulation of an evidence-based framework for the role of ID in PPCM, and a secondary data analysis of a hospital-based case-control study of heart failure among mothers in India. The association of PPCM with serum concentrations of five iron indices and prolactin was compared between 44 pregnant and postpartum women diagnosed with PPCM (cases) and 170 postpartum women with no heart failure (controls). The classification accuracy of each marker was also assessed by the area under the receiver operating characteristic curve.

Results: The previously reported association between prolactin concentrations and PPCM could not be replicated. However a new role for ID in PPCM was uncovered. Specifically, iron deficient women were significantly more likely to be diagnosed with PPCM than those without ID (OR=4.54, 95% CI: 2.02, 10.9). Women diagnosed with PPCM had lower levels of transferrin saturation (TSAT) (p=0.021), higher soluble transferrin receptor (sTfR) levels indicative of cellular iron depletion (p=0.015), and decreased expression of the iron homeostatic hormone hepcidin, indicative of a compensatory iron mobilisation response (p<0.001). Among anaemic mothers, the odds of PPCM were significantly greater for those with ID than for those who had non-ID anaemia (OR=2.90, 95% CI: 1.05, 8.51). Concentrations of sTfR and hepcidin had the greatest value for distinguishing between women with and without PPCM (AUC=0.74 for both), while TSAT performed the poorest (AUC=0.63).

Conclusions: This study is the first to characterise the iron status of women diagnosed with PPCM in any population. The results have important implications for public health policies targeting ID in pregnant and postpartum women in India, and can also inform future research and clinical guidelines for the prevention, diagnosis and treatment of PPCM in this population.

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All glory be to the Most High, who has always and continues to see in me someone worthy of grace and favour.

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List of abbreviations

ACE	Angiotensin I-converting enzyme
ANC	Antenatal care
AROC	Adjusted receiver operatic characteristic
AUC	Area under the curve
BMI	Body mass index
CCRF	Cardiovascular Clinical Research Facility
CI	Confidence interval
CINAHL	Cumulative Index to Nursing and Allied Health Literature
CRP	C-reactive protein
DAG	Directed acyclic graph
DMT-1	Divalent metal transporter-1
EMBASE	Excerpta Medica dataBASE
ESC	European Society of Cardiology
FPN	Ferroportin
Hb	Haemoglobin
HTA	Health technology assessment
IFA	Iron and folic acid
IL-6	Interleukin-6
IQR	Interquartile range
kDa	Kilodalton
LPL	Dr. Lal Path Labs
LVEDD	Left ventricular end diastolic diameter
LVEF	Left ventricular ejection fraction
MAR	Missing at random

MaatHRI	Maternal and perinatal Health Research collaboration, India
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
MEDLINE	Medical Literature Analysis and Retrieval System Online
miRNA-14a	Micro ribonucleic acid 14-a
MMP2	Metalloproteinase-2
MNAR	Missing not at random
NHLBI	National Heart, Lung, and Blood Institute
NIHR	National Institute for Health and Care Research
NT-proBNP	N-terminal (NT)-pro hormone brain natriuretic peptide
NYHA	New York Heart Association
OR	Odds ratio
OxTREC	Oxford Tropical Research Ethics Committee
PPCM	Peripartum cardiomyopathy
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
ROC	Receiver operating characteristic
sFe	Serum iron
sFLT1	Soluble fms-like tyrosine kinase-1
sFR	Serum ferritin
SMD	Standardised mean difference
STAT3	Signal transducer and activator of transcription 3
sTfR	Soluble transferrin receptors
sTfR-F	Soluble transferrin receptors-ferrtin index
TfR-1	Transferrin receptor-1
TSAT	Transferrin saturation
US	United States

Chapter 1: Introduction

1.1. Cardiovascular adaptations of pregnancy

The course of a pregnancy is marked by profound changes to a mother's physiology, in order to meet her own metabolic demands as well as those of her developing fetus. Adaptive changes occur in blood volume, vascularisation, heart rate, and myocardial contractility, all of which affect cardiac performance (1, 2).

Maternal cardiac output increases by 20% within the first two months of pregnancy (3), and may rise up to 50% above baseline by the end of gestation, due to significant increases in heart rate and stroke volume (4, 5). However, the greatest increase in cardiac demand occurs during labour and immediately after childbirth, as the sympathetic response to pain and anxiety cause the mother's heart rate to increase. Additionally, approximately 300-500 mL of blood is auto-transfused back into the mother's circulation with every contraction of the uterus, which causes the cardiac output to rise significantly during labour (1, 3). In the immediate postpartum period cardiac output can continue to rise up to 80% above pre-labour values, and approximately 150% above non-pregnant levels (4). Cardiac output typically decreases to pre-labour values within two days after birth, although levels may remain above pre-pregnancy values for several months postpartum (4).

Owing to these huge demands on the maternal cardiovascular system, pregnancy is often referred to as 'nature's stress test'. Mothers without underlying heart disease usually adapt well and adverse cardiac events rarely occur. However, in the presence of important structural or functional deficiencies, the increased cardiovascular demands of pregnancy can result in cardiac decompensation, arrhythmias or heart failure (2). Because of this, pregnancy may be contraindicated in women with existing heart conditions, as they may be unable to adequately increase their cardiac output to meet the requirements of gestation. In other women, the increased cardiac demands can expose hitherto unrecognised weaknesses in cardiac functioning, or even engender de novo cardiac dysfunction, leading to severe complications during pregnancy, labour and even after childbirth.

1.2. Epidemiology of heart disease in pregnancy

Cardiovascular diseases continue to be the most common cause of indirect (i.e. non-obstetric) maternal mortality and morbidity worldwide, despite being rarely observed during and after pregnancy. It has been estimated that cardiovascular diseases account for over 33% of pregnancy-related maternal deaths globally (6), although wide variations exist in the burden of disease between different countries and populations. Nevertheless, up to 68% pregnancy-related deaths caused by cardiovascular conditions are considered to be preventable (7).

In high income countries, maternal heart diseases complicate up to 4% of pregnancies, and up to 16% of pregnancies in women with previous cardiac conditions (8). Additionally, over half of maternal deaths from cardiovascular causes have been reported to occur in the postpartum period (8). Importantly, recent findings from the United Kingdom suggest that a minority of mothers who had died following major cardiovascular events were known to have a cardiac problem, while the majority appeared healthy before the onset of pregnancy (9). In high-income countries, the percentage of maternal deaths related to cardiovascular diseases has risen from 3% to 15% in the past 30 years, and is predicted to increase further (10). The increasing prevalence of cardiovascular risk factors (namely diabetes, hypertension, and obesity), as well as older maternal age have led to a parallel increase in the occurrence of cardiovascular diseases among pregnant women in higher income countries (5). In these regions of the world, myocardial infarctions, cardiomyopathies, and congenital heart diseases are currently the predominant causes of maternal mortality (5).

Data on the prevalence of cardiovascular disease and related death rates in pregnant women from low- and middle-income countries remain comparatively scarce. This is in part due to the prioritisation of conditions such as hypertensive disorders, postpartum haemorrhage and infections, which are often regarded as more pressing causes of maternal mortality in these countries and therefore typically receive more attention. A recent systematic review of maternal mortality due to cardiac disease in 29 low- and middle-income countries

found that the proportion of reported cardiac-related maternal deaths ranged from 0% in reports from Ghana to 24.8% in Sri Lanka (11). Importantly, although the proportion of cardiac maternal deaths appeared to be lowest among the lower income countries, cardiac related maternal mortality ratio was inversely related to a country's economic status. These findings likely reflect a lack of access to healthcare facilities or professionals with the adequate resources to diagnose cardiac problems, often resulting in delayed diagnosis or a misclassification of diagnosis, especially when the pregnant or postpartum woman presents with severe decompensated heart failure (11, 12). The under-diagnosis and underreporting of cardiovascular diseases in clinical practice likely result in an underestimation of the burden of diseases among pregnant and postpartum women from low- and middle-income countries.

The distribution of cardiovascular disorders in pregnant women from low- and middle-income countries also differs from higher income countries. Historically, rheumatic heart diseases have been the predominant cardiac lesions observed in low- and middle-income countries, followed by congenital heart diseases (13, 14). This has typically been attributed to the effect of poverty, infections, poor nutrition, and limited access to health services. However, recent evidence suggests that lower income countries may be experiencing a paradigm shift in the spectrum of heart diseases of pregnancy, which follows the trends observed in high income countries, albeit at a much slower pace (13). This shift may be led by improvements in health services and standards of living, increased antibiotic prophylaxis for streptococcal disease to reduce rheumatic heart disease, and an increase in the number of facilities with the capacity to diagnose conditions which may have been previously undetected or misclassified.

1.3. Peripartum Cardiomyopathy

Although cardiomyopathies remain relatively rare among pregnant women in low and middle income countries, mothers with cardiomyopathies typically have the highest mortality rate among all those with heart diseases in pregnancy (15, 16). The aetiology of pregnancy-associated cardiomyopathies includes inherited diseases (such as dilated, arrhythmogenic or hypertrophic cardiomyopathy), and acquired diseases such as peripartum cardiomyopathy

(PPCM). PPCM is the most common cardiomyopathy observed in pregnancy, and is defined by the European Society of Cardiology (ESC) as "heart failure secondary to left ventricular systolic dysfunction, usually shown by an left ventricular ejection fraction <45%, occurring towards the end of pregnancy (third trimester) or in the months following delivery without any other identifiable cause" (17).

1.3.1. Epidemiology and risk factors

Most of the data on the epidemiological profile of PPCM available to date originates from studies conducted in the United States, South Africa, Nigeria and Haiti. The findings from these studies suggest that the global incidence of PPCM varies widely by geographical region. The highest reported incidences have been in low and middle income countries such as Nigeria (one in 100 live births) (18) and Haiti (one in 300 live births) (19), while higher income countries such as Japan have reported incidences as low as one in 20,000 live births (20). Recent findings also suggest that the prevalence of PPCM may be increasing, with one study in the United States reporting a 39% increase between 2004 and 2011 (21). This increase in prevalence is likely due in part to a growing awareness of PPCM as an important complication of pregnancy, and a subsequent rise in diagnosis.

Risk factors for PPCM also show some geographical variation, but suggested determinants of the disease include maternal age greater than 30 years, poor socioeconomic status, malnutrition, pre-eclampsia and obesity (22). Multiparity and multiple gestation are also commonly observed risk factors. Other reported associations include African ancestry (23), thyroid deficiency dysfunction (24), and prolonged tocolysis (25). Associations with primipara status, tobacco, and alcohol use have also been reported, although the evidence remains equivocal (26). Recent studies have found that the risk of PPCM increases exponentially with the presence of each additional risk factor (23).

1.3.2. Diagnosis

The first clinical definition of PPCM was proposed by Demakis and colleagues in 1971 (27). They identified PPCM as an idiopathic heart failure occurring in the absence of any determinable heart disease in the last month of pregnancy or during the first 5 months postpartum. To prevent over-reporting, the diagnosis later also included echocardiographic evidence of left ventricular dysfunction, typically with left ventricular ejection fraction (LVEF) reduced at least below 45% and often left ventricular dilatation. Echocardiography may also show signs of left ventricular systolic dysfunction, bi-atrial enlargement, mitral and tricuspid regurgitation, and pulmonary hypertension (26). The majority of women with PPCM present with severe symptoms (New York Heart Association class III or IV) (28). Physical examination typically reveals evidence of both left and right sided congestion, tachycardia, sinus rhythm, pulmonary oedema and occasionally pleural effusions (26, 29).

PPCM is generally viewed as a diagnosis of exclusion, with a differential diagnosis including pre-existing cardiomyopathies, such as dilated cardiomyopathy. In the past, PPCM had often been seen as an instance of dilated cardiomyopathy triggered during pregnancy, owing to the many features shared by the two conditions (such as arrhythmia, ventricle dilation, and reduced systolic function). However, there are important differences in the presentation and features of these two cardiomyopathies, which suggest that they are in fact distinct clinical entities (30). The differential diagnosis for PPCM may also include congenital heart diseases, pulmonary arterial hypertension, history of myocarditis, and valvular diseases. However, women with these conditions tend to present with symptoms of heart failure earlier in pregnancy than do women with PPCM, although heart failure caused by valvular disease or pre-existing cardiomyopathy may also sometimes present later in pregnancy.

1.3.3. Clinical presentation and comorbidities

While PPCM may present at any point in late pregnancy, labour, birth or in the postpartum months, most cases develop in the first month after childbirth (26). Nevertheless,

a considerable number present in the third trimester of pregnancy and a few cases have been reported as early as the second trimester (29). Women with PPCM typically present with non-specific symptoms of heart failure (including fatigue, shortness of breath, and oedema), which may be difficult to distinguish from the symptoms of a normal pregnancy. Moreover, women who develop PPCM in the postpartum period may present with few physical signs, despite significant cardiac dysfunction (28). This increases the likelihood that PPCM is missed at the initial evaluation. Thus, a low threshold of suspicion of heart failure is generally recommended for women presenting with suggestive clinical features.

Reported comorbidities include diabetes mellitus, anaemia, obesity and hypertensive disorders, notably pre-eclampsia and eclampsia (31). While hypertensive disorders complicate as many as 5–10% of pregnancies worldwide, the prevalence of hypertensive disorders is generally higher among women diagnosed with PPCM. Namely, over 20% of cases have been reported to develop pre-eclampsia during the index pregnancy (28). However, while it may appear that PPCM might simply be a manifestation of severe pre-eclampsia, there is now concrete evidence suggesting that the two conditions are in fact distinct entities with a possible shared pathophysiological pathway (32). A significant number of women diagnosed with severe pre-eclampsia do not develop PPCM, and conversely, up to 80% of women with PPCM do not have a history of pre-eclampsia (32).

1.3.4. Pathophysiology of PPCM

To date, no specific diagnostic biomarkers have yet been identified for PPCM, although recent research has begun to elucidate some of the mechanisms underlying the disease. PPCM is likely to be caused and driven by multiple pathological mechanisms resulting in cardiac dysfunction. Suggested mechanisms for the development of the disease have included inflammation and auto-immune responses, albeit unlikely to be driven by viral infections (26). Other potential pathways may involve oxidative stress, vascular imbalance, and more recently, impaired neuro-hormonal signalling (28). Previous research has also implicated an imbalance in the production of the breast-milk producing hormone prolactin in the pathogenesis of PPCM,

implying that the inhibition of prolactin might be useful for therapeutic purposes. Some reports have even suggested that several of the mechanisms previously suggested for the pathogenesis of PPCM may converge on this common pathway (28). The current paradigm for the development of PPCM favours a "two hit" model, whereby PPCM results from endothelial dysfunction and damage caused by the hormonal environment of late-gestation, coupled with an underlying predisposition, which induce cardiomyopathy in some women (33). However, evidence for this model is still preliminary, and it is unclear whether the role of prolactin in PPCM varies across different populations, like other risk factors.

1.4. Rationale for the project

1.4.1. The case for India

To contribute to this body of research, this DPhil project was designed to conduct an epidemiological investigation of PPCM among pregnant and postpartum women in India. It has been estimated that heart diseases of pregnancy account for 6-10% of maternal deaths among Indian women (11, 34, 35). However, the exact contribution of PPCM to this burden of disease among Indian women is poorly studied. A recent review article identified seven single-centre observational studies of PPCM conducted across India over the past fifteen years (36). The largest of these studies had enrolled 56 women diagnosed with PPCM over the course of 10 years (37). The pooled analysis of these seven studies estimated an average incidence of 1 per 1,340 live births over a 20-year period (36). Most women with PPCM were young women (mean age of 27 years) in their first pregnancy, presenting in the postpartum period with severe left ventricular dysfunction and NYHA class III & IV symptoms of heart failure (36). The most commonly observed comorbidities among Indian women included hypertensive disorders (46%), anaemia (45%), gestational diabetes (9%) and hypothyroidism (8%) (36). Overall, the seven studies observed that PPCM was associated with a case fatality between 14-16% (38, 39). However, none of the studies previously conducted among Indian women provided estimations of risk factors for the disease, as they have all been non-comparative case-series investigations. Thus, the current scarcity of evidence from India on PPCM

significantly limits the accurate assessment of the burden of disease and population specific risk factors, and it also impedes the formulation of novel diagnostic approaches and management strategies for women diagnosed with PPCM in India.

Importantly, India is a large country with a widely heterogeneous population of 1.1 billion people. The immense socio-economic and cultural diversity that exists within and between the different regions of the country translates into important variations in lifestyle practices and in the systemic determinants of health. Thus, although cardiovascular risk factors are generally prevalent in India, there is significant heterogeneity in the prevalence of the different risk factors across regions of the country. For instance, while diabetes appears to be more prevalent in the southern states of India, hypertensive disorders are more commonly observed in the Northeast region (40). Currently, knowledge of the epidemiological profile of PPCM in India is derived from a few studies conducted in different parts of the country. The evidence derived from these sporadic investigations of PPCM in India is unlikely to be readily generalisable to the entire Indian subcontinent (41). Therefore, in the absence of large national-level studies, which are likely to be time-consuming and costly, there is a need to generate more epidemiological investigations of PPCM across different regions of the country, in order to have an adequate picture of the significance of this disease in Indian women.

1.4.2. Maternal iron deficiency and PPCM

One potential cause of cardiac dysfunction that has not yet been investigated in PPCM is iron deficiency. Iron deficiency is the single most prevalent nutritional deficiency in the world, reaching epidemic proportions in some low and middle income countries. According to the latest guidelines from the ESC, iron deficiency, with or without anaemia, is also one of the most common non-cardiovascular comorbidities observed in patients with heart failure, occurring in 50-80% of acute heart failure patients (17). In human studies, serum markers of iron status are commonly detected at iron deficiency levels in patients with heart failure (42, 43). Indices of myocardial iron status (such as the concentration of iron in cardiac cells) have also been shown to be decreased in failing human hearts compared to non-failing ones (44-

46), and these levels were not associated with the inflammation or renal insufficiency observed in heart failure (45).

If it is not corrected in time, iron deficiency can become severe enough to restrict erythropoietic activity, resulting in insufficient haemoglobin concentrations in the blood (a state also known as anaemia), and leading to decreased oxygen delivery to tissues. Anaemia is one of the most commonly observed comorbidities of PPCM, with the reported prevalence ranging from 16% to over 40% (23, 47, 48). The odds of PPCM has been reported to be up to five fold higher in anaemic pregnant women than among non-anaemic pregnant women (23, 24, 49). Although iron deficiency is only one of the many possible aetiologies of anaemia, several mechanisms associated with iron deficiency, including inflammation, autoimmune reactions and a pathological response to oxidative stress, have been proposed as underlying mechanisms for the pathophysiology of PPCM (50-52). Moreover, emerging evidence suggests that a deficiency in iron, which may occur regardless of concomitant anaemia, could directly contribute to cardiac dysfunction (53, 54).

Maternal iron deficiency anaemia is an important public health concern across the world, but especially in India where it exerts a heavy toll on mortality, disability, and productivity (55, 56). After over half a century of national prevention programmes, the burden of iron deficiency anaemia in India remains well above the global average. Between 2019 and 2020, over half of Indian women of reproductive age were reported to be anaemic, which represents an increase from previous years (56-58). Indian women are particularly susceptible to having inadequate iron stores due to high rates of malnutrition, a high-prevalence of vegetarianism and endemic infections such as malaria, hookworms and viral infections, which lead to an overall inadequate intake, increased loss of iron and poor absorption of iron from the diet (55, 56). A recent study reported that up to 83% of pregnant women in India are unable to meet the recommended daily intake of iron of 15-18 mg/day (57).

Thus, if indeed a relationship between maternal iron status and the onset of heart disease in pregnancy and the postpartum period exists, mothers in India may be at a particularly higher risk of severe cardiac complications. Elucidating the role of iron status in PPCM could have important implications for not only improving knowledge and understanding of this disease of pregnancy, but also for improving maternal health in India.

1.5. Aim and objectives of the thesis

The aim of this DPhil was to assess the contribution of iron deficiency to the epidemiology of PPCM among Indian women. The following objectives were defined to achieve this aim:

1. Systematically review and synthesise the literature on the biomolecular mechanisms that have been suggested for PPCM to:
    a) assess the epidemiological evidence of altered iron and prolactin metabolism in PPCM
    b) formulate an evidence-based framework for the possible role of iron deficiency in the pathophysiology of PPCM
2. Profile the epidemiology of PPCM in a population of Indian women,
3. Investigate the association between maternal iron status and PPCM among Indian women (both independently and in the context of anaemia),
4. Compare serum concentrations of prolactin to maternal iron status as a determinant of PPCM in Indian women.

Chapter 2: A systematic review and meta-analysis of the molecular profile of Peripartum Cardiomyopathy

2.1. Background and scope

Peripartum cardiomyopathy (PPCM) is increasingly recognized as an important cause of heart failure in pregnant women without pre-existing cardiovascular diseases, however, the aetiology and molecular profile of the disease are still being established. The primary molecular hypothesis for the pathogenesis of PPCM is a prolactin mediated mechanism, which suggests that PPCM may result from an imbalance in the breast-milk inducing hormone, prolactin (59, 60). This hypothesis is supported by mechanistic evidence from animal studies and preliminary clinical trials showing that a reduction in blood prolactin levels through the administration of Bromocriptine seems to improve symptoms and prognosis in women diagnosed with PPCM (61-63). However, several other mechanisms have been proposed for the pathophysiology of PPCM, some of which can be reconciled with the prolactin hypothesis and others which cannot (22, 52).

It is well established that iron deficiency is a co-morbidity in heart failure, and it has been suggested that a deficiency can have a causal and direct effect in cardiomyopathy (17). However, as the evidence on the molecular profile of women diagnosed with PPCM has not yet been systematically appraised, it is still unclear whether iron deficiency contributes to the pathology of this specific heart disease.

The literature on the biomolecular mechanisms that have been suggested for PPCM was systematically reviewed with the aims of: 1) critically appraising the current evidence for the prolactin hypothesis, and 2) determining if there is evidence of impaired iron metabolism in women diagnosed with PPCM.

2.2. Materials and methods

The protocol for this Systematic Review was registered in PROSPERO (CRD42019121716) and is available in full on the NIHR HTA programme website (https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=121716)

The systematic review was also published and is available as an open access article in the Open Heart journal (64).

2.2.1. Search Strategy

The search strategy for the systematic review was a mix of automated and manual searches and included both published and unpublished/grey literature. It was first developed and piloted in MEDLINE by applying the inclusion and exclusion criteria to a sample of papers to check its reliability in classifying the studies correctly. The search strategy was then modified for use in the other databases.

The following databases were searched for published literature: MEDLINE, EMBASE, CINAHL (Cumulative Index to Nursing and Allied Health Literature), Global Health Library. Additionally, the 'Proquest Dissertations & Theses' database was searched for relevant unpublished dissertations, reports and conference proceedings. The search also included ongoing trials registered on the Clinical Trials website (www.ClinicalTrials.gov). The search terms included a combination of terms for "peripartum cardiomyopathy", "heart failure" and "pregnancy" (an example of the search strategy used in this review can be found in **Appendix Table A1**)

2.2.2. Inclusion/Exclusion criteria

2.2.2.1. Type of studies

The review included all articles published since the database inception, from all countries and in any languages. All observational studies (cohort, cross-sectional and case-control) comparing biochemical markers in women with PPCM to women who had neither PPCM nor any other pre-existing cardiac diseases were included. Descriptive studies without comparator groups (such as case-reports or case-series) were not included in the review. Abstracts were also excluded, unless an article describing the original study was available. Systematic reviews and meta-analyses were not included in the final analysis, but their reference lists were reviewed and screened for any relevant original research articles.

2.2.2.2. Definition of PPCM

PPCM diagnosis was based on the definition established by the Heart Failure Association of the European Society of Cardiology (ESC) Working Group, which states that PPCM is "an idiopathic cardiomyopathy presenting with heart failure secondary to left ventricular systolic

dysfunction towards the end of pregnancy or in the months following birth, where no other cause of heart failure is found. The left ventricle may not be dilated but the ejection fraction is nearly always reduced below 45%" (65). To adhere to this definition of PPCM, studies of non-pregnant women and women who were more than six months postpartum, and studies of pregnant women with known pre-existing cardiac problems were excluded.

2.2.2.3. Definition of biomarkers

Biomarkers were defined as cellular, biochemical or molecular factors that are measurable in biological media such as human tissues, cells, or fluids. The review included papers reporting laboratory measurements of any biomarkers in blood, urine or tissue. Because the focus of this review was on markers that might be associated with the development of PPCM, papers measuring prognostic biomarkers (i.e. determinants of progression of PPCM and its outcomes) were excluded unless measurements of the biomarker(s) before or at the time of PPCM diagnosis were also available.

2.2.3. Screening & data extraction

The titles and abstracts of the search results were critically screened based on the inclusion and exclusion criteria. Two reviewers independently reviewed the full text of the articles generated from that screen and extracted data from the included studies on the means and standard deviation of the biomarker levels in all study groups. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart was used to report the results of the selection process**.**

Data from the included articles were extracted using a data extraction tool modelled on the Cochrane Collaboration and the Centre for Reviews and Dissemination, which was developed specifically for the extraction of quantitative research data **(see Appendix Table A2).** The tool was modified to extract data on the nature and purpose of the biomarker analysed, the biological media in which it was measured and the assay used. Any papers that were not available in English or other languages spoken or understood by the reviewers were reviewed and extracted with the help of native speakers.

2.2.4. Risk of bias assessment

Risk of bias across studies was assessed using the US National Heart, Lung and Blood Institute's (NHLBI) Quality Assessment Tool for the appropriate study design (66), which was modified to specifically account for and give proper representation to the assessment of the quality of biomarker measurements **(see Appendix Table A3)**. The reliability and validity of the specific criteria and methods used by the authors to establish PPCM diagnosis was also evaluated as part of the assessment of the quality of the paper. The overall quality of each paper was judged as "Poor", "Fair" or "Good" based on a critical appraisal of the risk of bias in each paper, as recommended by the tool (66). Additionally, the proportion of "Yes" and "Not applicable" answers out of the 15 questions assessed in the tool was measured and reported for each paper. The presence and extent of any publication bias was assessed visually by funnel plot asymmetry.

2.3. Statistical analysis

The level of inter-reviewer agreement on the inclusion of papers was assessed using a Cohen's Kappa statistic (a measure of chance-corrected agreement) (67). Any disagreements were resolved through discussion with a third reviewer. The level of agreement on the papers reviewed by the thesis author and the second reviewer was "Moderate" (Kappa=0.574; 95% CI: 0.382-0.766).

A meta-analysis was conducted for biomarkers with reported mean and standard deviation values for PPCM cases and healthy controls. For markers where only medians and interquartile ranges were reported, the study authors were contacted to obtain the mean values. If this data was unavailable, the mean and standard deviations were estimated using the quartile method described by Wan et al.(68). Given the small sample size of the included articles, the meta-analysis was only conducted for biomarkers reported in at least 5 different study populations. Where possible, biomarkers that were biologically similar (e.g. natriuretic peptides) were grouped for the analysis following the advice of clinical experts.

Due to the use of different units of measurements for the biomarkers, Hedges' g was used to compute pooled Standardised mean differences (SMD) as summary estimates for the

differences between PPCM cases and healthy controls. A random-effects model was fitted to account for the statistical, clinical and methodological heterogeneity of the studies included in the analysis. The I^2 statistic was used as a measure of the percentage of variation across studies that is due to heterogeneity rather than chance. The significance of the heterogeneity across studies was estimated using Cochran's Q-tests for residual heterogeneity (69).

2.4. Results

2.4.1. Description of the included studies.

The results of the study selection process are summarized in **Figure 2.1.** Forty-nine papers were excluded at the full-text screening stage. Most papers were excluded for not reporting any biomarkers according to the definition used in this review. The justification for exclusion is reported in **Appendix Table A4** for each excluded article**.**

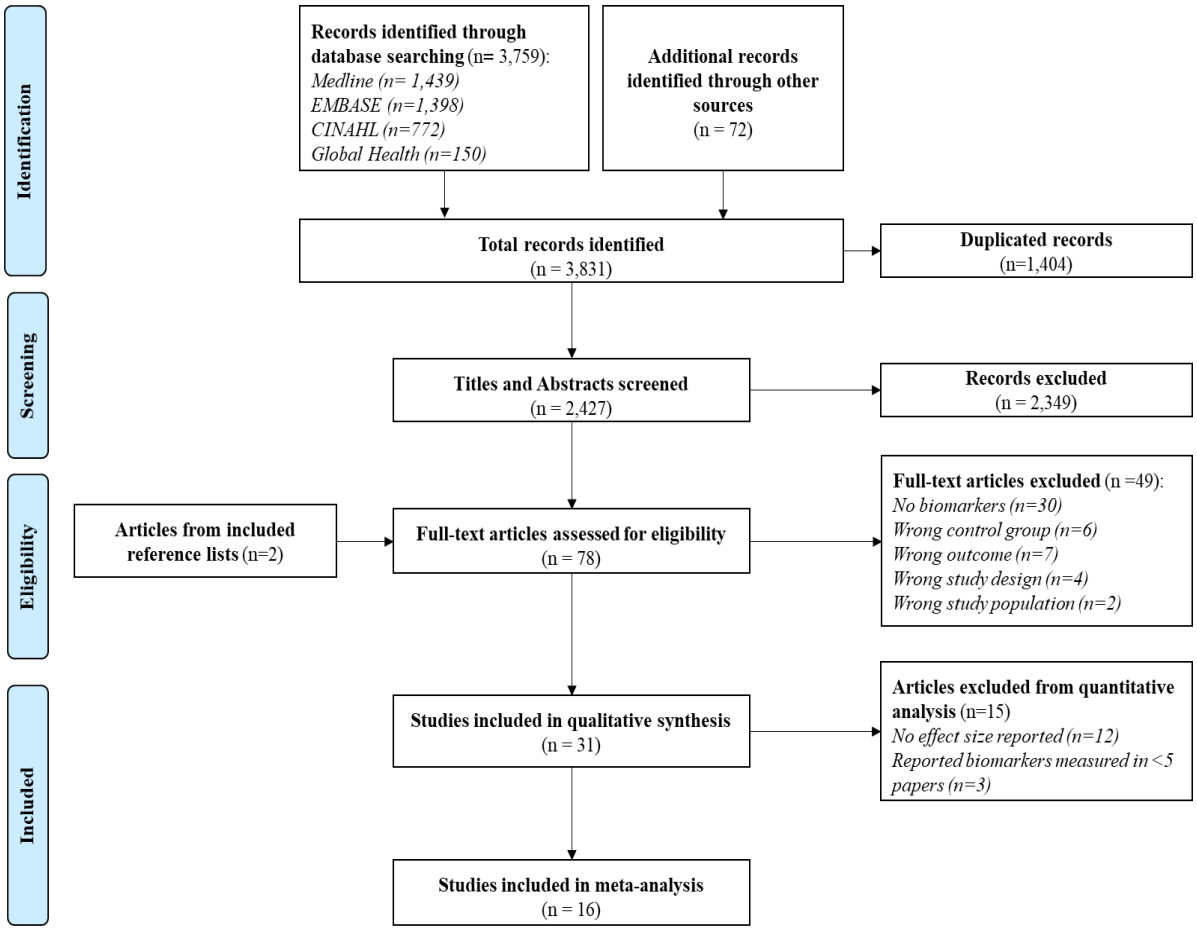


Figure 2.1. PRISMA flow diagram of the study selection process

The initial search from the four databases and the unpublished literature yielded 3,829 unique research articles, which were assessed for inclusion based on their titles and abstracts. From this initial screening and the reference list of review articles, 80 papers were selected for full text review, 31 were found eligible for inclusion and 16 of these were included in the meta-analysis. The authors, number and country of origins of the cases, as well as the list of biomarkers analysed in each paper are summarised in **Table 2.1.**

Table 2.1. Summary of included articles (N=31)

First author (Publication year)	N cases	Country of origin of cases	Biomarkers analysed
Adesanya et al. (1991)(70)	7	Benin	Albumin; Aldosterone; ANP; PRA; Sodium (urine)
Azibani et al. (2020)(71)	151	South Africa, Germany	Galectin-3; OPN, PINP; PIIINP; sST2
Cenac et al. (1990)(72)	28	Nigeria	Circulating immune complexes; Heart muscle autoantibody; IgA; IgG; IgM
Cenac et al. (1992)(73)	21	Niger	Selenium
Cenac et al. (1996)(74)	18	Niger	Albumin; Copper; Pre-albumin; Retinol binding protein; Selenium; Zinc
Cenac et al. (2000)(75)	12	Niger	Chlamydia (IgA; IgG; IgM)[a]
Cenac et al. (2004)(76)	11	Mali	Selenium
Cenac et al. (2009)(77)	24	Niger	Selenium
Ellis et al. (2005)(78)	28	Haiti	Anti-HSP60; Anti-HSP70; CRP; Endothelin-1; GM-CSF; IFN-g; IL-10; IL-1a; IL-1b; IL-4; proBNP; sCD40L; TGF-b; TNF-a
Fett et al. (2002)(79)	32	Haiti	Beta-carotene; Selenium; Vitamin A; Vitamin B12; Vitamin C; Vitamin E
Forster et al. (2008)(80)	35	South Africa	CRP; Fas/Apo-1; IFN-g; IL-1b; IL-6; MMP-2; MMP-9; NT-proBNP; oxLDL; prolactin; TGF-b1; TNF-a; VEGF
Haghikia et al. (2013)(62)	37	Germany	ADMA; Cathepsin-D; miRNA-146a (plasma); NT-proBNP
Haghikia et al. (2015)(81)	38	Germany	AAB: MHC & TnI
Halkein et al. (2013)(60)	38	Germany	ERBB4 mRNA (tissue); miRNA-146a (plasma); miRNA-146a(tissue)
Hilfiker-Kleiner et al. (2007)(59)	5	Germany	Cathepsin-D; oxLDL; prolactin (16 kDa); prolactin (26 kDa); STAT3

First author (Publication year)	N cases	Country of origin of cases	Biomarkers analysed
Huang et al. (2010)(82)	39	China	ADV-IgG; Albumin; AMA-IgG; CBV-IgG; cTnI; hs-CRP; Neutrophils; WBC
Huang et al. (2012)(83)	39	China	Albumin; cTnI; hs-CRP; NT-proBNP; WBC
Karaye et al. (2015)(84)	39	Nigeria	Albumin; Ceruloplasmin; Creatinine; Haemoglobin; Selenium; Sodium (blood)
Karaye et al. (2016)(85)	42	Nigeria	Creatinine; Potassium; Sodium (blood)
Liu et al. (2014)(86)	45	China	AAB: B1R; AAB: M2-R; NT-proBNP
McTiernan et al. (2018)(87)	50	USA	Macrophages[b]; Monocytes[c]; NK cells[d]; T-cells[e]
Mebazaa et al. (2017)(88)	52	South Africa, France	Copeptin; MR-proADM; NT-proBNP; PlGF; Relaxin-2; sFLT1; sST2; VEGF
Nonhoff et al. (2017)(89)	54	Germany	NT-proBNP; Relaxin-2
Patten et al. (2012)(90)	55	USA	sFLT1
Ricke-Hoch et al. (2019)(91)	64	Germany	CRP; IL-1b; IL-6; NT-proBNP; PAI-1; TnT; uPA
Sagy et al. (2017)(92)	70	Israel	Albumin; Alkaline Phosphatase; ALT; ANA; AST; Bilirubin; Calcium; CPK; Creatinine; CRP; D-dimer; GGT; Glucose; Haemoglobin; Phosphorous; Platelets; Potassium; Sodium (blood); TnT; TSH; Urea; Uric acid; WBC
Walenta et al. (2012)(93)	82	South Africa, Germany	EMP[f]; LMP; MMP; PMP[g]
Wang et al. (2018)(94)	83	China	Albumin; ALT; BNP; CK-MB; Creatinine; CRP; Haemoglobin; TnI; WBC
Xia et al. (2016)(95)	100	China	ALT; AST; B7-H1 (mRNA); C3; C4; CK-MB; Creatinine; Fasted blood glucose; Haemoglobin; IFN-g; IgA; IgG; IgM; IL-4; PD-1 (mRNA); proBNP; T-cells; TnI
Xia et al. (2017)(96)	115	China	Albumin; ALT; AST; Creatinine; B7-H1 (mRNA); B7-H1 (protein); CK-MB; proBNP; Fasted blood glucose; haemoglobinA1c; Haemoglobin; hs-CRP; IFN-g;

First author (Publication year)	N cases	Country of origin of cases	Biomarkers analysed
			IL-4; PD-1 (mRNA); PD-1 (protein); Platelets; RBC; TnI; WBC
Yaqoob et al. (2018)(97)	29	India	ACE polymorphism: DD; ID; II

ANP: Atrial natriuretic peptide, **PRA:** plasma renin activity, **OPN**: osteopontin, **PINP**: procollagen type-1 N-terminal propeptide, **PIIINP**: procollagen type-3 N-terminal propeptide, **sST2:** Soluble suppression of tumorigenicity 2, **IgA**: Immunoglobulin-A, **IgG**: Immunoglobulin-G, **IgM**: Immunoglobulin-M, **anti-HSP60**: anti-Heat shock protein 60, **anti-HSP70:** anti-Heat shock protein 70, **CRP**: C-reactive protein, **GM-CSF**: Granulocyte-macrophage colony-stimulating factor, **IFN-g:** Interferon gamma, **IL-10**: interleukin-10, **IL-1a:** Interleukin-1 alpha, **IL-1b**: Interleukin-1 beta, **IL-4**: interleukin-4, **proBNP:** pro B-type natriuretic peptide, **sCD40L**: soluble CD40 ligand, **TGF-b**: Transforming growth factor beta, **TNF-a**: Tumour Necrosis Factor alpha, **Fas/Apo-1**: apoptosis antigen 1, **IL-6**: interleukin-6, **MMP-2:** matrix metalloproteinase-2, **MMP-9**: matrix metalloproteinase-9, **NT-proBNP**: N-terminal pro B-type natriuretic peptide, **NK cells**: natural killer cells, **oxLDL**: Oxidized low-density lipoprotein, **VEGF**: Vascular endothelial growth factor, **miRNA-146a**: micro ribonucleic acid 146a, **ADMA**: asymmetric dimethylarginine, **AAB:MHC & TnI**: auto-antibody: myosin heavy chain & troponin-1, **ErbB4:** erb-B2 receptor tyrosine kinase 4, **STAT3**: signal transducer and activator of transcription 3, **ADV-IgG**: adenovirus immunoglobulin-G, **AMA-IgG**: anti-mitochondrial antibody immunoglobulin-G, **CBV-IgG:** coxsackie b virus immunoglobulin-G, **cTnI:** cardiac Troponin-1, **hs-CRP**: high sensitivity C-reactive protein, **WBC**: white blood cells count, **AAB:B1R**: auto-antibody: β1-adrenergic receptor, **AAB:M2R**: auto-antibody: M2-muscarinic receptor, **MR-proADM:** mid regional pro-adrenomedullin, **PlGF**: placental growth factor, **sFTL1**: soluble fms-like tyrosine kinase-1, **PAI-1**: plasminogen activator inhibitor-1, **TnT:** troponin-T, **uPA:** urokinase, **ALT:** alanine aminotransferase, **ANA**: antinuclear antibodies, **AST:** aspartate transaminase, **CPK:** creatine phosphokinase, **GGT:** gamma-glutamyl transpeptidase, **TSH**: thyroid stimulating hormone, **EMP**: endothelial microparticles, **LMP**: leukocyte-derived microparticles, **MMP**: monocyte-derived microparticles, **PMP**: Platelet-derived microparticles, **BNP:** B-type natriuretic peptide, **CK-MB:** creatine kinase myocardial band, **B7-H1:** B7 homolog 1, **C3**: complement component 3, **C4:** complement component 4, **PD-1**: programmed cell death protein 1, **ACE**: Angiotensin Converting Enzyme, **DD/ID/II**: double-deletion/insertion-deletion/double-insertion.
[a]**Including**: Chlamydia pneumonia: Chlamydia trachomatis and Chlamydia psittaci, [b]**Macrophages included**: CD14+CD16+: CD14+CD16+CD38+: and CD14+CD16+HLA-DR+, [c]**Monocytes included**: CD14+: CD14+CD16-: CD14+CD16-CD38+: and CD14+CD16-HLA-DR+, [d]**NK cells included**: CD3-CD56+CD16-: CD3-CD56+CD16+: CD3-CD56+CD16+CD38+: and CD3-CD56+CD16+HLA-DR+, [e]**T-cells included**: CD8+: CD3+: CD4+: CD3+CD4+: CD3+CD4+CD25+: CD3+CD4+CD38+: CD3+CD4+CD8+: CD3+CD4-CD8-: CD3+CD4-CD8-CD25+: CD3+CD4-CD8-CD38+: CD3+CD4-CD8-HLA-DR+: CD3+CD56+: CD3+CD56+CD8+: CD3+CD8+: CD3+CD8+CD25+: CD3+CD8+CD38+ and CD3+CD8+HLA-DR+, [f]**EMPs included**: CD62E+: CD144+AV+: CD31+AV+ and C D144+CD31+AV+, [g]**PMPs included**: CD42b-AV+: CD62P+ and CD62P+CD46b+AV+

The number of PPCM cases studied ranged from five to 115 and came from 18 different study populations. Africa was the most represented geographical region in this sample, with six different cohorts included in 13 studies. The Asian populations were represented in six papers from China, and one from India (n=29).

The clinical definition of PPCM used in the included articles was heterogeneous **(see Appendix Table A5)**. The three main differences in the diagnosis criteria for PPCM were in the

window of diagnosis, the cut-off value for the left ventricular ejection fraction (LVEF), and the explicit exclusion of other causes of heart failure.

2.4.2. Quality Assessment

Since all the included articles followed a case-control design, the quality assessment was performed using the modified NHLBI Quality Assessment tool for Case-control studies (as discussed above). **Table 2.2.** summarises the results of the assessment, as well as the proportion of "Yes" and "Not Applicable" answers for each article.

As PPCM is a rare condition, the few papers included in this review tended to have small numbers of participants (ranging from five to 115 cases), and as illustrated in the quality assessment tool, they all failed to meet criterion 3, which was to provide evidence of a sample size justification. Almost none of the included articles provided evidence of a random sampling for the comparator group, thus implying a moderate risk of selection bias (criterion 7). Additionally, few papers reported that the assessors of biomarker status were blinded to the participant's PPCM diagnosis when interpreting the biomarker test results (as assessed by criterion 11 of the tool). Finally, except for 5 articles, the included papers did not report any relative measures of risk (risk ratio, odds ratio or hazard ratio), and no assessment of confounding to account for the influence of different factors on the association of interest (criterion 12). However, in many of those studies confounding was addressed to some extent at the design phase using matching.

Overall, the articles included in this review had a moderate level of bias, and thus the overall quality of the sources for the review was "Fair". Additionally, the visual assessment of the funnel plot suggested significant publication bias across all the biomarkers measured.

Table 2.2. Quality of included studies by assessment tool criteria

	Bias criteria*															
First author (Year)	1	2	3	4	5	6	7	8	9	10.a	10.b	10.c	10.d	11	12	**Overall quality**
Adesanya et al. (1991)	Y	N	N	U	U	U	N	Y	N	Y	Y	Y	Y	U	N	Poor
Azibani et al. (2020)	Y	Y	N	Y	U	Y	Y	Y	NA	Y	Y	U	U	U	N	Fair
Cenac et al. (1990)	Y	N	N	Y	U	Y	N	Y	NA	Y	N	N	Y	U	N	Poor
Cenac et al. (1992)	Y	Y	N	Y	U	Y	U	Y	NA	Y	Y	Y	Y	U	N	Good
Cenac et al. (1996)	Y	Y	N	Y	U	Y	Y	Y	N	Y	Y	Y	Y	N	N	Fair
Cenac et al. (2000)	Y	N	N	Y	U	Y	N	Y	Y	Y	Y	Y	Y	U	N	Poor
Cenac et al. (2004)	Y	Y	N	N	U	N	U	Y	NA	Y	Y	Y	N	N	N	Poor
Cenac et al. (2009)	Y	Y	N	Y	U	Y	Y	Y	NA	Y	U	U	Y	U	N	Fair
Ellis et al. (2005)	Y	Y	N	Y	U	U	U	Y	NA	Y	Y	Y	Y	U	NA	Good
Fett et al. (2002)	Y	Y	N	Y	Y	U	U	Y	NA	Y	Y	Y	Y	U	N	Good
Forster et al. (2008)	Y	Y	N	Y	U	Y	U	Y	NA	Y	Y	Y	Y	U	N	Good
Haghakia et al. (2013)	N	Y	N	U	U	U	Y	Y	NA	Y	Y	Y	Y	U	NA	Fair
Haghakia et al. (2015)	Y	Y	N	U	U	U	Y	Y	NA	Y	Y	Y	Y	U	N	Fair
Halkein et al. (2013)	N	N	N	U	U	U	U	Y	NA	Y	Y	U	Y	U	N	Poor
Hilfier-Kleiner et al. (2007)	N	N	N	U	U	U	U	Y	Y	Y	Y	U	Y	U	N	Poor
Huang et al. (2010)	N	Y	N	Y	Y	Y	U	Y	N	Y	Y	U	Y	U	Y	Fair
Huang et al. (2012)	Y	Y	N	U	U	Y	U	Y	N	Y	Y	Y	Y	U	Y	Fair
Karaye et al. (2015)	Y	Y	N	Y	N	Y	Y	Y	U	Y	N	U	Y	U	Y	Fair

Karaye et al. (2016)	Y	Y	N	Y	U	Y	Y	Y	N	Y	Y	Y	Y	U	NA	Good
Liu et al. (2014)	Y	Y	N	U	U	U	U	Y	NA	Y	U	U	Y	U	Y	Fair
McTiernan et al. (2018)	Y	Y	N	U	U	U	U	Y	NA	Y	U	U	Y	U	N	Poor
Mebazaa et al. (2017)	Y	N	N	U	U	Y	U	Y	NA	Y	Y	Y	Y	Y	NA	Good
Nonhoff et al. (2017)	Y	Y	N	Y	U	Y	Y	Y	NA	Y	Y	U	Y	U	N	Good
Patten et al. (2012)	Y	N	N	U	U	Y	U	Y	NA	Y	Y	Y	Y	U	N	Poor
Ricke-Hoch et al. (2019)	Y	Y	N	U	U	Y	Y	Y	Y	Y	Y	U	Y	U	N	Fair
Sagy et al. (2017)	Y	Y	N	Y	NA	Y	Y	Y	U	N	U	U	Y	U	Y	Fair
Walenta et al. (2012)	Y	Y	N	U	U	U	U	Y	NA	Y	U	Y	Y	Y	N	Fair
Wang et al. (2018)	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	U	Y	U	N	Good
Xia et al. (2016)	Y	Y	N	U	U	Y	Y	Y	NA	Y	Y	Y	Y	U	N	Good
Xia et al. (2017)	Y	Y	N	U	U	Y	Y	Y	NA	Y	Y	Y	Y	U	N	Good
Yaqoob et al. (2018)	Y	Y	N	Y	U	Y	Y	Y	Y	Y	Y	U	Y	U	NA	Good

Y: Yes (meets criteria), **N**: No (does not meet criteria), **U**: Unclear, **NA**: Not Applicable

*Bias criteria: **1:** Research question clearly stated; **2:** Study population specified and defined; **3:** Sample size justified; **4:** Case and control groups recruited from the same population; **5:** Random selection of study participants; **6:** Enrolment of concurrent controls; **7:** Inclusion and exclusion criteria pre-specified and applied uniformly; **8:** Clear definition of cases and controls; **9:** Exposure assessed prior to outcome measurement; **10.a:** Clear definition of biomarker assessment methods; **10.b:** Valid biomarker assessment; **10.c:** Reliable biomarker assessment; **10.d.:** Biomarker assessment performed uniformly across study groups; **11:** Blinding of exposure assessors; **12:** Adjustment for confounding.

2.4.3. Molecular mechanisms for the development of PPCM

This review included 31 case-control studies which reported the levels of 117 biomarkers in the biological media of PPCM patients and pregnant or postpartum women without heart failure. **Figure 2.2.** illustrates a summary of the main biomarkers investigated according to the proposed mechanisms of effect. The most commonly investigated mechanisms for the pathogenesis of PPCM were inflammation, myocardial damage and vascular imbalance. N-terminal prohormone of brain natriuretic peptide (NT-proBNP), C-reactive protein (CRP), uric acid, anti-myocardial antibodies, cardiac receptor antibodies and the D allele of the angiotensin converting enzyme (ACE) gene were all reported to be associated with an increased odds of PPCM in pregnancy, although with insufficient samples to perform a quantitative analysis **(see Table 2.3).**

Table 2.3. Biomarkers associated with PPCM

First author (Year)	Biomarker	OR	95% CI
Liu (2014)(86)	Cardiac receptor antibodies (B1R & M2R)	18.8	1.93, 183
Huang (2010)(82)	Anti-myocardial antibodies (AMA-IgG)	2.68	1.19, 4.85
Huang (2012)(83)	NT-proBNP	1.92	1.12, 4.15
Huang (2012)(83)	C-reactive protein	1.86	1.08, 3.90
Sagy (2017)(92)	Uric Acid	1.30	1.05, 1.61
Yaqoob (2018)(97)	II polymorphism of the ACE gene	0.25	0.11, 0.56

OR: odds ratio, **CI:** Confidence Interval, **B1R**: β1-adrenergic receptor; **M2R**: M2-muscarinic receptor; **AMA-IgG:** anti-mitochondrial antibody immunoglobulin-G; **NT-proBNP:** N-terminal pro B-type natriuretic peptide; **II:** double insertion; **ACE**: angiotensin converting enzyme

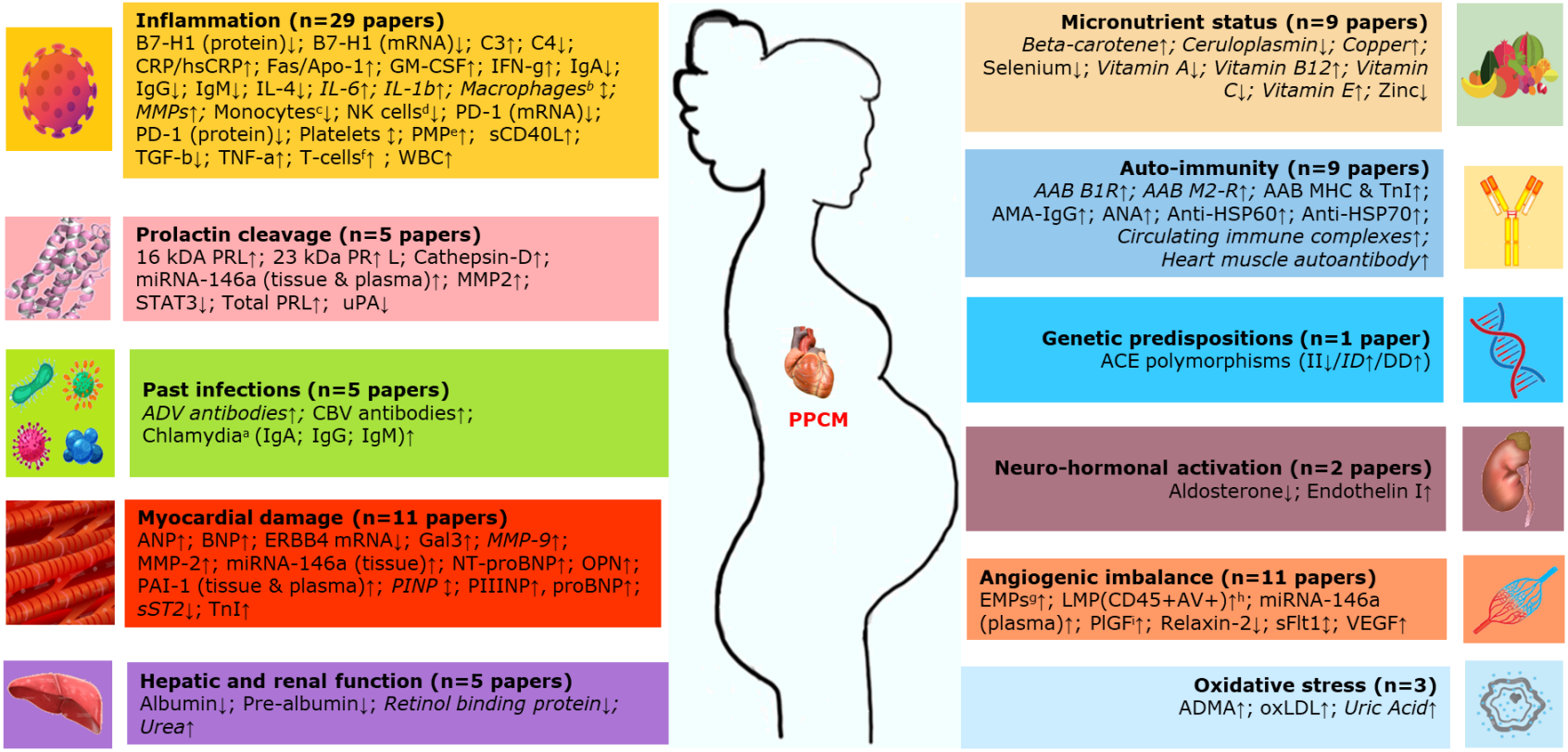


Figure 2.2. Laboratory biomarkers measured in women with and without PPCM according to their putative mechanism of effect.

B7-H1: *B7 homolog 1,* ***C3:*** *complement component 3,* ***C4****: complement component 4,* ***CRP/hs-CRP:*** *C-reactive protein/high sensitivity C-reactive protein,* ***Fas/Apo-1****: apoptosis antigen 1,* ***GM-CSF:*** *granulocyte-macrophage colony-stimulating factor,* ***IFN-g:*** *interferon gamma,* **IgA**: Immunoglobulin-A, **IgG:** Immunoglobulin-G, **IgM:** Immunoglobulin-M**,** ***IL-4:*** *interleukin-4,* ***IL-6:*** *interleukin-6,* ***IL-10****: interleukin-10,* ***IL-1a****: interleukin-1 alpha,* ***IL-1b:*** *interleukin-1 beta,* ***MMPs:*** *monocyte-derived microparticles,* ***PD-1****: programmed cell death protein 1,* ***PMP:*** *platelet-derived microparticles,* ***sCD40L:*** *soluble CD40 ligand,* ***TGF-b:*** *transforming growth factor beta,* ***TNF-a****: tumour necrosis factor alpha,* ***NK cells****: natural killer cells,* ***WBC:*** *white blood cells count,* ***PRL:*** *prolactin,* ***miRNA-146a:*** *micro ribonucleic acid 146a,* ***MMP-2****: matrix metalloproteinase-2,* ***STAT3****: signal transducer and activator of transcription 3,* ***uPA:*** *urokinase,* ***ADV:*** *adenovirus,* ***CBV:*** *coxsackie b virus,* ***ANP****: atrial natriuretic peptide,* ***BNP:*** *B-type natriuretic peptide,* ***ErbB4:*** *erb-B2 receptor tyrosine kinase 4,* ***Gal3:*** *galectin-3,* ***MMP-9****: matrix metalloproteinase-9,* ***NT-proBNP****: N-terminal pro*

B-type natriuretic peptide, ***ONP:*** *osteopontin,* ***PAI-1:*** *plasminogen activator inhibitor-1,* ***PINP****: procollagen type-1 N-terminal propeptide,* ***PIIINP****: procollagen type-3 N-terminal propeptide,* ***proBNP****: pro B-type natriuretic peptide,* ***sST2****: soluble suppression of tumorigenicity 2,* ***TnI****: cardiac troponin-1,* ***AAB:B1R****: auto-antibody: β1-adrenergic receptor,* ***AAB:M2R****: auto-antibody: M2-muscarinic receptor,* ***AAB:MHC & TnI****: auto-antibody: myosin heavy chain & troponin-1,* ***AMA****: anti-mitochondrial antibody,* ***ANA:*** *antinuclear antibodies,* ***anti-HSP60:*** *anti-Heat shock protein 60,* ***anti-HSP70****: anti-Heat shock protein 70,* ***ACE:*** *angiotensin converting enzyme,* ***DD/ID/II****: double-deletion/insertion-deletion/double-insertion,* ***EMP:*** *Endothelial microparticles,* ***LMP:*** *leukocyte-derived microparticles,* ***PlGF:*** *placental growth factor,* ***sFTL1:*** *soluble fms-like tyrosine kinase-1,* ***VEGF****: vascular endothelial growth factor,* ***ADMA****: asymmetric dimethylarginine,* ***oxLDL****: oxidized low-density lipoprotein.*

↑ Biomarkers with increased expression in the serum of PPCM cases compared to pregnant or postpartum women without HF.

↓ Biomarkers with decreased expression in the serum of PPCM cases compared to pregnant or postpartum women without HF.

↕ Biomarkers for which the direction of the difference between levels in PPCM cases and pregnant or postpartum women without HF is conflicting

Biomarkers italicised in the figure represent non-significant findings.

[a]Including: Chlamydia pneumonia, Chlamydia trachomatis and Chlamydia psittaci;

[b]Macrophages included: CD14+CD16+, CD14+CD16+CD38+, and CD14+CD16+HLA-DR+;

[c]Monocytes included: CD14+, CD14+CD16-, CD14+CD16-CD38+, and CD14+CD16-HLA-DR+;

[d]NK cells included: CD3-CD56+CD16-, CD3-CD56+CD16+, CD3-CD56+CD16+CD38+, and CD3-CD56+CD16+HLA-DR+;

[e]T-cells included: CD8+, CD3+, CD4+, CD3+CD4+, CD3+CD4+CD25+, CD3+CD4+CD38+, CD3+CD4+CD8+, CD3+CD4-CD8-, CD3+CD4-CD8-CD25+, CD3+CD4-CD8-CD38+, CD3+CD4-CD8-HLA-DR+, CD3+CD56+, CD3+CD56+CD8+, CD3+CD8+, CD3+CD8+CD25+, CD3+CD8+CD38+ and CD3+CD8+HLA-DR+;

[f]EMPs included: CD62E+, CD144+AV+, CD31+AV+ and C D144+CD31+AV+;

[g]PMPs included: CD42b-AV+, CD62P+ and CD62P+CD46b+AV+

2.4.3.1. Prolactin cleavage

Prolactin levels in PPCM cases and controls without heart failure were compared in two papers. Forster et al.(80) reported significantly higher levels of total prolactin (p<0.001) in the serum of PPCM patients (median = 24.7 ng/mL) compared to healthy controls (median=7.4 ng/mL). Hilfiker-Kleiner et al. detected the angiogenic 23 kDa isoform of prolactin in both lactating women with PPCM (n=5) and healthy lactating women (n=5) (59). However, while the angiostatic 16 kDa isoform was expressed in three of the five women with PPCM, it was barely detectable in the control group. No quantitative measurement of either isoforms were reported.

The serum of women with PPCM had altered concentration of other biomarkers suggested to be part of the prolactin-cleavage pathway. Results from these studies found that levels of miRNA-146a (the main by-product of 16 kDa prolactin production) were significantly increased in both the tissue and plasma of the PPCM group compared to healthy controls. Additionally, levels of prolactin cleavage factors (i.e. Cathepsin-D and MMP2) were significantly higher in cases than in controls. Finally, STAT3 was expressed at lower levels in the hearts of cases compared to those of controls. This biomarker protects cardiomyocytes from the oxidative stress environment that promotes the cleavage of prolactin.

2.4.3.2. Iron metabolism

Haemoglobin (Hb) was one of the most commonly reported markers in association with PPCM. Across all the included articles, the authors found that PPCM mothers had lower levels of Hb compared to their healthy postpartum counterparts, although no author reported a statistically significant difference between the study groups. The review found no original research papers that directly assessed markers of iron status (such as serum iron, ferritin, transferrin saturation, or soluble transferrin receptors) in pregnant women with PPCM.

Nevertheless, serum concentration of other markers involved in iron homeostasis were altered in women with PPCM **(Figure 2.3)**. Levels of the inflammatory cytokine interleukin-6 (IL-6), which regulates the iron homeostatic hormone hepcidin were increased in PPCM cases (98). Additionally, the vasoconstrictor endothelin-1 which is typically upregulated in iron deficiency

(99) was also significantly higher in PPCM cases compared to controls. Finally, serum Vitamin C, which facilitates gastrointestinal iron absorption, was lower than in healthy controls.

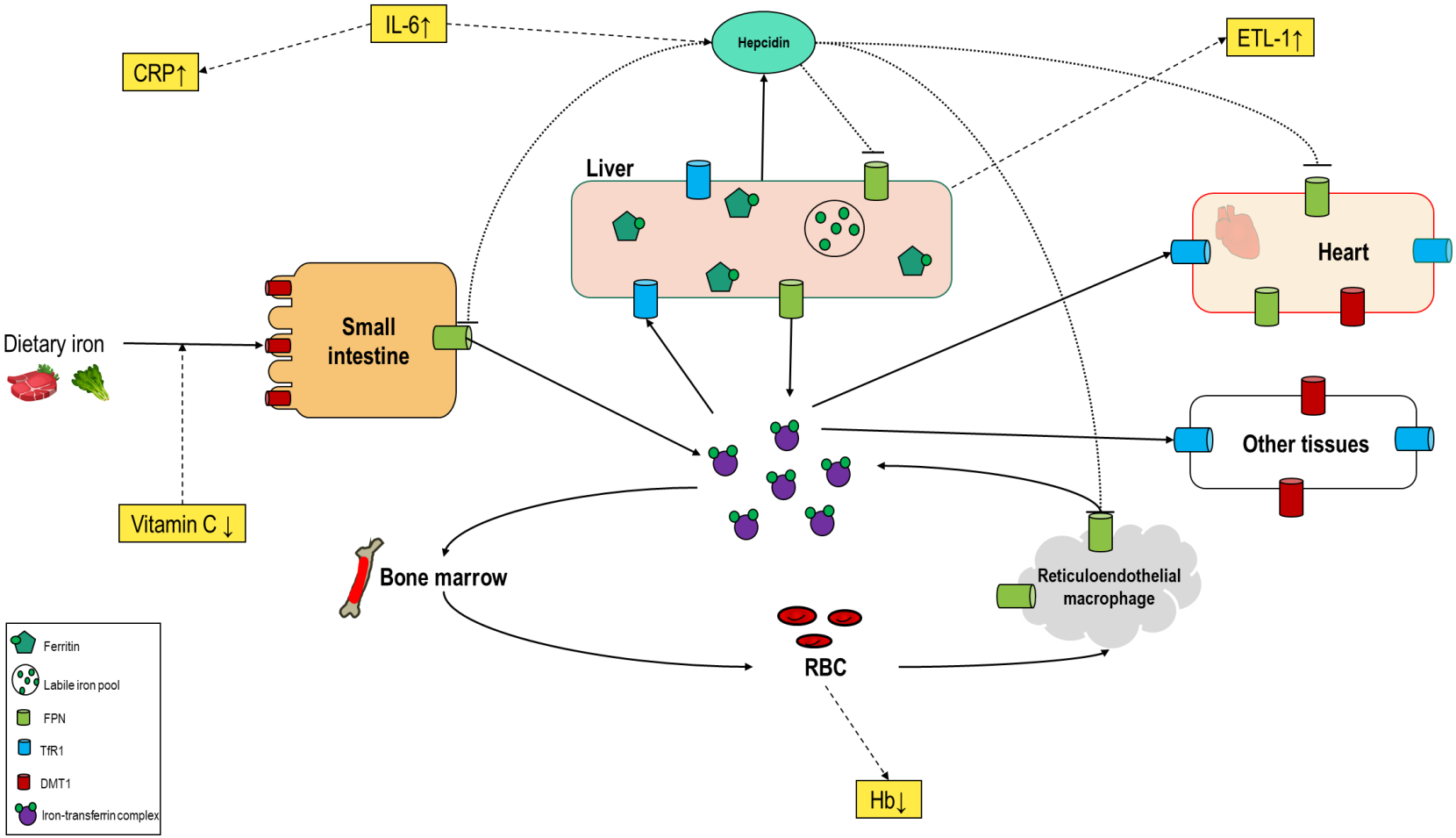


Figure 2.3. Markers related to iron metabolism studied in women with PPCM and their role in iron homeostasis. Vitamin C facilitates the absorption of dietary iron in the intestine. CRP is increased in inflammation through the action of IL-6. IL-6 regulates hepcidin which controls the mobilisation of iron from biological pools. Expression of the vasoconstrictor ETL-1 increases in iron deficiency. Iron restricted erythropoiesis leads to a decrease in Hb levels. ***CRP**: C-reactive protein, **IL-6**: interleukin-6, **ETL-1**: endothelin-1, **Hb**: haemoglobin, **RBC**: red blood cells, **FPN**: ferroportin, **TfR1**: transferrin receptor-1, **DMT1**: divalent metal-ion transporter-1. ↑ Markers with increased expression in the serum of PPCM cases compared to pregnant or postpartum women without HF. ↓ Markers with decreased expression in the serum of PPCM cases compared to pregnant or postpartum women without HF.*

2.4.4. Meta-analysis

The meta-analysis included 16 studies that compared and reported mean levels of biomarkers in women with and without PPCM. These papers investigated serum concentrations of natriuretic peptides, CRP, cardiac troponins, albumin, haemoglobin, creatinine, selenium and white blood cells in both study groups.

On average, women diagnosed with PPCM had significantly lower levels of haemoglobin (SMD: -0.449, 95%CI: -0.639; -0.259) compared to healthy controls. The SMD in haemoglobin is reported in the forest plot in **Figure 2.4.**

Additionally, serum levels of natriuretic peptides (SMD: 3.453, 95% CI: 2.174; 4.695) and creatinine (SMD: 0.513, 95% CI: 0.33; 0.694) were higher on average among women with PPCM, while levels of albumin were lower (SMD: -0.662, 95% CI: -0.971; -0.352) than in women without PPCM (see **Appendix Table A6**).

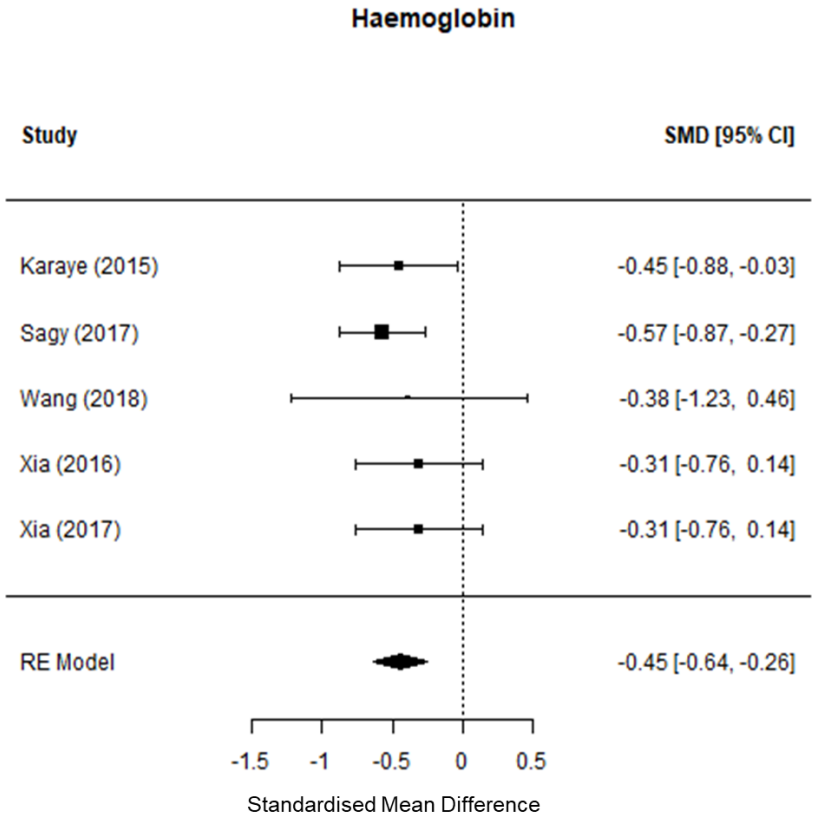


Figure 2.4. Forest plot of the standardised mean difference in haemoglobin concentrations between women with and without PPCM. ***SMD:*** *standardised mean difference,* ***CI:*** *confidence interval,* ***RE:*** *random effects*

2.5. Discussion

In this review, 117 biomarkers were extracted from 31 case-control studies comparing molecular markers in women diagnosed with PPCM to those in women without heart failure. The included articles had a moderate level of bias. The most commonly studied mechanisms for the development of PPCM were inflammation, myocardial damage and vascular imbalance. There was also limited evidence to support the notion that serum concentrations of prolactin in the postpartum period are different in women with PPCM compared to women without heart failure. However, the review found no studies in the literature investigating the iron profile of women diagnosed with PPCM.

The results of this systematic review support the evidence from previous studies which have identified anaemia as an important comorbidity of PPCM (21). Indeed, the meta-analysis of mean Hb concentrations in serum indicated a marginal but statistically significant pooled difference between PPCM cases compared to controls. The mean Hb concentration in cases met the WHO definition of anaemia in pregnancy (100).

However, as this review demonstrated, the nature of the association between anaemia and PPCM has not been fully elucidated and it is still unclear which of the various aetiologies of anaemia drives this association. Iron deficiency is the most commonly observed cause of anaemia in pregnancy, and there is growing evidence to suggest that a deficiency in iron can directly undermine cardiac functioning, even in the absence of concomitant anaemia (101, 102). Although, the search of the literature found no studies that assessed markers of iron status in women with PPCM, other markers involved in iron homeostasis were identified in association with the disease. Specifically, the increase in inflammatory markers (such as IL-6) and decreased concentrations of vitamin C observed in women diagnosed with PPCM suggest that iron absorption could be altered in this disease. However, the evidence is very limited and more studies are needed to accurately characterise the role of iron status in the development of PPCM, both independently and in the context of anaemia.

On the other hand, it has been suggested that the prolactin-mediated mechanism for the pathogenesis of PPCM underlies several mechanisms that have been proposed for PPCM. Namely, the physiological inflammation and oxidative stress of pregnancy may lead to the subsequent cleavage of prolactin into the angiogenic 16 kDa form, which in turn causes vascular impairment and myocardial damage (22). This hypothesis is the basis for recent clinical trials of Bromocriptine for the treatment of PPCM (62, 65, 89), and the current recommendation from the ESC that Bromocriptine may be considered as adjunct therapy for the treatment of PPCM(5).

However, while there was some evidence to suggest that biomarkers involved in the prolactin-cleavage pathway are expressed at significantly different levels in PPCM cases compared to healthy controls, this review only found two papers that directly measured and compared levels of prolactin in the blood of the study participants. One paper showed that women diagnosed with PPCM in the postpartum period had serum levels of prolactin three times higher than the median observed in healthy controls after childbirth. However, the reported concentrations fell within reported normal limits in non-pregnant women (103, 104). Moreover, the authors did not report any information on the postpartum window or the breastfeeding status of the study population at the time of serum analysis (both of which significantly affect prolactin levels in the postpartum period). Thus, more robust studies in human subjects are needed to better understand the metabolism of prolactin and its potential role in the pathophysiology in PPCM.

2.6. Strengths & limitations

This is the first comprehensive review of the molecular mechanisms underlying PPCM. By keeping the search terms relatively broad and manually screening for reports of biomarkers in the study of PPCM, this review attempted to capture all available evidence on the metabolism of iron and prolactin in women diagnosed with PPCM. Moreover, the screening, data extraction and quality assessment in this review were independently performed by two reviewers, which attests to the overall robustness of the review process.

Nevertheless, this review suffered from some limitations. First, the strict inclusion and exclusion criteria limited the analysis to papers that compared levels of biomarkers in women with PPCM to those in pregnant or early postpartum women without heart failure. Although some biomarkers in the molecular profile PPCM may have been missed due to this requirement, a comparison group was essential to be able to assess, compare and pool the level of effect of the biomarkers across different studies. Additionally, a control group of women without heart failure or cardiac problems allowed for an estimation of the baseline values of the biomarkers in a healthy pregnant population, which is important for understanding the unique biological profile of this disease.

Secondly, the standardised mean difference for some markers had to be estimated from median and interquartile range values. This may have resulted in some level of bias due to the non-normal distribution of the biomarker data. However, the quantile method of estimation used as described by Wan et al. has been found to be sensitive to the skewness of non-normal data (68). This method is contrasted to others that include maximum and minimum values in the calculation, which may be outliers and subsequently lead to worse estimations. Although the quartile method may result in some overestimation, the resulting estimates are relatively reliable.

As the results of the meta-analysis illustrated, there was considerable heterogeneity in the reported measures. However, since conducting a meta-regression analysis of the data was beyond the scope of this review, another limitation of this review was that the exact source of that heterogeneity was not assessed.

Finally, since the focus of this review was on identifying biomarkers that might have a plausible causal link with PPCM, the analysis of prognostic markers of PPCM was beyond the scope. However, prognostic markers are important in clinical settings since they can be useful indicators of disease progression and treatment effectiveness. Therefore, their investigation can be the focus of future reviews of the clinical profile of PPCM.

2.7. Chapter summary

The aim of this chapter was to systematically appraise the literature on the biomolecular mechanisms that have been suggested for PPCM. Overall, the current evidence on the molecular determinants of PPCM is limited by small study population numbers, and a lack of diversity in the populations and biomarkers studied. While iron metabolism is increasingly recognised as an important determinant of cardiac health, there are currently no studies of iron metabolism in women diagnosed with PPCM. Additionally, the review illustrated the limitations of the evidence for an association between PPCM and prolactin imbalance on which current recommendations for the treatment of PPCM are based. Thus, the results of this chapter have demonstrated the need for more population-based studies investigating iron and prolactin metabolism in women with PPCM. Given the current lack of evidence for this association, the following chapter will seek to establish the biological plausibility of a role for iron deficiency in the development of PPCM.

Chapter 3. A theoretical framework for the role of iron deficiency in Peripartum Cardiomyopathy

3.1. Background and scope

According to the systematic review and meta-analysis performed in the previous chapter, women diagnosed with peripartum cardiomyopathy (PPCM) are reported to have marginally lower levels of haemoglobin than their counterparts without heart failure. Previous studies have even identified anaemia as an important risk factor for the development of PPCM, with the risk of PPCM being up to five times greater among anaemic women (23, 49). Most cases of anaemia that occur in pregnant and lactating women can be attributed to a deficiency in iron (105). Iron deficiency has long been recognised as an important comorbidity of heart failure observed in both chronic and acute cases (17), and emerging evidence suggests that iron deficiency itself may also contribute to the development of incident cardiomyopathy even in the absence of anaemia (106-108). However, as the findings of the previous chapter demonstrated, no studies have previously investigated the association between iron deficiency *per se* and PPCM.

The aim of this chapter was to determine the biological plausibility of a pathogenic mechanism for PPCM involving iron deficiency. Current knowledge on the pathophysiology of iron deficiency-induced cardiomyopathy was reviewed from human and animal studies to formulate an evidence-based conceptual framework for understanding how iron deficiency may contribute to the development of PPCM in pregnant and postpartum women.

3.2. Sources and search strategy

The systematic review conducted in the previous chapter found limited evidence for the association between anaemia and PPCM, and no results for the role of iron deficiency in PPCM. Therefore, a scoping review of the literature was conducted to summarise the epidemiological and mechanistic evidence for the association between iron deficiency and PPCM.

The National Library of Medicine (MEDLINE), PubMed, and Web of Science were searched to identify original studies, case series, or abstracts published in English that assessed the association of cardiomyopathy or PPCM specifically with anaemia and iron deficiency. The search included MeSH terms for: “iron”, “iron deficiency anaemia”, “serum ferritin”, “transferrin

receptors", and "hepcidin". These were searched in association with the following: "Cardiac myocytes", "heart diseases", "heart failure", "myocardium", and "cardiomyopathies". To focus the results on recent understanding of the role on iron in cardiomyopathy, only studies in animals and humans published in English since 2010 were included. Terms for "haemochromatosis", "iron overload", "thalassemia", and studies not in adult populations were excluded. Studies on myocardial infarction were also excluded to more specifically focus the review on the role of iron status in cardiomyopathies.

3.3. Summary of systemic iron metabolism

The principles of human iron homeostasis are well established and have been extensively summarised in various comprehensive reviews (109-111).

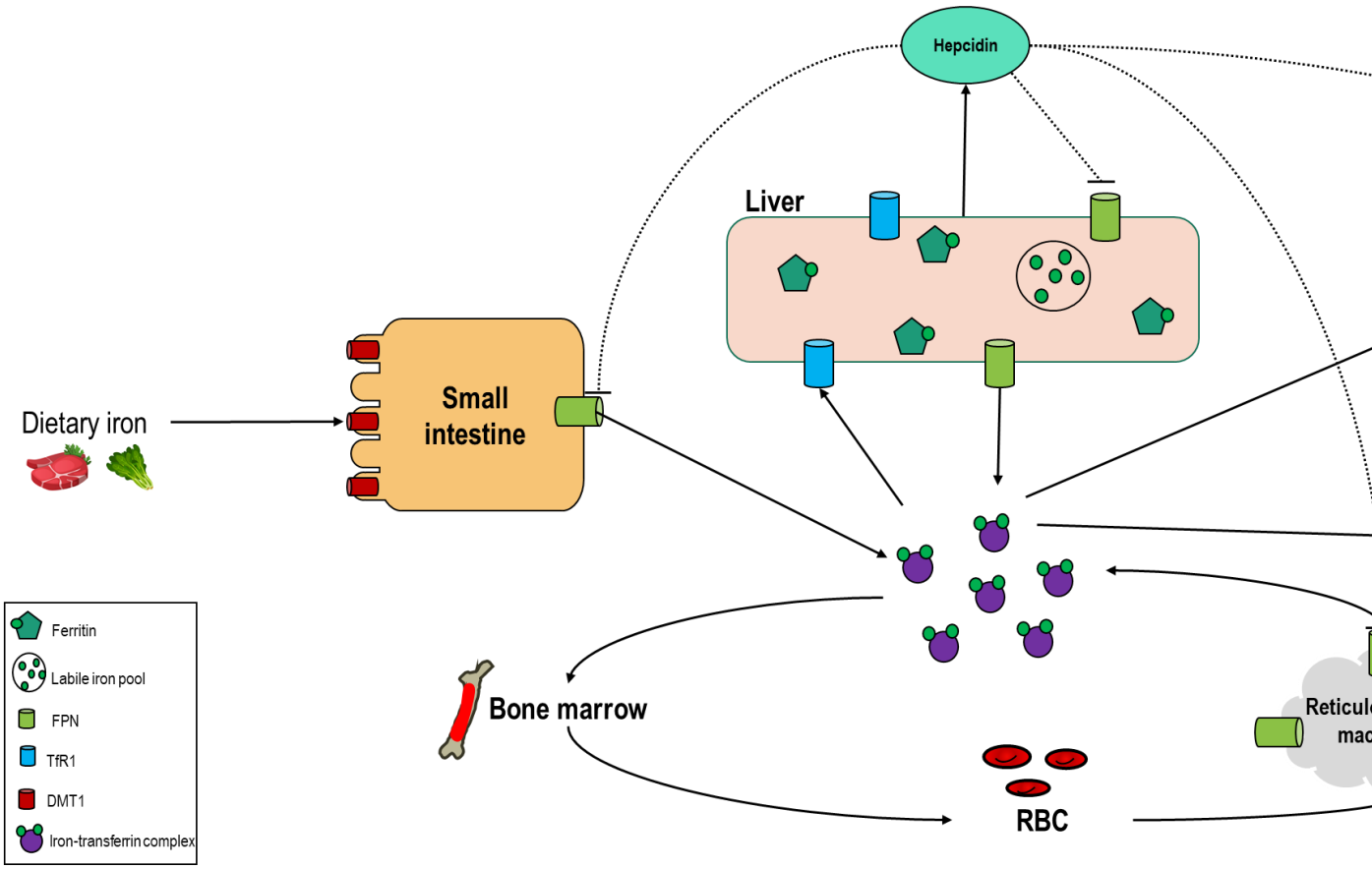


Figure 3.1. summarises the current understanding of the processes that govern the balance of iron supply and demand in the human body.

Iron is a functional component of proteins involved in oxygen transport and storage, oxidative metabolism, cell proliferation, and immune activation. However, while the micronutrient is necessary for maintaining normal cellular functioning, excess levels of free iron are also toxic to the human body due to its propensity to contribute to the production of reactive oxygen species, the substrate of oxidative stress. Consequently, mammals have developed a complex system to ensure that iron levels are adequate to support cellular metabolic functions, while remaining below the threshold at which oxidative stress occurs. Since the body cannot endogenously synthesise iron, all of the total iron present (~2.5-3g in the average adult woman (112)) is constantly being moved throughout various biological pools. Typically, only about 1 mg of 'new iron' is absorbed from the diet by gut enterocytes every day. Most of the of iron demand is supplied by splenic macrophages recycling haemoglobin from old red blood cells (113). The remaining iron is either circulating in the plasma as iron-transferrin complexes or stored within peripheral tissues.

In peripheral tissue, iron is stored in macromolecular cages called ferritin, or bound in heme and iron sulphur groups, which are particularly vital for mitochondrial metabolism. A small percentage of iron in cells is unbound to ferritin, heme or iron sulphur clusters, and forms the labile iron pool. Hepatocytes are the central store of iron in the body, containing large quantities of ferritin, with the typical levels of iron stored in the liver of a healthy adult being around 1g.

Most of the iron in the body is being used in the bone marrow for erythropoiesis. When cellular iron levels are insufficient, iron regulatory proteins increase the translation of divalent metal transporter-1 (DMT-1) and transferrin receptor-1 (TfR1) to facilitate the uptake of iron from the blood into the cell (109, 113). Systemic iron regulation is controlled by the hepatic secretion of the hormone hepcidin and its action on FPN (98). Hepcidin production by the liver is acutely regulated by both the levels of hepatic iron stores and the saturation of transferrin in the blood, such that hepcidin expression decreases when levels of iron are low. This compensatory mechanism leads to an increase in gastrointestinal absorption, and the mobilisation of iron out of hepatocytes and splenic macrophages into the circulation. The bone marrow also exerts control

over hepcidin production in the liver, as an increase in erythropoietic activity suppresses hepcidin secretion by means of bone-marrow derived erythroferrone (114). This ensures that red blood cell production is accompanied by an appropriate increase in iron availability for the synthesis of haemoglobin, which is necessary for the effective transport of oxygen to the body. On the other hand, when circulating levels of iron are adequate, hepcidin production increases to bind and internalise FPN transporters, which effectively prevents the influx of additional iron from the sites of iron absorption into the blood stream (98). Importantly, it has been shown that inflammatory cytokines such as IL-6 may also directly induce hepcidin production, independently of iron availability (115).

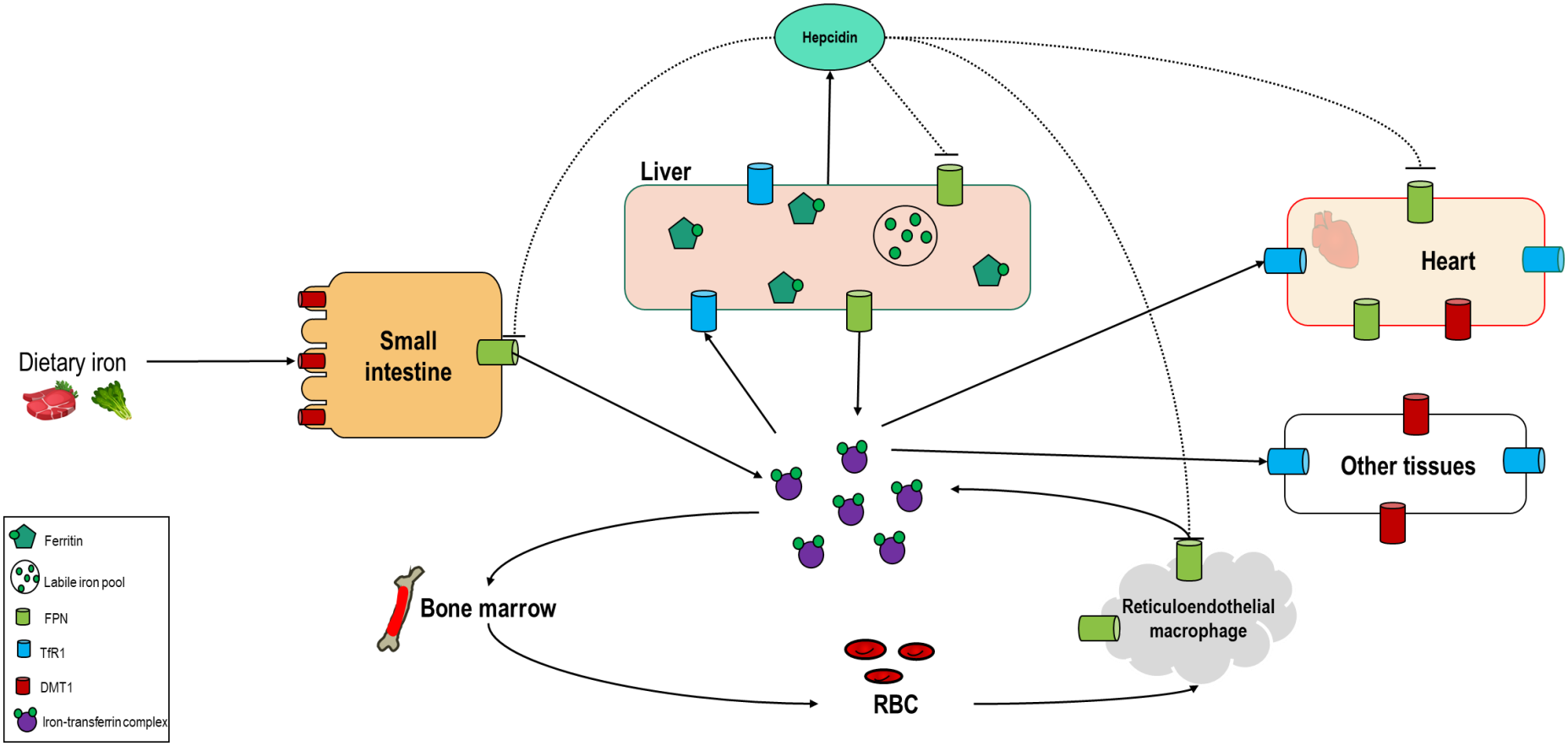


Figure 3.1. Summary of iron homeostasis in humans. Iron is necessary for the normal functioning of all cells in the body. Some of this iron is obtained through the absorption of non-haeme iron from the diet via DMT1 and FPN1 channels found on the surface of enterocytes. However, the main source of iron in the circulation comes from the recycling of senescent erythrocytes by reticuloendothelial macrophages. In the blood stream, iron is found complexed to transferrin and is distributed to cells throughout the body via TfR1 to be used in cellular metabolism. Most of the iron found in the body is used in the bone marrow to produce haemoglobin for new red blood cells. The liver is the main site of iron storage in the body, where iron is stored either bound as ferritin or unbound in labile iron pools. Systemic iron levels are regulated by the liver-derived hormone hepcidin, which acts through the inhibition of FPN expression, and is in turn regulated by iron availability. When iron stores are low, hepcidin levels decrease, allowing iron to leak out of the biological pools through the FPN transporters. On the other hand, when hepcidin levels are high (either in the presence of iron repletion or inflammation), iron export through these channels is inhibited, leading to retention of iron within cells and out of the circulation. ***RBC****: red blood cells,* ***FPN****: ferroportin,* ***TfR1****: transferrin receptor-1,* ***DMT1****: divalent metal-ion transporter-1*

3.4. Assessment of iron deficiency

Iron deficiency is a metabolic state that occurs when a disruption to one or more of the mechanisms that regulate cellular and systemic iron levels leads to a state of reduced iron availability. Iron deficiency is said to be 'absolute' when it results from depleted hepatic iron stores (for example due to poor dietary iron content, impaired iron uptake, blood loss or a combination of those), and is typically accompanied by a compensatory reduction in hepcidin levels to increase absorption and mobilisation from the biological pools. Alternatively, 'iron restriction' occurs either because of excessive erythropoietic activity (for instance in the case of haemolytic anaemias), or as a result of chronic inflammation when iron mobilisation is restricted due to raised levels of hepcidin.

Given the complexity of iron metabolism and homeostasis, iron deficiency is best diagnosed through an assessment of the complete iron profile of an individual, including iron storage, concentrations in the circulation, cellular availability and erythropoietic activity (116). However, since this may not always be feasible in clinical practice, iron deficiency is often diagnosed based on one or two biomarkers. The World Health Organisation considers ferritin the best indicator for measuring the effectiveness of iron trials in adults, as concentrations of this marker have been shown to correlate strongly with both total hepatic iron reserves and iron stores in bone marrow (117). Thus, in the absence of inflammation or chronic disease, iron deficiency is typically diagnosed in healthy non-pregnant adults at serum concentrations of ferritin <15-30 ng/mL (118-120). However, ferritin is an acute-phase protein whose concentrations can be increased in inflammatory states (including heart failure), which can make assessing the iron status of heart failure patients complex and uncertain. Consequently, higher cut-off values have been used for the definition of iron deficiency in patients diagnosed with heart failure. The European Society of Cardiology recommends defining iron deficiency in heart failure patients as having serum ferritin concentration <100 ng/mL, or 100–299 ng/mL with transferrin saturation <20% (17). Additionally, there are several physiological changes that happen during

pregnancy that may affect the ferritin thresholds used to define iron deficiency in pregnant women, including plasma volume expansion during the second trimester, and a physiological rise in inflammatory markers in the third trimester of pregnancy (121). To account for some of these changes, several studies have previously used a lower threshold of ferritin (12 ng/mL) to define iron deficiency in pregnant women. However, there is currently little evidence to justify the use of these lower cut-points (119).

Alternatively, soluble transferrin receptors (sTfR) may also be a useful indicator of tissue iron depletion in patients diagnosed with heart failure and in pregnant women (17). Transferrin receptors present in serum are largely derived from developing red blood cells, thus, their concentration is proportional to the number of red cell precursors, as well as their iron status. In the absence of erythropoietic disorders, serum concentration of sTfR rises when red blood cell production is restricted by the inadequate supply of iron (122). This biomarker of iron availability shows little intrapersonal variability, and unlike ferritin, its synthesis is not affected by the acute-phase effect. Thus, sTfR may be particularly useful for identifying cellular iron depletion in individuals experiencing inflammation. Recent studies have demonstrated that high serum concentration of sTfR can be useful for stratifying heart failure patients by their risk of dying, even beyond the typical prognostic variables (17). Likewise, previous studies have reported that elevated serum concentrations of sTfR has a sensitivity of 70–78% and a specificity of 46–100% for the detection of iron deficiency in pregnant women, as diagnosed by other iron indices (117). It has been suggested that the assessment of both ferritin and sTfR may better capture the full range of tissue iron depletion than either marker alone, especially when expressed as the ratio sTfR/ferritin (117). However, the use of sTfR for the diagnosis of iron deficiency is currently limited as it is not commonly available in clinical settings, and may be more costly than other iron tests. Additionally, since it is a relatively new marker, the measurements of sTfR across different assays has not yet been standardised (122). This presents a challenge to the interpretation of this biomarker, since normal reference values and diagnostic cut-points are likely to vary depending on the laboratory method used. Thus, the accurate assessment of iron

deficiency status among heart failure patients and pregnant women remains an area of active investigation.

3.5. Iron metabolism in pregnancy

Pregnant women are at increased risk of developing iron deficiency due to the increased demand for iron to cover their own basal iron needs, the development of the fetoplacental unit, as well as the increase in the maternal red blood cell mass (123). In the first trimester of pregnancy, iron requirements are lower than prior to pregnancy due to the lack of menstruation. Iron stores may even appear to increase slightly, although not enough to compensate for any pre-existing deficiency. Around the 16th week of pregnancy, maternal blood volume and red cell mass expand by about 20%, causing iron requirements to increase and iron stores to drop significantly during that time (124). As the pregnancy progresses, iron requirements for foetal growth rise steadily in proportion to the weight of the foetus, with more than 80% of the foetal iron uptake occurring after the 30th week of gestation (124, 125). Parturition also contributes to maternal iron depletion, as the loss of maternal blood during birth averages to the equivalent of 250 mg of iron, and a further 90 mg are lost with the placenta and umbilical cord (125). Maternal iron stores are expected to go back to normal in the puerperium period owing to the contraction of the expanded maternal red blood cell mass, the reduction in iron demand and lactational amenorrhoea. However, it has been shown that mothers may remain iron deficient for up to six months postpartum, especially if they are not supplemented with iron (126).

The total iron requirement for a 55-kg woman can be as high as 1,040 mg during pregnancy, with as much as 10 mg of iron needed to be absorbed per day in the last six to eight weeks of pregnancy alone (124, 125). These extra needs of pregnancy are met by adaptive changes to maternal iron metabolism, including the increased absorption of dietary iron in the intestines, and the mobilisation of iron from biological pools (125, 127). These adaptive changes are thought to be partly enabled by the suppression of hepcidin (127). However, even with an optimal diet rich in bioavailable iron, the amount of iron required for a healthy pregnancy is typically more than the average woman can achieve through dietary absorption alone (123, 125).

Hence, a mother should ideally have enough iron stores at the start of her pregnancy to ensure that she can meet these demands over the course of gestation. Yet, it is estimated that only about one-half of the women in high income countries have the necessary iron reserves to sustain a healthy pregnancy, and this proportion is even lower in low- and middle-income countries (124). Thus, by the middle of the second trimester, most women will have exhausted their iron reserves and many will develop anaemia over the course of their pregnancy secondary to severe iron-depletion. Without adequate supplementation to replenish their iron reserves, mothers can be at risk for developing various complications during pregnancy and the puerperium.

Iron deficiency during pregnancy has been consistently associated with increased risk of adverse pregnancy outcomes including low infant birthweight, pre-term birth, intrauterine growth restriction, as well as cognitive and behavioural impairments in infants and children (128, 129). The effect of iron deficiency on maternal health and outcomes during pregnancy are less studied. Some studies suggest that mothers suffering from iron deficiency anaemia during pregnancy are at risk of developing perinatal infections, pre-eclampsia and haemorrhage (130). However, few studies to date have investigated how iron deficiency can also affect cardiac functioning in the mother.

3.6. Pathophysiology of iron deficiency-induced cardiomyopathy

3.6.1. Iron deficiency anaemia

When iron deficiency is allowed to progress beyond subclinical depletion, iron deficiency anaemia may develop over time, as erythropoietic activity in the bone marrow becomes compromised by the insufficient supply of iron for haem production. Anaemia can also result from iron restriction as elevated levels of hepcidin prevent mobilisation of iron out of cellular stores for use in erythropoiesis (as observed in anaemia of chronic inflammation). Anaemia is an important comorbidity of heart failure occurring in approximately 30% of stable heart failure and 50% of hospitalized heart failure patients (131). In a study of 432 end-stage renal disease patients, it was shown that each 1 g/dL decrease in mean haemoglobin was independently

associated with a 28% increase in the risk of developing *de novo* heart failure, suggesting that anaemia may precede the development of cardiac dysfunction (132).

The mechanisms that lead to cardiomyocyte dysfunction as a consequence of anaemia are in part related to inadequate cellular oxygenation (i.e. hypoxia). Evidence from animal models of iron deficiency show that mice fed an iron deficient diet readily develop anaemia and exhibit increased expression of hypoxia inducible factors and regulation of HIF-target genes in the cardiomyocytes (133). The decrease in red blood cell count can lead to a reduction in systemic vascular resistance, and the reduction in haemoglobin levels may result in vasodilation. These systemic vascular changes can affect cardiac function either directly as the amount of oxygen available for metabolic use within the cardiomyocytes becomes limited (134), or indirectly as cardiac output is increased to maintain adequate oxygen delivery throughout the body. Over time the myocardium may become unable to withstand the sustained increase in workload, which leads to myocardial fatigue and eventually heart failure (106, 131).

3.6.2. Anaemia-independent pathways

Iron deficiency may nevertheless lead to the development of cardiomyopathy and incident heart failure, regardless of concomitant anaemia. In one study of patients with heart failure with preserved ejection fraction, 68% of patients with iron deficiency did not present with anaemia (135). In another study of patients with acute decompensated heart failure, 78.7% of women had iron deficiency without anaemia and 48.9% of those had absolute iron deficiency (136).

Like other cells in the body, cardiomyocytes meet their cellular iron demands primarily through the uptake of transferrin bound iron via TfR1 receptors. With very few exceptions, FPN transporters and hepcidin are primarily expressed in cells involved in systemic iron homeostasis (i.e. duodenal enterocytes, splenic macrophages and liver hepatocytes). Yet, recent evidence from animal models have shown that local iron regulation in cardiomyocyte is achieved through a cardiac hepcidin/FPN axis which acts semi-autonomously from the systemic hepcidin/FPN axis to protect the heart from systemic fluctuations in iron availability (98, 137). Although the precise

mechanism of the direct effects of iron insufficiency on cardiac function is poorly understood, several anaemia independent mechanisms that rely on this cardiac hepcidin/FPN axis have been demonstrated in experimental models. These mechanisms differ depending on the aetiology of the iron deficient state.

3.6.2.1. Absolute iron deficiency

In absolute iron deficiency, the global depletion of iron reserves may lead to alterations in cardiac structure and function due to local iron depletion at the cardiomyocyte level. Additionally, as hepcidin levels remain low in response to absolute iron deficiency, the expression of cardiac FPN transporters may paradoxically cause iron to leak out of cardiomyocytes (133). Both of these mechanisms would be associated with decreased concentrations of iron biomarkers in the cardiomyocytes of iron deficient heart failure patients. Indeed, evidence from human studies have found that not only are patients with heart failure more likely to have depleted iron stores than healthy controls (as measured by serum ferritin or sTfR), they also show significantly lower levels of myocardial ferritin and myocardial iron, even in the absence of anaemia (44-46). These results are supported by evidence from several experimental studies which show that animals fed iron deficient diets over time have significantly decreased levels of cardiac iron markers (including cardiac ferritin and cardiac iron), suggesting that cardiac iron homeostasis is affected in response to overall depleted iron stores (138-140). In every case, myocardial iron depletion was accompanied by significant alterations in cardiac structure as well disturbances in cardiac function (138, 139, 141). One study in particular found that mice fed an iron deficient diet over 3 weeks showed a clear decline in left ventricular function even before developing anaemia (140). Cardiac iron content was significantly decreased and was associated with severe alterations in cardiac structure. Overall, this evidence suggests that absolute iron deficiency can directly induce perturbations in cardiac function and morphology, via mechanisms of systemic iron insufficiency or through the direct effects of low hepcidin on heart cells, either of which would lead to myocardial iron depletion.

3.6.2.2. Iron restriction

Inflammation-induced iron restriction can also lead to a significant decrease in the systemic supply of iron as the increase in hepcidin concentrations restrict the supply of iron in the circulation. As in absolute iron deficiency, this may result in generalised cellular depletion of iron throughout the body, and lead to alterations in the structure and function of cardiac cells. A recent study of asymptomatic patients with chronic heart failure (NYHA functional class I) found that even in the absence of anaemia, study participants showed evidence of iron restriction characterised by elevated serum levels of hepcidin accompanied by high ferritin concentrations (54). Although no mechanism was suggested for this association, it is likely that the systemic restriction of iron that results from high levels of hepcidin may have led to cardiac iron depletion in these patients, ultimately resulting in the development of overt symptoms of heart failure. However, since the authors found no sign of significant inflammation in these patients, they hypothesised that this upregulation of hepcidin may have affected the myocardium directly (54). The current understanding of cardiac iron homeostasis suggests that an increase in hepcidin may directly cause cardiac FPN transporters to degrade, leading to iron retention in heart cells. Cardiac dysfunction may have then resulted from iron toxicity at the tissue level (133).

In uncomplicated pregnancies, maternal hepcidin levels are naturally reduced to accommodate the increased demand in iron (125, 127, 142). Importantly, hepcidin has been found to be elevated in pregnancies accompanied by inflammatory conditions (infections, pre-eclampsia or obesity) compared to healthy pregnancies (142, 143), and these conditions have previously been identified as specific risk factors for PPCM (22). However, it is still unclear precisely how and to what extent systemic hepcidin concentrations influence cardiac FPN expression. Thus, while biologically plausible, any direct effect of systemic hepcidin concentrations on the heart is likely to be secondary to the consequences of reduced iron availability in the body.

3.7. Formulation of the research questions

Figure 3.2. summarises the theoretical framework developed from the evidence summarised in this review. The framework illustrates how maternal iron deficiency can lead to PPCM through anaemia dependent pathways (resulting from inadequate oxygenation) or through anaemia independent pathways. In both absolute iron deficiency and inflammation-induced iron restriction, insufficient iron in the circulation can cause cardiac dysfunction due to inadequate supply of iron to the cardiomyocytes. Additionally, when hepcidin levels are low (as in absolute iron deficiency), cardiomyocyte iron depletion could also result from iron leaking out of heart cells due to the local effect of hepcidin on the expression of cardiac FPN transporters. Alternatively, an increase in hepcidin levels (as observed in iron restriction) may cause iron to accumulate inside cardiac cells, also leading to cardiac dysfunction. Based on this framework, the flowing research questions were formulated:

1. Is maternal iron deficiency associated with a greater risk of PPCM?
2. Does the effect of iron deficiency exacerbate the association between anaemia and PPCM?
3. What mechanism of iron deficiency (absolute iron deficiency or iron restriction) underlies this association?

Additionally, the association between iron deficiency and PPCM could be further characterised by assessing whether plasma concentrations of different markers of iron metabolism can be used to distinguish between Indian women with PPCM and those without PPCM. This assessment would not only provide insight on which element(s) of iron metabolism may be most relevant to the pathology of PPCM, but also help to determine if these biomarkers could be used in clinical practice to improve the diagnosis of PPCM.

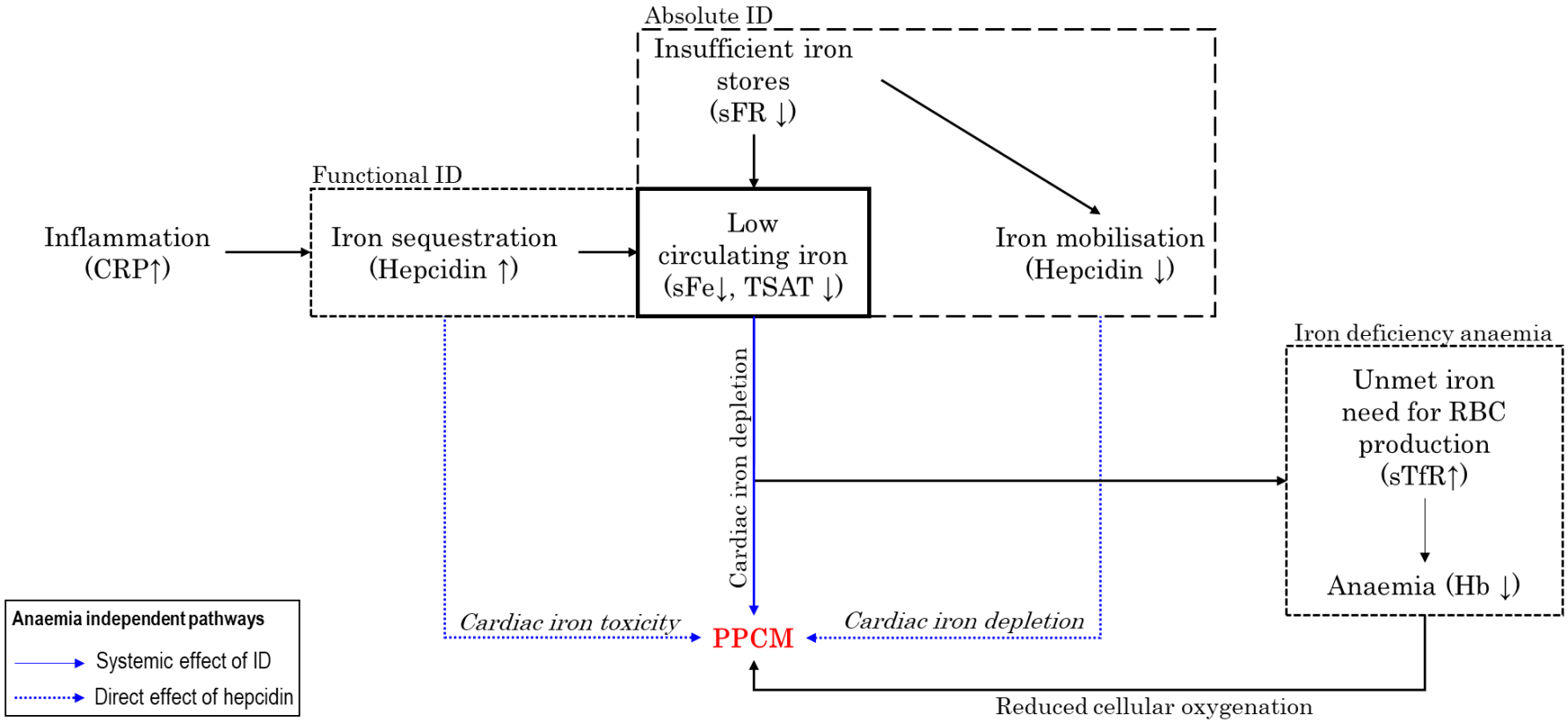


Figure 3.2. Conceptual framework for the association between iron deficiency and PPCM. ***ID:*** *iron deficiency,* ***sFR****: serum ferritin,* ***sFe****: serum iron,* ***TSAT****: transferrin saturation,* ***RBC****: red blood cell,* ***sTfR****: soluble transferrin receptors,* ***Hb****: haemoglobin,* ***CRP****: C-reactive protein.*

3.8. Chapter summary

Iron deficiency has been recognised as an important comorbidity in cardiovascular diseases, and emerging evidence from both animal and human studies is beginning to provide a clearer picture of the importance of iron homeostasis for myocardial function. Although the role of iron deficiency in heart failure is unknown, several possible mechanisms may underlie the association between iron deficiency and cardiomyopathy, including anaemia dependent and independent pathways. While pregnancy is known to significantly increase the risk of iron deficiency in the mother, it remains unclear if iron deficiency plays a role in the pathogenesis of PPCM. Based on evidence from the literature, research questions were formulated for the potential association between iron deficiency and PPCM, which will be investigated in the following chapters.

Chapter 4. A Case-Control study of Peripartum Cardiomyopathy in Northern India: *materials and methods*

4.1. Background & Scope

Between February 2019 and October 2021, a hospital-based unmatched case-control study of heart failure in pregnancy was conducted among women in the Northeast of India through the Maternal and perinatal Health Research collaboration, India (MaatHRI). This DPhil project used data from this study to investigate the research questions formulated in the previous chapter. This chapter describes the data source, case definition and inclusion criteria for the data analyses conducted for the DPhil project. The specific exposure and confounder variables, as well as the statistical analyses performed to address the thesis objectives will be reported in each of the corresponding subsequent chapters.

4.2. MaatHRI heart failure study

MaatHRI is a hospital-based collaborative research platform established in 2018. It is a collaboration between the University of Oxford and 15 public and private hospitals across five states in India (Assam, Meghalaya, Uttar Pradesh, Himachal Pradesh and Maharashtra). The platform is adapted from the UK Obstetric Surveillance System (UKOSS) and built on a pilot model (IndOSS-Assam) to conduct large epidemiological studies in maternal and perinatal health (144). Its goal is to improve maternal and infant health outcomes in resource-limited settings within India through equitable partnership and co-production of knowledge.

Four studies are being conducted through this hospital-based research platform, one of which is an unmatched case-control study to investigate the risk factors, clinical characteristics, and maternal outcomes of heart failure occurring during pregnancy or after childbirth. Twelve of the collaborating hospitals participated in the heart failure study **(**Error! Reference source not f ound.**).**

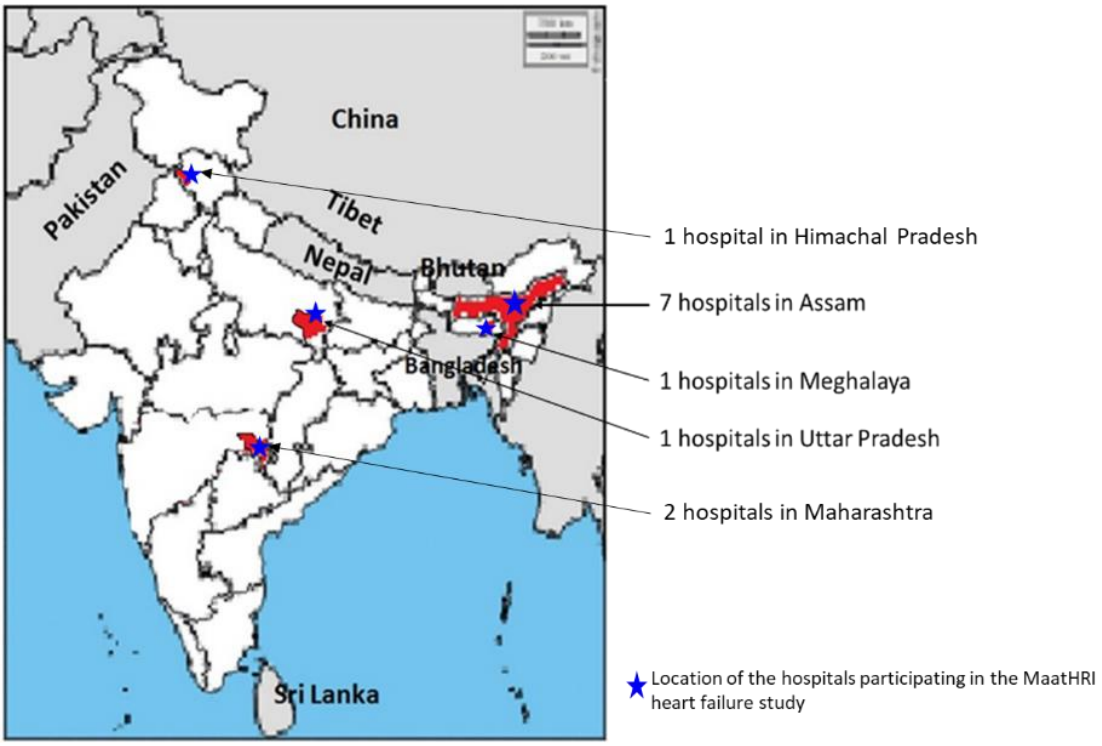


Figure 4.1. Regions in India where the MaatHRI heart failure study was conducted

4.2.1. Screening and enrolment

Figure 4.2. presents a flowchart of the study procedures for the MaatHRI heart failure study. Between February 2019 and October 2021, 188,970 hospital births were reported across the participating hospitals. Women presenting with heart failure at the obstetric or emergency department of collaborating hospitals at any point during pregnancy, childbirth, and up to 12 months postpartum, were approached to participate in the study. Heart failure was suspected if a pregnant or postpartum women presented with breathlessness (≥15 breaths per minute) with or without one or more of the following clinical signs: 1) elevated jugular venous pressure, 2) a cardiac murmur/gallop rhythm or 3) signs of pulmonary oedema (crackles in the lung/ pink frothy sputum). Women included in the study were 1) aged 18 years or above, 2) pregnant or within 1 year postpartum, and 3) willing to give informed consent to participate in the study. Participants were excluded if they were under 18 years old, or if they were unwilling to provide written informed consent. Controls were women of at least 18 years of age, who were not diagnosed with heart failure and who had given birth within two days of the case presenting at the hospital. Any woman who was willing and able to give informed consent for participating in

the study was included. Over the 30 months of enrolment, 298 pregnant and postpartum women suspected of having heart failure participated in the MaatHRI heart failure study.

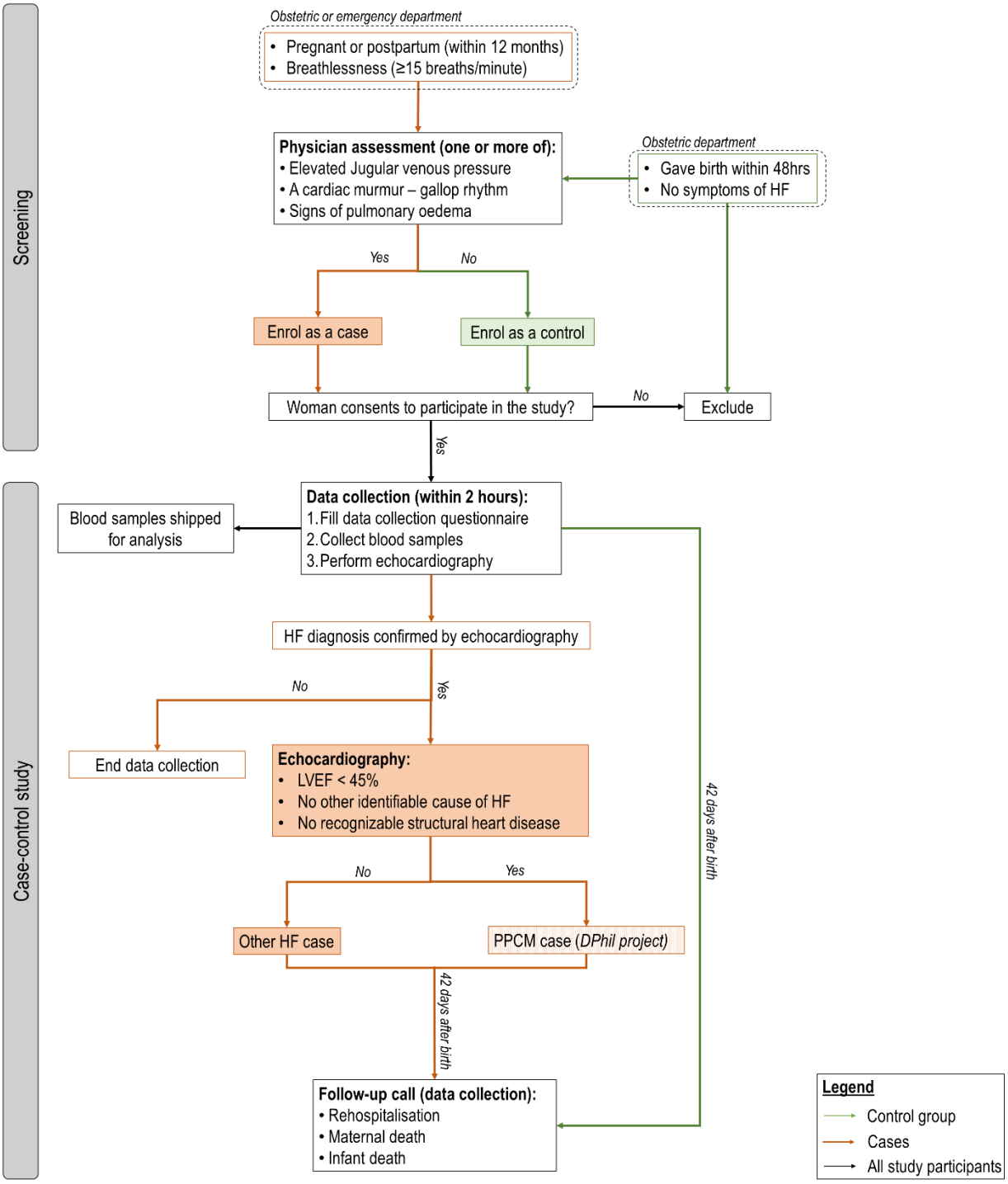


Figure 4.2. Flowchart of the procedures for the MaatHRI heart failure study. ***HF:*** *heart failure,* ***LVEF:*** *left ventricular ejection fraction*

4.2.2. Data Collection

Electronic questionnaires were filled by research nurses to collect baseline information from the study participants on sociodemographic, lifestyle and clinical factors. Information relating to the management and treatment of heart failure was also collected for pregnant or postpartum women with a confirmed heart failure diagnosis within 48 hours of enrolment into the study or before discharge.

4.2.3. Echocardiography images

Women who were successfully enrolled in the study had three standard views of their heart (parasternal long axis, parasternal short axis, and apical four chamber) captured by the attending obstetrician. The 2-Dimensional and Colour-flow images were obtained using a hand-held transthoracic echocardiography machine. Experts in cardiac imaging at the Cardiovascular Clinical Research Facility (CCRF) Core Lab at the University of Oxford and the Makunda Christian Leprosy and General Hospital in India independently interpreted the images to identify cardiac phenotypes. Any discrepancy in interpretation was resolved through consensus.

4.2.4. Biological samples

4.2.4.1. Collection and analysis

A research nurse collected 15 ml of blood from cases and controls immediately following enrolment into the study (for cases this was within 12 hours of admission to the hospital). Blood samples were centrifuged on site at 3700 revolutions per minute for 10 minutes using centrifuge machines of the same make and model in all study hospitals. Aliquots were also prepared from the supernatant plasma. A standard test requisition form was completed for each biological sample, which only included the participant iron deficiency, age and a barcode to ensure participant confidentiality and blinding of laboratory staff to minimise reporting bias.

All blood samples were shipped to and processed at the National Reference laboratory of Dr Lal PathLabs (the MaatHRI laboratory partner). Dr Lal PathLabs (LPL) is an accredited private laboratory with a pan-India network of regional laboratories and specimen collection centres (144). The blood tests, laboratory assays and systems used for the analyses conducted in the study population are reported in **Table 4.1.**

Table 4.1. Blood investigations for the MaatHRI heart failure study in pregnant women

Analyte (units)	Assay method	System used for the analysis
Cardio C-reactive protein (mg/L)	Immunoturbidimetry	ATELLICA
Creatinine (mg/dL)	Compensated Jaffe's reaction, IDMS traceable	Beckman Coulter AU 5800 series
Ferritin (ng/mL)	CLIA	Siemens ADVIA Centaur
Haemoglobin (g/dL)	Photometry	DxH -800 (Beckman coulter)
Haemoglobin electrophoresis	HPLC	Variant II Hemoglobin testing system (BIO-RAD)
Haematocrit (%)	RBC Pulse height detection	DxH -800 (Beckman coulter)
Hepcidin (ng/mL)	EIA	DRG International Inc
Iron (ug/dL)	Immunoturbidimetry	ATELLICA
Potassium (mEq/L)	Indirect ISE	Beckman Coulter AU 5800 series
Prolactin (ng/mL)	CLIA	ATELLICA
Sodium (mEq/L)	Indirect ISE	Beckman Coulter AU 5800 series
Soluble transferrin receptor (mg/L)	Nephelometry	BN II
Total Iron Binding Capacity (ug/dL)	Immunoturbidimetry	ATELLICA
TSH (uIU/mL)	CLIA	Siemens ADVIA Centaur
Urea (md/dL)	Urease UV	Beckman Coulter AU 5800 series
Vitamin B12 (pg/mL)	CLIA	Siemens ADVIA Centaur

IDMS: isotope dilution mass spectrometry, **CLIA**: chemiluminescence immunoassay, **HPLC**: high performance liquid chromatography, **RBC**: red blood cell, **EIA**: enzyme-linked immunosorbent assay, **ISE**: iron selective electrode, **UV**: kinetic ultraviolet

4.2.4.2. Assay standardisation and quality control

Two of the most important measures of an assay method's quality are traceability and measurement uncertainty. Traceability is an index of measurement consistency over space and time, which is obtained by linking the measurement results to common references (such as national or international standards) (145, 146). Establishing metrological traceability of laboratory results ensures that the results obtained from sample analysis can be compared between different laboratories or at different times (146).

The methods, traceability and performance characteristics of the assays used in the heart failure study were reviewed by experts at the University of Oxford's Wolfson laboratory and LPL before being included in the study. **Table 4.2.** shows the traceability references and performance characteristics for the assays used to analyse the markers measured in the heart failure study.

In the event that a blood sample was compromised in terms of quantity, stability, temperature or other quality indicator, the sample was discarded and the research nurse was advised to collect a fresh sample where possible.

Additionally, the LPL regularly runs quality control checks for each assay and monitors their mean coefficient of variation and standard deviation. This helped to standardise the laboratory procedures as well as ensuring quality control (144).

Table 4.2. Assay traceability and performance characteristics

Analyte (units)	Calibrator traceability reference	Laboratory reportable range	Normal reference range [a]	Biological variation (%)	Measurement uncertainty (%)
Cardio C-reactive protein (mg/L)	IRMM CRM-470	0.16 - 10 mg/L	<1.0	42.2	2.95
Creatinine (mg/dL)	NIST SRM 909b L2	0.1-25	0.51-0.95	5.95	9.0
Ferritin (ng/mL)	WHO 3rd International Standard 94/572	0.5-16500	10.00-291.00	14.2	22.5
Haematocrit (%)	Calculated	n/a	36.00-46.00	1.6	3.1
Haemoglobin (g/dL)	1:250 dilution in NCCLS2 recommended reagent for the hemiglobincyanide cyanmethemoglobin	1-25	11.50-15.00	2.5	4.9
Haemoglobin electrophoresis	NGSP Certification for A2/F	HbF-1.3-99.8% HbA2-1.6-18.7 %	HbF- <1.5 % HbA2-1.5-3.5 %	HbF-5.6 % HbA2-4.4 %	HbF-10.97 % HbA2-8.62 %
Hepcidin (ng/mL)	Internal standard	0.153-81 ng/ml	0.25-47.66 ng/ml	NA	NA
Iron (ug/dL)	NIST SRM 937	2 - 1000 µg/dl	50.00-170.00	26.5	1.25
Potassium (mEq/L)	NIST SRM 2201	2.5-7	3.50-5.10	4.6	3.5
Prolactin (ng/mL,)	WHO 3rd International Standard for human Prolactin (IRP 84/500)	0.3 - 1000 NG/ML	9.70-208.50	6.9	7.3
Sodium (mEq/L)	NIST SRM 2202	110-160	136.00-146.00	0.6	2.94
Soluble transferrin receptor (mg/L)	ERM®- DA470k/IFCC	0.14 - 4.4 mg/L	0.76-1.76	NA	5.6
Total Iron Binding Capacity (ug/dL)	Internal standard	40 - 670 µg/dl	250.00-425.00	NA	1.88
TSH (uIU/mL)	WHO 3rd International Standard for human TSH (IRP 81/565)	0.008-750	0.550-4.780	19.3	12.3
Urea (md/dL)	NIST SRM 909b L1	5-400	17.00-43.00	12.1	9.0
Vitamin B12 (pg/mL)	Internal standard manufactured using USP material	<45, >2000	211.00-911.00	14	17.0

[a]In adult women not pregnant

IRMM-CRM: Institute for Reference Materials and Measurements Certified Reference Material, **NIST SRM**: National Institute of Standards and Technology Standard Reference Materials, **WHO**: World Health Organization, **NCCLS2**: National Committee for Clinical Laboratory Standards 2, **HbF**: haemoglobin F, **HbA2**: haemoglobin A2, **NGSP**: National Glycohemoglobin Standardization Program, **IRP**: International Reference Preparation, **ERM**: European Reference Materials, **IFCC**: International Federation of Clinical Chemistry and Laboratory Medicine, **TSH**: thyroid stimulating hormone, **USP**: United States Pharmacopeia, **NA**: not applicable

4.3. Ethical Approval

The MaatHRI platform and the associated observational studies have been approved by the Oxford Tropical Research Ethics Committee (OxTREC), University of Oxford, UK (OxTREC Ref: 7-18), and the Indian government's Health Ministry's Screening Committee, the Indian Council of Medical Research, New Delhi (ID number 2018-0152).

Additionally, the platform and studies have also received approval from the institutional review boards (IRB) of each coordinating Indian institution, namely: Srimanta Sankaradeva University of Health Sciences, Guwahati, Assam (No.MC/190/2007/Pt-1/126); Nazareth hospital, Shillong, Meghalaya (Ref No. NH/CMO/IEC/COMMUNICATIONS/18-01); Emmanuel Hospital Association, New Delhi (Ref.Protocol No.167); Mahatma Gandhi Institute of Medical Sciences, Sevagram, Maharashtra (Ref No. MGIMS/IEC/OBGY/118/2017); and the Institute of Medical Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh (No.Dean/2018/EC/290).

4.4. Impact of the COVID-19 pandemic

Enrolment into the heart failure study began in February 2019. In the first year of recruitment, the enrolment rate for the heart failure study was at about 18 cases per month, such that about 200 women with heart failure had been enrolled in the study in that time. The COVID-19 pandemic affected recruitment for the heart failure study in all participating hospitals from March 2020 until January 2021. During that time, hospital births in the region had decreased by more than 35% and access to national laboratories was prohibited by national restrictions (147). Additionally, the overall case fatality of severe maternal complications had increased significantly during the pandemic. Specifically, the case fatality for heart failure among pregnant and postpartum women doubled from 29% to 58%, which further challenged enrolment into the study. Overall, the MaatHRI heart failure study experienced a 50% decrease in enrolment across all study hospitals over the course of the pandemic.

4.5. DPhil data

4.5.1. Study population

The DPhil study population included all heart failure cases from the MaatHRI study who met the criteria for PPCM as defined by the European Society of Cardiology (ESC) (65). The echocardiographic images included in the MaatHRI heart failure study helped to reliably exclude women with pre-existing cardiac pathology such as rheumatic heart disease or congenital heart disease, which further improved case ascertainment. Among the cases of heart failure enrolled in the MaatHRI study, 44 women (15%) met the criteria for PPCM diagnosis_(65). As a comparison, previous studies of PPCM conducted in India have included sample sizes of 9-56 cases of PPCM (36). Two women diagnosed with PPCM were enrolled in the study before the 28th week of pregnancy **(Figure 4.3).** About half of the cases were enrolled within the first three days after childbirth, and the majority (67%) were enrolled within two weeks postpartum.

The final study population of the DPhil project also comprised 170 postpartum controls without heart failure. Of the 170 controls enrolled in the study, approximately 40% were enrolled on the day of childbirth. The remainder were enrolled within 48 hours following childbirth.

4.5.2. Variables analysed

This DPhil project analysed the data collected on the demographic, lifestyle, pregnancy, clinical and biological characteristics of the study population, as described above. **Figure 4.3.** Distribution of the PPCM cases by gestational or postpartum period at enrolment.

Table 4.3**.** provides a summary of the variables included in the analyses that will follow**.** A more in depth description of the variables and their use in the analyses will be provided as they are analysed in the corresponding chapters.

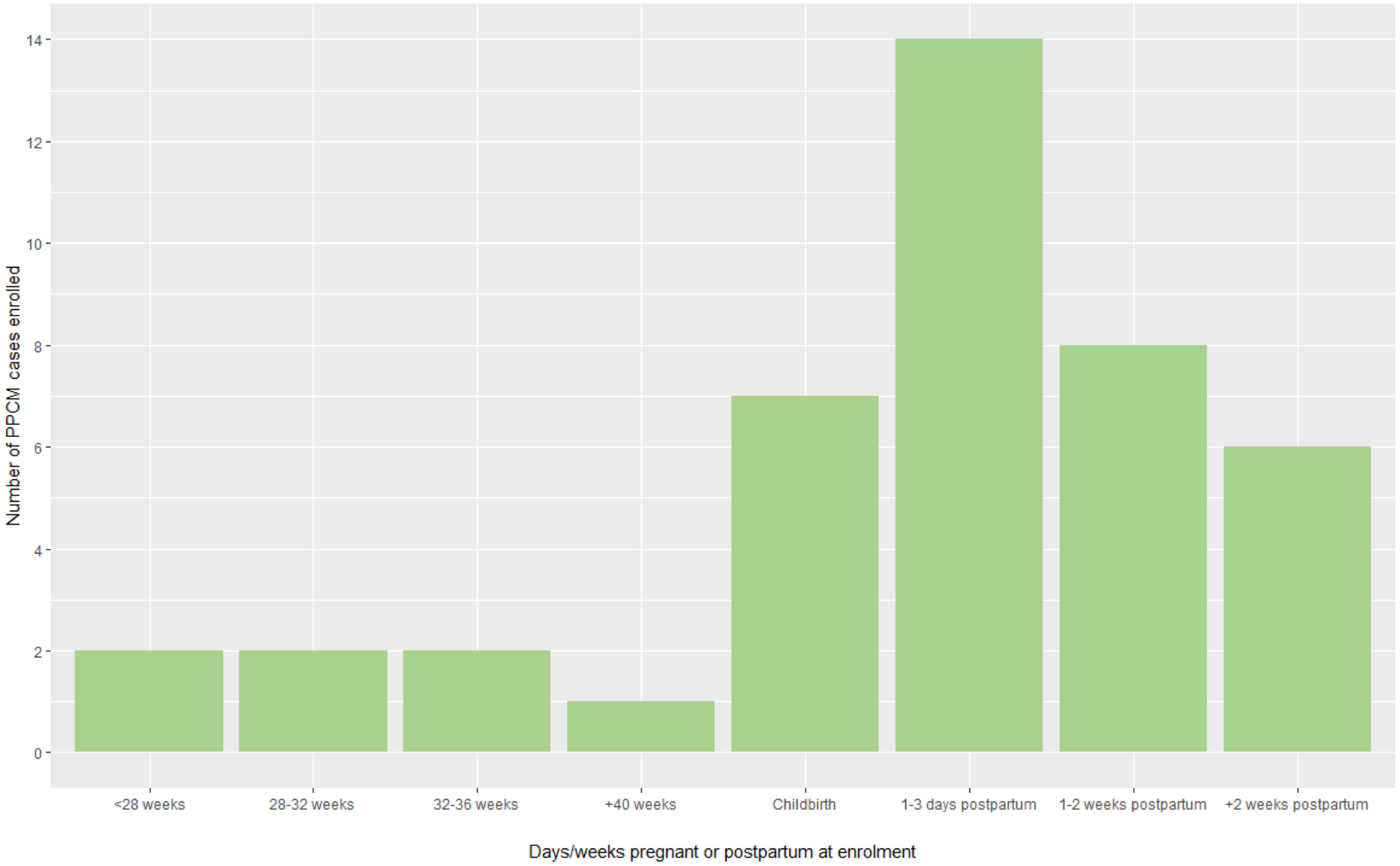


Figure 4.3. Distribution of the PPCM cases by gestational or postpartum period at enrolment.

Table 4.3. Population variables investigated in the DPhil project

Demographic & lifestyle factors	ANC & Clinical factors	Heart failure features	Pregnancy characteristics & outcomes	Biomarkers
• Maternal age • Education • Poverty • Residence • Alcohol use • Tobacco use • Religion • Betel-nut chewing • Fermented fish consumption	• Number of ANC visits • Start and duration of IFA supplementation • Hypertension/hypertensive disorders of pregnancy[a] • BMI • Diabetes[b] • Anaemia • Blood transfusion	• Gestational stage at HF onset • Gestational age/Postpartum days at onset • Systolic & diastolic blood pressure • NYHA classification • Echocardiographic features	• Parity • Multifetal pregnancy • Mode of birth • Blood loss • Infant birthweight • Pre-term birth • Stillbirth • Miscarriage • Maternal death • Infant death	• sFR • sFe • TSAT • sTfR • sTfR-F index • hepcidin • hs-CRP • Hb • MCV • MCH • Hb types[c] • RBC[d] • Prolactin

ANC: antenatal care, **IFA**: iron and folic acid, **BMI**: body mass index, **HF**: heart failure, **NYHA**: New York heart association, **sFR**: serum ferritin, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F index** = sTfR/log(sFR), **hs-CRP**: high sensitivity C-reactive protein, **Hb**: haemoglobin, **MCV**: mean corpuscular volume, **MCH**: mean corpuscular haemoglobin, **RBC**: red blood cell

[a]Including gestational hypertension, pre-eclampsia and eclampsia

[b]Including pre-existing and gestational diabetes

[c]Including HbA, HbA2, HbA2+E, and other non-specific Hb types

[d]Including red blood cell count, red cell distribution width, and haematocrit

4.6. Chapter summary

The quantitative analysis for the DPhil project was undertaken using data collected from an unmatched case-control study of heart failure among pregnant and postpartum Indian women, through the MaatHRI platform. The study population for the main analysis of this thesis included 44 women diagnosed with PPCM according to the ESC guidelines, and 170 postpartum women without heart failure. While small, this sample size is similar to previous studies of PPCM among Indian women. The following chapter will elucidate the epidemiological profile of PPCM in this population of Indian women.

Chapter 5. The epidemiological profile of Peripartum Cardiomyopathy among Indian women

5.1. Background and scope

Although peripartum cardiomyopathy (PPCM) has been recognised as an important complication of pregnancy and the postpartum (27), much of the epidemiology of the disease is still unclear. The reported incidence of the disease varies widely with geographical regions, ethnic characteristics of the population and diagnostic criteria used. The highest reported incidence estimates have been in Nigeria (one in 100) (148) and Haiti (one in 300) (149), while values as low as one in 15,533 births have also been observed in Japan (150). Suggested risk factors for PPCM include older maternal age, gravidity, parity, multifetal pregnancies, and hypertensive disorders of pregnancy. Population-based data on PPCM are rare due in part to difficulties in diagnosis and a lack of awareness about the disease. Currently, most of the available data comes from studies in high-income western countries such as the United States and various European countries (26, 52, 151). Several studies have also been conducted in African countries, mostly in Nigeria and South Africa (18, 152). Investigations of PPCM in Asian populations remain comparatively scarce. A recent pooled analysis found only seven studies of PPCM which have been conducted among Indian women in the past 20 years (36), demonstrating the need for more investigation of PPCM in this large, heterogeneous population.

The goal of this chapter was to contribute to the current knowledge of the epidemiology of PPCM in India. The objectives were to describe the sociodemographic, behavioural, obstetric and clinical characteristics of PPCM, and identify factors that are associated with the condition in a population of Indian women.

5.2. Methods

5.2.1. Study population

This analysis used the data collected from the MaatHRI case-control study of heart failure in pregnant and postpartum Indian women, as described in Chapter 4. Briefly, cases were 44 pregnant and postpartum women with a confirmed PPCM diagnosis based on the diagnostic

criteria of the European Society of Cardiology (ESC) (65). Controls were a random sample of 170 postpartum women without heart failure who had given birth within two days of the case presenting at the hospital.

5.2.2. Study variables

The analysis included an investigation of population characteristics potentially associated with PPCM in this study population. These were sociodemographic and behavioural variables, heart failure presentation, obstetric factors and medical conditions. See **Appendix Table A7** for a detailed description of the variables and how they were derived.

5.2.2.1. Sociodemographic and behavioural factors

Suggested sociodemographic and behavioural risk factors for PPCM (148, 153) were considered as potential covariates and included: maternal age, education, poverty status, residence, alcohol use, smoking, and tobacco use. Additionally, religion, betel-nut chewing and fermented fish consumption were also included to capture risk factors specific to the study population.

Religion: The majority of India's population is Hindu, however, Muslims, Sikhs, and Christians are also represented in significant numbers in the country. Religion in India is tightly linked with socioeconomic status. For instance, the highest prevalence of rural poverty are reported among Muslims (27%) and Hindus (26%), while Christians (22%) and Sikhs (6%) have some of the lowest rates in the country(154, 155). Additionally, religious beliefs can also significantly affect behavioural factors related to health outcomes, such as lifestyle and dietary restrictions. Previous studies in Indian populations have found differences in the prevalence and nature of health outcomes among individuals belonging to the different religious groups. For instance, Christians have been reported to have higher rates of chronic diseases such as heart disease, diabetes and hypertension, compared to Hindus(154). Presumably this may be linked to the more meat based diets of Christian Indians compared to the vegetarianism more widely observed among those of the Hindu faith(156). Consequently, it was expected that

religion might be an important determinant of health in this study population and was therefore included as a potential cofactor of PPCM.

Betel nut: India has been reported to have the largest betel nut (or *areca*) consuming population in the world (157). Chewing betel nut has been classified as a confirmed carcinogen, being robustly associated with increased risk of oral and oesophageal cancers(158). However, recent studies are now also suggesting a potential association between betel nut use and risk of cardiovascular disease (CVD), including Coronary Artery Disease and Ischemic Heart Diease(159). Consequently, this lifestyle factor was assessed as a potential determinant of PPCM in the current analysis.

Fermented fish: The dietary culture of the north-eastern regions of India (where this study population originates) differs vastly from that of observed in other parts of the country. This region has a food culture that is particularly characterised by the consumption of fish products which are fermented through traditional methods of the region (160, 161). However, fermented fish is a food product known to contain an enzyme which has anti-thiamine activity, especially when consumed without heat treatment (162). Consequently, eating fermented fish might be a potential indicator for the risk of thiamine deficiency in this study population. Thiamine deficiency can affect cardiovascular functioning, leading to the condition called 'wet beriberi', but it has also been shown to contribute to many other CVD (163). Therefore, this unique dietary practice was considered a relevant covariate for this study population.

5.2.2.2. Heart failure presentation

The clinical features of heart failure in women with PPCM were assessed to characterise the presentation of the disease in the study population. Echocardiographic investigations were undertaken as part of the larger MaatHRI heart failure study (see Chapter 4), therefore, there was a unique opportunity to describe the echocardiographic features of the PPCM cases in this study population. The echocardiographic features were described in terms of left ventricle end-diastolic diameter (LVEDD), left ventricular ejection fraction (LVEF), abnormal left or right ventricular

wall motion, ventricular and atrial enlargements, the presence of valvular regurgitation or stenosis, evidence of pericardial effusion, and tachycardia. The New York Heart Association (NYHA) classification of the heart failure symptoms and the gestational or postpartum period at the onset of heart failure were also reported.

5.2.2.3. Obstetric factors and pregnancy outcomes

Characteristics of the index pregnancy were described in terms of the number of antenatal care (ANC) visits during the index pregnancy, the body mass index (BMI) at the first clinical visit, as well as the duration of iron and folic acid (IFA) supplementation during the pregnancy. The mother's parity and the number of multifetal pregnancies was also reported. Parity was defined as the number of times a woman had given birth to a fetus with a gestational age of 28 weeks or more.

The number of miscarriages in the study population was reported, and the following pregnancy outcomes among the women who gave birth were described: mode of birth, amount of blood lost at birth (heavy bleeding defined as having lost more than 500 mL of blood during vaginal birth or more than 1000 mL of blood during caesarean section (164)), infant birthweight, preterm birth (gestational age at birth < 37 weeks), stillbirth, miscarriage (defined as a pregnancy loss at <28 weeks of gestation), maternal death and infant death.

5.2.2.3. Medical conditions

The medical conditions investigated in this chapter included essential hypertension, hypertensive disorders of pregnancy (including gestational hypertension and pre-eclampsia/eclampsia), diabetes mellitus (pre-existing and gestational) and whether the woman had received a blood transfusion as a treatment for severe antenatal anaemia in the index pregnancy. The association between antenatal anaemia and PPCM will be investigated separately in Chapter 7.

5.3. Statistical Analyses

5.3.1. Descriptive analyses

The characteristics of the study population were summarised using medians and interquartile ranges (IQR) for continuous variables, and proportions for categorical variables. Women diagnosed with PPCM were compared to women without PPCM using Pearson's chi-square tests for categorical variables and Wilcoxon rank-sum tests for continuous variables to account for non-normal distributions. For categorical variables with small cell sizes (i.e. fewer than five observations), a Fisher's exact test was performed instead to compare the proportions between the two groups.

5.3.2. Factors associated with PPCM

Univariable logistic regression models were fitted to examine the association between each explanatory variable and PPCM in the study population. Where appropriate, variables were tested for deviations from linearity by fitting first-degree fractional polynomials. All continuous variables showed a linear relationship with PPCM, and consequently were included without transformation in the regression models.

A multivariable regression model for PPCM was constructed by including risk factors that were significantly associated with PPCM during univariable analysis. The model sequentially adjusted for each category of explanatory variables, starting distal followed by intermediate then proximal factors. The order in which the variables were added was based on the theoretical association between the different risk factors and PPCM illustrated in **Figure 5.** 1**.** Thus, the model first adjusted for the sociodemographic factors, followed by the behavioural, then obstetric factors, and finally the medical conditions. At each step, factors that were not significantly associated with PPCM (Wald $p>0.05$) were dropped from the model, such that each subsequent multivariable model only adjusted for variables that were significantly associated with PPCM in the previous model. Thus, the final model included all significant socioeconomic, behavioural, obstetric and medical determinants of PPCM in the population.

Due to the small sample size of this study, the number of parameters included in the adjusted logistic regression models was an important factor to consider. Including too many factors in a model could cause issues with overfitting, unreliable confidence intervals, and model convergence (165). Recent simulation studies have shown that logistic regression models with at least five outcome events per predictor variable are able to yield adequate confidence interval coverage, minimal type I error and relatively little bias (166, 167). This ratio is less stringent than the 1:10 or 1:20 rule of thumb often used in practice, which may not always be feasible for the analysis of small data sets such as the one used in these analyses. Hence, at any point in the analyses, multivariable models included no more than one explanatory variable for every five outcome event.

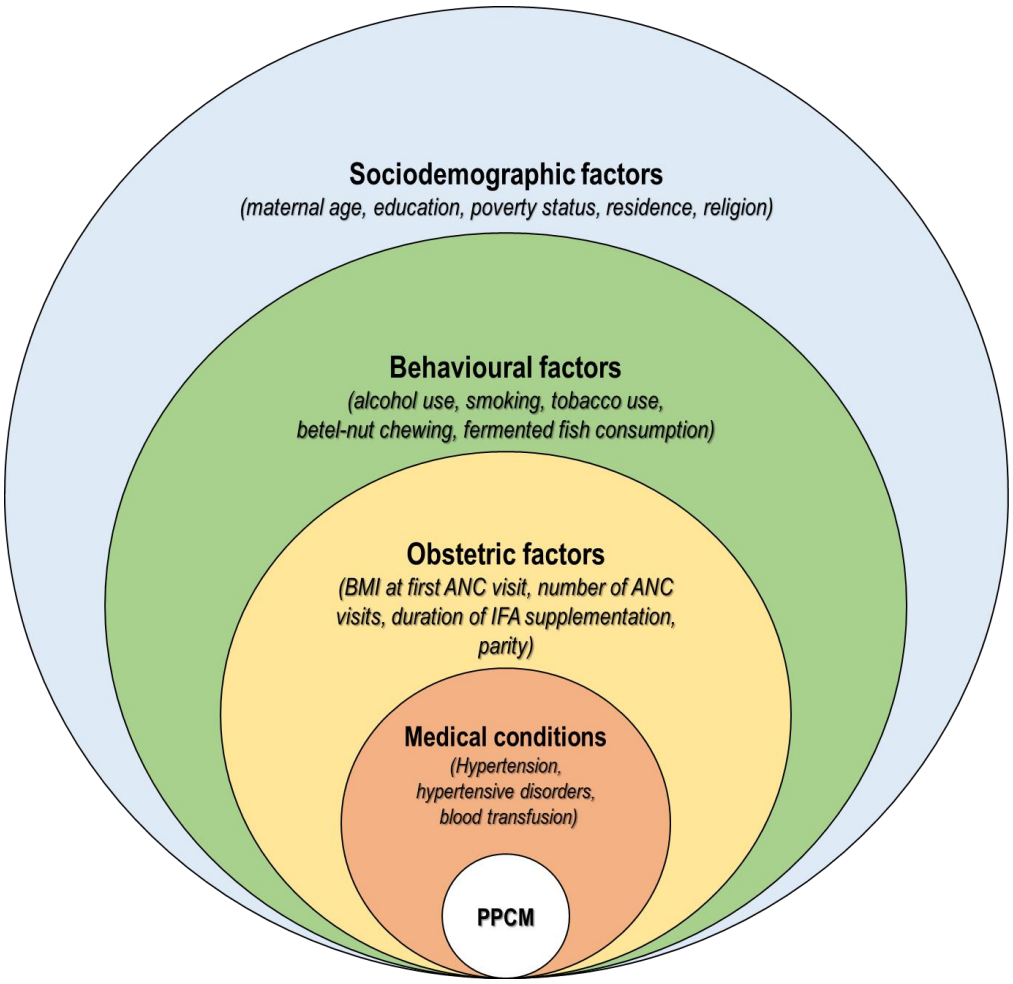


Figure 5. 1. Schema of the hypothesised relationship between the risk factors for PPCM. ***BMI:*** *body mass index,* ***ANC:*** *antenatal care,* ***IFA:*** *iron and folic acid.*

5.3.3. Sensitivity analyses

5.3.3.1. PPCM cases with postnatal onset

Because the cases in this study were enrolled between late pregnancy (late second or third trimester) and several months after childbirth, certain factors measured in the study could only be considered risk factors for mothers who had an onset of disease after having given birth. These included: duration of gestation, mode of birth and postpartum blood loss. Therefore, these factors were assessed as potential risk factors for PPCM in a subset of the cases whose onset of heart failure occurred in the postnatal period.

5.3.3.2. Missing data

Data is said to be missing at random (MAR) when there is no systematic relationship between the probability of an observation being missing and the missing value itself (168). If there is a relationship between the propensity of an observation to be missing and its value, the data is said to be missing not at random (MNAR) (169, 170). Data that is MNAR may not be easily estimated with statistical methods, as the missing data mechanism itself has to be identified and modelled. Although several tests have been proposed to determine if data is MAR or MNAR, these tests are not widely used, and their practical value is uncertain (171). The only meaningful way of distinguishing between MNAR and MAR data is to use substantive knowledge of the data collection process.

In this study, missing data on the questionnaire assessments were most likely to be missing due to incomplete interviews or non-availability of information in the hospital records. An interview may not have been completed because of the severity of the patient's illness or a data entry error. Since the risk factors were not likely to have been associated with incomplete interviews or non-availability of information, the missing data was assumed to be MAR. A sensitivity analysis was carried out where the missing observations were estimated using multiple imputation with chained equations, and the analyses were repeated with a complete dataset.

5.4. Results

5.4.1. Sociodemographic and behavioural characteristics of women with PPCM

The population incidence of PPCM in this population was 1 in 4,317 hospital births. PPCM cases represented 15% of all heart failure cases enrolled in the study. The women diagnosed with PPCM were less likely than healthy postpartum women to have a secondary school education (p<0.001), and more likely to have a rural residence (p=0.035) **(Table 5.1).** None of the women with PPCM reported using alcohol during the pregnancy. Additionally, there were no smokers in this study population and tobacco use was uncommon during pregnancy (1 case and 1 healthy control). Fifty-seven percent of the women diagnosed with PPCM reported chewing betel-nut during pregnancy compared with 39% of the controls (p=0.038). In this population, there was no statistically significant association between PPCM and maternal age, poverty status, religion, or the consumption of fermented fish.

Table 5.1. Distribution of the sociodemographic and behavioural characteristics of the study population by PPCM status

	Confirmed Cases (N = 44)	**Controls (N = 170)**	**p-value[a]**
	Median (IQR)		
Maternal age (year)	23.5 (20.0, 28.5)	24.0 (21.0, 27.0)	0.6
	Frequency (%)		
Secondary school education or higher			**<0.001**
Yes	24 (55)	147 (86)	
No	20 (45)	23 (14)	
Poverty status			0.14
Certified BPL	33 (75)	112 (66)	
Not BPL	8 (18)	51 (30)	
Missing	3 (7)	7 (4)	
Religion			0.084
Hindu	24 (55)	116 (68)	
Muslim	15 (34)	47 (28)	
Other	5 (11)	7 (4.1)	
Residence			**0.035**
Rural	40 (91)	130 (76)	
Urban/Suburban	4 (9.1)	40 (24)	
Chewed betel nut			**0.038**
Never/before pregnancy	19 (43)	103 (61)	
During pregnancy	25 (57)	67 (39)	

Consumes fermented fish			0.4
Yes	38 (86)	137 (81)	
No	6 (14)	33 (19)	

IQR: interquartile range, **BPL**: below poverty line

[a]Wilcoxon rank sum test for the difference in medians and Chi-square or Fisher's exact tests (for variables containing cells <5 observations) for the difference in proportions

5.4.2. Heart failure presentation in women with PPCM

While the majority of the women diagnosed with PPCM were enrolled within the first two weeks after birth, over half reported an onset of heart failure symptoms during pregnancy. The mean gestational age of antenatal presentation was at 34 ±7 weeks**.** In the postpartum period, the median onset of symptoms was 10 days after childbirth. Two women had an onset of symptoms in the second trimester (at 20 and 24 weeks of gestation), and one woman was diagnosed with PPCM seven months after childbirth.

The majority (57%) of cases reported severe symptoms of heart failure (NYHA class IV) and 23% had severe left ventricular dysfunction (i.e. LVEF < 30%) **(Table 5.2).** Most cases (66%) also showed reduced LVEDD, and about half of the women had pericardial effusion.

Table 5.2. Heart failure and echocardiographic features of PPCM cases (N=44)

	Frequency (%)
Onset of heart failure	
Antenatal	24 (55)
Postnatal	19 (45)
NYHA class	
I	1 (2.3)
II	9 (21)
III	9 (21)
IV	25 (57)
LVEDD (cm)	
≤5.3	29 (66)
>5.3	15 (34)
LVEF (%)	
<30	10 (23)
30-44	34 (77)
Left ventricular enlargement	
Yes	16 (36)
No	28 (64)
Right ventricular enlargement	
Yes	2 (5)
No	41 (93)
Missing	1 (2)

Left atrial enlargement	
Yes	12 (27)
No	32 (73)
Right atrial enlargement	
Yes	2 (5)
No	42 (95)
Valvular regurgitation	
Yes	16 (39)
No	25 (61)
Pericardial effusion	
Yes	21 (48)
No	23 (52)
Tachycardia	
Yes	10 (23)
No	34 (77)

NYHA: New York heart association, **LVEDD**: left ventricular end diastolic diameter, **LEVF**: left ventricular fraction

[a]Chi-square or Fisher's exact test for the difference in proportions.

5.4.3. Obstetric factors and pregnancy outcomes of women with PPCM

A greater proportion of women diagnosed with PPCM had had fewer than three ANC visits during the index pregnancy, compared to women who did not have PPCM (p<0.001) **(Table 5.3)**. Women with PPCM had a lower median BMI at their first ANC visit than postpartum controls (*p*=0.037). PPCM cases also reported taking IFA supplementation for a median of 60 days, which was half as long as the median duration of supplementation among the control group (*p*<0.001). The majority of cases (64%) and controls (66%) were primiparous. All the women in the control group had singleton pregnancy, but three twin pregnancies were reported among the cases.

Table 5.3. Distribution of obstetric factors in the study population by PPCM status

	Confirmed Cases (N = 44)	**Controls (N = 170)**	**p-value[a]**
	Median (IQR)		
BMI at first ANC (kg/m2)	20.9 (18.3, 22.4)	21.6 (20.0, 23.8)	**0.037**
N missing	5	4	
Duration of IFA supplementation (days)	60 (30, 120)	120 (90 ,150)	**<0.001**
	Frequency (%)		
Number of ANC visits			**<0.001**
Fewer than 3	15 (34)	14 (8)	
3 or more	24 (55)	153 (90)	
Missing	5 (11)	3 (2)	
Parity			0.9
Primiparous	28 (64)	110 (65)	
Multiparous	16 (36)	60 (35)	

IQR: interquartile range, **BMI:** body mass index, **ANC**: antenatal care, **IFA**: iron and folic acid
[a]Wilcoxon rank sum test for the difference in medians and Pearson's Chi-square tests for differences in proportions.

There were seven miscarriages among the women diagnosed with PPCM. From the eight stillbirths reported in the study population, six were to women who were not diagnosed with PPCM. Thirty-five women with PPCM had a livebirth at the time of enrolment in the study. Among all women who had given birth, caesarean sections was more common in women with a PPCM diagnosis than in those with no heart failure (p<0.001) **(Table 5.4).** No women in the study experienced heavy bleeding during birth, and the amount of blood lost during parturition did not significantly differ between the two groups. Mothers diagnosed with PPCM had a higher frequency of low birth weight babies than did mothers without heart failure (p<0.001). There were no infant deaths reported in the study population, but the maternal case fatality was 20%.

Table 5.4. Outcomes of the pregnancies among women who had given birth by PPCM status

	Confirmed Cases (N = 35)	**Controls (N = 170)**	**p-value[a]**
	Median (IQR)		
Postpartum blood loss (mL)[c]	265 (150, 388)	200 (200, 260)	0.14
N missing	13	0	
	Frequency (%)		
Method of birth			**0.001**
Caesarean section	11 (31)	19 (11)	
Vaginal	22 (63)	151 (89)	
Missing	2 (6)	0 (0)	
Preterm birth[d]			0.11
Yes	10 (29)	29 (17)	
No	25 (71)	141 (83)	
Low birthweight[e]			**0.002**
Yes	15 (48)	40 (24)	
No	14 (45)	130 (76)	
Missing	6 (17)	0 (0)	

IQR: interquartile range
[a]Wilcoxon rank sum test for the difference in medians or Fisher's exact test for the difference in proportions
[b]Women who had given birth at the time of enrolment in the study
[c]Values reported are: mean (standard deviation)
[d]Defined as a livebirth at < 37 weeks' of gestation
[e]Defined as birthweight < 2500 grams

5.4.4. Medical conditions in women with PPCM

There were no reports of diabetes or obesity among the women diagnosed with PPCM in this study population. However, these women had a significantly higher frequency of hypertension and hypertensive disorders of pregnancy than the postpartum controls without heart failure (p<0.001). Seven women with PPCM were diagnosed with pre-eclampsia or eclampsia in the index pregnancy, compared with just one postpartum woman without heart failure. Additionally, women diagnosed with PPCM reported having received a blood transfusion as a treatment for severe antenatal anaemia more frequently than did women without heart failure (p=0.006).

5.4.5. Factors associated with PPCM

5.4.5.1. Sociodemographic and behavioural factors

In univariable analyses, PPCM was significantly associated with having lower than secondary school education (p<0.001), having a rural residence (p=0.023), and chewing betel nut during pregnancy (p=0.038). However, maternal age, poverty, religion, and fermented fish consumption were not significant risk factors for PPCM in this study population.

A mother's level of education was the only sociodemographic factor that remained significantly associated with PPCM in the multivariable model. After adjusting for betel-nut use, women with less than secondary school education were 3.43 times more likely to be diagnosed with PPCM **(Table 5.5).** Neither rural residence nor betel nut use during pregnancy were significant behavioural risk factors for PPCM after adjusting for a mother's level of education.

Table 5.5. The association between PPCM and sociodemographic and behavioural factors

Sociodemographic factors		
	Unadjusted OR (95% CI)	*Adjusted OR (95% CI)†*
Maternal age (years)	0.99 (0.92, 1.06)	--
Secondary school education		
Yes	1 (ref)	1 (ref)
No	**5.33 (2.55, 11.2)**	**3.43 (1.36, 8.48)**
Poverty status		
Not BPL	1 (ref)	--
Certified BPL	1.88 (0.84, 4.63)	--
Religion		
Hindu	1 (ref)	--

Muslim	1.54 (0.73, 3.18)	--
Other	3.45 (0.95, 11.8)	--
Residence		
Urban/Suburban	1 (ref)	1 (ref)
Rural	**3.08 (1.15, 10.7)**	2.14 (0.69, 8.25)
Behavioural factors		
	Unadjusted OR (95% CI)	*Adjusted OR (95% CI)‡*
Chews betel nut		
Never/before pregnancy	1 (ref)	1 (ref)
During pregnancy	**2.02 (1.04, 4.00)**	1.26 (0.54, 2.90)
Consumes fermented fish		
No	1 (ref)	--
Yes	1.53 (0.63, 4.28)	--

OR=Odds Ratio, **CI**=Confidence Interval, **BPL**: below poverty line
†Multivariable logistic regression model adjusting for sociodemographic factors that were significantly associated with PPCM in the univariable analyses.
‡Multivariable logistic regression model adjusting for education level

5.4.5.2. Obstetric factors

Neither maternal BMI at the first antenatal visit, nor parity were significant risk factors for PPCM in this study population. While the number of ANC visits was associated with PPCM in the univariable model (OR=6.83, 95% CI: 2.94, 16.1), this variable was no longer a significant risk factor for PPCM after adjustment for education level and duration of IFA supplementation. However, the duration of IFA supplementation during the index pregnancy was significantly associated with PPCM. After full adjustment, the odds of disease decreased by 8% with every additional week of IFA supplementation (OR=0.92, 95% CI: 0.87, 0.97) **(Table 5.6).**

Table 5.6. The association between PPCM and obstetric factors

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
BMI at first ANC (kg/m^2)	0.91 (0.81, 1.01)	--
Duration of IFA supplementation (days)[a]	**0.90 (0.85, 0.94)**	**0.92 (0.87, 0.97)**
Parity		
Primiparous	1 (ref)	--
Multiparous	1.05 (0.52, 2.07)	--
Number of ANC visits		
3 or more	1 (ref)	1 (ref)
Fewer than 3	**6.83 (2.94, 16.1)**	2.62 (0.95, 7.03)

OR=Odds Ratio, **CI**=Confidence Interval, **BMI:** body mass index, **ANC:** antenatal care, **IFA**: iron and folic acid
[a]For every 7 days of IFA supplementation
†Multivariable logistic regression, adjusting for education level and the other significant obstetric factor(s)

5.4.5.3. Medical conditions

In unadjusted models, PPCM was significantly associated with hypertension or hypertensive disorders of pregnancy (OR=11.2, 95% CI: 4.63, 28.8), and having received a blood transfusion as a treatment for severe antenatal anaemia in the index pregnancy (OR=4.11, 95% CI: 1.53, 11.0) **(Table 5.7).** After adjusting for education level and the duration of IFA supplementation, being diagnosed with hypertension or a hypertensive disorder was associated with nearly nine times the odds of PPCM in the multivariable model (OR=8.69, 95% CI: 3.28, 24.3). However, having received a blood transfusion as a treatment for severe anaemia was no longer a significant risk factor for PPCM in the multivariable model.

The final model included education level (OR= 3.85, 95% CI: 1.62, 9.14), duration of IFA supplementation (OR=0.92, 95% CI: 0.88, 0.97) and hypertension/hypertensive disorders in the index pregnancy (OR=8.75, 95% CI: 3.32, 24.3) as independent risk factors for PPCM in this population.

Table 5.7. The association between PPCM and medical conditions

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
Severe anaemia treated with blood transfusion		
No	1 (ref)	1 (ref)
Yes	**4.11 (1.53, 11.0)**	2.76 (0.86, 8.78)
Hypertensive disorders[a]		
No	1 (ref)	1 (ref)
Yes	**11.2 (4.63, 28.8)**	**8.69 (3.28, 24.3)**

OR=Odds Ratio, **CI**=Confidence Interval
†Multivariable logistic regression, adjusting for education level and duration of IFA supplementation
[a]Including essential hypertension and hypertensive disorders of pregnancy (gestational hypertension, pre-eclampsia and eclampsia)

5.4.6. Sensitivity analyses

In the sensitivity analysis conducted among women who were diagnosed with PPCM after having given birth, there was no association observed between the amount of blood loss at childbirth and developing PPCM in the postpartum period (adjusted OR=1.00, 95% CI: 1.00, 1.01) **(Appendix Table A8).** Likewise, neither the duration of gestation (OR=1.02, 95% CI: 0.89, 1.13)

nor birth by caesarean section (OR=0.99, 95% CI: 0.15, 3.87) were significantly associated with a higher risk of PPCM.

The results of the regression analyses using the imputed data were not materially different from the complete case analyses presented here (**Appendix Table A9, Table A10,** and **Table A11**).

5.5. Discussion

In this population of pregnant and postpartum women in India, PPCM was a rare complication of pregnancy with a 20% case fatality. Most women diagnosed with PPCM reported severe symptoms of heart failure (NYHA class IV), which developed during the third trimester of pregnancy. Echocardiographic investigations showed that about a quarter of these cases had severe left ventricular dysfunction (LVEF < 30%). In this study population, maternal age, BMI, poverty, religion, parity, fermented fish consumption and the number of ANC visits were not associated with PPCM. Instead, significant risk factors for PPCM included the mother's level of education, the duration of IFA supplementation during pregnancy, and having hypertension or a hypertensive disorder in the index pregnancy.

The precise incidence of PPCM in India is still unknown, but is generally reported to be between one in 1,300 and 1,500 live births (36, 38, 172). In the present study, the incidence of PPCM was about one in 4,317 hospital births; the prevalence among live births is likely to be higher. While a rare disease, PPCM diagnoses made up 15% of cases of heart failure observed in this population of pregnant and postpartum Indian women. This is twice the proportion that has been previously reported among Indians in the literature (13). This discrepancy may be due in part to increased awareness and targeted screening for PPCM in this study compared to previous investigations of heart disease among Indian women.

Risk factors previously suggested for PPCM include factors common to all cardiovascular diseases (such as older age, hypertension, smoking, diabetes and obesity) (173), but also others more specific to the pregnancy state including multi-parity, multifetal pregnancies and pre-

eclampsia (174). In the current study, there were no reports of smoking or diabetes, and maternal age did not differ between women diagnosed with PPCM and controls without heart failure. Although it has previously been suggested that maternal age over 25 years is a risk factor for the development of PPCM (175), the median age of women diagnosed with PPCM in this population was 23.5 years. Results from other studies of PPCM in Indian populations have also observed that Indian mothers diagnosed with PPCM were younger than PPCM patients from other countries (36). However, this may simply reflect the differences in the age of structure of the pregnant population of high income versus lower-and-middle income countries (5). Indeed, similar results to those observed in the present study have been reported by prospective studies conducted in Haiti and South Africa (153, 176), which generally conflict with evidence from high income countries such as the United States (21, 49, 177).

Likewise, women in low income countries are generally reported to have higher fertility rates than women from high income countries. While the fertility rate in India has been declining in recent years, at the time of this study the country's fertility rate was reported to be 2.2 compared to the average 1.5 reported in high income countries(178). Since PPCM has previously been associated with multiparity, this was expected to be a strong determinant of the disease in the present population. Yet surprisingly, 66% of the cases in this study population were primiparous, and no significant effect of parity was observed on the risk of PPCM in the current study. Similar results were reported in other studies of PPCM in India, which found that 56% of Indian women diagnosed with PPCM were primigravida (36). On the other hand, the EURObservational Research Programme which enrolled PPCM patients from different European countries found that only 18.6% of PPCM patients enrolled were primiparous (151).

Similarly, while obesity (BMI≥30 kg/m^2) has been suggested as a predisposing factor for PPCM in studies of high income populations (179, 180), this risk factor was uncommon in this study population and not observed at all among the women with PPCM. Instead, having a low BMI may be associated with an increased odds of the disease, although the results from the present study were not statistically significant.

Consistent with evidence from previous studies among Indian women, hypertension and hypertensive disorders were significantly associated with PPCM in this study population (36). In particular, pre-eclampsia/eclampsia was observed in 15% of women diagnosed with PPCM. PPCM has consistently been associated with hypertensive disorders, but also specifically with disorders of pregnancy such as pre-eclampsia and pregnancy-induced hypertension (32, 52). A meta-analysis of 22 studies including 979 cases of PPCM reported a pooled prevalence of pre-eclampsia of 22% (95% CI: 16% to 28%), which is more than four times the 5% average rate of pre-eclampsia in pregnancy reported globally (32). It has been suggested that PPCM and pre-eclampsia share a common underlying pathophysiologic mechanism possibly related to antiangiogenic factors such as soluble fms-like tyrosine kinase-1 (sFLT1), which are secreted by the placenta during pregnancy (88, 181). However, since systolic left ventricular function is usually preserved or even improved in pregnant women with hypertension and pre-eclampsia (148, 182), hypertensive disorders of pregnancy are generally considered strongly associated conditions but not aetiologic determinants of PPCM (182).

In this population, lower maternal education was found to be a significant risk factor for PPCM, consistent with evidence from previous investigations (153). Education is commonly used as a measure of socioeconomic status (SES) along with wealth, and SES has long been identified as an important risk factor for cardiovascular diseases (183). This is typically attributed not only to access to adequate healthcare and other health related resources, but also because economic status is robustly correlated with behaviours and lifestyle choices that impact cardiovascular health (such as smoking, drug and alcohol use, exercise, or diet quality) (184). It is well accepted that education is an essential determinant of socioeconomic status, especially for women in low-income countries (185). Generally, the more literate and educated a women is, the better her socioeconomic prospects, and consequently, the better her chances of having a healthy lifestyle. However, the findings of this study showed that maternal education rather than poverty status alone was a significant determinant of PPCM. Previous studies in low- and middle-income countries have also shown that low educational was a stronger and more consistent determinant

of cardiovascular disease and mortality in these populations than wealth (186). This suggest that education may specifically capture factors beyond financial capital that relate to knowledge and understanding of health, disease and treatment. In this population, women diagnosed with PPCM were more likely to have less than a secondary school education, which has previously been associated with low health literacy (187, 188). Health literacy has been linked to several lifestyle and behavioural risk factors for cardiovascular disease, including the differential use of health care services (188), as well as poorer quality of primary and secondary prevention received for cardiovascular diseases (186). Thus, the association observed between lesser education and PPCM in this population could be mediated not only by the effects of wealth, but also by the degree to which a mother is able to access and process health information and services, and thus participate in decisions that would influence her likelihood of developing PPCM.

Finally, this analysis found that every additional week of IFA supplementation was associated with an 8% reduction in the risk of PPCM. The National Nutritional Anaemia Prophylaxis Programme in India recommends daily IFA supplementation for a minimum of 100 days, starting after 12 weeks of pregnancy to prevent the development of birth defects, low birth weight, and prematurity in the neonate, as well as iron deficiency anaemia and puerperal sepsis in mothers (189, 190). Although PPCM has previously been associated with some nutritional deficiencies, namely selenium, zinc and vitamin C (see Chapter 2), this is the first time that a potential association with iron has been suggested. As discussed in Chapter 3, anaemia secondary to iron deficiency may lead to cardiac dysfunction through pathways relating to hypoxia but also cardiac iron depletion. This association will be further explored in the following chapters.

Overall, the findings of this study suggest that there are significant differences in the features of PPCM depending on the socio-economic profile of the country and the study population. This suggests potential differences in the mechanisms underlying PPCM in European high income countries compared to lower income populations. This is an important point that needs to be taken into account when evaluating other suggested risk factors for PPCM, and

emphasises the need for more epidemiological studies of this disease across different populations.

5.6. Strengths and Limitations

This study suffered from some limitations. First, the results are limited by the small sample size of PPCM cases which reduces the power to observe statistically significant associations. Nevertheless, this is one of the few and largest epidemiological investigation of PPCM in an Indian population to date. As such, this study provides valuable insights into the features of this disease in a poorly studied population, which is important given that the profile of PPCM is known to vary wildly by geographic region. Another limitation of the study comes from its retrospective and observational nature. Certain variables such as the onset of heart failure symptoms, duration of IFA supplementation or the number of antenatal visits were self-reported, which makes these measurements susceptible to both recall and participant bias. This bias is most likely to lead to an overestimation of the true duration of supplementation or number of antenatal visits, which would appear to dampen the true association. Additionally, the exact date of blood transfusion, which could affect the outcome of interest, was not recorded and therefore the temporality of the effect of this risk factor cannot be established. These limitations notwithstanding, the design of the study ensured a robust collection of objective data from clinical examinations, laboratory measurements and echocardiographic investigations.

5.7. Chapter summary

This chapter investigated the demographic, lifestyle, pregnancy and clinical characteristics of a population of Indian women diagnosed with PPCM. Notably, it was observed that women with PPCM reported taking IFA supplementation for a shorter duration during their pregnancy compared to women without heart failure. Along with education level and having a hypertensive disorder, the duration of IFA supplementation in the index pregnancy was a significant determinant of PPCM in this population. The results of this chapter hint at a potential role of iron in PPCM, which will be further characterised in the next chapter.

Chapter 6. The iron profile of Indian women diagnosed with Peripartum Cardiomyopathy

6.1. Background and Scope

In the previous chapter, it was demonstrated that the duration of iron and folic acid (IFA) supplementation during pregnancy was independently associated with PPCM in a population of Indian women. While PPCM has previously been associated with other nutritional deficiencies, this was the first suggestion of a possible role of iron in the pathology of this disease. Iron deficiency is a common comorbidity observed in heart failure patients, which is associated with poorer prognosis (17). Yet, the systematic review conducted in Chapter 2 revealed that the iron profile of women diagnosed with PPCM has not been characterised, and consequently, the association between iron deficiency and PPCM remains unknown. The conceptual framework formulated from animal and human studies in chapter 3 demonstrated how iron deficiency itself may directly contribute to the development of cardiomyopathy (138, 139, 141).

The current chapter aimed to determine if PPCM is associated with iron deficiency in a population of Indian women, and if so what mechanism of iron deficiency (absolute or functional) underlies this association (see Chapter 3: research questions #1 & #3). The primary objectives were to: 1) characterise the iron profile of women diagnosed with PPCM, and 2) assess the association between iron deficiency and PPCM. Additionally, this chapter also investigated the association between iron status and heart failure severity in women with PPCM, and assessed which marker of iron metabolism might be most useful for identifying PPCM in this study population.

6.2. Methods

6.2.1. Study population

The analysis was conducted using data from the MaatHRI case-control study of heart failure in pregnant and postpartum Indian women, as described in Chapter 4. The final study population comprised 44 confirmed case of PPCM and 170 postpartum controls without heart failure enrolled within two days of the case presenting at the hospital.

6.2.2. Laboratory investigations

The blood investigations included in the analyses of this chapter were a panel of five iron markers that included: serum concentrations of ferritin (sFR), iron (sFe), transferrin saturation (TSAT), soluble transferrin receptors (sTfR) and hepcidin. Additionally, since inflammation can directly influence the levels of several iron markers, the measurements of high-sensitivity C-reactive protein (hs-CRP) were also analysed as an indicator of the level of inflammation that the participants were experiencing. All blood samples were collected non-fasted at the time of enrolment in the study. See Chapter 4 for details on the assays and instruments used to analyse these biomarkers.

6.2.3. Study variables

6.2.3.3. Iron deficiency

As mentioned previously (see Chapter 3), the latest guidelines from the European Society of Cardiology (ESC) recommends defining iron deficiency in heart failure patients as a sFR concentration <100 ng/mL, or 100–299 ng/mL with TSAT <20% (17). In the absence of inflammation or chronic disease, iron deficiency is typically diagnosed in healthy non-pregnant adults at serum concentrations of ferritin <15-30 ng/mL (118-120). Therefore, to accurately characterise iron deficiency in the current population, iron deficiency was defined as sFR < 30 ug/L in women without heart failure (control group), and the ESC definition was used for the group of women diagnosed with PPCM (cases).

When detailed iron studies are not possible, microcytic-hypochromic anaemia is also often used as an indicator of possible iron deficiency in clinical practice. Microcytic-hypochromic anaemia is typically diagnosed when anaemia is accompanied by a mean corpuscular volume (MCV) <80 fL (191) and a mean corpuscular haemoglobin (MCH) < 27 pg (192, 193). For this analysis, microcytic-hypochromic anaemia was considered as an alternative definition for iron deficiency in the analysis, with anaemia defined as having haemoglobin levels < 10 g/dL.

6.2.3.1. Iron metabolism markers

Maternal iron status was further characterised by analysing serum concentration of the other four iron biomarkers. sFe and TSAT were used as estimates of the amount of iron circulating

in the blood stream, which is available for cellular intake. sTfR and the sTfR-F index were used as estimates of intracellular and erythropoietic iron demand. The sTfR index was calculated from sTfR and sFR concentrations using the following formula developed by Cook et al. (194, 195): $\frac{sTfR}{\log_{10}(sFR)}$. Finally, levels of hepcidin were used as a measure of the body's regulation of iron absorption and mobilisation from biological pools.

6.2.3.4. Heart failure severity

The severity of heart failure among women diagnosed with PPCM was defined by the New York Heart Association (NYHA) class, left ventricular ejection fraction (LVEF) and survival status of the mother. NYHA class was categorised as classes I, II, III or IV, and LVEF was dichotomised as <30% or 30-44%.

6.3 Statistical analyses

6.3.1. Descriptive analyses

The prevalence of iron deficiency based on the definitions described above was estimated and compared between the two study groups using Pearson's Chi-square tests for differences in proportions. Additionally, the serum concentration of each iron biomarker was described using medians and interquartile ranges. The values were then compared between women with PPCM and women without heart failure using Wilcoxon rank sum tests, to account for non-normal distributions. The distribution of the biomarkers across the gestational/postpartum periods at enrolment is also presented in **Appendix Figure A1-Figure A6.**

6.3.2. Main effect of iron status on PPCM

A directed acyclic graph (DAG) was developed to identify potential confounders of the association between iron deficiency and PPCM **(Figure 6.1.)**. Variables that were significantly associated with PPCM in the multivariable analyses conducted in Chapter 5 were considered potential risk factors for PPCM in this analysis. These were education level, duration of iron and folic acid (IFA) supplementation during pregnancy, and hypertensive disorders. Risk factors for iron deficiency were identified from literature searches and clinical knowledge, and included education level, maternal age, body mass index (BMI), poverty, postpartum blood loss, iron

supplementation, parity and duration of gestation (196-199). Education level and iron supplementation were common risk factors for both PPCM and iron deficiency. However, since the effect of iron supplementation on PPCM was likely to be mainly through the development of iron deficiency, iron supplementation was not considered to be a true confounder of the association between iron deficiency and PPCM.

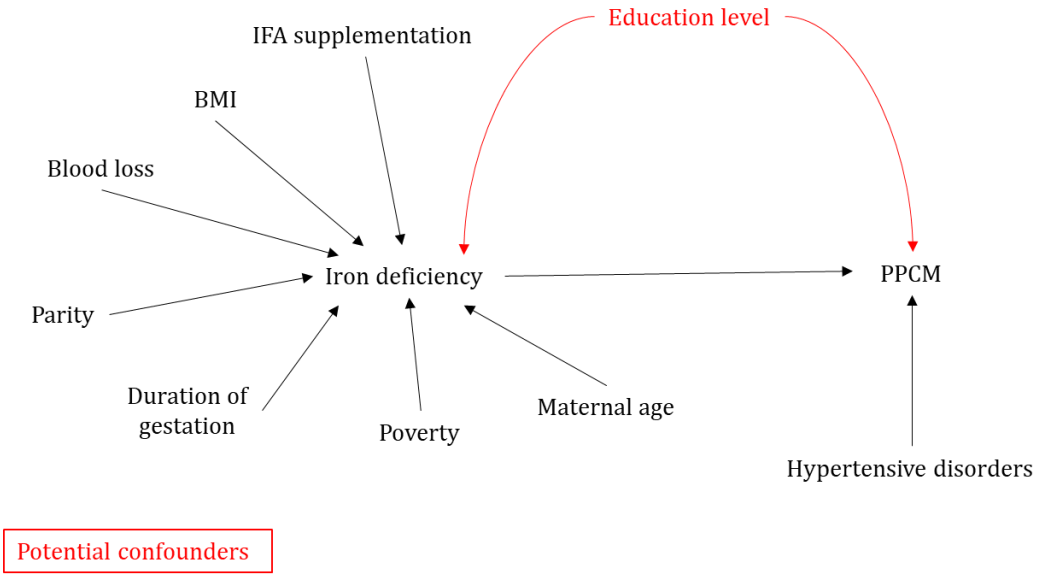


Figure 6.1. Directed acyclic graph for the association between iron deficiencyand PPCM. Risk factors associated with iron deficiency and PPCM. In red are factors that are associated with both, and were included in the regression models as potential confounders of the association between iron deficiency and PPCM. ***BMI**: body mass index, **IFA:** iron and folic acid.*

The association between iron deficiency and PPCM was measured in separate unadjusted and adjusted logistic regression models for each of the definitions of iron deficiency mentioned above. The multivariable regression models were adjusted for the effect of education as a potential confounder of the exposure-outcome association.

The shape of the association between PPCM and each iron marker was assessed and represented graphically using first-degree fractional polynomial models. The final polynomial model was chosen iteratively using a closed-test procedure wherein the best fitting first-degree fractional polynomial model was compared against the linear model using a chi-square test for difference in deviance, with three degrees of freedom. If the test was not significant at a pre-

specified alpha value of 0.05, the linear model was chosen over the first degree transformation (200).

The association between the iron indices and PPCM was then assessed in separate logistic regression models for each iron marker. Serum concentrations of the iron biomarkers were treated as continuous variables in their respective regression models. Serum iron and TSAT demonstrated significantly non-linear associations with PPCM **(see Appendix Figure A7 and Figure A8),** and therefore were included in their respective model as first degree polynomials: sFe^{-1} and $TSAT^{-2}$.

Since serum biomarker concentrations of zero are not biologically plausible, and given their skewed distribution, the main effect of the iron markers were presented as a unit change from the median. Since the effect sizes were very small, the associations with sFR and sFe were presented as the effects associated with a 10 unit decrease from the median concentration of sFR (35.5 ug/mL) and sFe (60 ug/dL), respectively, for ease of interpretation . The association with TSAT was expressed as the effect of a unit decrease from the median (13.8%), the effect size for hepcidin was presented for a unit decrease from 11.78 ng/mL, and the results for sTfR and sTfR-F index were presented as a unit increase from 1.67 mg/L and 1.1, respectively.

6.3.3. Correction for inflammation

It has been shown that levels of sFR and hepcidin in the blood can be increased by the effects of inflammation (115). Likewise, the protein transferrin is a negative acute phase protein whose concentrations in the serum are known to decrease in the presence of inflammation, which may translate to higher TSAT values than expected in individuals experiencing high inflammation (201, 202). To account for this effect, the concentration values of these biomarkers were corrected for inflammation using a linear regression adjustment for CRP on a continuous scale, based on the following equations (203, 204):

$$\ln(marker)_{adjusted} = \ln(marker)_{crude} - \beta_1\left(lnCRP_{obs} - lnCRP_{ref}\right)$$

, where β1 is the linear regression coefficient for CRP with the marker as the outcome variable. The resulting correction was only applied to participants with observed CRP above the internal reference cut off for inflammation (CRP_{ref} = 5 mg/L) (204) to prevent overcorrection for low levels of inflammation.

6.3.4. Secondary analyses

6.3.4.1. Association with heart failure severity

A secondary analysis was conducted among the women diagnosed with PPCM to determine whether serum concentrations of the iron markers were also associated with the severity of heart failure. The median and interquartile range of the iron markers were compared in women with PPCM across NYHA class, LVEF group and maternal death status using Wilcoxon rank sum tests for differences in medians.

6.3.4.2. Classification accuracy of iron markers

The classification accuracy of a biomarker is its ability to distinguish between individuals in a population who have the outcome of interest and those who do not. The receiver operating characteristic (ROC) curve is a visual representation of the true positive and false positive rates associated with classification rules that are based on a range of values of the biomarker. The area under the ROC curve (AUC^{ROC}) provides an aggregated measure of the accuracy of the biomarker across all possible classification thresholds. The AUC ranges from 0 to 1, and is interpreted as the probability that a randomly chosen woman with PPCM is correctly rated as being more likely to have the disease than a randomly chosen woman without PPCM, based on her value of the biomarker (205).

A secondary analysis was conducted to compare the classification accuracy of the different iron markers, and determine which one was most useful in distinguishing women diagnosed with PPCM from those without PPCM. To account for the non-normal distribution of the iron markers, non-parametric ROC curves were generated for each marker model (206). Although education level was a potential confounder of the association between iron status and PPCM in this study population, the classification accuracy of the markers was not expected to vary by education status. Thus, it was unnecessary to adjust the ROC curve for education in this

analysis (207). Hence, the crude AUC^{ROC} (AROC) and 95% confidence intervals were estimated for each of the ROC curves (206).

The Youden index is a summary statistic of the ROC curve that is typically used to generate a clinically meaningful classification criterion using a biomarker. The Youden index is a function of sensitivity and specificity: $(\frac{sensitivity}{100} + \frac{specificity}{100}) - 1$, and represents the maximum vertical distance between the ROC curve and the diagonal chance line (208, 209). The optimal cut-point for a biomarker is the threshold value at which this maximum value is achieved, and therefore is the value that provides the best trade-off between true and false positive rates when equal weight is given to sensitivity and specificity. The optimal cut-points, corresponding sensitivities, specificities and Youden indices were estimated for each iron marker in this analysis.

6.3.5. Power calculations

For the fixed sample of 44 cases and 170 controls, the study had 80% power (alpha 5%) to detect an odds ratio (OR) of ≥2.55 for the association between iron deficiency (as defined by concentrations of sFR) and PPCM, assuming the prevalence of iron deficiency was 40% in the general pregnant population (117, 124). This expected OR was later compared to the observed effect size to assess the power achieved in this analysis.

6.3.6 Sensitivity analyses

6.3.6.1. Confounder adjusted ROC curves

Education level was assumed to have no contribution to the discriminatory accuracy of the iron biomarkers. However, a sensitivity analysis was conducted to test this assumption by estimating the confounder-adjusted ROC (AROC) curves for each of the iron markers.

6.3.6.2. Missing data

The main regression models in this study were generated using a complete-case analysis. Based on knowledge of the data collection process in the MaatHRI heart failure study, the biomarker data were likely to be missing for the following reasons: 1) the severity of the disease which prevented complete data collection, 2) samples being discarded due to issues with quality, and 3) the inability to analyse biological data due to laboratory closures during the COVID-19

pandemic. Since the severity of iron deficiency may increase the severity of heart failure symptoms, the missing biomarker values may have been associated with the values themselves (i.e. the data was missing not at random). If the results of the heart failure severity analysis indicated otherwise, a sensitivity analysis would be carried out where the missing data would be estimated using multiple imputation with chained equations**.**

6.4. Results

6.4.1. Prevalence of iron deficiency and association with PPCM

This analysis included 214 women (n=44 PPCM cases and n=170 controls without PPCM). There were no significant differences in the median blood lost before specimen collection between women with PPCM (175 mL) and controls (200 mL) (p=0.2).

Over half of the women diagnosed with PPCM in this study population met the ESC definition for iron deficiency **(Table 6.1).** The frequency of iron deficiency among these women was significantly greater than what was observed among women without heart failure (p<0.001). Likewise, the prevalence of microcytic-hypochromic anaemia was greater in the group of women diagnosed with PPCM (p=0.027).

Table 6.1. Prevalence of iron deficiency in the study population by iron deficiency definition

	Confirmed Cases (N=44)	**Controls (N=170)**	**p-value[a]**
	Frequency (%)		
Depleted iron stores[b]			**<0.001**
Iron deficient	24 (55)	57 (34)	
Not iron deficient	10 (23)	111 (65)	
Missing	10 (23)	2 (1)	
Microcytic-hypochromic anaemia[c]			**0.042**
Yes	7 (16)	10 (6)	
No	35 (80)	159 (93)	
Missing	2 (4)	1 (1)	

[a]Statistical tests performed: Pearson's Chi-square tests for differences in proportions
[b]Defined as sFR < 30 ng/mL in controls, and sFR < 100 ng/mL or 100–299 ng/mL with transferrin saturation <20% in cases
[c]Defined as MCV < 80 fL and MCH < 27 pg

After adjusting for education, women with depleted iron stores were more than four times more likely to be diagnosed with PPCM (OR=4.54, 95% CI: 2.02, 10.9) **(Table 6.2)**. In the

unadjusted model, PPCM was also associated with microcytic-hypochromic anaemia (OR=3.18; 95% CI: 1.09, 8.87), although the association was no longer significant after adjusting for level of education.

Table 6.2. Association between iron deficiency and PPCM

	Unadjusted model		**Adjusted model[a]**	
	N cases	*OR (95% CI)*	*N cases*	*OR (95% CI)*
Depleted iron stores[b]	***34***		***34***	
Not iron deficient	*10*	1 (ref)	*11*	1 (ref)
Iron deficient	*24*	**4.67 (2.15, 10.9)**	*23*	**4.54 (2.02, 10.9)**
Microcytic-hypochromic anaemia[c]	***42***		***41***	
No	*35*	1 (ref)	*35*	1 (ref)
Yes	*7*	**3.18 (1.09, 8.87)**	*7*	3.05 (0.97, 9.23)

OR = Odds Ratio, **CI** = Confidence Interval
[a]Models adjusted for education level.
[b]Defined as sFR<100 ug/dL or 100-299 with TSAT < 20% for cases and <30 ug/dL for controls
[c]Defined as anaemia (Hb < 10 g/dL) with MCV < 80fL and MCH<27pg

6.4.2. Iron profile of the study population

Women diagnosed with PPCM appeared to have higher absolute concentration of sFR (median = 76 ug/mL) compared to the women without PPCM (43 ug/mL) (p=0.018). The median concentration of the marker among the cases was reduced after correcting for CRP levels but remained higher than concentrations among women without PPCM (p=0.022) **(Table 6.3).**

However, those diagnosed with PPCM had a lower concentration of serum iron (p<0.001) and correspondingly lower TSAT levels (p=0.021) compared with women with no heart failure **(Table 6.3).** Inadequate levels of intracellular iron was also more prevalent among the cases, as demonstrated by higher median concentrations of sTfR (p<0.001) and higher sTfR-F index compared to the controls (p=0.015). This was accompanied by lower median concentrations of hepcidin (p<0.001), indicative of a compensatory increase in iron mobilisation and absorption compared to women without heart failure.

Table 6.3. Indices of iron status in PPCM cases and controls without heart failure

	Confirmed Cases (N = 43)	**Controls (N = 170)**	**p-value[a]**

	Median (IQR)		
sFR (ug/mL)[b]	58 (23, 145)	33 (18, 66)	**0.022**
N missing	10	4	
sFe (ug/dL)	40 (22, 65)	65 (44, 105)	**<0.001**
N missing	10	1	
TSAT (%)[b]	11 (6, 22)	14 (9, 24)	**0.021**
N missing	10	2	
sTfR (mg/L)	2.50 (1.72, 5.43)	1.56 (1.19, 2.40)	**<0.001**
N missing	9	3	
sTfR-F index	1.64 (0.84, 3.86)	1.06 (0.72, 1.83)	**0.015**
N missing	10	6	
Hepcidin (ng/mL)[b]	3 (1, 10)	13 (3, 31)	**<0.001**
N missing	13	16	

IQR: interquartile range, **sFR:** serum ferritin, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F:** sTfR-ferritin**, hs-CRP**: high sensitivity C-reactive protein

[a]Statistical tests performed: Wilcoxon rank-sum test for difference in median

[b]Values corrected for inflammation (CRP>5 mg/L)

After correction for inflammation, a 10 ug/mL increase from the median sFR values was associated with a 4% higher odds of PPCM among the women in this population (OR=0.96, 95% CI: 0.94, 0.99) (**Table 6.4**). However, the adjusted regression models for the other iron markers indicated a significant association between having reduced iron availability and being diagnosed with PPCM **(Table 6.4).** The odds of PPCM increased by 14% with every 10 ug/mL decrease from the median concentration of sFe (OR=1.14, 95% CI: 1.06, 1.24). A unit decrease from the median TSAT level was associated with a small increase in the odds of disease (OR=1.02, 95% CI: 1.01, 1.04). Every unit increase from the median sTfR concentrations or the sTfR-F index was associated with 42% and 33% greater odds of the disease, respectively (OR=1.42, 95% CI: 1.19, 1.74 and OR=1.33, 95% CI: 1.08, 1.66). Accordingly, every unit decrease in hepcidin was associated with a 63% increase in the odds of being diagnosed with PPCM (OR=1.63, 95% CI: 1.17, 2.53).

Table 6.4. Association between markers of iron availability and PPCM

	Unadjusted model		**Adjusted model[a]**	
	N cases	*OR (95% CI)*	*N cases*	*OR*
sFR (ug/mL)[b]	*34*	**0.96 (0.93, 0.99)**	*34*	**0.96 (0.94, 0.99)**
sFe (ug/mL)[b]	*34*	**1.16 (1.08, 1.25)**	*33*	**1.14 (1.06, 1.24)**
TSAT (%)[c,d]	*34*	**1.02 (1.01, 1.04)**	*33*	**1.02 (1.01, 1.04)**
sTfR (mg/L)[e]	*35*	**1.49 (1.25, 1.80)**	*34*	**1.42 (1.19, 1.74)**
sTfR-F index[e]	*34*	**1.39 (1.14, 1.73)**	*33*	**1.33 (1.08, 1.66)**
Hepcidin (ng/mL)[c,d]	*31*	**1.78 (1.28, 2.77)**	*30*	**1.63 (1.17, 2.53)**

OR: Odds Ratio, **CI**: Confidence Interval, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F**: sTfR-ferritin index
[a]Models adjusted for education level.
[b]For a 10 unit decrease from the median
[c]Values adjusted for inflammation (CRP > 5 mg/L)
[d]For a 1 unit decrease from the median
[e]For a 1 unit increase from the median

6.4.3. Association with heart failure severity

Table 6.5. presents a summary of the distribution of heart failure severity among the PPCM cases, according to NYHA class, LVEF and survival status of the mother.

Table 6.5. Distribution of heart failure severity among PPCM cases (N=44)

	Frequency (%)
NYHA class	
I-II	10 (23)
III	9 (20)
III	25 (57)
LVEF (%)	
<30	10 (23)
30-44	34 (77)
Maternal survival status[a]	
Died	9 (20)
Survived	24 (55)
Unknown	11 (25)

NYHA: New York Heart Association, **LVEF**: left ventricular ejection fraction
[a]Survival at 6 weeks follow-up

Serum concentrations of iron markers were not associated with NYHA class of heart failure symptoms among women diagnosed with PPCM (**Appendix Table A12)**. However, PPCM cases severe left ventricular dysfunction (LVEF < 30%) had significantly lower concentrations of sFe (p=0.023) and TSAT (p=0.045) than cases with LVEF between 30-44% **(Table 6.6).** PPCM cases with LVEF < 30% also had significantly higher sTfR levels than controls (p=0.028). Women diagnosed with PPCM who died in the first six weeks after birth had lower concentrations of sFe, lower TSAT, higher concentrations of sTfR and higher sTfR-F index than those who survived, but these differences were not statistically significant (**Appendix**

Table A13**).**

Table 6.6. Serum concentration of iron markers in women diagnosed with PPCM by left ventricular ejection fraction

	LVEF < 30% (N = 10)	LVEF 30-44% (N = 34)	p-value[a]
	Median (IQR)		
sFe (ug/dL)	24 (14, 30)	44 (24, 79)	**0.023**
N missing	1	9	
TSAT (%)[b]	6 (3, 9)	13 (7, 23)	**0.045**
N missing	1	9	
sTfR (mg/L)	5.91 (2.40, 7.13)	2.27 (1.57, 4.05)	**0.028**
N missing	1	8	
sTfR-F index	2.66 (1.18, 4.78)	1.20 (0.79, 2.84)	0.086
N missing	1	9	
Hepcidin (ng/mL)[b]	2 (1,3)	3 (2, 16)	0.2
N missing	2	11	

IQR: interquartile range, **LVEF:** left ejection fraction, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F:** sTfR-ferritin**, hs-CRP**: high sensitivity C-reactive protein

[a]Statistical tests performed: Wilcoxon rank-sum test for difference in median

[b]Values corrected for inflammation (CRP>5 mg/L)

6.4.4. Classification accuracy of iron markers

Overall, serum concentrations of iron markers had moderate accuracy when discriminating between women diagnosed with PPCM and women without heart **(Table 6.7)**. Serum sTfR (AUC=0.74, 95% CI: 0.65, 0.82) and hepcidin (AUC=0.74, 95% CI: 0.64, 0.83) had the greatest diagnostic value among the iron markers investigated, while TSAT performed the worst (AUC=0.63, 95% CI: 0.51, 0.75). Concentrations of sTfR above 1.79 mg/L (sensitivity = 74%) and hepcidin levels below 8.86 ng/mL (sensitivity = 77%) provided the best sensitivity, although the specificity was only moderate (62% and 61%, respectively). Serum sFe concentrations below 30.5 ug/mL and sTfR-F index above 2.69 resulted in the highest specificity for PPCM (90%), but the associated sensitivities were suboptimal (48% and 36%, respectively). Similarly, TSAT had a high specificity of 86%, but a low sensitivity of 42%.

Table 6.7. Area under the curve, Sensitivity, specificity, and the Youden index for each iron marker

Iron marker	AUC (95% CI)[a]	Optimal cut-point	Sensitivity	Specificity	Youden index
sFe (ug/mL)	0.70 (0.56, 0.82)	30.50	0.48	0.90	0.38
TSAT (%)	0.63 (0.51, 0.75)	7.22	0.42	0.86	0.29
sTfR (mg/L)	0.74 (0.65, 0.82)	1.79	0.74	0.62	0.36
sTfR-F index	0.64 (0.52, 0.74)	2.69	0.36	0.90	0.26

Hepcidin (ng/mL)	0.74 (0.64, 0.83)	8.86	0.77	0.61	0.38

AUC: area under the curve, **CI**: confidence interval, **sFR**: serum ferritin, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F**: sTfR-ferritin index
[a]AUC of the unadjusted non-parametric single marker receiver operating characteristic (ROC) curve

6.4.5. Power estimation

It was estimated that this study of 214 women had 80% power to identify an OR of at least 2.55 for the association between iron deficiency and PPCM, using the sFR definition of iron deficiency. The unadjusted OR observed in this analysis was greater than this value (OR=4.54, 95% CI: 2.02, 10.9), suggesting that the analysis was adequately powered to observe a significant association between PPCM and depleted maternal iron stores.

6.4.6. Sensitivity analyses

In sensitivity analyses, there were no marked difference between the AUC of the confounder-adjusted ROC curves for the iron markers and the unadjusted AUCs reported here **(see Appendix**

Table A14**).**

Since there was evidence that serum concentrations of iron markers was associated with the severity of disease, the missing biomarker data may have been MNAR. Therefore, a multiple imputation analysis was not appropriate to estimate the missing data.

6.5. Discussion

This study investigated for the first time the role of iron status in PPCM. In this study population of Indian women, PPCM was significantly associated with an overall reduction in iron availability. Specifically, over half of the Indian women diagnosed with PPCM had depleted iron stores at the end of their pregnancy, and this was associated with a four-fold increase in the odds of PPCM. The molecular profile of PPCM was characterised by low levels of circulating iron and unmet erythropoietic iron demand, accompanied by increased iron absorption and mobilisation. These observations are consistent with the notion that PPCM is significantly associated with absolute iron deficiency. There was some indication that the severity of iron deficiency may be associated with heart failure, particularly with severe left ventricular dysfunction. Concentrations

of sTfR and hepcidin had the greatest value for distinguishing between women with and without PPCM, while TSAT performed the poorest. However, iron biomarkers were only moderately useful overall for identifying PPCM in the population.

In many resource-limited countries, iron investigations beyond haemoglobin are not part of routine clinical care, as these assays can be costly and not always readily available. Moreover, their interpretation can be challenging in populations prone to inflammatory conditions. Consequently, the exact prevalence of iron deficiency in low-income countries is difficult to ascertain. While the WHO estimates that about 50% of anaemias observed in women are likely to be due to a deficiency in iron (210), recent population studies suggest that the contribution of iron deficiency to anaemia could be closer to 37% (211). This is consistent with the findings of the present analysis that about 30% of the Indian women without heart failure in this study had depleted iron stores. There are currently no other studies reporting the prevalence of iron deficiency in Indian women with PPCM with which to compare the results of this study. However, in patients with acutely decompensated heart failure, the reported prevalence of iron deficiency ranges from 72% to 83% (107). In comparison, only about half of the women diagnosed with PPCM in this study population met the ESC definition for iron deficiency. The differences in values could be due to the different iron deficiency definitions used, as well as differences in the characteristics of the populations investigated (namely pregnancy or anaemic status).

While sFR is often used as a measure of iron status in clinical practice, it is an acute-phase protein that is elevated in the presence of infection or systemic inflammation irrespective of iron stores. This inflammatory response has been shown to mask iron deficiency in individuals with heightened inflammatory status such as would be expected in heart failure patients or in populations where certain infections (such as malaria, HIV and tuberculosis) are endemic. Consistent with this, women in this study who were diagnosed with PPCM were observed to have higher absolute concentration of sFR compared to the women without PPCM, even though over half of these women met the ESC definition for iron deficiency in heart failure patients. When estimating the prevalence of iron deficiency based on ferritin levels in a population with high

inflammation, previous studies have recommended that at least two markers of inflammation (eg. CRP, albumin or α1-acid glycoprotein) should be measured to account for the direct effects of inflammation on the values of sFR (203, 212). And even then, the prevalence of iron deficiency may still be underestimated. Thus, it is likely that the statistical correction for CRP concentrations in this analysis may not have been enough to account for this phenomenon. In the absence of additional indicators of inflammation, the absolute serum concentration of ferritin was not a reliable marker of iron stores in women with PPCM in this population.

On the other hand, the ESC definition of iron deficiency used in this study (i.e. sFR<100 ug/mL or 100-299 ug/mL with TSAT < 20) has been reported to have a sensitivity of 94% and a specificity of 72% for identifying iron deficiency among heart failure patients (213). A classification based on serum concentrations of TSAT and sFR can provide a more useful assessment of the iron stores of women diagnosed with PPCM, as demonstrated by the findings of this study. One limitation of the ESC definition of iron deficiency is that it focuses on patients with low iron stores (absolute iron deficiency), but excludes cases where sFR levels may be normal while circulating levels of iron are low (iron restriction). Hence, the prevalence of iron deficiency observed among women with PPCM in this population may in fact be greater than what has been measured in the study.

In clinical practice, iron deficiency is often diagnosed based solely on low levels of haemoglobin and red blood cell indices such as MCV and MCH. While these indices are expected to change very little across the perinatal period in an iron-replete woman (214), they are typically reduced in iron deficiency. However, the red blood cell markers lack specificity for the diagnosis of the condition (105). Indeed, both MCV and MCH can be significantly decreased in certain haemoglobinopathies (such as beta-thalassaemia), as well as in the anaemias of chronic infection and inflammation (105). Since these conditions are common in many low and middle-income countries, microcytic-hypochromic anaemia is generally a poor indicator of iron deficiency in these populations. Moreover, this definition of iron deficiency depends on the presence of concomitant anaemia, and therefore excludes individuals with milder levels of iron deficiency

where the erythropoiesis process may not have yet been affected. Thus, the prevalence of microcytic-hypochromic anaemia among the women diagnosed with PPCM in this population was significantly lower than the prevalence of depleted iron stores. However, although the association was borderline significant, women with PPCM were more likely to present with microcytic-hypochromic anaemia than were women without. This suggests that microcytic-hypochromic anaemia can still be of some use in identifying iron deficiency in women diagnosed with PPCM when further iron studies may not be possible.

One aim of this study was to characterise the iron status of women diagnosed with PPCM and determine which mechanism of iron deficiency (absolute or functional) could be underlying the association with PPCM. In this study population, women diagnosed with PPCM had lower levels of hepcidin than those without PPCM, which indicates that they were experiencing increased iron absorption and mobilisation from their biological pools. Because this effect was accompanied by significantly lower levels of circulating (sFe and TSAT) and cellular iron (sTfR, sTfR-F index), it is likely that the decrease in hepcidin was a compensatory response to unmet iron needs. These results suggest that PPCM may be associated with the mother's inadequate iron stores (absolute iron deficiency), rather than impaired iron mobilisation (iron restriction). Furthermore, since only four women with PPCM were pregnant at the time of blood collection (the rest of the cases being postpartum), this decrease in hepcidin levels is unlikely to be a result of the normal adaptations of pregnancy to meet the iron demands of the fetus. Thus, the pathology of PPCM may involve mechanisms of cellular iron depletion.

Consistent with these findings, serum concentrations of sTfR (an indicator of iron demand in red blood cells) were increased in women diagnosed with PPCM. Although this biomarker was only able to classify the PPCM status of women in this study population with moderate accuracy, it performed better than traditional cardiovascular biomarkers, such as NT-proBNP, which has previously been reported to classify PPCM women with poor accuracy (AUC= 0.55, 95% CI: 0.45, 0.67) (88). Concentrations of sTfR above 1.79 mg/mL had 74% sensitivity and 62% specificity for identifying women with PPCM in the study population. Reference values for the diagnosis of iron

deficiency in pregnant women are highly debated, and the subject of many recent investigations (117, 215). Consequently, it may be challenging to interpret these statistically estimated cut-points, as they may or may not be biologically meaningful. Nevertheless, the optimal cut-point estimated for sTfR is similar to the laboratory specified 1.76 ng/mL cut-off for iron deficiency in non-pregnant adult populations. Serum concentrations of sTfR are not expected to change in pregnancy relative to non-pregnant values, except in the presence of significant iron restricted erythropoiesis in the mother (127). Thus, although, iron-replete mothers may experience a mild increase in sTfR concentration in the third trimester (owing to a natural increase in erythropoeitic activity), concentrations significantly above reference values are typically indicative of iron deficiency.

On the other hand, while a TSAT < 20% is generally used in clinical practice as an indication of iron deficiency in non-pregnant adults, a lower cut-off of 16% has been used to diagnose iron deficiency in pregnant women to account for the natural reduction in TSAT levels that occurs during gestation (117, 216). Notably, the estimated cut-point for TSAT in this study was more than half of this suggested value, which provides further evidence to support the importance of severe iron deficiency in the presentation of PPCM among women in this study population.

Future models using these markers in combination with other relevant risk factors could be built to predict the risk of PPCM in populations similar to the one investigated in this study. The measurement of iron markers in prospective cohort studies could also help to better understand how maternal iron status affects the risk of PPCM over the course of pregnancy. However, such in depth longitudinal assessments of a panel of biomarkers are likely to be long and costly, as PPCM is a rare disease. Alternatively, a retrospective study with measurements of biomarkers before the onset of disease could help to substantiate the associations suggested by the findings of the present study. Nevertheless, it is important to note that these iron markers (especially newer ones such as sTfR) may not always be readily available in resource-limited clinical settings. Thus, most population-based investigations of these iron biomarkers are likely

to be cross-sectional in nature. These population studies could be supplemented by laboratory investigations of animal models designed to investigate the causal effect of these and other markers on the pathogenesis of PPCM, and more clearly describe the pathophysiological mechanisms underlying iron deficiency in this disease.

6.6. Strengths and limitations

This is the first study to date which has investigated iron metabolism in relation to PPCM in any population. The main strengths of this study are the thorough measurement, high ascertainment and reporting of perinatal iron status, and the examination of a panel of iron indices, which provided a well-rounded picture of maternal iron metabolism in the study population.

Nevertheless, the small number of patients included in this study should be considered an important limitation. While this analysis was powered to observe statistically significant differences in levels of sFR between cases and controls, it was not specifically powered to investigate five different markers of iron metabolism. Additionally, there was not enough power to investigate the association between iron deficiency and heart failure severity in this study. Therefore, the results from this chapter should be interpreted with caution, and should serve as a hypothesis-generating starting point for further mechanistic investigations addressing the underlying pathogenesis of PPCM.

In this study, women diagnosed with PPCM were enrolled between the third trimester of pregnancy and six months postpartum, while all women in the control group were enrolled within two days of parturition. This difference in gestational and postpartum time at enrolment may rightly raise some concern regarding the comparability of the marker measurements between the two study groups, especially with respect to the effects of parturition on maternal iron markers. While childbirth is indeed expected to affect maternal iron status, parturition is likely to exacerbate iron depletion owing to the loss of blood and expulsion of the placenta and umbilical cord (125). Over the course of the puerperium period, maternal iron stores are expected to

progressively go back to normal, due to the contraction of maternal red blood cell mass and the significant decrease in maternal iron demand during lactation (126). Thus, any bias resulting from the differences in enrolment periods between the study groups would have pushed the effect sizes towards the null, resulting in an underestimation of the associations investigated.

Importantly, the definition of iron deficiency used in this study was based on differing cut-offs of sFR in cases and controls, to account for the effect of inflammation on measurements of this marker. This definition assumes that all PPCM cases but none of the controls would have been experiencing increased inflammation. However, this assumption may not have held true for all the study participants. In that case, the different cut-offs may have potentially resulted in the misclassification of the cases who did not experience inflammation as being iron deficient, while the controls who experienced more inflammation than anticipated would have been classified as iron replete. Overall, this definition may have resulted in an apparently stronger association between iron deficiency and PPCM status in this population.

Another notable study limitation is the lack of data on maternal diet coupled with the fact that the participants were not in a fasted state at the time of blood draw. Some of the iron markers analysed in this study are known to be subject to diurnal variation and can be acutely affected by the dietary supplementation of iron (particularly sFe, TSAT and to some extent hepcidin). However, while diurnal changes in iron markers may affect their absolute plasma concentration, these natural fluctuations were not expected to reflect significantly different overall iron status, and were unlikely to have affected the cases and controls differently.

The measurement of serum sTfR, which has been shown to have low intrapersonal variability (117), supports the conclusions derived from the other markers and further illustrates the value of having a complete panel of iron indices in the analysis. Nonetheless, one limitation of the sTfR measurements, is the lack of standardization of the measurements across different assays. Indeed, sTfR can be measured using different methods (such as enzyme-linked immunosorbent assay, immunonephelometry and immunoturbidimetry), which can hinder the

comparison of the absolute values of this marker across different studies. Thus, the optimal cut-point estimated for sTfR in this analysis is only interpretable for investigations of this marker using a nephelometry assay. Nevertheless, since this study also provides evidence for the effect of a single unit change in the concentrations of sTfR, the associations reported here are interpretable and relevant regardless of the assay method used.

Finally, because the biomarkers were measured only at the point of recruitment into the study, the causal relationship between disease status and the levels of the biomarkers could not be established. Thus, the associations reported here are merely correlational. Nevertheless, the depletion of adequate iron stores is a long process that occurs over the course of the pregnancy. Additionally, since heart failure is not known to acutely affect levels of iron indices, it was expected that the measurements of these markers at enrolment would not have been influenced by the acute condition itself, and thus could reasonably be assumed to reflect the maternal iron status before diagnosis.

6.7. Chapter summary

The results of this chapter uncovered a new role for iron deficiency in PPCM. The analysis found that over half of Indian women diagnosed with PPCM had iron deficiency in the perinatal period, and showed evidence of unbalanced iron status. Specifically, PPCM was significantly associated with negative tissue iron balance and a compensatory, albeit if insufficient increase in iron mobilisation (through the suppression of the hormone hepcidin). Markers of iron status were moderately useful indicators of PPCM in this population, and could be useful for the prediction of future risk of developing PPCM. The next chapter will investigate how iron status can modify the association between anaemia and PPCM.

Chapter 7. The role of iron availability in the association between anaemia and Peripartum Cardiomyopathy

7.1. Background and Scope

The European Society of Cardiology (ESC) has identified anaemia as one of the most important non-cardiovascular comorbidities of heart failure (17). Consistent with that evidence, anaemia is commonly observed in women diagnosed with peripartum cardiomyopathy (PPCM), occurring in up to 40% of cases (23, 47, 48). In low- and middle-income countries, anaemia is a multifactorial condition with various aetiologies (including nutritional deficiencies, infections and genetic disorders), which may require different modalities of treatment. Thus, determining the extent to which iron deficiency contributes to anaemia observed in women diagnosed with PPCM will be important to ensure they receive the most appropriate treatment. The previous chapter demonstrated that Indian women who were diagnosed with PPCM were more likely to have microcytic-hypochromic anaemia, which is most commonly caused by iron deficiency. This evidence suggest a potential association between iron deficiency anaemia and PPCM, which has not yet been investigated. The theoretical framework developed in Chapter 3 illustrated how the observed association between anaemia and PPCM may be driven by the effect of iron deficiency on the heart, which exacerbates the hypoxic effects of anaemia (Chapter 3, research question #2).

The goal of this chapter was to investigate this hypothesis by 1) determining the prevalence of iron deficiency among anaemic Indian women diagnosed with PPCM, and 2) comparing the risk of PPCM in anaemic women with depleted iron stores to the risk in women with non-iron deficient anaemia. Additionally, this chapter assessed whether combining haemoglobin and iron marker measurements improved the diagnostic accuracy of haemoglobin for PPCM in this population.

7.2. Methods

7.2.1. Study population

This analysis was conducted using data from the MaatHRI case-control study of heart failure in pregnant Indian women, as described in Chapter 4. The analysis investigated all 44 women diagnosed with PPCM between the third trimester of pregnancy and six months

postpartum, and the 170 women without PPCM who gave birth within two days of the case presenting at the hospital.

7.2.2. Laboratory analyses

All blood samples were collected non-fasted at the time of enrolment in the study. The blood investigations analysed in this chapter included: haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), haemoglobin types (including HbA, HbA2, HbA2+E, and other non-specific Hb types), and the panel of iron indices investigated in the previous chapter (i.e. serum iron (sFe), transferrin saturation (TSAT), soluble transferrin receptors (sTfR), the sTfR-FR index and serum hepcidin). See Chapter 4 for more details on the laboratory assays used to measure these markers.

Although vitamin B12 concentrations were measured in the heart failure study, some patients who were known to be deficient in vitamin B12 had been given supplements to correct the deficiency. However, it was unclear whether this corrective supplementation had taken place before or after blood investigations. Consequently, the measurements of vitamin B12 were unreliable, and therefore, this biomarker was not analysed in the present study.

7.2.3. Study variables

7.2.3.1. Anaemia

A previous study of maternal outcomes associated with anaemia in a similar study population found that moderate to severe anaemia (Hb < 10 g/dL) but not mild anaemia (Hb 10-10.9 g/dL) was a strong independent risk factor for adverse maternal and infant outcomes (217). Therefore, anaemia was defined as haemoglobin levels below 10 g/dL in this study. Furthermore, anaemia severity was categorised following the World Health Organization's (WHO) guidelines: moderate (Hb= 7-9.9 g/dL), and severe (Hb< 7 g/dL) (120).

7.2.3.2. Characteristics of anaemia

The analysis compared the risk of PPCM associated with different characteristics of anaemia in the study population. These included: microcytic-hypochromic red blood cells, iron deficiency, iron loading anaemia, and haemoglobinopathies.

Microcytic-hypochromic anaemia

Microcytic-hypochromic anaemia was defined as anaemia accompanied by MCV < 80 fL (195) and MCH levels < 27 pg (192, 193).

Iron deficiency

For women diagnosed with PPCM, iron deficiency was defined following the latest ESC guidelines, which recommend defining iron deficiency in heart failure patients as a sFR concentration <100 ng/mL, or 100–299 ng/mL with TSAT <20% (17). In the postpartum women without heart failure, iron deficiency was diagnosed at sFR < 30 ng/mL.

Iron loading anaemias

Although iron deficiency is the most common cause of anaemia, certain cases of anaemia are instead characterized by ineffective erythropoiesis, leading to excessive iron absorption and secondary iron overload (e.g. thalassaemic syndromes, sickle cell or other congenital anaemias) (218). In these so called 'iron loading anaemias', serum levels of TSAT are usually expected to be above 50% (219). TSAT values were adjusted for the effects of inflammation using CRP measurements, as described previously (see Chapter 6). Study participants with corrected TSAT values > 50% were identified as potential cases of iron loading anaemia.

Haemoglobinopathies

Haemoglobinopathies refer to a range of disorders affecting the structure or production of Hb that may lead to anaemia. Inherited Hb disorders (particularly sickle cell disorders, Hb E trait and thalassaemic syndromes), are common in the eastern half of the Indian sub-continent, where most of the study population originated (220). For the current analysis, haemoglobinopathies were suspected in the absence of a normal haemoglobin electrophoresis pattern, which was defined according to the laboratory specifications as HbA > 80%, HbA2<4%, HbA2+E <50% or other Hb variants < 10%.

7.3. Statistical analyses

7.3.1. Descriptive analyses

The concentration of the erythropoietic parameters were summarised using medians and interquartile ranges, and compared between women with PPCM and women without heart failure

using non-parametric Wilcoxon rank sum tests. The prevalence of anaemia and anaemia severity were estimated and compared between cases and controls using Pearson's Chi-square tests for differences in proportions. The median and interquartile range of Hb concentrations in PPCM cases and controls across the gestational/postpartum periods at enrolment is also presented in **Appendix Figure A9.**

7.3.2. Main effect of anaemia on PPCM

A directed acyclic graph (DAG) was drawn to identify potential confounders of the association between anaemia and PPCM **(Figure 7.1)**. Based on the results of Chapter 5, risk factors for PPCM included level of education, duration of iron and folic acid (IFA) supplementation during the index pregnancy, and hypertensive disorders. Risk factors for anaemia were identified from clinical knowledge and evidence from a previous study of maternal outcomes of pregnancy associated with anaemia, which used data collected through the MaatHRI platform (217). Of the factors identified in the previous study, the following were included in the DAG: maternal age, body mass index (BMI), level of education, religion, below poverty line status, parity, number of antenatal visits, postpartum blood loss and iron and folic acid (IFA) supplementation during the index pregnancy. Education level and the duration of IFA supplementation in the index pregnancy were common risk factors for both PPCM and anaemia. However, the association between IFA supplementation and PPCM was thought to be mainly through the effect of iron deficiency anaemia, consequently, only education level was considered as potential confounder of the association between anaemia and PPCM (indicated in red in **Figure 7.1**).

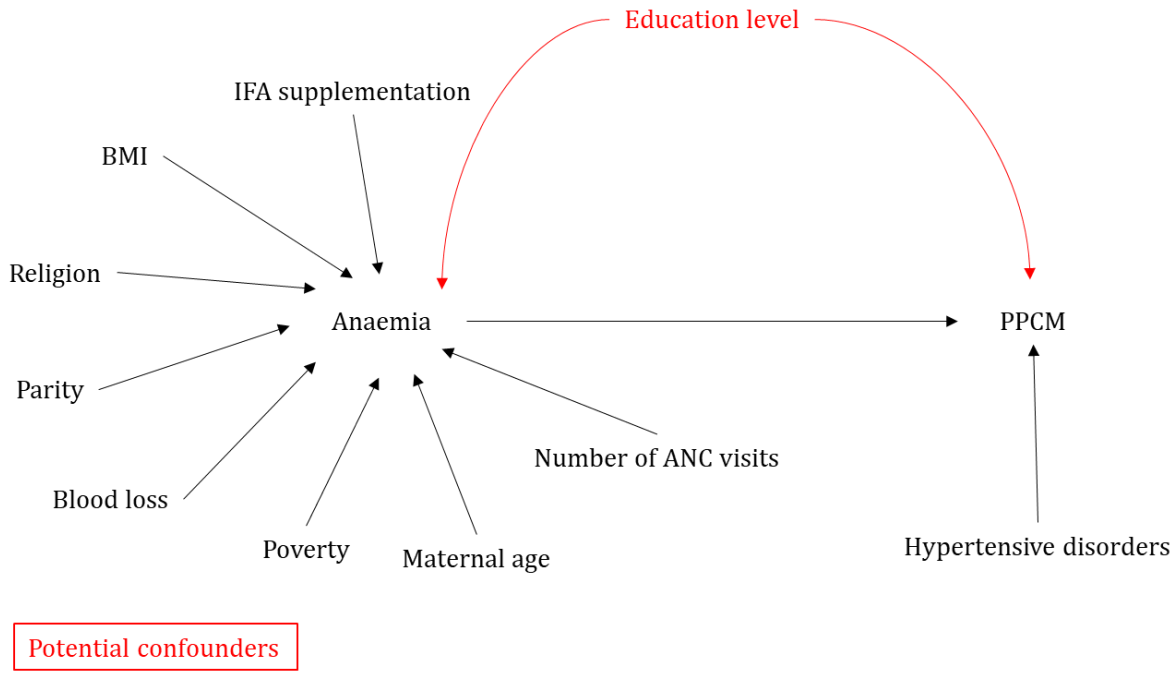


Figure 7. 1. Directed acyclic graph for the association between anaemia and PPCM. Risk factors associated with anaemia and PPCM. In red are factors that are associated with both, and were treated as potential confounders of the association between anaemia and PPCM. ***BMI:*** *body mass index,* ***ANC:*** *antenatal care.*

Hb concentrations at enrolment were tested for deviation from linearity using univariable fractional polynomials. Fitting a first-degree fractional polynomial showed an overall linear relationship between this variable and the link function for the odds of PPCM. Therefore, regression models were generated for the effect of a one g/dL decrease in serum levels of Hb, with Hb concentrations included as an untransformed continuous variable centred at the median (10.4 g/dL). The association of anaemia and anaemia severity with PPCM were also assessed. Multivariable models were adjusted for the level of education as a confounder of the exposure-outcome association.

7.3.3. Effect modification by iron deficiency status

The modifying effect of iron deficiency on the association between anaemia and PPCM was assessed by generating a dummy variable, which combined both anaemia and iron deficiency status into a single variable with the following four levels: anaemia with iron deficiency, anaemia only, iron deficiency only, and neither anaemia nor iron deficiency.

First, the prevalence of each of these categories was represented visually and compared between women diagnosed with PPCM and those without heart failure using a Pearson's Chi-square test for differences in proportions. The dummy variable was then included in a multivariable logistic regression model to compare the odds of PPCM associated with each of these categories. Study participants who were neither anaemic nor deficient in iron were treated as the reference group.

The magnitude of the effect modification was assessed on both the multiplicative and additive scale. Multiplicative interaction was estimated by: $\frac{OR_{11}}{OR_{10}*OR_{01}}$, where OR_{11} refers to the odds ratio for women who are both anaemic and iron deficient, OR_{10} indicates the odds ratio for those who are anaemic only and OR_{01} for those who are only iron deficient. Values >1 would indicate that the effect of both exposure together exceeds the product of their individual effects. The Relative Excess Risk due to Interaction (RERI), given by: $OR_{11} - OR_{01} - OR_{10} + 1$, was used as the measure of additive interaction. A positive interaction on the additive scale was inferred from a RERI > 0. The 95% CI for both measures of interaction were also estimated.

7.3.4. Secondary analyses

7.3.4.1. Other contributors to anaemia

In a secondary analysis among anaemic women only, the frequency of the different potential contributors to anaemia detailed above were estimated and compared between women with PPCM and women without PPCM using Fisher's exact tests to account for small cell sizes. The unadjusted effect of these other features of anaemia on the odds of PPCM was estimated by logistic regression.

7.3.4.2. Incremental value of the iron markers

Another secondary analysis was carried out to assess the improvement in classification performance gained by adding iron marker measurements to Hb values. Each iron marker was included with Hb in a separate regression model without an interaction term, and the non-parametric unadjusted receiver operating characteristic (ROC) curves for the combination of Hb and each iron maker was assessed. The corresponding area under the curves (AUC) and 95%

confidence interval were compared to the AUC for the ROC curve of Hb only, using DeLong's test for the comparison between ROC curves (221).

7.3.6. Power calculations

For the fixed sample of 44 cases and 170 controls, the study had 80% power (alpha 5%) to detect an OR ≥2.81 assuming a prevalence of anaemia of 52.2% in the general pregnant population (58). This expected OR was later compared to the observed effect size to assess the power achieved in this analysis.

7.3.7. Sensitivity analyses

7.3.7.1. Antenatal anaemia

The prevalence of antenatal anaemia and its association with PPCM was also assessed in a sensitivity analysis. Serum concentrations of haemoglobin measured at the first antenatal visit were dichotomised as described above. The odds ratio (OR) and 95% confidence interval after adjustment for the level of education were estimated as a measure of the association between antenatal anaemia and PPCM.

7.3.7.2. Missing data

Based on knowledge of the data collection process in the MaatHRI heart failure study, any laboratory data was likely to be missing for the following reasons: 1) the severity of the disease which prevented complete data collection, 2) samples being discarded due to issues with quality, and 3) the inability to analyse biological data due to laboratory closures during the COVID-19 pandemic. Because anaemia severity may have increased the severity of heart failure symptoms, the data may have been missing not at random (MNAR). If Hb concentrations among cases with severe left ventricular dysfunction (ejection fraction < 30%) did not differ from concentrations among cases with moderate dysfunction (33-44%), a sensitivity analysis would be carried out where the missing data is estimated using multiple imputation with chained equations**.**

7.5. Results

7.5.1. Erythropoietic characteristics

The erythropoietic characteristics of 44 women diagnosed with PPCM were compared to those of 170 postpartum women without heart failure **(Table 7.1)**. Median Hb levels at enrolment

were significantly lower in women with PPCM (p<0.001). The erythropoietic profile of the cases was also characterized by lower median concentrations of MCV (p=0.031), MCH (p<0.001), and haematocrit (p=0.021) compared to women without PPCM. Consistent with this profile, anaemia was both more prevalent (p<0.001) and more severe (p<0.001) in women diagnosed with PPCM compared to controls without heart failure. Women diagnosed with PPCM also had significantly higher RDW values compared to controls (p<0.001). As a measure of the degree of variation in the size of red blood cells, RDW is expected to be elevated in iron deficiency anemia, but also in macrocytic anemia secondary to vitamin B deficiencies (222). However, there was no significant differences observed in the red blood cell count of the two study groups.

Table 7.1. Summary of erythropoietic characteristics according to PPCM status

	Confirmed Cases, N = 44	**Controls, N = 170**	**p-value[a]**
	Median (IQR)		
Enrolment Hb (g/dL)	9.00 (7.85, 10.95)	10.50 (9.60, 11.40)	**<0.001**
N missing	1	0	
MCV (fL)	88 (81, 100)	95 (87, 105)	**0.031**
N missing	2	2	
MCH (pg)	25.9 (22.6, 28.7)	28.9 (26.0, 30.8)	**<0.001**
N missing	2	1	
RBC count (mill/mm3)	3.61 (3.03, 4.25)	3.74 (3.38, 4.06)	0.6
N missing	4	1	
RDW (%)	21.4 (17.8, 24.7)	16.6 (15.2, 18.9)	**<0.001**
N missing	8	3	
Haematocrit (%)	32 (27, 37)	35 (31, 37)	**0.021**
N missing	1	0	
	Frequency (%)		
Anaemia status[b]			**<0.001**
Not anaemic	14 (31)	109 (64)	
Anaemic	29 (69)	61 (36)	
Missing	1 (2)	0 (0)	
Anaemia severity[c]			**<0.001**
No anaemia	14 (31)	109 (64)	
Moderate anaemia	22 (52)	53 (31)	
Severe anaemia	7 (17)	8 (5)	
Missing	1 (2)	0 (0)	

IQR: interquartile range, **Hb**: haemoglobin; **MCV**: mean corpuscular volume, **MCH**: mean corpuscular haemoglobin, **RBC**: red blood cell count; **RDW**: red cell distribution width

[a]Statistical tests performed: Wilcoxon rank-sum test for difference in median, Chi square test for difference in proportions

[b]Hb < 10 g/dL

[c]Hb≥10 g/dL (no anaemia), Hb: 7-9.9 g/dL (moderate), Hb<7g/dL (severe)

7.5.2. Main effect of anaemia on PPCM

One woman in the study population was missing Hb measurement at enrolment, therefore, the regression analyses were conducted on 43 women. After adjusting for the mother's level of education, the odds of PPCM increased by 24% with every one g/dL decrease from the median Hb concentration (OR=1.24, 95% CI: 1.03, 1.51) **(Table 7.2).** Being anaemic at enrolment was associated with a threefold increase in the odds of PPCM (OR=3.10, 95% CI: 1.49, 6.65). The risk of being diagnosed with PPCM also appeared to increase with increasing anaemia severity. Moderate anaemia was associated with almost three times the odds of disease (OR=2.91, 95 % CI: 1.35, 6.44), while being severely anaemic was associated with over four times the odds of PPCM (OR=4.12, 95% CI: 1.16, 14.4).

Table 7.2. The association between anaemia and PPCM

	Unadjusted model		**Adjusted model[a]**	
	N cases	*OR (95% CI)*	*N cases*	*OR (95% CI)*
Hb levels[c]	***43***	**1.36 (1.14, 1.64)**	***43***	**1.24 (1.03, 1.51)**
Anaemia status[d]	***43***		***43***	
No Anaemia	*14*	1 (ref)	*14*	1 (ref)
Anaemia	*29*	**3.70 (1.85, 7.72)**	*29*	**3.10 (1.49, 6.65)**
Anaemia severity[e]	***43***		***43***	
No anaemia	*14*	1 (ref)	*14*	1 (ref)
Moderate	*22*	**3.23 (1.55, 6.95)**	*22*	**2.91 (1.35, 6.44)**
Severe	*7*	**6.81 (2.25, 24.0)**	*7*	**4.12 (1.16, 14.4)**

[a]Models adjusted for education level
[b]Statistical test performed: Wald test for regression coefficients
[c]For a 1 g/dL decrease from the median
[d]Defined as Hb < 10 g/dL
[e]Severe: Hb < 7g/dL; Moderate: Hb 7-9.9 g/dL

7.5.3. Effect modification by iron deficiency status

Anaemia was accompanied by iron deficiency in 47% of women diagnosed with PPCM compared to 14% among women without heart failure (p<0.001) (**Figure 7.2**).

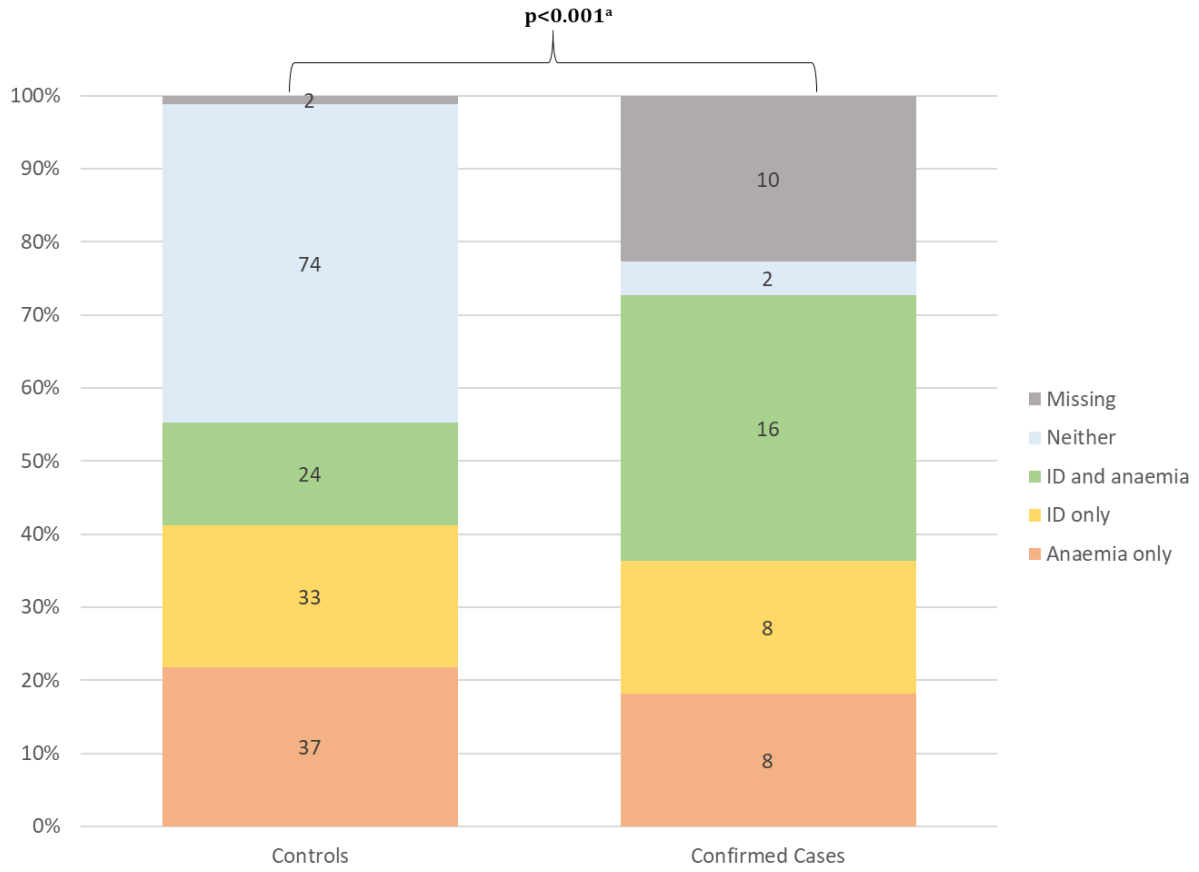


Figure 7. 2. Prevalence of anaemia with iron deficiency in the study population. [a]Chi-square test for the difference in proportions between women diagnosed with PPCM and women without heart failure. ***IDA****: iron deficiency and anaemia,* ***ID****: iron deficiency*

After adjusting for their level of education, anaemic mothers with depleted iron stores had a significantly higher odds of being diagnosed with PPCM than mothers who were only anaemic (OR=2.90, 95% CI: 1.05, 8.51). This was confirmed by a positive, albeit non-significant interaction on the additive scale (RERI = 5.27, 95% CI: -12.30, 22.8) but not on the multiplicative scale (OR=0.32, 95% CI: 0.04, 1.97) **(Table 7.3).**

Table 7.3. The modifying effect of iron status on anaemia in PPCM

	N cases	***OR (95% CI)[a]***
ID and anaemia status	***34***	
Neither	2	1 (ref)
Anaemia with ID	*16*	**20.5 (5.16, 137)**
ID only	*8*	**9.14 (2.10, 63.7)**
Anaemia only	8	**7.05 (1.62, 49)**
Interaction terms		
Multiplicative	*34*	0.32 (0.04, 1.97)
Additive (RERI)	*34*	5.3 (-12.3, 22.8)

OR: odds ratio, **CI**: confidence interval, **ID**: iron deficiency, **RERI**: relative excess risk attributable to interaction
[a]Model adjusted for education level
[b]Statistical test performed: Wald test for regression coefficients

7.5.4. Other contributors of anaemia in PPCM

Fifty-five percent of anaemic women diagnosed with PPCM met the ESC definition for iron deficiency in patients with heart failure, which was significantly greater than the prevalence observed among controls (p=0.023) **(Table 7.4)**. After iron deficiency, the most prevalent contributors of anaemia in these women were: microcytic-hypochromic red blood cells (24%), and abnormal Hb variants (17%). 10% of the women showed evidence of excess iron (TSAT>50%). However, there were no statistically significant differences in the prevalence of these contributors of anaemia between the two study groups.

Table 7.4. Other characteristics of anaemia by PPCM status (anaemic women only)

	Confirmed Cases with anaemia, N = 29	**Controls with anaemia, N = 61**	**p-value[a]**
	Frequency (%)		
Microcytic-hypochromic RBCs[b]			0.3
No	21 (72)	51 (84)	
Yes	7 (24)	10 (16)	
Missing	1 (3)	0 (0)	
Iron deficient[c]			**0.023**
Not iron deficient	8 (28)	37 (61)	
Iron deficient	16 (55)	24 (39)	
Missing	5 (17)	0 (0)	
Iron loading[d]			0.5
Not iron loading	21 (72)	46 (75)	
Iron loading	3 (10)	14 (23)	
Missing	5 (17)	1 (2)	
Haemoglobinopathies[e]			0.2
No	18 (62)	36 (59)	
Suspected	5 (17)	22 (36)	
Missing	6 (21)	3 (5)	

RBC: red blood cells
[a]Statistical tests performed: Chi square or Fisher's exact test for the difference in proportions
[b]MCV<80 fL and MCH<27 pg
[c]Ferritin < 100 ug/mL or 100-299 with transferrin saturation < 20% in cases, and ferritin < 30 ug/mL in controls
[d]Transferrin saturation > 50%. Values adjusted for inflammation (CRP > 5 mg/L).
[e]Absence of normal haemoglobin electrophoresis pattern (HbA > 80%, HbA2<4% or HbA2+E < 50% or other Hb variants <10%)

In unadjusted regression models, PPCM was significantly associated with being iron deficient among anaemic women (OR=3.08, 95% CI: 1.17, 8.67) **(Table 7.5)**. Consistent with that, anaemic women with microcytic-hypochromic red blood cells were more likely to have PPCM, although the association was not statistically significant (OR=1.70, 95% CI: 0.55, 5.04). No significant association was observed between the other potential contributors of anaemia and the odds of PPCM.

Table 7.5. Unadjusted odds of PPCM in anaemic women by anaemia contributors

	N cases	Unadjusted OR (95% CI)
Microcytic-hypochromic RBCs[a]	***28***	
No	*21*	1 (ref)
Yes	*7*	1.70 (0.55, 5.04)
Iron deficiency[b]	***24***	
Not iron deficient	*8*	1 (ref)
Iron deficient	*16*	**3.08 (1.17, 8.67)**
Iron loading[c]	***24***	
Not iron loading	*21*	1 (ref)
Iron loading	*3*	0.47 (0.10, 1.63)
Haemoglobinopathies[d]	***23***	
No	*18*	1 (ref)
Suspected	*5*	0.58 (0.19, 1.63)

RBC: red blood cells, **OR**: odds ratio, **CI**: confidence interval

[a]MCV < 80 fL and MCH<27 pg

[b]Ferritin < 100 ug/mL or 100-299 with transferrin saturation < 20% in cases, and ferritin < 30 ug/mL in controls

[c]Transferrin saturation > 50%. Values adjusted for inflammation (CRP > 5 mg/L).

[d]Absence of normal haemoglobin electrophoresis pattern ((HbA > 80%, HbA2<4%, HbA2+E < 50% or other Hb variants < 10%)

7.5.5. Incremental value of iron markers

Serum concentrations of Hb were moderately useful for identifying women with PPCM in this study population (AUC=0.68, 95% CI: 0.57-0.78). The addition of any iron biomarker to Hb measurements appeared to improve the classification accuracy for PPCM, although the changes in accuracy were not statistically significant **(Table 7.6).** The combination of Hb and sFe resulted in a 13 % improvement in diagnostic accuracy compared to Hb measurements alone. Likewise, the addition of hepcidin measurements improved the diagnostic accuracy of Hb by 11%.

Table 7.6. Area under the receiver operating characteristic curves for the joint accuracy of Hb and iron markers in diagnosing PPCM

	Hb + iron marker	**Comparison to Hb only AUC[ROCa]**
Marker	*AUC (95% CI)[b]*	*DeLong p-value[c]*
sFe	0.78 (0.69-0.87)	0.18
TSAT	0.75 (0.66, 0.84)	0.42
sTfR	0.74 (0.64, 0.84)	0.30
sTfR-F index	0.72 (0.62-0.83)	0.51
Hepcidin	0.76 (0.66-0.86)	0.35

Hb: haemoglobin, **AUC**: area under the curve, **ROC:** receiver operating characteristic, **CI**: confidence interval, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F index**: sTfR/log(ferritin)

[a] AUC=0.68, 95% CI: 0.57-0.78

[b]AUC of the receiver operating characteristic (ROC) curve for Hb and the iron marker without an interaction term

[c]DeLong's test comparing the AUC for the Hb+iron marker curve to the AUC for the Hb only curve

7.5.6. Power estimations

The unadjusted OR for the association between anaemia and PPCM observed in this analysis was 3.99 (95% CI: 1.97, 8.47), which was greater than the minimum detectable OR at 80% power (expected OR=2.81). Thus, this analysis was adequately powered to observe a significant association between PPCM and anaemia in the study population.

7.5.7 Sensitivity analyses

On average, participants had their first antenatal haemoglobin measurement at the end of the second trimester (27 weeks of gestation). Women with PPCM had significantly lower median haemoglobin concentrations at the first antenatal measurement (10.50 g/dL) compared to postpartum controls (p=0.007). This was reflected in a higher frequency of antenatal anaemia among the cases (p=0.004). After adjusting for the mother's level of education, antenatal anaemia was associated with nearly three times the odds of PPCM (OR=2.75, 95% CI: 1.30, 5.99).

The sensitivity analysis revealed that the median Hb concentration among cases with severe left ventricular dysfunction (8.70 g/dL, IQR: 8.30, 9.70) did not differ significantly from the median concentrations among cases with moderate left ventricular dysfunction (9.00 g/dL, IQR: 7.70, 10.90) (p>0.9). Therefore, the Hb observations were assumed to be missing at random. The results of the imputed analyses for the association between anaemia and PPCM were similar to the complete-cases analyses presented in this chapter **(Appendix Table A15).** However, after

imputing the missing data**,** there was a non-significant but positive interaction between anaemia and iron deficiency on both the multiplicative scale and the additive scales **(Appendix**

Table A16**)**.

7.6. Discussion

This analysis demonstrated that lower maternal Hb levels in the perinatal period were associated with an increased odds of PPCM in this study population. This chapter expands upon the current evidence on the association between anaemia and PPCM by investigating different features of anaemia in PPCM. Specifically, it was observed that anaemia was accompanied by a deficiency in iron in nearly half of the women diagnosed with PPCM. Moreover, being iron deficient significantly increased the risk of PPCM among anaemic. The odds of the disease associated with having iron deficiency was greater than the odds associated with other contributors of anaemia. Combining of Hb measurements with sFe or hepcidin values allowed for the detection of up to 13% more PPCM cases in the population than would have otherwise been captured by Hb concentrations alone. However, this improvement in classification accuracy was not statistically significant.

Despite a long history of policy commitment and interventions (56), the prevalence of anaemia among pregnant women in India is among the highest in the world (223). It is estimated that about 20% of maternal deaths in India are directly related to anaemia, and another 50% are associated with the condition (56). As a complication of pregnancy, anaemia has been associated with various adverse outcomes in both mothers and infants, including low infant birthweight, pre-term birth, and intrauterine growth restriction (128, 129). In this analysis, having moderate to severe anaemia at the first antenatal care visit was associated with a nearly threefold increase in the odds of developing PPCM, which is consistent with results from previous studies (23, 24, 49). These findings hint at a potentially causal role of anaemia in this disease, which should be investigated in future studies.

While pregnancy is associated with normal haemodynamic changes that can induce anaemia, the causes of anaemia vary wildly, ranging from nutritional deficiencies to genetic abnormalities. Previous studies have shown that most instances of anaemia in pregnancy and heart failure are due to inadequate iron availability in the body (105). The results of this analysis confirm these results, as iron deficiency was the most common contributor of anaemia in this study population, although the prevalence observed here was lower than what has been reported in the literature (57). This may be due in part to the fact that most cases of anaemia in pregnancy are generally assumed to be caused by and thus treated for iron deficiency, even though the additional lab test beyond haemoglobin that are needed to confirm this diagnosis are not always performed. However, no studies to date have explicitly investigated the exact aetiology of anaemia in PPCM. For the first time, this study showed not only that anaemia was associated with PPCM, but also that most cases of anaemia in women with PPCM were accompanied by a deficiency in iron. Previous studies have proposed that iron deficiency itself may be involved in the development of cardiomyopathy even in the absence of anaemia (54, 135, 136). The results of the current analysis are consistent with this theory. Importantly, nearly one quarter of women diagnosed with PPCM in this population were deficient in iron without being anaemic. Moreover, there was a suggestion of a positive (albeit not statistically significant) interaction between anaemia and iron deficiency, such that having iron deficiency increased the odds of disease by almost three time in mothers who are anaemic. However, the present interaction analysis only answers whether the effect of anaemia on the risk of PPCM varies depending on a mother's iron deficiency status, suggesting that anaemic mothers who are also iron deficient are at an increased risk compared to those with non-iron deficient anaemia. The results do not answer the question of whether the effects of iron deficiency exist independently of the effects of anaemia. Thus, the findings of this study incite further investigations into the direct role of iron deficiency itself on the risk of PPCM in the absence of the effects of anaemia, through mechanistic mediation analyses.

Potential contributors to the development of anaemia include nutritional deficiencies in folate and vitamin B12. While it was not possible to measure the association between vitamin

B12 and the risk of PPCM in this analysis, previous studies have found that vitamin B12 deficiency was relatively rare in heart failure patients (224-226). Instead, elevated serum B12 levels have been observed in heart failure cases, which authors have attributed to increased secretion from damaged liver cells (225, 226). Higher concentrations of vitamin B12 in heart failure patients were positively correlated with New York Heart Association (NYHA) functional class and NT-proBNP levels, but were not associated with prognosis (225, 226). Future studies will be needed to expand upon the findings of this chapter, and elucidate the potential contribution of vitamin B12 deficiency to the association between anaemia and PPCM.

In some cases anaemia may be caused by inherited genetic conditions resulting in a change in the structure, quality and quantity of haemoglobin produced in the blood. Globally, South East Asian populations are the most affected by haemoglobinopathies, with India having some of the highest carrier frequencies for some Hb variants (220). In the state of Assam, where a large portion of this study population originates, carrier frequency have been reported as high as 51% for HbE trait (220). In the current study population, approximately one quarter of the women showed haematological evidence for abnormal Hb variants. Yet, having a haemoglobinopathy was not associated with an increased odds of PPCM in this population. Further studies would be needed to further substantiate this evidence.

7.7. Strengths and Limitations

The results of this study add to the current literature on PPCM by investigating for the first time the specific aetiology of anaemia in this disease, and reporting the prevalence of iron deficiency among anaemic women diagnosed with PPCM in India. The main strength of this study was the investigation of the different aetiologies and erythropoietic characteristics of anaemia using robust laboratory analyses.

On the other hand, the main limitation of this study was the small sample size which resulted in a lack of power to detect a statistically significant interaction effect. Generally, even larger sample sizes are needed to detect causal interactions, which would additionally require

strong assumptions regarding uncontrolled confounding. Thus, while the magnitude and direction of the observed effects provided some crucial insights into the potential importance of the interaction between anaemia and iron deficiency, it was not possible to assess whether the observed effect of heterogeneity among iron deficiency strata was due to the iron deficiency state itself in this study. Likewise, although the addition of iron markers measurements seemed to have improved the classification accuracy of haemoglobin concentrations, there was likely insufficient power to detect a statistically significant change in diagnostic accuracy.

Importantly, the laboratory analyses of haemoglobin which were analysed in this chapter were conducted at the time of enrolment in the study. This cross-sectional assessment of the biomarker at the time of disease diagnosis presents a challenge to establishing the direction of the association between anaemia and PPCM. Namely, anaemia may have resulted from inflammation induced iron deficiency, instigated by the heart failure condition itself (i.e. reverse causality). However, the results of the previous chapter showed strong evidence that PPCM was associated with depleted iron status rather than inflammation itself (see Chapter 6). Thus, it was estimated that reverse causality would have been unlikely (though not impossible) source of bias in this analysis. The results of the sensitivity analysis of antenatal anaemia provide further evidence for the temporality of the association between anaemia and PPCM, since measurements of haemoglobin in the antenatal period tended to precede the onset of heart failure symptoms in the population. Regardless of the aetiology, anaemia represents a state of impaired erythropoeitic activity, which is expected to develop progressively over time. Thus, it was reasonable to assume that the moderate to severe anaemia observed among the women in this population would have developed prior to the acute symptoms of heart failure that characterise PPCM.

However, the more severe the anaemia, the more likely it is to be caused by a combination of factors. One potential cause of anaemia that could not be investigated in this project was a deficiency in vitamin B12, as the measurements of the marker were unreliable. This was an important limitation because the most common cause of severe vitamin B12 deficiency worldwide is pernicious anaemia; anaemia resulting from an auto-immune disease that affects

the gastro-intestinal absorption of Vitamin B12 (227). Therefore the contribution of vitamin B12 deficiency to the association between anaemia and PPCM could not be compared to that of iron deficiency in this study. Thus, while the results of this analysis may suggest an important role for iron deficiency, several different mechanisms may actually be at play in the association between anaemia and PPCM, which could not be assessed within the scope of the present study. Further mechanistic analyses are needed to elucidate the effect attributable to iron deficiency itself in the observed association between anaemia and PPCM.

7.8. Chapter summary

Consistent with previous evidence, the analyses of this chapter demonstrated that in the perinatal period, anaemia is associated with an increased odds of PPCM. For the first time, the aetiology of anaemia in PPCM was investigated. The most prevalent cause of anaemia in this population of Indian women was a deficiency in iron, and moreover, women with anaemia accompanied by iron deficiency had higher odds of developing PPCM compared to those with non-iron deficient anaemia. There was some preliminary evidence that maternal iron status might modify the effect of anaemia on the odds of PPCM in this study population. When assessed together, serum concentrations of Hb and markers of iron status were moderately useful for identifying women with PPCM in this population of Indian women. The following chapter will assess whether an Indian mother's serum concentrations of prolactin can provide more useful information about her PPCM status than her iron profile.

Chapter 8. Serum concentration of prolactin in Indian women with Peripartum Cardiomyopathy

8.1. Background & Scope

Several mechanisms have previously been proposed for the pathophysiology of PPCM, including inflammation, oxidative stress and vascular imbalance. Recently, it has been suggested that these various aetiologies of PPCM may converge on one common pathway involving elevated levels of the 16 kDa fragment of prolactin (59, 60). However, the review of the literature (Chapter 2) demonstrated that the evidence for prolactin imbalance in women diagnosed with PPCM compared to women without PPCM is limited. To date, the utility of prolactin for diagnosing women with PPCM has never been assessed. Moreover, given the wide variation in the prevalence of risk factors for PPCM across different geographical regions, the prolactin mechanism may or may not be relevant for Indian women diagnosed with PPCM.

Therefore, the aim of this chapter was first to ascertain whether an association exists between serum prolactin concentrations and PPCM among Indian women. Second, the chapter sought to assess whether serum concentrations of prolactin or maternal iron status was a greater determinant of PPCM status, by comparing the diagnostic accuracy of these different biomarkers.

8.2. Methods

8.2.1. Study population

This secondary analysis was conducted on the data collected from the MaatHRI case-control study of heart failure in pregnant Indian women, as described in Chapter 4. Women diagnosed with PPCM were enrolled in the study during pregnancy and up to six months postpartum. Controls were postpartum women without heart failure who had given birth within two days of the case presenting at the hospital.

Previous studies have suggested that in women with healthy pregnancies, prolactin levels rise steadily during gestation to reach peak levels at birth for up to three days after birth (228, 229). Thereafter, prolactin levels are expected to decrease but remain above normal for several weeks, especially in breastfeeding mothers (230, 231). To take these variations into account, two women diagnosed with PPCM in the second trimester of pregnancy (gestational age <28 weeks) were excluded from the analysis. Furthermore, the study population was categorised into the

following groups corresponding to the pregnancy stage of the mother at the time of enrolment into the study: 1) late pregnancy (defined as > 28 weeks of gestation), 2) the first three days postpartum, and 3) more than three days postpartum. The final dataset investigated in this chapter comprised 34 women diagnosed with PPCM, and 165 women not diagnosed with heart failure, for whom measurements of serum concentrations of prolactin were available.

8.2.2. Biomarkers

Serum concentrations of prolactin were measured by chemiluminescence immunoassay (ATELLICA). The iron indices investigated in this analysis were serum iron (sFe), transferrin saturation (TSAT), soluble transferring receptors (sTfR), the sTfR-F index and hepcidin. All blood samples were collected non-fasted at the time of enrolment in the study. See Chapter 4 for more details on the laboratory assays for the iron markers.

8.3. Statistical analyses

8.3.1. Descriptive analyses

The distribution of prolactin levels in the study population was slightly right skewed. Therefore, median values and interquartile ranges were calculated and compared between women with PPCM and women without PPCM in the different pregnancy periods, using non-parametric Wilcoxon rank sum tests.

8.3.2. Association between prolactin concentrations and PPCM

In the study, cases were enrolled throughout pregnancy and postpartum while the control group was exclusively enrolled within the first two days after birth. To ensure the prolactin measurements between the two groups were comparable, the analysis for the association between prolactin and PPCM was carried out only among the participants enrolled in the first three days postpartum. Thus, 21 women diagnosed with PPCM and 170 controls were included in the analysis of the association between serum concentrations of prolactin and PPCM.

Prolactin levels were tested for deviation from linearity using univariable fractional polynomials. Fitting a second-degree fractional polynomial showed a non-linear relationship between this variable and the link function for the odds of PPCM. However, upon further

investigation this was determined to be due to the effect of one influential outlier; a women diagnosed with PPCM with abnormally low serum concentration of prolactin. The outlier was kept in the dataset, but serum levels of prolactin were included in the regression model as an untransformed continuous variable.

Reported determinants of prolactin levels in the early postpartum period include mode of birth and breastfeeding duration (232). Breast feeding duration was not measured in the heart failure study, and mode of birth was previously shown to have no association with PPCM in this study population (see Chapter 5). Therefore, these factors were not controlled for in this analysis. However, it has also been reported that hypoxia may inhibit the production of the 16 kDa form of prolactin (233), which has been directly implicated in mechanistic pathways leading to PPCM (59). Therefore, anaemia was considered a potential confounder of the association between PPCM and prolactin, and the analyses were adjusted for this factor.

For ease of interpretation, the association between continuous values of prolactin and the odds of PPCM was presented as the effect associated with a 100 ng/mL increase from the median concentration of prolactin (171.96 ng/mL), to account for the small effect sizes.

8.3.3. Classification accuracy of prolactin

In the event of a significant association between prolactin concentrations and PPCM, a non-parametric receiver operating characteristic (ROC) curve was to be generated to assess the accuracy of prolactin for identifying the PPCM status of women in the first three days after birth. The anaemia-adjusted ROC curve (AROC) for prolactin with its corresponding adjusted AUC^{ROC} (aAUC) and 95% confidence interval would be estimated to account for the potential effect of anaemia on prolactin (207). The aAUC can be interpreted as the weighted average accuracy of the marker across values of the confounding variable, with weights corresponding to the proportion of cases in each level of the variable (207, 234). The aAUC for prolactin would be compared to the AUC for each of the iron indices investigated previously for women in the first three days after birth (221).

8.3.4. Power calculations

For the fixed sample of 21 cases and 170 controls enrolled in the first three days after birth, the study had 80% power (alpha = 5%) to detect a 13.74 ng/mL difference in the mean concentrations of prolactin between PPCM cases and controls without heart failure. This expected difference in means was compared to the observed difference to assess the power achieved in this analysis.

8.3.5. Sensitivity analyses

A sensitivity analysis was conducted to assess the effect of the outlier (described in Section 8.3.2) on the results of the analysis, by removing the outlier from the data.

An additional sensitivity analyses was done for the association between prolactin and PPCM by including prolactin measurements for women enrolled in the third trimester of pregnancy (n=5).

As mentioned previously, blood parameters were primarily missing for women who died after enrolment but before blood samples could be collected. Since high prolactin concentrations may have theoretically increased the severity of heart failure symptoms among cases, the missing prolactin observations may not be missing at random. Thus, the prolactin concentrations among cases with severe left ventricular dysfunction (ejection fraction < 30%) were compared to concentrations among cases with ejection fraction between 30-44%. In the absence of any association between prolactin concentrations and the severity of left ventricular dysfunction, the complete-case analyses presented here were repeated with a dataset with the missing values imputed by chained equations.

8.4. Results

8.4.1. Association between prolactin concentrations and PPCM

After excluding two women whose symptoms of heart failure started before the third trimester, the prolactin levels of 42 women diagnosed with PPCM were compared to those of 170 women without heart failure. Among those with PPCM, six women were in their third trimester at the time of enrolment, 21 were enrolled within three days of childbirth and 15 were enrolled

more than three days after birth. In the first three days after birth, the median prolactin levels of women diagnosed with PPMC was 40 ng/mL higher than that of women without heart failure, however this difference was not statistically significant (p=0.57) **(Figure 8.1)**. PPCM cases who were more than three days postpartum had significantly lower levels of prolactin than all others in the study population.

The regression analyses for the association between prolactin and PPCM in the first three days postpartum included 18 women diagnosed with PPCM. Among the women enrolled in first three days after birth, there was the indication of a small and non-significant positive association between concentrations of prolactin and PPCM in the unadjusted model (OR=1.05, 95% CI; 0.74, 1.42). After adjusting for anaemia, every 100 ng/mL increase from the median serum prolactin concentration was associated with a 12% increase in the odds of PPCM, but the association was not statistically significant (OR=1.12, 95% CI: 0.77, 1.54).

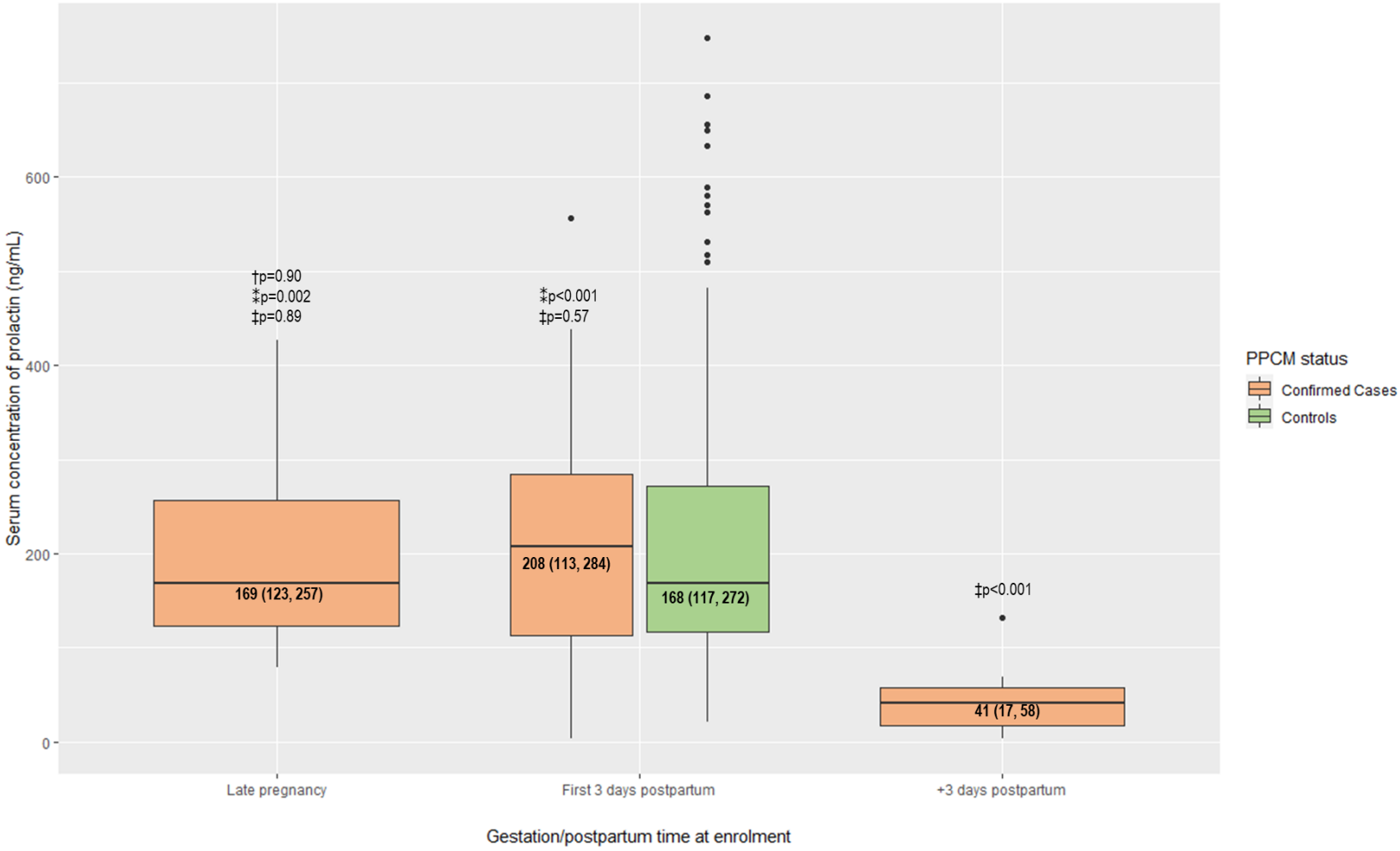


Figure 8. 1. Medians and interquartile ranges of prolactin concentrations by PPCM status and pregnancy period. Values represent medians and interquartile ranges for the corresponding group of women. p-values are given for the Wilcoxon rank sum test for the difference in medians: †comparing to first 3 days postpartum (cases), ⁑ comparing to 3+ days postpartum, ‡ comparing to first 3 days postpartum (controls).

8.4.2. Classification accuracy of prolactin

Since an association could not be established between serum concentrations of prolactin and PPCM in this population, it was unnecessary to estimate the classification accuracy of the biomarker for PPCM status.

8.4.3. Power estimations

This analysis had only 9.6% power to compare serum concentrations of prolactin between cases and controls. The difference observed between the mean prolactin concentration of women diagnosed with PPCM and women without PPCM in the first three days after birth was 11.8 ng/mL. This difference was smaller than the value expected to be observed at 80% power (13.74 ng/mL).

8.4.4. Sensitivity analyses

After removing the outlier from the dataset, a 100 ng/mL increase in serum prolactin concentrations was associated with an 18% increase in the odds of PPCM status (OR=1.18, 95% CI: 0.82, 1.63) in the adjusted model, which represented a marginal increase (5%) in the effect size.

When the cases enrolled during the last trimester of pregnancy were included in the analyses, the final results showed similar but slightly weaker associations between prolactin levels and PPCM status (adjusted OR=1.09, 95% CI: 0.79, 1.47).

Finally, prolactin concentrations did not appear to be associated with the severity of left ventricular dysfunction among the cases (difference in medians = 103 ng/mL, p=0.11). Thus, an imputation analysis was carried out to estimate the missing values. The results of the imputation analysis were similar to those from the complete-case analysis presented here (OR=1.10, 95% CI: 0.78, 1.54 for the adjusted association between prolactin and PPCM).

8.5. Discussion

According to the prolactin-mediated mechanism for the pathogenesis of PPCM, women diagnosed with the disease are believed to have elevated serum concentration of the hormone. Some clinical trials for the treatment of PPCM using the dopamine antagonist Bromocriptine have

provided evidence in support of this mechanism. Results from these trials found that a reduction of prolactin levels through the administration of Bromocriptine in addition to standard therapy was associated with greater recovery of left ventricular ejection fraction among women diagnosed with PPCM compared to controls (62, 63, 89). Although the epidemiology of PPCM is known to show large geographical variations, the majority of these trials have been conducted among high-income populations. It was therefore important to determine whether this mechanism was also relevant in a population of Indian women diagnosed with PPCM.

Over the course of a normal pregnancy, maternal prolactin levels increase steadily, peaking in the first 48 hours after birth as the inhibitory effect of the placental steroid hormones disappear following placental separation (235). Accordingly, it was observed in this study that women with PPCM in the antenatal period had serum concentrations of prolactin that were relatively lower than the levels observed in those who had recently given birth, although the difference was not statistically significant. However, in the first three days after birth, the Indian women diagnosed with PPCM appeared to have higher average concentrations of prolactin compared to women without PPCM. While this difference was not statistically significant, it is almost twice as large as what has previously been reported in a similarly sized study of postpartum women diagnosed with PPCM (236). It is unclear whether this difference is clinically meaningful, since the concentrations of prolactin reported among the women with PPCM were within the normal range expected for pregnant women (103, 104).

After birth, prolactin levels have been shown to naturally decrease over time, although levels surge with every initiation of breastfeeding (228). Concentrations generally remain elevated for up to six weeks in breastfeeding mothers, but they may lower to non-pregnancy levels by the third week postpartum in non-lactating mothers (230, 237). Thus, the reduction on prolactin concentrations observed in women diagnosed with PPCM later in the postpartum period may be attributed either to the natural decrease in prolactin secretion over time, or to difficulties in breastfeeding as a result of their heart failure symptoms. Nevertheless, since none of the women without PPCM were enrolled beyond the first three days after birth, it is unknown

from the current study whether these levels are higher than the normal values that would be expected at this point in the postpartum period. Further studies are therefore needed to better characterise the association between prolactin and PPCM, by taking into account the various factors affecting prolactin secretion in postpartum women. Prospective studies with longitudinal measurements of prolactin over the course of pregnancy and the postpartum period would help determine whether this association is observed during the antenatal period, or if it is specifically related to the postpartum surge in the hormone. It would also be useful to determine how long this effect remains into the postpartum period. However, such longitudinal biomarker studies are likely to be costly and lengthy, as they would require enrolling a large number of participants to investigate a rare disease such as PPCM.

The prolactin mechanism for the pathogenesis of PPCM suggests that the disease may be caused by an increased expression of the prolactin cleaving enzyme Cathepsin-D, which converts prolactin into its antiangiogenic 16-kDa form (59). A recent study has found that the secretion of Cathepsin-D is significantly decreased in hypoxic environments, which leads to a reduction in the generation of the 16-kDa isoform of prolactin (233). Tissue hypoxia is the main consequence of anaemia, and can lead to severe organ dysfunction. Consistent with this evidence, the odds ratio for the association between prolactin levels and PPCM was increased after adjusting for anaemia in the current analysis. This suggested that anaemia may mask some of the effect of prolactin on PPCM in this study population. Nevertheless, the previous chapter demonstrated that anaemia itself was associated with an increase in the odds of PPCM, and that association was three times greater than the effect of prolactin in this population. These results suggest that several mechanisms may be at play in this syndrome of heart failure among the Indian women investigated, potentially including an imbalance in prolactin but certainly including nutritional deficiencies. Future clinical trials comparing the utility of iron treatments to Bromocriptine among Indian women would help to clarify the relevance of this prolactin-mediated mechanism for PPCM in this population, compared to maternal iron deficiency and anaemia.

8.6. Strengths & Limitations

The current analysis suffers from several limitations that mainly stem from the small size of the study population and the design of the original heart failure study, which was not intended to specifically investigate PPCM or the prolactin mechanism. Consequently, certain elements of the study design, which would have been needed to ensure a robust investigation of the association between prolactin and PPCM, were not available.

First, given the expected changes in prolactin concentrations throughout the peripartum period, cases and the controls should have ideally been assessed at similar (if not the same) time points in their gestation or postpartum period. The difference in the time of enrolment resulted in a lack of comparability in the prolactin concentrations of the two study groups, which severely undermined the validity of the analysis. While the current study attempted to mitigate this by restricting the analyses to women who were enrolled within the same gestational window, this came at a significant cost to the sample size. Thus, the lack of significant findings were likely due to the limited power resulting from a small study population size, as opposed to the true absence of an association.

Additionally, not only are prolactin levels known to vary naturally throughout the day, but concentrations of the hormone also vary widely as a factor of breastfeeding duration and frequency (228, 238, 239). Thus, it is generally suggested that prolactin measurements be repeated at least three time to account for these large intra-person variation in the hormone. However, no data was collected on breastfeeding behaviour and prolactin was only measured once in this study. Consequently, the results of the current analysis must be interpreted with caution. In theory, matching the women diagnosed with PPCM and controls without heart failure on the time-period of pregnancy and postpartum could have address some of these comparability issues. Nonetheless, it would be difficult to exactly match the participants on their breastfeeding behaviours.

The systematic review conducted in Chapter 2 identified only two studies that investigated prolactin metabolism in women diagnosed with PPCM (103, 104). One of these

studies included five participants with PPCM and another included 21 participants, compared to the 18 cases included in the current analysis. Moreover, the authors in the previous studies did not report any information on the postpartum window or the breastfeeding status of the study population, and it was unclear if the prolactin analyses were performed more than once. Thus, in spite of its limitations, the present study adds to the scarce evidence currently available on the association between prolactin and PPCM, and therefore has inherent scientific value. Nevertheless, more studies are needed to improve on this evidence by addressing these limitations, and more robustly assess this association in different population.

8.7. Chapter summary

The current chapter explored the association between prolactin and PPCM in a population of Indian mothers. However, this suggested association could not be replicated. In light of this lack of evidence, the utility of the iron markers for classifying women by PPCM status could not be compared to that of prolactin. The study suffered from some important limitations, mainly a small sample size and lack of matching on pregnancy time-period and breastfeeding behaviour, which will need to be addressed in future studies examining the role of prolactin in PPCM.

Chapter 9. Discussion and conclusions

9.1 Summary of findings

In a population of women in Northeast India, peripartum cardiomyopathy (PPCM) was characterised by negative iron balance and increased iron mobilisation, consistent with absolute iron deficiency. Between the last trimester of pregnancy and the postpartum period, women with depleted iron stores were significantly more likely to be diagnosed with PPCM than those with adequate reserves. Furthermore, not only was antenatal anaemia an important risk factor for PPCM in this population, but the odds of disease were also significantly greater for anaemic women with depleted iron stores than for anaemic women with adequate iron stores. This is the first study to provide evidence that iron deficiency *per se* may contribute to the pathology of PPCM, perhaps even independently of the effects of anaemia. In contrast, there was limited evidence to suggest that PPCM was associated with an increase in serum concentrations of prolactin. This association was poorly investigated in published literature, and could not be established in the study population. While serum concentration of prolactin could not be used to detect PPCM in the population, markers of iron status such as soluble transferrin receptors (sTfR) were moderately useful in identifying women diagnosed with PPCM, especially at levels that were indicative of iron deficiency.

9.2. Impact of the COVID-19 pandemic

This project was largely a secondary data analysis of an ongoing hospital-based unmatched case-control study of heart failure in pregnant and postpartum women. The data analysed was collected between February 2019 and October 2021, in the northeast region of India. During the COVID-19 pandemic, hospital births in the region were significantly reduced and the case-fatality for heart failure increased to more than 60% (147). This led to a decrease in enrolment rates from March to December 2020 as women were either not coming to the hospitals or were dying soon after reaching the hospital. Overall, the MaatHRI heart failure study experienced a 50% decrease in enrolment across all study hospitals over the course of the pandemic. Given that the incidence of PPCM in the study population was approximately one in

over 4,000 hospital births, such a reduction in the enrolment rates meant a significant decrease in the size of the final study population, which was an important limitation of the project, as will be discussed below.

During the first wave of the COVID-19 pandemic, from March to August 2020, it was not possible to analyse blood investigations for women enrolled in the study, as national restrictions prohibited shipment of blood samples from hospitals to the national laboratory where all samples are analysed. Additionally, an overall increase in the case fatality for heart failure among pregnant and postpartum women led to a higher rate of missing biomarker data among the cases. In this study, there was evidence to suggest that women with severe iron deficiency may be more likely to have severe symptoms of heart failure (Chapter 6). Therefore, the missing data may not be missing at random, and is likely to have resulted in biased estimates. In the case of this project, the measured association between the concentration of iron markers and the risk of PPCM are likely to be an underestimation of the true relationship.

In this DPhil project, half of the women diagnosed with PPCM were enrolled after the start of the pandemic in March 2020. However, since the study was not initially designed to collect information about COVID infections, it is unclear how many of these women may have been infected with the virus at the time their iron status was investigated. There is growing evidence suggesting that severe infections with the COVID-19 virus are associated with inflammation-driven imbalances in iron homeostasis upon admission (240, 241). Specifically, patients diagnosed with severe COVID-19 infection present with hyperferritinemia and have higher hepcidin concentrations than other groups (240). Thus, any unknown COVID-19 infection in the study population could have caused an increase in serum concentration of ferritin and hepcidin, resulting in an attenuation of a true association with PPCM. However, there were no positive test results among the women recently enrolled in the MaatHRI projects who had been tested for COVID-19 infections. Although this does not completely preclude the possibility that some participants were infected at the time of enrolment, it was estimated that the effect of COVID-19

infections on the associations observed between iron deficiency and PPCM was a minor concern for the group of women included in the DPhil project.

9.3. Strengths and limitations

9.3.1. Strengths

The research presented here represents one of the few and largest epidemiological investigation of PPCM in an Indian population to date (242-244). As such, it provided valuable insights into the characteristics of this disease in a poorly studied population. The epidemiological data was derived from a robust multi-centre collection of data from clinical examinations, laboratory measurements, and echocardiographic imaging. Unlike the majority of studies that have previously investigated the molecular profile of PPCM, relative measures of risk for the association between the iron markers and PPCM were reported, which were also adjusted for confounding to account for the influence of different factors on the associations of interest. Additionally, the assessment of biomarker status was blinded to the participant's PPCM diagnosis by design, in order to prevent any measurement bias of the biomarker results (245). Thus, the DPhil project improved upon the studies currently reporting the molecular profile of PPCM by addressing some issues relating to the quality of previous investigations (64).

The project also adds to the current literature by investigating for the first time the iron profile of women diagnosed with PPCM. A robust review of the literature ensured the formulation of evidence-based hypotheses for the biological plausibility of an association between iron deficiency and PPCM. These hypotheses can be useful for informing the direction of future studies aiming at identifying risk factors for PPCM. In that way, the hypotheses generated in this project lay the foundation for formulating new policies, identifying points of intervention to address this complication of pregnancy, and improve maternal health. Moreover, while most studies investigating iron status in low-and-middle income countries rarely include assessments beyond the measurement of haemoglobin, this DPhil project coupled the measurement of erythropoietic markers with the thorough investigation of a panel of iron indicators. This provided a well-

rounded picture of maternal iron metabolism in the study population, which was fundamental for elucidating the exact nature of iron deficiency in women diagnosed with PPCM. Having a clear picture of the iron profile of these women is necessary for understanding the underlying mechanisms at play, and therefore, anticipating disease progression and formulating the most appropriate course of corrective treatment. Moreover, the results of this project provide actionable insights on the association between maternal iron status and PPCM by estimating cut-off values for the biomarkers. If validated in future population studies, these cut-points can be valuable in the future for assessing a pregnant woman's risk of being diagnosed with PPCM and guiding clinical decisions.

Finally, although it is the basis for several clinical trials (61-63) and expert recommendations for the treatment of PPCM (17), the results of the systematic review (Chapter 2 (64)) found that the role of prolactin metabolism in the pathology of PPCM was poorly investigated in population studies. This DPhil project contributes to the current evidence, and demonstrates that more work is still needed to investigate the putative association between prolactin and PPCM. Furthermore, although prolactin has been suggested to directly contribute to the pathogenesis of PPCM, no studies to date have investigated the utility of this biomarker as a diagnostic marker for PPCM. This DPhil project lays the foundation for this work by assessing for the first time the classification utility of prolactin for PPCM. While the results of this analysis were inconclusive, they illustrate the need for future studies to carry out similar investigations.

9.3.2. Limitations

The main limitation of the DPhil project was the small number of PPCM cases which often resulted in a disproportionate distribution of observations across levels of categorical variables and measures of effect with very wide confidence intervals. While this research project was adequately powered to conduct the primary investigation of the associations with iron deficiency and anaemia, there was not enough power to observe significant associations in secondary analyses, such as the interaction effects (Chapter 7) and the association with prolactin (Chapter

8). Additionally, the small sample size of the study also limited the assessment of the classification utility of the iron markers (Chapter 6), since the optimal cut-points estimated in the analyses could not be validated in the study population. The lack of precision in the associations estimated in these secondary analyses limits the strength of the conclusions that can be reached from the results. Thus, these analyses were largely exploratory in nature, and only provide a first indication of the nature of these associations. Nonetheless, these preliminary results can serve as hypothesis-generating starting point for further investigations.

The hospital-based design of the MaatHRI heart failure study also presents some potential limitations with respect to selection bias, as the study may have missed potential cases in the community who did not present at the hospital or died before reaching the hospital. An inherent problem in hospital-based case-control studies is that the controls may not be representative of the general pregnant population because women who came to the hospitals for childbirth may have pregnancy-related or medical complications including severe anaemia. However, in such cases, the effect sizes are more likely to be attenuated, thus leading to an underestimation of the true effect. In addition, a recent report found the rate of hospital births in the state of Assam to be at about 73% (246) therefore, it was expected that a majority of pregnant women in the population would have been captured by the network of hospitals involved in the MaatHRI project. It is also possible that some PPCM cases with milder symptoms may not have presented at the hospital, as symptoms of heart failure such as breathlessness and fatigue could be mistaken for normal pregnancy discomfort. This may have led to a bias in the study population towards more severe cases who are more likely to have other comorbidities such as nutritional deficiencies, hypertensive disorders or anaemia. However, as PPCM is a severe cardiac complication characterised by significantly reduced ejection fraction, it is uncommon for these cases to present with such mild symptoms of heart failure as to be confused with the symptoms of pregnancy. Instead, women diagnosed with PPCM would have likely sought emergency care for acute heart failure whenever possible. Indeed, a significant number of the PPCM cases enrolled in

this study presented postpartum at emergency departments with severe symptoms of heart failure.

Another important limitation was the cross-sectional assessment of the blood indicators in the original case-control study, which meant that the iron biomarkers were measured after the onset of heart failure, making it difficult to determine unequivocally the direction of the observed associations. In this DPhil project specifically, the outcome of interest could have reasonably influenced some of the exposures measured, as the heart disease itself may lead to iron deficiency through inflammatory mechanisms (102, 107). However, by characterising the iron profile of women diagnosed with PPCM, this DPhil project has demonstrated that PPCM was mainly associated with absolute iron deficiency, as opposed to inflammation-induced iron restriction. Moreover, it was reasonable to assume that cellular iron deficiency was unlikely to occur acutely at the time of enrolment, but rather would have been the result of long-term depletion, thus likely prior to the onset of acute heart failure. Nevertheless, the possibility of a reverse association cannot be ruled out.

The lack of matching on gestational or postpartum stage was another potential source of confounding in this project, and therefore an important limitation. Indeed, while women in the control group were enrolled within two days of having given birth, those diagnosed with PPCM were enrolled between the third trimester of pregnancy and up to six months postpartum. The natural haematological changes that occur over the course of pregnancy and the puerperium can significantly affect the results of the blood investigations. In particular, parturition can lead to important shifts in maternal blood indices, making it difficult or even inappropriate to compare the measurements between pregnant and postpartum women. However, evidence from the literature supported the assumption that several of the iron markers under investigation remain relatively stable during the perinatal period (especially soluble transferrin receptors (117)). Since the control group was recruited within 48 hours after childbirth,, serum concentrations of these markers were expected to remain low, even somewhat decreased compared to concentrations among PPCM cases who were still pregnant. Additionally, the PPCM cases who

were enrolled later in the postpartum period would have been more likely to experience an increase in iron availability as iron stores replenish steadily throughout the lactation period. Consequently, any potential confounding that may have resulted from the lack of matching was likely to lead to an underestimation of the association between iron deficiency and PPCM.

This may not have been the case for the prolactin analyses, however, as serum concentrations of prolactin have been shown to vary widely throughout the pregnancy and across the postpartum period (228, 238, 239). Thus, ideally, all women involved in this analysis should have been enrolled at the same gestational or postpartum stage in order to ensure that the prolactin measurements were comparable. That being said, since PPCM can occur at any time between the end of pregnancy and six months postpartum, it would have been difficult to accrue enough patients at a single time point to ensure adequate statistical power for a matched analysis. This issue was addressed by restricting the analysis to women enrolled within the same pregnancy window. However, this came at a cost to the statistical power, and also, there may have been some differences remaining between the study groups (namely in breastfeeding behaviour), which may have still affected the prolactin analyses.

The MaatHRI heart failure study also lacked information about certain factors that may have directly impacted the measurement of the biomarkers investigated. Namely, there was no information about the status, duration and frequency of breastfeeding in the postpartum women, which are the primary determinants of prolactin concentrations in pregnant and postpartum women. This was because prolactin was not part of the laboratory analyses of the original study, but added later for the DPhil project. Without information about these factors, the measurements of prolactin were largely uninterpretable as it was not possible to assess their effect on the values observed. In that way, the results of the analyses presented in Chapter 8 suffer from the same limitations as previous studies of prolactin in PPCM (Chapter 2). Yet, those same limitations make it difficult to compare results across studies.

In this study, the analysis of the iron markers was also potentially biased by the fact that the participants were not in a fasted state at the time of blood collection. Indeed, serum concentrations of iron, transferrin saturation, and to some extent hepcidin have been shown to be acutely affected by the recent ingestion of iron (202). Therefore, it is possible that for some women included in this study the measurement of these markers may have reflected the acute absorption of iron in the food eaten that day, rather than their true iron status. However, this effect is likely to have been random as it would not have systematically impacted one study group over the other. Thus, despite this limitation, the associations estimated in the analyses remain meaningful and significant.

9.4. Implications & future research

9.4.1. The association between iron deficiency and PPCM

The study of biomarkers of iron status is a rapidly evolving field and an active area of research. The assessment of iron deficiency in pregnancy in particular remains fraught with difficulties owing to the profound haemodynamic changes associated with pregnancy, which affect several indices of iron status. Consequently, the physiology of maternal iron homeostasis and the establishment of reference cut-off values for defining iron deficiency in pregnancy are the subject of many recent investigations (117, 215). The associations investigated in this DPhil and their interpretation are based on the current understanding of maternal iron homeostasis and the physiology of these different markers, some of which are still relatively new in relation to the assessment of iron deficiency in pregnancy (215, 247). Currently, iron deficiency screening in pregnant women is often initiated only in the presence of abnormal haemoglobin levels, especially in resource-limited settings. However, the results of this study show that nearly one quarter of women diagnosed with PPCM in this population were deficient in iron without being anaemic. Thus, a significant proportion of women who are at an increased risk of PPCM may be missed by current screening practices. This DPhil project highlights the importance of including

routine iron investigations in the clinical work-up of pregnant women, even before the onset of anaemia.

This DPhil has also generated new hypotheses, and provided preliminary evidence for a causal role of iron deficiency in the pathogenesis of PPCM. As discussed above, the investigation of these hypotheses was limited in this study. However, future studies that are able to further establish a causal relationship between iron deficiency and PPCM can help to substantiate the associations observed in the analyses presented here. While randomised clinical trials are considered the gold standard for establishing causality, they would not be feasible for investigating iron deficiency and PPCM, as it would be unethical to withhold iron therapy from pregnant mothers who are known to be deficient in iron. Prospective cohort studies of PPCM would also be challenging, as such studies would require a large number of participants enrolled over a long period of time to accrue enough incident cases for analysis. Moreover, the longitudinal measurement of iron biomarkers would prohibit the assessment of an association between iron deficiency and PPCM, since any women who demonstrate abnormal iron status at any point over the course of the study would need to receive treatment for iron deficiency irrespective of her PPCM status. Alternatively, nested case-control studies where the cases and controls are selected from individuals within an established cohort study that included iron investigations could also help to establish causality. However, because many of the iron markers investigated in this project are not routinely measured in clinical practice, finding such existing cohorts of Indian women would be challenging. There are also registries, which have recently been established for the study of PPCM, such as the EURObservational Research Programme (151). These registries are particularly helpful for capturing a large number of PPCM cases and investigating the characteristics of the disease in the population. However, they are ill-suited to the estimation of the relative importance of different factors for the development of PPCM, as they do not include a disease-free comparison group. Thus, in spite of their intrinsic limitations, cross-sectional investigations are the most practical study design for substantiating the associations observed in this project in human populations. Animal studies similar to those that have investigated the

association between prolactin and PPCM, could more directly investigate whether there are any biological pathways leading to PPCM in pregnant subject with iron deficiency. Although useful for establishing mechanisms of effect, these studies may not always be directly translatable to human populations. Nevertheless, when used in conjunction with evidence from population studies, they may provide support for the strong biological plausibility of the observed associations.

In the current project, it was demonstrated that anaemic women with depleted iron stores had a higher risk of disease than iron-replete women who are anaemic for other reasons. This suggests that the association between iron deficiency and PPCM may be relatively independent of the effects of anaemia. This is supported by previous studies, which have demonstrated that iron deficiency itself may impair functional capacity and promote cardiomyocyte dysfunction, irrespective of the hypoxic and hemodynamic effects of anaemia (53, 54). However, to determine whether these associations are truly independent from each other would have required mediation analyses, with anaemia treated as the mediator of the association between iron deficiency and PPCM. Mediation analyses rely on robust underlying assumptions regarding the causal association between exposure and outcome, appropriate adjustment for confounding and typically demand high levels of statistical power to observe significant direct and indirect associations (248). In this study, few women were iron deficient without being anaemic, which meant that there was insufficient statistical power to conduct a mediation analysis of the effect of iron deficiency. In the future, larger studies with a robust clinical assessment of the different aetiologies of anaemia in the population could be used to perform such mediation analyses, and determine the direct and indirect effects of iron deficiency on the risk of PPCM.

Finally, although the findings of the DPhil project provide evidence of an association with iron deficiency, it is important to note that a deficiency in iron is unlikely to occur in isolation. Because nutritional deficiencies often occur concomitantly, it can be difficult to tease apart the effects of one from another. Thus, women with PPCM have been shown to have decreased levels of several micronutrients including selenium (77, 79), vitamin C (79) and now iron. Generally, maternal nutritional deficiencies result from inadequate dietary intake of energy and unbalanced

diets (249). Consistent with this evidence, women with PPCM in the study population tended to have a lower BMI than women without heart failure on average. This was in contrast to previous evidence from high income countries (179, 180), but consistent with data from low income countries (250). Moreover, nutritional deficiencies are also associated with larger socioeconomic and lifestyle risk factors, such as poverty, education or religion. Thus, while the current project suggests an association between iron deficiency and PPCM, this association should be interpreted within the context of global malnutrition among pregnant women in low income settings.

9.4.2. Diagnosis and risk stratification of PPCM

Currently, PPCM is a diagnosis of exclusion based on nonspecific clinical and echocardiographic evidence, which presents an important challenge in the study of this disease. The diagnosis of heart failure is often difficult in a pregnant woman presenting with breathlessness for the first time, as the pregnancy state itself often leads to clinical symptoms very similar to those of heart failure. Moreover, studies have repeatedly shown a low sensitivity and specificity for making a clinical diagnosis of heart failure based on clinical signs and echocardiographic imaging alone (251). Echocardiographic imaging of pregnant patients beyond 20 weeks of gestation can also present additional technical challenges to physical examination, which may affect the reliability of the diagnosis (252). Thus, there is a pressing need for research focusing on improving the diagnosis of PPCM in the general pregnant population, the risk stratification of pregnant and postpartum women, and the prediction of incident PPCM.

As mentioned above, the prospective studies that are needed to estimate the risk of incident PPCM in a population are likely to be difficult, especially in settings with limited resources. This DPhil project has demonstrated that iron markers are only moderately useful for distinguishing women who developed PPCM from those who did not, although in some cases this accuracy can be improved by including the joint measurement of haemoglobin concentrations. Of all the iron markers investigated in this study, serum concentrations of sTfR and hepcidin show the most promise for future clinical use. Since the development of a risk stratification model for PPCM was outside the scope of this project, the added predictive value offered by the iron

markers is unknown, as there was no baseline risk model upon which to improve. Moreover, the small study population restricted the analyses to single biomarker models only, and such simple models are likely to lack accuracy for predicting the risk of PPCM. Larger population studies would enable the development of risk stratification models that combine multiple biomarkers with other clinical and sociodemographic factors, to more accurately predict the risk of PPCM. Such large-scale studies have previously generated reasonable estimates for the risk of cardiovascular diseases based on combinations of biomarkers (253). Moreover, if the cut-off values estimated for the iron biomarkers in this study are validated in similar study populations, they can then be used to develop clinical scores for the risk stratification of pregnant and postpartum women. The validated cut-points could be therefore invaluable for translating the results of this DPhil project into clinically actionable insights.

It is important to note that the iron markers investigated in this project may lack specificity for PPCM, as mechanisms of iron deficiency have been shown to be involved in the pathology of different cardiovascular diseases (53). Thus, while they may be useful as part of a diagnostic workup for individuals at high risk of PPCM, markers of iron status may be of limited use for screening asymptomatic healthy pregnant or postpartum women for PPCM. Future studies could compare the iron profile of PPCM to that of other non-pregnancy related cardiomyopathies that may arise in pregnant women, and determine if there are any underlying differences that may be exploited for clinical purposes. Alternatively, other markers such as soluble fms-like tyrosine kinase-1(sFlt1), Relaxin-2 and Placental Growth Factor (PlGF), which reflect processes that are directly related to the pregnancy state (90, 254), have been shown to have significant diagnostic value in distinguishing PPCM from healthy women and women with non-pregnancy-related acute heart failure (88). These markers may be preferable to the iron markers investigated in this project to screen for PPCM, and thus warrant further investigation in the future.

Finally, as the association between iron deficiency and PPCM continues to be investigated, it will also be important to ascertain the prognostic utility of iron markers for women diagnosed

with PPCM. These investigations would be useful for the design of future clinical trials, as prognostic markers may be useful for randomisation and risk stratification(255).

9.4.3. Treatment of PPCM

The European Society of Cardiology (ESC) currently states that Bromocriptine may be considered for the treatment of patients diagnosed with acute PPCM in addition to standard heart failure therapy (17). However, the ESC also recognises that the usefulness and efficacy of this treatment has not been well-established by the existing evidence (classified as Class IIb evidence) (256). The administration of Bromocriptine to mothers diagnosed with PPCM should therefore be considered with caution, especially given that the effects of this drug inhibit production of breastmilk in postpartum women. It is now well-established that breastfeeding is associated with long-term benefits for both the mother and the baby. The promotion of exclusive breastfeeding for at least the first six months of life continues to be an important public health effort to improve maternal and neonatal health, especially in low- and middle-income countries (257). The implications of administering Bromocriptine to pregnant and postpartum women are therefore significant.

On the other hand, iron therapies are routinely given to pregnant and postpartum women, and are generally considered to be safe for both the mother and baby, although to a lesser extent in populations where malaria is endemic (258, 259). Previous clinical trials have shown that intravenous iron supplementation with ferric carboxymaltose can also improve symptoms, exercise tolerance, quality of life, and mortality rates in iron deficient patients with heart failure (17). Given the findings of the current research on the role of iron deficiency in PPCM, randomised clinical trials could be designed to compare the usefulness of iron therapy alone to that of iron with Bromocriptine for the treatment of heart failure in women diagnosed with PPCM. The results of these studies may provide important evidence to support a less problematic and more effective treatment for this complication.

The results of this project revealed that Indian women who were supplemented with iron and folic acid tablets for fewer than three months were significantly more likely to be diagnosed with PPCM. Specifically, the odds of disease were significantly reduced with every additional week of IFA supplementation during pregnancy. Thus, oral iron supplementation during pregnancy may be a simple way of reducing the risk of developing PPCM among pregnant women. However, oral iron therapy may be of limited use for improving acute symptoms of heart failure in women diagnosed with PPCM. Typically, oral iron therapy is not recommended for the treatment of iron deficiency in patients with heart failure, as it has been shown to be ineffective for iron repletion and did not improve exercise capacity in patients with heart failure with reduced left ventricular ejection fraction (260). The limited responsiveness to oral iron treatment is usually attributed to impaired iron absorption resulting from the chronic inflammatory response to heart failure (260). Yet the results of this project demonstrated clearly that despite inflammation, women diagnosed with PPCM had significantly decreased serum levels of hepcidin, which is indicative of increased iron absorption and mobilisation. These findings suggest that women with PPCM may be more receptive to oral iron treatment than other heart failure patients.

Importantly, intravenous iron has been shown to be a faster and more effective way of correcting iron deficiency, since its effects are not inhibited by hepcidin (261, 262). The more severe the symptoms of iron deficiency, the more likely the individual will benefit from intravenous rather than oral iron therapy (262). However, parenteral iron therapy requires greater clinical supervision, more medical resources and a risk of adverse reactions to the transfusions than oral iron (263, 264). Moreover, current data on the effect of intravenous iron on maternal and infant health outcomes are lacking. Thus, further studies are needed to compare the usefulness of intravenous and oral iron for the treatment of PPCM symptoms, and to also investigate the maternal and infant outcomes associated with each iron preparation.

9.4.4. The prolactin hypothesis for PPCM

It has been suggested that cardiac dysfunction in PPCM may result from an increase in the concentration of prolactin cleavage products and the subsequent production of anti-angiogenic factors (22). In this DPhil project, no significant association was observed between serum concentration of prolactin and the risk of PPCM in pregnant and postpartum Indian women. However, this lack of association does not necessarily translate to a lack of evidence for the prolactin hypothesis.

In human blood, prolactin is expressed in several isoform of varying lengths and biological reactivity. The mature 23-kDa variant constitutes the majority of the prolactin measured in the circulation, and is responsible for the maturation of the mammary glands during pregnancy, as well as the induction of breastmilk synthesis during the postpartum period (265, 266). However, other variants that result from the post-translational cleavage of the mature protein are also present, among which is the 16-kDa fragment. According to the proposed prolactin mechanism for the development of PPCM, the harmful effects of the hormone are directly attributable to the unique angiostatic and pro-apoptotic activity of this 16 kDa isoform. However, most of the automated immunoassays which are currently used for the measurement of prolactin are only able to measure the total concentration of all prolactin in the blood. It is estimated that between 60-85% of the prolactin measured by these assays is likely be the mature 23-kDa variant of the hormone (267). Furthermore, the different assays currently available for measuring prolactin in the blood show considerable variations in their ability to detect the different isoforms of the hormone (265). Thus, adequate assessment of the prolactin hypothesis for the pathogenesis of PPCM would require the profiling of the different prolactin variants present in the blood of women diagnosed with PPCM.

To date, only one study has attempted to conduct such an analysis in a sample of five lactating PPCM patients. The authors used a western blot assay, which allowed them to visually determine which of the different prolactin fragments were present in the blood of the participants

(59). In that study, the 16-kDa prolactin fragments were observed in three out of the five PPCM patients, whereas these fragments were "barely detectable" in the healthy lactating women (59). On the other hand, the 23-kDa form of prolactin was readily detectable in both groups of women. In this study, the authors neither assessed nor compared the concentration of these fragments in the two groups, thus the association between the presence of the 16-kDa prolactin fragments and PPCM could not be estimated. New methods are now available to conduct the quantitative characterisation of different prolactin isoforms found in human blood (268, 269). Future studies using these assays to specifically measure serum concentrations of the 16-kDa variant of prolactin, and estimating the risk of PPCM associated with levels of this isoform could provide better evidence for the prolactin cleavage hypothesis. However, the World Health Organization's (WHO) reference material that is widely used for the standardisation of these assays is exclusively based on the 23 kDa monomeric prolactin (270). Therefore, although these phenotyping assays can show good within-method agreement, the reference intervals are often assay dependent and vary considerably between methods(265). Consequently, the interpretation of these analyses may not always be comparable across studies. These assays are also likely to be relatively inaccessible, expensive and challenging to carry out for large population studies, especially in countries with limited laboratory resources.

It has been suggested that several of the proposed mechanisms for the development of PPCM converge into the prolactin mediated pathway. Despite the associations observed between PPCM and iron deficiency in this project, it is still unclear whether iron deficiency could also contribute to this prolactin cleavage pathway. While some evidence suggest that iron deficiency may be associated with lower levels of prolactin and impaired lactation (271-273), there is also evidence that depleted iron stores increase prolactin levels possibly through a decrease in dopamine synthesis (which occurs through iron-dependent enzymes) (274, 275). The latter would suggest that severe depletion of maternal iron stores during pregnancy could lead to increased production of prolactin beyond what is expected in a normal pregnancy, ultimately causing LV dysfunction and heart failure in women with no pre-existing cardiovascular diseases.

Furthermore, previous human studies both in pregnant and non-pregnant populations have found that high prolactin levels were associated with a higher incidence of anaemia, which is consistent with the observation that anaemia is a common comorbidity of PPCM (276-278). However, there is also emerging evidence to suggest that the hypoxic effects of anaemia may directly lead to a decrease in prolactin-cleaving enzymes, and consequently a reduction in the production of the 16-kDa form of the hormone (233). Mechanistic studies (likely using animal models) could be used to elucidate potential associations between these pathways, and establish the biological plausibility for a unified pathophysiology of PPCM that encompasses mechanisms of iron deficiency, anaemia and prolactin cleavage.

9.4.5. Importance of postpartum iron deficiency

Iron deficiency anaemia has long been recognised as an important condition affecting pregnant women, and it remains an important public health concern in India (223). Several adverse maternal and infant outcomes have been associated with this condition, including maternal anaemia, puerperal sepsis, low birth weight, and preterm birth (279, 280). Consequently, there has been a long history of policy commitment and interventions targeting routine iron supplementation for pregnant women in India (56). It is generally recommended that all pregnant women be supplemented daily with iron and folic acid during pregnancy to prevent adverse outcomes in both the mother and the baby (281).

In contrast, the puerperium period is conventionally thought to be a time of low iron deficiency risk for the mother, since maternal iron requirements radically decline with the birth of the baby (126, 282). Iron losses are also significantly reduced by postpartum amenorrhea and relatively little iron is lost through breast milk (126). Yet, recent investigations of low-income populations have shown that maternal iron stores may remain depleted for several months following childbirth, especially if the mother was deficient at the end of her pregnancy and remains unsupplemented following parturition (126, 282). Given the limited data on the long-term health implications of postpartum iron deficiency for the mother, the WHO only cautiously

recommends that oral iron supplementation be provided to postpartum women for 6–12 weeks following childbirth (282). However, the results of this research project showed not only that over one third of Indian women were iron deficient after birth, but being iron deficient was also significantly associated with a greater risk of developing PPCM. Since PPCM can occur up to six months after birth, iron deficiency is an important risk factor for the development of severe maternal cardiac complications in the postpartum period. Maternal iron supplementation during pregnancy should undoubtedly remain a priority for public health policy, especially in countries such as India where iron deficiency in pregnancy is still an important public health issue. This DPhil project illustrates the importance of also monitoring and correcting maternal iron deficiency after childbirth.

Although it was beyond the scope of this DPhil project, it would be important to also identify the determinants of maternal iron deficiency among these Indian women. In this study population, insufficient IFA supplementation was an important risk factor for iron deficiency. Other factors which were not measured in this study, but that could be particularly relevant for this population, include the interval between consecutive pregnancies and maternal diet. Indeed, shorter birth interval is commonly observed among women in low income countries, largely owing to unmet needs for family planning (283). Previous publications have consistently shown that non-anaemic women with less than a year interval between consecutive pregnancies are at a higher risk of iron depletion (284). There is now ample evidence to suggest that consecutive pregnancies can lead to a cumulative depletion of iron stores, especially if there has not been sufficient time for iron repletion in between the pregnancies. Individuals who adhere to a vegetarian diet are also known to be at a higher risk of iron deficiency (285). That is because, even though a vegetarian diet is likely to contain similar amounts of iron than an omnivorous diet, animal-based haeme-iron is more readily absorbed than plant-based non-haeme iron (55, 125). About 20% of the adult population in the North-eastern region of India (where this study primarily took place) consume some form of a vegetarian diet (286). Thus, future studies should investigate these factors as well as other population-specific determinants of maternal iron status

among postpartum women, as they may be key intervention points for future policies targeting the prevention of iron deficiency and the cardiac complications associated with it during the postpartum period.

9.5. Conclusions

Despite limitations, this DPhil research is the first study to date to characterise the iron status of women diagnosed with PPCM in any population. For the first time, it has been demonstrated that being iron deficient at the end of pregnancy and into the postpartum period puts mothers at a higher risk of developing PPCM. This project provided a thorough and robust assessment of the association between iron deficiency and PPCM in a population of Indian women. The findings offer valuable insights into the pathology of this severe complication in a hitherto poorly studied population, and generated important hypotheses that can inform future research. The results have extended understanding of the epidemiology of PPCM and have important implications for policies targeting iron deficiency in pregnant and postpartum women in India.

Appendices

Table A1. Search strategy for Medline (Ovid) 1946 to present

1	*Peripartum Period/ or *Pregnant women/ or Pregnancy Complications, Cardiovascular/ or *Pregnancy Outcome/ or *pregnancy/
2	exp animals/ not humans.sh.
3	1 not 2
4	(pregnan* or labour or labor or peripartum or perinatal or prenatal or antenatal or matern* or mother* or "pregnant wom*" or "expect* mother*").ti.
5	heart failure/ or *cardiomyopathies/
6	limit 5 to pregnancy
7	6 not 2
8	*heart diseases/ or *cardiovascular diseases/
9	limit 8 to pregnancy
10	9 not 2
11	("heart failure*" or "congestive heart failure" or "chf" or "chronic heart failure" or "decompensated heart failure*" or "acute heart failure*" or "acute decompensat* heart failure*" or "peripartum cardiomyopath*" or "PPCM" or "cardiomyopath*" or "cardiac complication*" or "heart complication*" or "cardiac event" or "major cardiac event*" or "major adverse cardi* event*" or "MACE").ti,ab.
12	10 and 11
13	3 or 4
14	7 or 11 or 12
15	13 and 14
16	15 and (murine or rat or rats or mouse or mice or sheep).ti.
17	15 not 16
18	limit 17 to case reports
19	17 and ("report" or "case adj report").ti.
20	18 or 19
21	17 not 20
22	21 and (fet$ or foet* or neonat$ or newborn* or chil* or infan* or pediatri* or paediatri* or offspring*).ti.
23	21 and ((fet$ or foet* or neonat$ or newborn* or chil* or infan* or pediatri* or paediatri* or offspring*) adj1 (mother* or matern$ or wom$)).ti.
24	22 not 23
25	21 not 24
26	limit 25 to (comment or editorial or letter)
27	25 and (author* adj1 reply).ti.
28	26 or 27
29	25 not 28

Table A2. Data extraction tool for the systematic review

General Information

Date form completed (dd/mm/yyyy)	
Initial of reviewer extracting data	
Reference citation (APA style)	
Notes:	

Study Eligibility

	Eligibility criteria	**Eligibility Criteria met?** (Y/N/Unclear)	**Location In text**
Study Type	Any analytical study with at least one comparator group		
Study population	All pregnant women or postpartum women within 5 months of delivery		
Comparator group	Pregnant women or postpartum women within 5 months of delivery with no pre-existing cardiovascular diseases AND without heart failure/PPCM; OR Pregnant women or postpartum women within 5 months of delivery with no pre-existing cardiovascular diseases AND not exposed to the Risk Factor of interest		
Disease/Outcome of Interest	Peripartum Cardiomyopathy		
Outcome measures reported	Levels and/or presence of molecular biomarkers		
Eligibility Decision (with reason for exclusion)			

Notes:

DO NOT PROCEED IF STUDY IS EXCLUDED FROM REVIEW

Methods

	Description	**Location in text**
Aim of the study		
Main outcome(s)		
Secondary outcome(s)		
Study Design		
Sampling Method		
Study period:		
Notes:		

Population and Setting

	Description	**Location in text**
Population description		
Cases description		
Comparator group description		
Country of origin		
Source/setting of the population		
Sample size		
Number of cases		
Definition of PPCM used:		
Method of PPCM Diagnosis:		
Exclusion criteria		
Gestation stage of cases at enrolment		
Any treatment provided? (if yes, specify)		
Clusters (if applicable: # of clusters, type, # of people/cluster**)**		
Withdrawals and exclusions		
Notes:		

Variables Measured

Demographic variable(s) measured		
Co-morbidities measured		
Risk factor(s)/Predictor(s) of HF or PCCM measured (must have OR, RR or HR reported)		
Laboratory biomarker(s) measured		

Notes:

Biomarker measurements

Biomarker 1	**Description**	**Location in text**
Name		
Nature of the biomarker: E.g. hormone, antibody, enzyme, cytokine, ionic compound etc.		
What is the biomarker measuring?		
Source: urine, blood (specify if plasma, serum, RBC etc.), saliva, etc.		
Method of detection: ELISA, cytometry, western blot etc.		
Unit of measurement		
Time point measured		
Notes:		

Results and findings

Biomarker 1	**Description**	**Location in text**
Name		
Measurement reported: mean, median, change in mean/median		
Value of the measurement reported (Cases)		
Value of the measurement reported (Controls)		
Overall Significance (95% CI and/or p-values)		
Value of the measurement reported (clusters) if applicable		
Clusters Significance (95% CI and/or p-values)		
Key conclusions of study authors		
Notes:		

Table A3. Quality assessment tool for case-control studies

General Information

Date form completed (dd/mm/yyyy)	
Initial of reviewer completing the Q&A	
Reference citation	

Summary of risk of bias

Risk of bias Domain	Definition	Yes/No
Research question & study population	Can we be confident that the researchers had a well-defined study goal and population?	
Control group selection	Can we be confident that the control group is a representative sample of the general population?	
Case definition	Can we be confident that the case definition did not lead to misclassification on the target condition?	
Assessment of exposure	Can we be confident in the conduct and/or interpretation of the biomarker results?	
Confounding	Can we be confident that the authors accounted for other relevant factors that could potentially explain the association between exposure and outcome.	

Overall quality rating (Poor/Fair/Good):_____

Criteria	Yes/No/NA/Unclear (cd, nr)	Text location
Domain 1: Research question & study population		
1. Was the research question or objective in this paper clearly stated and appropriate?		
2. Was the study population clearly specified and defined?		
3. Did the authors include a sample size justification?		
Domain 1 Notes:		
Domain 2: Control group selection		
4. Were controls selected or recruited from the same or similar population that gave rise to the cases (including the same timeframe)?		
5. If less than 100 percent of eligible cases and/or controls were selected for the study, were the cases and/or controls randomly selected from those eligible?		
6. Was there use of concurrent controls?		
Domain 2 Notes:		
Domain 3: Case definition		
7. Were the definitions, inclusion and exclusion criteria, algorithms or processes used to identify or select cases and controls valid, reliable, and implemented consistently across all study participants?		
8. Were the cases clearly defined and differentiated from controls?		
Domain 3 Notes:		
Domain 4: Assessment of exposure/biomarker(s)		
9. Where possible, were the investigators able to confirm that the biomarker exposure occurred prior to the development of the condition or event that defined a participant as a case?		
10a. Were the biomarker measurements clearly defined?		
10b. Were biomarker measurements valid?		
10c. Were biomarker measurements reliable?		
10d. Were the biomarker measurements implemented consistently across all study participants? (including the same time period)		
11. Were the assessors of exposure blinded to the case or control status of participants?		
Domain 4 Notes:		
Domain 5: Confounding		
12. Were key potential confounding variables measured and adjusted statistically in the analyses? If matching was used, did the investigators account for matching during study analysis?		
Domain 5 Notes:		

Table A4. Summary of excluded articles

Index	First author (Year)	Title	Reason for exclusion
1	Afana *et al.* (2016)	Characteristics and In-Hospital Outcomes of Peripartum Cardiomyopathy Diagnosed During Delivery in the United States From the Nationwide Inpatient Sample (NIS) Database	No biomarkers reported
2	Akinwusi *et al.* (2013)	Hospital-based incidence of maternal heart failure during pregnancy in Nigeria	Wrong study design
3	Aoyama *et al.* (2018)	Temporal Variations in Incidence and Outcomes of Critical Illness Among Pregnant and Postpartum Women in Canada: A Population-Based Observational Study	Wrong study design
4	Aziz *et al.* (2019)	Maternal Outcomes by Race during Postpartum Readmissions	No biomarkers reported
5	Barasa *et al.* (2017)	Heart Failure in Late Pregnancy and Postpartum: Incidence and Long-Term Mortality in Sweden From 1997 to 2010	No biomarkers reported
6	Beaton *et al.* (2018)	Impact of heart disease on maternal, fetal and neonatal outcomes in a low-resource setting	No biomarkers reported
7	Bollen *et al.* (2017)	Myofilament Remodeling and Function Is More Impaired in Peripartum Cardiomyopathy Compared with Dilated Cardiomyopathy and Ischemic Heart Disease	Wrong comparator group
8	Bourjeily *et al*. (2017)	Obstructive sleep apnea in pregnancy is associated with adverse maternal outcomes: a national cohort	No biomarkers reported
9	Brar *et al*. (2007)	Incidence, mortality, and racial differences in peripartum cardiomyopathy	Wrong comparator group
10	Briasoulis *et al.* (2016)	Longitudinal systolic strain profiles and outcomes in peripartum cardiomyopathy	No biomarkers reported
11	Cenac *et al.* (2003)	Are anti-Chlamydia pneumoniae antibodies prognosis indicators for peripartum cardiomyopathy?	Wrong study design

Index	First author (Year)	Title	Reason for exclusion
12	Chang *et al.* (2017)	Epidemiological profile and obstetric outcomes of patients with peripartum congestive heart failure in Taiwan: A retrospective nationwide study	No biomarkers reported
13	Dotters-Katz *et al.* (2013)	Medical and infectious complications associated with pyelonephritis among pregnant women at delivery	No biomarkers reported
14	Ertekin *et al.* (2016)	Ventricular tachyarrhythmia during pregnancy in women with heart disease: Data from the ROPAC, a registry from the European Society of Cardiology	Wrong study population
15	Gelson *et al.* (2015)	Maternal cardiac and obstetric performance in consecutive pregnancies in women with heart disease	Wrong outcome
16	Gentry *et al.* (2010)	African-American women have a higher risk for developing peripartum cardiomyopathy	No biomarkers reported
17	Goland *et al.* (2013)	Differences in clinical profile of african-american women with peripartum cardiomyopathy in the United States	Wrong comparator group
18	Grotegut *et al.* (2014)	Medical and obstetric complications among pregnant women aged 45 and older	No biomarkers reported
19	Gunderson *et al.* (2011)	Epidemiology of peripartum cardiomyopathy: incidence, predictors, and outcomes	No biomarkers reported
20	Hameed *et al.* (2001)	The effect of valvular heart disease on maternal and fetal outcome of pregnancy	Wrong outcome
21	Hameed *et al.* (2015)	Pregnancy-related cardiovascular deaths in California: beyond peripartum cardiomyopathy	Wrong outcome
22	Hildebrand *et al.* (2015)	Characteristics and outcomes of AKI treated with dialysis during pregnancy and the postpartum period	No biomarkers reported

Index	First author (Year)	Title	Reason for exclusion
23	Huang *et al.* (2011)	Clinical analysis of pregnancy outcome of pregnancy combined with hyperthyroidism	No biomarkers reported
24	Irizarry *et al.* (2017)	Comparison of clinical characteristics and outcomes of peripartum cardiomyopathy between African American and non-African American women	Wrong comparator group
25	Isogai *et al.* (2018)	Clinical features and peripartum outcomes in pregnant women with cardiac disease: a nationwide retrospective cohort study in Japan	No biomarkers reported
26	Kampman *et al.* (2017)	Uteroplacental Doppler flow and pregnancy outcome inÂ women with tetralogy of Fallot	No biomarkers reported
27	Kao et al. (2013)	Characteristics, adverse events, and racial differences among delivering mothers with peripartum cardiomyopathy	No biomarkers reported
28	Khan et al. (2018)	The spectrum of heart disease in pregnancy and its outcome in patients visiting a tertiary care centre of North East: A prospective study	No biomarkers reported
29	Kharbanda et al. (2013)	Inactivated influenza vaccine during pregnancy and risks for adverse obstetric events	No biomarkers reported
30	Lee et al. (2018)	Incidence, Risk Factors, and Clinical Characteristics of Peripartum Cardiomyopathy in South Korea	No biomarkers reported
31	Lima et al. (2015)	Clinical characteristics and outcomes in pregnant women with Ebstein anomaly at the time of delivery in the USA: 2003-2012	No biomarkers reported
32	Lima et al. (2016)	Association of cardiomyopathy with adverse cardiac events in pregnant women at the time of delivery	Wrong study population
33	Lin et al. (2011)	Effect of pre-eclampsia/eclampsia on major cardiovascular events among peripartum women in taiwan	No biomarkers reported

Index	First author (Year)	Title	Reason for exclusion
34	Mogos et al. (2018)	Heart Failure in Pregnant Women: A Concern Across the Pregnancy Continuum	No biomarkers reported
35	Nizamuddin et al. (2018)	Hypertensive Diseases of Pregnancy Increase Risk of Readmission With Heart Failure: A National Readmissions Database Study	No biomarkers reported
36	Prameswari et al. (2018)	Hypertension in pregnancy as the most influential risk factor for PPCM	No biomarkers reported
37	Roos-Hesselink et al. (2013)	Outcome of pregnancy in patients with structural or ischaemic heart disease: Results of a registry of the European Society of Cardiology	No biomarkers reported
38	Santacesaria et al. (2016)	Pregnancy in women with cardiovascular disease in the guidelines era: An Italian single-center experience	Wrong outcome
39	Sarojini et al. (2013)	Inflammatory markers-serum level of c-reactive protein, tumor necrotic factor-alpha, and interleukin-6 as predictors of outcome for peripartum cardiomyopathy	Wrong comparator group
40	Schlichting et al. (2017)	Pregnant women with congenital heart disease: Pregnancy outcomes and proximity to care	No biomarkers reported
41	Shani et al. (2015)	Peripartum cardiomyopathy - risk factors, characteristics and long-term follow-up	No biomarkers reported
42	Singh et al. (2019)	Brain natriuretic peptide in pregnant women with heart disease	Wrong outcome
43	Thomas et al. (2017)	Pulmonary hypertension and pregnancy outcomes: Insights from the national inpatient sample	No biomarkers reported
44	Tong et al. (2018)	Impact of frequent premature ventricular contractions on pregnancy outcomes	No biomarkers reported
45	Umazume et al. (2015)	Prospective study on changes in blood variables in pregnant women at higher risk of peripartum cardiomyopathy	Wrong outcome

Index	First author (Year)	Title	Reason for exclusion
46	Vinas Oliva et al. (2003)	Perinatologic results of cardiopathy and pregnancy's service in 2000-2001, in "Ramon Gonzalez Coro" Hospital, Havana	Wrong study design
47	Warraich et al. (2005)	Impact of pregnancy-related heart failure on humoral immunity: Clinical relevance of G3-subclass immunoglobulins in peripartum cardiomyopathy	Wrong outcome
48	Xu et al. (2012)	Investigation of cystatin C and cystatin C based estimated glomerular filtration rate in pregnant patients with heart failure	Wrong comparator group
49	Zhou, B.H. (1993)	Analysis on the causes of maternal death with pregnancy induced hypertension in Shanghai from 1981 to 1990. [Chinese]	No biomarkers reported

Table A5. Criteria used for the definition of PPCM in included papers

First author (Publication year)	PPCM definition used
Adesanya et al. (1991)(70)	Not reported
Azibani et al. (2020)(71)	2010 ESC Working Group on Peripartum Cardiology
Cenac et al. (1990)(72)	Left or global heart failure appearing in the last month of pregnancy or five months after childbirth, with ventricular dilation and hypokinetic syndrome (LVEF <50%)
Cenac et al. (1992)(73)	Diagnosis of left or global heart failure in the last trimester of pregnancy or within six months postpartum
Cenac et al. (1996)(74)	Left global heart failure presenting symptoms that appeared during the third trimester of pregnancy or during the 6 months after delivery, or with global cardiomegaly with important and global alteration of ventricular contraction detected echocardiographically or by fluoroscopy, or by both.
Cenac et al. (2000)(75)	Not reported
Cenac et al. (2004)(76)	(1) Dilated heart with low ventricular contractility, (2) normal ventricular thickness, (3) heart failure first developing during the third trimester of pregnancy or in the first six months postpartum.
Cenac et al. (2009)(77)	Diagnosis of left or global heart failure, appearing in the last month of pregnancy or in the 5 months of the post-partum period, with ventricular dilation and hypokinetic syndrome (failed left ventricular ejection fraction)
Ellis et al. (2005)(78)	(1) Onset of congestive heart failure during the period of one month before delivery through five months postpartum; (2) absence of pre-existing heart disease; (3) no other identifiable cause of congestive heart failure and (4) evidence of left ventricular dilatation with diminished contractility characterized by an ejection fraction less than 45% or fractional shortening less than 30% and end-diastolic dimension more than 2.7 cm/m^2
Fett et al. (2002)(79)	(1) Onset of CHF during the time period of 1 month antepartum to 5 months postpartum, (2) no evidence of pre-existing heart disease, and (3) no other cause identified for the presenting CHF, (4) evidence of left ventricular dilatation and dysfunction.
Forster et al. (2008)(80)	(1) NYHA functional class II–IV, (2) symptoms of congestive heart failure that developed in the last month of pregnancy or during the first 5 months postpartum, (3) no other identifiable cause for heart failure, (4) LVEF 40% by transthoracic echocardiography, (5) sinus rhythm.
Haghikia et al. (2013)(62)	(1) Heart failure secondary to LV systolic dysfunction towards the end of pregnancy or in the months following delivery, where no other cause of heart failure is found; (2) diagnosis of exclusion with a LVEF nearly always reduced below 45 % but not always associated with LV dilatation.

First author (Publication year)	PPCM definition used
Haghikia et al. (2015)(81)	2010 ESC Working Group on Peripartum Cardiology
Halkein et al. (2013)(60)	(1) Symptoms of congestive heart failure that developed in the last month of pregnancy or during the first month postpartum, (2) no other identifiable cause for heart failure, (3) LVEF <35% by transthoracic echocardiography.
Hilfiker-Kleiner et al. (2007)(59)	Not reported
Huang et al. (2010)(82)	LVEDD >5.0 cm and LVEF <45%.
Huang et al. (2012)(83)	Heart failure symptoms in late pregnancy or within the first five months postpartum. Left ventricular EF of less than 45%.
Karaye et al. (2015)(84)	2010 ESC Working Group on Peripartum Cardiology (with LV systolic dysfunction as LVEF <50%)
Karaye et al. (2016)(85)	2010 ESC Working Group on Peripartum Cardiology (with LV systolic dysfunction as LVEF <50%)
Liu et al. (2014)(86)	(1) NYHA functional classes II–IV, (2) symptoms of congestive heart failure developed in the last month of pregnancy or during the first 5 months post-partum, (3) no other identifiable causes for heart failure, (4) LVEF <45%, (5) LV fractional shortening <30%
McTiernan et al. (2018)(87)	(1) No previous history of cardiac disease, (2) estimated clinical LVEF ≤0.45, (3) an evaluation consistent with non-ischemic cardiomyopathy
Mebazaa et al. (2017)(88)	(1) Symptoms of congestive heart failure that developed in the last months of pregnancy or during the first 5 months postpartum, (2) no other identifiable cause for heart failure, (3) LVEF ≤45% by transthoracic echocardiography; (4) sinus rhythm.
Nonhoff et al. (2017)(89)	2010 ESC Working Group on Peripartum Cardiology
Patten et al. (2012)(90)	Not reported
Ricke-Hoch et al. (2019)(91)	2010 ESC Working Group on Peripartum Cardiology
Sagy et al. (2017)(92)	2010 ESC Working Group on Peripartum Cardiology
Walenta et al. (2012)(93)	Not reported
Wang et al. (2018)(94)	Idiopathic cardiomyopathy conferred with heart failure secondary to LV systolic dysfunction near the end of a pregnancy or during the months following the delivery, where no other cause of heart failure can be found; it is a diagnosis of exclusion, with the LVEF almost always reduced to below 45% but not consistently associated with LV dilatation.
Xia et al. (2016)(95)	(1) Development of congestive heart failure in the last month of pregnancy or within the first five months of delivery, (2) absence of recognizable heart disease before pregnancy, (3) no other identifiable cause for cardiac

First author (Publication year)	PPCM definition used
	failure, (4) LV systolic dysfunction demonstrated by echocardiographic analysis such as depressed LVEF (<0.55).
Xia et al. (2017)(96)	(1) Development of congestive heart failure in the last month of pregnancy or within the first 5 months of delivery, (2) absence of recognizable heart disease before pregnancy, (3) no other identifiable cause for cardiac failure, (4) LV systolic dysfunction demonstrated by echocardiographic analysis such as depressed LVEF (< 0.55)
Yaqoob et al. (2018)(97)	(1) Presentation of heart failure in last month of pregnancy or first 5 months postpartum, (2) LVEF <0.45 or M-mode fractional shortening <30% (or both) and LVEDD >2.7 cm/m2.

ESC: European Society of Cardiology, **LVEF:** Left ventricular ejection fraction, **CHF:** congestive heart failure, **NYHA:** New York Heart Association, **LV:** left ventricular, **LVEDD:** Left ventricle end-diastolic diameter

Table A6. Pooled standardised mean difference (SMD) for biomarkers compared between PPCM cases and healthy controls

Marker	N studies	N cases	Pooled SMD (95 % CI)	$I^{2\dagger}$	p-value[a]
Natriuretic peptides[b]	10	435	3.77 (0.71; 6.82)[c]	0.995	<0.001
Albumin	8	296	-0.67 (-1.01; -0.32)	0.77	0.001
CRP/hsCRP	7	328	1.67 (0.22; 3.12)[c]	0.99	<0.0001
Selenium	6	165	-0.73 (-1.58; 0.12)	0.93	<0.0001
Troponin	6	263	1.06 (0.33; 1.80)	0.94	<0.0001
Creatinine	5	225	0.51 (0.33; 0.69)	0	0.72
WBC	5	184	0.44 (0.07; 0.82)	0.76	0.01
Haemoglobin	5	168	-0.45 (-0.64; -0.26)	0	0.85

SMD: Standard Mean Difference, **CI**: Confidence Interval, **CRP/hs-CRP**: C-reactive protein/high sensitivity C-reactive protein; **WBC**: White Blood Cells**;**

[†]Index of heterogeneity: percentage of variation across studies that is due to heterogeneity rather than chance

[a]Statistical test performed: Cochran's Q-test for residual heterogeneity

[b]Naturetic peptides included: ANP, BNP, proBNP and NT-proBNP

[c] Mean values estimated from median and interquartile ranges

Table A7. Variables assessed in the PPCM analysis and the questionnaire variables used to derive them

New variable	Values	Questionnaire variable(s) used	Values
Socio-demographic and lifestyle			
Maternal age	age in year	Maternal age	age in years
Education	Secondary school or higher: Yes/No	Education level	Illiterate/ Literate up to 5th class/ 6th to 12th class/ Beyond 12th class/ Not known
Religion	Hindu/Muslim/ Other	Religion	Hindu/Muslim/Christian/Sikh/Other
Poverty status	Certified BPL/ Not BPL	Below poverty line status	BPL Certified/Not BPL/Not known
Alcohol use	Never or before pregnancy/ During pregnancy	Alcohol use	Never/Gave up prior to pregnancy/Current/Gave up during pregnancy
Smoking	Never or before pregnancy/ During pregnancy	Smoking	Never/Gave up prior to pregnancy/Current/Gave up during pregnancy
Tobacco use	Never or before pregnancy/ During pregnancy	Tobacco use	Never/Gave up prior to pregnancy/Current/Gave up during pregnancy
Chews betel nut	Never or before pregnancy/ During pregnancy	Chewing betel nut	Never/Gave up prior to pregnancy/Current/Gave up during pregnancy
Consumes fermented fish	Yes/No	Consumes fermented fish	Yes/No
Heart failure features			
Systolic blood pressure	mmHg	Systolic blood pressure	mmHg
Diastolic blood pressure	mmHg	Diastolic blood pressure	mmHg
Gestational stage at onset of HF	Antenatal/Labour or Delivery/Postpartum	Onset of HF	Antenatal/Labour and delivery/Postpartum
Gestational age at onset (antenatal)	weeks	HF onset (antenatal)	weeks

New variable	Values	Questionnaire variable(s) used	Values
Days postpartum at onset (post-partum)	days	1) Date of blood test 2) Delivery date/Pregnancy loss date	YYYY/MM/DD
NYHA class	I/II/III/IV	NYHA	I/II/III/IV
Treatment for HF	Yes/No	Therapeutic management of heart failure	List of drugs if any
Echocardiography			
LVEDD	<=5.3 cm/>5.3cm	LVEDD (cm)	3.9-5.3cm/5.4-5.7cm/5.8-6.1cm/>6.1cm
LVEF	>55%/45-54%/30-44%/<30%	LVEF (%)	>70%/55-69%/45-54%/30-44%/<30%
Abnormal left ventricular wall motion	Yes/No	Regional left wall motion abnormality	Present/Absent
Abnormal right ventricular wall motion	Yes/No	Regional right wall motion abnormality	Present/Absent
Enlarged left ventricle	Yes/No	Enlarged Left ventricle	Yes/No
Enlarged right ventricle	Yes/No	Right ventricle basal diameter (RVD1) >4.2 cm	Present/Absent
Enlarged left atrium	Yes/No	Left atrium enlarged	Yes/No
Enlarged right atrium	Yes/No	Right atrium enlarged	Yes/No
Aortic stenosis	Yes/No	Aortic stenosis	None/Mild/Moderate/Severe
Aortic regurgitation	Yes/No	Aortic regurgitation	None/Mild/Moderate/Severe
Mitral stenosis	Yes/No	Mitral stenosis	None/Mild/Moderate/Severe
Mitral regurgitation	None or Mild/ Moderate or Severe	Mitral valve Regurgitation	None/Mild/Moderate/Severe
Tricuspid stenosis	None or Mild/ Moderate or Severe	Tricuspid stenosis	None/Mild/Moderate/Severe
Tricuspid regurgitation	None or Mild/ Moderate or Severe	Tricuspid valve Regurgitation	None/Mild/Moderate/Severe
Pericardial effusion	Yes/No	Pericardial effusion	Present/Absent
Thrombus	Yes/No	Thrombus	Yes/No
Cardiac mass	Yes/No	Cardiac Mass	Yes/No

New variable	Values	Questionnaire variable(s) used	Values
Tachycardia	Yes/No	Tachycardia	Yes/No
Clinical factors			
BMI	kg/m2	1) Height 2) Weight at first ANC	1) cm 2) kg
Obesity	Yes (≥30 kg/m2)/ No(<30 kg/m2)	1) Height 2) Weight at first ANC	1) cm 2) kg
First antenatal Hb	In g/dL	Antenatal haemoglobin 1	In g/dL
Gestational age at first antenatal Hb	Weeks	1) Estimated Date of Delivery 2) Antenatal haemoglobin 1 date	YYYY/MM/DD
Blood transfusion	Yes/No	Received a blood transfusion (for the treatment of anaemia in pregnancy)	Yes/No
Antenatal anaemia	Yes/No	Antenatal haemoglobin 1	< 10 g/dL or ≥10 g/dl
Diabetes Mellitus	Yes/No	1) Pre-existing medical problems 2) Other pre-existing medical problems 3) Medical problems in current pregnancy 4) Other medical problems in current pregnancy	1) Rheumatic fever (without heart disease)/Rheumatic heart disease/Congenital heart disease/Other cardiac problems/Essential hypertension/**Diabetes**/HbE homozygous/Sickle cell trait or disease/Thalassemia/Other haemoglobinopathy/Beri beri/Malaria/Chronic renal disease/**Others** 2) Text: "Diabetes" 3) Anaemia/Cardiac problems/High blood pressure/Antepartum

New variable	Values	Questionnaire variable(s) used	Values
			haemorrhage/Pre-eclampsia/Eclampsia/Sepsis/Uterine rupture/**Gestational diabetes**/Miscarriage (<28 weeks)/Placental problems/**Others**/None 4) Text: "Diabetes"
Essential hypertension	Yes/No	1) Pre-existing medical problems 2) Other pre-existing medical problems	1) Rheumatic fever (without heart disease)/Rheumatic heart disease/Congenital heart disease/Other cardiac problems/**Essential hypertension**/Diabetes/HbE homozygous/Sickle cell trait or disease/Thalassemia/Other haemoglobinopathy/Beri beri/Malaria/Chronic renal disease/**Others** 2) Text: "Hypertension" or "High blood pressure"
Hypertensive disorders of pregnancy	Yes/No	1) Any medical problems in current pregnancy 2) Medical problems in current pregnancy 3) Other medical problems in current pregnancy	1) Yes/No 2) Anaemia/Cardiac problems/**High blood pressure**/Antepartum haemorrhage/**Pre-eclampsia/Eclampsia**/Sepsis/Uterine rupture/Gestational diabetes/Miscarriage (<28 weeks)/Placental problems/**Others**/None

New variable	Values	Questionnaire variable(s) used	Values
			3) Text entry: "Severe PIH" or "Gestational hypertension" or "High blood pressure" or "Eclampsia" or "Pre-eclampsia/eclampsia" or "Pre-eclampsia"
Pregnancy characteristics and outcomes			
Parity	Primiparous/ Multiparous	Number of completed pregnancies ≥28 weeks	Count
Gestational age at birth	weeks	1) Estimated date of delivery 2) Birth date/pregnancy loss date	YYYY/MM/DD
Mode of birth	C-section/Vaginal	Birth by C-section	Yes/No
Miscarriage	Yes/No	1) Pregnancy loss (<28 weeks) 2) Medical problems in current pregnancy 3) Other medical problems in current pregnancy	1) Yes/No 2) Anaemia/Cardiac problems/High blood pressure/Antepartum haemorrhage/Pre-eclampsia/Eclampsia/Sepsis/Uterine rupture/Gestational diabetes/**Miscarriage (<28 weeks)**/Placental problems/**Others**/None 3) Text entry: "Miscarriage" or "Pregnancy loss"
Postpartum blood loss	mL	Blood loss	
Premature birth	Yes/No		
Infant birth weight	grams	Infant birth weight	grams
Infant stillborn	Yes/No	Infant stillborn	Yes/No
Infant death	Yes/No	Infant death	Yes/No
Maternal death	Yes/No	Maternal death	Yes/No

New variable	Values	Questionnaire variable(s) used	Values

BPL: below poverty line, **HF:** heart failure, **NYHA:** New York Heart Association, **LVEDD:** left ventricular end diastolic diameter, **LVEF:** left ventricular ejection fraction, **BMI:** body mass index, **Hb:** haemoglobin, **ANC:** antenatal care, **PIH:** pregnancy induced hypertension, **RVD1:** Right ventricular basal diameter at end-diastole, **HbE:** haemoglobin E

Table A8. Univariable regression models of factors associated with PPCM cases with postnatal onset

	N cases	OR	95% CI	p-value[a]
Postpartum blood loss	***7***	1.00	1.00, 1.01	0.17
Duration of gestation	***20***	1.02	0.89, 1.13	0.8
Mode of birth	***18***			
Vaginal	*16*	1 (ref)	1 (ref)	>0.99
C-section	*2*	0.99	0.15, 3.87	

OR= Odds Ratio, **CI**= Confidence Interval

[a]Wald-test or type-III analysis-of-variance for the regression parameter.
***p<0.01, **p<0.05, *p<0.1 after false discovery rate correction for multiple testing.

Table A9. The association between PPCM and socioeconomic and behavioural (imputed data)

	Sociodemographic factors	
	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
Maternal age (years)	0.99 (0.93, 1.07)	--
Secondary school education		
Yes	1 (ref)	1 (ref)
No	**5.33 (2.53, 11.2)**	**3.30 (1.38, 7.81)**
Poverty status		
Not BPL	1 (ref)	--
Certified BPL	1.84 (0.78, 4.32)	--
Religion		
Hindu	1 (ref)	--
Muslim	1.54 (0.74, 3.21)	--
Other	3.45 (1.00, 11.9)	--
Residence		
Urban/Suburban	1 (ref)	1 (ref)
Rural	**3.08 (1.03, 9.18)**	2.77 (0.91, 10.7)
	Behavioural factors	
	Unadjusted OR (95% CI)	Adjusted OR (95% CI)‡
Chews betel nut		
Never/before pregnancy	1 (ref)	1 (ref)
During pregnancy	**2.02 (1.03, 3.97)**	1.29 (0.58, 2.86)
Consumes fermented fish		
No	1 (ref)	--
Yes	1.53 (0.59, 3.93)	--

OR=Odds Ratio, **CI**=Confidence Interval, **BPL**: below poverty line
†Multivariable logistic regression, adjusting for the other variables in the model. The final models excludes variables that were not significantly associated with PPCM in the univariable analysis.
‡Multivariable logistic regression adjusting for significant sociodemographic factor(s)

Table A10. The association between PPCM and obstetric factors (imputed data)

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
BMI at first ANC (kg/m^2)	0.91 (0.81, 1.04)	--

Duration of IFA supplementation (days)[a]	**0.90 (0.85, 0.94)**	**0.92 (0.87, 0.97)**
Parity		
Multiparous	1 (ref)	--
Primiparous	1.05 (0.52, 2.10)	--
Number of ANC visits		
3 or more	1 (ref)	1 (ref)
Fewer than 3	**6.87 (2.88, 16.4)**	1.98 (0.70, 5.41)

OR=Odds Ratio, **CI**=Confidence Interval, **BMI:** body mass index, **ANC**: antenatal care, **IFA**: iron and folic acid

[a]For every 7 days of IFA supplementation

†Multivariable logistic regression, adjusting for education level and other significant obstetric factor(s).

Table A11. The association between PPCM and medical conditions in the index pregnancy (imputed data)

	Unadjusted OR (95% CI)	Adjusted OR (95% CI)†
Received blood transfusion		
No	1 (ref)	1 (ref)
Yes	**4.11 (1.55, 10.9)**	2.78 (0.91, 8.36)
Hypertension/hypertensive disorder[a]		
No	1 (ref)	1 (ref)
Yes	**11.0 (4.42, 27.4)**	**8.80 (3.34, 24.5)**

OR=Odds Ratio, **CI**=Confidence Interval

[a]Including high blood pressure, pregnancy-induced hypertension, pre-eclampsia and eclampsia

†Each multivariable logistic regression model adjusts for education and duration of IFA supplementation in the index pregnancy, which were significantly associated with PPCM in the previous model

Table A12. Serum concentration of iron markers in women diagnosed with PPCM by NYHA class

	NYHA I-II (n=10)	**NYHA III (n=9)**	**NYHA IV (n=25)**	**p-value**[a]
		Median (IQR)		
sFR (ug/mL)[b]	40 (14, 44)	98 (16, 105)	108 (24, 301)	0.061
N missing	2	4	4	
sFe (ug/dL)	32 (24, 72)	30 (24, 43)	44 (21, 75)	0.9
N missing	2	4	4	
TSAT (%)[b]	8 (6, 16)	8 (6, 11)	14 (7, 23)	0.6
N missing	2	4	4	
sTfR (mg/L)	3.10 (1.81, 3.54)	1.72 (1.58, 2.59)	2.50 (2.03, 7.07)	0.4
N missing	2	3	4	
sTfR-F index	2.40 (1.12, 3.01)	0.84 (0.78, 2.44)	1.49 (0.90, 4.41)	0.7
N missing	2	4	4	
Hepcidin (ng/mL)[b]	4 (2, 15)	3 (3, 9)	2 (1, 7)	0.7
N missing	2	4	7	

NYHA: New York heart association, **IQR:** interquartile range, **sFR:** serum ferritin, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F:** sTfR-ferritin**, hs-CRP**: high sensitivity C-reactive protein

[a]Statistical tests performed: Wilcoxon rank-sum test for difference in median
[b]Values corrected for inflammation (CRP>5 mg/L)

Table A13. Serum concentration of iron markers in women diagnosed with PPCM by survival status

	Died (N = 9)	**Survived (N = 23)**	**Unknown (n=11)**	**p-value[a]**
		Median (IQR)		
sFR (ug/mL)[b]	148 (111, 268)	39 (22, 68)	108 (56, 254)	0.064
N missing	3	3	4	
sFe (ug/dL)	24 (22, 38)	44 (23, 63)	30 (26, 71)	0.8
N missing	3	3	4	
TSAT (%)[b]	6 (5, 15)	10 (6, 15)	16 (8, 25)	0.4
N missing	3	3	4	
sTfR (mg/L)	3.72 (2.43, 5.58)	2.61 (1.81, 4.47)	1.81 (1.48, 3.43)	0.3
N missing	3	3	3	
sTfR-F index	2.14 (1.29, 2.64)	1.77 (0.89, 3.94)	1.00 (0.54, 2.87)	0.5
N missing	3	3	4	
Hepcidin (ng/mL)[b]	4 (1, 7)	2 (1, 20)	2 (1, 3)	0.6
N missing	4	3	6	

IQR: interquartile range, **sFR:** serum ferritin, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F:** sTfR-ferritin**, hs-CRP**: high sensitivity C-reactive protein
[a]Statistical tests performed: Wilcoxon rank-sum test for difference in median
[b]Values corrected for inflammation (CRP>5 mg/L)

Table A14. Comparison of the crude and adjusted area under the receiver operating characteristic curves of the iron biomarkers

Iron marker	**Crude AUC (95% CI)[a]**	**Adjusted AUC (95% CI)[b]**
sFe (ug/mL)	0.70 (0.56, 0.82)	0.70 (0.59, 0.81)
TSAT (%)	0.63 (0.51, 0.75)	0.62 (0.50, 0.73)
sTfR (mg/L)	0.74 (0.65, 0.82)	0.73 (0.63, 0.81)
sTfR-F index	0.64 (0.52, 0.74)	0.63 (0.51, 0.73)
Hepcidin (ng/mL)	0.74 (0.64, 0.83)	0.68 (0.56, 0.78)

AUC: area under the curve, **CI:** confidence interval, **sFe**: serum iron, **TSAT**: transferrin saturation, **sTfR**: soluble transferrin receptors, **sTfR-F:** sTfR-ferritin
[a]AUC of the crude receiver operating characteristic (ROC) curve
[b]AUC of the receiver operating characteristic (ROC) adjusted for education level

Table A15. The association between late pregnancy anaemia and PPCM (imputed data)

	Unadjusted Model	**Adjusted Model[a]**
	OR (95% CI)	*OR (95% CI)*
Hb levels[b]	**1.36 (1.13, 1.65)**	**1.26 (1.02, 1.55)**
Anaemia status[c]		
No Anaemia	1 (ref)	1 (ref)
Anaemia	**3.99 (1.97, 8.47)**	**3.41 (1.54, 7.53)**
Anaemia severity[d]		

No anaemia	1 (ref)	1 (ref)
Moderate	**3.48 (1.65, 7.62)**	2.94 **(**1.29, 6.71**)**
Severe	**7.34 (2.25, 24.0)**	6.33 **(**1.67, 24.0**)**

OR: odds ratio, **CI:** confidence interval, **Hb**: haemoglobin
[a]Model adjusted for residence and the number of ANC visits.
[b]For a 1 g/dL decrease from the mean
[c]Defined as Hb < 10 g/dL
[d]Severe: Hb < 7g/dL; Moderate: Hb 7-9.9 g/dL

Table A16. The modifying effect of iron status on anaemia in PPCM (imputed data)

	OR (95% CI)[a]
ID and anaemia status	
Neither	1 (ref)
Anaemia with ID	**12.9 (4.45, 43.8)**
ID only	3.13 (0.92, 11.5)
Anaemia only	2.59 (0.76, 9.47)
Interaction terms	
Multiplicative	1.59 (0.31, 8.32)
Additive (RERI)	8.13 (-2.97, 19.23)

OR: odds ratio, **CI**: confidence interval, **ID**: iron deficiency, **RERI**: relative excess risk attributable to interaction
[a] Model adjusted for residence and the number of ANC.
[b]Defined as sFR<100 ug/mL for cases or <15 ug/mL for controls

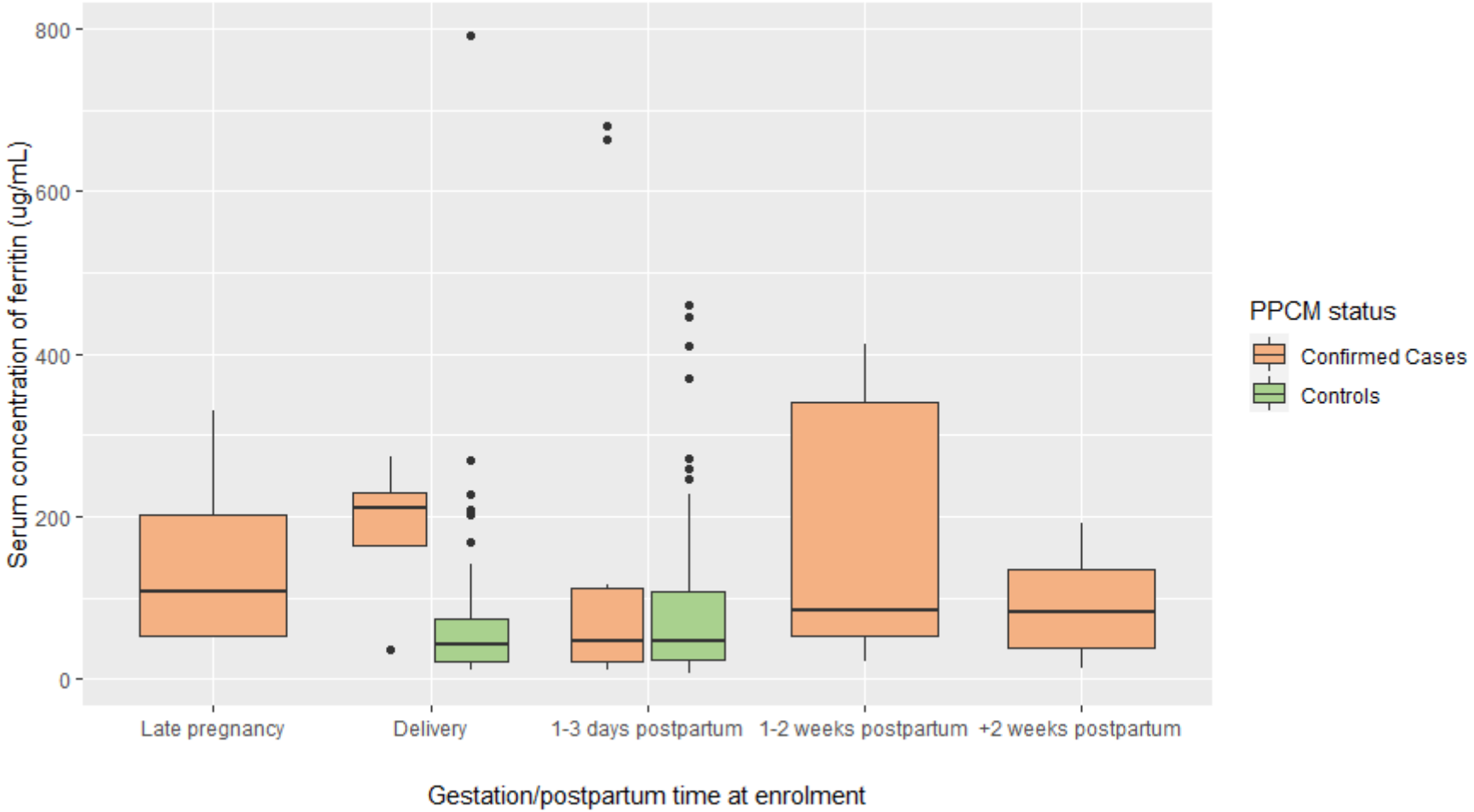


Figure A1. Distribution of serum concentrations of ferritin by gestation and postpartum time at enrolment and PPCM status

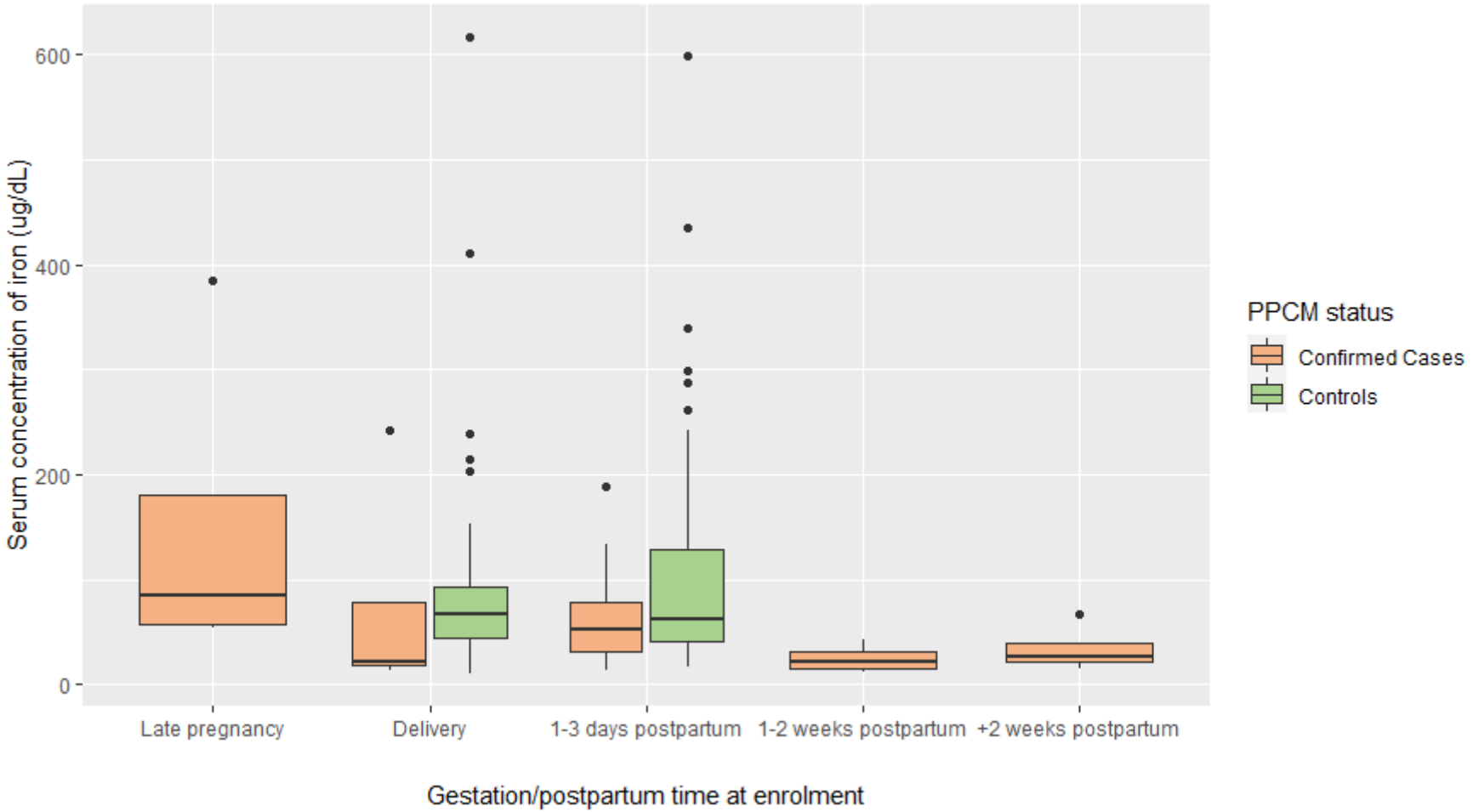


Figure A2. Distribution of serum concentrations of iron by gestation and postpartum time at enrolment and PPCM status

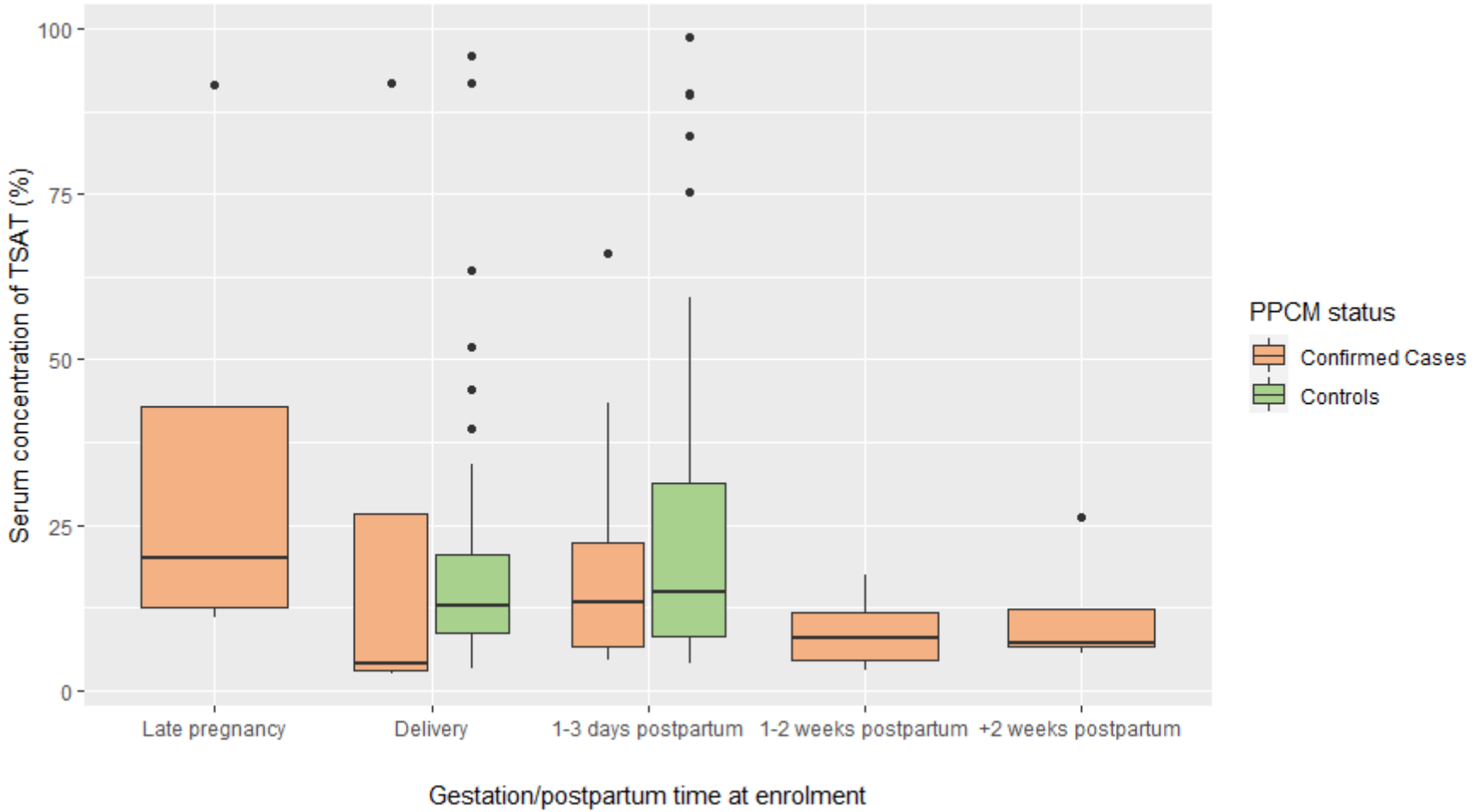


Figure A3. Distribution of serum transferrin saturation (TSAT) by gestation and postpartum time at enrolment and PPCM status

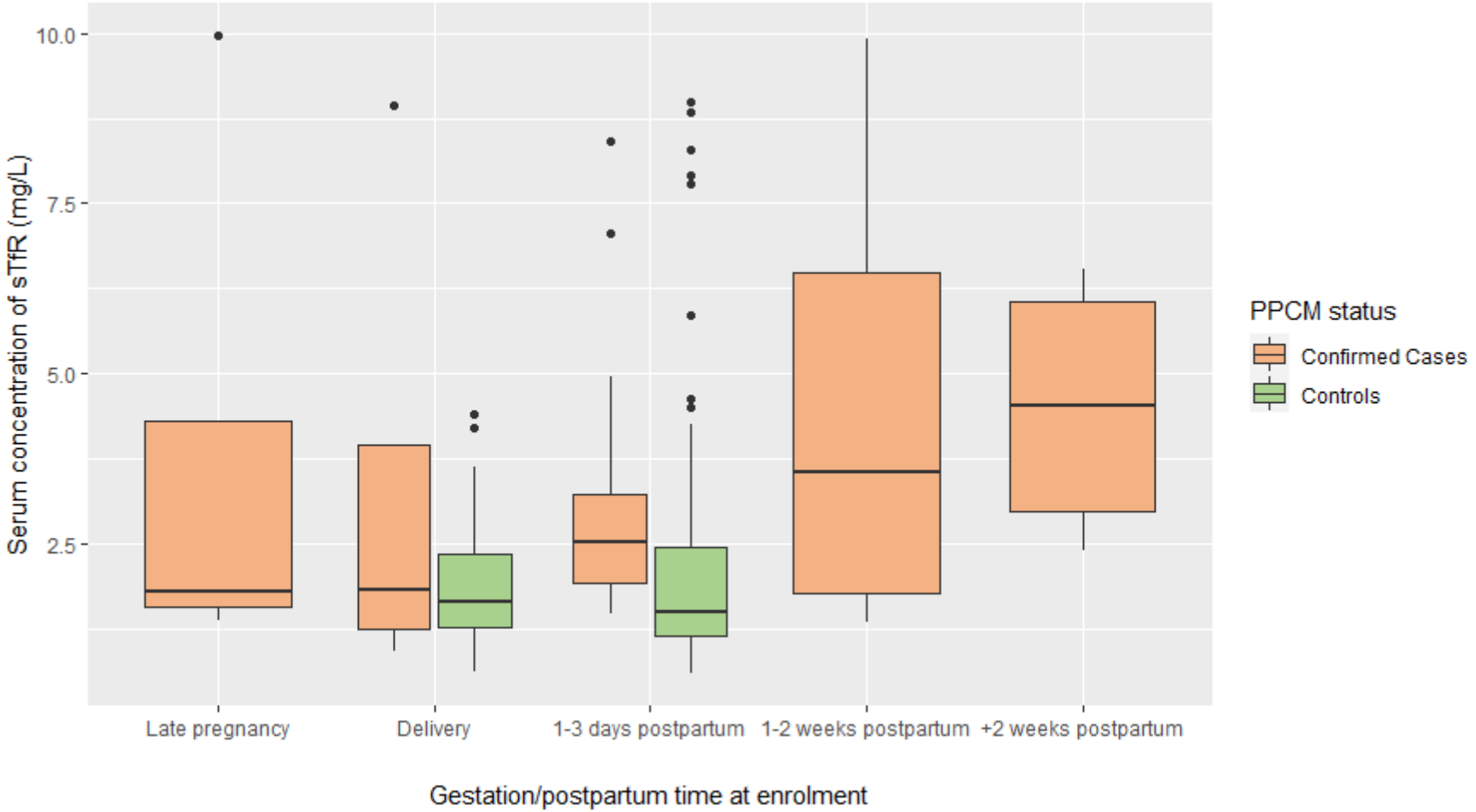


Figure A4. Distribution of serum concentration of soluble transferrin receptors (sTfR) by enrolment time at enrolment and PPCM status

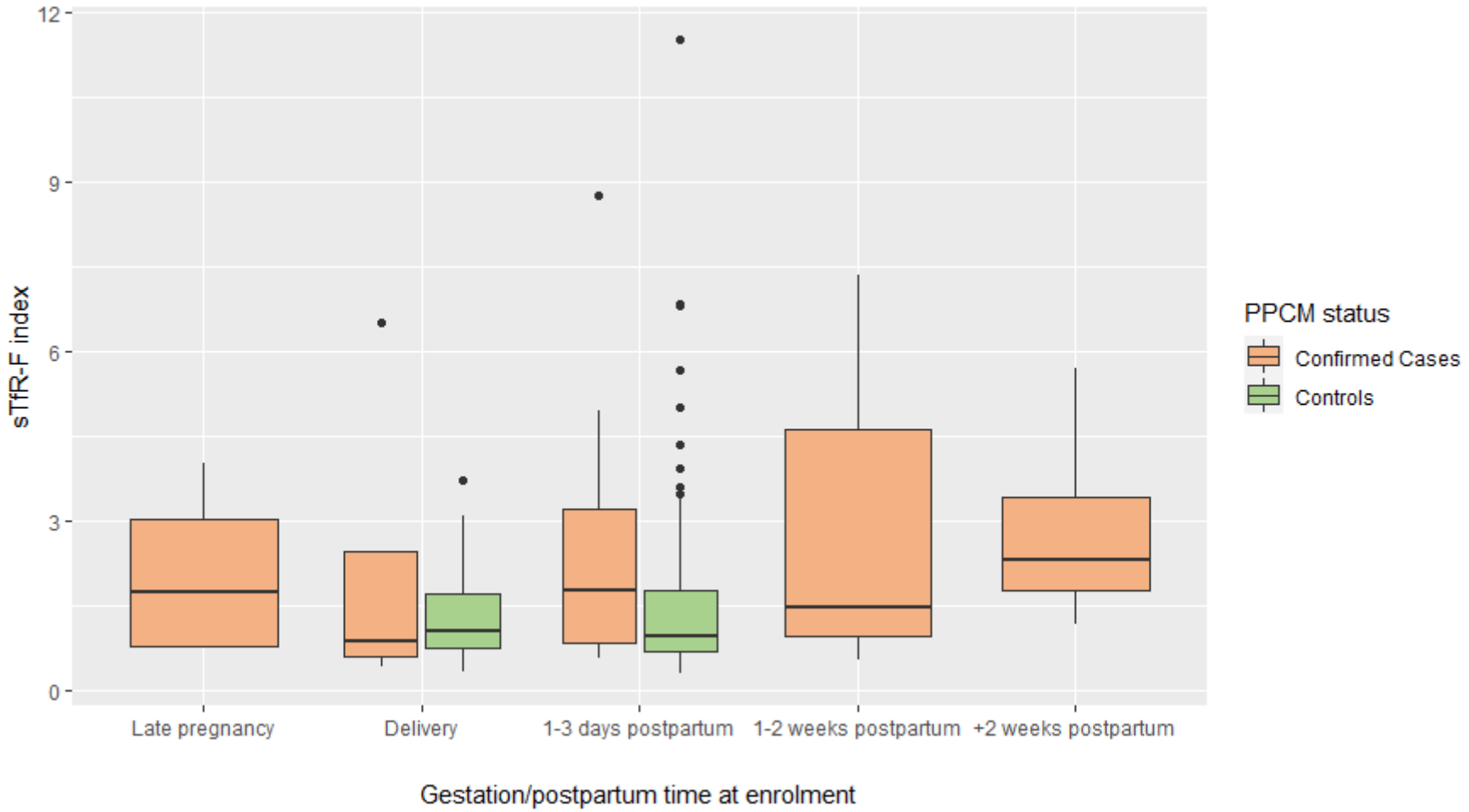


Figure A5. Distribution of soluble transferrin receptors-ferritin index (sTfR-F) values by enrolment time at enrolment and PPCM status

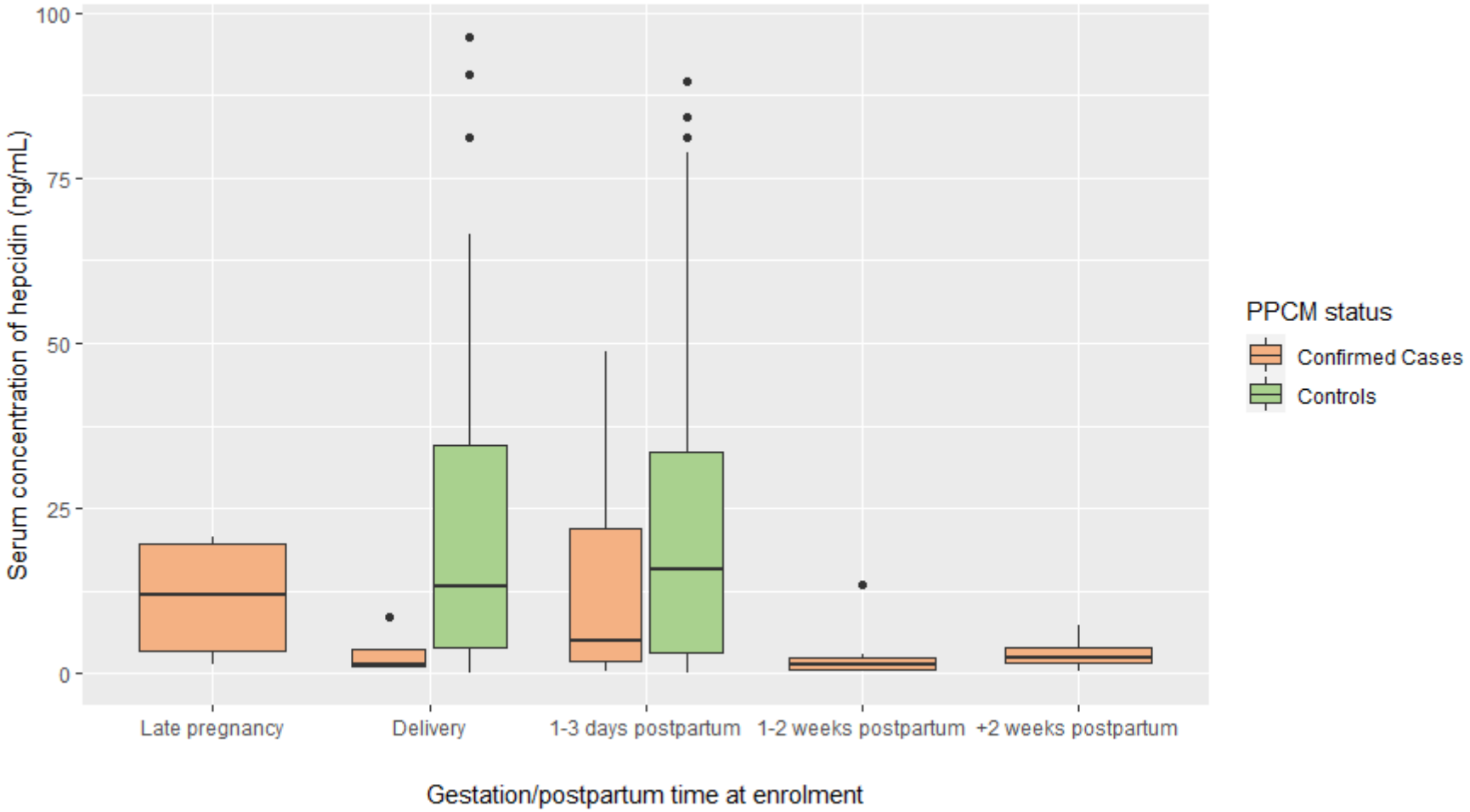


Figure A6. Distribution of serum concentrations of hepcidin by enrolment time at enrolment and PPCM status

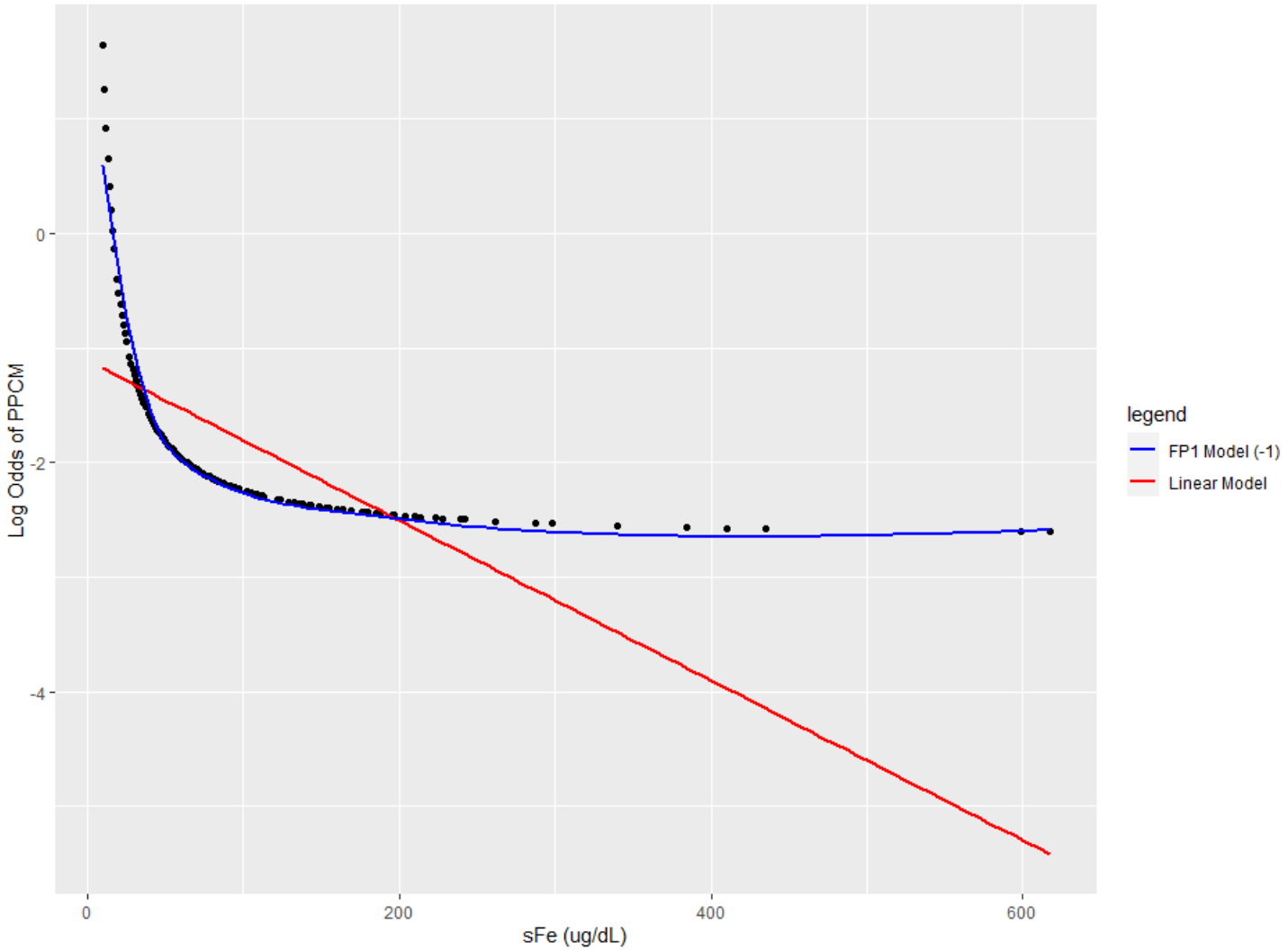


Figure A7. Shape of the association between serum iron and the log odds of PPCM

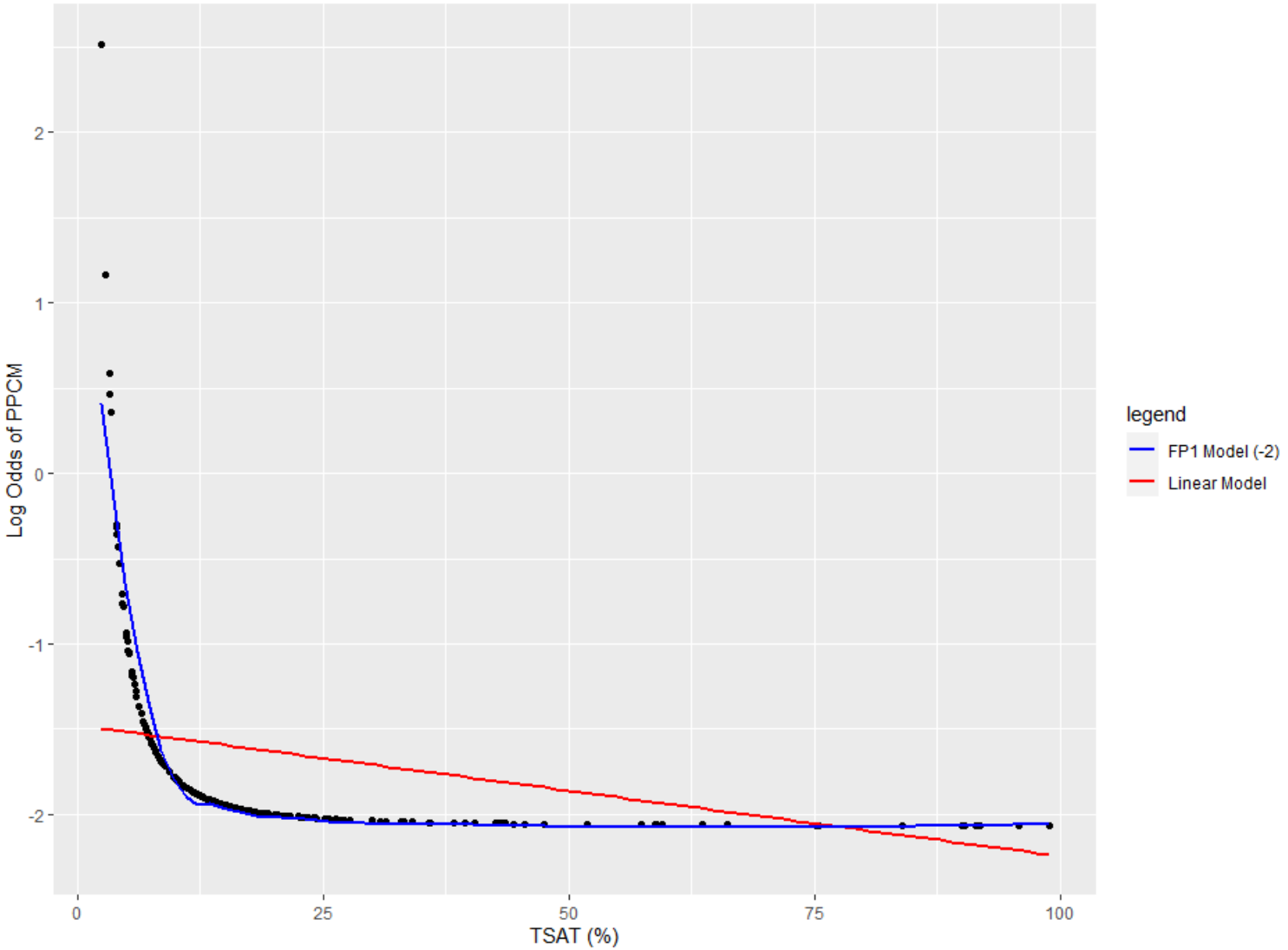


Figure A8. Shape of the association between transferrin saturation and the log odds of PPCM

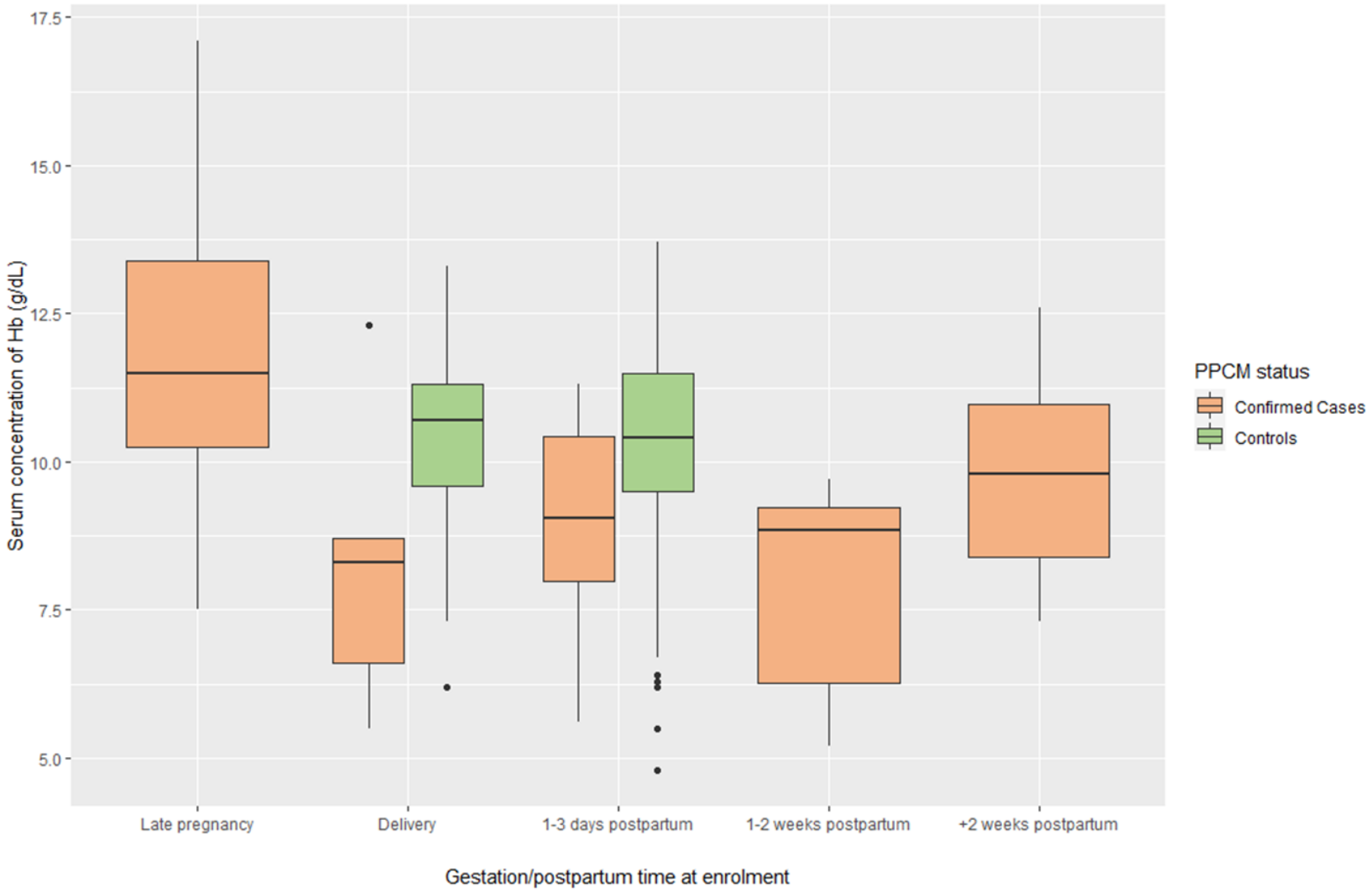


Figure A9. Distribution of serum concentrations of haemoglobin by enrolment time and PPCM status

Publications and conference proceedings

Publications related to the thesis

- **Cherubin S**, Peoples T, Gillard J, Lakhal-Littleton S, Kurinczuk JJ, Nair M. Systematic review and meta-analysis of prolactin and iron deficiency in peripartum cardiomyopathy. Open heart. 2020 Oct 1;7(2):e001430. https://openheart.bmj.com/content/7/2/e001430.abstract

Conference presentations

- *A systematic review of biomarkers for the risk of Peripartum Cardiomyopathy*. Conference lightning talk. Presented at the Nuffield Department of Population Health Annual Symposium, Oxford, United Kingdom. (January 2020).
- *Molecular biomarkers in Peripartum Cardiomyopathy: a systematic review and meta-analysis*. Conference talk. Presented at the University of Cambridge Society for Social Medicine & Population Health, Annual Scientific Meeting 2020, Cambridge, United Kingdom. (September 2020).
- *Iron deficiency in peripartum cardiomyopathy: an epidemiological investigation in Northern India.* Conference talk. Presented at the Maternal and perinatal Health Research collaboration, India annual conference, Goa, India. (May 2022).
- *Investigating the iron profile of Indian women diagnosed with Peripartum Cardiomyopathy*. Conference talk. Accepted for presentation at the European Iron Conference 2022, Oxford, United Kingdom. (July 2022).

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