BIOLOGY OF IRON IN RBC REGENERATION: UPCOMING PLAYERS OF REGENERATIVE MEDICINE

SREENITHI.S

PhD THESIS

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A THESIS SUBMITTED BY

SREENITHI.S

TO

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY, TRIVANDRUM.

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF

DOCTOR OF PHILOSOPHY

2024

DECLARATION BY THE STUDENT

I, Sreenithi.S, hereby certify that I had personally carried out the work depicted in the thesis titled, "Biology of Iron in RBC regeneration: Upcoming players of regenerative medicine". No part of this thesis has been submitted for the award of any other degree or diploma prior to this date.

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WITH GOD, ALL THINGS ARE POSSIBLE. Mathew 19:26.

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SREENITHI

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RBC	Red Blood Cell
HSCs	Hematopoietic Stem Cells
EPO	Erythropoietin
PLP-E	Placental Prolactin Like-E
TDT	Transfusion dependent thalassemia
NTDT	Non-transfusion dependent thalassemia
PV	Polycythemia Vera
Jak2	Janus Kinase 2
HIFs	Hypoxia-Inducible Factors
TF	Transferrin
SCF	Stem Cell Factor
BFU	Burst-Forming Units
CFU	Colony-Forming Units
sTfR	Serum Transferrin Receptors
IDA	Iron Deficiency Anemia
GDF15	Growth Differentiation Factor 15
TFR1	Transferrin Receptor 1
TFR2	Transferrin Receptor 2
ERFE	Erythroferrone
LIP	Labile Iron Pool
DMT1	Divalent Metal Transporter 1
FPN	Ferroportin
WHO	World Health Organization
ELISA	Enzyme-Linked Immunosorbent Assay
RNA	Ribonucleic Acid
DNA	Deoxyribonucleic Acid
LT-HSCs	Long-Term Hematopoietic Stem Cells
ST-HSCs	Short-Term Hematopoietic Stem Cells
MPPs	Multipotent Progenitors
CMPs	Common Myeloid Progenitors
CLPs	Common Lymphoid Progenitors

GMPs	Granulocyte-Macrophage Progenitors
MEPs	Megakaryocyte-Erythrocyte Progenitors
TFs	Transcription Factors
HSPCs	Hematopoietic Stem and Progenitor Cells
FACS	Fluorescence-Activated Cell Sorting
LCR	Locus Control Region
EBI	Erythroblastic Island
ALA	δ-aminolevulinic acid
ALAS	ALA synthase
IRP	Iron-Responsive Protein
CPgenIII	Coproporphyrinogen III
PPIX	Protoporphyrin IX
FECH	Ferrochelatase
MFRN1, MFRN2	Mitoferrin1, Mitoferrin2
IGF-1	Insulin-Like Growth Factor 1
IL-3, IL-6	Interleukin-3, Interleukin-6
Stat5	Signal Transducer and Activator of Transcription 5
PI3K/Akt	Phosphoinositide-3 Kinase/Akt
MAPK	Mitogen-Activated Kinase
miRNAs	MicroRNAs
EMP	Erythroblast Macrophage Protein
MAEA	Macrophage Erythroblast Attacher
ICAM-4	Intracellular Adhesion Molecule 4
VCAM-1	Vascular Cell Adhesion Molecule 1
Fe	Iron
Dcytb	Duodenal cytochrome b
DMT1	Dimetal transporter 1
FTH	Ferritin heavy chain
FTL	Ferritin light chain
HEPH	Hephaestin
HAMP	Hepcidin
BMP	Bone morphogenetic protein
SMAD	Small mother against decapentaplegic

TMPRSS6	Transmembrane serine protease 6
IRE	Iron-responsive element
ROS	Reactive oxygen species
NTBI	Non-transferrin bound iron
ZIP	Zrt- and Irt-like protein
HO-1	Heme oxygenase 1
PCBP	Poly(rC) binding protein
STAT5	Signal transducer and activator of transcription 5
HRE	Hypoxia-responsive element
SNX3	Sorting nexin 3
EXOC6	Exocyst complex component 6
FLVCR1	Feline leukemia virus subgroup C cellular receptor 1
FTMT	Mitochondrial ferritin
GDF15	Growth Differentiation Factor 15
TWSG1	Twisted gastrulation BMP signaling modulator 1
DFO	Deferoxamine
DFP	Deferiprone
DFX	Deferasirox
IFNα	Interferon alpha
MF	Myelofibrosis
MCV	Mean Corpuscular Volume
PIH	Pregnancy-Induced Hypertension
IREB2	Iron-Responsive Element Binding Protein 2
PBSC	Peripheral Blood Stem Cells
FBS	Fetal Bovine Serum
TIBC	Total Iron-Binding Capacity
ACO1	Aconitase 1
PGF	Placental Growth Factor
HIF1A	Hypoxia Inducible Factor 1 Subunit Alpha
STEAP3	Six Transmembrane Epithelial Antigen of the Prostate 3
SLC46A1	Solute Carrier Family 46 Member 1
TWSG1	Twisted Gastrulation BMP Signaling Modifier 1
SP1	Specific Protein 1

TP53	Tumor Protein 53
FAC	Ferric ammonium citrate
LRP1	Low-density lipoprotein receptor-related protein 1
FPN1B	Non-IRE bearing form of ferroportin 1
TFRC	Transferrin receptor
GATA1	GATA binding protein 1
KLF1	Krüppel-like factor 1
Esr1	Estrogen receptor 1(mice)
plp-e	Prolactin-like protein E (mice)
hamp	Hepcidin antimicrobial peptide (mice)
IO	Iron overload
ID	Iron deficient
NP	Non-pregnant

BIOLOGY OF IRON IN RBC REGENERATION: UPCOMING PLAYERS OF REGENERATIVE MEDICINE

SREENITHI.S

SYNOPSIS

CHRISTIAN MEDICAL COLLEGE,
VELLORE

For Ph.D. Degree of

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY,

TRIVANDRUM

Background

Erythropoiesis is a complex and multistep process highly influenced by iron regulation. Iron homeostasis and erythropoiesis are inextricably linked, and a delicate balance is essential since disruption of either leads to iron deficiency or anaemia. Erythropoiesis either decrease or increase in response to a physiological or pathological condition. Physiological upregulation of erythropoiesis is noted in pregnancy, whereas pathological upregulation (chronic stress erythropoiesis) is seen in beta-thalassemia and Polycythemia vera.

During pregnancy, increased erythropoiesis occurs in the second trimester because of dynamic alterations in a maternal environment in response to developing the placentofetal compartment. A net cost of 1000mg of iron is required for the increased maternal erythrocyte mass expansion and the developing fetal-placental unit. Nearly one-third of iron is utilised for establishing adequate iron stores in neonates at birth. Earlier studies on erythropoiesis in pregnancy stated that erythropoietic activity did not depend on erythropoietin levels during anaemia and early pregnancy. A study on animal models suggested that iron deficiency may significantly induce erythropoietin production due to the production of RBCs in pregnancy. However, there is no clear evidence of factors related to iron, erythropoietin, or any other factors that stimulate erythropoiesis in pregnancy.

Chronic stress erythropoiesis in beta-thalassemia (BT) is due to the absence or decreased production of beta-globin chains of Hb tetramer. It leads to the accumulation of excess α- globin chains, which leads to hemolysis. BT is characterised by ineffective erythropoiesis, splenomegaly, extramedullary expansion, apoptosis of erythroid precursors and shortened mature RBC survival. Often, the chronic state of anaemia is treated by RBC transfusions, which in turn

results in iron overload owing to the production of reactive oxygen species that are highly toxic. The mechanism underlying high erythroid iron demand and blood transfusions resulting in iron overload is still not precise.

Polycythemia Vera (PV) is a myeloproliferative disorder characterised by unlimited proliferation of erythrocytes, megakaryocytes and granulocytes. WHO defined PV as Hb>18.5g/dL for men and 16.5g/dL for women; also, the occurrence of Janus kinase 2(Jak2) mutation is mainly related to V617F allele in exon 14 or exon 12 In PV, therapeutic phlebotomies limit the total body iron accumulation, causing iron deficiency. There is no clear evidence on regulating iron and erythroid under chronic stress erythropoiesis.

Identifying factors that trigger increased erythropoiesis in pregnancy will aid in a better understanding of physiological and pathological erythropoiesis.

Objectives

1. Elaborate on how iron plays an essential role in RBC regeneration. We will study the role of iron and various regulators of erythropoiesis in physiological (pregnancy) and pathological (PV) states of increased erythropoiesis using healthy pregnant women at different time points and PV patients.
    - ❖ To evaluate the role of iron and various regulators of erythropoiesis in pregnant women at gestational weeks – 16,21,27 and 36.
    - ❖ To elucidate the influence of maternal iron status on the regulation of placental iron transport and fetal supply.
    - ❖ To evaluate the role of iron and various regulators of erythropoiesis in pathological states of erythropoiesis - Polycythemia Vera, β-thalassemia.

2. Study the differentiation of CD34 progenitor stem cells and expand them in-vitro into the erythroid lineage and study their behaviour.
3. Comprehensive analysis of iron homeostasis regulators in erythropoiesis during pregnancy using iron-replete and iron-deficient mice models.
4. To develop an ex vivo experimental model for RBC regeneration.

Experimental methods used in the study:

Study population

The study was approved by the Institutional Review Board (Ethics Committee) of Christian Medical College (CMC) at Vellore, India, and informed written consent was obtained from all the study participants.

Cohort 1: Pregnant women who visited the antenatal clinic at the Department of Community Health and Development (CHAD), CMC, for childbirth were screened, and subjects who fulfilled the inclusion criteria were included in the study (Age-18-35 years, primi gravida, gestational age ≤16 weeks). Pregnant women with age > 35 years, gravida>1, gestational Age >16 weeks, gestational diabetes and pregnancy-induced hypertension (PIH). This study was conducted between the year 2019-2022. Daily oral iron supplementation with 60 mg of elemental iron was recommended for all pregnant women who visited our antenatal clinic. Maternal peripheral blood samples were collected at gestational weeks- 16,21,26 and 36 weeks.

Cohort 2: This cross-sectional study was conducted on pregnant women between 2016 and 2018. Subjects who visited CHAD for childbirth were screened, and subjects who fulfilled the inclusion criteria were included in the study (Age 18-35 years and gestational age (GA) of ≥ 36 weeks). Pregnant women with gestational diabetes, pregnancy-induced hypertension (PIH), hypothyroidism, previous caesarean section, bacterial or viral infections during the onset of labour, and twin

pregnancies who received transfusion during delivery were not included in the study. Daily oral iron supplementation with 60 mg of elemental iron was recommended for all pregnant women who visited our antenatal clinic.

Maternal peripheral blood samples were collected at admission (GA≥38 weeks) before or after delivery. The umbilical cord was clamped and cut during delivery, and cord blood was collected. Placental tissue was obtained and processed within an hour of delivery.

For the analysis, Iron deficiency anemia in pregnancy (IDA) was defined as a Hb level of <10.5g/dl with a ferritin level of <30ng/ml; iron-replete subjects (control) Hb>10.5g/dl and ferritin >30ng/ml.

Cohort 3: Patients who visited the Haematology department at CMC for Polycythemia Vera (PV) between 2019 and 2022 were included in the study. Newly diagnosed PV patients (n=50) were recruited based on WHO criteria- Hb >16.5 g/dL in men, >16.0 g/dL in women, or Hct >49% in men, >48% in women and with the presence of JAK2V617F mutation in exon14. **Cohort 4**: β thalassemia major patients with age of ≤ 10 years and ≤ 50 transfusions; HbE-β thalassemia patients with age of ≤ 10 years were included in the study. Patients who underwent hydroxyurea treatment were excluded from the study.

Male voluntary blood donors (n=50) with normal haematological parameters and adequate iron stores were included as a control group.

Animal Studies

8-10 weeks old C57BL/6 mice were obtained from the Centre for Animal Facility, CMC and the study was approved by the Institutional Animal Ethics Committee (IAEC) in 2020. Mice were kept in the in-house animal facility maintained at 22±3°C and 55±15%relative humidity with a 12-hour light-dark

cycle. Male mice were introduced in the female cage for timed pregnancies, and within 24 hours, female mice were checked for the presence of a copulation plug visually and labelled as Day 0 of pregnancy. Then, male mice were removed, and plugged females were housed in groups of 4 throughout gestation. Mice were fed three different diets and divided into control, iron deficient, and iron overload groups.

Cohort 1: C57BL/6J females received a standard diet for two weeks prior to mating and throughout gestation.

Cohort 2: Mice were maintained on an iron-deficient diet (4ppm, Research Diets, USA) for four weeks before mating and throughout gestation.

Cohort 3: Iron overload diet (10000ppm, Research Diets, USA) was given for two weeks prior to mating and during gestation.

Pregnant mice were sacrificed on embryonic day 0, 10.5, 12.5 and 18.5. Tissue samples collected from the liver, spleen, kidney, duodenum, fetal liver and placenta were stored in RNA later at -80°C.

Cell Culture

Erythroid differentiation from hematopoietic progenitor cells:

According to the manufacturer's instructions, $CD34^+$ cells were purified from healthy stem cell donors by positive selection using magnetic-activated cell sorting. The purity of isolated $CD34^+$ cells was 95% to 98%. The cell culture procedure was comprised of 3 phases. The composition of the base culture medium was a serum-free stem-span medium (Stem Cell Technologies, USA). In the first phase (day 0 to day 6), $CD34^+$ cells at a concentration of 10^5 /mL were cultured in the presence of 10 ng/mL stem cell factor, 1 ng/mL IL-3, and 3 IU/mL erythropoietin. In the second phase (day 7 to day 11), IL-3 was omitted from the culture medium. In the third

phase, which lasted until day 21, the medium for this phase was the base medium plus 3 IU/mL erythropoietin, and the transferrin concentration was adjusted to 1 mg/mL. The cells were cultured at 37°C in 5% CO2.

Ter119+ cell sorting

For bone marrow cell suspension preparation from C57BL/6 mice, cells from the mice's tibia and femur were flushed with PBS/2 mM EDTA. Cells were centrifuged at 300*g* for 5 minutes, and the pellet was suspended in RPMI medium. Ter119+ cells labelled with FITC-conjugated antibodies were isolated from the bone marrow cell suspension using EasySep™ FITC Positive Selection Kit. The purity of isolated Ter119+ cells was >90%. Cells were stored in trizol at - 80°C.

Placenta collection and processing

The placenta collected during delivery was processed within 1 hour following delivery. Amniotic membranes were removed from the placenta, and tissue of 0.5-0.8cm thickness was incised from red cotyledons. Below the amniotic membrane side of the placenta, a deep cut was avoided. The dissected tissues were stored in RNAlater (Ambion) at -80°C until analysis.

Haematological and Biochemical Assessment

Complete blood counts (CBC) were performed on maternal peripheral and cord blood samples using an automated haematology analyser (Sysmex KX21). Serum ferritin was analysed using a chemiluminescence immunoassay using the Advia Centaur, Siemens XPI. According to the manufacturer's protocol, serum hepcidin was quantified using an enzyme immunoassay method from DRG, GmbH. GDF15 was quantified in serum using an ELISA method (R&D Systems, Inc. MN, USA).

Flow cytometry

Human and mouse erythroid cells were stained with markers such as FITC-

labelled CD71(human and mouse), APC-labelled mouse Ter119 and APC-labelled human CD235. Apoptosis staining with annexin V and FITC labelled 7-AAD were performed as described in the kit's manual.

RNA extraction and PCR Arrays

Total RNA was extracted from the reticulocytes isolated from human peripheral blood samples. Tissue RNA extraction was done for frozen human placental tissue and mice samples (liver, spleen, fetal liver, placenta) using the Protein and RNA Isolation (PARIS) kit (Qiagen). RNA was reverse transcribed and converted into cDNA using RT^2 first strand Kit (QIAGEN). Real- time PCR was performed on the 7500 QPCR System (Applied Biosystems model) and used SYBR green detection (human cDNA) and Taqman probe detection (mice cDNA). Plate-to- plate variation was controlled by normalising gene expression to β-actin/GAPDH and control by using the $2^{-\Delta\Delta Ct}$ method.

Protein expression of placental Fe transporters

According to the manufacturer's protocol, the placental tissues were lysed by homogenisation in cell disruption buffer (PARIS kit, Ambion). Protein concentration was quantified using Bradford assay. All samples were prepared in Lamaelli buffer with reducing agent β- mercaptoethanol. 30µg of samples were boiled at 100°C for 5 mins. Tissue lysates were separated by SDS-PAGE gels (4%-12%) and transferred to polyvinylidene difluoride fluorescence membranes (Millipore, Billerica, MA, USA). Non-fat dry milk (NFDM-10%) was used to block the membranes and probed with primary antibody diluted in 5% NFDM diluted in TBS buffer with 0.1% Tween20 and kept at 4°C overnight. Membranes were rinsed and probed with secondary antibodies for 1.5 hours in NFDM blocking buffer containing 0.1% Tween20. The bands were visualised using a chemiluminescence

ECL system (Super Signal West femto, Thermo Scientific). The FluorChem E system detected protein bands using digital darkroom software. Band intensities were quantified by densitometric analysis using ImageJ software.

Statistical Analysis

Statistical data analysis was carried out using the software SPSS, version 22. For categorical data, the Chi-square test was used. Appropriate statistical tests were used, including t-test for continuous variables, analysis of variance (ANOVA) for comparison of groups, Mann– Whitney, and Kruskal–Wallis for nonparametric data.

Salient findings and their significance:

- A progressive increase in erythropoietin and a decrease in ferritin levels from the initial to third trimester suggest the utilization of mobilized iron for enhanced erythropoiesis, supporting placental and fetal development. Under maternal iron insufficiency, the expression of placental iron transporters DMT1, FPN1, and GDF15 was upregulated at the protein level. Placental GDF15 and ferroportin protein had an association with fetal iron status. Placental iron traffickers respond to maternal iron deficiency by increasing their expression and allowing sufficient iron to pass to the fetus.
- In the mice model, erythropoietin, DMT1 (iron transporter) and erythroferrone mRNA expression from erythroid cells exhibited greater sensitivity to iron depletion at E12.5 (second trimester). The expression of Hepcidin (Hamp mRNA) was significantly suppressed throughout gestation in iron-deficient mice. This coincides with decreased liver iron content.Placental prolactin Plp-e, identified to regulate maternal erythropoiesis, was highly expressed at E12.5 in the placenta. Fetal consumption of iron increased in the third trimester in maternal iron-deficient mice.

- The in vitro terminal erythroid differentiation process from hematopoietic stem cells obtained from healthy stem cell donors displayed a significant decrease in the maturation of erythroid cells when subjected to an iron-deficient environment. Decreased mRNA expression of iron regulators such as ferroportin, DMT1 revealed that iron deficiency impairs terminal erythroid maturation.
- Estradiol treatment in hemin-treated HEL cells showed a significant decrease in CD71% and CD 235% at 48 and 72 hrs, respectively. Thus, estradiol treatment impairs erythroid differentiation by suppressing erythroid maturation.
- In thalassemia, ineffective erythropoiesis was associated with systemic iron overload.
- During 16-21 gestational weeks, a decrease in TFRC mRNA expression suggests the presence of systemic iron overload. KLF1 involved in terminal erythroid maturation has increased expression in thalassemia.
- In polycythemia vera, pathological expansion of erythropoiesis was associated with reduced iron stores. Decreased hepcidin levels indicated increased iron mobilisation for an increased rate of erythropoiesis. The elevated sTfR-ferritin index confirms the functional iron deficiency. Relatively low serum ferritin levels and normal IL-6 levels observed in PV patients suggested that inflammation does not significantly contribute to the dysregulated iron metabolism. Ferroportin isoform FPN1B transcript that lacks a 5'IRE (iron-responsive element), activated under iron-deficient conditions, contributes iron into the circulation.

Implications of the findings:

- In our study, 22% of pregnant women between 20 and 35 years old had iron deficiency anemia at delivery despite iron supplementation. Increased expression of DMT1 and FPN1 in the placenta is most likely to represent an increased amount of iron transfer to the fetus despite the mother being iron deficient. Fetal iron metabolism seems to be independent of maternal iron status.
- Estradiol, placental prolactin (PLP-e), and iron regulators are essential in regulating erythropoiesis in pregnancy. Validating their mechanism at the protein level might help in understanding the tight regulation of increased erythropoiesis during pregnancy.
- In pregnancy and polycythemia vera, hemoglobin levels could be sustained at either normal or high levels due to the enhanced supply of iron for hemoglobin production. On the other hand, in thalassemia, defect in hemoglobin synthesis results in erythropoiesis overriding the regulation of iron levels. In the hierarchy of processes, erythropoiesis can sustain hemoglobin levels even when iron stores are low.

1.0 Introduction

Blood transfusion is an essential therapy for various clinical conditions (Li et al., 2013). The diverse forms of blood donation yield three principal blood components: red blood cell (RBC) concentrates, platelet concentrates, and therapeutic plasma (Hardwick, 2008). India faces a blood transfusion shortage of 10% relative to its requirement (Ray et al., 2000). A large quantity of blood is needed for patients with thalassemia, hemophilia, sickle cell anemia, and for road accident surgeries and pregnancy complications (Mammen et al., 2022). Due to a deficient number of blood donors and potential contamination with undetectable pathogens, it is crucial to have alternative blood sources for transfusions (Spahn and Goodnough, 2013).

Studies have demonstrated an imbalance in blood supply and demand. The aging of the population reduces the number of healthy donors and increases the incidence of diseases that require transfusion (Mortazavi et al., 2023). *in vitro* generation of universal blood substitutes aim to reduce reliance on blood donations in clinical settings and address unmet clinical requirements. (Seo et al., 2019).

In such a scenario, stem cells could provide a better alternative solution for blood transfusion as it has the unique ability to develop into specialized cell types in the body. Hematopoietic stem cells (HSCs) give rise to blood cell lineages, including red blood cells, platelets, and white blood cells. HSCs could be an effective substitute as it differentiates into erythroid lineage and generate red blood cells (RBCs). Researchers have attempted to develop an in vitro system for RBC regeneration on a large scale. However, the limited proliferation ability of HSCs, late maturation, and enucleation of erythroid cells restricted the usage of these cells (Bresnick et al., 2018). Studying the mechanisms of an increased erythropoietic state will lead to a better understanding of RBC regeneration.

Nearly 20mg of iron is required to produce 200 billion erythrocytes daily. Burst-forming units (BFU) are primary immature precursors devoted to the erythroid lineage. BFU requires growth factors and stem cell factors (SCF) for proliferation and differentiates into colony-forming units (CFU). CFU-Es depend on erythropoietin for further differentiation into different stages of proerythroblast, basophilic, polychromatophilic, Ortho chromatophilic erythroblast, and mature erythrocyte (Muckenthaler et al., 2017). Iron, vitamin B12, and folate are required nutrients for the dynamic process of erythropoiesis. Iron is recruited at its maximum during the basophilic stage for hemoglobin synthesis (Koury and Ponka, 2004).

To gain maximal iron, erythroid cells depend on transferrin-bound iron. Transferrin receptor (TFR1) mediated ferric-transferrin complex is endocytosed in the erythroid cell, wherein in an acidic environment, iron is released from the TFR-Tf-Fe^{3+} complex. Ferric iron is reduced to ferrous form by STEAP3(six-transmembrane epithelial antigen of the prostate) and exported via dimetal transporter (DMT1) to the cytoplasm. Extracellular ferritin released from macrophages moves into erythroid cells and may lend iron through pinocytosis, but it cannot substitute iron mediated by TFR. TFR2 senses plasma iron levels to regulate the erythropoietin receptor signaling. Iron is acquired by cells and mobilized to a cytosolic pool called a labile iron pool (LIP). Iron from LIP is either stored in ferritin or incorporated into mitochondria via mitoferrin for heme synthesis and Fe-S (Sulphur) clusters or efflux through ferroportin (FPN) and oxidized on the extracellular side (Muckenthaler et al., 2017).

Hepcidin is a major regulator of systemic iron homeostasis, controls iron balance, and limits iron absorption. High erythroid activity suppresses hepcidin either by stress erythropoiesis or pathological ineffective erythropoiesis. However, the mechanism behind erythroid stimulation to suppress hepcidin still remains elusive. Erythroid-derived hormones such as Growth Differentiation factor (GDF15) and twisted gastrulation homolog 1(TWSG1) were considered as hepcidin suppressors in the setting of increased erythropoiesis. Erythroferrone (ERFE),

another emerging suppressor candidate, might link increased erythropoiesis and hepcidin suppression, but it is still not clear. Several mechanisms have been proposed, but the exact mechanism by which erythropoiesis induces hepcidin suppression is still obscure (Pasricha et al., 2016a). In pregnancy, hepcidin level decreases in the second and third trimesters, and it is a useful diagnostic test for iron deficiency anemia in pregnancy (Bah et al., 2017). The relationship between hepcidin and increase in erythropoiesis' physiological/pathological states is currently unclear.

A classical regulator of increased erythropoiesis is hypoxia. Hypoxia-inducible factors (HIFs) induce modifications in specific cell type-gene expression, leading to the production of EPO in the kidney and liver. In addition, it increases iron absorption to facilitate erythroid progenitor production, maturation, and proliferation. HIF-2 specifically regulates the synthesis of renal and hepatic EPO and intestinal iron absorption (Haase, 2013). HIF2 undergoes post-transcriptional modification and is regulated by intracellular iron levels. A decrease in intracellular iron levels inhibits the translation of HIF2α and limits HIF2-stimulating EPO synthesis and erythropoiesis. Consequently, the presence of iron is a prerequisite for the process of erythropoiesis.

Notably, in pregnancy, hematopoietic stem cells (HSCs) actively contribute to enhanced red blood cell (RBC) regeneration for the developing placentofetal compartment. Enhanced iron levels are required to sustain hemoglobin synthesis in such a scenario. These requirements are mostly fulfilled by recycling iron through erythrophagocytosis by macrophages. Increased RBC regeneration in pregnancy occurs in a physiological manner. It involves erythropoiesis expansion, causing an increase in red blood cell mass and plasma volume during the second and third trimesters up to term. The total blood volume escalates up to 1.5 liters to support the newly developing fetus, and loss recurs in delivery (Kepley et al., 2023).

Earlier studies on erythropoiesis in pregnancy stated that erythropoietic activity did not depend on erythropoietin levels during early pregnancy (Howells et al., 1986). Based on elevated transferrin receptor levels, it was also observed that iron deficiency did not interfere with erythropoietic activity in early pregnancy (Beguin et al., 1991). However, a recent study on animal models suggested that iron deficiency may significantly induce erythropoietin due to the production of RBCs during pregnancy (Horiguchi et al., 2005). Invitro studies demonstrated the expansion of erythrocyte precursors in the spleen during the mid-gestational period (Bustamante et al., 2008). Erythropoietin (EPO) and serum transferrin receptors (sTfR) levels were measured in pregnant women to quantitatively assess the absolute rate of erythropoiesis (Beguin, 2003). Even a moderate increase in EPO level would initiate the production of erythroid progenitor cells. However, increased erythropoietic activity in early pregnancy did not appear to depend upon increased plasma EPO levels (Choi and Pai, 2001). Interestingly, animal studies have demonstrated that erythropoietic activity is suppressed by estrogen (MIRAND and GORDON, 1966). It was evidenced that elevated estrogen levels sustained during pregnancy have induced stem cell mobilization and red cell production. (Nakada et al., 2014).

Expectant mothers exhibit increased plasma Epo levels(Cotes et al., 1983), although these levels are lower than those observed in nonpregnant women experiencing a similar degree of anemia(Beguin et al., 1990). Thus gestational anemia did not influence the rate of erythropoiesis (Choi and Pai, 2001). Iron deficiency is a well-known micronutritional deficiency causing severe anemia in maternal women and increasing the risk of neuronal impairment in neonates(Delaney et al., 2019). Iron deficiency anemia (IDA) affects 1.7 billion people globally; among them, pregnant women are the most vulnerable population (Rahman et al., 2019). In India, iron deficiency is the most common cause of anemia in 50% of pregnant women (Kaushal et al., 2022).

There has been minimal research on erythropoiesis during pregnancy, and the existing studies have not addressed the underlying mechanisms. Currently, there is a lack of definitive evidence regarding the factors that impact increased erythropoiesis in pregnancy.

Moreover, increased erythropoiesis is evident in conditions like Beta thalassemia (BT) major and polycythemia vera (PV). BT is characterized by ineffective erythropoiesis, splenomegaly, extramedullary expansion, apoptosis of erythroid precursors, and shortened mature RBC survival. It manifests in three forms based on severity and genotype, including beta-thalassemia trait, thalassemia intermedia, and thalassemia major. The β globin mutation results in accumulation excess α- globin chains, which leads to hemolysis (Cao and Galanello, 2010). Often, the chronic state of anemia is treated by RBC transfusions, which in turn results in iron overload. Several studies inline have investigated the crosstalk between iron and erythropoiesis in beta-thalassemia with the focus on hepcidin as a therapeutic target for beta thalassemia (Ramos et al., 2012). Patients with beta-thalassemia major had higher hepcidin levels than beta-thalassemia intermedia patients (Eshagh Hossaini and Haeri, 2019). The mechanism responsible for ineffective erythropoiesis and the resulting iron overload remains unclear; therefore, further exploration is warranted.

Polycythemia Vera is a chronic myeloproliferative disorder characterized by unlimited proliferation of erythrocytes, megakaryocytes, and granulocytes. WHO defined PV as Hb>18.5g/dL for men and 16.5g/dL for women; also, the occurrence of Janus kinase 2(Jak2) mutation is mostly related to V617F allele (Lu and Chang, 2023). Clinical trials such as polycythemia vera study group, European Collaboration on Low-dose Aspirin in Polycythemia Vera (ECLAP) trial have recommended hydroxyurea as a drug of choice for PV treatment

(Ferrari et al., 2019a). A system of invitro erythroid expansion of peripheral blood mononuclear cells of PV patients had exhibited hyperproliferation of erythroid precursors and rapid maturation between 9- 14 days with a deviant expression of EPO receptors (Bruchova et al., 2009a). Jak2 mutation is evidenced in more than 90% of PV patients, which results in increased EPO signaling. However, several studies have witnessed that Jak2 mutation is not an initial event occurring in PV (Kralovics et al., 2006). In sixteen polycythemia vera patients, hepcidin and GDF15 levels were measured; higher GDF15 levels were observed in Jak2V617F mutant subjects. No significant changes in hepcidin levels was seen in spite of high erythroid activity (Tarkun et al., 2013). Therefore, it is crucial to investigate the molecular mechanisms regulating erythropoiesis in PV.

1.1 Rationale of the study

Several research groups have successfully generated red blood cells (RBCs) in the laboratory and demonstrated their therapeutic potential using animal models. With the foundational understanding of RBC production, researchers focus on refining the procedure to acquire an ample quantity of functional RBCs in a convenient and cost-effective manner.

Hence, it is imperative to study the response of HSCs in a state of increased erythropoiesis, such as pregnancy, to achieve a better understanding of red blood cell (RBC) regeneration.

Pregnancy increases steady-state erythropoiesis due to the development of the placental-fetal compartment. The maternal environment undergoes dynamic alterations to support fetal growth, including increased blood supply to the new vascular bed, which supports fetal hematopoiesis (*The Global Library of Women's Medicine*, n.d.). Physiologic alteration results in elevated plasma volume and red cell mass in the second and third trimesters to peak at term leading to physiological anemia. In nonpregnant women, anemia typically serves as a key stimulant for Epo production. However, during pregnancy, it does not function as a significant

driving force. This is evident from the lack of a close correlation between serum Epo levels and hemoglobin (Hb) levels, as observed in nonrenal anemia patients(Huch and Huch, 1993; Riikonen et al., 1994). These observations suggest that other factors play more crucial roles in triggering Epo production during pregnancy. High erythroid activity also occurs in pathological conditions of beta-thalassemia (BT) and polycythemia Vera (PV). However, the factors influencing the chronic state of erythropoiesis are unknown.

Iron and erythropoiesis are inextricably linked; hence iron regulators might function as a stimulant to induce and regulate erythropoiesis. Here, we analysed iron regulators and erythroid factors in the acceleration of erythropoiesis in pregnancy as well as in PV and BT. Animal studies were performed to demonstrate the mechanism of iron-related factors in the induction of increased red blood cell production during pregnancy. The present study focused on identifying various regulators involved in accelerated erythropoiesis, thereby providing an understanding of the molecular mechanism of erythropoiesis.

1.2 Aim of the Study

To evaluate the iron regulators and numerous factors involved in stimulating increased erythropoiesis in pregnancy and stress erythropoiesis, such as Beta-thalassemia and Polycythemia Vera.

1.3 Objectives

1. To elaborate on how iron plays an essential role in RBC regeneration: We will study the role of iron and various regulators of erythropoiesis in pregnancy (physiological) and in pathological conditions.
    a. To evaluate the role of iron and various regulators of erythropoiesis in pregnant women at gestational weeks – 16,21,27 and 36.

b. To elucidate the influence of maternal iron status on the regulation of placental iron transport and fetal supply.
c. To evaluate the role of iron and various regulators of erythropoiesis in pathological states of erythropoiesis - Polycythemia Vera, β-thalassemia.

2. To study the differentiation of haematopoietic stem progenitor cells and expand them *in vitro* into the erythroid lineage and study their behaviour.
3. Comprehensive analysis of iron homeostasis regulators in erythropoiesis during pregnancy using iron-replete and iron-deficient mice models.

1.4 A brief overview of chapters

1.4.1. Literature Review

Following the introduction, rationale, and objectives of the study, the subsequent important chapter is the literature review. This section provides a brief overview of hematopoiesis, and the ontology of erythropoiesis is presented, followed by a concise discussion of the regulation of erythropoiesis and iron homeostasis. The primary focus centers on exploring the mechanism of iron and various factors involved in regulating increased erythropoiesis, particularly in conditions such as pregnancy, beta-thalassemia, and polycythemia vera.

1.4.2. Materials and Methods

This section provides a detailed description of the study participants, the recruitment process, and the criteria for inclusion. It elucidates the various techniques and methods employed in conducting experiments and analyzing data. Hematological and biochemical parameters and molecular analyses are thoroughly discussed within this segment. Additionally, details are provided on gene expression, RNA sequencing, ELISA, and protein techniques utilized. The section also summarizes the establishment of pregnant mice models subjected to different iron diets, cell culture techniques, immunophenotyping by flow cytometry, and the statistical analysis conducted.

1.4.3. Results

This section provides a detailed exploration of iron and erythroid regulation under different conditions of increased erythropoiesis. The functions of iron, erythroid, and placental factors in various tissues are investigated using pregnant mice models exposed to both iron deficiency and overload. The changes in the expression of iron and erythroid regulators at each stage of erythroid differentiation were discussed, considering characteristics such as cell morphology, immunophenotype, and proliferation capacity. The final segment evaluates the mechanisms underlying various factors at different stages of erythropoiesis.

1.4.4. Discussion

This section provides a detailed description of the key results obtained in the study and elucidates their significance. The findings of the study have been analyzed in connection with existing scientific information, and their clinical relevance has been discussed.

1.4.5. Summary and Conclusion

In this chapter, the primary observations and proposed mechanisms from the doctoral work have been summarized. The section also features a comprehensive figure depicting and discussing the findings. Furthermore, the bibliography includes a list of references cited in the text.

2.0 Review of Literature

2.1. Hematopoiesis

Haematopoiesis serves as a model for understanding the biology of tissue stem cells and their involvement in aging, disease, and oncogenesis (Orkin and Zon, 2008). Stem cells play a crucial role throughout an individual's life, as mature blood cells have a relatively short lifespan, necessitating the replenishment of multi-lineage progenitors and precursors committed to specific hematopoietic lineages (Orkin, 2000). Hematopoietic stem cells (HSCs) are rare cells in the bone marrow of adult mammals and occupy the top tier of a hierarchy of progenitors that gradually differentiate into several or single lineages. Franz Neumann and Alexander Maximow, proposed the idea in the 1800s that a multipotent hematopoietic stem cell (HSC) in the bone marrow has the commitment and differentiation processes that result in the development of all blood cells(Pinho and Frenette, 2019). Although the bone marrow is the predominant site for hematopoiesis in adults (medullary), it also occurs in other locations, such as the liver, thymus, and spleen (extramedullary)(Lim et al., 2014). It is estimated that an adult human produces approximately 4-5 x 10^{11} hematopoietic cells, highlighting the remarkable efficiency of this intricate system(Kaushansky, 2006).

2.2. HSC Regeneration

HSCs are characterized by their self-renewal capacity and ability to produce different progenitors that proliferate and become mature blood cells(Laurenti and Göttgens, 2018). Regenerative capability of hematopoietic system arises from the presence of multipotent stem cells, ensuring the continuous replacement of millions of aging blood cells with new ones (Rieger and Schroeder, 2012). HSC pool is the heterogenous and consists of long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs).In steady state, LT-HSCs are predominantly in

quiescence state with highest self-renewal potential, thereby prevents exhaustion of stem cell function (Orford and Scadden, 2008). ST-HSCs, characterized by their short-term repopulation potential, tend to exit quiescence faster than their long-term counterparts, LT-HSCs, upon mitogenic activation. (Laurenti et al., 2015).

In the classical hierarchical model of hematopoiesis, HSCs undergo self-renewal and multipotent differentiation, ultimately giving rise to all blood and immune cell types (Rieger and Schroeder, 2012). LT-HSCs has 3-4 months of reconstitution capacity, whereas ST-HSCs has 1 month of reconstitution capacity (Yang et al., 2005). LT-HSCs are characterized by $CD34^-$ expression, while ST-HSCs are characterized by $CD34^+$ expression. LT-HSCs differentiates into ST-HSCs, which in turn produce multipotent progenitors (MPPs). MPPs differentiate into common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). With declining multilineage potential, CMPs differentiate into myeloid-erythroid lineage, whereas CLPs differentiate into the lymphoid lineage. CMPs generate granulocyte-macrophage progenitors (GMPs), which, in turn, give rise to granulocytes, macrophages, and dendritic cells. Additionally, CMPs differentiate into megakaryocyte-erythrocyte progenitors (MEPs), leading to the development of megakaryocytes and erythrocytes. Meanwhile, common lymphoid progenitors (CLPs) segregate to form T, B, NK, and dendritic cells. This intricate process establishes a balanced and tree-like hierarchy of cell populations (Figure 2.2.1.1.). The precise orchestration of this stepwise differentiation from hematopoietic stem cells (HSCs) to mature blood cells is closely regulated by intrinsic transcription factors (TFs) and extrinsic cytokines(Cheng et al., 2020). Researchers have identified different stages of hematopoietic stem and progenitor cells (HSPCs) by analyzing their functional properties and surface marker expression. This allows for the efficient isolation of these cells using fluorescence-activated cell sorting (FACS) (Rieger and Schroeder, 2012).

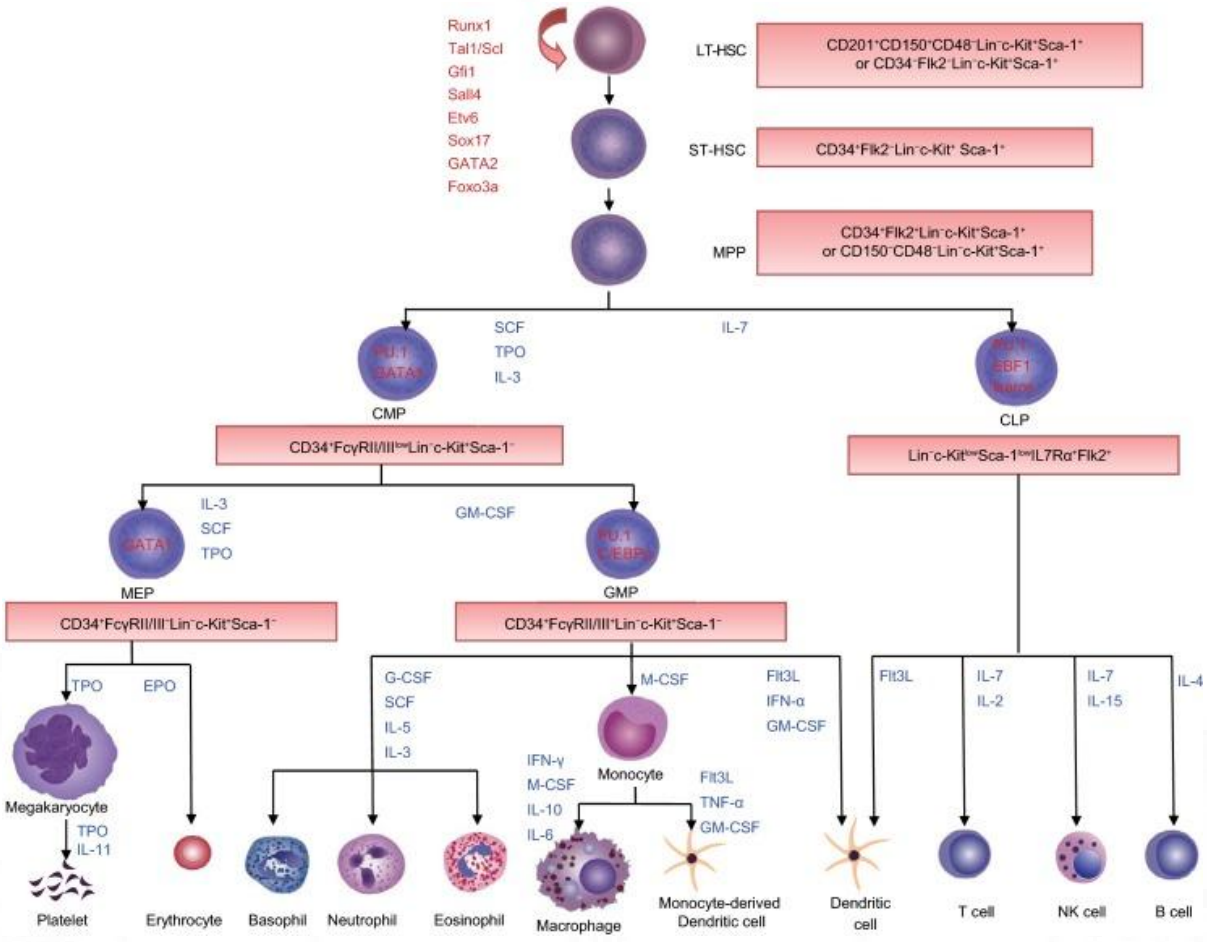


Figure 2.2.1.1: Classical hierarchy of hematopoiesis (Rieger and Schroeder, 2012)

2.3. Erythropoiesis

Erythropoiesis involves a complex series of events within the bone marrow, where HSCs undergo proliferation and transform into erythroid committed progenitors and ultimately produce mature red blood cells (RBCs) (Ebrahimi et al., 2020). These cells have a vital function in delivering oxygen and are essential throughout life, from embryonic to adult stages During the process of erythrocyte production, factors such as cytokines, transcriptional factors, and miRNAs play a crucial role in regulating erythroid differentiation.

2.3.1. Ontogeny of erythropoiesis

The process of erythropoiesis in humans occurs in two distinct phases, namely primitive (embryonic) and definitive (adult), each taking place in different anatomic locations. Primitive erythroid cells originate in the yolk sac on embryonic day E7.5. The first wave of definitive erythropoiesis also commences in the yolk sac and placenta at E9, but the cells complete maturation in the fetal liver (Figure 2.3.1.1a). Subsequently, a second wave of definitive erythropoiesis emerges from multipotent hematopoietic stem and progenitor cells (HSPCs) produced from aorta –gonad –mesonephros (AGM) at E10.5, undergoes expansion in the fetal liver, and eventually migrates to the bone marrow (Figure 2.3.1.1b). During the postnatal stage, bone marrow becomes the permanent site for erythropoiesis throughout adult life and spleen also is a site of erythroid cells differentiation. Primitive erythrocytes are large, nucleated cells, whereas definitive erythrocytes are smaller, lack a nucleus, and express distinct hemoglobin compared to primitive erythroid cells (Baron et al., 2012; Dzierzak and Philipsen, 2013). In definitive erythropoiesis in adults, HSPCs with declining multipotential ability, differentiates into burst-forming unit (BFU-E) and colony-forming unit (CFU-E). CFU-E then further differentiates into proerythroblasts, leading to the formation of basophilic, polychromatophilic, orthochromatic erythroblasts, reticulocytes, and eventually red blood cells (RBCs). Reticulocytes, which are slightly larger than mature erythrocytes, undergo transformation into erythrocytes within 1-2 days upon entering the bloodstream. This is achieved by shedding additional plasma membrane and cellular organelles, allowing them to become smaller and adopt the characteristic biconcave disc shape of erythrocytes. These cells circulate for around 120 days before being cleared by the reticuloendothelial system, primarily in the spleen and liver (Shah et al., 2014).

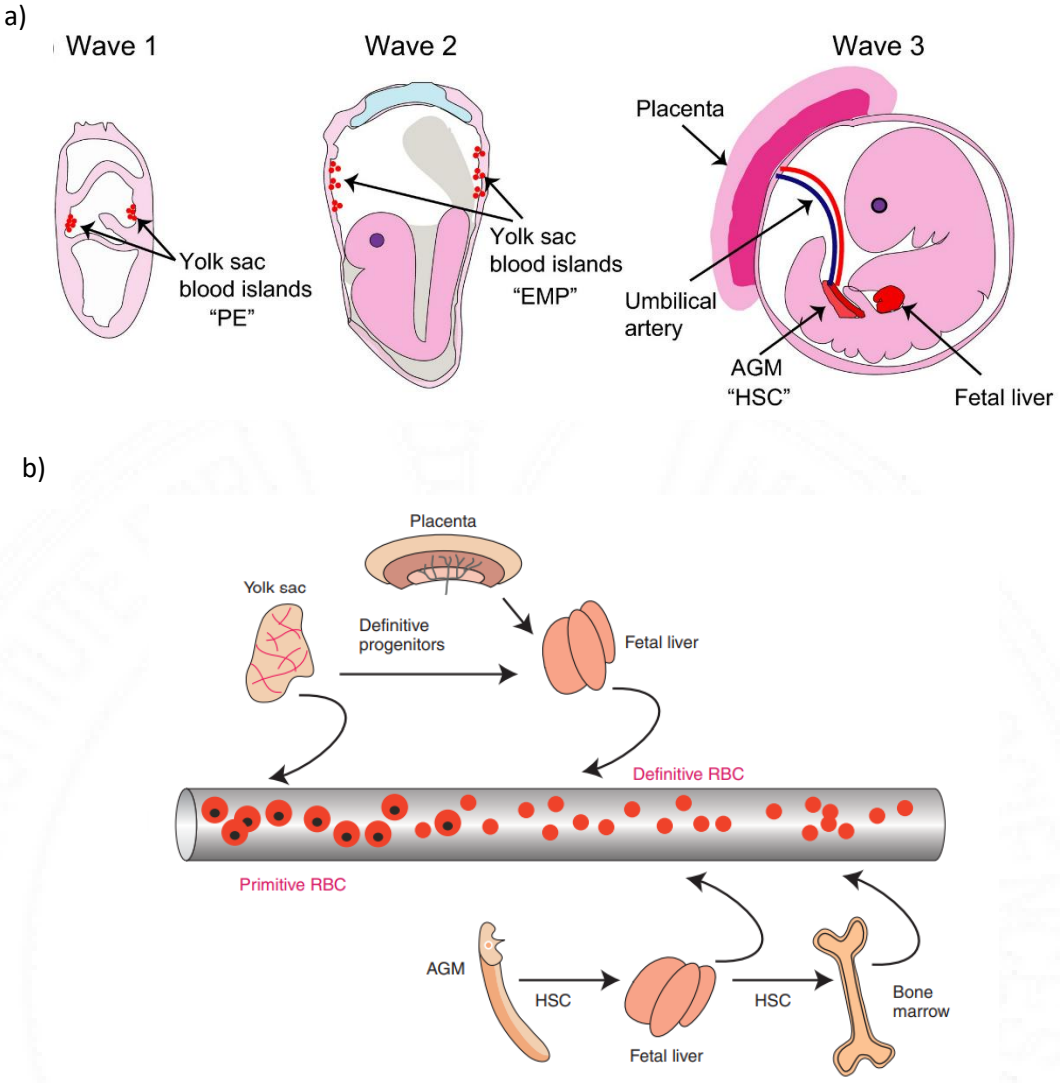


Figure 2.3.1.1. a&b) Ontogeny of erythropoiesis (Dzierzak and Philipsen, 2013)

2.3.1.1. Ontogeny of hemoglobin synthesis

The hemoglobin molecule is composed of two α-like globin peptide chains and two β-like globin peptides, which are located on chromosomes 16 and 11, respectively. Each chain is accompanied by a heme moiety, which is essential for its oxygen-carrying capacity. The β-like globin molecules are produced through developmental regulation of the human β-globin locus present on chromosome 11(Sankaran and Nathan, 2010).

The β-like globin genes are arranged in a cluster and are expressed in a specific order during development. The Locus Control Region (LCR), consisting of multiple enhancers positioned at a distance, collectively enhances the rate of transcriptional elongation, leading to the high-level expression of these genes(Brand, 2015).

In the early weeks of first trimester, the yolk sac-derived primitive erythrocytes have a significant expression of an embryonic form of a β-like globin known as ε-globin. During fetal liver development, γ-globin is the main type of β-like globin produced when the first enucleated definitive erythrocytes are formed. The γ-globin chains are the result of two duplicated genes located within the β-globin gene cluster. The production of adult hemoglobin (HbA) initiates at a minimal level around 9-12 weeks of gestation, and there is indication that the fetal liver can produce both HbA and HbF (Wood and Weatherall, 1973). They combine with adult α-globin chains, creating a stable tetramer called fetal hemoglobin (HbF). HbF remains the dominant form of hemoglobin for a sizable portion of gestation. After birth, the switch from HbF to HbA occurs as definitive erythroid progenitors shift their transcriptional activity from γ- to β-globin (Sankaran and Orkin, 2013) (Figure 2.3.1.1.1).

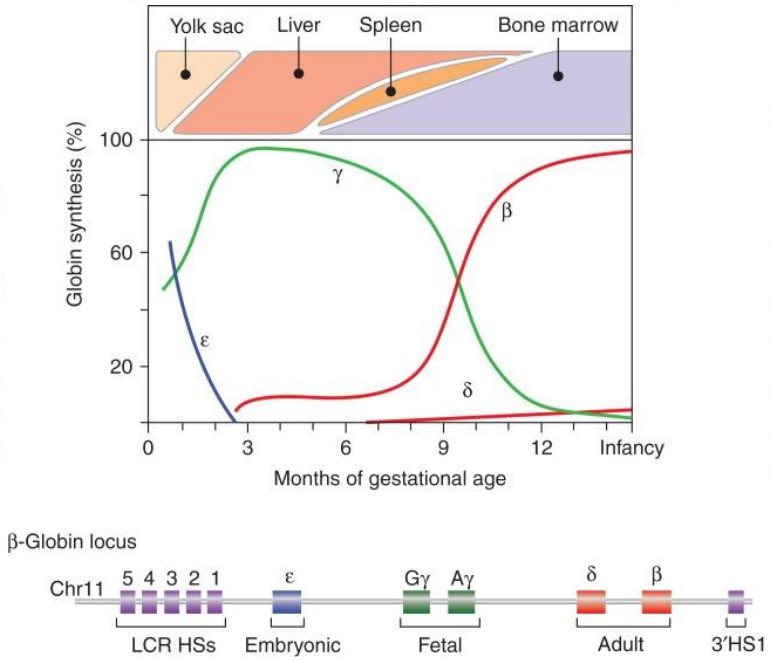


Figure 2.3.1.1.1. Fetal to adult hemoglobin switch (Sankaran and Orkin, 2013)

2.3.2. Stages of erythropoiesis

During the differentiation of hematopoietic stem cells (HSCs) into the erythroid lineage, erythrocytes undergo four major stages such as progenitor stage, pre-erythroid cells stage, reticulocyte stage and erythroid maturation stage (Figure 2.3.2.1).

2.3.2.1. *Progenitor stage:* BFU-E cells represent the initial stage of erythrocyte development, characterized by high proliferation and differentiation activity. They are larger in size, form larger erythroid colonies, and have a high nucleus to cytoplasm ratio. BFU-E differentiates into CFU-E which eventually gives rise to small erythroid cell colonies. BFU-E and CFU-E are erythroid progenitors, distinguished by surface markers. BFU-E is characterized by c-kit+CD45+CD71low, while CFU-E is characterized by c-kit+CD45–/dimCD71hi (Zhu et al., 2023).

2.3.2.2. *Erythroid cells stage:* This stage is composed of three types of cells: proerythroblast basophilic, polychromatophilic (mid-juvenile erythrocytes), and Ortho chromatophilic (late juvenile erythrocytes). Cell division and the expansion of erythroid cells occur in the initial stages, particularly at the progenitor and proerythroblast levels. And mitosis is not observed beyond the polychromatic erythroblast stage. There is a gradual condensation of the nucleus, which is completed by the end of the late juvenile phase (Cooling, 2014).

2.3.2.3. *Reticulocyte stag*e: In orthochromatic erythroblasts, the hyperchromatic nucleus aligns itself along the RBC membrane, followed by the formation of a membranous actin ring. This leads to the nucleus being extruded and subsequently engulfed by the erythroblastic macrophage. During this stage, there is a gradual increase in the synthesis of hemoglobin and other erythroid-specific proteins, cytoplasm of erythroblast becomes red in appearance.

2.3.2.4. *Erythroid maturation stage:* After the removal of the nucleus, the resulting reticulocyte displays a distorted, multilobulated structure with noticeable membrane pitting and surface

motility. Over the subsequent 2–3 days, reticulocyte undergoes maturation into a mature red blood cell (RBC). This maturation process entails vesiculation and the elimination of residual RBC organelles, such as mitochondria, RNA, and ribosomes. Additionally, there is a reorganization of the cell cytoskeleton and membrane, enhancing membrane stability and deformability, contributing to the distinctive biconcave shape of RBCs.

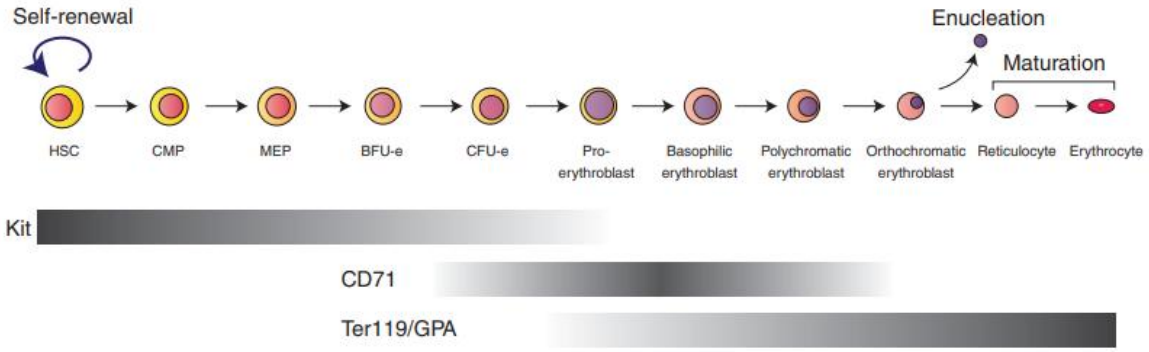


Figure 3.2.1.1. Erythroid differentiation in Bone marrow (Dzierzak and Philipsen, 2013)

2.3.3. Regulation of erythropoiesis

2.3.3.1. Growth factors

During erythroid differentiation, specific transcription factors and cytokines play a crucial role in supporting the erythroid differentiation. BFU-E depends on stem cell factor (SCF), with cKIT as its ligand, other hormones such as insulin like growth factor 1 (IGF-1), glucocorticoids and interleukin-3 (IL-3), and interleukin-6 (IL-6) (Hattangadi et al., 2011). SCF is required for self-renewal, proliferation and prevents BFU-E from exhaustion. c-Kit expression decreases from the proerythroblast stage, indicating that later erythroid stages are not dependent on SCF. From CFU-E and proerythroblast stage, erythroblasts entirely depend on erythropoietin (Epo) for differentiation and proliferation. Erythropoietin (Epo) is synthesized by interstitial cells in the kidney, adjacent to the proximal tubules. It is essential for maintaining the number of circulating erythrocytes. In response to oxygen levels in the peripheral blood, the enhancer

within the Epo gene locus is activated by hypoxia-inducible transcription factor 2 (HIF 2)(Jelkmann, 2011).

EPO induces the differentiation of CFU-E into proerythroblast, subsequently resulting in the creation of an erythroblastic island (EBI) around the central macrophage, distinguished by the expression of the $CD169^{+}$ surface marker (Gomes and Gomes, 2016). EBI supports erythroblast cell division and differentiation, as well as engulfment of nuclei expelled by reticulocytes, known as pyrenocytes, during erythroblast enucleation (Soni et al., 2006). Limberg et al., reported that iron recycled in EBI serves as an iron source for heme synthesis (Leimberg et al., 2008).

Epo binds to erythropoietin receptor (EpoR) on the surface of proerythroblasts and activates multiple intracellular transduction pathways. These pathways encompass the activation of Janus Kinase (Jak2), signal transducer and activator of transcription 5 (Stat5), phosphoinositide-3 kinase/Akt (PI3K/Akt), and mitogen-activated kinase (MAPK)(Lodish et al., 2009). Disruption of either of the initial two pathways results in substantial apoptosis of early progenitors and a diminished production of erythrocytes (Ghaffari et al., 2006).

MicroRNAs (miRNAs) are single-stranded noncoding RNAs that regulate gene expression by targeting mRNAs for translational repression or degradation. miRNAs including miR-142, miR-144, miR-451, miR-221/222, and miR-155, have essential role in erythroid differentiation, and the maintenance of erythrocyte metabolism. miRNAs target both positive and negative regulators of erythropoiesis. In negative regulators, the deletion of miR-142 leads to the development of abnormally shaped red blood cells, impaired elasticity, and shortened lifespan. In a positive regulator, miR-155 overexpression leads to a reduction in erythroid precursors by targeting the erythroid transcription factors PU-1 and CEBP(Li et al., 2023).

2.3.3.2. Transcriptional factors

GATA transcription factors are a group of zinc finger proteins known for their specific recognition of the DNA sequence (T/A)GATA(A/G). GATA1 acts as a master regulator of erythroid gene expression and promotes erythroid differentiation. GATA1 interacts with other transcription factors (TF) including FOG-1, EKLF and PU1. GATA-1 binds FOG-1 at specific binding sites within the myeloid-erythroid progenitors, directing the commitment to the erythroid lineage (Mancini et al., 2012). Mathematical modelling of erythroid transcription factors exhibited synergistic mechanism for erythroid lineage commitment. GATA1, SCL/TAL1 ((stem cell leukemia/T-cell acute lymphoblastic leukemia) and KLF1 (Kripple like factor-1) forms transcriptional network to regulates erythropoiesis while PU1 represses their activity, acting as a negative regulator of terminal erythroid maturation (Wontakal et al., 2012).

Another transcription factor from GATA family is GATA2, which regulates erythroid progenitors proliferation and maintenance (Bresnick et al., 2012; Fujiwara et al., 2004). Thus, GATA1 and GATA2 exhibit opposite expression patterns during erythropoiesis (Moriguchi and Yamamoto, 2014). In human, $CD34^{+}$ cells differentiated into erythroid cells, both GATA1 and GATA2 expressions were observed in sorted BFU-E while only GATA1 expression was observed in CFU-E (Li et al., 2014). GATA2 expression is suppressed by GATA1 during erythroid differentiation, leading to GATA factor switching in terminal erythroid maturation (Kaneko et al., 2010).

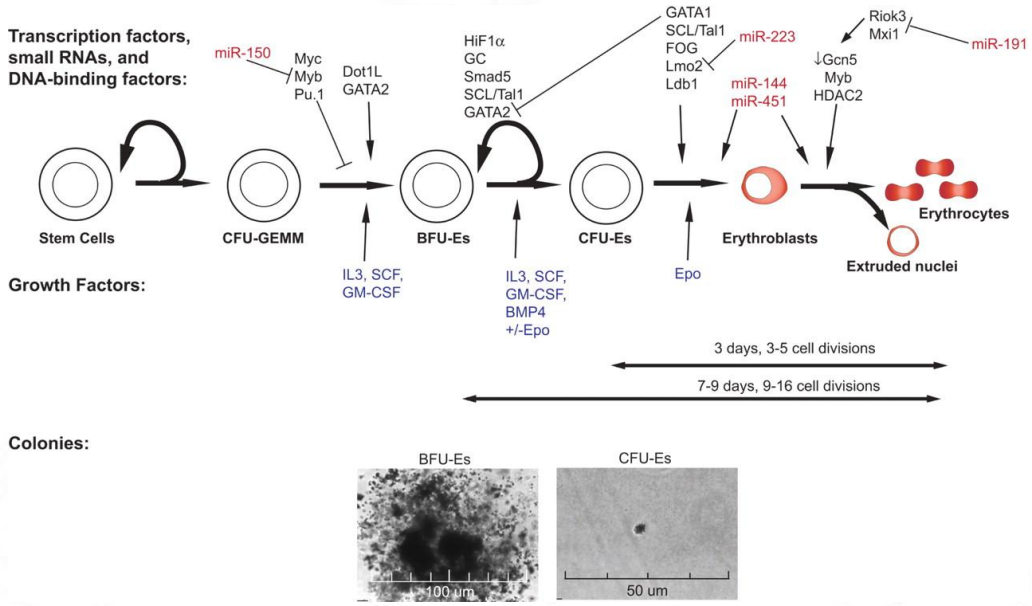


Figure 2.3.3.2.1. Transcription regulation of erythropoiesis (Hattangadi et al., 2011)

2.3.3.3. Heme synthesis

The synthesis of heme occurs in the mitochondria of erythroid precursors. The process involves the formation of δ-aminolevulinic acid (ALA) from succinyl-CoA and glycine, which is catalyzed by ALA synthase (ALAS). ALAS2 is an isoform of ALAS, specifically expressed in erythroid cells, with predominant expression in mature RBC. In the post-transcriptional regulation of ALAS2, iron regulatory element (IRE) situated at the 5' UTR of ALAS2 mRNA interacts with iron-responsive protein (IRP), thereby stabilizing ALAS2 mRNA. ALA is transported to the cytosol, where it undergoes a multistep process to be converted into coproporphyrinogen III (CPgenIII). After being imported back into the mitochondrial intermembrane space, CPgenIII undergoes conversion to protoporphyrinogen IX by coproporphyrinogen oxidase (Chiabrando et al., 2014).

Protoporphyrinogen IX is further oxidized to protoporphyrin IX (PPIX) by the enzyme protoporphyrinogen oxidase (PPOX) (Figure 2.3.3.3.1). Subsequently, ferrous iron is incorporated into PPIX, resulting in the formation of heme within the mitochondrial matrix. This process is catalyzed by the iron sulfur protein, ferrochelatase (FECH). Thus, iron is crucial for heme synthesis and the regulation of ALAS2 and FECH. Moreover, iron within erythroid cells is transported to the mitochondrial inner membrane by mitoferrin1 (MFRN1) and mitoferrin2 (MFRN2), contributing to the synthesis of heme and iron-sulfur clusters (Yien and Perfetto, 2022).

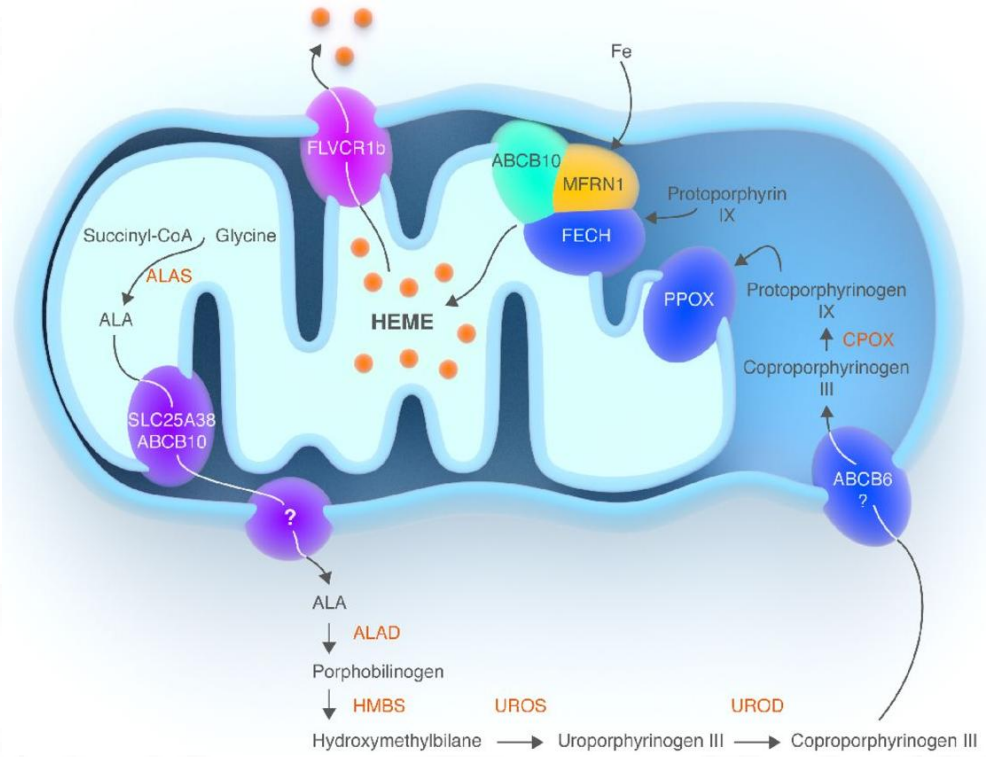


Figure 2.3.3.3.1. Heme synthesis in erythroid cells (Chiabrando et al., 2014)

2.3.3.4. Erythroblastic island

Erythroblastic islands are a distinctive microenvironment characterized by a central macrophage that surrounds a ring of neighboring erythroblasts with cytoplasmic protrusions. Within EBI, erythroid cells at various stages of erythropoiesis undergo expansion and differentiation. The immature EBI acts as a nurse cell, providing nutrients, growth factors for

proliferative and survival signals to the erythroblasts (Chasis and Mohandas, 2008; Li et al., 2021). Additionally, mature EBI supplies iron for heme synthesis and engulfs the extruded nuclei of erythroblasts and promotes enucleation. Increasing evidence suggests that macrophages facilitate the enucleation by itself (Li et al., 2021). Widely reported surface markers for EBI macrophage are F4/80, VCAM1, and CD169 (Romano et al., 2022).

The optimal function of EBI depends on the adhesive interactions between erythroblasts and the central macrophage (Figure 2.3.3.4.1). As erythroblasts undergo differentiation, they express numerous adhesion molecules which enable them to adhere to extracellular matrix proteins like fibronectin and laminin and attach to the central macrophage. Erythroblast macrophage protein (EMP) also known as macrophage erythroblast attacher, MAEA, facilitates interaction between erythroblast and the macrophage via homophilic interaction contributing to the maintenance of EBI integrity. Adhesive molecule α4β1 integrin present on erythroblast interacts with vascular cell adhesion molecule (VCAM-1) located on central macrophages (Manwani and Bieker, 2008). Also, interacts with αV integrin expressed on central macrophages is the complementary receptor for erythroid cells ICAM-4 (intracellular adhesion molecule 4). Studies have reported that disruption of ICAM-4 interaction with αV integrin decreases the formation of EBI islands (Lee et al., 2006). Another adhesion motif expressed on erythroblast is CD163, known to scavenge hemoglobin- haptoglobin complexes, facilitates interaction between macrophage and erythroid cells for proliferation and survival (de Back et al., 2014).

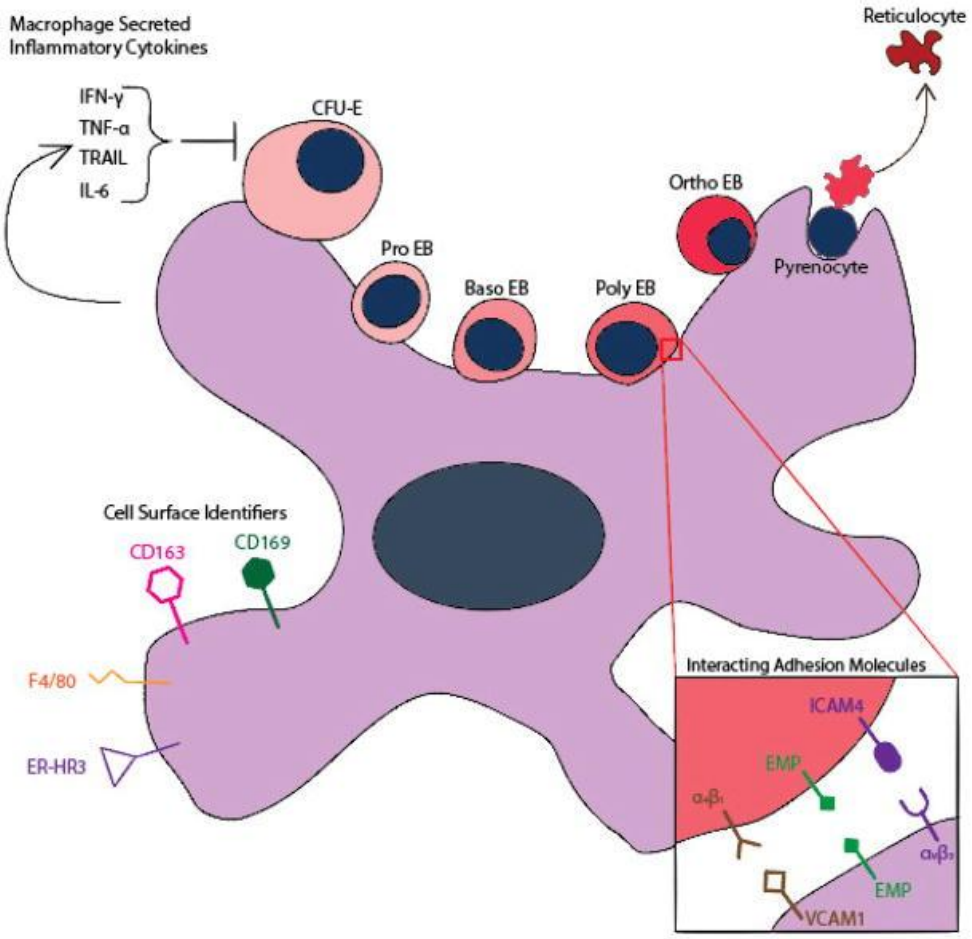


Figure 2.3.3.4.1. Erythroblastic island (Hom et al., 2015)

2.4. Disorders affecting erythropoiesis.

The regulation of the erythropoiesis process involves a complex network encompassing cytokines, oxygen sensors, transcription factors, iron regulators, and numerous factors. This regulatory network tightly controls both steady-state and pathological erythropoiesis. The demand for erythropoiesis escalates during pregnancy or in high-altitude conditions, prompting the regulatory network to adapt according to the physiological requirements. The regulatory network, however, is impaired in pathological conditions, leading to either erythrocytosis or anemia (Valent et al., 2018).

Stress-induced erythropoiesis in polycythemia vera ($JAK2^{V617F}$ mutation) is characterized by the expansion of erythroid progenitors, extramedullary erythropoiesis in the spleen, and elevated RBC production(Ramos et al., 2013).

Ineffective erythropoiesis is characterized by the increased proliferation of immature erythroid cells and the apoptosis of mature erythroid cells during the later stages of erythropoiesis. In such stressed conditions, anemia develops due to heightened erythropoiesis, affecting both the total count of erythroid cells and the utilization of iron by these cells. Despite this expansion, the production of red blood cells remains inadequate. Ineffective erythropoiesis in the genetic disorder beta-thalassemia (resulting in impaired β-globin synthesis) leads to secondary iron overload and anemia (Cazzola, 2022). Several other conditions, such as sideroblastic anemia (associated with low protoporphyrin synthesis) and megaloblastic anemia (where DNA synthesis is hindered due to a deficiency in either vitamin B12 or folate), exhibit ineffective erythropoiesis. In these types of anemia, the hemoglobin concentration is low which in turn activates erythropoietin, leading to increased erythropoiesis.

Decreased erythropoiesis results in a reduced number of erythroid precursors, leading to anemia due to decreased RBC production. Factors contributing to the decline in RBC production include poor iron absorption causing iron deficiency anemia, depletion of erythroid precursors due to an autoimmune reaction in conditions like aplastic anemia and acquired pure red cell aplasia, as well as erythropoietin deficiency seen in chronic renal failure anemia (Manchanda, 2020).

2.5. Iron Homeostasis

Iron is an essential micronutrient necessary for the survival of most organisms. Due to its distinctive redox chemistry, iron plays a critical role in biochemical processes within the cells.

Iron metabolism is tightly controlled because both deficiency and excess can result in detrimental effects (Das et al., 2020).

2.5.1. Systemic iron homeostasis

The average adult human contains 3-4 g of iron, with most of it being found in hemoglobin, which accounts for 2-3 g of iron. Iron is stored in hepatocytes and in macrophage in a specialized cytoplasmic iron storage protein called ferritin. Every cell contains lower concentrations of iron-containing proteins that are crucial for energy production, and other essential functions. Iron is transported to tissues through the bloodstream, where it is bound to the transferrin, with blood plasma containing only 2–4 mg iron(Ganz, 2013). Reticuloendothelial macrophages phagocytose senescent RBCs and free iron from heme moiety is released into circulation. Around 20mg of recycled iron is utilized for new cycle of red cell synthesis (Knutson et al., 2005). In hepatocytes, about 1000mg of iron is stored and 1800mg within the red cells. 1-2mg of iron is lost every day through the shedding of skin and intestinal lining. Rest (400mg iron) is distributed to other proteins like myoglobin and cytochromes. Enterocytes contribute an additional ∼1–2 mg of iron to the plasma by absorbing dietary iron (Figure 2.5.1.1.1). Hepcidin, the key regulator of iron metabolism, functions by inhibiting the release of iron in enterocytes, splenic macrophages, and mobilization from hepatic stores. This inhibition occurs through the internalization of ferroportin (FPN) and degradation.

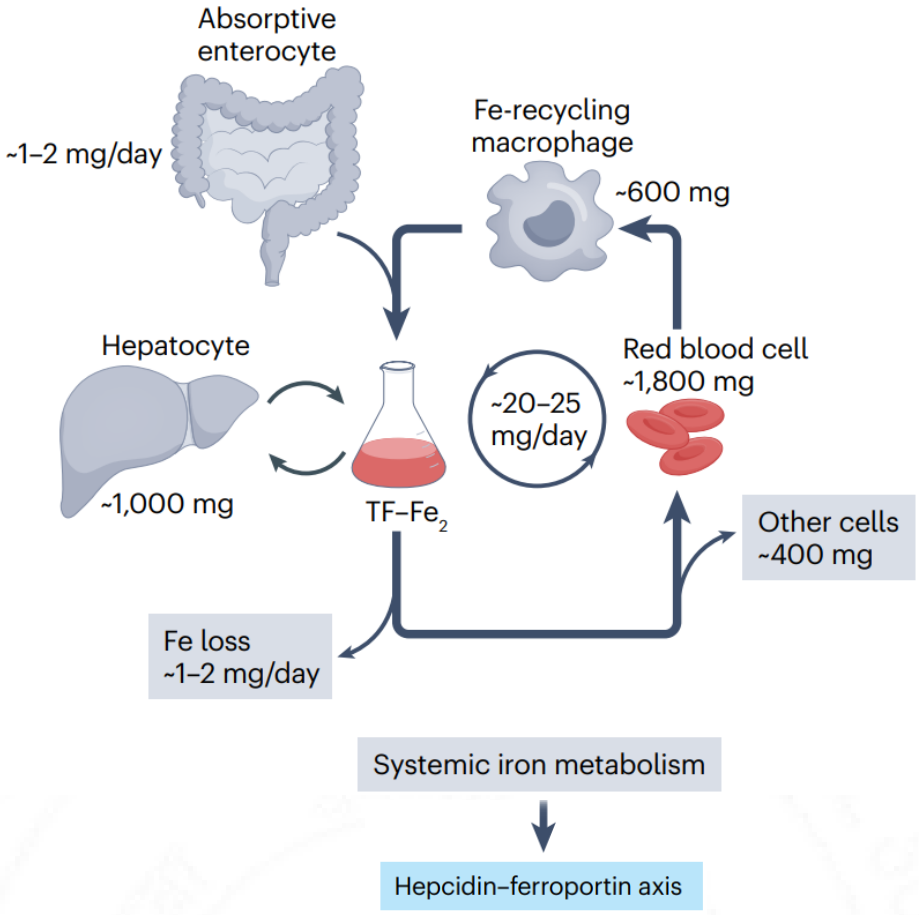


*Figure 2.5.1.1.1. Systemic iron homeostas*is (Galy et al., 2023)

2.5.2. Cellular iron metabolism

Iron absorption is facilitated by duodenal enterocytes through a systemic and local regulatory system. Dietary iron Fe^{3+} is oxidized to Fe^{2+} by ferrireductases such as duodenal cytochrome b (Dcytb) and a proton generated by Na^{+}/H^{+} exchanger-3 in the apical membrane of duodenal enterocytes and transported via dimetal transporter 1(DMT1) (Shawki et al., 2015, 2016). In enterocytes, excess ferrous iron is stored in cytoplasmic ferritin, until it is required for biological processes. This iron storing protein is a heteropolymer composed of heavy chain (FTH) and light chain (FTL) subunits. The iron detoxification is facilitated by the H subunit, which has a catalytic ferroxidase center for the swift oxidation of Fe^{2+}. On the other hand, the L subunit is responsible for activities such as mineralization, and long-term iron storage (Carmona et al., 2013). Further iron required for metabolic process is exported via ferroportin

(FPN), iron efflux pump located across the basolateral membrane of enterocytes. FPN belongs to the solute carrier family of transporters, characterized by dual six-helix transmembrane bundles that form a central cavity, facilitating the outward efflux of iron. The structure of ferroportin undergoes a conformational flip-flop, enabling the export of iron through an alternating access mechanism. This process allows the FPN to carry iron across the membrane as it cycles between inward and outward-facing conformations (Ganz, 2021). The exported Fe^{2+} is oxidized to Fe^{3+} by multicopper oxidases such as hephaestin (HEPHL1) or ceruloplasmin.

In plasma, Fe^{3+} is bound to the transferrin molecule and transported to different organs like bone marrow, liver, and spleen. Transferrin (TF), a glycoprotein, consists of binding sites for two ferric ions. It can deliver iron in its monomeric form, with only less than 50% of transferrin typically bound to iron in healthy persons (Parrow et al., 2019). Diferric TF has higher affinity to bind to transferrin receptor 1(TFR1) expressed on the plasma membrane of erythroid cells and hepatocytes.

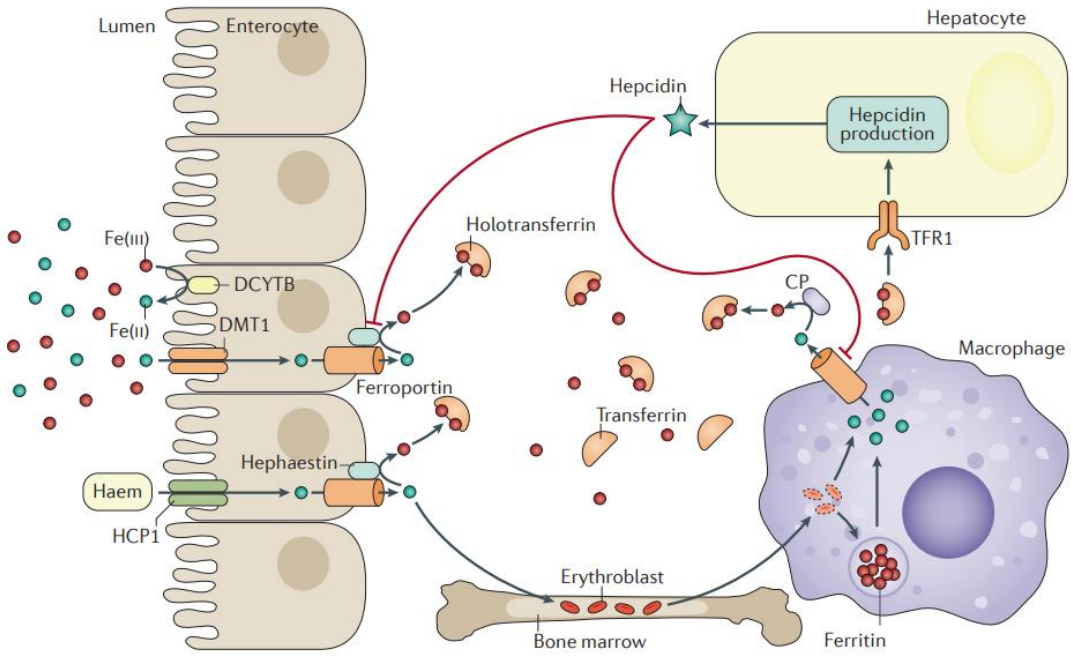


Figure 2.5.2.1. Cellular iron metabolism (Brissot et al., 2018)

2.5.3. Hepcidin-ferroportin axis

Hepcidin, a liver-synthesized peptide hormone, is transcriptionally regulated by iron signals. The gene HAMP, responsible for hepcidin encoding, is activated through the BMP-SMAD pathway. Dimeric ligands BMP2 and BMP6 synthesized by liver endothelial cells, interact with BMP type II receptors and coreceptor hemojuvelin, leading to transphosphorylation of BMP type I receptors on the hepatocyte membrane. Subsequently, this activates a protein cascade, with BMPRI phosphorylating SMAD 1/5/8, forming a complex with SMAD4 for nuclear translocation. This orchestrated process upregulates hepcidin transcription(Xiao et al., 2020).

During iron deficiency, hepcidin is downregulated, and its transcription is hindered by transmembrane serine protease 6 (TMPRSS6). Under inflammation or iron overload conditions, hepcidin is upregulated (Ganz, 2011).

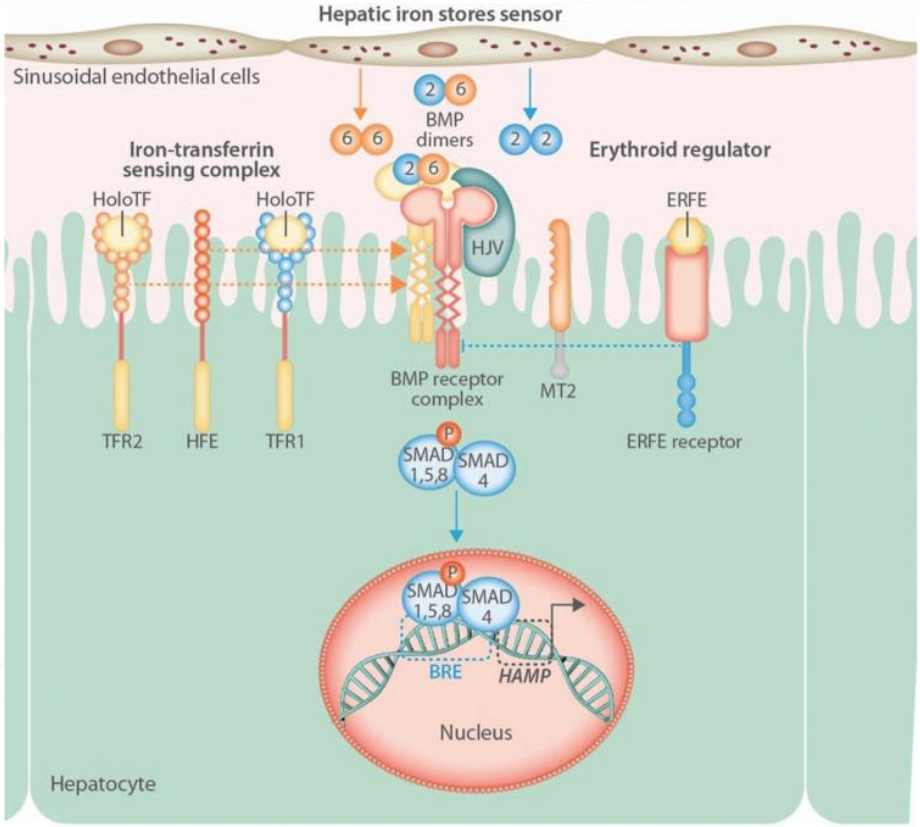


Figure 2.5.3.1. Hepcidin transcriptional regulation by BMP-SMAD pathway (Coffey and Ganz, 2018)

The Hepcidin-ferroportin axis regulates systemic iron levels, maintaining a balance in plasma iron for cellular needs. Hepcidin inhibits iron absorption in enterocytes, release of recycled iron from macrophage and iron mobilized from hepatocytes via FPN degradation. Hepcidin prompts the rapid ubiquitination of lysine residues in the cytoplasmic domain connecting the N-lobe and the C-lobe of FPN, mediated by ring finger protein 217 (RNF217). This process leads to the internalization and subsequent degradation of FPN. Ubiquitination mechanism is absent in erythrocytes, where hepcidin directly inhibits iron efflux via FPN (Aschemeyer et al., 2018).

2.5.4. Cellular iron regulation

Iron metabolism genes including TFRC, FPN, DMT1 are post-transcriptionally regulated by iron regulatory proteins, IRP1 and IRP2. IRPs bind iron responsive elements (IRE) located at untranslated region (3′ UTR or 5′ UTR), forming stem-loop structures. Under iron deficiency, the IRE motif in the 5′-UTR of FTL, FTH1, and FPN mRNAs becomes stabilized because of the IRE-IRP interaction, leading to subsequent inhibition of translation. The diminished expression of ferritin and FPN lowers the iron storage and efflux. This, in turn, increases the availability of free iron for cellular utilization (Pantopoulos, 2004). However, in iron replete condition, the binding of iron to IRPs induces a conformational change, which promotes the detachment of IRPs from binding to IRE motif, facilitating the translation of the target mRNA. Under low iron levels, IRP interaction with IRE motif at 3′ UTR in DMT1 and TFRC prevents mRNA from endonuclease cleavage and prolongs the half-life of transcripts and facilitates the translation(Zhou and Tan, 2017). Whereas in iron replete cells, detachment of IRP-IRE at 3′ UTR triggers the mRNA degradation, resulting in translational repression. Of note, IREs located at the 5′ end of HIF2α and ALAS2 transcripts, interacts with IRP for EPO regulation and hemoglobin production(Galy et al., 2023).

IRP2 is regulated by leucine-rich repeat 5 F-box protein (FBXL5) which induces ubiquitination and degradation of IRP2. In iron deficient cells, IRP2 remains stable and attaches to IREs, whereas in iron-replete cells, it is degraded (Galy et al., 2023).

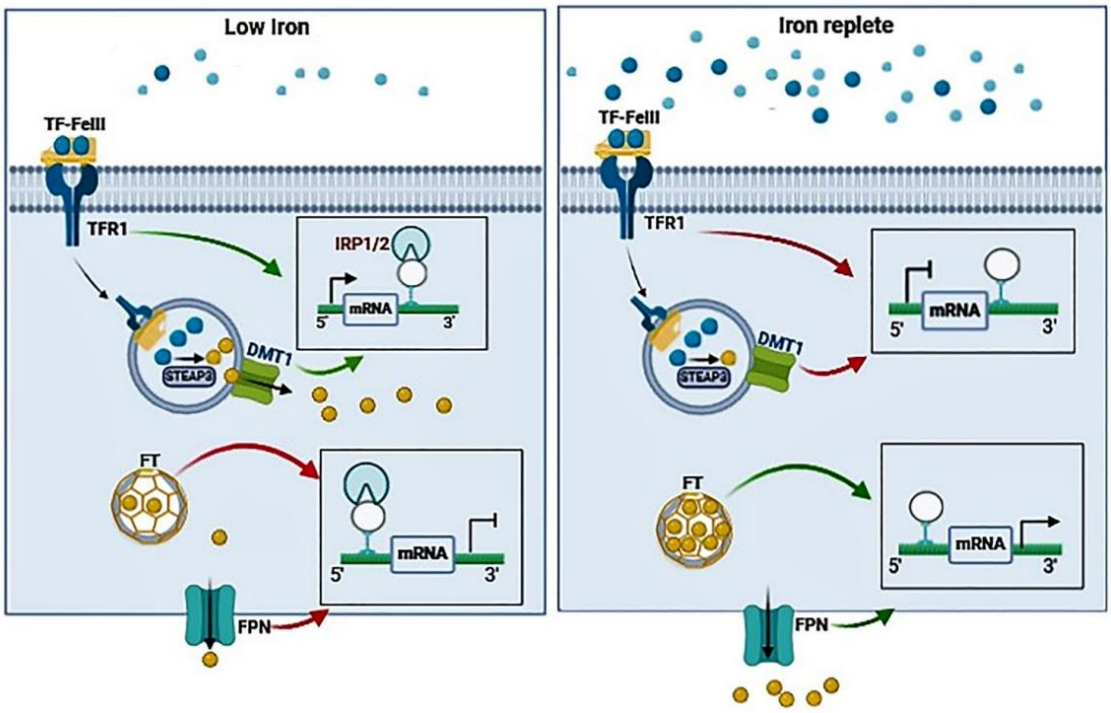


Figure 2.5.4.1. Cellular iron regulation in iron deficient and replete cells (Santhakumar and Edison, 2024)

2.5.5. Cellular iron acquisition mechanism

Cells can acquire iron either through clathrin-mediated endocytosis, transporters, or phagocytosis of other cells.

2.5.5.1. Iron acquisition by transferrin receptor

Transferrin receptor, a membrane glycoprotein, forms homodimers that bind with high affinity to transferrin at each subunit. Transferrin receptor 1 (TFR1) transcription is partially regulated by erythropoietin (EPO) and hypoxia-inducible factor (HIF-1α), as indicated by STAT5 binding elements and hypoxia-responsive element (HRE) present in promoter region, respectively. In plasma, ferric iron bound to TF is protected from producing reactive oxygen

species (ROS) and keeps it inaccessible to extracellular iron-dependent pathogens. Once assembled at the cell surface, the TF–Fe_2–TFR1 complex undergoes internalization through clathrin-mediated endocytosis. Endosomal acidification releases iron from TF, while apo-TF (TF without iron) remains attached to TFR1. TF-TFR1 complex in early endosomes were recycled to the cell surface, where TF dissociated from TFR1 is readily available for ferric iron binding (Richard and Verdier, 2020).

TFR2, homolog of TFR1 exhibits lower affinity for holo-TF and is not post transcriptionally regulated by IRE-IRP system. TFR2 stability is regulated by body iron levels to modulate HAMP expression and to adjust erythropoietic signaling (Galy et al., 2023).

2.5.5.2. Non-transferrin bound iron acquisition.

Non-transferrin bound iron (NTBI) is found in plasma, when transferrin saturation is greater than 60-70% and remains undetectable in healthy individuals (Porter et al., 2014). NTBI is transported into cells by divalent metal transporters including ZIP14, ZIP8, and DMT1. Recent study has reported that in iron overload, NTBI stimulates BMP6 expression in liver sinusoidal endothelial cells, which in turn promotes hepcidin synthesis (Charlebois et al., 2023).

2.5.5.3. Cellular iron handling by macrophages

Macrophages of the reticuloendothelial system of the reticuloendothelial system retrieve iron from aging red blood cells through erythrophagocytosis and release it back into circulation for reutilization. When RBCs are phagocytosed, hemoglobin undergoes proteolysis and heme moiety gets detached. Further, heme is degraded by heme oxygenase-1 (HO-1) to release iron, which is transported to cytoplasm via DMT1. In cytoplasm, iron is stored in ferritin by cytoplasmic chaperones of the poly(rC) binding protein (PCBP) family. Otherwise, iron is released into circulation by FPN. Iron is stored in ferritin by cytoplasmic chaperones of the

poly(rC) binding protein (PCBP) family. Otherwise, iron is released into circulation by FPN (Figure 2.5.5.3.1).

During hemolysis, macrophages phagocytose hemoglobin to prevent the deleterious effects of cell-free hemoglobin and heme. Hemoglobin binds to its carrier protein, haptoglobin (Hp) and free heme binds to hemopexin. Hb-Hp complex and heme-Hpx complex were scavenged by CD163 (cysteine-rich type 1 protein M130) and LRP1 (prolow-density lipoprotein receptor-related protein 1), respectively into the macrophages and hepatocytes (Hindson, 2020).

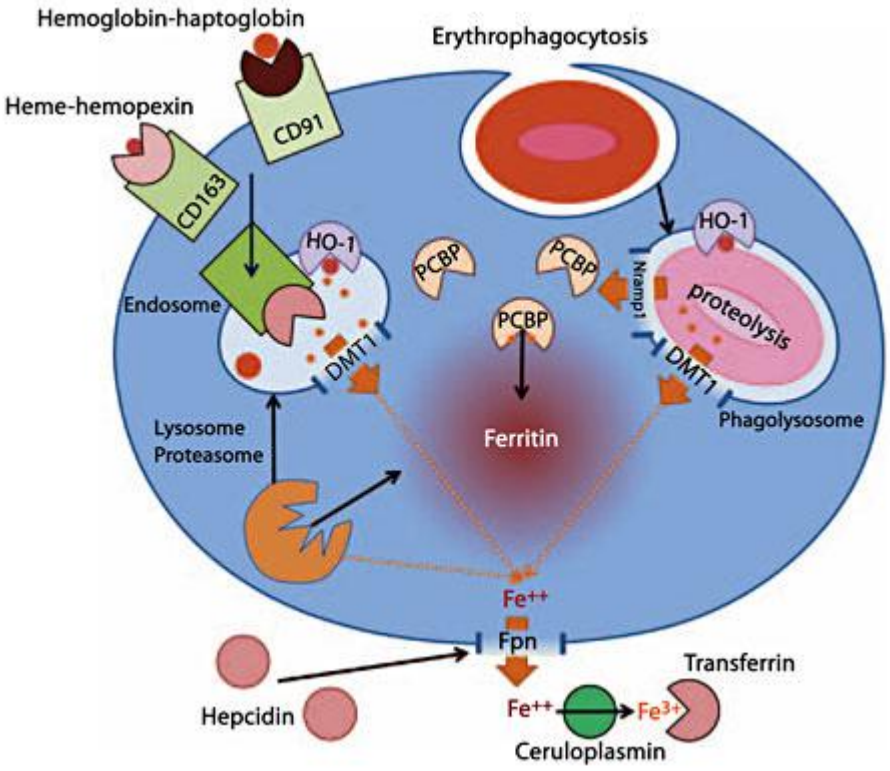


Figure 2.5.5.3.1. Iron recycling in macrophages (Ganz, 2012)

2.5.6. Iron in erythropoiesis

The body produces 200 billion RBCs every day, with a requirement of 2×10^{15} iron atoms per second to support steady-state erythropoiesis. These quantities result in the production of 20 mL of blood every day, comprising 6 g of Hb and 20 mg of iron(Slusarczyk and Mleczko-Sanecka, 2021). Most of the iron is utilized for Hb synthesis. Iron uptake in erythroid cells

acquired through binding of TF-$Fe^{3+}_{(2)}$-TFR1 on the cell surface of erythroid precursors. The TF-$Fe^{3+}_{(2)}$-TFR1 complex undergoes endocytosis, and within the acidic environment of early endosomes, iron dissociates from transferrin (TF). Released iron is reduced to ferrous state by STEAP3 (six-transmembrane epithelial antigen of prostate 3) and transported to cytosol by DMT1. Concurrently, the Apo-TF-TFR1 complex undergoes recycling to the cell surface for optimal iron uptake. Sorting nexin 3 (SNX3) is crucial for directing TF/TFR1 complexes into recycling endosomes, while the transportation of the TF/TFR1 complex from recycling endosomes to the cell surface depends on EXOC6 (a constituent of exocyst protein complex). The labile iron pool present in the cytosol serves various functions, including its involvement in heme synthesis, the synthesis of iron-sulfur clusters, storage within ferritin, and export via FPN (Figure 2.5.6.1). In the heme synthesis pathway, iron is transported into mitochondria through mitoferrin. Within the mitochondria, iron combines with the protoporphyrin complex, leading to the production of heme.

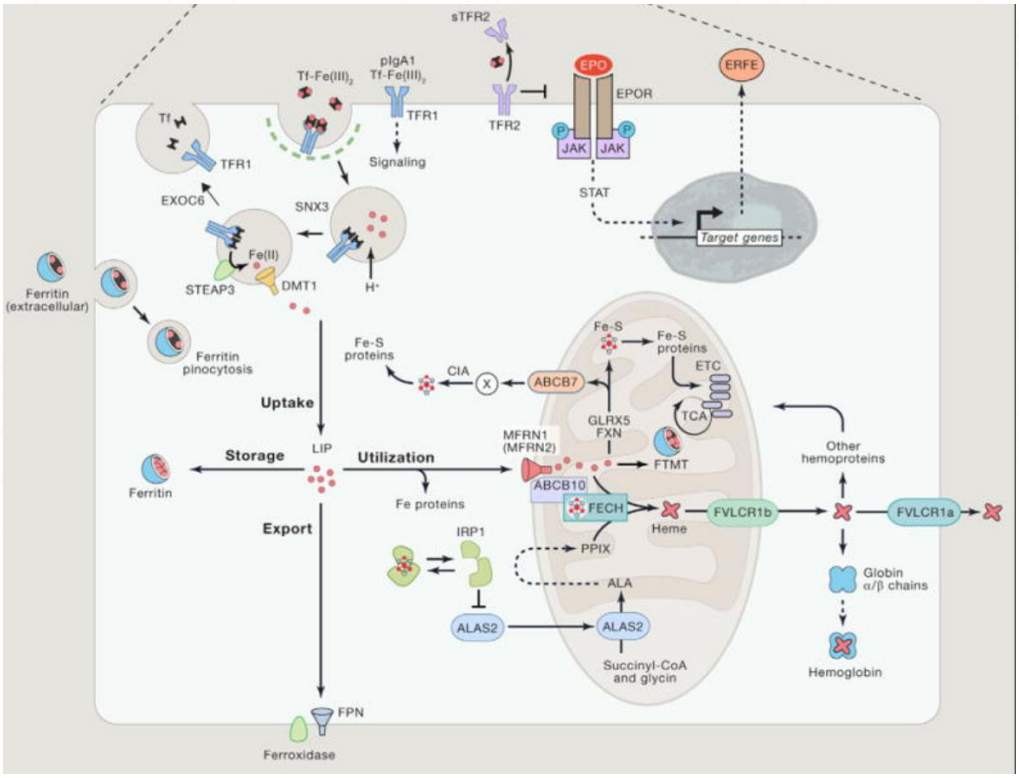


Figure 2.5.6.1. Mechanism of iron in erythroid cells (Muckenthaler et al., 2017)

Subsequently, FLVCR1 (feline leukemia virus subgroup C cellular receptor) facilitates the transportation of heme outside the mitochondria. Any surplus iron within the mitochondria can be stored in the organelle-specific form of ferritin (FTMT) (Muckenthaler et al., 2017).

2.5.6.1. Hepcidin regulation by erythroid factors

Hepcidin suppression occurs in response to iron deficiency and increased erythroid activity. Experimental studies in humans and animals have shown that hepcidin suppression is influenced by erythroid factors. Growth differentiation factor 15 (GDF15) and twisted gastrulation BMP signaling modulator 1 (TWSG1) were identified as possible hepcidin suppressors (Pasricha et al., 2016b). Studies examining the effects of induced erythropoiesis through phlebotomy in GDF-15 knockout mice revealed no reduction in hepcidin suppression. In a thalassemia mouse model, there was no increase in gdf-15 mRNA expression in the bone marrow(Casanovas et al., 2011). Increased TWSG1 expression was observed in bone marrow and spleen of a thalassemia mouse model, but phlebotomy did not induce the same effect in mouse bone marrow (Casanovas et al., 2013). Evidence on the involvement of GDF-15 and TWSG1 in regulating hepcidin during erythropoiesis is inconclusive.

Kautz et al. demonstrated the potential erythroid regulator, erythroferrone by examining genes in mouse bone marrow that showed differential expressions in response to phlebotomy. Findings revealed an increase in ERFE mRNA levels at time points prior to hepcidin suppression caused by phlebotomy(Kautz et al., 2014). Several studies have demonstrated that erythropoietin (EPO) upregulates the transcription of ERFE within erythroblasts. ERFE inhibits the activation of the hepatic BMP–SMAD signaling pathway, resulting in HAMP suppression (Arezes et al., 2018; Mastrogiannaki et al., 2012; Wang et al., 2020).

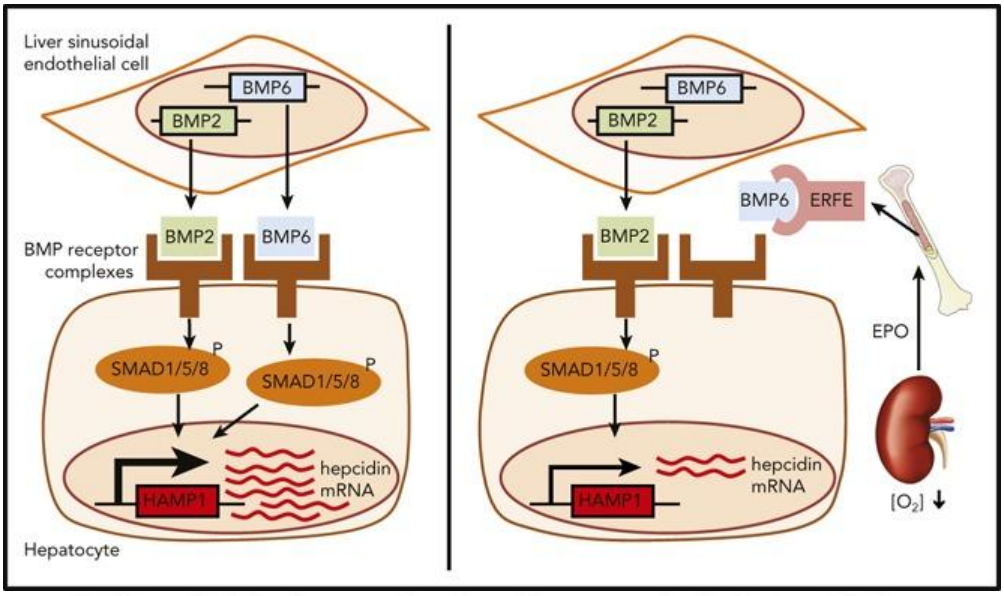


Figure 2.5.6.1.1. Erythroferrone suppresses hepcidin transcription by inhibiting BMP/SMAD signaling(Arezes et al., 2018)

2.6. Pregnancy

2.6.1. Iron requirement in pregnancy

During pregnancy, the occurrence of physiological anemia arises from the expansion of blood volume and the escalating demands from the growing placenta and fetus. To meet these physiological requirements in the initial stages of gestation, a pregnant woman necessitates an augmented supply of iron from her iron reserves. However, women commence pregnancy with insufficient iron stores. Global estimates reveal that 33% of non-pregnant women, 40% of pregnant women, and 42% of children are at risk of iron deficiency anemia (IDA)(WHO guidance helps detect iron deficiency and protect brain development, n.d.). Infants with IDA may experience neurodevelopmental deficits, cognitive impairments, and delayed behavioral development (Georgieff, 2020). In developing countries, the prevalence of iron deficiency in neonates before six months of age suggests that numerous newborns might not have optimal iron stores at birth (Yang et al., 2009).

Iron plays a pivotal role in the development of the placenta and the fetus, contributing significantly to fetal survival. Failing to adequately meet the demands of this physiological adaptation can lead to adverse outcomes, including premature birth, reduced birth weight, cognitive abnormalities in the offspring, and an elevated risk of maternal mortality. Therefore, maintaining adequate iron supply throughout the gestational period is paramount (Cao and Fleming, 2016). At birth, a neonate's body contains 1g of iron acquired from the mother. Of which, 600mg of iron is obtained from the maternal diet and the discontinuation of menstruation, with an additional 400mg sourced from the iron reserves within the mother(Gunes et al., 2016).

During the first trimester, the demand for iron is approximately 0.56mg/day as menstruation ceases. This requirement gradually rises to 4mg/day and 6mg/day in the subsequent trimesters. As pregnancy progresses into the second and third trimesters, maternal iron reserves are utilized to support fetal growth. Approximately 40% of pregnant women start their pregnancy with insufficient iron reserves (Bothwell, 2000).

2.6.2. Placental iron regulation

The placenta adopts a unique cellular iron homeostasis pattern responding solely to systemic and local regulatory signals from the maternofetal environment. Maternal circulation facilitates the uptake of non-heme iron through TF-$Fe^{3+}_{(2)}$-TFR1 complex on the apical side of syncytiotrophoblasts. TF-$Fe^{3+}_{(2)}$-TFR1 complex undergoes endocytosis and iron dissociates from apo-TF-TFR1 complex. Freed iron is reduced to ferrous form by STEAP3 and transported to cytosol by transporters like DMT1 and Zrt and Irt-like protein 14 (ZIP14). In the cytoplasm, iron is either integrated into ferritin, a protein strongly expressed in the stroma (Bastin et al., 2006), or it is transported to the basal side of syncytiotrophoblasts through FPN. Iron oxidized to ferric form by an unknown ferroxidase in the placenta. The characterization of iron transport

across the fetal capillary endothelium is obscure. Although there is some evidence indicating the expression of transferrin receptor in fetal capillary endothelium, further investigation is warranted (Parrow and Fleming, 2020) (Figure 2.6.2.1).

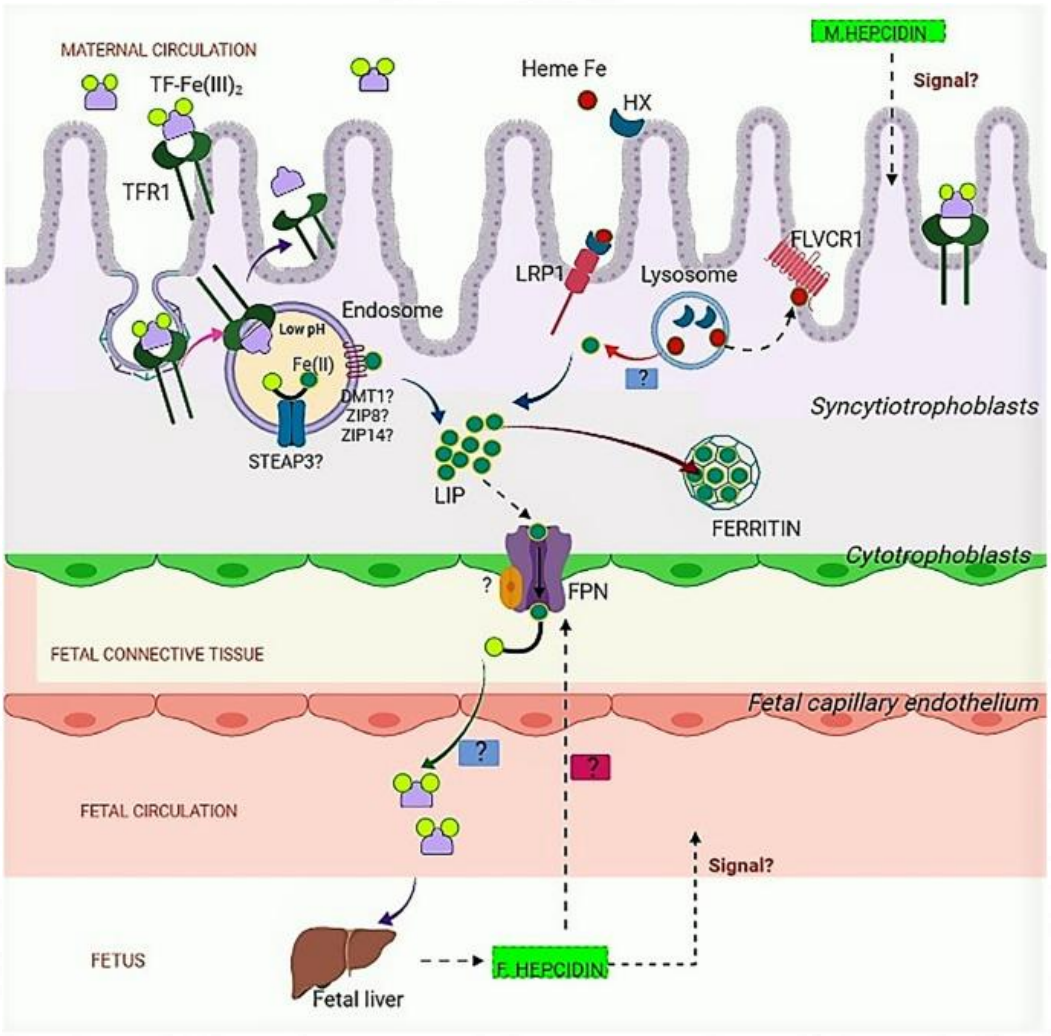


Figure 2.6.2.1. Placental iron trafficking (Santhakumar and Edison, 2024)

2.6.3. Fetal iron utilization

Several studies suggest that placental FPN exports iron into the fetal circulation(Donovan et al., 2005; Drakesmith et al., 2015a). A total knockout of FPN in mice resulted in embryonic lethality, whereas the targeted inactivation of FPN in all tissues except the placenta allowed normal embryonic development(Drakesmith et al., 2015b). Animal studies have demonstrated that fetal hepcidin regulates the maternal iron transfer to the fetal circulation(Cao and Fleming,

2022). Supporting this, studies in rat models have demonstrated a physiological link between fetal liver iron and the expression of maternal transferrin receptors (Gambling et al., 2009). This implies that the fetal liver could regulate maternal iron supply to the fetus. At birth, neonatal DMT1 knockout mice had excess liver iron stores and maintained optimal iron stores even without DMT1(Gunshin et al., 2005).

2.6.4. Regulators of iron homeostasis in pregnancy

Hepcidin levels decrease during pregnancy to increase iron absorption and mobilization towards maternal erythroid cells, to support increased erythropoiesis. Additionally, lower maternal hepcidin concentration facilitates direct iron delivery to the fetus, ensuring that newborns have optimal iron stores at birth. Studies reported that fetal hepcidin plays a regulatory role in determining the iron supply to the placental-fetal unit (Kulik-Rechberger et al., 2016a; McDonald et al., 2022). Further studies have collectively suggested that fetal hepcidin can directly impact the amount of iron transported to the fetus (Fisher and Nemeth, 2017; Kämmerer et al., 2020).

In humans, fetal hepcidin levels in cord blood at delivery are notably higher than maternal levels. (Gunes et al., 2016; Kulik-Rechberger et al., 2016a). A study investigating potential hepcidin suppressors in healthy pregnant women proposed that maternal ferritin, soluble hemojuvelin, and EPO repress hepcidin transcription (Finkenstedt et al., 2012). ERFE levels were higher in neonates than in mothers and it increased in response to EPO and had negative association with cord blood hepcidin (Delaney et al., 2021).

2.7. Beta thalassemia

2.7.1. Pathophysiology and iron overload

Beta thalassemia is a recessively inherited disorder characterized by absence or decreased production of beta globin chains of Hb tetramer (Sanchez-Villalobos et al., 2022). It manifests in three forms based on severity that includes β-thalassemia major (TDT), β-thalassemia intermedia (NTDT), and β-thalassemia minor (Cao and Galanello, 2010). The β-globin mutation leads to accumulation of excess α- globin chains which leads to hemolysis. β-thalassemia is characterized by ineffective erythropoiesis, splenomegaly, apoptosis of erythroid precursors and shortened mature RBC lifespan (Li et al., 2017). Primary overload, characterized by increased iron stores and liver iron concentration. The chronic state of anemia, treated by RBC transfusions results in secondary iron overload owing to the production of reactive oxygen species (Figure 2.7.1.1).

TDT patients are characterized by severe anemia from an early age who require lifelong periodic blood transfusions along with iron chelation therapy. NTDT patients exhibit a spectrum of anemia, ranging from mild to severe, and typically do not necessitate regular blood transfusions. Complications associated with NTDT include extramedullary hematopoiesis, iron overload, facial and bone deformities, and growth retardation. β-thalassemia minor or β-thalassemia trait, the less severe form with one mutated gene, is identified in individuals with asymptomatic anemia.(Viprakasit and Ekwattanakit, 2018).

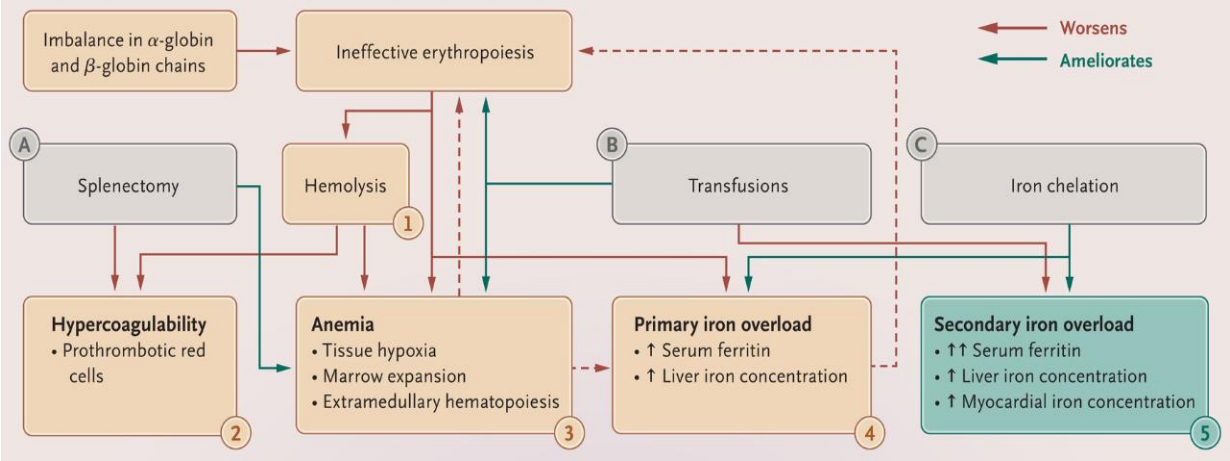


Figure 2.7.1.1. Pathophysiology of β-thalassemia (Taher et al., 2021)

2.7.2. Therapeutic options

TDT patients depend on regular blood transfusions combined with iron chelation therapy. Clinically approved iron chelators are deferoxamine (DFO) (clinically first approved in 1980), deferiprone (DFP) and deferasirox (DFX). Another treatment for TDT patients is splenectomy, which improves growth and survival in a few patients. Adverse outcomes of splenectomy include risk of infection and sepsis, and increased risk of thrombosis (Grech et al., 2022).

Hematopoietic stem cell transplant (HCT) is another treatment option for β-thalassemia patients with severe anemia. Outcomes of HCT in β-thalassemia patients depend on several factors including iron chelation status, hepatic fibrosis, and presence of hepatosplenomegaly. HCT treatment during childhood yields survival rates greater than 88% (Lucarelli and Gaziev, 2008). In our center, allogeneic stem cell transplantation of 63 beta thalassemia major patients revealed that augmenting the stem cell dosage corresponded to a diminished susceptibility to bacterial and fungal infections post transplantation (Mathews et al., 2014).

To improve ineffective erythropoiesis, several drugs and treatment strategies have been tried in the last decade. One of the novel therapeutic drugs is sotatercept, which increases mature RBCs by suppressing negative regulators of terminally differentiating erythroid cells. This drug has been under phase II clinical trial. Another drug under phase III clinical trial is luspartercept

which has been approved for the treatment of TDT patients in 2019. This drug improves splenomegaly and decreases ineffective erythropoiesis(Cappellini et al., 2019). Jak2 inhibitors are another potential drug target which improves erythropoiesis and reduces splenomegaly. Ruxolitinib, Jak2 inhibitor used in TDT patients presented with splenomegaly, effectively inhibited the proliferation of erythroid precursors and decreased spleen size (Taher et al., 2018).

Several studies inline have investigated iron overload in β-thalassemia. Mainly they focused on hepcidin as a therapeutic target for β-thalassemia (Nemeth, 2010; Therapeutic Target for β-Thalassemia Patients, n.d.). TDT patients had higher hepcidin and ferritin values compared to NTDT patients (Kaddah et al., 2017). Mini hepcidins are hepcidin agonists which reverse iron overload in β-thalassemia patients. And it has been under clinical trial in TDT and NTDT patients.

Iron restricted approaches have been applied in β-thalassemia to control chronic stress erythropoiesis. Such approach was observed in a study, where hepcidin expression was increased by the inhibition of TMPRSS6, a serine protease, thereby it reduced iron overload in β-thalassemia (Guo et al., 2013). To evaluate its efficacy, phase II clinical trial was conducted in NTDT patients using TMPRSS6 inhibitor IONIS TMPRSS6-LRx. Another potential therapeutic target for iron overload is ferroportin inhibitor. Inhibiting FPN levels will result in decreased iron absorption in the blood stream. FPN inhibitor (VIT-2763) efficacy and tolerability was evaluated in NTDT patients in an ongoing phase II clinical trial.

Several gene therapy clinical trials were conducted in β-thalassemia patients who require multiple blood transfusions since 2007 (Grech et al., 2022). It is a lentiviral mediated gene transfer used to correct the aberrant production of β-globin chains by isolating hematopoietic stem cells from β-thalassemia patient. Another novel approach is gene editing, which was employed to create small deletions in the promoter region of BCL11A gene. By targeting BCL11A, a transcription factor, its activation in the switch between fetal and adult globin genes

can be suppressed, leading to an increase in fetal hemoglobin. Currently, CRISRP/Cas9 gene editing (CTX001) is being used in ongoing phase I/II clinical trial for TDT patients, resulting in a notable increase in HbF expression from 10% to 99% (Frangoul et al., 2021).

2.8. Polycythemia Vera

2.8.1. Pathophysiology of polycythemia vera and iron deficiency

Polycythemia Vera (PV) is chronic myeloproliferative disorder characterised by unlimited proliferation of erythrocytes, megakaryocytes, and granulocytes. WHO defined PV as Hb>18.5g/dL for men and 16.5g/dL for women; also, the occurrence Janus kinase 2(Jak2) mutation mostly related to V617F allele (Bruchova et al., 2009a). the clinical trajectory is complex, marked by high incidence of thrombosis and a tendency to transform into acute myelogenous leukemia (AML) or myelofibrosis (MF)(Tefferi et al., 2021).

Patients with polycythemia vera (PV) are categorized as low risk if they are under the age of 60 and without a history of thrombosis. Conversely, those classified as high risk are aged over 60 and have a history of thrombosis. Phlebotomy or low-dose aspirin is recommended for low-risk PV patients, while high-risk patients, in addition to aspirin and phlebotomy, are suggested to have hydroxyurea (cytoreductive drug) treatment (Barbui et al., 2023). Several clinical trials have also recommended hydroxyurea as a drug of choice for PV treatment(Ferrari et al., 2019).

An *invitro* erythroid expansion of peripheral blood mononuclear cells of PV patients exhibited hyper proliferation of erythroid precursors and rapid maturation between 9-14 days with deviant expression of EPO receptors (Bruchova et al., 2009a). Predominantly Jak2 mutation is evidenced in more than 90% of PV patients, which resulted in increased EPO signalling. However several studies have witnessed that Jak2 mutation is not an initial event occurring in PV(Bruchova et al., 2009a; Kralovics et al., 2006).

In polycythemia vera (PV), the Jak2 driver mutation V617F has an impact on the JAK/STAT pathway within erythroid cells. Jak2, a tyrosine kinase, undergoes transphosphorylation upon the binding of erythropoietin (EPO) to the erythropoietin receptor (EPO-R) expressed on the cell membrane. Following activation, Jak2 initiates a cascade of signaling pathways, including STAT5 (signal transducer and activator of transcription 5), PI3K (phosphatidylinositol 3 kinase), and MAPK (mitogen-activated protein kinase), which play roles in erythroid proliferation, survival, differentiation, and the regulation of iron homeostasis. The Jak2 mutation results in continuous JAK/STAT signaling, independent of EPO (Ginzburg et al., 2018). This leads to increased expression of downstream JAK/STAT pathway genes (Figure 2.8.1.1).

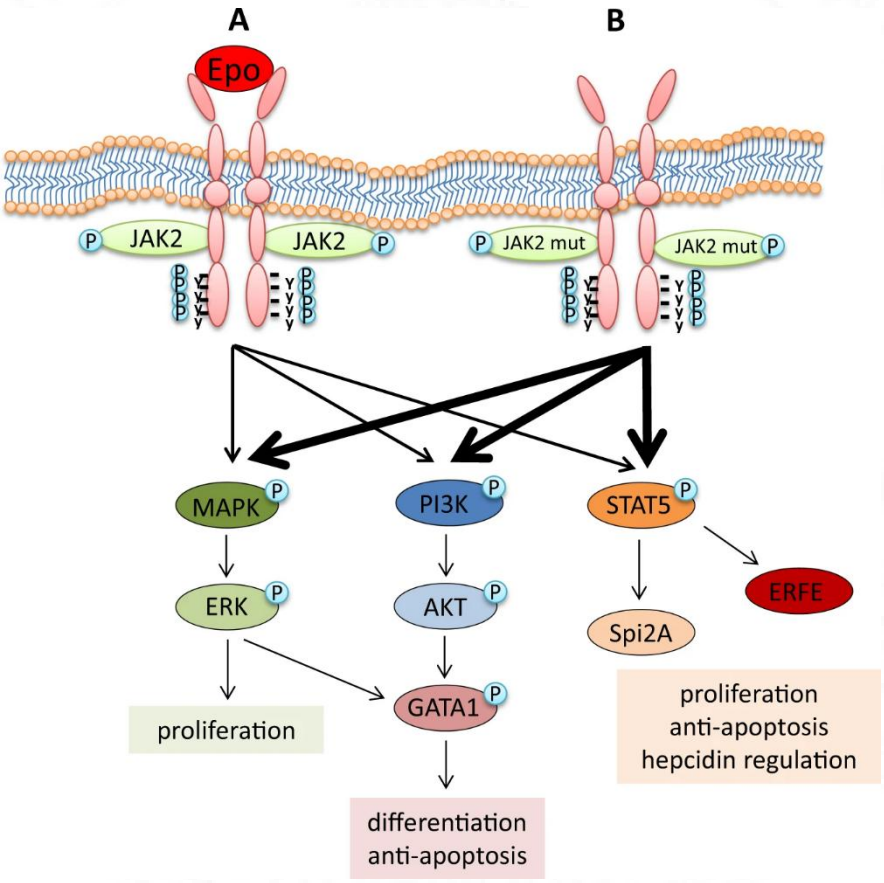


Figure 2.8.1.1. JAK/STAT pathway in Polycythemia Vera(Ginzburg et al., 2018)

Iron deficiency is common in PV patients at diagnosis, partly due to therapeutic phlebotomy. Recent study have shown that 60.5% of patients with PV suffer from iron deficiency (Randrianarisoa et al., 2023). Hence it is vital to assess ferritin, transferrin saturation and MCV levels in these patients.

Increased erythropoiesis in PV leads to augmented utilisation of iron from the plasma. Some authors proposed the involvement of hepcidin in this process, noting elevated hepcidin levels in individuals with PV(Albayrak et al., 2019; Ginzburg et al., 2018). However Ginz et al. have suggested that hepcidin levels were declined by erythropoiesis (Figure 2.8.1.2)(Ginzburg et al., 2018).

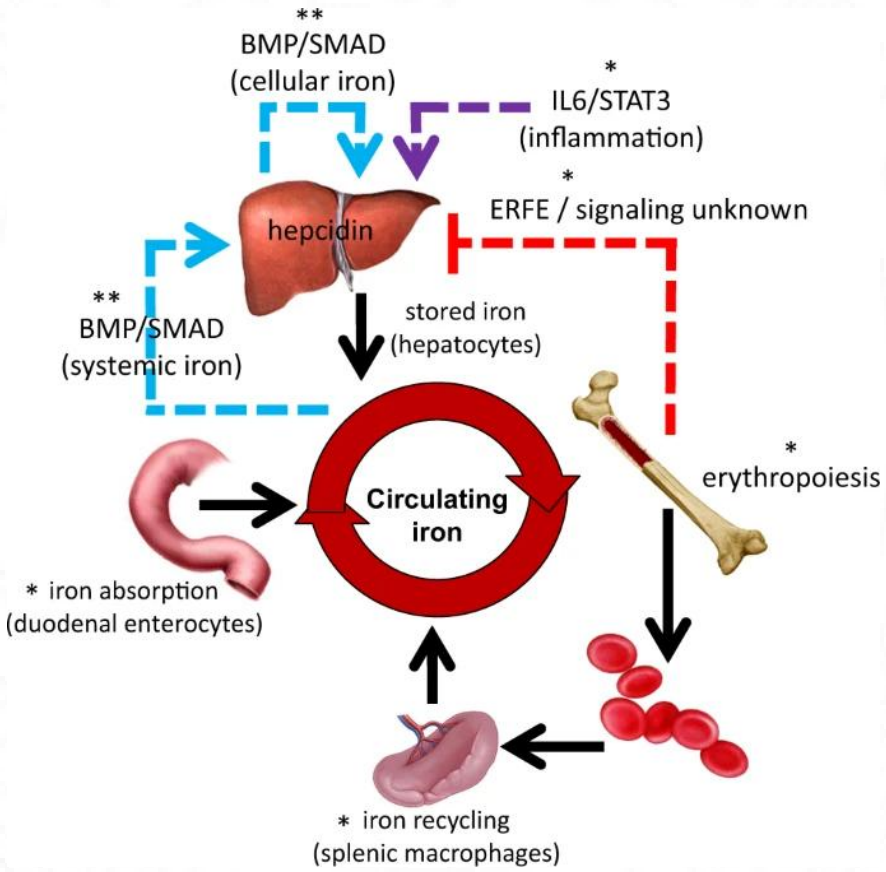


Figure 2.8.1.2. Systemic iron homeostasis in PV (Ginzburg et al., 2018)

2.8.2. Treatment strategies

Initial studies in untreated patients with PV had an increased frequency of thrombotic events and 18 months survival post-diagnosis. The use of cytoreductive treatments to address blood

hyper viscosity, including methods like phlebotomy or chemotherapy, has markedly decreased the occurrence of thrombotic events. Despite these interventions, transformations in hematologic aspects leading to acute leukemia remain substantial contributors to mortality(Marchioli et al., 2005). Recognizing the potential involvement of myelosuppressive drugs in long-term complications, the current treatment options should be customized based on the anticipated thrombosis risk for each individual patient (Barbui and Finazzi, 2006).

Interferon alpha (IFNα) therapy is one of the treatments used in PV. IFNα inhibits the proliferation of hematopoietic progenitors and directly suppresses bone marrow fibroblast progenitor cells and counteracts the activity of cytokines. PV patients administered with pegylated IFNα had reduced burden of Jak2 mutant allele. However further studies are required to under its clinical relevance (Finazzi and Barbui, 2007).

A comprehensive understanding of how hepcidin is regulated by iron status, inflammation, and erythropoiesis in polycythemia vera (PV) may lead to innovative treatments. Studies with JAK2 V617F mice suggest that hepcidin mimetic agents can reduce erythropoiesis, splenomegaly, and iron accumulation in splenic macrophages. Similar to beta thalassemia, hepcidin mimetics, such as endogenous hepcidin or mini-hepcidin, ERFE antagonists, and ferroportin inhibitors, are used in PV patients at different stages of clinical trials(Casu et al., 2018) . These agents are intended to stimulate the production of red blood cells with a limited iron supply.

2.9. International Status:

In earlier studies on erythropoiesis during pregnancy, it was noted that erythropoietic activity seemed to be independent of erythropoietin levels in the early stages of pregnancy and anemia. (Beguin et al., 1991). Invitro studies reported the expansion of erythroid cells in spleen during mid-gestational period (Fu et al., 2023). Even a moderate increase in EPO levels could initiate

the production of erythroid progenitor cells. However in early pregnancy, increased erythropoietic activity did not seem to depend on elevated plasma EPO levels (Fuchs et al., 1992). Interestingly, animal studies have demonstrated that erythropoietic activity is suppressed by estrogens (MIRAND and GORDON, 1966). It was evidenced that increased estrogen levels during pregnancy has induced stem cell mobilization and red cell production in splenomegaly (Nakada et al., 2014). While few studies have explored erythropoiesis in pregnancy, the underlying mechanisms leading to increased remain unexplored.

Several studies have investigated the interplay between iron and erythropoiesis in beta thalassemia. Beta thalassemia major patients demonstrated higher levels of hepcidin, and ferritin compared to those with beta thalassemia intermedia. Iron restricted approaches have been applied in beta thalassemia to reduce stress erythropoiesis. In one study, hepcidin expression was decreased by the inhibition of TMPRSS6, leading to decreased iron overload and improved anemia in beta thalassemia (Guo et al., 2013). However, the underlying mechanism behind iron dysregulation and its role in ineffective erythropoiesis remains unclear and requires further exploration.

In sixteen polycythemia vera patients, hepcidin and GDF15 were measured and found that Jak2-V617F mutation individuals had higher GDF15 level and no significant changes in hepcidin levels despite high erythroid activity(Albayrak et al., 2019). However, several analyses need to be carried out to minimize iron utilization, thereby reducing chronic erythropoiesis. Therefore, exploring the molecular mechanism of erythropoiesis in polycythemia vera is essential.

2.10. National status: In pregnancy, iron deficiency anemia is a major public health issue and studies have been directed mainly to understand the etiology and the magnitude of the problem. In India, the approach to managing iron deficiency anemia during pregnancy involves dietary modifications, oral iron supplementation, intravenous iron administration, and blood

transfusions. (Natekar et al., 2022). However, there is no study on iron metabolism and erythropoiesis during pregnancy. Iron parameters have been evaluated in cases of beta thalassemia (MISHRA and TIWARI, 2013). Few case studies on polycythemia vera have been reported (Singh et al., 2022). However, no data is available on iron dysregulation in beta thalassemia and polycythemia vera.

In pregnancy, the physiological factors that upregulate erythropoiesis has not been explored. The precise role of factors such as erythropoietin, iron regulators, or numerous factors that might drive increased erythropoiesis during pregnancy is unknown. Identifying these factors would help in stimulating red blood cell production invitro. In pathological erythropoiesis, polycythemia vera and beta thalassemia exhibit dysregulated iron metabolism. However, to date, there has been a lack of studies investigating iron regulation and how stress erythropoietic activity manages iron supply in these conditions. The identification of regulatory factors in physiological erythropoiesis holds the potential for therapeutic targeting in pathological states of erythropoiesis in polycythemia vera and beta thalassemia.

3.0 Materials and Methods

3.1. Study population

3.1.1. Ethical Review

The study was approved by the Institutional Review Board (Ethics Committee) of Christian Medical College (CMC) at Vellore, India (IRB Min. No. 11480 dated 22.08.2018, IRB Min. No. 12460 dated 18.12.2019, IRB Min. No. 15137 dated 25.01.2023) and informed written consent was obtained from all the study participants.

3.1.2. Subject recruitment

The study included four groups of patients and healthy control. The inclusion criteria for each group are listed below. Only patients who were willing to sign the informed consent and met the inclusion criteria were included in the study.

Pregnancy- Cohort 1: Pregnant women who visited the antenatal clinic at the Department of Community Health and Development (CHAD), Christian Medical College (CMC), Vellore, were screened, and subjects who fulfilled the inclusion criteria were included in the study (Age-18-35 years, primi gravida, gestational age ≤16 weeks). Pregnant women with age > 35 years, Gravida>1, Gestational Age >16 weeks, gestational diabetes, and pregnancy-induced hypertension (PIH) were excluded from the study. This study was conducted between the years 2019 and 2022. Daily oral iron supplementation with 60 mg of elemental iron was recommended for all pregnant women who visited our antenatal clinic. Maternal peripheral blood samples were collected at gestational age of 16,21,26 and 36 weeks.

Pregnancy- Cohort 2: This study was conducted on pregnant women between 2016 and 2018. Only subjects who visited the antenatal clinic at the Department of Community Health and Development, CMC, Vellore, for childbirth and met the inclusion criteria were included in the study. The inclusion criteria were women between the ages of 18 and 35 with a gestational age (GA) of at least 36 weeks. Pregnant women with gestational diabetes, pregnancy-induced

hypertension (PIH), hypothyroidism, previous caesarean section, bacterial or viral infections during the onset of labour, and twin pregnancies who received transfusion during delivery were excluded from the study. All pregnant women who visited the antenatal clinic were recommended to take daily oral iron supplementation with 60 mg of elemental iron. A detailed proforma was recorded, including type of delivery, placenta size, and newborn details such as sex and baby weight.

Maternal blood samples were collected either before or immediately after delivery for women with a GA of at least 36 weeks. The umbilical cord was clamped and cut during delivery, and cord blood was collected. Placental tissue was obtained and processed within an hour of delivery.

Polycythemia Vera - Cohort 3: Patients who visited the Department of Hematology at CMC with Polycythemia Vera (PV) between 2019 and 2022 were included in the study. Newly diagnosed PV patients were recruited based on WHO criteria- Hb >16.5 g/dL in men, >16.0 g/dL in women, or Hct >49% in men, >48% in women and with the presence of JAK2V617F mutation in exon14. The study did not include patients who were under hydroxyurea treatment. For *in vitro* erythroid differentiation from PV HSCs, 50 to 60 mL of blood was obtained from 400 mL therapeutic phlebotomy units from PV patients.

β thalassemia-Cohort 4: β thalassemia major patients with age of ≤ 10 years and ≤ 50 transfusions; HbE-β thalassemia patients with age of ≤ 10 years were included in the study. Patients who were on hydroxyurea were excluded from the study.

Healthy Controls Cohort: Male voluntary blood donors (n=50) with normal hematological parameters and adequate iron stores were included as a control group. Peripheral blood samples were collected, and serum samples were stored at -80°C.

Mobilized peripheral blood (PBSC) of healthy stem cell donors (N=12) were used for *in vitro* erythroid differentiation.

Definitions: Iron deficiency anemia in pregnancy (IDA) was defined as a hemoglobin level of <10.5 g/dL with a ferritin level of < 30 ng/ml. Iron-replete subjects (control) were defined as having a hemoglobin level of>10.5 g/dL and a ferritin level of > 30 ng/ml.

Polycythemia Vera patients were recruited based on WHO criteria, as Hb >16.5 g/dL in men, >16.0 g/dL in women, or Hct >49% in men, >48% in women, and with the presence of JAK2V617F mutation in exon14.

TDT: Transfusion dependent thalassemia is a term used for beta thalassemia major patients who require regular blood transfusions. These patients present clinically within two years of life, exhibiting symptoms indicative of severe anemia (Hb<7 g/dL) (El-Beshlawy et al., 2024).

NTDT: Non-transfusion-dependent thalassemia (NTDT) is a term used for beta thalassemia patients who do not require regular red blood cell transfusions for survival; NTDT encompasses β-thalassemia intermedia, hemoglobin E/β-thalassemia (mild and moderate forms) with Hb<10 g/dL(Saliba et al., 2023).

Male voluntary blood donors with Hb>12g/dl and Ferritin>15ng/ml served as healthy Controls.

3.1.3. Animal Studies

8-10 weeks old C57BL/6 mice were obtained from the Centre for Animal Facility, CMC and the study was approved by the Institutional Animal Ethics Committee (IAEC) in 2020. Mice were kept in the in-house animal facility maintained at 22±3°C and 55±15% relative humidity with a 12-hour light-dark cycle. Male mice were introduced in the female cage for timed pregnancies, and within 24 hours, female mice were checked for the presence of a copulation plug visually and labelled as Day 0 of pregnancy. Then, male mice were removed, and plugged females were housed in groups of 4 throughout gestation. Mice were fed three different diets and divided into control, iron deficient, and iron overload groups.

- **Cohort 1:** C57BL/6 females received a standard diet for two weeks before mating and throughout gestation.

- **Cohort 2:** Mice were maintained on an iron-deficient diet (4ppm, Research Diets, USA) for four weeks before mating and throughout gestation.
- **Cohort 3:** Iron overload diet (10000ppm, Research Diets, USA) was given for two weeks before mating and during gestation.

Pregnant mice were sacrificed on embryonic day 0, 12.5 and 18.5 (Figure 3.1.3.1). Tissue samples collected from the liver, spleen, kidney, duodenum, fetal liver, and placenta were stored in RNA later at -80°C.

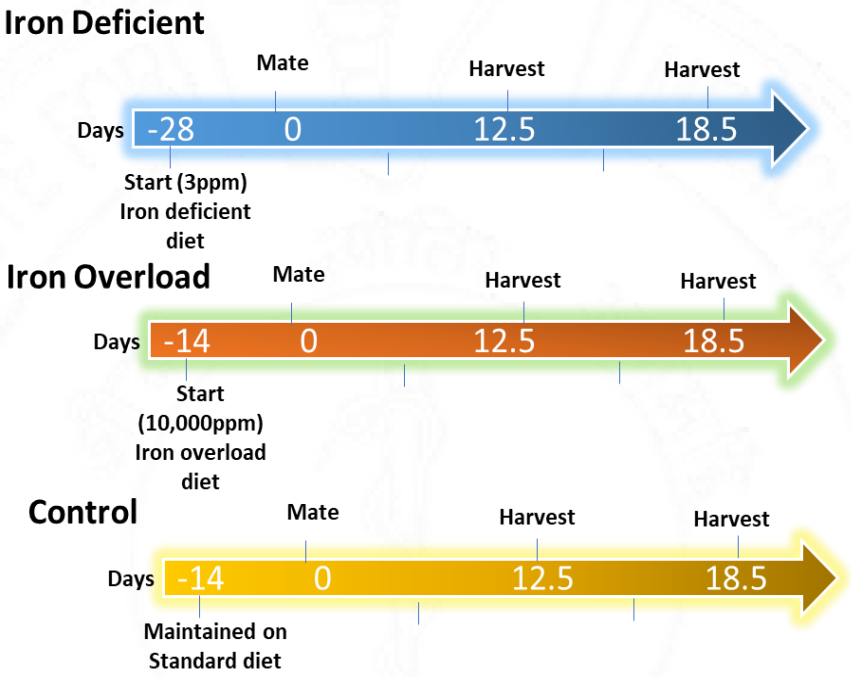


Figure 3.1.3.1. Iron status of C57BL/6 female mice was altered using iron diets and sacrificed at embryonic day 12.5 and 18.5

3.2. Isolation of Mononuclear cells

Mononuclear cells (MNCs) were isolated from the mobilized peripheral blood (PBSC) of healthy stem cell donors and phlebotomized peripheral blood of patients with polycythemia vera (PV).

MNCs were isolated through gradient centrifugation using lymphocyte separation medium (LSM) from MP Biomedicals in Irvine, CA, USA. Initially, samples were diluted with 1X PBS,

gently layered onto LSM at a ratio 1:2 and spun at 400g for 30 minutes with a gradual acceleration and deceleration. Subsequently, the separated MNCs underwent red blood cell (RBC) lysis, followed by a PBS wash, and were prepared for subsequent use.

3.3. $CD34^+$ cells isolation

The MNCs were enriched using the EasySep Human CD34 positive selection kit (Stem Cell Technologies in Vancouver, Canada). In brief, the isolated mononuclear cells (MNCs) at a concentration of $1x10^7$ cells/100µl were suspended in an enrichment medium (PBS containing 2% FBS and 1 mM EDTA). The cells were then incubated with CD34 microbeads (EasySep™) and Positive Selection Cocktail at room temperature for 10 minutes. Then, EasySep™ Dextran Rapid Spheres™ 50100 was added and incubated for 5 minutes. The cells were resuspended in the enrichment media (volume up to 2.5ml) and passed through the EasySep™ magnet for 3 minutes. The isolated cells were separated from the supernatant by inverting the magnet in one continuous motion. After this, the tube was removed from the magnet, and the cells were washed thrice with phosphate buffer saline (PBS). In the final wash, the cells were suspended in PBS and centrifuged to obtain the cell pellet. The number of cells was determined using a cell counter. An aliquot of the sample was stained with an APC-conjugated CD34 antibody (BD Pharmingen, San Diego, CA, USA) antibody to assess the enrichment. The $CD34^+$ cells' purity was between 95% to 98% (Figure 3.3.1.1).

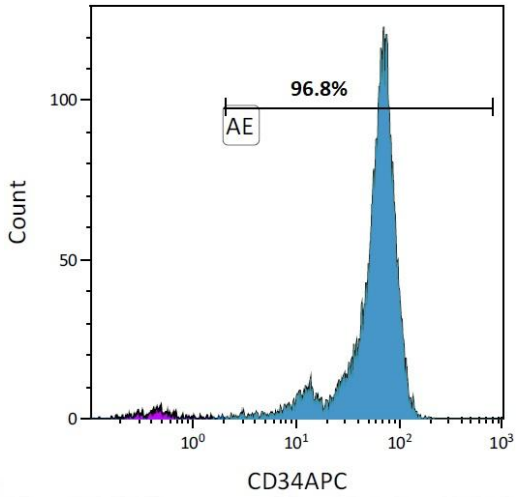


Figure 3.3.1.1: Purity assessment of CD34$^+$ cells by flow cytometry

3.3.1 CD34$^+$ cells expansion

CD34$^+$ cells were cultured in serum-free StemSpan SFEM Medium supplemented with StemSpan CD34$^+$ expansion supplement (StemCell Technologies) for 6 days. The expansion supplement includes FLT-3 Ligand (Flt-3L), Stem cell factor (SCF), Interleukin-3 (IL-3), Interleukin-6 (IL-6) and thrombopoietin (TPO).

3.4. Erythroid differentiation from hematopoietic progenitor cells

Erythroid differentiation can be divided into three phases. The base culture medium used was a serum-free stem-span medium from Stem Cell Technologies, USA.

During the first phase, which lasted from day 0 to day 6, CD34$^+$ cells purified from healthy stem cell donors and PV patients were cultured in a medium containing 10 ng/mL stem cell factor from Peprotech, 1 ng/mL IL-3 from Stem Cell Technologies, and 3 IU/mL erythropoietin from Zyrop 4000IU. In the second phase, which lasted from day 7 to day 11, IL-3 was not included in the culture medium.

The third phase started on day 11 and lasted until day 21. During this phase, the cells were cultured in IMDM medium from Thermo Fisher, supplemented with 3 IU/mL erythropoietin

and 1 mg/mL holotransferrin (Sigma-Aldrich) (Figure 3.4.1.1). The cells were kept at 37°C in 5% CO2 incubator (Thermo Fisher Scientific), and the media was changed on alternative days.

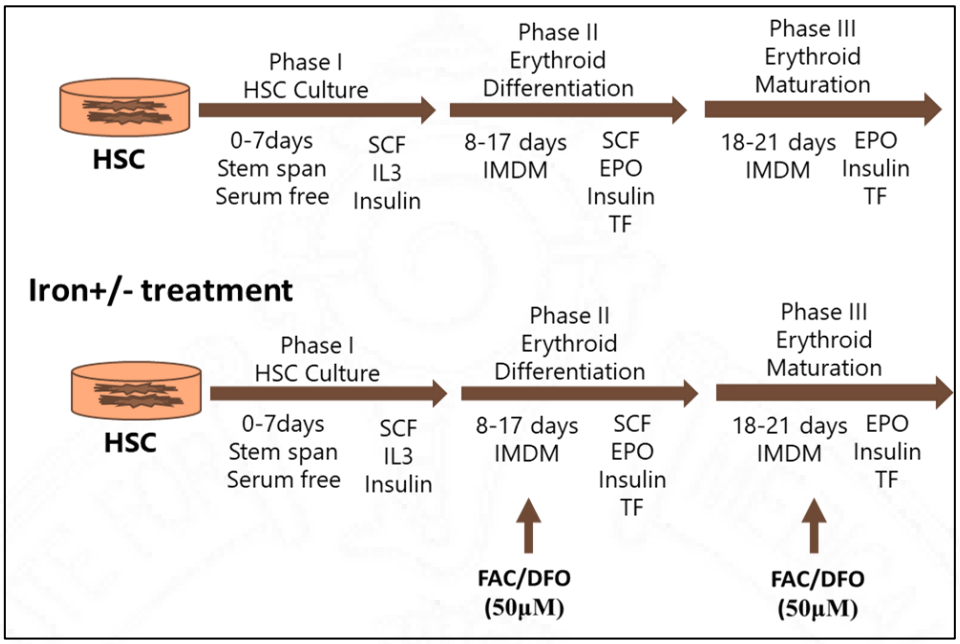


Figure 3.4.1.1. Time course of CD34⁺cells in vitro erythroid differentiation with the list of used cytokines at each stage.
(SCF-stem cell factor, IL3-interleukin-3, EPO-erythropoietin, TF-holo-transferrin, DFO-deferoxamine, FAC-ferric ammonium citrate)

3.4.1. Erythroid Differentiation from Polycythemia Vera

The $CD34^+$ cells were enriched from 50 to 60 mL of blood procured from 400 mL of therapeutic phlebotomy units obtained from patients diagnosed with PV. This methodology involved layering peripheral blood onto Ficoll-Hypaque (MP Biomedicals) and subsequently separating mononuclear cells (MNC) via centrifugation. The enrichment of $CD34^+$ cells from MNC was facilitated using the EasySep, following the manufacturer protocol. MNCs were subjected to flow cytometric analysis to quantify the percentage of $CD34^+$ cells. We were able to isolate $1x10^6$ $CD34^+$ cells from a single phlebotomy unit. CD34+ cells, isolated through a magnetic separation with a purity ≥ 95%, were used for erythroid differentiation, as mentioned above.

3.5. Placenta collection and processing

The placenta collected during delivery was processed within one hour after the delivery. First, the amniotic membranes were removed from the placenta. Then, a tissue of 0.5-0.8cm thickness was carefully cut from the red cotyledons, avoiding a deep cut below the amniotic membrane side of the placenta. The dissected tissues were later stored in RNAlater (Ambion) and kept at -80°C until they were ready for analysis.

To ensure that there was no maternal contamination in the placental tissues, we conducted a variable number tandem repeat (VNTR) analysis using five markers. We extracted DNA from maternal peripheral blood, cord blood, and placental tissues and performed a multiplex PCR for five short tandem repeat (STR) markers (ACTBP2, FES, THO1, VWF, and F13A1) using fluorescently labelled primers followed by capillary electrophoresis.

3.6. Reticulocyte Isolation

The reticulocyte fraction from the peripheral blood was extracted using Bresnick's protocol(Pal et al., 2004). Firstly, the plasma and buffy coat were removed through centrifugation. The plasma was frozen at -80°C, and DNA was extracted from the buffy coat. The reticulocyte-rich fraction was then suspended in phosphate-buffered saline and placed on top of a cellulose (Sigma) column consisting of two parts cellulose and one-part microcrystalline cellulose. The reticulocytes were extracted by gently spinning the column at 1500 rpm for 5 minutes. The leucodepleted elute was washed with PBS and stored in Trizol at -80°C until RNA extraction.

3.7. Ter119^{+} cell sorting

To prepare the bone marrow cell suspension from C57BL/6 mice, the cells from the tibia and femur of the mice were flushed with PBS/2 mM EDTA. The cells were centrifuged at 300g for 5 minutes, and the pellet was suspended in RPMI medium. Ter119^{+} cells were isolated from the bone marrow cell suspension using the EasySep™ FITC Positive Selection Kit (Stemcell

Technologies) and labelled with FITC-conjugated antibodies. The purity of the isolated Ter119^{+} cells was over 90% (Figure 3.7.1.). Finally, the cells were stored in Trizol at -80°C.

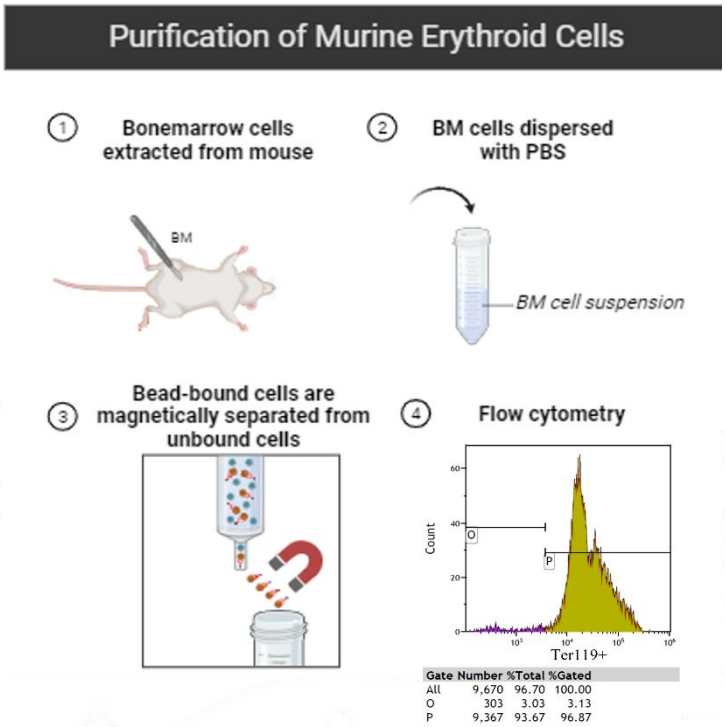


Figure 3.7.1. Purification of murine erythroid cells

3.8. Estradiol treatment of erythroid-differentiated HEL Cells

Human erythroleukemic cell line (HEL) was cultured in RPMI 1640 medium, and the cells were grown at 37℃ in humidified air containing 5% CO_2. HEL cell line ($1*10^6$ cells) was treated with 30µM hemin (Sigma) for 96 hrs according to the previously described protocol (Smith et al., 2000). Hemin-treated cells were treated with 17 β-estradiol (Sigma) after 24hrs and continued treatment every 24hrs (Figure 3.8.1). The culture medium was changed at every 48 hrs with hemin. The cells were collected at 24, 48, 72, and 96 hrs. Flow cytometry was performed for CD235 and CD71 erythroid surface markers. The cell pellet was stored in trizol at -80℃.

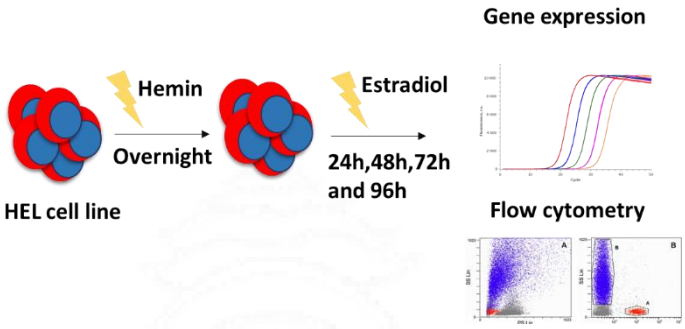


Figure 3.8.1: Schematic representation of erythroid differentiation in HEL cell line induced by hemin and subsequent estradiol treatment.

3.9. Haematological and Biochemical Assessment

Complete blood counts (CBC) were performed on maternal peripheral and cord blood samples using an automated haematology analyser (Sysmex KX21). Serum ferritin, soluble transferrin receptor, erythropoietin, estradiol, Interleukin-6 (IL6), and UIBC were analysed using a chemiluminescence immunoassay using the Advia Centaur, Siemens XPI. CRP (Immulite 2000 siemens Xpi) levels analysed using chemiluminescent immunoassay. The transferrin saturation was calculated as (serum iron/total iron-binding capacity [TIBC]) × 100. Serum vitamin B12 and folate were measured by Roche Electro chemiluminescence immunoassay in Roche Modular E170 system.

3.9.1. Measurement of Serum Hepcidin-25

Hepcidin-25 in serum was measured using an Enzyme Immunoassay following the manufacturer's protocol (DRG, GmbH, Germany). The assay is a solid-phase enzyme-linked immunosorbent assay that uses competitive binding. The intensity of the developed colour is inversely proportional to the concentration of hepcidin present in the sample. The samples were analyzed in duplicates, and quality controls with high and low values were used at each run. The results were calculated using a 4PL curve fit (Spectramax M4 plate reader, CA, USA).

3.9.2. Measurement of Serum GDF15

Serum GDF15 was measured using an ELISA method (R&D Systems, Inc., MN, USA). The amount of serum GDF15 present was directly proportional to the color developed. Samples were diluted with assay diluent and tested in duplicates. A standard curve was generated using 4PL curve fit, and concentrations were calculated using the Spectramax M4 plate reader from CA, USA.

3.10. Flow cytometry

Human and mouse erythroid cells were stained for 20 minutes with anti-human and anti-mouse pre-conjugated antibodies such as FITC-labelled CD71(human and mouse), APC-labelled mouse Ter119 and APC-labelled human CD235 (BD Pharmingen, San Diego, CA, USA). The cells were washed in PBS supplemented with 0.5% NaN3 (Sigma-Aldrich) and 0.1% FBS and acquired on a Navios flow cytometer (Beckman Coulter). Apoptosis staining with annexin V and FITC labelled 7-AAD (BD Pharmingen, San Diego, CA, USA) was performed as described in the kit's manual. The data analysis was performed using the Kaluza software (Beckman Coulter).

3.11. Molecular Analysis

3.11.1.DNA Extraction

DNA was extracted from peripheral blood mononuclear cells using the Qiagen Gentra kit (Qiagen GmbH, Hilden, Germany) method according to the manufacturer's instructions and stored at 4°C until use.

3.11.2. RNA Extraction

Total RNA was extracted from human reticulocytes using the Trizol reagent (Invitrogen, CA, USA) according to standard protocol and stored at -80°C. To extract RNA, the cells were lysed in trizol reagent and 0.2 ml chloroform was added per 1 ml of trizol reagent. After 15 minutes of incubation, the mixture was centrifuged at a speed of 13000g for 15 minutes. The aqueous phase was collected slowly and transferred into a fresh tube. An equal volume of isopropanol was added to the mixture and incubated for 10 minutes. After centrifugation at 13000rpm for 10 minutes, the RNA was precipitated and washed with 75% ethanol. The RNA pellet was air-dried, suspended in RNase-free water, and stored at -80°C for future use. The concentration and purity of RNA were assessed using a nanodrop microvolume UV-Vis spectrophotometer (Thermo Scientific, USA) by calculating the absorbance ratio of 260/280, which ranged from 1.9 to 2.2.

From human placental and animal tissues stored in RNA Later (Spleen, liver, placenta, and fetal liver), total RNA was extracted using the Protein and RNA Isolation (PARIS) kit (Qiagen) following the manufacturer's instructions. Cell disruption buffer, around 100-600µl (6-8 volumes/tissue mass) volume was added to a 10-30mg tissue sample on ice and homogenised. To the homogenised lysate, an equal volume of 2X lysis/binding solution and ethanol was added. And a 700µl sample mix was added to a filter cartridge and centrifuged at13000rpm for 1 minute. The sample mix was washed using a wash solution and spun at 13000rpm for 1 minute. Then the elution buffer, used to elute RNA was heated at 95°C and RNA was collected in the same tube and stored at -80°C.

3.11.3. cDNA Synthesis

The RT^2 First Strand kit (QIAGEN) was used for cDNA synthesis and genomic DNA elimination. The genomic DNA elimination mix was prepared for each RNA sample according to Table 3.11.3.1.

Table 3.11.3.1: Genomic DNA elimination mix	
RNA	25ng-5µg
Buffer GE	2µl
RNase-free water	Variable
Total volume	10 µl

Genomic DNA elimination mix was incubated for 5 min at 42°C, then placed immediately on ice for at least 1 min. 4. Prepared the reverse-transcription mix according to Table 3.12.3.2.

Table 3.11.3.2: Reverse-transcription mix	
Volume	**1 reaction**
5x Buffer BC3	4µl
Control P2	1 µl
RE3	2 µl
RNase-free water	3 µl
Total volume	10 µl

Reverse-transcription mix (10 µl) was added to each tube containing 10 µl genomic DNA elimination mix. Incubated at 42°C for 15 minutes and immediately stopped the reaction by incubating at 95°C for 5 min. 91 µl RNase-free water was added to each reaction and stored at -20C for further downstream applications.

3.11.4. Polymerase Chain Reaction (PCR)

The synthesised cDNA products from tissues and reticulocyte RNA samples were analysed qualitatively for the GUS (*GUS*: β-glucuronidase) and beta-actin housekeeping genes using PCR. PCR products were analysed using 2% agarose gel. Representative cDNA amplified products of placental tissue samples of IDA and control are given below. The primer sequence (Table 3.11.4.1), master mix and PCR conditions are given in Table 3.11.4.1.

Table 3.11.4.1. Primer sequences for GUS gene

Gene Name	Primer sequence	Amplicon Size
GUS	Forward 5`-CTGTGACCTTTGTGAGCAA-3`	600 bp
	Reverse 5`- TCTGCCGTGAACAGTCCA-3	

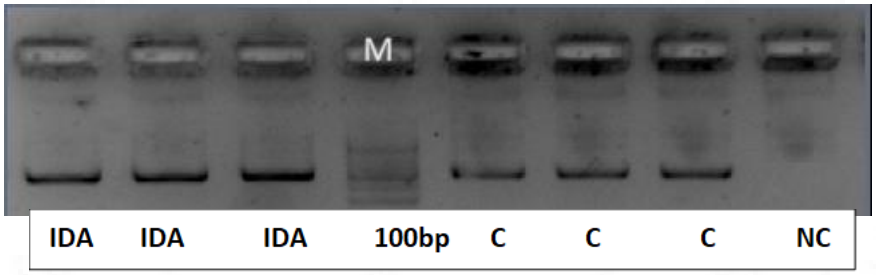


Figure 3.11.4.1.1: Agarose gel electrophoresis showing amplification of GUS from cDNA synthesized (Lane M: 100bp marker, Lane1-3, 5-7: cDNA samples showing amplification of GUS gene in IDA – Iron Deficiency Anemia, C – control and NC-negative control, respectively).

3.11.5. PCR Arrays

The RT2 Profiler PCR Array (Qiagen) was designed to analyse a panel of iron metabolising genes related to placental iron trafficking pathway. The array was a 96-well- plate containing gene-specific primer assays for iron metabolising genes. Each RT^2 Profiler PCR array also included a control element for genomic DNA contamination detection. Array was used for

placental tissue cDNA samples. The average genomic DNA CT value was greater than 35 in all the samples, thus eliminating the genomic DNA contamination. Gene layout used for iron metabolising genes were given below (Table 3.11.5.1).

Table 3.11.5.1: Gene array for iron metabolising genes

	1	2	3	4	5	6	7	8	9	10	11	12
A	GDF15 1	ACO1 9	TFR2 17	GDF15 1	ACO1 9	TFR2 17	GDF15 1	ACO1 9	TFR2 17	GDF15 1	ACO1 9	TFR2 17
B	SLC11A2 2	IREB2 10	TWSG1 18	SLC11A2 2	IREB2 10	TWSG1 18	SLC11A2 2	IREB2 10	TWSG1 18	SLC11A2 2	IREB2 10	TWSG1 18
C	SLC40A1 3	PGF 11	TFRC 19	SLC40A1 3	PGF 11	TFRC 19	SLC40A1 3	PGF 11	TFRC 19	SLC40A1 3	PGF 11	TFRC 19
D	HEPHL1 4	SP1 12	ACTB 20	HEPHL1 4	SP1 12	ACTB 20	HEPHL1 4	SP1 12	ACTB 20	HEPHL1 4	SP1 12	ACTB 20
E	FTL 5	SLC46A1 13	GAPDH 21	FTL 5	SLC46A1 13	GAPDH 21	FTL 5	SLC46A1 13	GAPDH 21	FTL 5	SLC46A1 13	GAPDH 21
F	FLVCR1 6	HFE 14	HGDC 22	FLVCR1 6	HFE 14	HGDC 22	FLVCR1 6	HFE 14	HGDC 22	FLVCR1 6	HFE 14	HGDC 22
G	HIF1A 7	CD163 15	RTC 23	HIF1A 7	CD163 15	RTC 23	HIF1A 7	CD163 15	RTC 23	HIF1A 7	CD163 15	RTC 23
H	TP53 8	LRP1 16	PPC 24	TP53 8	LRP1 16	PPC 24	TP53 8	LRP1 16	PPC 24	TP53 8	LRP1 16	PPC 24

TaqMan probes from Thermo Fisher were used for mouse iron, erythroid and placental specific genes in Ter119^{+} cells, liver, placenta, and fetal liver tissues (Table 3.11.5.2).

Table 3.11.5.2: Iron and erythroid related genes in mouse

Genes	
Iron metabolising genes	dmt1, tfrc, hamp(hepcidin),irp1,2(iron responsive proteins)
Erythroid genes	Jak2, scara5(Scavenger Receptor Class A Member 5), epor(erythropoietin receptor), Erfe (erythroferrone), Gdf15(growth differentiation factor), vcam1(Vascular cell adhesion protein 1), icam1 (intracellular adhesion molecule 1), estrogen receptors- Esr1,esr2, Estradiol isoforms- E-alpha, E-beta
Placenta	PLP-E (Prolactin-like protein E)

3.11.6. Real-Time qPCR

Real-time PCR was performed on the 7500 QPCR System (Applied Biosystems). SYBR green detection (Takyon) was used for human samples. For murine samples, Real-time PCR was

performed on the Quant studio 5 (Applied Biosystems model), and TaqMan probe detection (Thermo Fisher) was used. For the PCR array, all data was analysed by SA Bioscience's PCR array data analysis web portal. Plate-to-plate variation was controlled by normalising gene expression to β-actin by using the $2^{-\Delta\Delta Ct}$ method.

3.11.7. Alternative transcripts of placental iron traffickers

We selected eighteen iron metabolising genes involved in placental iron homeostasis, and their alternative transcript data were retrieved from the Ensembl website. Genes include DMT1, TFRC, FPN1, STEAP3, SLC46A1, HIF1A, ACO1, IREB2, GDF15, TWSG1, SP1, TP53, GAPDH, HFE, CD163, LRP1, FLVCR1, PGF. Forty primer sets were designed to amplify these genes' main and alternative transcripts. Of these 40 primer sets, 23 transcripts were found to be expressed in the placental tissue by qualitative PCR. Selective amplification of these transcripts was qualitatively confirmed using two controls and IDA samples. Quantitative PCR was performed for 15 transcripts of eight genes. Relative quantification was done by using the $2^{-\Delta\Delta Ct}$ method. Representative main and alternative transcripts amplified products of placental tissue samples are given below.

Sno	Genes	Sequence
1	DMT1A	Fwd:5'-GTG TTC TAC TTG GGT TGG CA-3' Rev:5'-AAA CAC ACT GGC TCT GAT GG-3'
2	DMT1B	Fwd:5'-CTT CCT GGA CTG TGG GCA TA-3' Rev:5'-TGA ACA CCC TTC CCA TGA GG-3'
3	FPN1A	Fwd:5'-CTC CCA AAC CGC TTC CAT-3' Rev:5'-AAC ACA GAC ACC GCA AAG TGC-3'

4	FPN1B	Fwd:5'-CCG AGG TGC TAT CTC CAG TT-3' Rev:5'-TTG CAG AGG TCA GGT AGT CG-3'
5	IRP2-201	Fwd:5'-GCC TTA CCC CTC GTG AAT TC-3' Rev:5'-ACA TCT AGC GTC TGT CCT GA-3'
6	IRP2-208	Fwd:5'-GGG GTT GGA GGC ATT GAA ACA-3' Rev:5'-ATG AAC CCA AAT CCC TGC TC-3'
7	TFRC-201	Fwd:5'-CTC AGA GCG TCG GGA TAT C-3' Rev:5'-TAT CGC CAT CTA CTT GCC GA-3'
8	TFRC-202	Fwd:5' – CTA GTG TGA GTG CGG GCT T-3' Rev:5'- CCA TCA TTC TGA ACT GCC ACA-3'
9	TFRC-203	Fwd:5'-CGC GCT AGT GTT CTT CTG TG-3' Rev:5'-CTG TGC TGT CCA GTT TCT CC-3'
10	Actin	Fwd:5'- GGC GGC ACC ACC ATG TAC CCT-3' Rev:5'-AGG GGC CGG ACT CGT CAT ACT-3'
11	SLC46A1-207	Fwd:5'-CCA GTC CTG ATG TCC ACC TT-3' Rev:5'-CTA GTC ATG CCC TGG TCT CC-3'
12	SLC46A1-208	Fwd:5'-CAC GCC TCT CAT GTT CAC AG-3' Rev:5'-CAT CCC AAT CAG AAC AGC CG-3'
13	HIF1A-202	Fwd:5'-GCG GCG CGA ACG ACA AGA AAA AGA-3' Rev:5'- CCA GCA TCC AGA AGT TTC CTC ACA-3'
14	HIF1A-204	Fwd:5'-GAG GTT GAG GGA CGG AGA TT-3' Rev:5'- TGG CTG CAT CTC GAG ACT T-3'
15	GDF15- 201	Fwd:5'-CGG ATA CTC ACG CCA GAA GT-3' Rev:5'- GGT CTT GCA AGG CTG AGC-3'
16	GDF15- 203	Fwd:5'-CTA GGA CCC TCG GAC AAG C-3'

		Rev:5’- CAG CCT GGG AGT CTG TGC-3’
17	TWSG1	Fwd:5’-GCG CCT TAT TCC AGT GAC AAA GAA C-3’ Rev:5’- GCT TTG GTT TGC ATT TGT CTT CT-3’
18	SP1-201	Fwd:5’-ATG AGC GAC CAA GAT CAC TC-3’ Rev:5’- TGG GTG ACT CAA TTC TGC TG-3’
19	SP1-202	Fwd:5’-AGC CTA CCC TTC CTG CAT TT-3’ Rev:5’- CCA ACC CCT GTG AAT GCA TC-3’
20	TP53-202	Fwd:5’-GTG TTT GTG CCT GTC CTG G-3’ Rev:5’-TGG CCG GAA ATG TTT TCT GA-3’
21	TP53-204	Fwd:5’-AGG ACC AGA CCA GCT TTC AA-3’ Rev:5’-CAG CTC TCG GAA CAT CTC GA-3’
22	TP53-206	Fwd:5’-GAG CAC TGC CCA ACA ACA C-3’ Rev:5’-TGG GCA TCC TTG AGT TCC AA-3’
23	TP53-218	Fwd:5’-TGA CAC GCT TCC CTG GAT TGG CA-3’ Rev:5’-AGA GCA GAA AGT CAG TCC CA-3’
24	GAPDH-201	Fwd:5’-AAG ACC TTG GGC TGG GAC-3’ Rev:5’-AAT CCG TTG ACT CCG ACC TT-3’
25	GAPDH-202	Fwd:5’-ATC TTC TTT TGC GTC GCC AG-3’ Rev:5’-CCA TGT AGT TGA GGT CAA TGA AGG GGT C-3’
26	GAPDH-203	Fwd:5’-GAT GGG TGG AGT CGC GTG-3’ Rev:5’- CCA TGT AGT TGA GGT CAA TGA AGG GGT C-3’
27	HFE-202	Fwd:5’-GAA AAT CAC AAC CAC AGC AAG G-3’ Rev:5’- AGG TTC GAA CTC CTT GGC A-3’
28	HFE-204	Fwd:5’-AGC TGT AGG GTG ACT TCT GG -3’

		Rev:5’-GTG TGG GAC TGC AGC AAG-3’
29	HFE-208	Fwd:5’-AAG CGG AGA TTT AAC GGG GA-3’ Rev:5’-ACG GCG ACT CTC ATG ATC AT-3’
30	CD163-201	Fwd:5’-ACT CCG CCT CCA TAT GTA GC-3’ Rev:5’-AGT CAG CAG ATC CAG AGT CT-3’
31	CD163-202	Fwd:5’-AAG GGA CAG GGT TAG GGA GT-3’ Rev:5’-TTT CCA AAA CGT GCT CCT CC-3’
32	LRP1-201	Fwd:5’-GCA TCC TGA TTG AGC ACC TG-3’ Rev:5’-TGG TCG TTT TCA CAG GCA TG-3’
33	LRP1-202	Fwd:5’-CCT CAG TCT GCA CCT GTG T-3’ Rev:5’-GGG CAT GGT TAG CTT TCA CA-3’
34	LRP1-207	Fwd:5’-CGT CGA CAG CAA GAT TGT GT-3’ Rev:5’-AGACTCAGAGAACGGTTCCC-3’
35	PGF-202	Fwd:5’-CAC ATG TTC AGC CCA TCC TG-3’ Rev:5’-TGA AGT CAC CAG GCA TGT CT-3’
36	PGF-203	Fwd:5’-TGT CAC CAT GCA GCT CCT AA-3’ Rev:5’-AAT AGA GGG CAG GTA CCA GC-3’
37	TFR2-202	Fwd:5’-CTG ACG TCT ACT GCC CCT AC-3’ Rev:5’-GCC TTT GAG CTT CCT TCT GG-3’
38	TFR2-205	Fwd:5’-CTG GAA GGC TGG ACT GAG G-3’ Rev:5’-GTT GTT GCG CTC TCT GGA AT-3’
39	FLVCR1-202	Fwd:5’-TGA CAC AAA TCT CCT GGC TTG-3’ Rev:5’-AGG CAC CAG TCA TGA TAC CA-3’
40	FLVCR1-201	Fwd:5’-TGA CAC AAA TCT CCT GGC TTG-3’ Rev:5’-AGG CAC CAG TCA TGA TAC CA-3’

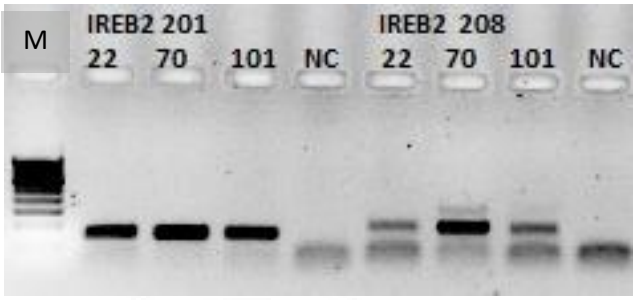


Figure 3.11.7.1: Agarose gel electrophoresis showing amplification of GUS from cDNA synthesized (Lane M: 100bp marker, Lane1-3, 5-7: cDNA samples showing amplification of GUS gene in IDA and control, respectively; Lane NC-negative controls)

3.11.8. Immunoblotting

Placental tissues were lysed by homogenisation in cell disruption buffer (PARIS kit, Ambion) according to the manufacturer's protocol. Protein concentration was quantified using Bradford assay. All the samples were prepared in Lamaelli buffer with reducing agent β-mercaptoethanol. 50µg of samples used for FPN1 were not pre heated. For DMT1, samples were prepared in Lamaelli buffer without reducing agent and was not pre-heated. For all other proteins, 30µg of samples were boiled at 100°C for 5 mins. Primary and secondary antibodies were listed in Table 3.12.8.1. Protein size markers (Bio-Rad precision plus protein standards) was loaded without heating. Tissue lysates were separated by SDS-PAGE gels (4%-12%) and transferred to polyvinylidene difluoride fluorescence membranes (Millipore, Billerica, MA, USA). Non-fat dry milk (NFDM-10%) was used to block the membranes and probed with primary antibody diluted in 5% NFDM diluted in TBS buffer with 0.1% Tween20 and kept at 4°C for overnight. Membranes were rinsed and probed with secondary antibody for 1.5hr in NFDM blocking buffer containing 0.1% Tween20. The primary and secondary antibodies are listed in Supplemental Table 2. The bands were visualised using a chemiluminescence ECL system (Super signal West femto, Thermo Scientific). The FluorChem E system detected the protein bands using digital darkroom software. Band intensities were quantified by densitometric analysis using ImageJ software.

Table 3.11.8.1. Primary and Secondary antibodies used in western blot.

Target Protein	Primary antibody	Dilution
FPN1	Rabbit polyclonal antibody (NBP1-21502, Novus biologicals)	1:1000
DMT1	Rabbit polyclonal antibody (Cat no: A10231, Abclonal)	1:1000
TFR1	Rabbit polyclonal antibody (Cat no: A18083, Abclonal)	1:1000
GDF15	Mouse monoclonal antibody (sc-515675, Santa cruz biotechnology)	1:1000
β-Actin	Mouse monoclonal antibody (sc-47778, Santa cruz biotechnology)	1:1000

Secondary antibody
Anti-mouse IgG HRP (Santa cruz biotechnology, sc-2005)
Anti-rabbit IgG HRP (Cell Signaling #7074)
Anti-goat IgG HRP (Santa cruz biotechnology, sc-2020)

3.12. Statistical Analysis

The statistical data analysis was carried out using the SPSS software version 21. Prevalence data was analyzed using frequencies. Chi-square test was employed for categorical data. To ensure accuracy, appropriate statistical tests such as t-test for continuous variables, analysis of variance (ANOVA) for group comparison, Mann–Whitney, and Kruskal–Wallis for non-parametric data were utilized. Pearson or Spearman correlation was also used to determine the correlation between variables. Statistical significance assumed as $p<0.05$. Associations were evaluated using both univariate and multivariate linear regression. Multiple logistic regression models were developed, and the risk factors associated with anemia were evaluated using odds ratio (OR).

4.0. Results

4.1.1. Role of iron and various regulators of erythropoiesis in the physiological state of increased erythropoiesis in pregnant women

4.1.1.1 Baseline characteristics of the subjects

In this cross-sectional study, we enrolled 320 expectant mothers at 16±1 weeks of gestation during their first antenatal visit. The mean age of pregnant women was 24 ±3.5 years. We collected follow-up samples from 212 participants at 21±1 weeks, 161 participants at 26±1 weeks, and 126 participants at 36±1 weeks. Among them, sample collection was accomplished throughout all four gestational weeks in 99 pregnant women.

The mean age of 99 pregnant women at their first visit (16±1weeks) was 23±3 yrs. During this initial visit, around 10.1% of the pregnant women (N=10) had iron deficiency anemia (Hb<10.5mg/dl & ferritin ≤15 ng/ml). In addition, IDA was observed in 4%, 7%, and 11% of pregnant women at 21±1weeks, 26±1weeks, and 36±1 weeks, respectively. Nearly 24% (N=24) of subjects had iron deficiency consistently throughout the gestational period (Ferritin≤15ng/ml).

Hematological and biochemical parameters were tabulated in Table 4.1.1.1.1. Mean hemoglobin, hematocrit, and MCV levels were normal throughout the gestational age (Figure 4.1.1.1.2). A significant drop in hemoglobin was typically 0.2g/dL by 26 weeks compared to 21 weeks (p=0.042) and stabilized thereafter in the 36 weeks. A significant increase in MCV was observed during the entire gestational period.

Table 4.1.1.1.1. Laboratory parameters of the study

Group N=99	Hb (g/dL)	MCV (fL)	Serum Ferritin (ng/mL)	Serum iron (µmol/L)	TIBC (µmol/L)	sTfR (mg/L)	Vitamin B12 (pg/mL)	Folic acid (ng/mL)	IL-6 (pg/mL)
Gestational Age									
16 ±1weeks	12.4 (7.2-16.3)	78.5 (55-87.6)	23 (5-150)	62 (22-479)	394.5 (275-695)	3.2 (1.68-21.8)	205 (50-785)	13.9 (2.1-20)	2.3 (1.5-5.63)
21±1 weeks	12.4 (9-16)	82 (61-91.2)	23 (3.2-280)	74 (27-189)	442 (296-620)	3 (1-10.21)	177 (50-858)	16 (2.4-20)	2.5 (1.5-9.22)
26±1 weeks	12.2 (9.4-16.4)	83.1 (62.9-94)	25.2 (4.6-171.5)	71 (24-302)	459 (294-612)	3.45 (1.16-8.38)	162 (37.6-427.8)	16 (2-20)	2.8 (1.5-9)
36±1 weeks	12.4 (7.6-16)	83.8 (61.5-99)	26.9 (7.2-232.2)	90 (19-423)	482 (317-763)	3.45 (1.43-11.59)	150.7 (50-434)	15.6 (2.57-20)	3.5 (1.5-9.93)

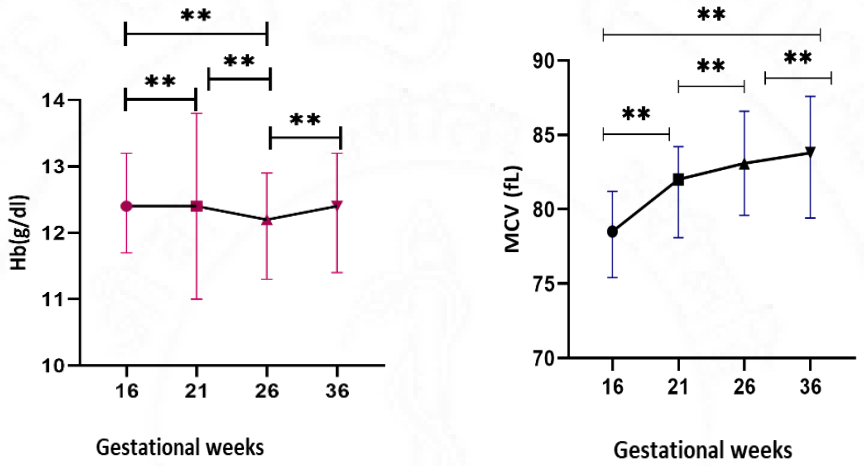


Figure 4.1.1.1.2. Change in hematological parameters from baseline.

4.1.1.2. Kinetics of iron and erythroid parameters

Serum ferritin significantly decreased across the gestational period (Figure 4.1.1.2.1). Serum iron and transferrin saturation showed a similar pattern of increase at 16-21 weeks and reduced at 26 weeks. TIBC levels increased gradually throughout the pregnancy. sTfR levels significantly increased from the second trimester and stabilized in the third (Figure 4.1.1.2.2). Erythropoietin demand was significantly elevated from 21 weeks ($p<0.001$) and stabilized at 26-36 weeks. Vitamin B12 levels were significantly reduced throughout the pregnancy (Figure 4.1.1.2.3). No significant changes were observed in the folate levels. A significant increase in

interleukin-6 (IL-6) levels was observed during pregnancy; however, the levels were within the normal limit.

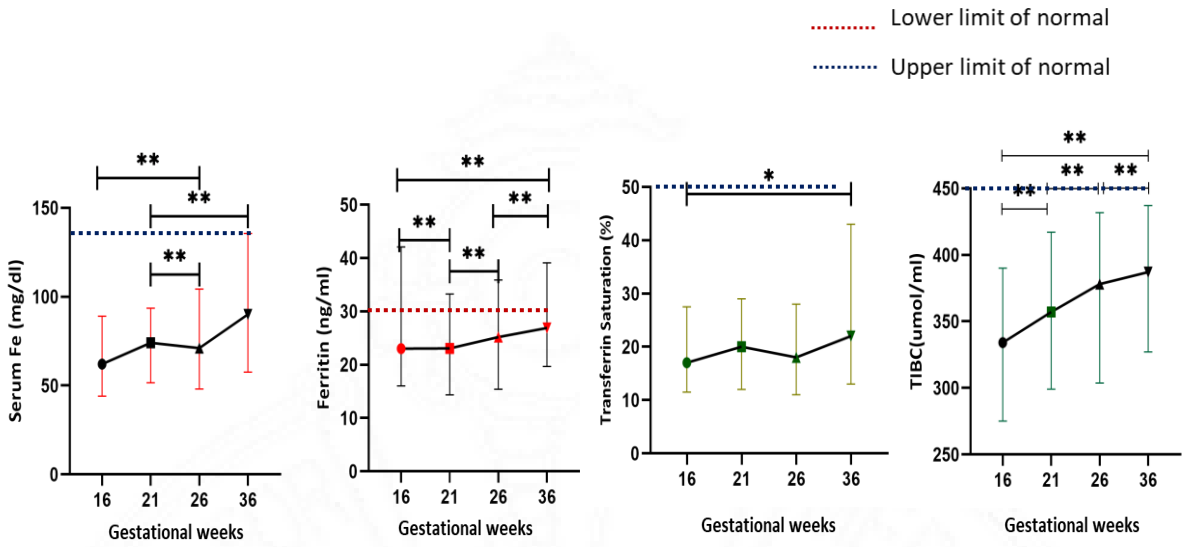


Figure 4.1.1.2.1. Kinetics of serum iron and related parameters during pregnancy

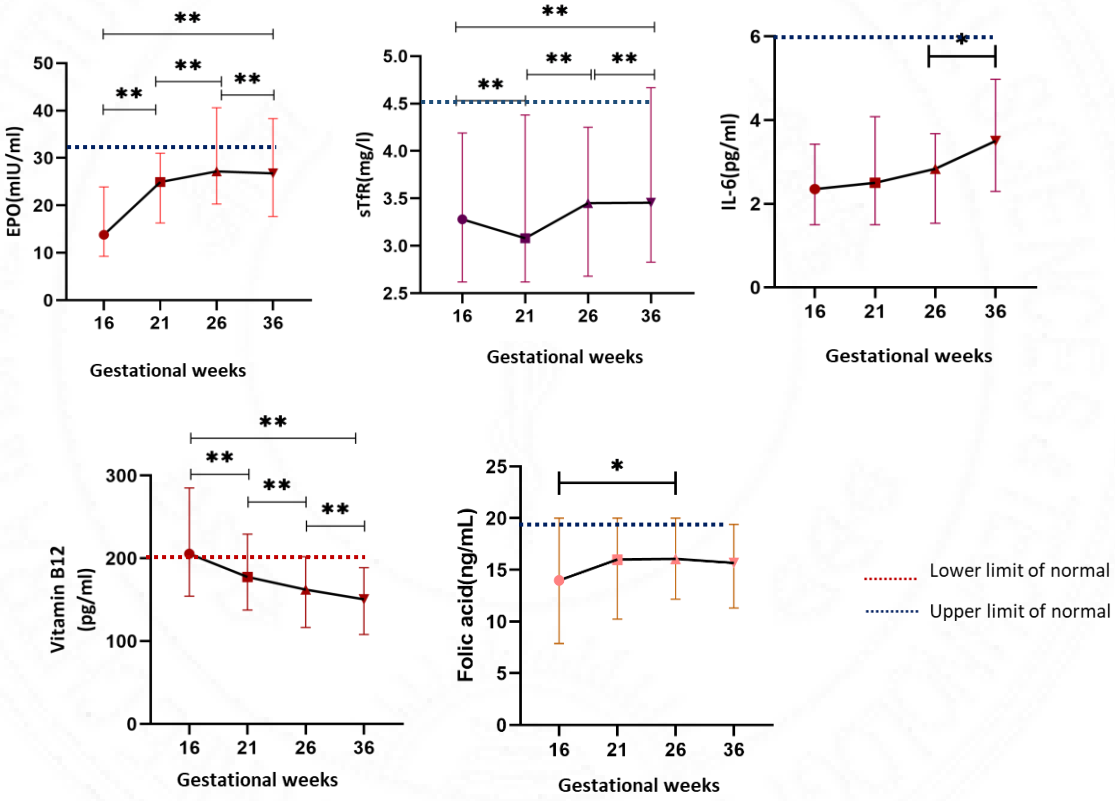


Figure 4.1.1.2.2. Kinetics of erythroid parameters during pregnancy

4.1.1.3. Association of iron and erythroid parameters

The negative correlation of sTfR with serum ferritin level was consistent throughout gestation (Figure 4.1.1.3.1). Nearly 16% of pregnant women (N=16) had iron deficient erythropoiesis (ferritin≤15ng/ml and sTfR>4.5mg/l) throughout the gestational period. sTfR had a negative association with hemoglobin at 16 and 36 gestational weeks (p=0.001; p=0.053).

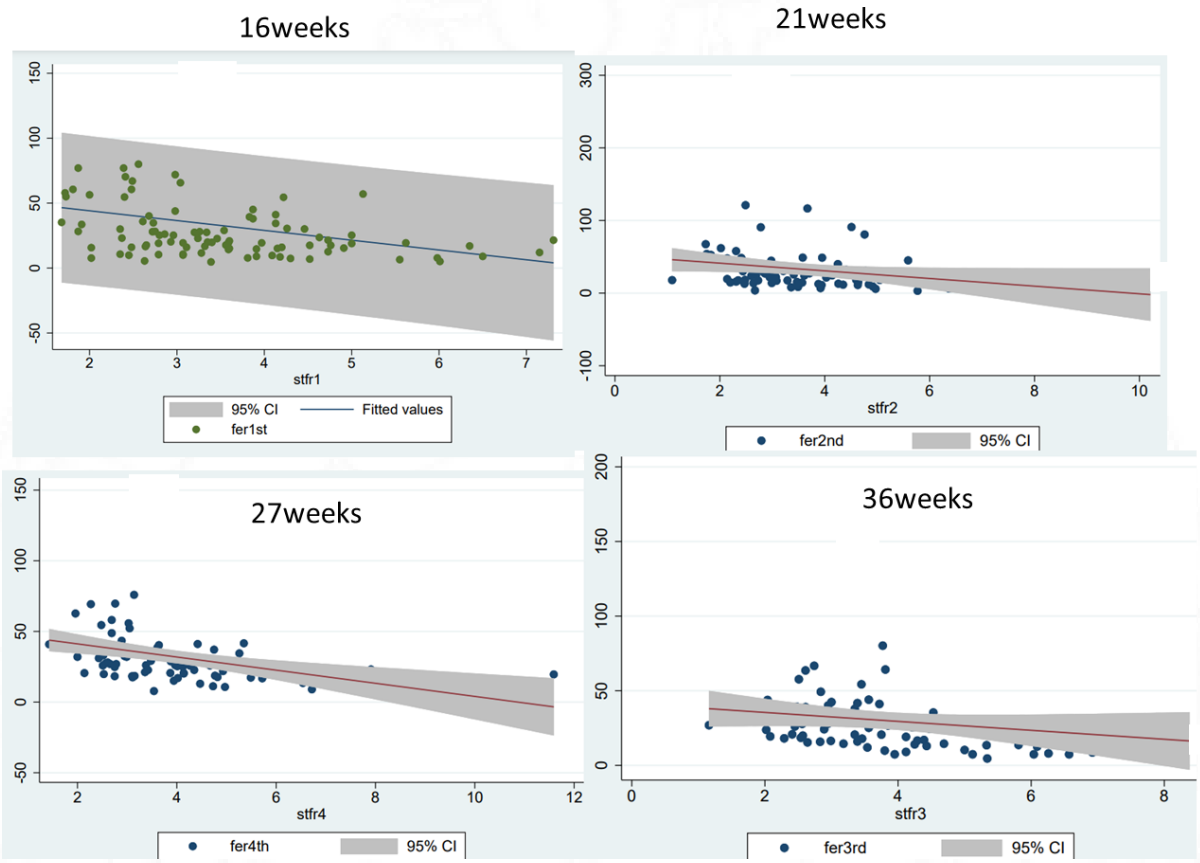


Figure 4.1.1.3.1. Correlation of soluble transferrin receptor and ferritin levels

Using logistic regression analysis, we found four-fold increased risk of anemia (Hb<10g/dl) in pregnant women a weeks (relative risk ratio (RRR) = 4.93, 95% CI 1.26,19.1), which could result in increased risk of low iron availability to the bone marrow (Figure 4.1.1.3.2). The findings indicate that the iron demand escalates because of an increase in the production of red blood cells.

Serum sTfR >4.5mg/L								
Anemia (Hb<10g/dl)	16w		21w		26w		36w	
	RRR (95% CI)	p-value	RRR (95% CI)	p-value	RRR (95% CI)	p-value	RRR (95% CI)	p-value
	4.93 (1.26-19.1)	<0.01	2.46 (0.38-15.7)	0.34	8.66 (0.74-18.7)	0.085	1.2 (0.21-6.61)	0.83

Figure 4.1.1.3.2 Relationship between anemia and iron biomarkers in pregnant women

In multivariate analysis, Hb levels had a negative association with EPO levels at 16 weeks, suggesting that anemia causes increased EPO secretion as a response to low hemoglobin concentration (β=-0.402, p=0.049). At 21 weeks and 26 weeks, EPO was positively associated with sTfR levels (β =0.627, p=0.005 & β =0.544, p=0.016, respectively) (Figure 4.1.1.3.2**)**. Thus, erythropoietin increases the rate of erythropoiesis in the second trimester.

Serum iron and ferritin levels were negatively associated with sTfR levels at 36 weeks (β =-1, p=0.017 & β =-0.404, p=0.052, respectively) (Figure 4.1.1.3.3**)**. This association indicates that erythropoiesis decreased in the third trimester. Elevated iron levels indicate the utilization of iron to meet fetal demand.

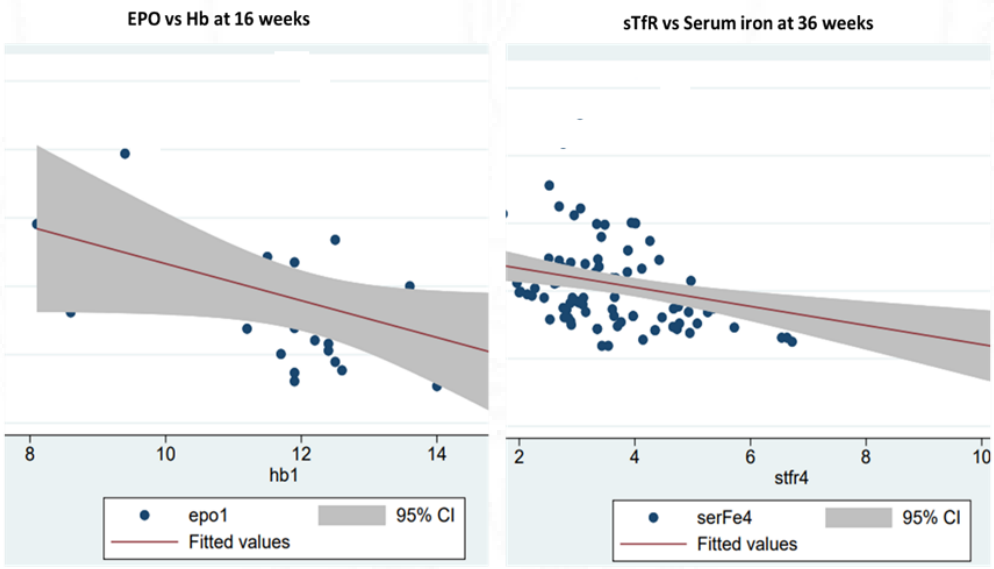


Figure 4.1.1.3.3 Relationship between iron biomarkers, erythropoietin, and hemoglobin in pregnant women

4.1.1.4. Erythrocyte gene expression during gestation

The reticulocyte mRNA expression of iron and erythroid genes such as FPN1B, IRP2, TFRC, GATA1, and KLF1 demonstrated pregnancy-associated expression (Figure 4.1.1.4.1). Non-IRE bearing form FPN1B significantly decreased at 26 weeks compared to 21 weeks. TFRC mRNA expression significantly reduced in the third trimester compared to the early gestational period (16 weeks). Decreased TFRC expression suggests reduced iron uptake towards the 3rd trimester as the rate of erythropoiesis remains constant.

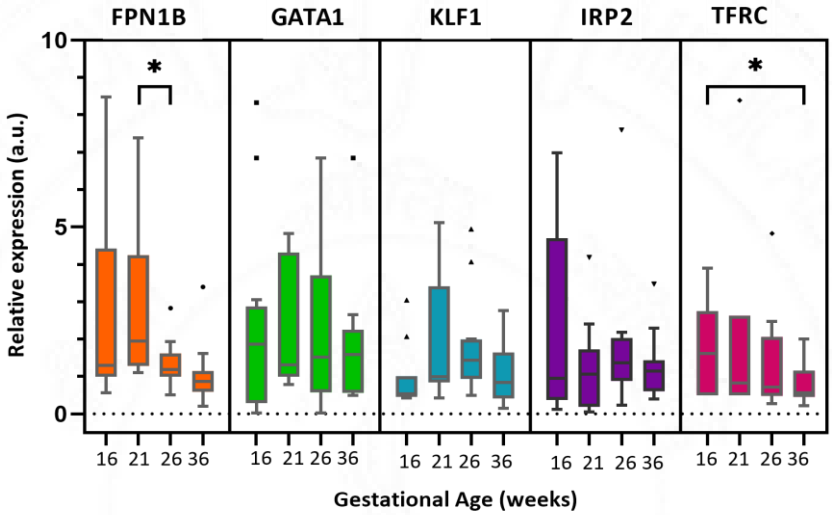


Figure 4.1.1.4.1. mRNA expression of iron metabolizing genes at 16,21,26 and 36 weeks.

4.1.2. To elucidate the influence of maternal iron status on the regulation of placental iron transport and fetal supply

4.1.2.1 Baseline characteristics of the subjects

In this prospective study, a total of 138 pregnant women attending the antenatal clinic were initially enrolled. Fourteen subjects were excluded due to the unavailability of either maternal or cord blood serum samples. All participants received iron supplements (60mg elemental Fe/day until delivery) regardless of their hemoglobin levels at the initial visit. Among the 124 pregnant women included in the analysis, 47% were primigravida, 44% were second gravida,

and 9% were multigravida. The mean gestational age at the first visit and delivery was 12.8±2.72 weeks and 39±1.65 weeks, respectively. Six percent of the pregnant subjects delivered preterm (<37 weeks of gestation), 45% were born early term (37-39 weeks), 42.8% were delivered at full term (39-42 weeks), 7% delivered late term (41-42 weeks), and one person delivered post-term (≥42 weeks). Regarding fetal gender, there were sixty-nine males and fifty-five females.

The main objective of the study was to investigate placental iron transport in pregnant women with iron deficiency anemia and compare it with healthy controls. Participants were recruited based on their hemoglobin levels upon admission to the labor ward. Groups were categorized as iron deficiency anemia (IDA) and healthy controls based on hemoglobin and ferritin levels at the time of delivery.

Based on the inclusion criteria, among 124 subjects, 28 subjects had IDA (Hb≤10.5g/dL and ferritin values ≤30ng/L), and 52 were iron replete (Hb>10.5g/dL and ferritin >30ng/L). Forty-four pregnant women who either had high ferritin(>30ng/mL) or Hb (>10.5g/dL) were excluded from the downstream analysis [Table 4.1.2.1.1]. All further analysis was based on the IDA and the iron-replete group (Controls).

Table 4.1.2.1.1. Study participants were classified into separate groups based on hemoglobin and ferritin levels

Pregnant women N=124	Iron Replete (Hb>10.5g/dL and S.Ferritin>30ng/ml) N=52	Anaemia (Hb≤10.5g/dL) N=23	Iron deficient (S.Ferritin≤30ng/ml) N=21	Iron deficiency anaemia (Hb≤10.5g/dL and S.Ferritin≤30ng/ml) N=28
Maternal				
GA (weeks)	39.1±1.25	38.8±1.34	38.7±1	39±1.2
Hb (g/dl)	12.7±1.4	9.4±0.79	12.1±1.5	9.3±0.9
Ferritin (ng/ml)	66 (31.1-207)	46.4 (31.2-261)	20 (6.6-29.2)	15.4 (0.8-28.3)
Hepcidin (ng/ml)	9.8 (0.9-92.5)	7.4 (0.9-18.7)	4.4 (0.8-15.4)	7.5 (0.9-31.7)
Fetal				
Hb (g/dl)	14.9±1.7	14±2	14.8±1.8	14.5±1.6
Ferritin (ng/ml)	143 (13.1-461)	196 (39.5-544)	122 (30.2-267.9)	139 (15.4-300)
Hepcidin (ng/ml)	12.2 (0.7-56.2)	12 (0.7-56.2)	11 (1.7-34.9)	9.1 (0.1-74.7)

In the IDA group, the maternal mean age was 24±3 years, while it was 25±3 years in the control group. At delivery, the mean gestational age in the IDA group was 273±9 days, and in the control group, it was 274±8.8 days. The mean birth weight of term neonates was 2.95±0.34 kg in the IDA group and 3±0.44 kg in the control group.

Most subjects (34 out of 50; 68%) were classified as having mild anemia. A significant decrease in hemoglobin levels at delivery was observed in the IDA group (p=0.000) compared to the levels at the first antenatal visit (Table 4.1.2.1.2).

In term neonates from the IDA group, the mean Hb concentration was 14.5±1.6 g/dl, and the mean MCV was 81.4±9.3 fL. The cord serum ferritin in the IDA group (n=28) was 139 (15.4-300) ng/ml. The demographic and laboratory parameters of the mothers and their fetuses are detailed in Table 4.1.2.1.2.

Table 4.1.2.1.2. Hematological and iron parameters in mother and cord blood

Cohort	Maternal								Fetal					
	Age (Years)	Gestational Age (days)	Hb at first visit for ANC	Hb (g/dL)	MCV (fL)	HCT (%)	Ferritin (ug/L)	Hepcidin (ug/L)	Hb (g/dL)	MCV (fL)	HCT (%)	Ferritin (ug/L)	Hepcidin (ug/L)	Mean Birth weight (kg)
IDA (N=28)	24±3	273±9	10.7±1.01	9.3± 0.9	81.4±9	30±3.5	15.4 (0.8-28.3)	6.9 (0.9-19.5)	14.5±1.6	108.7 ±8.2	47.6±6	139 (15.4-300)	9.1 (0.2-74.7)	2.95± 0.34
Control (N=52)	25±3	274±8.8	11±0.96	12.7± 1.4	93.2±6	40±5	66 (31.1-207)	7.6 (0.9-19.3)	14.9±1.7	110.8 ±8.7	46.7±6	143 (13.1-461)	11.6 (0.9-54.8)	3± 0.44
P value	0.321	0.729	0.375	0.000	0.000	0.016	0.000	0.512	0.375	0.310	0.83	0.721	0.686	0.694

4.1.2.2. Evaluation of iron status in normal and iron deficient anemia in pregnancy

The median maternal hepcidin levels were 6.9 (0.9-19.5) ng/ml for the iron deficiency anemia (IDA) group and 7.6 (0.9-19.3) ng/ml for the control group (p=0.512). Fetal hepcidin levels had a median of 9.1 (0.2-74.7) ng/ml in the IDA group and 11.6 (0.9-54.8) ng/ml in the controls (p=0.686). Elevated GDF15 levels were noted in both groups, with a median of 36040 (11910-66255) pg/ml in iron-deficient mothers and 31070 (11477-67330) pg/ml in the control group (p=0.365). Normal GDF15 levels were found in the cord blood of both IDA and controls [3840 (1880-7187) pg/ml and 3957 (2435-8542) pg/ml, respectively (p=0.396)] (Figure 4.1.2.2.1).

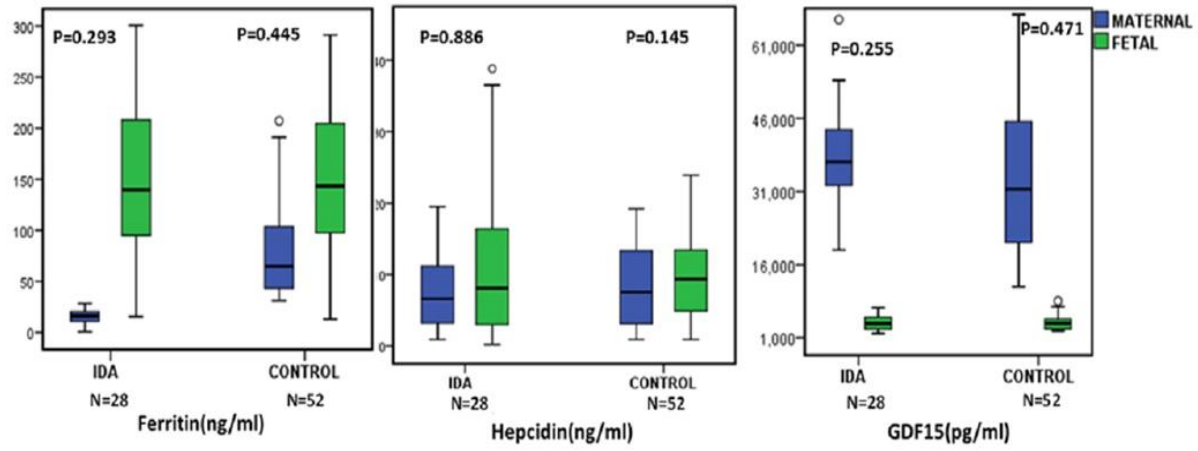


Figure 4.1.2.2.1: Ferritin, hepcidin, and GDF15 levels in maternal and cord blood serum

4.1.2.3. Association of maternal and fetal iron status indicators

In IDA, fetal cord blood Hb and ferritin levels were markedly higher than maternal Hb and serum ferritin levels (p=0.000; p=0.000) (Table 4.1.2.1.2.). A positive correlation was noted between serum ferritin and maternal Hb levels in IDA (r=0.421, p=0.026). Both hepcidin and ferritin levels were independent of each other in maternal and cord blood.

Through univariate regression analysis, we identified that fetal hemoglobin was linked to fetal ferritin levels (β=-0.360; p<0.05). The maternal hepcidin: ferritin ratio was significantly higher in IDA than in controls (p=0.000). However, there was no discernible association between

maternal and cord blood hepcidin: ferritin ratios. Conversely, in the control group, increased maternal hepcidin was correlated with increased fetal hepcidin and fetal hepcidin: ferritin ratios, respectively (r=0.442, p=0.001; r=0.379, p=0.006). Although the association between fetal ferritin and fetal hepcidin showed a trend toward significance (r=0.273, p=0.052), logarithmic fetal hepcidin was related to maternal ferritin (β=0.385; p=0.047). Fetal GDF15 demonstrated an association with the fetal hepcidin-ferritin ratio (β=0.476; p=0.014). Multigravida pregnant women exhibited significantly lower maternal hepcidin levels than primigravida (p=0.014).

In both groups, GDF15 levels were significantly higher in maternal serum than in cord blood levels (p=0.000). GDF15 did not influence hepcidin and ferritin levels in both mothers and fetus. Intriguingly, maternal GDF15 demonstrated a negative association with the fetal hepcidin: ferritin ratio in cases of IDA (r= -0.439; p=0.025).

4.1.2.4. VNTR analysis of placental tissue

To ensure that there was no maternal contamination in the placental tissues, we conducted a variable number tandem repeat (VNTR) analysis using five markers. We extracted DNA from maternal peripheral blood, cord blood, and placental tissues and performed a multiplex PCR for five short tandem repeat (STR) markers (ACTBP2, FES, THO1, VWF, and F13A1) using fluorescently labelled primers followed by capillary electrophoresis. Our analysis confirmed that all collected placental tissue samples had fetal origin, as shown in Figure 4.1.2.4.1.

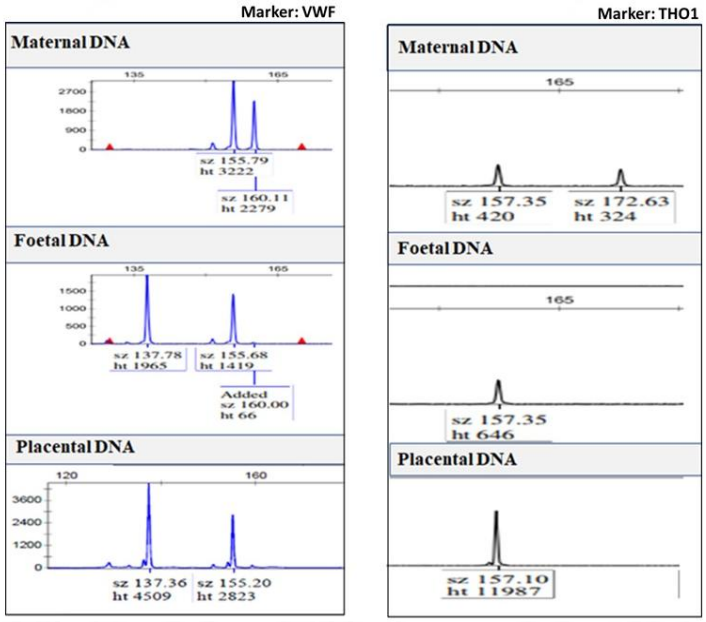


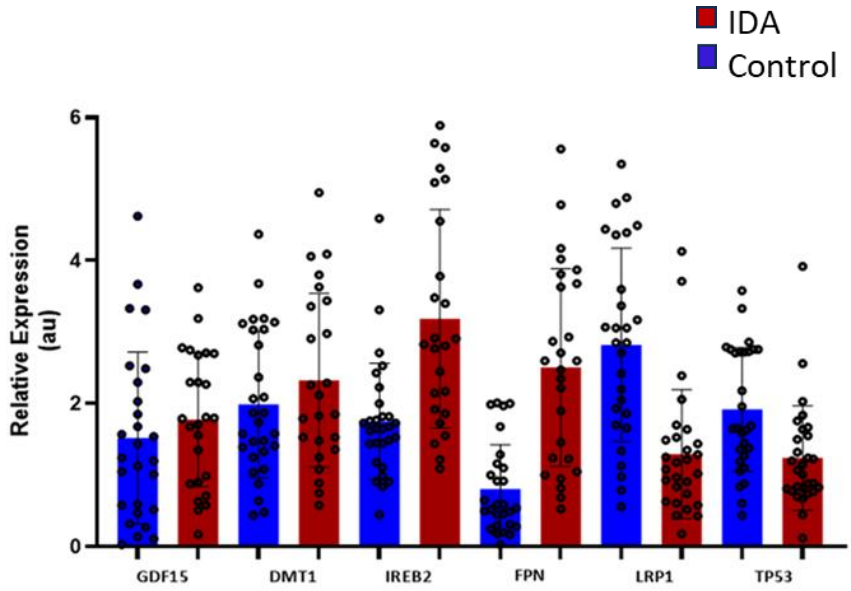
Figure 4.1.2.4.1. Pattern of short tandem repeat (STR) markers- von Willebrand Factor (vWF) and Tyrosine hydroxylase intron 1 (THO1) indicating the fetal origin of placental tissue as compared to maternal and cord blood DNA. Two representative DNA samples of normal pregnant women are shown.

4.1.2.5. Assessment of Placental iron transporters and regulators

The mRNA and protein expression of iron metabolizing genes was analyzed in placental maternal and fetus. Among the six differentially expressed genes, iron transporters (DMT1, FPN1), cellular iron regulator IREB2, the recognized hepcidin suppressor GDF15, and its transcription factor SP1 were upregulated in cases of iron deficiency anemia (IDA) (Figure 4.1.2.5.1).

Tumor suppressor gene TP53, essential for maintaining the intracellular iron pool, and the heme scavenger LRP1 were found to be downregulated in IDA compared to controls. No significant correlation was observed between the mRNA expressions of the mentioned iron transporters and their respective downstream protein expressions.

a)



b)

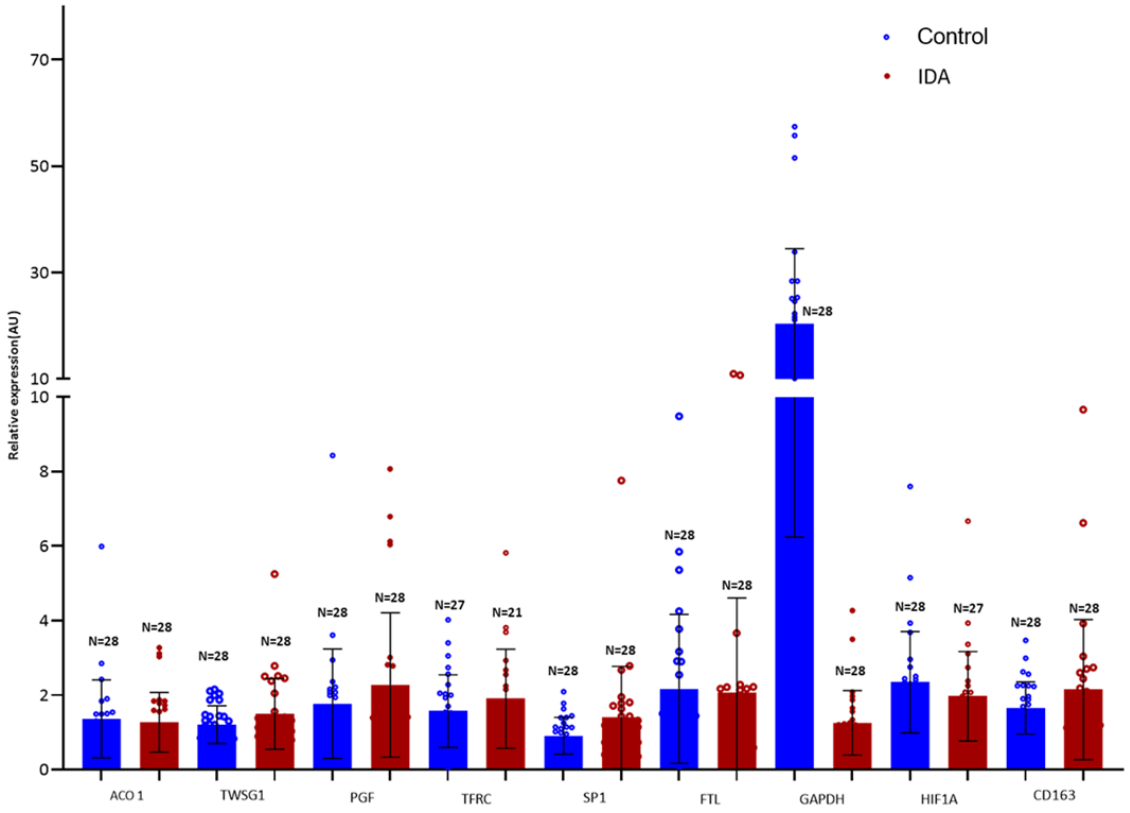


Figure 4.1.2.5.1. Placental mRNA expression of iron traffickers in IDA and control. a) mRNA expression of six differentially expressed genes. b) mRNA expression of genes that are not differentially expressed.

In cases of iron deficiency anemia (IDA), there was no significant difference in placental TFRC at the mRNA level. However, under low iron conditions (IDA), an increase in DMT1 mRNA

expression was observed. A positive correlation was identified between IREB2 mRNA expression and maternal serum ferritin (r=0.519, p=0.027). Elevated cellular FPN1 mRNA levels were associated with increased expression of IREB2 mRNA (r=0.635, p=0.005).

At the protein level, GDF15, DMT1, TFRC, and FPN1 were found abundantly in the placenta (Figure 4.1.2.5.2). FPN1 protein displayed a trend toward a significant association with maternal Hb levels (r=0.583; p=0.060). DMT1, FPN1, and GDF15 proteins were significantly increased in IDA (p=0.019, p=0.051, p=0.033), respectively. Positive associations were evident between placental GDF15, DMT1, and TFRC proteins.

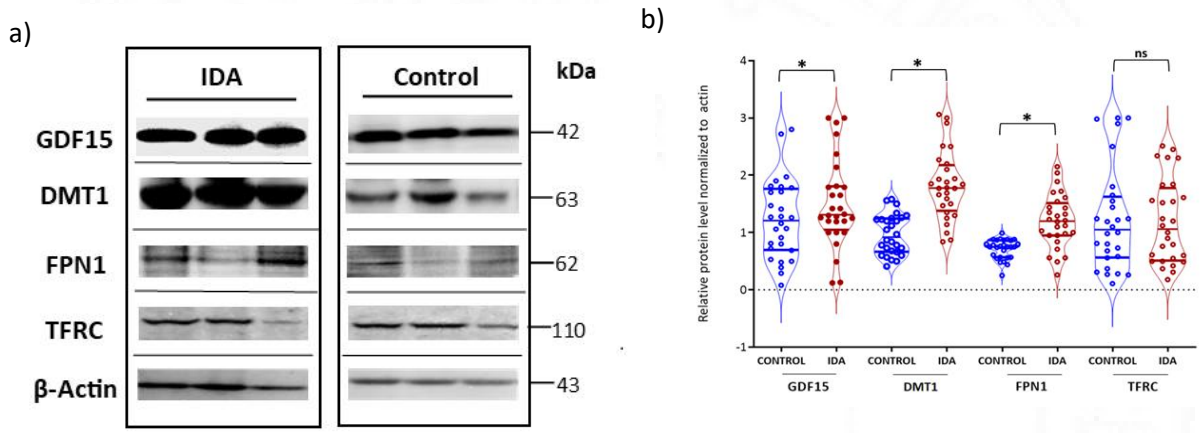


Figure 4.1.2.5.2. a & b) Changes in placental iron transport expression resulting from maternal iron deficiency anemia: western blot demonstration of placental protein expression of iron traffickers in IDAP and control group.

No correlation was observed between maternal iron status indicators and placental iron transporters at the mRNA level (Table 4.1.2.5.3). Furthermore, no significant associations were found between DMT1 protein expression and maternal and fetal iron status indicators (Table 4.1.2.5.3).

	Mother				Cordblood			
Placental Fe transporters	Hb	Ferritin	Hepcidin	GDF15	Hb	Ferritin	Hepcidin	GDF15
mRNA DMT1	0.722	0.091	0.541	0.893	0.820	0.775	0.414	0.854
mRNA TFRC	0.912	0.101	0.486	0.958	0.766	0.852	0.151	0.405
mRNA FPN	0.698	0.250	0.772	0.723	0.645	0.468	0.937	0.854
mRNA GDF15	0.977	0.210	0.747	0.958	0.763	0.480	0.268	0.778
mRNA IREB2	0.797	0.027	0.580	0.657	0.868	0.801	0.918	0.499
Protein DMT1	0.679	0.734	0.158	0.986	0.541	0.587	0.217	0.853
ProteinTFRC	0.609	0.824	0.703	0.761	0.602	0.725	0.736	0.642
Protein FPN	0.060	0.450	0.958	0.417	0.769	0.048	0.626	0.244
Protein GDF15	0.479	0.155	0.252	0.589	0.687	0.017	0.193	0.128

Table 4.1.2.5.3. Correlation matrix of mRNA expressions and protein abundance of placental Fe transporters with maternal and cord blood iron markers. Significantly correlated values are highlighted in blue.

On the contrary, heme uptake facilitated by the placental heme receptor LRP1 exhibited differential expression in IDA compared to controls (p=0.004). The mRNA expression of LRP1 was significantly influenced by maternal ferritin levels (r=0.470; p=0.049), suggesting that placental heme utilization supports fetal iron demands. However, the heme scavenger CD163 and the exporter FLVCR1 did not show differential expressions.

Several studies have highlighted the role of TP53 in maintaining iron homeostasis, indicating its influence on hepcidin and ferritin levels. Zhang et al. observed elevated serum iron levels in iron-overloaded mice with decreased TP53 levels (Zhang and Chen, 2019). Our study revealed a significant elevation in placental TP53 mRNA expression in the iron-deficient group. Interestingly, placental TP53 mRNA expression positively correlated with placental GDF15 mRNA and maternal hepcidin concentration (r=0.642, p=0.004; r=0.492, p=0.038), suggesting a potential relationship that requires further investigation.

In IDA, gestational age (39±1.2 weeks) had a positive impact on placental iron traffickers, such as TFRC and LRP1 (r=0.568, p=0.017; r=0.625, p=0.007). However, the expression of iron

transport molecules in the placenta did not impact neonatal birth weight. Maternal and fetal hemoglobin levels were not associated with the expression of placental iron transporters.

In the control group, a significant finding revealed a negative correlation between placental GDF15 mRNA and fetal hemoglobin (Hb) (r=-0.446, p=0.022).

4.1.2.6. Transport of Iron to Fetus by Placental Iron Transporters

Fetal ferritin was associated with the protein abundance of GDF15 (β=0.516; p=0.050) and ferroportin (β=0.719; p=0.019). Additionally, the fetal hepcidin-ferritin ratio was associated with placental FPN1 mRNA (β=0.532; p=0.028). These findings suggest that fetal iron status plays a regulatory role in influencing placental iron transporters to transport iron to the fetus.

4.1.2.7. Alternatively spliced transcripts in placental iron transport

We analyzed all splice variant transcripts of targeted iron transporters detected in placental tissue. We qualitatively confirmed the presence of alternative splice variants for DMT1, FPN1, TFRC, SP1, and SLC46A1. Differentially expressed isoforms of DMT1 and FPN1 were observed in IDA. FPN1 mRNA isoforms had increased expression in the iron-deficient cohort. Specifically, FPN1 with 5'-Iron Regulatory Element (IRE) [FPN1A] expression was elevated in IDA compared to FPN1B. The DMT1A mRNA isoform containing IRE was stabilized under iron-deficient conditions in IDA (p=0.05) (Figure 4.1.2.6.1.). DMT1A exhibited a positive association with IREB2 (r=0.512, p=0.018) and FPN1A (r=0.625, p=0.006). SP1, responsible for the transcriptional response to iron deprivation, showed a significant association with increased expression of both FPN1A (r=0.478; p=0.003) and FPN1B (r=0.625; p=0.006).

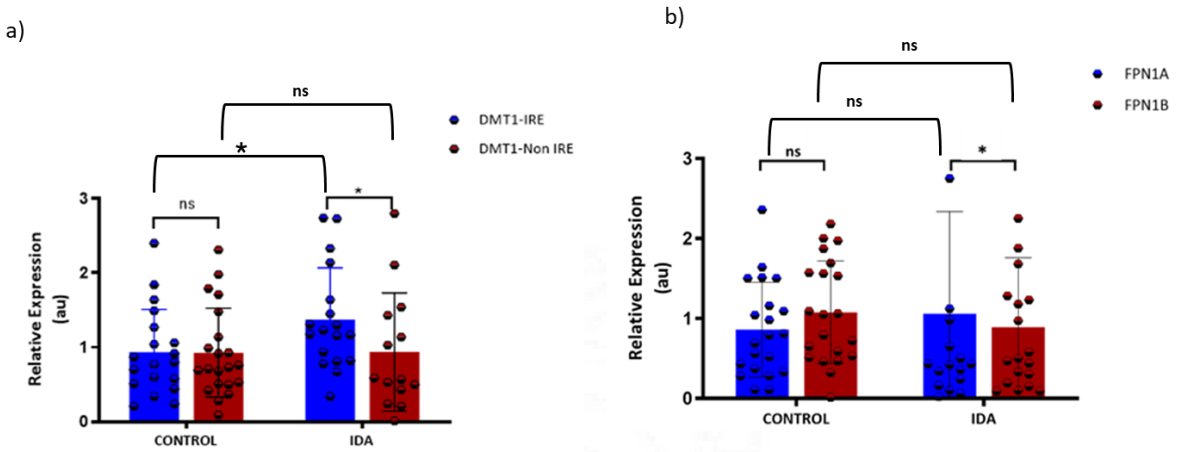


Figure 4.1.2.71. mRNA expression of a) DMT1 and b) FPN1 isoforms in IDA and control

4.1.3. To evaluate the role of iron and various regulators of erythropoiesis in pathological states of erythropoiesis - Polycythemia Vera, β-thalassemia

4.1.3.1. Iron regulators in stress erythropoiesis- β-thalassemia

In the β thalassemia group, we included 20 individuals diagnosed with thalassemia major (TDT), all of whom had received less than 50 transfusions and 20 subjects with thalassemia intermedia (NTDT). The healthy control group comprised of 50 male voluntary blood donors. The hematological and biochemical parameters are tabulated in Table 4.1.3.1.1.

Table 4.1.3.1.1. Hematological and biochemical parameters of the study

Cohort	Age (years)	Hb (g/dL)	HCT (%)	MCV (fL)	Ferritin (ng/mL)	Hepcidin (ng/mL)	H:F ratio	sTfR (mg/L)
β-thal Major N=22	4 (1-10)	9.4±1.8*	28.3±5.1	80±8	1800 (105-3150)	76.6 (42.9-80)	0.14 (0.01-7.7)	13 (2.78-37)
β-thal Intermedia N=26	8 (4-17)	8.6±2.8	26.7±8.6	66.8±9	612 (130-3284)	14.8 (4.7-23.2)	0.04 (0.01-2.36)	26.8 (9.19-45.8)
Control N=50	29 (19-52)	14.7±1.1	44.6±3.4	89±6.4	63.45 (46.7-574)	14.8 (4.6-36.3)	0.21 (0.01-0.43)	3.2 (1.7-4.65)

*Transfused patients

Serum ferritin levels were found to be significantly higher in patients with TDT ($p<0.001$) and NTDT ($p<0.001$) compared to the control group. Serum hepcidin levels were

significantly increased in TDT compared to controls (p=0.014). Increased soluble transferrin receptor levels in TDT (p<0.001) and NTDT (p<0.001) were evidence of increased erythropoietic activity (Figure 4.1.3.1.2).

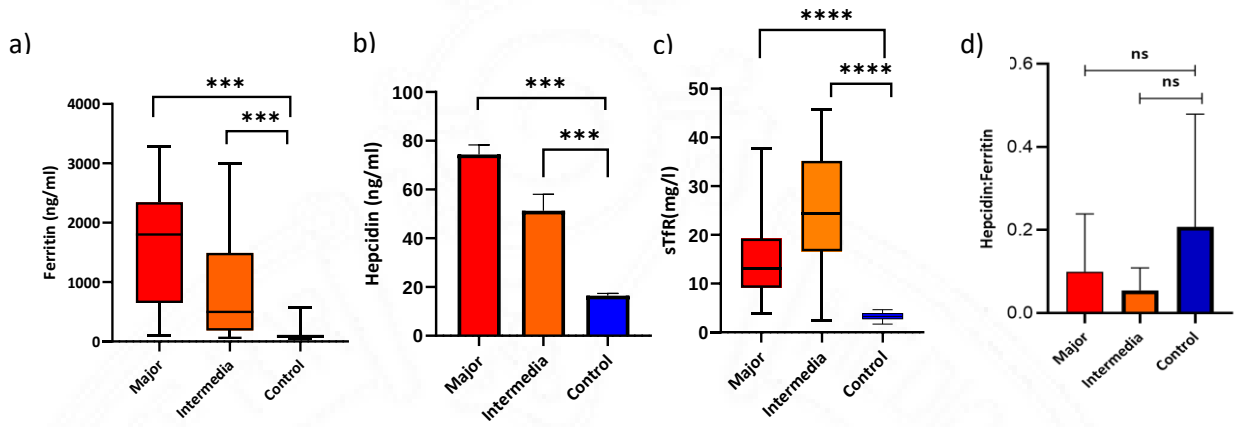


Figure 4.1.3.1.2. Serum ferritin, hepcidin, and soluble transferrin receptor level

4.1.3.1.2. Analysis of reticulocyte iron and erythroid regulators in β thalassemia

Iron and erythroid genes were assessed in reticulocytes of BT and the control group. Non-IRE FPN1B, TFRC, GATA1, and KLF1 mRNA were expressed in reticulocytes of TDT and NTDT (Figure 4.1.3.1.2.1). TFRC mRNA expression was significantly decreased in TDT (p=0.045) and NTDT (p=0.038) in comparison to controls. KLF1, involved in erythroid maturation, had significantly increased expression in TDT (p=0.001) and NTDT (p=0.001), but was not expressed in the control group.

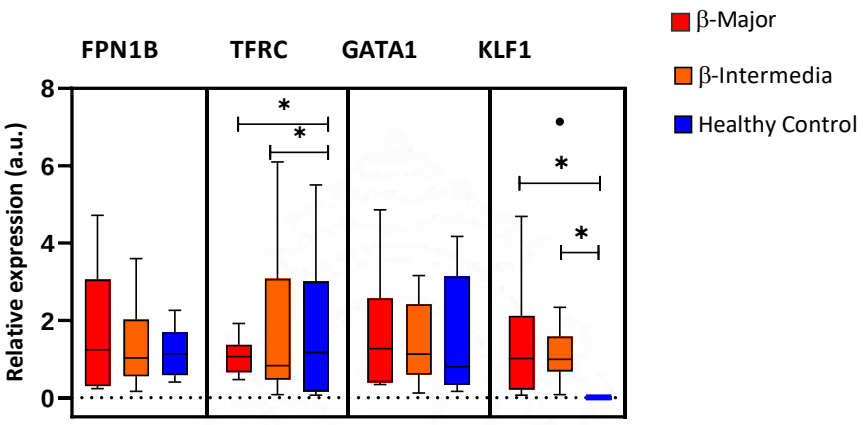


Figure 4.1.3.1.2.1. Iron and erythroid gene expression in reticulocytes

4.1.3.2. Iron regulators in stress erythropoiesis- Polycythemia vera

Twenty-five polycythemia vera patients with JAK2V617F mutation and without hydroxyurea treatment were investigated. Iron and erythroid parameters were compared between PV patients and 50 healthy controls. Patients with PV exhibited elevated hematocrit, hemoglobin levels, and ferritin levels within the mid-to-normal range (Figure 4.1.3.2.2). Sixteen percent of PV patients (N=4) had iron deficiency (Ferritin<15ng/ml). Demographic and laboratory parameters of PV and HC were tabulated in Table 4.1.3.2.1.

Table 4.1.3.2.1. Demographic and laboratory parameters of PV

Cohort	Age (years)	Hb (g/dL)	HCT (%)	MCV (fL)	Ferritin (ng/mL)	Hepcidin (ng/mL)	H:F ratio	sTfR (mg/L)
PV N=25	53 (29-57)	15.8±1*	50.1 ±4.3 (43-62.7)	79±8.6	36.1 (9.5-405)	8.5 (1-35)	0.41 (0.02-6.17)	16.4 (3-59.2)
Control N=50	29 (19-52)	14.7±1.1	44.6±3.4	89±6.4	79.2 (46.7-574)	14.8 (4.6-36.3)	0.21 (0.01-0.43)	3.2 (1.7-4.65)

Serum hepcidin concentration was significantly decreased in PV (p=0.006) (Figure 4.1.3.2.2). sTfR levels were significantly elevated in PV, indicating an increased rate of erythropoiesis. The ratio of hepcidin to ferritin was significantly increased in PV (p=0.001). There was no indication of inflammation in patients with PV, as reflected by the normal IL-6 levels. (Figure

4.1.3.2.2). Low serum ferritin levels and normal IL-6 levels observed in PV patients suggest that inflammation is not a major contributor to the dysregulated iron metabolism.

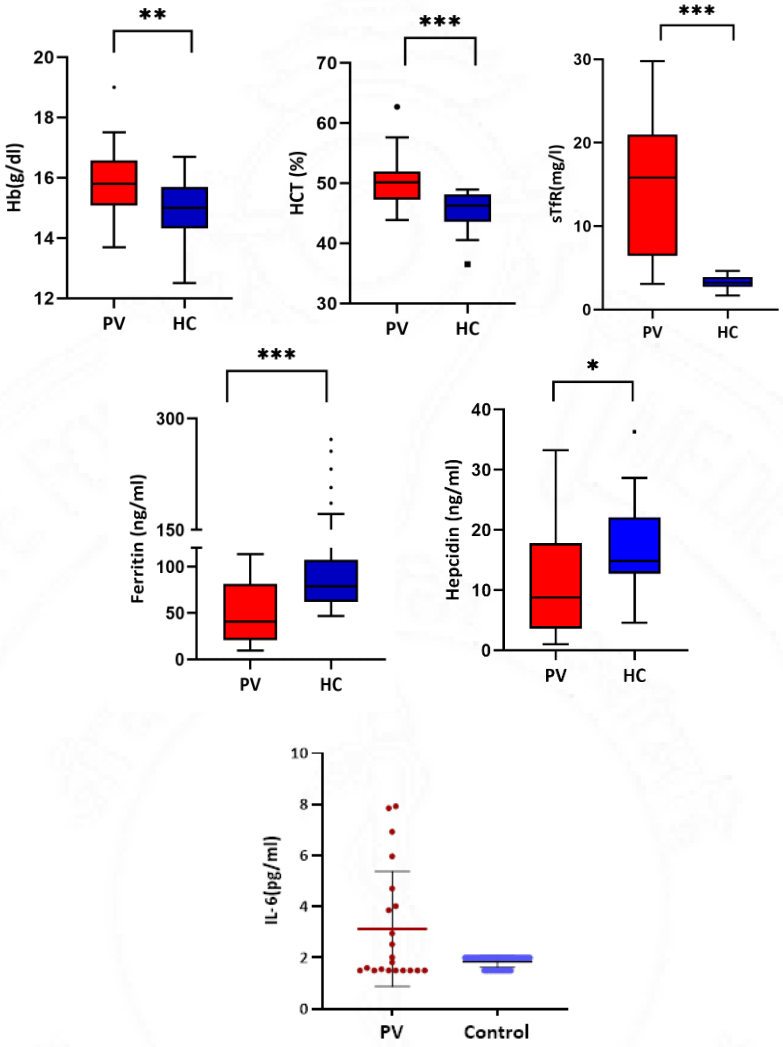


Figure 4.1.3.2.2. Iron and erythroid parameters in PV patients and Healthy controls

4.1.3.2.1. Association of iron status indicators in PV and controls

We observed a negative correlation between soluble transferrin receptor and ferritin levels in PV patients (R^2=-0.553, p=0.003), which was not observed in the control group (R^2=-0.224, p=0.114) (Figure 4.1.3.2.1.1).

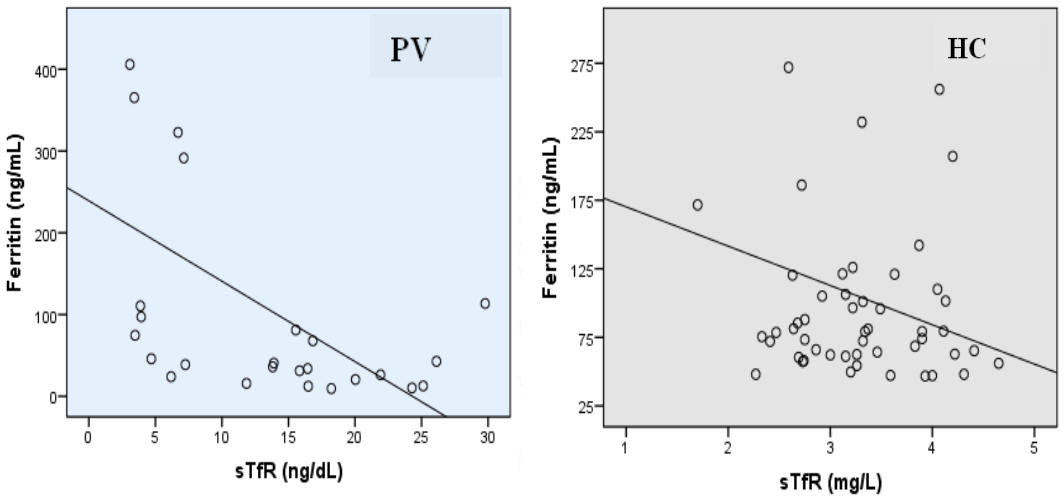


Figure 4.1.3.2.1.1. Association of serum soluble transferrin receptor and ferritin levels in PV and healthy control

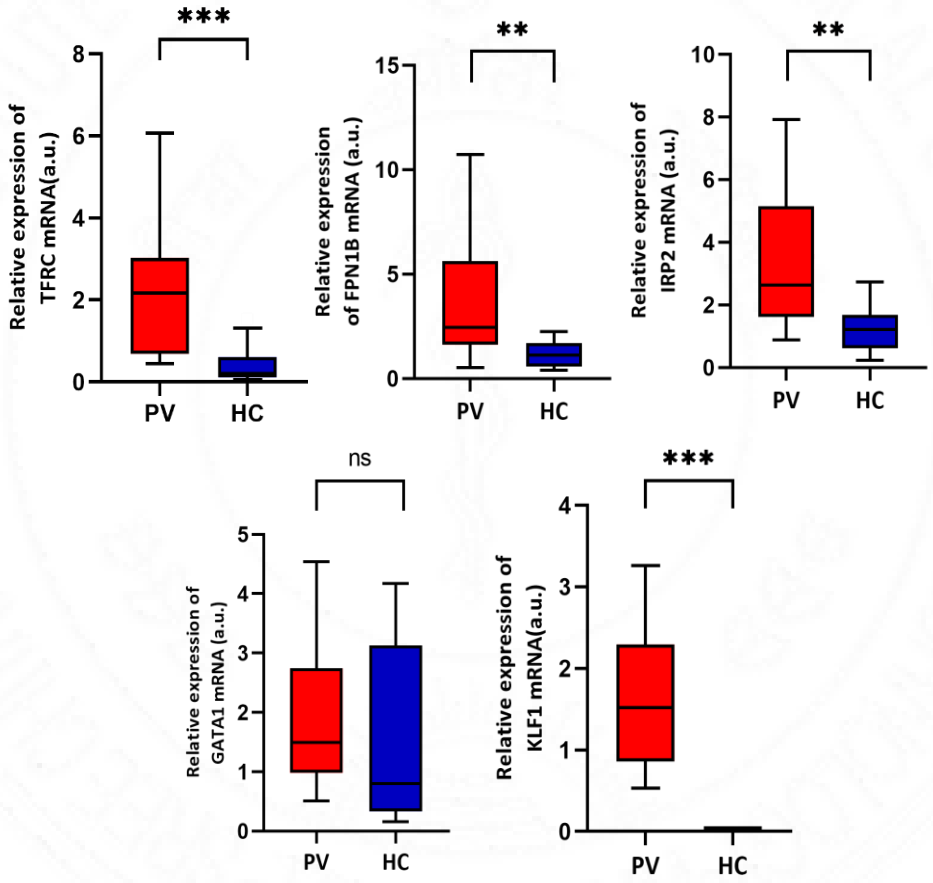


Figure 4.1.3.2.2.1. Iron and erythroid gene expression in reticulocytes of PV and controls

4.1.3.2.2. Iron and erythroid genes expression in PV

We evaluated the expression of iron and erythroid genes in reticulocytes from both the PV and control groups. Among iron metabolizing genes, TFRC, non-IRE FPN1b, and IRP2 mRNA were differentially expressed in PV compared to the controls. In the late erythroid stage, the expression of the erythroid transcriptional factor GATA1 mRNA was detected in both the PV and control groups. Notably, KLF1 mRNA exhibited differential expression in PV, whereas it was not expressed in the control group (Figure 4.1.3.2.2.1).

4.2. Study the differentiation of haematopoietic stem cells into the erythroid lineage and their characteristics.

4.2.1. Characterization of erythroid differentiation from HSCs

To induce the differentiation of erythroid cells, the hematopoietic stem and progenitor cells (HSPCs) harvested from healthy stem cell donors (HD) were expanded in serum-free media for 7 days. After this, it was cultured in the erythroid culture system (Figure 4.2.1.1a). During the expansion phase, HSPCs underwent various stages of erythropoiesis, producing cells at various stages, including pro-, basophilic, polychromatic, and orthochromatic erythroblasts, as well as enucleated reticulocytes. This process was marked by distinctive alterations in cell morphology, as shown in Figure 4.2.1.1b. Flow cytometric analysis performed on days 14 and 17 exhibited a decrease in the expression of $CD49d^+$ (α-integrin) and $CD71^+$ (TFRC) erythroid markers during the mature stage of erythropoiesis. The cells showed a 2-fold increase in proliferation on day 13, which gradually decreased as they progressed towards later erythroid differentiation stages, as depicted in Figure 4.2.1.1d. Furthermore, the pellet of differentiated cells, collected on days 7, 12, and 16, demonstrated a gradual transition towards a red coloration, indicating the production of hemoglobin.

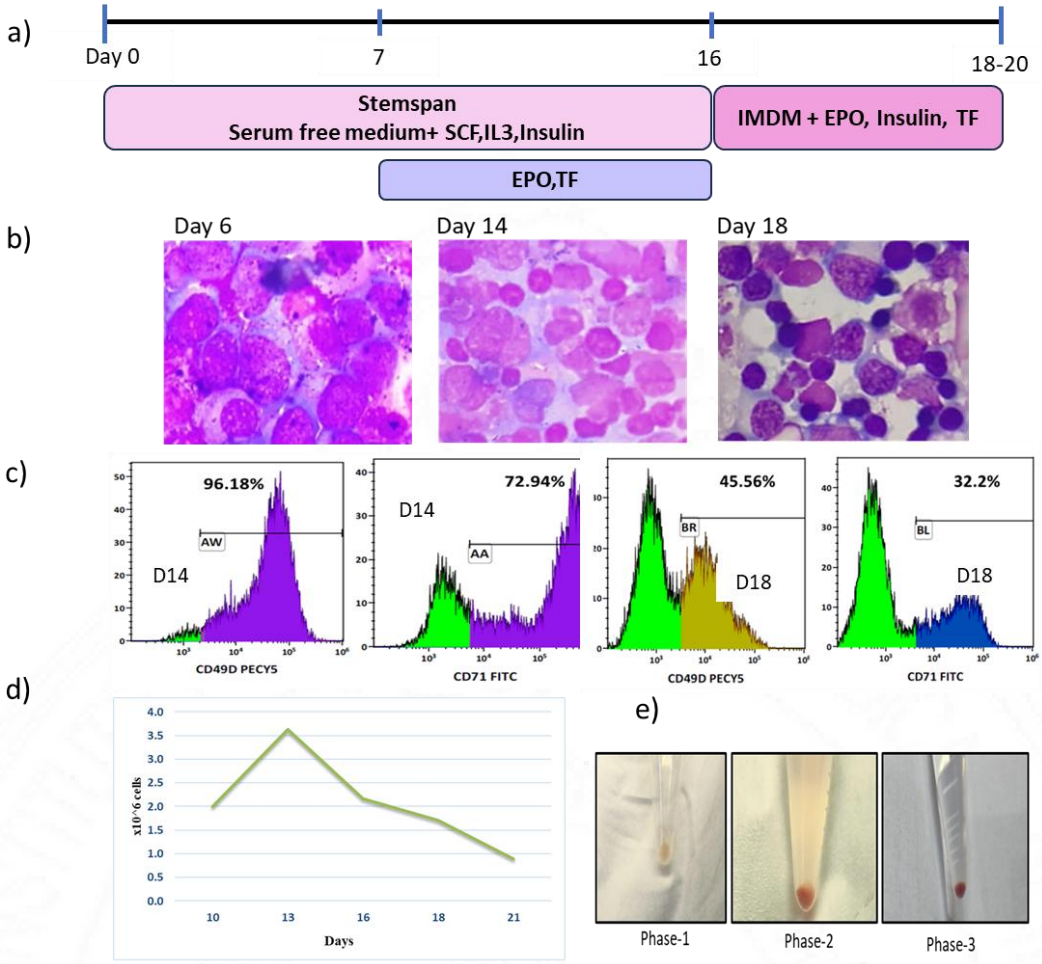


Figure 4.2.1.1. Schematic representation of erythroid differentiation from HSPCs (N=3)

4.2.2. Effect of iron overload and chelation on erythroid differentiation:

To investigate the effects of iron deficiency and overload, as well as various proteins involved in iron uptake and export, we conducted a series of experimental analyses using hematopoietic progenitor cells under various conditions.

Here, we induced the differentiation of $CD34^+$ cells toward the erythroid lineage using either 50µM ferric ammonium citrate (FAC) or 50µM iron chelator deferoxamine (DFO) during phases 2 and 3. In the presence of iron overload, there was no significant difference in $CD71^+$ expression in erythroid cells on days 12 and 18 compared to the control. Moreover, $CD235^+$ expression was not inhibited on day 18. Under iron deficiency, $CD71^+$ cells did not exhibit a

reduction compared to the control. However, there was a significant suppression of $CD235^+$ cells in mature erythroblasts on day 18 (Figure 4.2.1.2a). Under iron deficiency, the pellets containing mature erythroid cells from phase 3 differentiation showed a decreased intensity of red color, while there was no change in the color of pellets from cells treated with iron overload condition (Figure 4.2.1.2b).

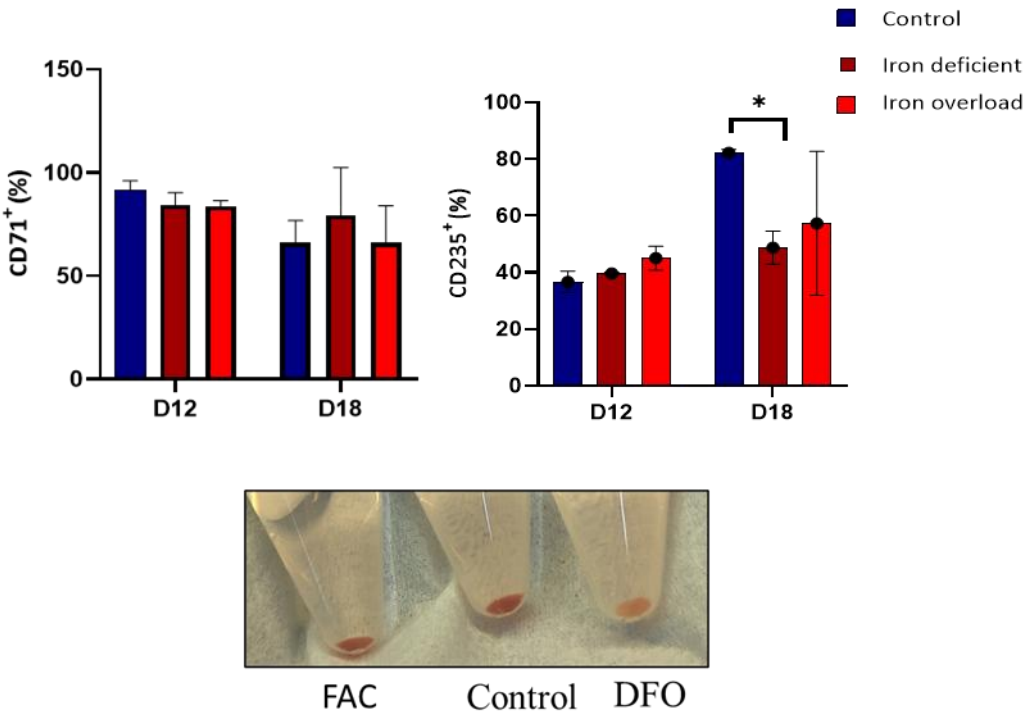


Figure 4.2.1.2. Expression of $CD71^+/235^+$ during erythroid differentiation (N=3 in each group)

4.2.3. Iron regulatory gene expression at each erythroid differentiation stage

Iron metabolizing genes such as TFRC, DMT1, IRP2, and FPN mRNA expression were assessed in erythroid cells at various stages of the erythroid lineage. In phase 2 (Days 8-12), FPN and DMT1 mRNA expression gradually increased, while TFRC expression decreased gradually. In the late erythroid stage, FPN and DMT1 mRNA expression were significantly reduced as compared to phase 2 (*p=0.025 & *p=0.043, respectively) (Figure 4.2.3.1).

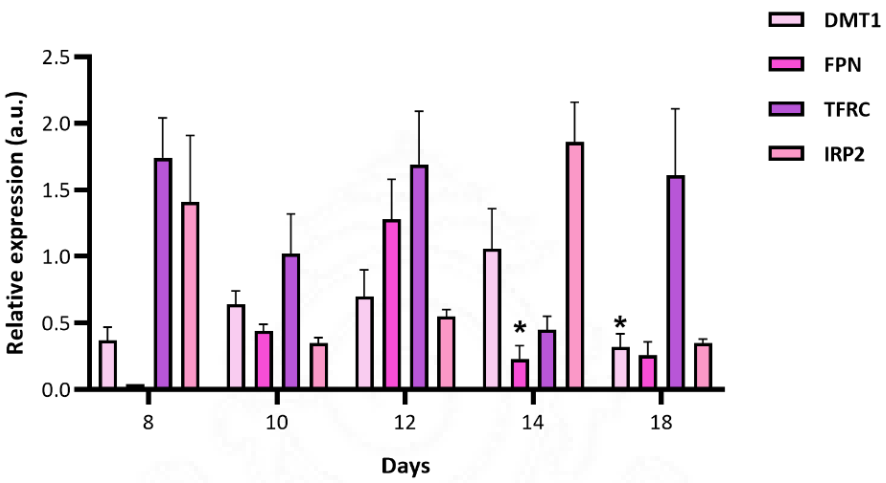


Figure 4.2.3.1. Iron regulatory gene expression at each erythroid differentiation stage (N=3)

Under iron deficiency and overload conditions, iron metabolising genes including TFRC, FPN, IREB2 and DMT1 mRNA expression were analysed. FPN mRNA expression was significantly decreased at phase 3 in iron-deficient cells compared to control cells (*p=0.025) (Figure 4.2.3.2)

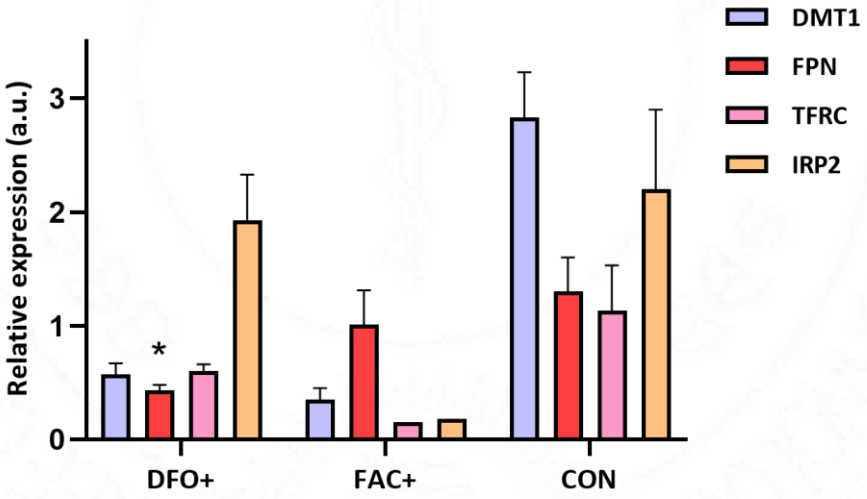


Figure 4.2.3.2. Iron metabolising genes expression in mature erythroid cells under iron+/- treatment (N=3 in each group)

4.2.4. Characterization of erythroid differentiation from PV-HSCs

To determine the iron regulation in pathological erythropoiesis, $CD34^+$ cells were isolated from 60 mL of blood retrieved from 400 mL of discarded therapeutic phlebotomy units collected from polycythemia vera (PV) patients. Like HSPCs erythroid differentiation from HD, PV $CD34^+$ cells were cultured in the erythroid culture system. During the second phase of erythroid expansion, there was a two-fold increase in the proliferation of erythroid cells as compared to HD (Figure 4.2.4.1b). Flow cytometric analysis revealed increased expression of $CD71^+$, and 50% of cells expressed $CD235^+$ in the presence of EPO. Further, iron regulatory gene expression was analyzed in phases 2 and 3, which represent immature and mature erythroid stages. We observed increased TFRC and FPN1B mRNA expression at phase 2 erythroid cells ($p=0.000$ & $p=0.005$, respectively). KLF1, a transcription erythroid factor known to regulate terminal erythroid differentiation, was highly expressed at phase 3 ($p=0.000$) (Figure 4.2.4.2).

Further, PV HSPCs were treated with DFO to examine the behavior of erythroid cells under iron deficiency. In both phases 2 and 3, we observed fewer cells in DFO treated as compared to untreated PV. Also, the expression of $CD71^+$ and $CD235^+$ were comparatively decreased (Figure 4.2.4.1d). This suggests that iron chelation reduces erythroid differentiation, like HD erythroid cells.

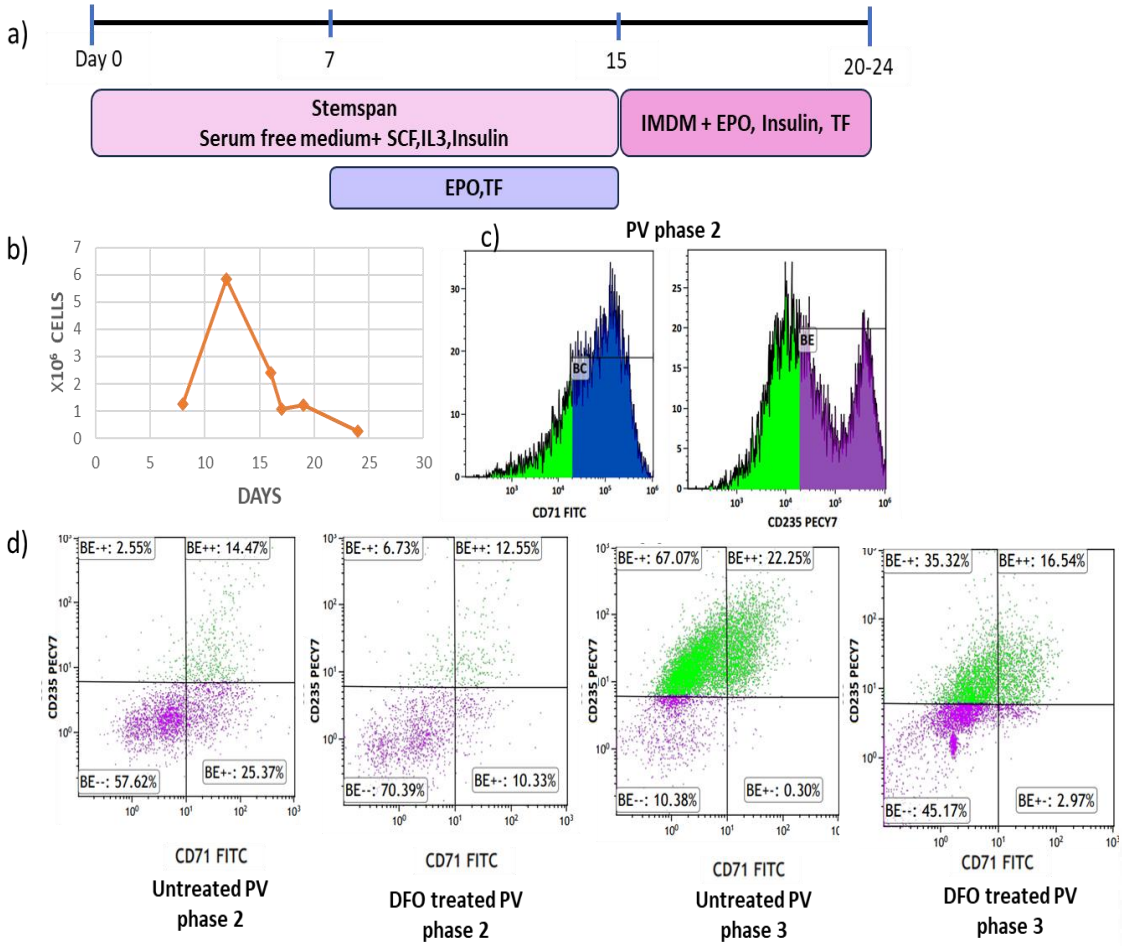


Figure 4.2.4.1. Erythroid Differentiation in Polycythemia Vera (N=3 in each phase)

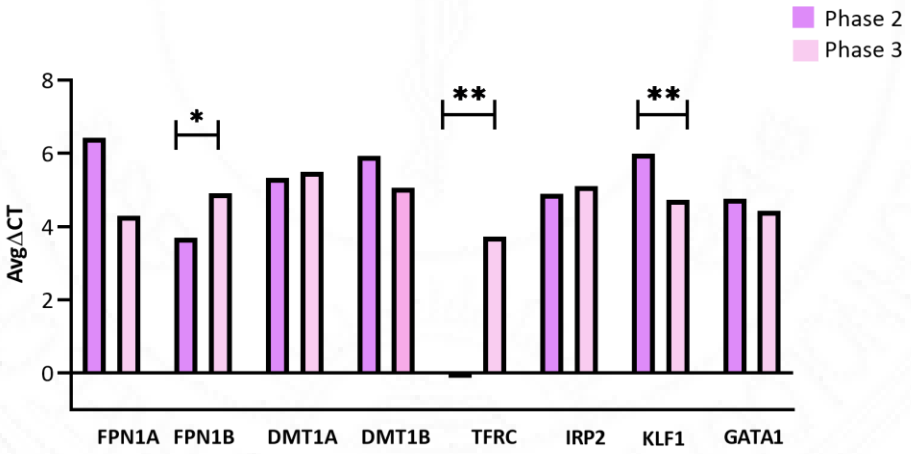


Figure 4.2.4.2. Iron and erythroid regulators expression in PV erythroid cells (N=3 in each phase)

4.3. Comprehensive analysis of iron homeostasis regulators in erythropoiesis during pregnancy using iron-replete and iron-deficient mice models

C57BL/6 female mice, aged 6 to 8 weeks, were provided either a standard diet (40 ppm iron), or a low iron diet (ID) (4 ppm iron) for four weeks before and during pregnancy. In the iron overload group, mice were fed an iron overload diet (20,000 ppm iron) for two weeks before gestation. During gestation, they were fed a diet of 10000 ppm iron since iron overload mice were not getting pregnant at 20000 ppm. Additionally, non-pregnant (NP) female mice following the standard dietary regimen were sacrificed before mating. Gene expression analysis was conducted for iron and erythroid genes in mice exposed to conditions of iron deficiency, iron overload, and control groups in the non-pregnant state and at mid-gestation (E12.5) and late gestation (E18.5) (Figure 4.3.1).

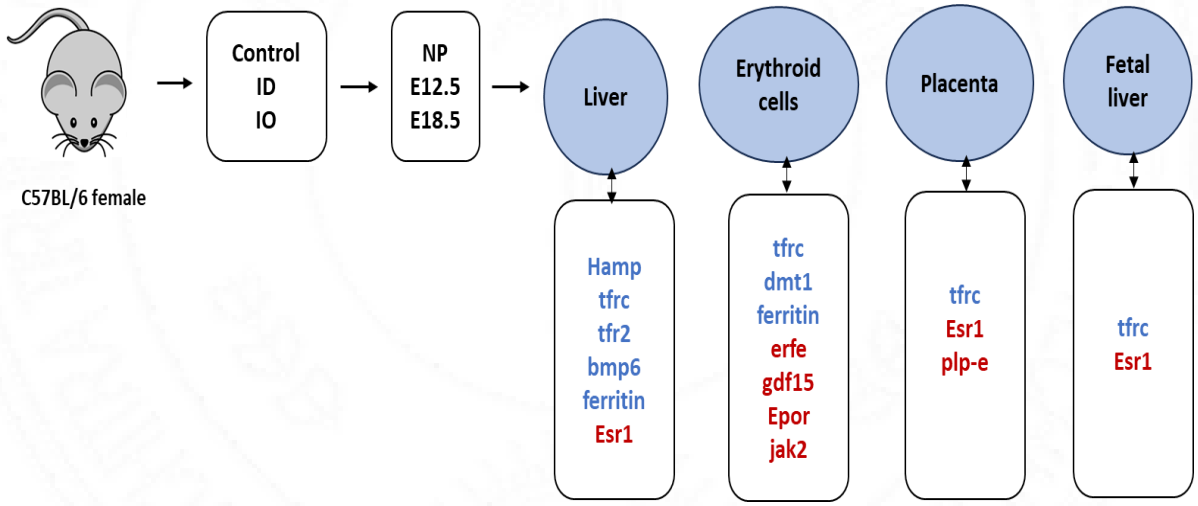


Figure 4.3.1. Schematic representation of iron (blue) and erythroid (red) genes expression analysed in different organs of mice fed with different iron diets. NP-non-pregnant mice, E12.5-embryonic day 12.5, E18.5-embryonic day 18.5, ID-Iron deficiency, IO-iron overload.

4.3.1. Iron-erythroid gene expression in hepatocytes of pregnant mice

Liver iron content significantly decreased in ID at E12.5 and E18.5 as compared to control (Figure 4.3.1.1b). Additionally, iron deposition in iron overload and control mice was assessed using Perl's staining. At 2 weeks, non-pregnant IO mice had grade II iron overload, and during pregnancy, IO mice had grade I iron overload at embryonic stages E12.5 and E18.5 (Figure 4.3.1.1a).

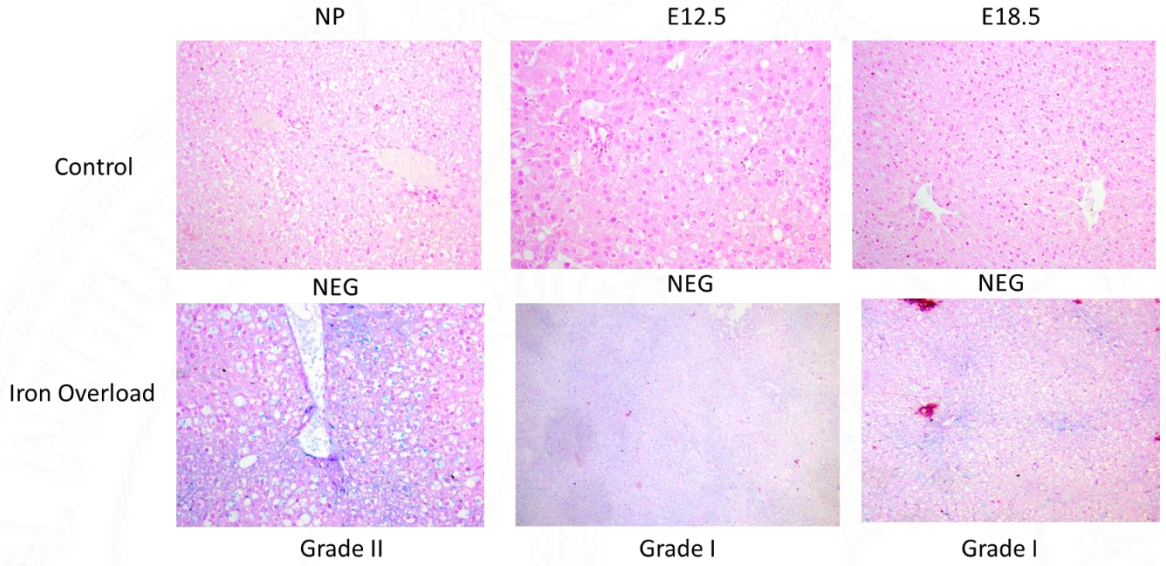


Figure 4.3.1.1a) Liver parenchyma with zonal distribution of iron granules (Perl's iron stain) 20x-control, 40x-iron overload

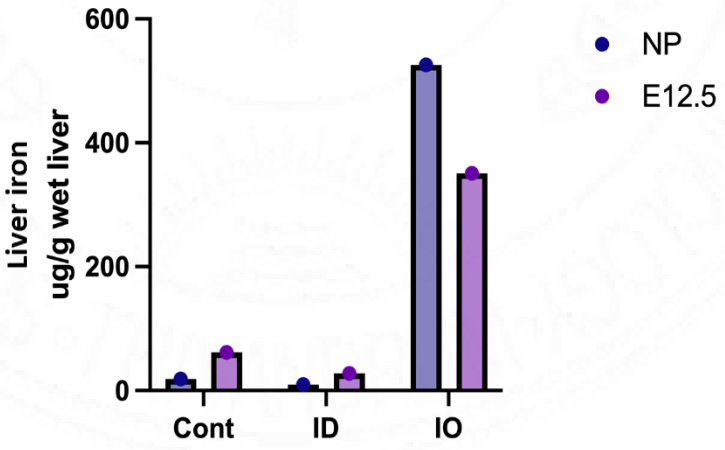


Figure 4.3.1.1b) Liver iron content measured by AAS

The mRNA expression of iron and erythroid hormone-related genes was analysed in all mice groups, and fold changes were tabulated (Table 4.3.1.1.1). Hepcidin expression undergoes consistent downregulation throughout the gestational period. As expected, hepatic Hamp mRNA was significantly reduced in ID and increased in IO at NP, E12.5, and E18.5 compared to control mice (Figure 4.3.1.2). In ID mice, tfrc had higher expression at mid-gestation. Further, we observed a significant reduction of ftl, bmp6, and tfr2 mRNA expressions at E12.5 in iron-deficient mice. Tmprss6, a known inhibitor of hepcidin under iron deficiency, was significantly increased in non-pregnant ID mice and at E18.5 in ID (Figure 4.3.1.2).

Table 4.3.1.1.1) Fold change of iron (blue) and erythroid (red) related genes in hepatocytes of control, iron-deficient and iron-overload mice.

NP: non-pregnant, black arrow indicates direction of the expression

	Control			ID			IO		
Genes	**NP**	**E12.5**	**E18.5**	**NP**	**E12.5**	**E18.5**	**NP**	**E12.5**	**E18.5**
Hamp	2.87	0.76	0.72	0.10 ↓	0.01 ↓	0.06 ↓	12.8 ↑	1.22	7.15 ↑
Tfrc	1.29	0.98	1.91	16.20 ↑	4.58 ↑	2.79	0.36 ↓	0.53	0.13 ↓
Tfr2	1.06	1.45	0.91	3.45	0.48 ↓	1.56	1.16	0.63 ↓	1.30
ftl	1.23	1.10	0.99	0.71 ↓	0.57 ↓	0.83	2.36	1.04	1.54 ↑
tmprss6	0.64	0.63	0.79	19.48 ↑	0.82	1.63 ↑	3.96 ↑	1.01	1.33 ↑
Bmp6	0.92	0.74	0.80	0.36 ↓	0.26 ↓	0.41	5.41 ↑	3.13 ↑	4.37 ↑
Eα	0.96	0.83	1.02	7.28 ↑	0.78	1.04	2.80	1.24	1.29
esr1	0.97	1.02	0.77	9.62 ↑	1.75	2.29 ↑	1.93	0.67	0.65

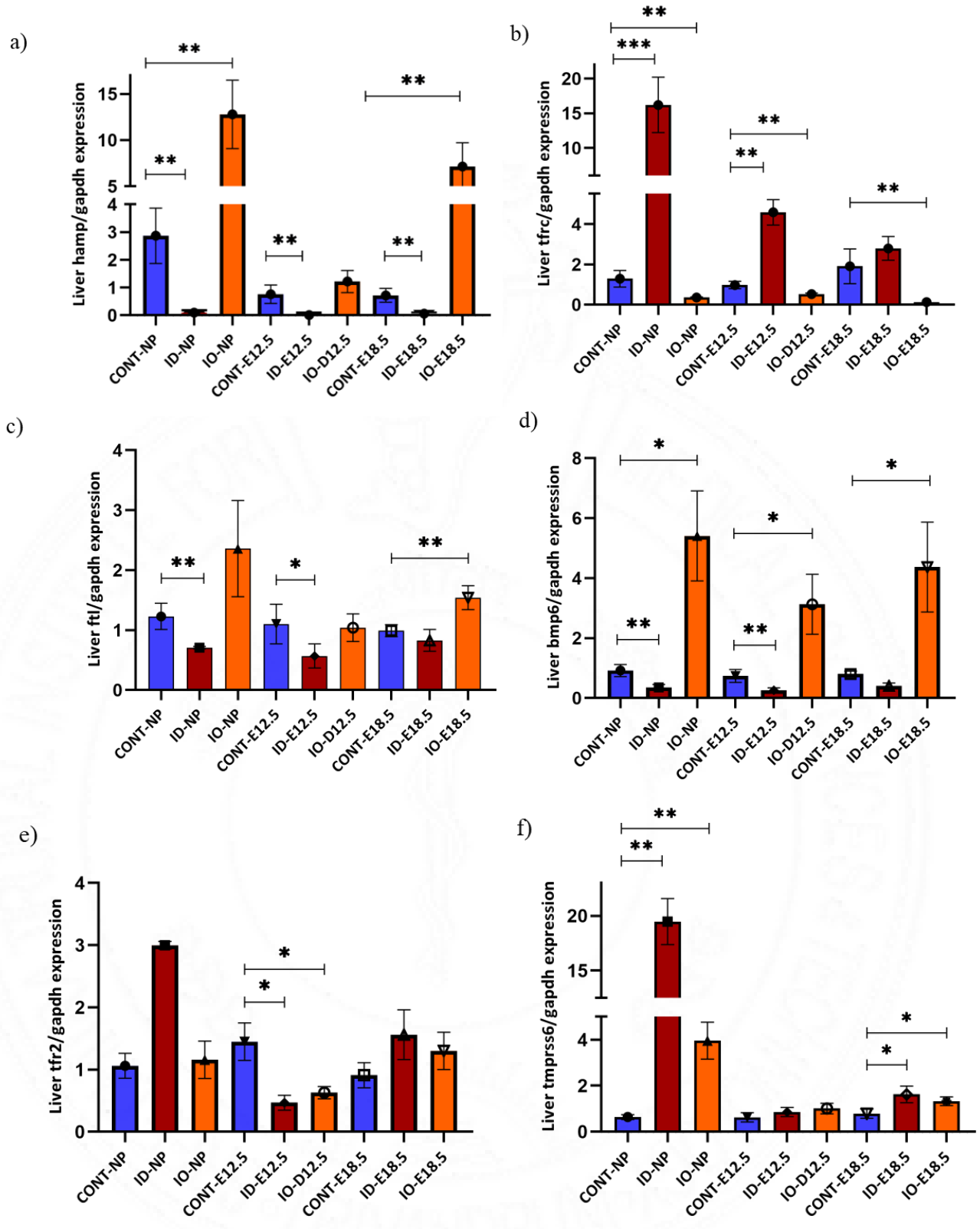


Figure 4.3.1.2) Hamp and iron-related gene expression were analyzed in non-pregnant and pregnant mice at E12.5 and E18.5 under normal, iron-deficient, and iron-overload conditions.

These findings indicate that during mid-pregnancy, liver iron stores and the mRNA expressions of tfrc, ftl, and bmp6 genes play a role in regulating hepcidin under iron deficiency. In turn, iron mobilization towards erythropoiesis increases at mid-gestation.

Estrogen, a steroid hormone known to inhibit hepcidin transcription and erythropoiesis, was evaluated in all mice groups. Notably, estrogen receptor 1 (esr1) was upregulated in non-pregnant ID mice (Figure 4.3.1.3).

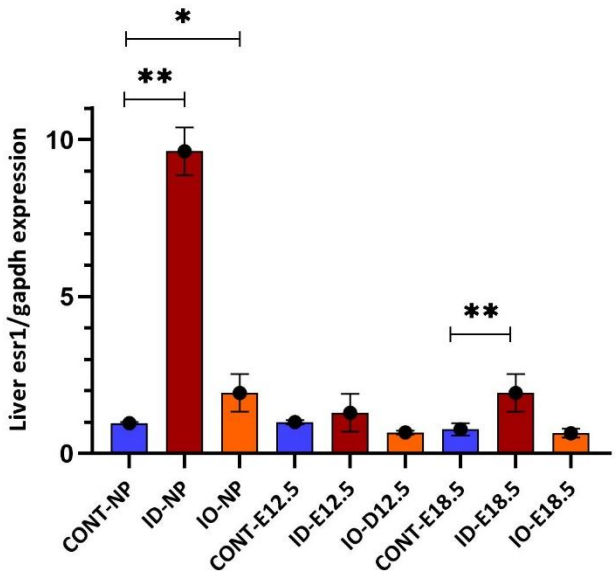


Figure 4.3.1.3. Estrogen receptor α (esr1) mRNA expression in hepatocytes

4.3.2. Iron and erythroid gene regulation in erythroid cells

From the second trimester, the iron requirement increases gradually to meet the demand for increased erythropoiesis in the mother and for placental-fetal development. To determine the regulation of iron and erythroid genes at mid and late gestation, we analyzed erythropoiesis in our pregnant mice. Maternal hemoglobin levels decreased in non-pregnant ID mice as compared to the control mice, whereas Hb levels were unchanged in non-pregnant IO mice. In pregnant ID, IO, and control mice, we did not observe significant changes in the Hb levels at E12.5 and E18.5 (Figure 4.3.2.1a). Mean cell volume decreased in ID mice at D0 and was

unchanged at E12.5 and E18.5 when compared to the control group. IO group had higher MCV at mid and late gestation (Figure 4.3.2.1b). RBC count was unchanged across the gestation in all the groups (Figure 4.3.2.1c).

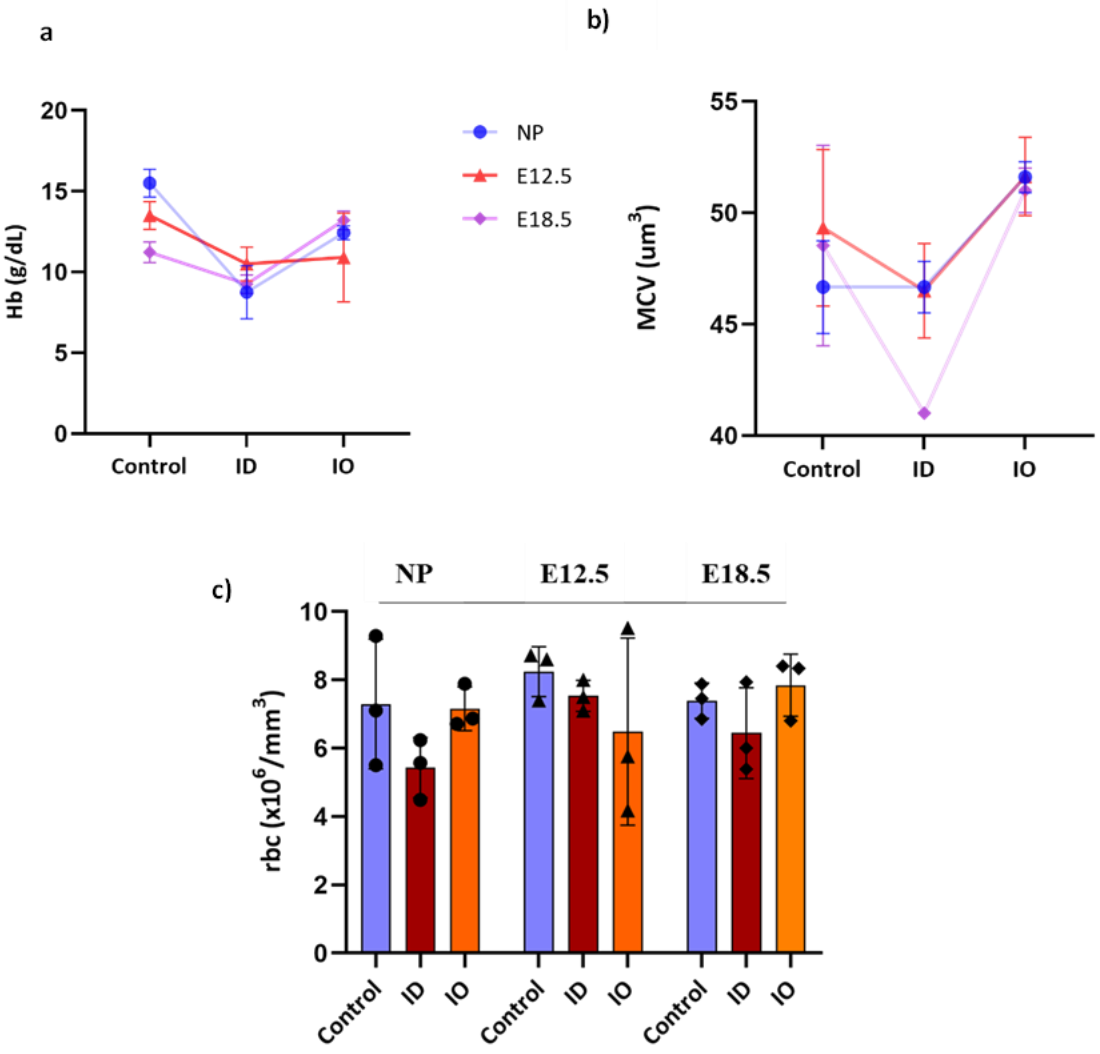


Figure 4.3.2.1. Mean value of hemoglobin, mass corpuscular volume and red blood cell count in iron$^{+/-}$ treated mice.

In non-pregnant ID mice, dmt1 and ferritin mRNA were significantly increased. At mid gestation, iron transporters tfrc and dmt1 were significantly increased in ID and there was no differential expression in IO mice. However, in late gestation, iron transporters were downregulated in both ID and IO mice (Figure 4.3.2.2).

These data demonstrate that under iron deficiency, iron uptake and transport increased in erythroid cells. However, in iron-loaded mice, there were no significant alterations observed in erythropoiesis compared to the control group.

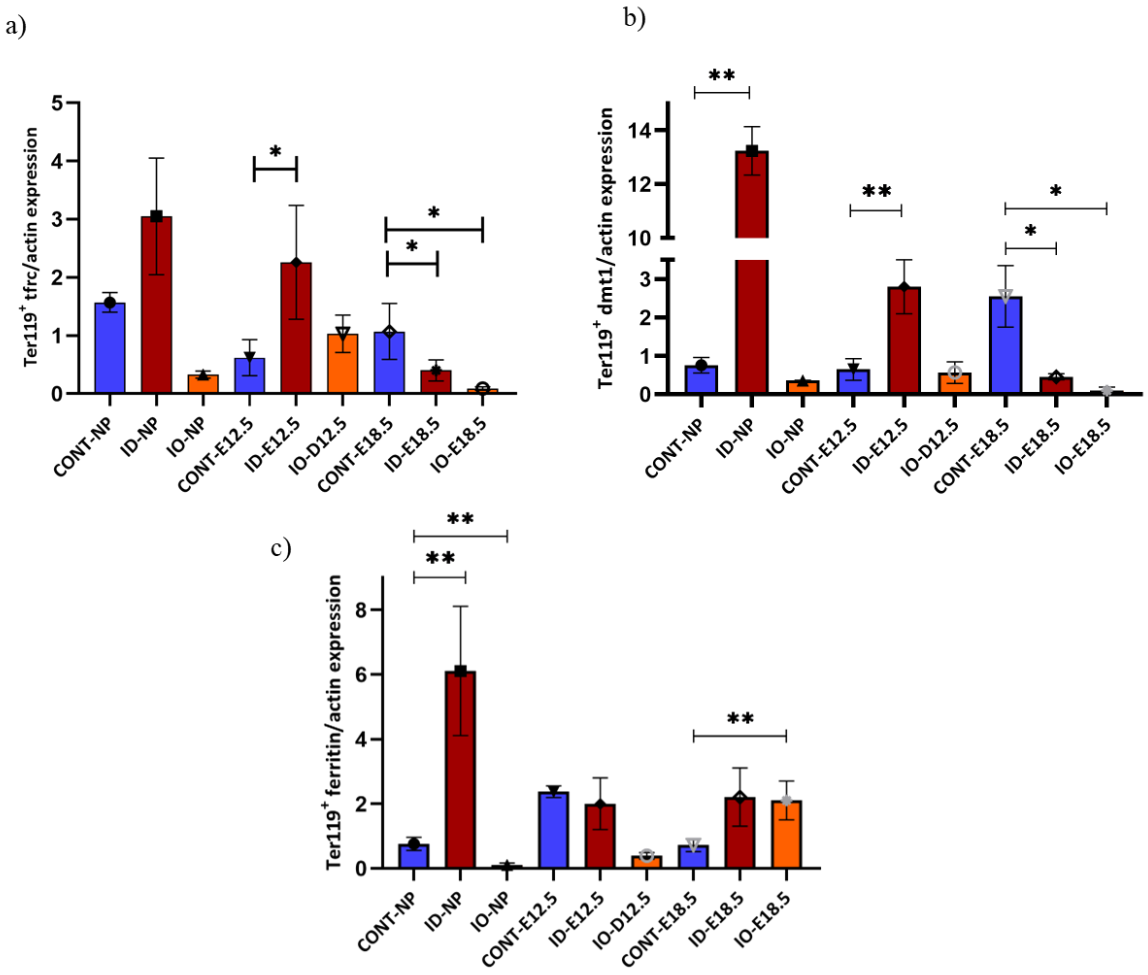


Figure 4.3.2.2. Iron-related gene expression in erythroid cells

To determine the increased erythropoietic activity during pregnancy, we analysed the expression of erythroid genes, erythropoietin receptor (epor) and janus kinase 2 (jak2). At E12.5, there was a notable increase in the expression of epor and Jak2 in ID mice (Figure 4.3.2.3a, b). However, by E18.5, a significant decrease in their expression was observed in both the IO and ID groups, indicating a decrease in erythropoietic activity at the late-gestational period.

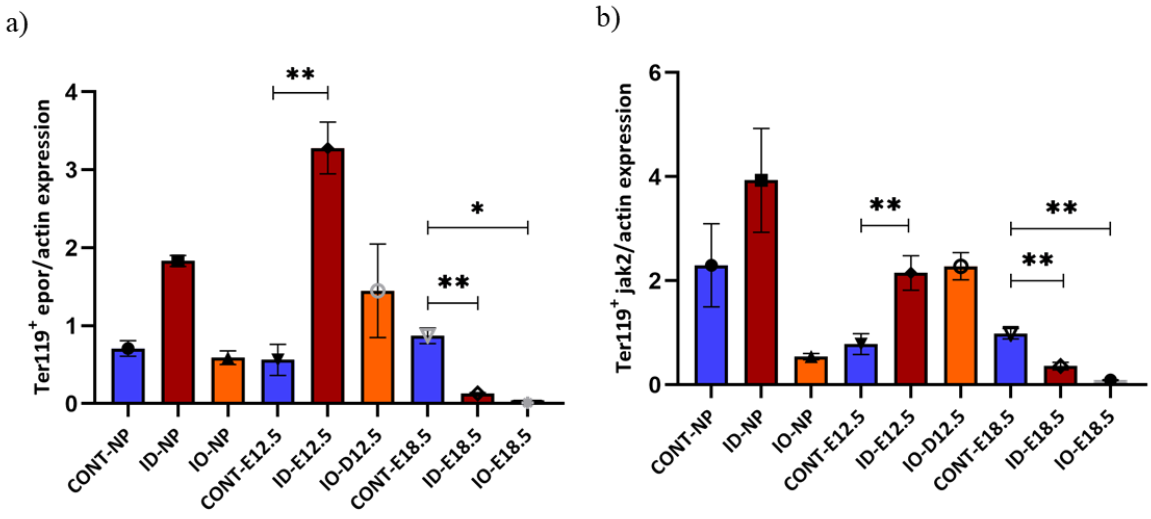


Figure 4.3.2.3 a&b) Erythropoietic activity determined by epor and jak2 mRNA expression in ID and IO mice.

The expression of growth differentiation factor(gdf15) and erythroferrone (*Erfe*), known as hepcidin suppressors secreted by erythroblasts, were also significantly increased at E12.5 in erythroid cells of ID mice (Figure 4.3.2.4). Conversely, no alterations in their expression were noted in iron-overloaded mice.

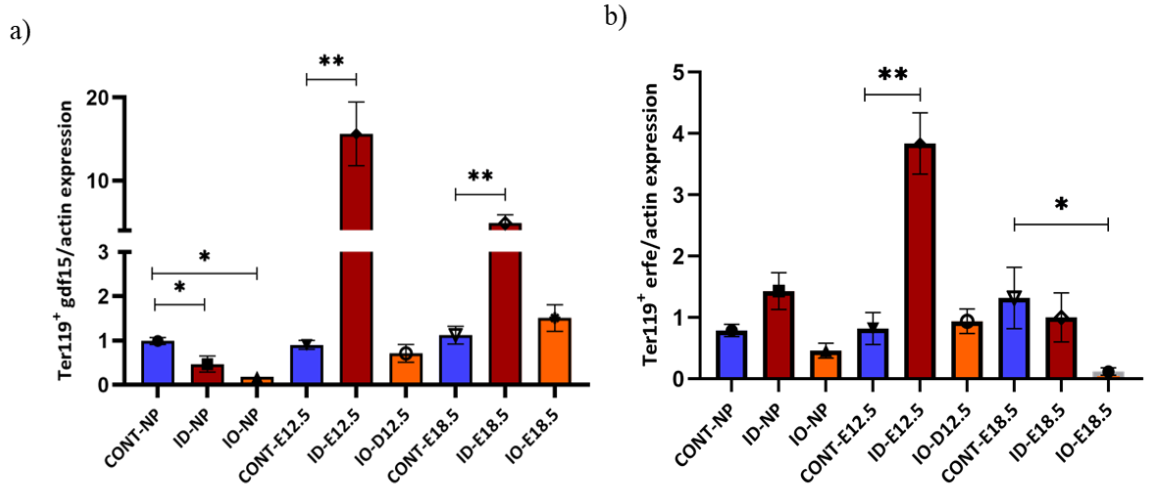


Figure 4.3.2.4(a&b) Hepcidin suppressors expression in erythroid cells

4.3.3. **Expression of placental hormones in iron deficient/overload mice**

During pregnancy, the placenta serves as an active endocrine gland, producing various hormones that induce physiological responses in the mother. Hormones encompass steroid hormones such as estrogen and progesterone, the prolactin gene family, and growth hormones(Napso et al., 2018). We observed increased expression of plp-e at E12.5 under iron deficiency (Figure 4.3.3.1a). The expressions of estrogen receptors Eα and Eβ in the placenta were examined, and Eα was found to be expressed in all groups of mice. In contrast to plp-e, there was no differential expression of Eα (Figure 4.3.3.1b). Placental iron transporter tfrc mRNA had differential expression at E12.5 in ID mice, indicating increased iron absorption at mid-gestation (Figure 4.3.3.1c).

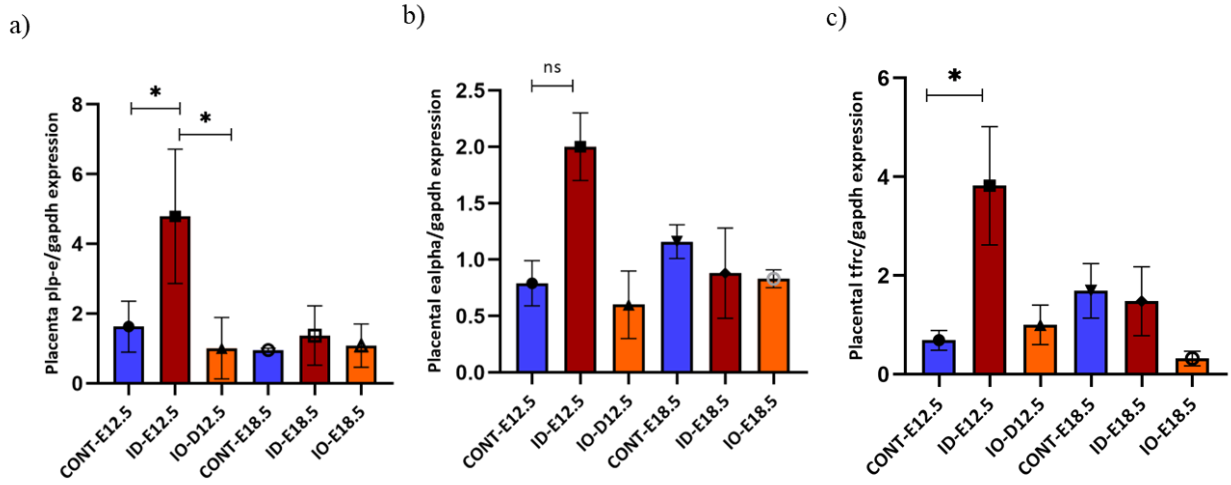


Figure 4.3.3.1. Expression of placental hormones and iron transporter in ID and IO mice

4.3.4. **Maternal iron deficiency perturbs fetal erythropoiesis in mice.**

To determine iron consumption in fetal liver, we evaluated fetal liver iron content in maternal ID and IO mice. Fetuses from mice that were fed an iron-deficient diet displayed an iron-deficient phenotype, while fetuses of IO mice exhibited iron overload phenotype (Figure

4.3.4.1). At E12.5, fetal iron content was notably decreased in mice with iron deficiency and remained unchanged in mice with iron overload (Figure 4.3.4.1).

Figure 4.3.4.1. a) Phenotype of fetus of control, ID, and IO at E18.5 b) fetal iron content at E12.5

During mid-gestation, the fetal liver serves as the primary organ for erythropoiesis. Studies have shown that estrogen suppress erythropoiesis by competing with effects of erythropoietin in the bone marrow, to sustain a steady state erythropoiesis (Bleiberg and Perah, 1975; DUKES and GOLDWASSER, 1961). We observed increased expression of estrogen receptor alpha (esr1) mRNA in ID mice at E12.5 and remained unchanged at E18.5. Notably, fetal liver had differentially expression of tfrc mRNA at E18.5 in ID, indicating consumption of iron increases in third trimester in maternal iron deficient mice.

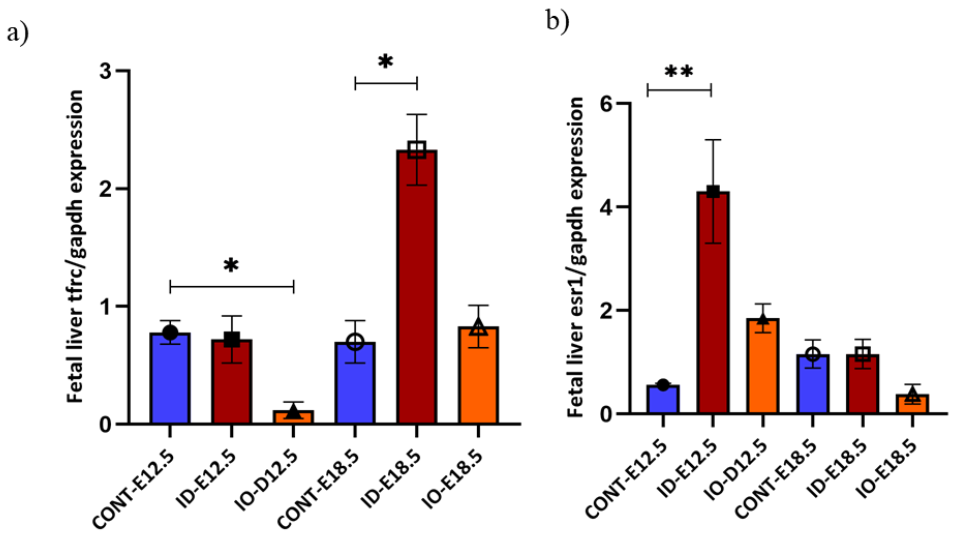


Figure 4.3.4.1. a) Transferrin receptor b) estrogen receptor 1 expression in ID and IO mice fetus at mid and late gestational period.

4.3.5. Estradiol treatment during erythroid differentiation in HEL cell line

In our animal experiment, we observed the expression of estrogen receptors in the placenta, maternal liver, and fetal liver. Subsequently, we aimed to investigate the impact of estrogen on erythropoiesis. Since it was not feasible to induce estrogen in pregnant mice due to their elevated estrogen levels, we conducted *in vitro* experiments to elucidate the effects of estrogen on erythropoiesis. To induce erythropoiesis in human erythroleukemic cell line (HEL), cells were subjected to hemin treatment for 96 hours. Subsequently, the hemin-treated cells were exposed to estradiol. The results indicated a notable reduction in $CD71^+$ % at 48 hours and $CD235^+$ % at 72 hours in cells treated with estradiol. This suggests that the estradiol treatment impairs erythroid differentiation by suppressing the maturation of erythroid cells.

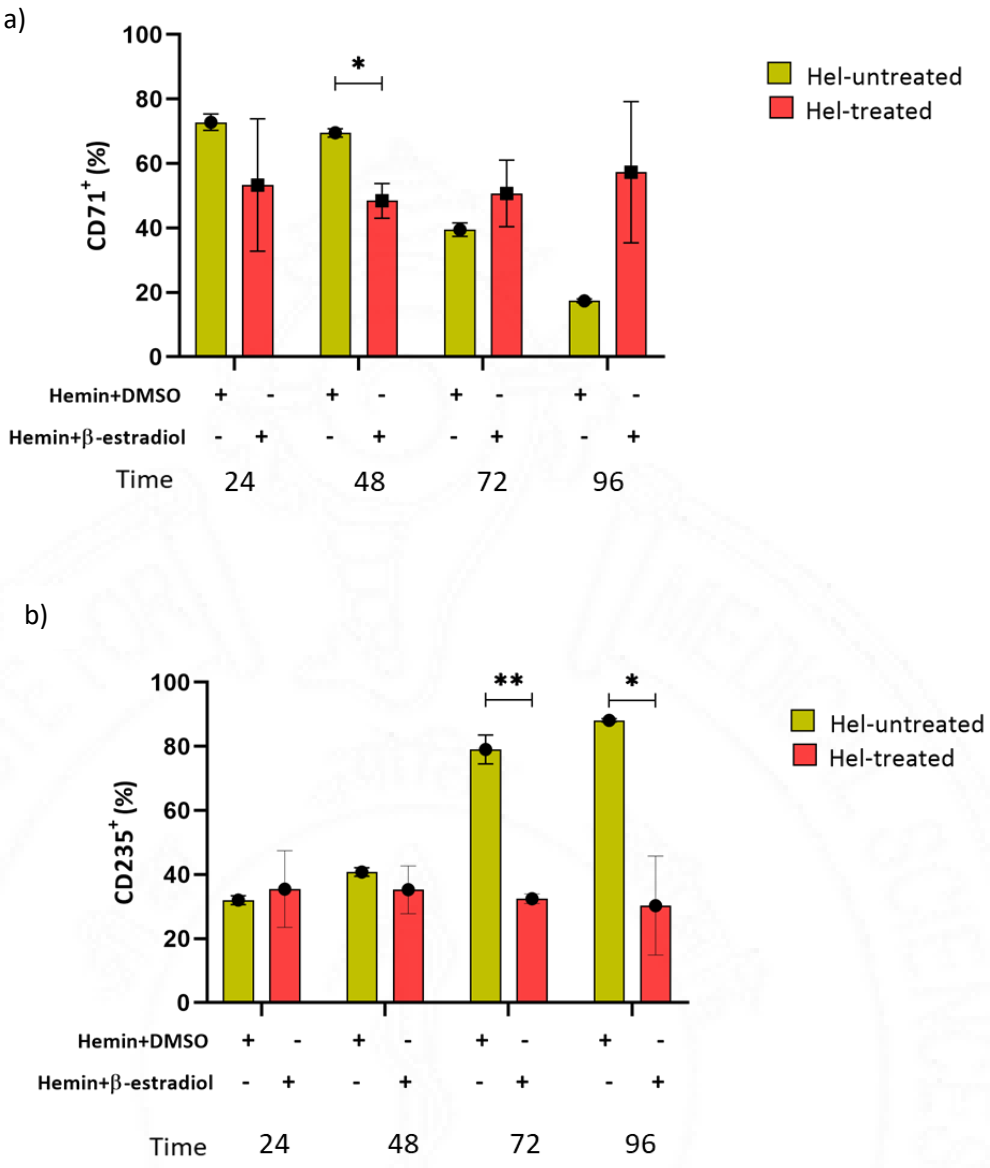


Figure 4.3.5.1. Expression of erythroid markers during erythroid differentiation examined in HEL cells treated with estradiol.

5.0 Discussion

Erythropoiesis is an intricately regulated process, originating from a multipotent stem cell in the bone marrow and maturing into an enucleated erythrocyte. Erythropoietin synthesized in the kidney binds to the erythropoietin receptor present on the erythroid progenitor membrane and triggers the JAK/STAT5 pathway, subsequently resulting in the proliferation, differentiation, and maturation of erythroid progenitors. The temporal regulation of erythropoiesis by EPO spans from the CFU-E stage to the polychromatophilic stage. Iron is crucial for the synthesis of hemoglobin, and erythroid cells acquire it through the transferrin receptor. To ensure an abundant iron supply, critical actions like duodenal iron absorption and the release of recycled iron from mitochondria contribute to circulation. This iron is then transported by the carrier protein transferrin (Zivot et al., 2018). Thus, erythropoiesis and iron homeostasis are intricately interconnected.

Increased erythropoiesis is a normal physiological occurrence during conditions like pregnancy and at high altitudes. In pathologically increased erythropoiesis status, alterations in red cell production can arise from direct impairment in erythropoiesis, as observed in Beta-thalassemia and Polycythemia Vera.

During pregnancy, iron deficiency anemia emerges as a significant public health concern, and studies have been directed mainly to understand the etiology and the magnitude of the problem. Although increased erythropoiesis is well-known, it often results in iron deficiency anemia during pregnancy. Extensive research has been carried out to understand ineffective erythropoiesis in beta-thalassemia and has identified hepcidin suppression by erythropoietic factors like GDF15 and erythroferrone as the primary cause of iron dysregulation. However, the understanding of the regulators of iron homeostasis and the various factors influencing

erythropoietic activity in beta-thalassemia remains incomplete. In polycythemia vera, decreased iron levels are observed and hepcidin regulation is not fully understood.

Here, we investigated various factors that upregulate erythropoiesis and modulate iron metabolism in pregnancy, β-thalassemia, and polycythemia vera.

For physiological model of increased erythropoiesis, we chose pregnancy as the model; erythropoiesis increases from second trimester in pregnancy to meet the demands of fetal and placental development. During this period, iron requirement escalates from 0.56mg/day (GA-<12 weeks) to 4mg/day (GA-12-21weeks). Physiological adaptation of maternal environment during pregnancy allows unknown factors to stimulate increased erythropoiesis at mid gestation. Hence, we investigated pregnant womens' peripheral blood and serum samples at different gestational timepoints.

A good study of erythropoiesis requires erythroid progenitors. Since it is unethical to collect bone marrow samples of pregnant women, we developed mice models of pregnancy exposed to different iron diets to study factors stimulating increased erythropoiesis.

For pathological erythropoiesis model, we studied Beta thalassemia and Polycythemia vera, as both have dysregulated iron homeostasis. In Beta thalassemia, the combination of high erythroid iron demand and blood transfusions results in iron overload and in PV, therapeutic phlebotomies limit the total body iron accumulation. How iron and erythroid are regulated under chronic stress erythropoiesis is not known.

Thus, identifying regulatory factors associated with iron homeostasis will aid in a better understanding of the physiological and pathological states of erythropoiesis.

5.1. Iron and Erythropoiesis Regulation in Pregnancy

Our study cohort was homogenous, with the mean age of 23±3.5yrs. The women were from our town and adjacent villages. In our study, despite iron supplementation, 24% had iron deficiency throughout the gestational period. Remarkably, nearly all pregnant women, in line with observational studies, remained non-anemic throughout the duration of pregnancy (Bothwell, 2000). The concentrations of hemoglobin and hematocrit reached their lowest point by the end of the second trimester, and then exhibited an upward trend during the third trimester. Laflamme et al. have reported a similar trend of Hb and HCT levels during pregnancy (Laflamme, 2011). We observed a significant drop in Hb and Hct levels at 21 weeks, due to plasma expansion. These findings align with results reported in other studies (Kumar et al., 2013; Lone et al., 2004; Moghaddam Tabrizi and Barjasteh, 2015).

Using logistic regression analysis, we identified an increased risk of anemia in pregnant women at 16 weeks with increased sTfR levels. This escalation is associated with a fourfold rise in the likelihood of inadequate iron supply to the bone marrow. Further statistical analysis confirmed that anemia causes increased EPO secretion as a response to low hemoglobin concentration. These findings imply that the iron demand is associated with an increased production of red blood cells.

A key observation in our study is the consistent rise in EPO levels and a concurrent decline in ferritin levels from the initial to the third trimester. This pattern implies the utilization of mobilized iron to support elevated erythropoiesis and contribute to placental and fetal development. Increased iron levels in the third trimester were associated with lower sTfR levels, suggesting that erythropoiesis decreased in the third trimester. Elevated iron levels during this period indicate utilization for fetal endowment. Studies suggested that the elevated

levels of sTfR and EPO during pregnancy are predominantly caused by increased erythropoietic activity (Choi et al., 2000; Flores-Quijano et al., 2016).

We then studied the gene expression of iron and erythroid-related genes (n=15) in reticulocytes. Decreased mRNA expression of FPN1B and TFRC at the onset of the third trimester indicates a decline in iron transport to plasma. This is due to decreased erythropoiesis at third trimester.

One of the strengths of our study is the focus on iron biomarkers in substantial number of participants with uncomplicated pregnancies. These findings suggest that erythropoiesis increases from the second trimester. This shift corresponds to the regulation of increased iron from plasma toward the bone marrow to support erythropoiesis. In the third trimester, the rate of erythroid activity stabilizes, and during this period, a massive portion of iron is utilized for fetal iron endowment (Figure 5.1.1).

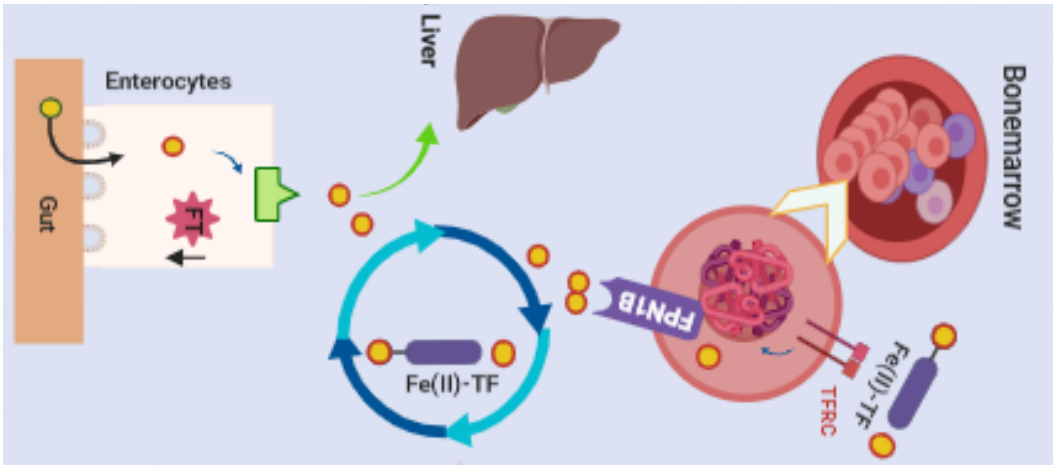


Figure 5.1.1. Proposed mechanism of iron homeostasis and erythropoietic iron regulation during second trimester.

Physiological increase in erythropoietic activity occurs at 21 weeks of gestation, which causes increased iron transport from the plasma towards the erythroid cells. TFRC, responsible for incoming iron in erythroid cells facilitates increased acquisition and FPN1B, lacking iron responsive element, contributes iron to the circulation at late erythroid maturation stage.

5.2. Maternal-fetal iron transfer

During pregnancy, placental mechanisms ensure that sufficient iron is made available for fetal erythropoiesis. Here we investigated how maternal and fetal iron status relate to placental iron transporters' expression. We assessed serum iron parameters hepcidin, ferritin, GDF15 and hematological parameters in pregnant women with iron deficient anemia and compared them to iron replete pregnant women.

At delivery, 22% pregnant women aged between 20-35 years had iron deficiency anemia, even with continuous iron supplementation. Similarly, 18.7% of Turkish pregnant women had IDA regardless of iron supplementation(Kavak and Kavak, 2017). The majority experienced mild anemia, similar to the findings in a study involving Iranian pregnant women(Moghaddam Tabrizi and Barjasteh, 2015). Notably, risk factors such as maternal age, gestational age at delivery, gravida, and consanguinity did not show significant effects on IDA in this study. We found that neonates born to mothers with IDA showed normal cord blood ferritin levels, confirming a normal iron status in the newborns.

Hepcidin, the regulator of systemic iron homeostasis, was decreased in IDA as compared to controls, consistent with earlier observations (Kulik-Rechberger et al., 2016b). There was no association between maternal or fetal ferritin and maternal hepcidin in the IDA group. Similar patterns of depleted iron stores and hepcidin concentration at term pregnancy were reported in Finland women (Rehu et al., 2010).

Additionally, we did not witness direct effects of IDA on fetal iron status; instead, we observed an association with placental iron transporters. In IDA, there was an increase in the placental protein expression of TFRC, a similar observation reported by Sangkhae et al (Sangkhae et al., 2020). Furthermore, both DMT1 and FPN1 mRNA expressions significantly increased in

placenta. Together these results suggest that maternal iron deficiency redirects iron from the placenta towards the fetus.

GDF15 is a known suppressor of hepcidin, was decreased in cases of preeclampsia and miscarriage. Consistent with other studies, we found elevated GDF15 concentrations in pregnant women (Finkenstedt et al., 2012). No association was found between hepcidin/ferritin with GDF15 in mother and fetus, but robust GDF15 expression was observed in placenta at mRNA and protein levels. GDF15 protein expression correlates positively with TFRC, DMT1, SP1, and TP53 proteins, emphasizing its crucial role in placental iron trafficking. Transcript factors SP1 and TP53, were reported to increase GDF15 in erythroid cells (Tanno et al., 2010). Thus, our analysis suggests that SP1 and TP53 might contribute to the augmented placental GDF15 expression.

We propose that fetal ferritin potentially regulates placental GDF15 and FPN to ensure the optimal iron transfer to fetus, as indicated by their positive associations. Nevertheless, Further characterization is necessary to comprehend the role of placental GDF15 in iron regulation.

Through post-transcriptional mechanisms, alternative splicing generates multiple mRNA transcripts from a single gene (Black et al., 2019). Our study is the first to evaluate isoforms of different non-heme iron transport proteins in human placental tissue among IDA and control groups. The isoforms DMT1A and FPN1A, which include the IRE motif, exhibit a significant increase in IDA, leading to increased iron absorption. The expression pattern of placental FPN1A aligns with recent findings from Sangkhae and colleagues. (Sangkhae et al., 2020).

Consistent with several authors, we have also noticed a positive correlation between hepcidin and ferritin levels in healthy pregnant women (Kulik-Rechberger et al., 2016; van Santen et al., 2013; Lorenz et al., 2014). In iron-deficient pregnant women, our current findings reveal

that lower iron status and reduced hepcidin levels induces an augmented mobilization of iron from stores. Placental iron transporters respond to maternal iron deficiency by upregulating their expression, facilitating adequate iron transfer to the fetus ensuring normal iron stores in the fetus at birth. Fetal iron status was independent of maternal iron status.

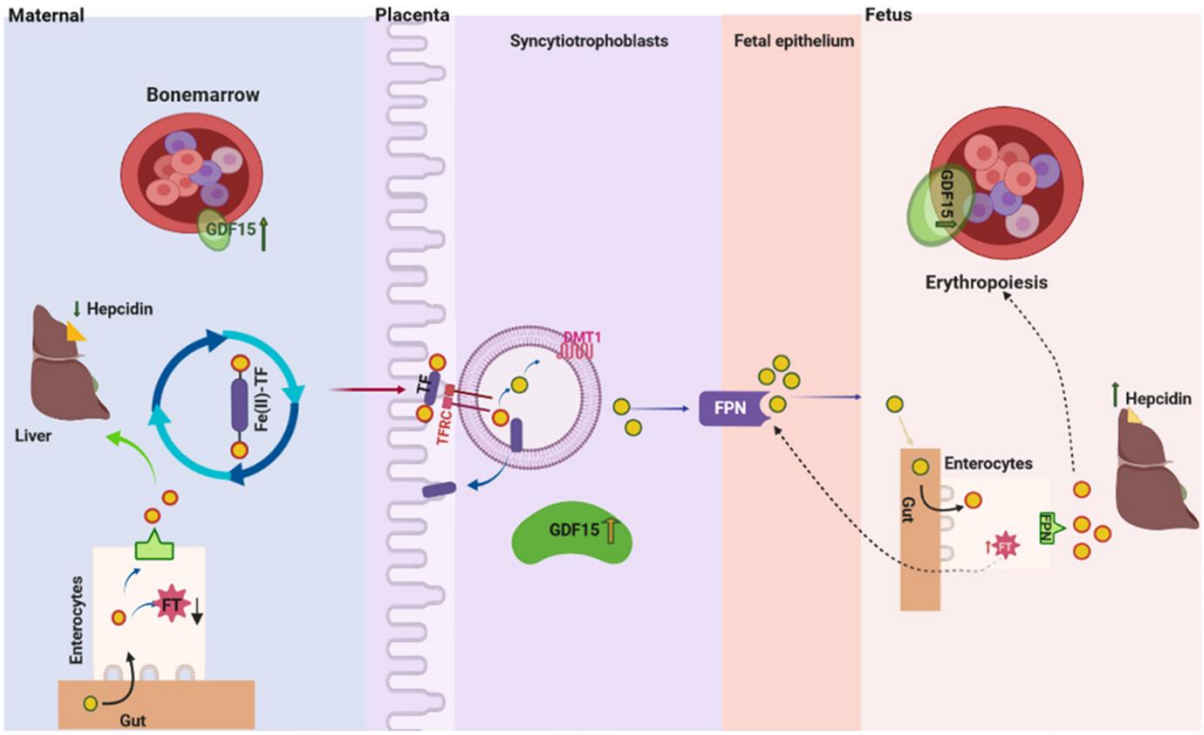


Figure 5.2.1: Proposed mechanism of iron and erythroid regulation in pregnancy. Under maternal iron deficiency, duodenal ferritin and hepatocytic hepcidin levels decrease. GDF15, recognized as a hepcidin suppressor, significantly increases during pregnancy. Transferrin-transferrin receptor complex facilitates the transport of maximal maternal iron to the placenta. Iron is released via DMT1 and effluxed by FPN1 into the fetal circulation. The placental abundance of GDF15 may also play a role in iron transport regulation. Fetal iron stores regulate placental FPN and GDF15, ensuring an increased iron endowment and maintaining fetal iron homeostasis.

5.3. Interplay of Iron and erythroid activity during pregnancy in mice

To determine the factors involved in stimulating increased erythropoiesis during pregnancy, we performed a comprehensive evaluation of iron and erythroid regulators in iron-deficient and overloaded mice models during pregnancy.

5.3.1. Changes in iron and erythropoietic activity during pregnancy

During gestation, blood volume progressively increases, reaching levels 20% above nonpregnant levels by day 12. Fowler et al. reported a rise in hemoglobin around gestational day 12, followed by a decline towards term in control mice (Fowler and Nash, 1968). In contrast, our observations did not reveal significant changes in hemoglobin levels in all groups except for non- pregnant iron deficient mice, which exhibited lower hemoglobin values.

To the best of our knowledge, this is the first study to analyse the mRNA expression of iron and erythroid genes in erythroid cells of mice. In erythroid cells, a decline in ferritin expression at E12.5 during iron deficiency indicates a preference for utilizing iron in hemoglobin synthesis under maternal iron deficiency. Furthermore, there was a notable increase in mRNA expression of iron transporters tfrc and dmt1 at E12.5, succeeded by a decrease at E18.5 in iron-deficient mice. Conversely, iron-loaded mice demonstrated reduced expression of iron regulators (tfrc and dmt1). These findings emphasize that under iron deficiency, iron is predominantly directed towards erythropoiesis during mid-gestation. However, in iron-loaded mice, no significant changes in erythropoiesis were observed compared to the control group.

During pregnancy, a major factor regulating increased erythropoiesis is erythropoietin (Jepson and Lowenstein, 1968). We analyzed the expression of genes involved in erythropoietic signaling. There was a marked increase in the expression of epor and jak2 in the erythroid cells at mid-gestation in ID mice. However, by late gestation, a significant decrease in their expression was observed in both IO and ID groups, indicating a decline in erythropoietic activity during the late gestational period.

Helman et al. reported increased erfe mRNA expression in the bone marrow of control mice at mid-gestation(Helman et al., 2023). In comparison, erfe mRNA had augmented expression at mid-gestation in iron-deficient mice compared to the control group. Another known hepcidin

suppressor, gdf15, also showed a similar increase at mid-gestation in ID mice. In iron deficiency, hepcidin needs to be suppressed efficiently to increase iron absorption and availability. Increased expression of erfe and gdf15 may play an instrumental role in suppressing hepcidin.

5.3.2. Stimulated erythropoiesis causes a decrease in hepcidin expression.

In agreement with other studies, we confirmed suppression of maternal hepcidin expression in iron deficient and its augmented expression in iron overload as compared to controls (Helman et al., 2023; Sangkhae et al., 2021). This corresponds to a reduction in liver iron stores, transferrin receptor, and bmp6 expression at E12.5, key regulators of hepcidin production. The decrease in hepcidin indicates an increased demand for iron during this period, primarily attributed to increased erythropoiesis. Within each group, hepcidin continued to be suppressed in comparison to nonpregnant females. Ferritin was decreased at mid-gestation as compared to controls. Recent animal studies have revealed that the erfe hormone inhibits hepcidin by binding to bmp6 and thereby hindering the SMAD signaling pathway(Helman et al., 2023; Wang et al., 2020). The iron overload observed in β-thalassemia leads to elevated levels of BMP6, suggesting a possible requirement for increased efre to inhibit bmp6 activity (Frazer et al., 2012).

Nevertheless, our study revealed a diminished bmp6 expression at E12.5, implying that a lower efre level might be adequate to inhibit bmp6 activity. At mid-gestation, we observed elevated erfe expression in erythroid cells, which could effectively contribute to suppressing the inhibition of BMP6 signaling.

The involvement of TMPRSS6 in hepcidin regulation during stimulated erythropoiesis cannot be dismissed. We noted increased expression in non-pregnant ID mice, which did not show

differential expression during gestation. However, it's essential to validate tmprss6 mRNA at the protein level, as it is modulated at the post-transcriptional level.

5.3.3. Effects of placental hormone and fetal liver iron on erythropoiesis

During pregnancy, the placenta serves as an active endocrine gland, producing various hormones that induce physiological responses in the mother. Hormones encompass steroid hormones such as estrogen and progesterone, the prolactin gene family, and growth hormones (Napso et al., 2018). Studies have reported that estrogen and prolactin gene PLP-E are involved in maternal erythropoiesis regulation (Bittorf et al., 2000; Jepson and Lowenstein, 1968). PLP-E stimulate expression of the adult beta major globin gene in mouse erythroleukemia cells(Bittorf et al., 2000). PLP-E acts on specific receptors of erythroid-committed murine and human cells by the activation of intracellular signaling pathways promoting cell growth and differentiation. In our study, ID mice had increased expression of placental plp-e at mid-gestation. Under maternal iron deficiency, plp-e could regulate increased maternal erythropoiesis at mid-gestation.

Placental iron transporter, tfrc mRNA had increased expression at mid-gestation in ID mice, suggesting increased iron absorption for fetal endowment. Similar observation was reported by Sangkhae et al.(Sangkhae et al., 2021). During mid-gestation, the fetal liver serves as the primary organ for erythropoiesis. Notably, fetal liver concentration was decreased at mid-gestation in ID and elevated in IO mice. Fetal liver had maximal tfrc expression at late gestation in ID, indicating iron consumption increases in third trimester in maternal iron deficient mice.

Thus, at mid-gestation maternal hepcidin suppression facilitates increased iron absorption, ensuring optimal iron transfer from maternal circulation to the fetus through placenta. Iron is utilized for fetal erythropoiesis, with decreased iron stored in the fetal liver. In IO mice,

maternal hepcidin is high and prevents fetal iron overload, thereby maintaining iron homeostasis and fetal erythropoiesis (Figure 5.3.1.).

5.3.4. Effects of estrogen during pregnancy

Steroid hormones estrogen and progesterone increase during pregnancy, has a role in plasma volume elevation(Aguree et al., 2020). Studies have shown that estrogen suppress erythropoiesis by competing with effects of erythropoietin in the bone marrow, to sustain a steady state erythropoiesis (Bleiberg and Perah, 1975; DUKES and GOLDWASSER, 1961; Horiguchi et al., 2005). In addition repeated administration of estrogen in rats exhibited increased TIBC levels (Horiguchi et al., 2005). When iron metabolism was perturbed in ovariectomized rats, estrogen serum level was increased (Haouari et al., 1993). Additionally, estrogen also known to inhibit hepcidin transcription by binding to estrogen responsive element located in promoter region (Yang et al., 2012).

Based on this evidence, we analysed estrogen receptors esr1 and esr2 expression in liver, erythroid cells, placenta, and fetal liver in all mice groups. In liver, esr1was upregulated in non-pregnant ID mice and was not altered during gestation. While esr1 was expressed in the placenta of all mice groups, there was no differential expression. Fetal liver had increased expression of esr1 mRNA in ID mice at mid-gestation. These results suggests that placental and fetal liver esr1 expressions may have potential role in maternal and fetal erythropoiesis, respectively. Further validation is required to fully understand these potential roles.

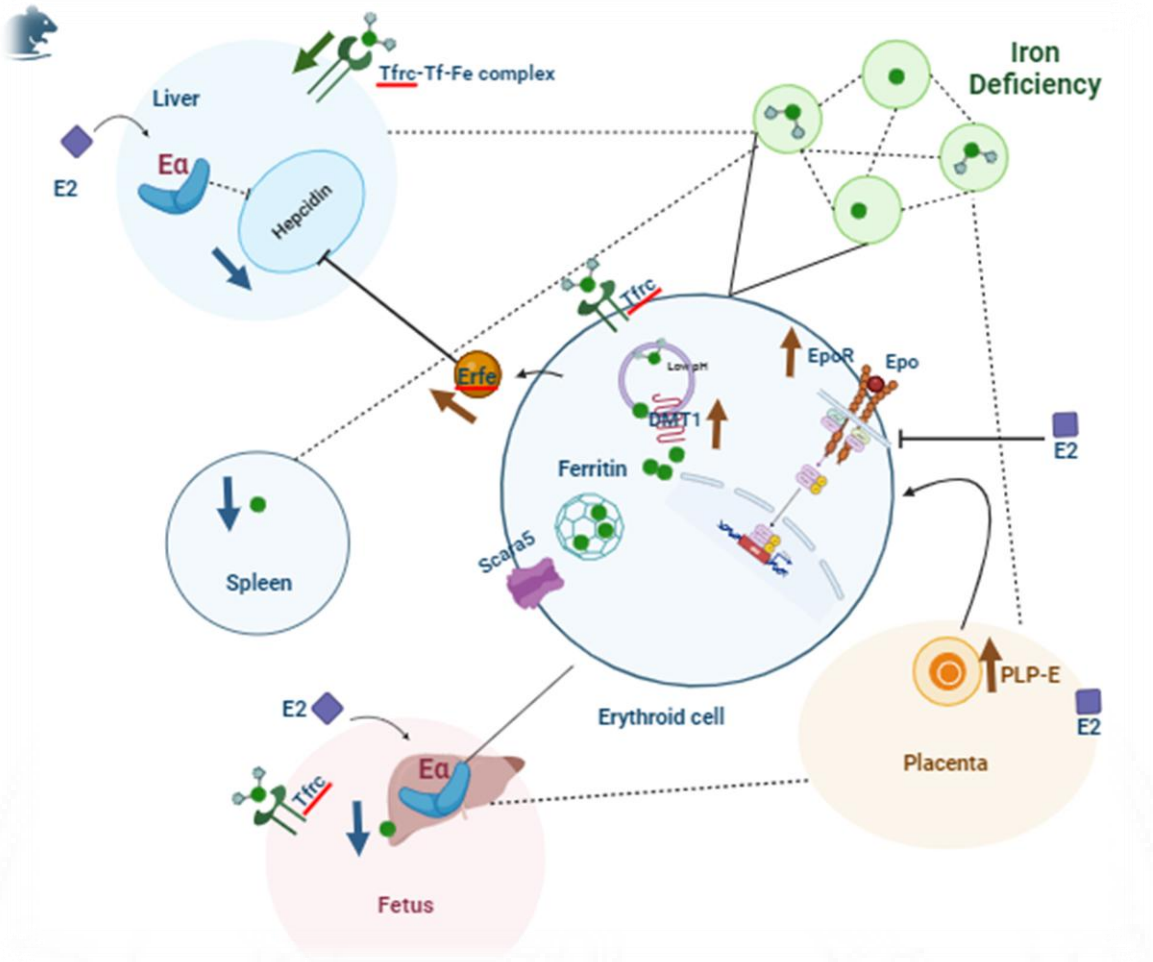


Figure 5.3.1. summarises the iron and erythroid regulators increase during mid-gestation in response to iron deficiency.

In iron deficient mice, at mid-gestation, increased erythroid activity demands additional iron. Within erythroid cells, iron loaded on to transferrin is transported via transferrin receptor and undergoes endocytosis. In acidic endosomes, iron is released through dimetal transporter 1 into cytoplasm, where it is either stored in ferritin or utilised for hemoglobin synthesis. Erythropoietin binds to erythropoietin receptor present on red cell membrane and activates downstream pathways for erythroid differentiation. Maternal erythropoiesis may be regulated by Placental Prolactin-Like E (PLP-E) and estrogen. Despite low liver iron content in the fetus, iron uptake remains normal through increased tfrc expression. Estrogen receptor 1 (ERα) found in both maternal and fetal livers may play a role in modulating erythropoiesis.

These findings from animal experiments indicate that concurrent iron deficiency in human pregnancy could trigger augmented iron mobilization in response to increased erythropoiesis during the mid-gestational period. Additional factors such as plp-e and estrogen may potentially play a role in stimulating erythropoiesis in mid-gestation.

Further action of estrogen during erythroid differentiation was determined in vitro using HEL cell line. Erythroid differentiation was treated with estradiol for 24,48 and 96hours in HEL cells induced by hemin. Estradiol treatment in hemin treated HEL cells showed significant decrease in $CD71^{+}$% and $CD\ 235^{+}$% at 48hrs and 72 hrs, respectively. Estradiol treatment impaired erythroid differentiation by suppressing erythroid maturation. Thus, estradiol is essential for regulating erythropoiesis.

5.4. Erythroid activity modulates iron regulation in pathological erythropoiesis.

To understand iron dysregulation in pathological erythropoiesis, we studied the iron mechanism in β-thalassemia and Polycythemia Vera. β-thalassemia leads to ineffective erythropoiesis, causing the production of abnormal red cells and subsequent secondary iron overload. Early ferrokinetic studies have demonstrated that β-thalassemic patients exhibit a tenfold increase in plasma iron turnover, with reduced release of red blood cells and elevated oxidative stress due to excess iron (Ramos et al., 2010). Systemic iron overload with relatively increased hepcidin levels was noted in both β-thalassemia major (TDT) and intermedia (NTDT). However, the hepcidin ferritin ratio was lower compared to controls, suggesting a disproportionally reduced hepcidin level relative to the extent of iron overload. A similar finding was observed in β-thalassemic mice models (Gardenghi et al., 2007). The sTfR level was higher in NTDT in comparison to TDT, highlighting the role of transfusions in suppressing ineffective erythropoiesis in TDT(Pasricha et al., 2013). This is the first study to analyze iron

regulatory genes expression in reticulocytes of thalassemia patients. We observed lower TFRC expression in reticulocytes from individuals with TDT and NTDT showed compared to controls, indicating reduced iron acquisition by erythroid cells during the maturation stage of differentiation. The expression of KLF1, a transcription factor recognized for promoting erythroid differentiation in the terminal maturation stage, was elevated in the reticulocytes of both TDT and NTDT patients. These results imply that the iron requirements in the bone marrow are determined by ineffective erythropoiesis, impacting the regulation of hepcidin.

Polycythemia Vera (PV) is a myeloproliferative disorder characterized by increased erythropoiesis and concurrent iron deficiency. Limited research has shown iron deficiency in PV(Ginzburg et al., 2018). In our study, PV patients showed reduced hepcidin levels, suggesting that the decrease in hepcidin is a result of decreased circulating iron induced by chronic erythropoiesis. Around 16% of PV patients exhibited iron deficiency, while others had subnormal serum ferritin levels. The hepcidin-to-ferritin ratio was comparable between PV and control group. A recent study by Bennett et al. reported similar observations, except for reduced ferritin levels in their study (Bennett et al., 2023). Inflammatory marker, IL-6 levels remained within normal limits in PV. The presence of relatively low serum ferritin levels and normal IL-6 levels in PV patients suggests that inflammation is not a major contributor to the dysregulated iron metabolism. A negative association was observed between sTfR and ferritin levels in PV, which was not observed in controls. This suggests that increased erythropoiesis result in relatively lower ferritin levels. Among iron and erythroid gene analysed in reticulocytes of PV and control, TFRC, FPN1B, IRP2 and KLF1 had augmented expression in PV. Gene expression analysis reveals that enhanced iron acquisition and transport occurs during the final stages of erythroid maturation when iron reserves are reduced.

Hence in pathological erythropoiesis, erythropoietic activity determines amount of iron transfer to the bone marrow by modulating intracellular iron regulation in erythroid cells and influencing systemic iron level.

5.5. Effects of iron treatment during erythroid differentiation from human HSCs

Hematopoietic stem cells (HSCs) give rise to erythroid lineage through a multi-step process. The terminal phase of erythroid differentiation begins with morphologically identifiable proerythroblasts, which undergo both proliferation and differentiation to yield red cells (Hu et al., 2013). In the present study, we explored the regulation of iron transporters during erythroid differentiation by developing a serum-free *in vitro* model for erythropoiesis using human $CD34^+$ cells. We first performed a comprehensive analysis of modifications in the expressions of red cell membrane proteins such as transferrin receptor ($CD71^+$) and glycophorin A ($CD235^+$). Consistent with findings in other studies, we observed increased expression of $CD71^+$ in immature erythroid cells and $CD235^+$ in late-stage mature erythroid cells (Hu et al., 2013; Wangen et al., 2014). Around $1x10^6$ $CD34^+$ cells expanded for 6 days and in erythroid phase 2, cells proliferated from 2-3 $x10^6$ cells. In the late erythroid stage, cell number declined to 1 $x10^6$ cells. The cells exhibited a twofold increase in proliferation on day 13, which subsequently declined as they advanced through later stages of erythroid differentiation. Additionally, the pellet of differentiated cells, gathered on days 7, 12, and 16, exhibited a progressive shift towards a red coloration, signifying the synthesis of hemoglobin. Morphologically, various erythroid stages, such as basophilic, polychromatophilic, and ortho-chromatophilic erythroblasts, appeared. During the second phase of erythroid expansion, a substantial level of proliferation is sustained within the nucleated erythroid population. Wangen et al. reported that the increased cell proliferation was due to 50% of cells being in the S phase(Wangen et al., 2014).

We noted an elevated expression of TFRC and DMT1 mRNA during the intermediate stages of erythroid differentiation including basophilic and polychromatophilic, indicating its role in facilitating iron influx into erythroid cells. FPN 1A has been demonstrated in erythroid cells, where FPN1A, with the iron-responsive element (IRE) is functional during hemoglobin synthesis and restricts iron export. (Cianetti et al., 2010). In our study, increased FPN1A mRNA corresponds to the differentiation stage from erythroid progenitors to polychromatophilic erythroblasts. Further we studied the effects of iron chelation and overload on erythroid differentiation. Under iron deficiency, erythroid maturation stage was arrested, and cells exhibited lower FPN1A mRNA expression, indicating limited iron export. Despite reduced expression of iron transporters DMT1 and TFRC in iron overload condition, there was no significant changes in expression as compared to control. Collectively, these results suggest that intracellular iron regulators modulate their function depending on the iron requirements of erythroid cells at various stages of development.

5.6. Iron regulation in polycythemia vera erythroid differentiation

To investigate intracellular iron regulation in polycythemia vera, we performed in vitro erythroid differentiation using $CD34^+$ cells obtained from patients diagnosed with PV. In the second phase of erythroid expansion in the presence of EPO, PV erythroid cells had an accelerated proliferation and flow cytometric analysis revealed $CD235^+$ appeared on day 8, which is earlier than in normal erythroid differentiation. However, the late erythroid stage was similar when compared to healthy erythroid cells. This observation aligns with findings from other studies (Bruchova et al., 2009b; Ugo et al., 2004). Around $1x10^6$ $CD34^+$ cells expanded for 6 days and in erythroid phase 2, cells proliferated from 6 $x10^6$ cells. In the late erythroid stage, cell number declined to 0.5 $x10^6$ cells. The cells exhibited a four-fold increase in proliferation on day 13, as compared to HD proliferation. Further we evaluated iron regulatory

genes expression in PV. TFRC and FPN1B (non-IRE) had augmented expression in immature erythroid stage. Cianetti el al. demonstrated that FPN1B is expressed when precursor cells require iron accumulation within the cell (Cianetti et al., 2010). When PV erythroid cells were treated with iron chelator, proliferation and surface marker expression was decreased like normal erythroid differentiation. These findings suggest that increased iron uptake and accumulation occurs in erythroid precursor cells of PV for hemoglobin synthesis.

a)	**Physiological**	**Pathological**	
	Pregnancy	**β-thalassemia**	**Polycythemia vera**
sTfR	Normal	↑	↑
Ferritin	↓	↑	↓
Hepcidin	↓	↑	↓
Correlation			
sTfr vs Ferritin	-ve	ns	-ve
FPN1B vs KLF1 mRNA	+ve	ns	+ve
Reticulocyte mRNA expression			
FPN1B	↓	ns	↑
TFRC	↓	↓	↑
KLF1	ns	↑	↑

b)	**Erythroid Differentiation**				**Erythroid Differentiation Under iron deficiency**			
	Healthy HSCs		PV HSCs		Healthy HSCs		PV HSCs	
Surface Markers	IMM	MAT	IMM	MAT	IMM	MAT	IMM	MAT
$CD71^+$	↑	↓	↑	↓	↑	↓	↑	↓
$CD235^+$	↓	↑	↑	↑	↓	↓	↓	↑
Reticulocyte mRNA expression								
FPN	↑	↓	↑	↓	↑	↓	-	-
TFRC	↑	↓	↑	↓	ns	ns	-	-
DMT1	↑	↓	ns	ns	ns	ns	-	-

Figure 5.10.1: Summary of iron and erythroid regulation at different states of increased erythropoiesis. ns: non-significant, correlation: -ve, negative; +ve, positive, IMM-immature erythroid cells, MAT-mature erythroid cells

Overall, the regulation of iron in pregnancy and polycythemia vera exhibited similarities, except for the levels of soluble transferrin receptor. Moreover, statistical analysis indicated comparable associations between erythroid activity and iron status in both conditions. In the second trimester, iron uptake is reduced in reticulocytes, while increased iron acquisition is found in reticulocytes of PV patients. These findings imply that systemic iron regulation is influenced by erythropoietic activity. In the case of beta thalassemia, although iron regulators were elevated, a defect in hemoglobin production led to ineffective erythropoiesis, thereby surpassing the iron regulation (Figure 5.0.1a).

Compared to the erythroid differentiation observed in healthy hematopoietic stem cells (HSCs), polycythemia vera (PV) exhibited increased proliferation during the immature erythroid stage and $CD235^{+}$ expressed in earlier stage. Nevertheless, the expressions of iron transporters were similar in both conditions. In iron deficient erythroid cells, terminal erythroid maturation was halted in healthy HSCs, while PV demonstrated diminished erythroid differentiation (Figure 5.0.1b).

5.7. Limitations of the study

The study has limitations, including challenges in ensuring compliance among pregnant women undergoing iron supplementation, particularly given the consistent observation of iron deficiency throughout the gestational period. Gene expression analysis requires further validation, given the post-transcriptional modifications commonly observed in many iron-regulatory genes. The relatively small sample size of pregnant women with iron deficiency anemia at delivery is noteworthy, and ethical constraints prevented the implementation of radio-iron transfer in this group. Some correlations could have shed more light on functional studies like EMSA. Validation of animal experiments at the protein level is essential, and establishing associations between serum iron parameters and iron, as well as erythroid

regulatory genes, would enhance our understanding of the mechanism. To gain a clearer understanding of erythroid regulation, it is crucial to conduct functional studies on placental prolactin-like E and estrogen. The beta-thalassemia cohort exhibited heterogeneity due to the presence of various types of beta-thalassemia variants among the patients. Obtaining an untransfused patient subset for the study of iron and erythroid mechanisms was challenging, as the majority had a history of multiple transfusions. *in vivo* erythroid differentiation remains a challenge in achieving the complete maturation of erythroid cells as comparable to those observed in vivo. The *in vitro* environment may not provide all the necessary factors for optimal maturation, leading to differences in morphology, functionality, and lifespan compared to natural erythrocytes.

5.8. Future directions

Over the past decade, considerable progress in understanding iron metabolism has identified key regulators like hepcidin, erythroferrone, and ferroportin. These regulators, studied for their functions in red cell disorders, and their agonists/antagonists are being explored for new therapeutic options. Our study has provided fresh insights into how iron regulation operates during increased erythropoiesis. A more in-depth examination at the level of single-cell transcriptomics could reveal detailed characteristics of the various genes involved in both erythropoiesis and iron homeostasis. Recent research underscores the crucial role of iron in the broader function of hematopoietic stem cells. Investigating the interplay between hematopoiesis and iron homeostasis further could aid in identifying novel therapeutic targets for beta-thalassemia and polycythemia vera.

6. Summary and Conclusion

Regeneration of red blood cells (RBCs) through an *in vitro* system serves as a model addressing blood transfusion shortages and enhancing therapeutic options for hematological diseases. The pivotal regulation of iron metabolism, particularly in hemoglobin synthesis, is essential for erythroid regeneration. A physiological increase in erythroid production is observed in pregnancy during the second trimester. We studied the role of iron and erythroid regulators in pregnant women at different gestational time points and at delivery in pregnant women with iron deficiency anemia (IDA). Additionally, we established a pregnant mouse model exposed to various iron diets to observe the roles of erythroid, iron, and various regulators in different organs during non-pregnancy, mid-gestation, and late gestation. Furthermore, we explored the impact of iron deficiency on erythroid differentiation in human hematopoietic stem cells (HSCs) and HSCs of patients with polycythemia vera. Iron and erythroid regulators were also examined in conditions of pathologically increased erythropoiesis, such as beta-thalassemia and polycythemia vera.

Our findings illustrated a gradual increase in erythropoietin and a decline in ferritin levels from the initial to the third trimester, indicating the utilization of mobilized iron to support increased erythropoiesis for placental and fetal development. Statistical analysis revealed a rise in erythropoiesis from the second trimester. This aligns with the redirection of increased iron from plasma to the bone marrow to support erythropoiesis. In the third trimester, erythroid activity stabilizes, with a significant portion of iron devoted to fetal iron endowment. During delivery, placental iron transporters like ferroportin and GDF15 respond to maternal iron deficiency by increasing their expression, facilitating adequate iron passage to the fetus.

In the context of pathological erythropoiesis in polycythemia vera (PV), erythropoietic activity governs the amount of iron transferred to the bone marrow by modulating intracellular iron in

erythroid cells and systemic iron regulation. In thalassemia, iron requirements in the bone marrow are influenced by ineffective erythropoiesis, impacting hepcidin regulation.

Both animal and *in vitro* studies revealed that systemic and intracellular iron deficiency modulates erythropoiesis and erythroid differentiation, respectively. Observations from animal models indicated that placental prolactin and estrogen may contribute to the regulation of increased erythropoiesis in pregnancy. Maternal regulatory hormone hepcidin expression was found to be diminished in cases of iron deficiency and elevated in iron overload mice compared to controls. This corresponded with a reduction in liver iron stores, transferrin receptor, and bmp6 expression at mid-gestation, key regulators of hepcidin production. Our study also demonstrated diminished bmp6 expression at mid-gestation, suggesting that erythroferrone (erfe) secreted by erythroid cells might inhibit bmp6 activity. During mid-gestation, elevated erfe expression in erythroid cells was noted, potentially aiding in suppressing the inhibition of bmp6 signaling (Figure 6.1.1).

Our study suggests that systemic and intracellular iron transport for erythropoiesis depends on the rate of erythroid activity in pathological states.

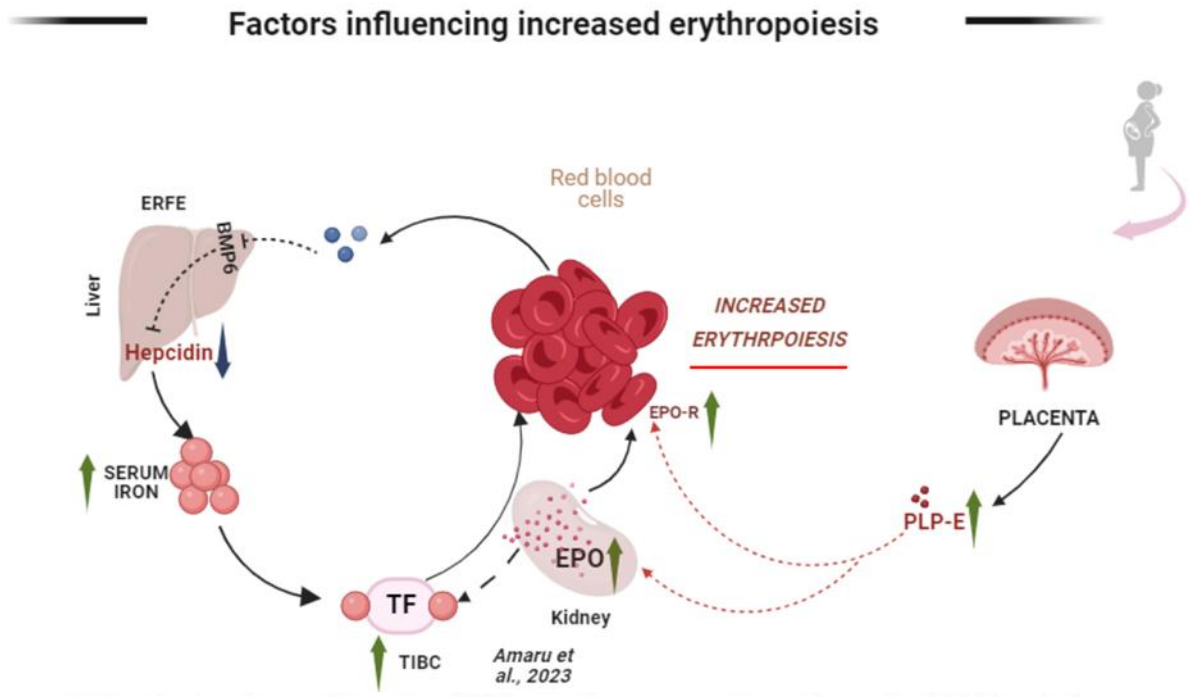


Figure 6.1.1. Summary of factors influencing increased erythropoiesis during pregnancy

During the 2nd trimester of pregnancy, increased iron demand causes decrease in hepcidin levels, which leads to increased iron mobilization towards bone marrow for erythropoiesis. Transferrin (transports iron) and erythropoietin levels escalated due to increased erythropoiesis and iron deficiency. These two factors in turn increase erythropoietic activity. Placental prolactin like -E (plp-e) is also involved in inducing increased erythropoiesis along with erythropoietin.

6.1. Significant findings of the study

- This is the first study to comprehensively evaluate the iron and erythroid regulation in pregnant women at various gestational stages and during delivery. Additionally, we established a pregnant mice model subjected to various iron diets to study in detail erythropoietic activity and iron homeostasis not only within the bone marrow but also in the placenta, maternal and fetal liver.
- To the best of our knowledge, this study stands as the first to draw comparisons between physiological and pathological erythropoiesis using human samples.

- In the second trimester, iron is predominantly utilized for elevated erythropoiesis. Erythroid activity reaches a stable state in the third trimester, and a significant portion of iron is dedicated to the endowment of fetal iron reserves.
- Increased expression of ferroportin and GDF15 in the placenta is most likely to represent an increased amount of iron transfer to the fetus despite the mother being iron deficient. Fetal iron metabolism is independent of maternal iron status.
- Estradiol, placental prolactin like -E (PLP-e), and iron regulators such as tfrc, dmt1, and hepcidin are essential in regulating increased erythropoiesis at the mid-gestational period. Validating their mechanism at the protein level might help in understanding the tight regulation of increased erythropoiesis during pregnancy.
- Insights from both animal and in vitro studies indicate that erythropoiesis is influenced by systemic iron deficiency, whereas intracellular iron deficiency plays a role in modulating erythroid differentiation.
- In pregnancy and polycythemia vera, hemoglobin levels could be sustained at either normal or elevated levels due to the enhanced supply of iron for hemoglobin production. On the other hand, in thalassemia, defects in hemoglobin synthesis result in erythropoiesis, overriding the regulation of iron levels.

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