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IN VITRO FERROPORTIN EXPRESSION IN NON-TRANSFUSION DEPENDENT THALASSEMIA DURING ERYTHROID DIFFERENTIATION

Dottorando : Laura SONZOGNI

Matricola N° R09544

TUTORE : Chiar.ma Prof.ssa Maria Domenica CAPPELLINI

COORDINATORE DEL DOTTORATO: Chiar.mo Prof. Mario CLERICI

SOMMARIO

INTRODUZIONE
Le β-talassemie sono una delle malattie genetiche più frequenti in tutto il mondo con 270 milioni di portatori e 350.000 nuovi nati affetti all'anno.
Questa malattia è geneticamente caratterizzata dalla perdita di produzione della catena β globinica dell'emoglobina adulta, dovuta a diverse mutazioni nel gene della β-globina. Poiché il gene beta è espresso su entrambi i cromosomi 11, possiamo avere due differenti tipi (e con differente gravità) di beta talassemia a seconda dell'assenza di entrambi o di un solo gene della beta globina: nel primo caso si ha la β talassemia MAJOR o trasfusione dipendente, nel secondo caso si ha la β talassemia MINOR o INTERMEDIA trasfusione indipendente. I nostri studi si concentrano su quest'ultima.
L'assenza della catena β globina comporta diverse conseguenze per l'organismo come:
- Eritropoiesi inefficace
- Il sovraccarico di ferro
- Il danno ossidativo
Finora sono stati condotti molti studi in diversi campi (genomico, proteomico e nel metabolismo ferro) per garantire una maggiore comprensione di questa malattia. Recentemente si è scoperta una nuova proteina che potrebbe essere un eventuale regolatore o responsabile del sovraccarico di ferro nella β - talassemia; questa molecola è la ferroportina.
La Ferroportina (FPN) è l'unico esportatore di ferro finora conosciuto. Essa è espressa in diversi tipi di cellule, tra cui gli enterociti duodenali, gli epatociti, i macrofagi e gli eritroblasti.
Pochi anni fa, è stata segnalata l'esistenza di due trascritti alternativi della FPN con o senza le Iron Responsive Elements (IRE) sul loro promotore (FPN1A e FPN1B rispettivamente). L'espressione delle diverse isoforme della ferroportina nonché i meccanismi che la regolano nelle cellule eritroidi della β talassemia non-trasfusione dipendente (NTDT) non sono ancora noti.

SCOPO
Studiare il profilo di espressione delle due isoforme della ferroportina durante il differenziamento eritroide in colture controllo e di NTDT e chiarire i meccanismi che regolano la loro espressione.

MATERIALI E METODI
Per questi studi è stato usato un modello di eritropoiesi in vitro derivato da cellule $CD34^+$ *provenienti da sangue periferico di volontari sani (controllo) e pazienti NTDT. Il profilo dell'espressione genica delle due isoforme (FPN1A e FPN1B) è stato valutato allo stadio basale (giorno 0) e al giorno 7 e 14 della cultura (stadio di pro eritroblasti e di eritrociti ortocromatici rispettivamente) mediante la tecnica di real-time PCR* (2^{-dCt})*. La percentuale relativa di ogni isoforma è stata calcolata sulla base dell'espressione della ferroportina totale (FPN1A + FPN1B). La concentrazione di ferro intra and extracellulare è stata analizzata utilizzando un kit di Ferro Assay (Biovision). In esperimenti indipendenti, colture di controllo e NTDT sono state trattate con: ferro (Ferro Ammonio Citrato [FAC] 100μM), Desferal (DFO, 4μM), protoporfirina (SNPP IX 50-20μM), eme (Emina 20-10μM) o perossido*

di idrogeno (H_2O_2 0,1mM) per indagare su un possibile ruolo di questi composti nella regolazione della ferroportina. L'espressione della FPN è stata valutata al 14esimo giorno in condizioni standard e nei trattati mediante la tecnica di real-time PCR (2^{-ddCt}; cellule non trattate utilizzate come calibratore).

RISULTATI

L'espressione ferroportina aumenta durante il differenziamento eritroide, raggiungendo il livello massimo di espressione allo stadio di eritroblasti (giorno 14 di coltura) sia nel controllo sia negli NTDT. La FPN1A è l'isoforma più espressa in entrambe le condizioni. La sua espressione è più elevata negli stadi iniziali e finali dell'eritropoiesi (giorno 0 e 14), mentre l'espressione della FPN1B è maggiore nella fase intermedia di differenziamento eritroide (giorno 7). Degno di nota, l'espressione della FPN1B, anche se inferiore rispetto alla 1A, è significativamente maggiore nelle culture NTDT rispetto ai controlli, in particolare al giorno 14. La concentrazione di ferro intracellulare è diminuita in modo significativo durante il differenziamento eritroide (dal giorno 7 al giorno 14), sia nei controlli sia negli NTDT, tuttavia, al giorno 7 (stadio di eritroblasti) i livelli di ferro nelle culture NTDT sono notevolmente inferiori rispetto ai controlli. L'aggiunta di FAC, DFO, SnPP IX ed Emina nei controlli e nelle colture di NTDT non ha modificato l'espressione della ferroportina rispetto ai non trattati. L'H_2O_2 aggiunto ai controlli aumenta l'espressione di entrambe le isoforme della ferroportina (FPN1A: cellule non trattate: 1; H_2O_2: 1.33 FPN1B: cellule non trattate: 1; H_2O_2: 2.04). I livelli di ferro intra ed extracellulari riflettono i risultati genetici: c'è stato un aumento di ferro extracellulare causa di un aumento di espressione FPN.

CONCLUSIONI

L'espressione della ferroportina aumenta durante il differenziamento eritroide sia nei controlli sia nelle culture NTDT, suggerendo il suo ruolo nell'esportare il ferro intracellulare in eccesso. In entrambe le condizioni, la FPN1A è l'isoforma più espressa. Tuttavia, l'espressione dell'isoforma 1B non responsiva al ferro, anche se minore rispetto a FPN1A, è significativamente maggiore nei NTDT rispetto ai CTRL. In colture di controllo, l'espressione della FPN, ed in particolare dell'isoforma 1B, sembra essere regolata dall'aggiunta di H_2O_2. Questi dati suggeriscono che lo stress ossidativo, particolarmente elevato nelle NTDT, potrebbe essere uno dei principali regolatori dell'espressione dell'isoforma 1B, generando così un'importante esportazione di ferro dalle cellule NTDT.

ABSTRACT

INTRODUCTION

β-Thalassemias are one of the most frequent genetic disorders worldwide with 270 million of carriers and 350.000 affected new-borns per year.

This disease is genetically characterized by the loss of production of the β globin chain of the adult haemoglobin, due to several mutation within the beta globin gene. Since the beta gene is expressed on both the chromosomes 11, we can have two different type (and severity) of beta thalassemia depending on the absence of both or just one beta gene: in the first case we have the β thalassemia MAJOR transfusion dependent, in the second case we have the β thalassemia MINOR or INTERMEDIA, transfusion independent. Our studies are focused on the last one.

The absence of the β globin chain implies different consequences for the organism like as:

- *Ineffective erythropoiesis*
- *Iron overload*
- *Oxidative damage*

Many studies have been conducted so far in different fields (genomic, protein expression and regulation, iron metabolism) in order to guarantee a major comprehension of this disease. Recently a new protein came out as a possible regulator/responsible for the iron overload in β thalassemia; this molecule is the FERROPORTIN.

Ferroportin (FPN) is the only know iron exporter protein. It is expressed in different cell types including duodenal enterocytes, hepatocytes, macrophages and erythroblast cells.

Few years ago it has been reported the existence of two alternative transcripts of FPN with or without an iron – responsive element (IRE) on their promoter (FPN1A and FPN1B respectively). The expression of the different ferroportin isoforms as well as the mechanisms regulating their expression in erythroid cells in non-transfusion dependent β thalassemia syndromes (NTDT) are not known yet.

AIM

To investigate the expression profile of ferroportin isoforms during erythroid differentiation in control and NTDT cell cultures and to elucidate the mechanisms regulating their expression.

MATERIALS AND METHODS

An in vitro model of erythropoiesis derived from human peripheral CD34+ cells from healthy volunteers (control) and NTDT patients was used. The expression profiling of FPN isoforms (FPN1A and FPN1B) was evaluated at baseline (day 0) and at day 7 and 14 of culture (pro erythroblasts and orthochromatic erythroblasts stage respectively) by real−time PCR (2^{-dCt}). The relative percentage of each isoform was calculated based on total ferroportin expression (FPN1A+FPN1B). The intracellular iron concentration was analyzed by using an Iron Assay Kit (Biovision). In independent experiments, control and NTDT cultures were treated with iron (Ferric Ammonium Citrate [FAC] 100μM), Desferal (DFO, 4μM), protoporfirin (SnPP IX 50-20μM), heme (Hemin 20-10μM) or hydrogen peroxide (H_2O_2 0.1mM) to investigate a possible role of these compounds in ferroportin

regulation; FPN expression was evaluated at day 14 in standard and treated conditions by real−time PCR (2^{-ddCt}; untreated cells used as calibrator).

RESULTS

The ferroportin expression increased during erythroid differentiation; with the highest level at the end of erythroblasts stage (day 14 of cultures) both in control and NTDT cultures. The FPN1A was the more expressed isoform in both conditions. Its expression was higher at the initial and final steps of erythropoiesis (day 0 and 14), while FPN1B expression was higher at the intermediate erythroblast stages (day 7). Noteworthy, the FPN1B expression, although lower compared to FPN1A, was significantly higher in NTDT cultures than in control ones, particularly at day 14. The intracellular iron concentration decreased significantly during erythroid differentiation (from day 7 to day 14) both in control and NTDT cultures, however, at day 7 (early erythroblasts stage) the iron levels in NTDT cultures were notably lower than in controls. The addition of FAC, DFO, SnPP IX and Hemin in control and NTDT cultures did not modify the ferroportin expression compared to untreated. H_2O_2 added to control cells increased the expression of both ferroportin isoforms (FPN1A: untreated cells: 1; H_2O_2: 1.33. FPN1B: untreated cells: 1; H_2O_2: 2.04). The intra and extracellular iron levels reflected the genetic results: there was an increase of extracellular iron due to an increase of FPN expression.

CONCLUSIONS

The ferroportin expression increases during erythroid differentiation either in control than in NTDT cultures, suggesting its role in exporting the excess intracellular iron. In both conditions, the FPN1A is the more expressed isoform. However, the expression of the non−iron responsive FPN1B isoform, although lower compared to FPN1A, is significantly higher in NTDT than in control conditions. In control cultures, FPN expression, and particularly the FPN1B isoform, seems to be up regulated by H_2O_2 addition. These data suggest that the oxidative stress, notably higher in NTDT conditions, could be one of the major regulator of FPN1B expression, with a major iron export from NTDT erythroblast cells.

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LIST OF SYMBOLS & FIGURES

Symbols	Description
AKT (PKB)	Protein Kinase B
Bach1	BTB and CNC homology 1, basic leucine zipper transcription factor 1
BCL11a	B-Cell CLL/Lymphoma 11a (zinc finger protein)
Bcl-XL	B-cell lymphoma-extra large (transmembrane protein in mitochondria)
BFUe	Burst Forming Unit erythroid
BSA	Bovine Serum Albumine
CFUe	Colony Forming Unit erythroid
CFU-GEMM	Colony Forming Unit – Granulocytes, erythrocytes, Monocytes. Megakariocytes
ChIP	Chromatin Immuno Precipitation
CTRL	Control
DFO	Desferal
DMT1	Divalent Metal Transporter 1
DNMT	DNA methyltransferase
EKLF	Erythroid Kruppel - like factor
EPO	Erythropoietin
EDTA	Ethylenediaminetetraacetic acid
FAC	Ferric Ammonium Citrate
FOG	Friend of GATA
FPN	Ferroportin
FPN1A	Ferroportin isoform 1A
FPN1B	Ferroportin isoform 1B
G-CSF	Granulocytes Colony Stimulating Factor
GM-CSF	Granulocytes Macrophages Colony Stimulating Factor
H2O2	Hydrogen Peroxide
HAMP	Hepcidin
Hb	Haemoglobin
HbA	Adult Haemoglobin
HbA2	A2 Haemoglobin
HbF	Foetal Haemoglobin
HDAC	Histone deacetylase
HIF1α	Hypoxia Inducible Factor 1α
HLA	Human Leucocyte Antigen
HRE	HIF Responsive Element
HSC	Human Stem Cell
IL-3	Interleukin
IRE	Iron Responsive Element
IRP	Iron Responsive Protein

JAK	Janus Kinase
KO	Knock – out
LCR	Locus Control Region
LMO2	LIM domain only 2 (rhombotin – like 1)
MAP	Mitogen Activated Protein
MARE	Maf Recognition Element
NF-E2 (NFR2)	Nuclear Factor Erythroid 2
Nramp2	natural resistance-associated macrophage protein 2
NTBI	Non Transferrin Bound Iron
NTDT	Non – transfusion Dependent Thalassemia
NuRD	Nucleosome Remodelling Deacetylase
PBS	Phosphate Buffered Saline
PI3	Inositol triphosphate
QTL	Quantitative Transcription Loci
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
SnPP IX	Protoporfirin IX
SOX6	SRY (sex determining region) – box 6
SP1	Specific protein 1
STAT	Signal Transducer and Activator of transcription
TAL1	T-cell acute lymphocytic leukemia protein 1
Tf	Transferrin
TfR	Transferrin Receptor
TI	Thalassemia Intermedia
TM	Thalassemia Major
TPO	Thyroid Peroxidase
U	Untreated
UTR	Untraslated Region

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INTRODUCTION

CHAPTER 1: THE IRON METABOLISM

1.1 The Iron

Iron is a significant and extremely important molecule for all the organisms survival since is a heme and myoglobin component, and is involved in numerous metabolic reactions (such as: ossido/reduction, respiratory chain, DNA synthesis). Its characteristics have a certain ambiguity because one side is observed the importance for the survival, on the other hand the ability to react with oxygen, causing the production of free radicals and thus be toxic to tissues.

This reaction, called *Fenton reaction*, is reported below:

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + OOH\cdot + H^+$$

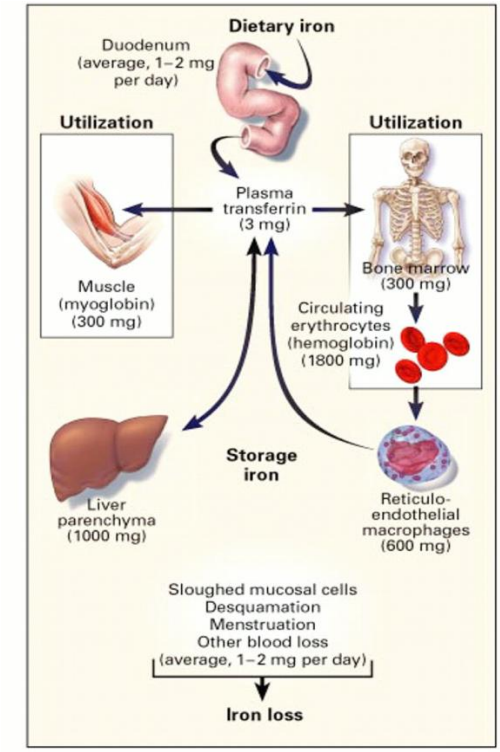
In solution, the iron may have two oxidation states: Fe (II) or Fe 2^+, and Fe (III), or Fe 3^+; is poorly soluble at physiological pH, especially when it is in the oxidized form Fe (III); then the organisms had to obtain various proteins capable of transporting the ion in biological fluids and across cell membranes and to store it in the tissues in a non-toxic form and easily metabolizable.

In physiological conditions, an adult body contains an average of 1.8 to 3.5 grams of iron that could be split up in a functional compartment represented by haemoglobin (about 3 grams), mioglobin (100-150 milligrams) and intracellular enzymes (8-10 milligrams), and another compartment of deposit represented by ferritin (300-1000 milligram) (Figure 1.1).

The heme content iron is continuously recycled and recovered through phagocytosis by the cycle of senescent red blood cells that are degraded by macrophages forming part of the reticuloendothelial splanchnic. This process allows the recovery of about 25-30 mg of iron daily, the quantity corresponding to that required to ensure erythropoiesis.

Intestinal absorption is the only way to introduce the ion in the body and is a process closely and finely tuned; at the level of the duodenal villi are absorbed daily about 1-2mg of iron. The elimination instead takes place through processes of epithelial desquamation, loss of blood, menstruations, all mechanisms not subjected to homeostatic regulation.

Figure 1.1 : Iron Absorption, Distribution and Elimination through the Organism



Source: Andrews NC. New England Journal of Medicine 1999; 1986-1995.

1.2 The Intestinal Absorption of Iron

Iron absorption is related to three main factors: 1) the content of iron in the diet: it is normally absorbed 5-10% of the iron present in food; 2) the bioavailability of the metal: the heme iron is more easily absorbed than non-heme, whose absorption can be influenced by several factors in a negative

(phosphates and tannates), or in a positive sense (ascorbic acid); 3) the integrity of the mechanisms of iron uptake from the intestinal lumen to the enterocyte and the transportation from the latter to the bloodstream.

Intestinal absorption is limited to the duodenum, the cells responsible for that are the enterocytes, placed on the surface of villus. The iron enters into the cell through the apical membrane and is transferred to the basal side before being exported to the plasma through the carrier called Ferroportin. Not all of the iron is introduced into the bloodstream, part of it remains into the enterocyte, bound to ferritin and it will be removed during the process of exfoliation of the intestinal wall.

The first step for the iron absorption is the reduction: there is the passage from the ferric state to the ferrous thanks to the action of a reductase placed on the apical membrane of the enterocyte. Once reduced (Fe (II)), the iron enters into the cell through the transporter Nramp2 / DMT1 (its synthesis is strongly enhanced by iron depletion).

Once reached the vascular bed, the iron binds to transferrin (Tf), the main transport protein, which provides the transfer of iron to various tissues. It is very important that the iron does not remain free in the cells or in the bloodstream, it has to be bound to proteins because free iron participates in a reaction that generates oxygen free radicals damaging the tissues.

1.3 The Hematic Iron Recycling

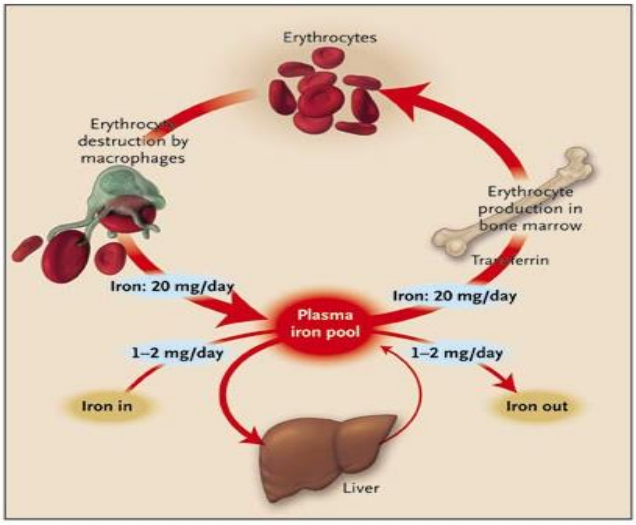
Most of the iron in the body is bound to haemoglobin and the phagocytosis of senescent erythrocytes by macrophages guarantees an efficient recycling of iron.

Daily, about 20-25 mg of iron are retrieved by this route of recycling and they will then be used for the production of new haemoglobin during the erythropoiesis in the bone marrow. (Figure 1.2)

Macrophages responsible of the degradation of senescent red blood cells are found mainly in the spleen, bone marrow and in the Kupffer cells. During their stay in the bloodstream (about 120 days), red blood cells undergo biochemical changes in the level of their membrane: externalization of phosphatidyl - serine, lipoprotein peroxidation, loss of sialic acid residues and antigen expression of senescence that are signals for macrophages ranging so to identify the cells that need to be degraded by a process of phagocytosis.

The heme group is destroyed and the iron bound to it is released into the cytoplasm of the monocyte, the ion can now take different paths: be stuck to the ferritin and remain within the cell, or to come out from it by the ferroportin action and then be picked up by the plasmatic transferrin.

Figure 1.2 : Hematic Iron Recycling



Source: Pietrangelo A. ; "Hereditary hemochromatosis--a new look at an old disease." N Engl J Med 2004

1.4 The Intracellular Iron Homeostasis: the IRE/IRP System

The synthesis of key proteins involved in iron metabolism, its transport, storage and use is controlled at the post-transcriptional level through the intracellular iron. This adjustment depends on the interaction between cytoplasmic proteins called "Iron Regulation Proteins" (IRPs), which act as sensors of the iron, and the "Iron Responsive Elements" (IREs), which are highly conserved motif of 30 nucleotides at the level of '3 'end or 5' mRNA having a steem - loop or ring conformation. (Figure 1.3)

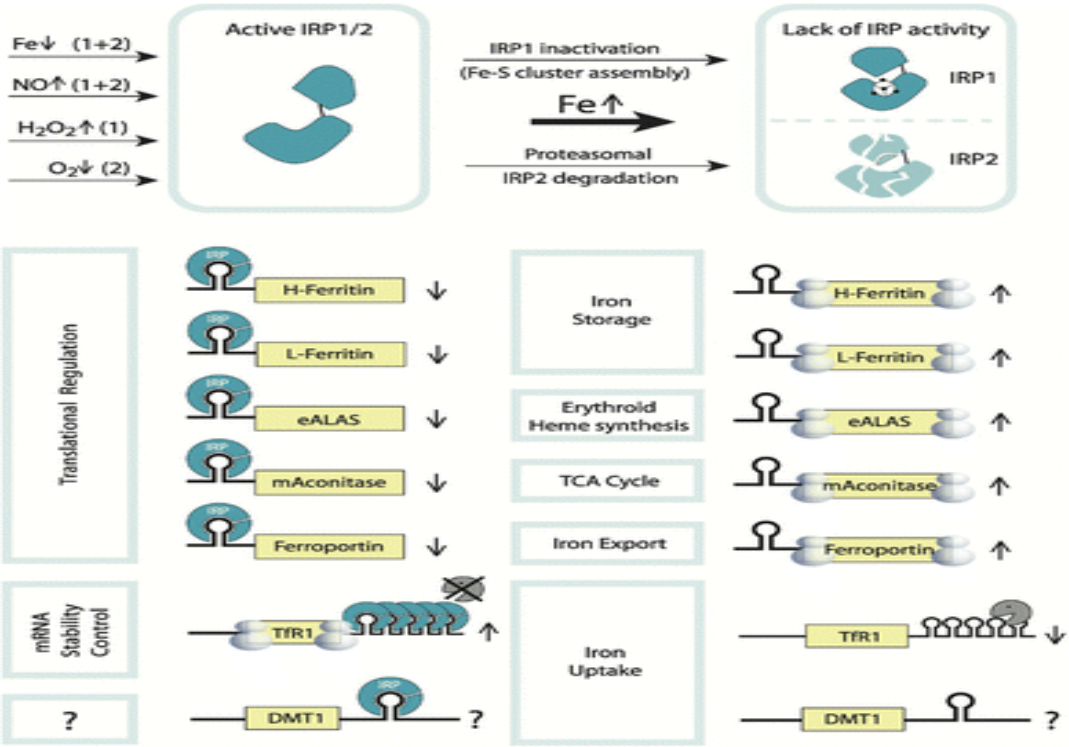
The loop is characterized by a consensus sequence as follow: 5 '- CAGUGN - 3' which will be recognized by the IRP.

Single IREs are present at the 5' non-coding region of the mRNA of the H or L subunit ferritin and ferroportin. A single IRE was found the 3' end of the mRNA of the protein responsible for the transport of iron (transferrin receptor TfR1 and DMT1). There are two distinct forms of IRP: IRP1 and IRP2 which have a high affinity for IREs regions. The entry of the iron in the cell causes a change of the conformation of IRP1 through the acquisition of a cluster iron - sulfur (4Fe-4S) and degradation of IRP2 due to oxidation.

If an IRP should recognize the IRE of ferritin mRNA, this bond would prevent the translation of the protein as it would be impossible for the ribosome binding to the 5' of the transcript; if the IRP binds the 3' end, this would ensure a better stabilization of the polyA tail resulting in an increased translation of the gene.

This post – transcriptional regulation iron - modulated allows to modify the cell's ability to absorb the ion according to the need.

Figure 1.3 : The Action of IRE/IRP



Source: Valenti L. presentation: "Metabolismo del Ferro"2010

1.5 The Iron Homeostasis at the Organism Level: the Role of Hepcidin and Ferroportin.

As already mentioned, there is no specific mechanism by which the body can eliminate the iron absorbed in excess; the iron overload can be avoided through the control of intestinal absorption and the macrophagic recycling ways.

In 2001 it was discovered a hepatic protein with antimicrobial function called hepcidin (hepcidin, hep = hepatocyte, idine = indicates the antimicrobial activity).

Hepcidin is a hepatic peptide hormone, its gene contains three exons and encodes a pro peptide of 84 amino acids having a signal peptide and a site for cutting by a furin that ensures the production of a functional protein.

Hepcidin is one of the acute phase proteins, it has a structure of 25 amino acids containing 8 cysteine residues that form four disulfide bonds. Humans possess a single copy of the gene for the hormone (HAMP), while mice have a tandem duplication of the gene sequence.

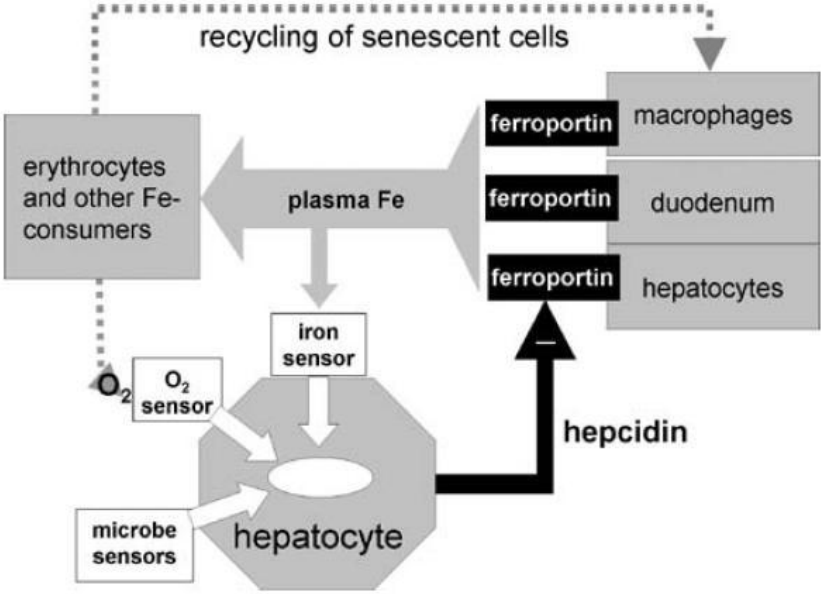
From the evolutionary point of view, there is a high conservation of the gene sequence, from fish to humans.

The role of hepcidin in the innate immune response is consistent with its regulation by inflammatory cytokines such as interleukin (IL) - 6, IL - 1α, IL - 1β.

In addition to its anti-inflammatory activity, hepcidin plays a key role in iron homeostasis, specifically in the control of its absorption.

The essential role of hepcidin in the context of iron metabolism has been established by the study of mouse models deficient for the peptide. The hepcidin KO mice showed a huge iron overload, with a substantial ion deposition in liver tissue, pancreas and splanchnic macrophages. These effects have led to the conclusion that hepcidin was directly involved in iron absorption in the gut and control of ion release by macrophages. [1] (Figure 1.4)

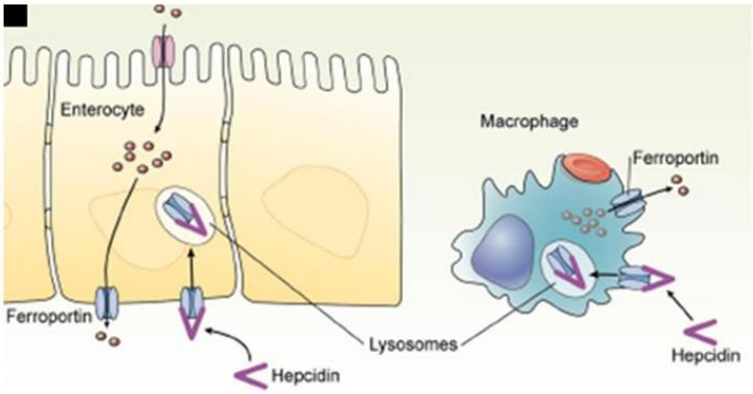
Figure 1.4 : A Model of Homeostatic Regulation of Iron by Hepcidin



Source: Nemeth E., Ganz T. : "regulation of iron metabolism by hepcidin" Annual Reviews Nutritional 2006

Hepcidin has a region to the N terminal, which consists of 5 amino acids that has high affinity for FERROPORTIN: their bond causes the internalisation and degradation of the latter, thus ensuring a brake in the export of iron absorbed by the basal membrane of the enterocyte to the blood stream. [2] (Figure 1.5)

Figure 1.5 : the Action of Hepcidin on Ferroportin



Source: Andrews NC. "Forging a field: the golden age of iron biology." Blood. 2008 Jul 15; 112(2):219-30.

It is well established that hepcidin reduces the amount of circulating iron, preventing its escape from the cells (mainly enterocytes and macrophages) and its entry into the bloodstream by the enterocyte; on the contrary its absence causes an increase of iron in the circulation due to the intestinal absorption and its release by macrophages.

1.6 The Ferroportin

Ferroportin is the sole iron exporter so far known in vertebrates. Studies in zebrafish and mice have shown that the complete loss of the exporter during embryonic development is lethal due to the inability of the ion transport across the placenta from the mother to the embryo.

The presence of ferroportin was detected in all tissues that have a greater influx of iron, such as duodenal enterocytes, macrophages and hepatocytes. Studies on mice with a selective silencing of the gene, which makes ferroportin expressed on the interface foetus - mother, but is inactive in other tissues, they observed the early development of iron depletion in the new offspring caused by the inability to export the iron from the gut into the bloodstream. [1]

Ferroportin (FPN, FPN1 or SLC40) is an exporter having 12 transmembrane domains, with both C and N terminals placed in the cytosolic side; it is present on the basolateral membrane of enterocytes, on the membranes of macrophages and non-polarized cells (red blood cells). It exports the ferrous ion ($Fe2^{+}$), which must be oxidized to the ferric state by a ferroxidase, also needed to stabilize the ferroportin at the level of the membranes, before the ion exported binds to transferrin.

1.6.1 Ferroportin and Erythropoiesis

An adequate intake of iron is important to have adequate erythropoiesis, it is known that about 70% of the iron in the body is included in the heme group. On the other hand, must be very careful that the iron does not accumulate in the erythroblasts during the process of maturation, causing cellular toxicity resulting in apoptosis (in this case, haemolysis); for this reason it is necessary that the cells of the erythropoietic lineage are equipped with an iron exporter such as FPN1. The discovery of FPN1 on the membrane of erythroblasts is surprising because it was thought that these cells did not need an iron exporter since it was assumed that the cellular ion leakage happen only during the process of degradation by macrophages.

Nevertheless, it seems that the expression of FPN1 on red blood cells (RBCs) is kept constant throughout the process of cellular maturation, even by subjecting the cells to iron chelation treatments or saturation; this leads to suppose that on RBCs is present, in addition to FPN1, another isoform that is not IRE dependent.

Currently it is possible to affirm that there are two isoforms of ferroportin: the IRE dependent: FPN1A and non - IRE: FPN1B.

It has been recently demonstrated by Cianetti and Zhang that both transcripts are expressed mainly during the intermediate step of erythropoietic differentiation in vitro (days 4-11) when we have

polychromatic erythroblasts in cell culture; at this stage there is an increased expression of the transferrin receptor, solely responsible for the entry of iron in erythroblasts. The FPN1B increases its expression when cells need iron to accumulate in them, it is thought that the cells during erythroid differentiation transcribe a constant level of FPN1B that remains insensitive to high levels of iron present in the erythroblasts.

The FPN1A instead is transcribed at high levels on day 0 of erythropoiesis, when we still have erythroid precursors in cell culture and at the final step of maturation (day 14) when we get mature RBCs, suggesting a possible role in these stages of differentiation.

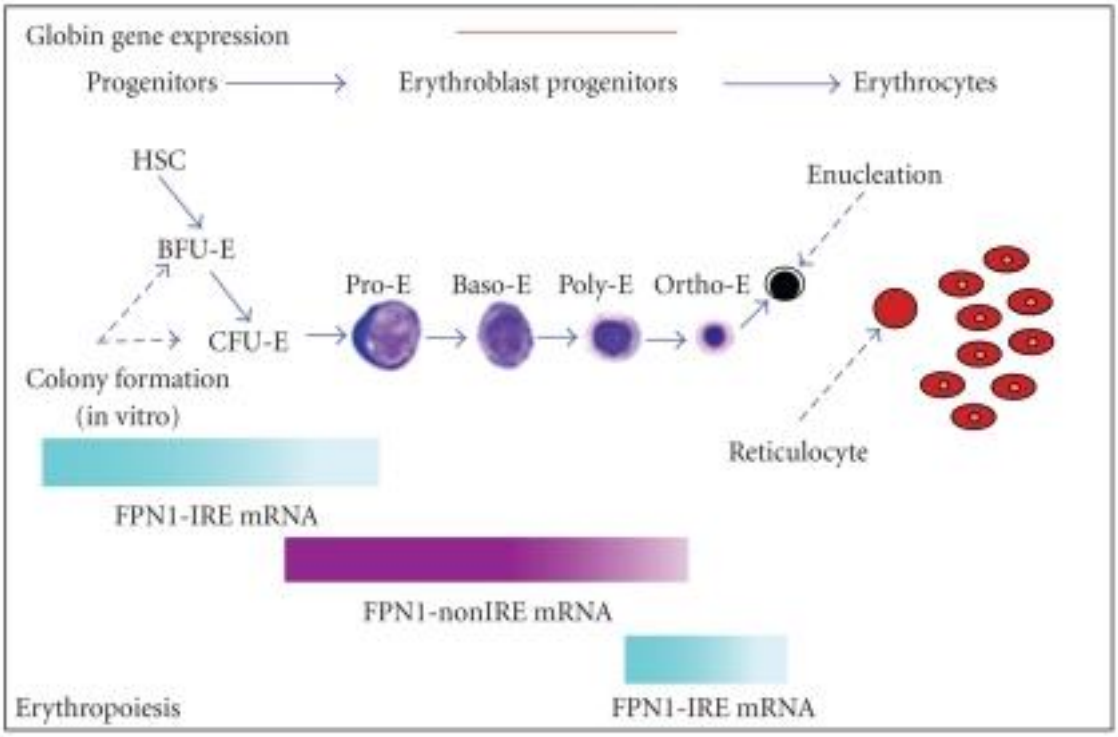
The lack of production of FPN1A at the intermediate step of erythroid maturation can be attributed to the fact that, at this level of differentiation, the cells are in need of a lot of iron, therefore it would be deleterious having an exporter that is expressed in function of martial concentrations; for this reason the cells of the erythroid lineage express the isoform 1B in a constant manner throughout the differentiation, since the protein has the role to protect the cellular toxicity caused by extremely high concentrations of iron. [3] (Figure 1.6)

The two isoforms of FPN1 arouse much interest, a recent study has suggested that the functionality of the exporter, located on the erythrocytes membrane, is to ensure a partial suppression of erythropoiesis if the non erythropoietic tissues risk the development of iron depletion. This explains why situations characterized by iron deficiency (anaemia) are the main conditions that herald the manifestation of iron depletion in mammals. [4]

The discovery of FPN1B may have important physiological implications. Since the FPN1 is locked by hepcidin, the exporter in the erythroblasts is functional only when there is a situation of iron depletion (in which hepcidin is poorly expressed). The expression of FPN1B in the erythroblasts can represent a development of a mechanism to prevent an erroneous

distribution of iron. In conditions of wide availability of the ion at the systemic level, the down-regulation of FPN1 ensures the retention of the iron inside the erythroblasts, which will require large amounts to ensure the production of heme and, consequently, to allow erythropoiesis. In contrast, in conditions of iron deficiency, erythroblasts allow other tissues (such as heart and brain) to have priority regarding the iron uptake, preventing these organs to be iron deficient. Whereas erythroblasts have an efficient mechanism for the recovery of iron (the transferrin receptor is highly expressed on the surface of these cells) and that only 1% of the erythrocyte population is recycled daily, is decidedly better for the body to decrease the erythropoietic activity during a condition of iron depletion and thus avoid the risk of shortage of the ion in the vital organs. The connection between the expression of hepcidin and ferroportin at the level of erythroblasts may explain why anaemia is commonly the first symptom of a systemic iron deficiency. [4][5]

Figure 1.6 : FPN1 Expression During Erythropoiesis



Source: Cianetti L. Gabbianelli M. Sposi N.M.:" Ferroportin and Erythroid Cells: an Update". Advance in Hematology, 2010

1.6.2 Transcriptional Regulation of FPN by non-Iron Molecules

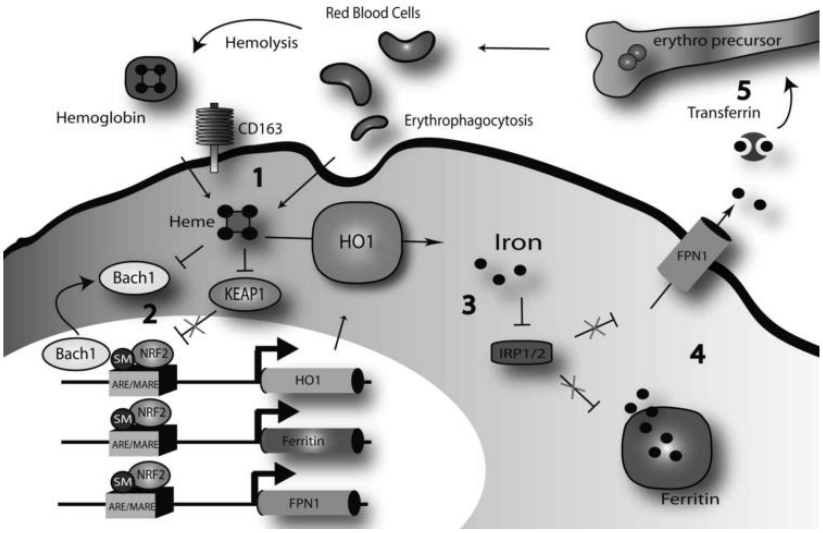
- Heme

Recent studies conducted by Marro, lead to the conclusion that, in the macrophages, there is another mechanism of regulation of FPN1 non-iron related. They discovered that an increasing of haemoglobin caused an increase of FPN1 expression, with the possible conclusion that the haemoglobin can directly regulate the transcription of the iron exporter through a system involving the Btb And Cnc Homology 1 (Bach1), Nuclear Factor Erythroid 2-like (NFR2) and the Maf Recognition Elements (MAREs). (Figure 1.7)

Heme controls the transcription of the iron exporter FPN1 involving Bach1 activity, Nrf2 nuclear accumulation and a highly conserved MARE/ARE enhancer element located at the FPN1 promoter. This finding suggests that iron recycling from heme involves a single transcription control mechanism that regulates heme catabolism, iron storage and detoxification, as well as iron export in a coordinated manner. [6]

In the macrophage cell, heme and/or haemoglobin induced FPN1 transcription may thus enhance systemic iron availability to sustain erythropoiesis under conditions when intact erythrocytes are limiting.

Figure 1.7: How Heme/Haemoglobin Can Regulate the FPN1 Transcription



Source: Marro S., Chiabrando D., Messana E., Stolte J., Turco E., Tolosano E., Muckenthaler MU.: "Heme controls ferroportin1 (FPN1) transcription involving Bach1, Nrf2 and a MARE/ARE sequence motif at position -7007 of the FPN1 promoter". Haematologica, 2010

- HIF1α

One of the major physiological cues for increased iron absorption is hypoxia/anaemia. Increased erythropoiesis leads to increased iron absorption and elevated levels of FPN mRNA. McKie et al.'s discovery of FPN was based on increased expression of its mRNA in the duodenum of the hypotransferrinemic mouse [7]. This mouse is severely anaemic and shows increased hypoxic response. Hypoxia leads to wide changes in transcription including genes involved in iron [8] The Fpn promoter contains HIF-Responsive Elements (HRE) and an Fpn reporter construct responded to low oxygen (studies confirmed by ChIP on the Fpn promoter).

1.6.3 Post – Transcriptional and Post - Translational Regulation of FPN

The FPN mRNA was identified by its ability to bind to Iron Regulatory Protein 1 (IRP1). Subsequent studies showed that translation of FPN or FPN reporter constructs containing the IRE was inhibited by low iron and increased by high iron. The importance of the 5′-IRE was underscored by the discovery of a mouse that had a mutation that deleted the IRE. A radiation-induced mutation in mice was identified, resulting in a 58bp micro deletion in the Fpn promoter region [8]. This deletion altered the transcription start site and eliminated the 5′-IRE, resulting in increased duodenal and hepatic Fpn protein levels during early postnatal development. It is unclear why Fpn mRNA levels were decreased. Iron overload in adult mice was attributed to increased Fpn protein expression due to the absence of the 5′-IRE. Age-related anaemia was ascribed to a loss of splenic stromal cells. The reason for this loss was not determined. Analysis of mRNA transcripts in mouse duodenum and erythroblasts and in human erythroblasts identified a FPN transcript which lacked the 5′-IRE, termed FPN1B. The translation of FPN1B is insensitive to iron. The reading

frame of the protein, however, is identical to that of the IRE-containing transcript and the protein can transport iron and respond to hepcidin. FPN1B accounted for 25% of total FPN mRNA in duodenum but constituted a much higher percent of FPN transcripts in erythroblasts [5]. It was suggested that the FPN1B in the intestine might still export iron even under conditions of iron deficiency. In the developing erythroblast, iron-insensitive translation of FPN might make erythropoiesis sensitive to hepcidin levels and thus sensitive to systemic iron deficiency [4].

1.6.4 Post - Translational Regulation of FPN

The importance of FPN1 is put in further evidence when it is related to hepcidin, because the iron exporter is also the ligand of the hepatic hormone. Hepcidin binds to ferroportin and promotes its internalization through phosphorylation of amino acids placed at the level of the intracellular loop of the exporter. The complex FPN1 - hepcidin is so internalized and promoting the ubiquitination and lysosomal degradation of both proteins. [9][10](Figure 1.8)

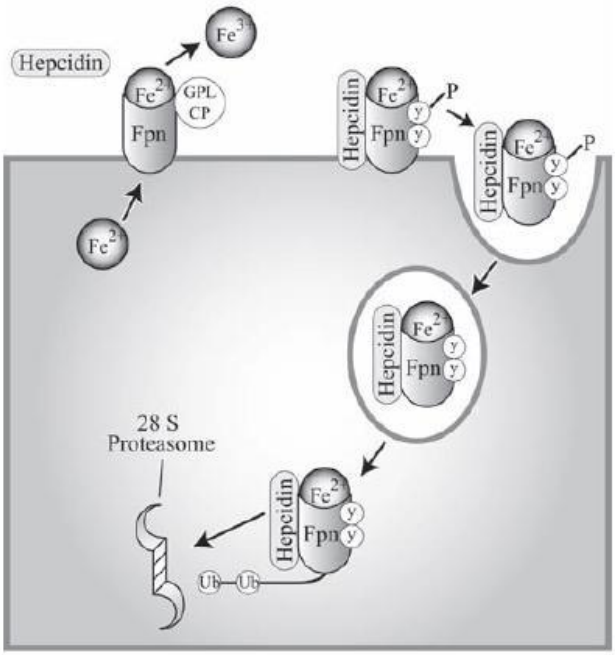
The roles of hepcidin and FPN were cemented by the striking observation that mice with a targeted deletion in Hamp were massively iron-loaded [11]. In contrast, overexpression of hepcidin in mice led to severe iron deficiency [12]. A study in humans showed that overexpression of hepcidin, as seen in a severe example of the Anaemia of Chronic Inflammation, resulted in hypoferremia [13]. In contrast, the recessive iron overload diseases were due to decreased levels of hepcidin [14]. These studies showed that hepcidin affected entry of iron into blood. How this occurred was elucidated by Nemeth et al., who demonstrated that hepcidin bound to and induced the internalization of FPN expressed in cultured cells [2]. The observation that FPN levels were affected by hepcidin explained both recessive iron overload disorders resulting from decreased hepcidin and iron deficiency disorders resulting from increased levels of hepcidin. The relationship

between hepcidin and intestinal FPN, however, remains cloudy. Chronically high levels of hepcidin lead to loss of intestinal FPN, but studies have shown that acute changes in hepcidin have a modest effect on intestinal FPN levels. At the same time, however, acute changes in hepcidin have a significant effect on FPN levels in splenic macrophages [15][16]. Analogous results were reported in intestine- like cell lines; acute hepcidin exposure had little effect on FPN levels but did affect iron transport activity. The mechanism of hepcidin-mediated FPN internalization in macrophages and many other cultured cell types has been described. The binding site for hepcidin has been identified as an extracellular loop in FPN in which the residue Cysteine 326 is absolutely required for hepcidin binding [17]. Mutation of C326 leads to hepcidin- resistant hemochromatosis in patients.

The importance of FPN in iron homeostasis is supported by the fact that it is regulated at many different levels. Many of aspects of that regulation remain to be clarified. The transcriptional regulation of FPN in response to iron or to inflammation requires identification of the critical transcriptional activators or repressors. What also requires clarity is the reason for the cell type variation in transcriptional regulation of FPN and in the subcellular location of FPN. Why do different FPN mutants show differences in subcellular localization in different cell types?

This lead us to further investigate about the role of this protein in the big puzzle of the iron metabolism.

Figure 1.8 : Internalization and Degradation of the Hepcidin – Ferroportin Complex



Source: Mackenzie E.L., Iwasaki K., Tsuji Y. "Intracellular Iron Transport and Storage: From Molecular Mechanisms to Health Implications" Antioxidant and Redox Signaling; Volume 10, Number 6, 2008

CHAPTER 2: THE THALASSEMIC SYNDROMES

The thalassemias are a heterogeneous group of Mendelian disorders, which are characterized by a lack or a decreased synthesis of α or β chain of haemoglobin A (α2β2). [18] On the basis of the chain missing it is possible to distinguish between α-thalassemia and β-thalassemia. The thalassemias are one of the most common genetic disorders in the world with an estimated number of bearers more than 270 million and more than 350,000 people affected; they are particularly affected the populations of the Mediterranean, Africa, India and the Orient. In Italy are prevalent forms of β-thalassemia, which is endemic mainly in Sicily and Sardinia. [19]

2.1 The β - Thalassemia

The β-thalassemia is characterized by the deficient synthesis of β chains, while the synthesis of α chains remains unchanged. The altered production of β chains is due to the presence of point mutations or small deletions at the level of the β globin cluster on chromosome 11. Such mutations may cause a failure or reduced synthesis of β chains; we speak respectively of β^0 and β^+ thalassemias. [18] [20]

Genotypically the β thalassemias are very heterogeneous; up to date more than 200 mutations in the beta globin gene causing partial deficiency (α^+ or β^{++}) or total (β^0) of synthesis of beta globin chain are known. Defects in transcription, maturation and translation of messenger RNA have been identified. Defects by deletion of the gene of beta globin are rare and limited to sporadic families.

Here is a list of the major flaws of expression of the beta globin gene:

- **Defects in transcription**: in most cases, they are point mutations within the promoter region, at the 5' end of the gene. Simple nucleotide substitutions are able to reduce of 30-50% the transcription of the beta gene and mutations are defined β^+ as there is a residual activity of

transcription. The hematologic phenotype is very mild, sometimes completely silent. Small deletions at the 5' end of the gene responsible for complete inactivation of the transcription process have been detected in few cases. In this case, the defects are defined β^0.

- **Defects in the maturation of messenger RNA**:

1) *Alterations of splicing*: about half of the thalassemia mutations fall into this group. It is required, for a proper splicing, that at the junction between introns and exons, there is the presence of 2 pairs of dinucleotides unchanged: GT at the 5' and AG at the 3' of the intron. Intronic mutations can delete a normal site of splicing by destroying one of the two nucleotides unchanged. In Italy the most frequent mutations of this type are IVSI - 1 G/A and IVSII-1 G/A both β^0. Mutations that reduce the efficiency of splicing by altering its consensus sequence are also known. Among these, the most frequent in Italy is the IVSI - 6 T/C, that leaves a residual activity and it is defined β^+ mutation. In this context, other mutations in β^+ (with low residual activity) are those that create another splice site by producing the GT or AG nucleotides in a site that competes with the normal one. The most common mutation in Italy are IVSI – 110 and IVSII - 745.
2) *Mutations in the cutting site and at the polyadenylation site of pre-RNA*: in the process of maturation of the primary transcript of the RNA messenger, or pre-RNA, it is important to cut the terminal part in correspondence with the sequence AATAAA and the addition of a tail of residues of adenylic acid (poly-A) which increase the speed of translation and the stability of the messenger. If the sequence AATAAA is changed, the two processes occur much more downstream and the mRNA produced is longer and unstable. These mutations lead to defects of type β^+.

- **Defects of translation of RNA messenger**:

1) *Mutations in the start codon of translation*: all give rise to defects of type β^0, and are sporadic mutations.

2) *Non-sense mutations*: they consist in the substitution of a purine base in the codon of coding RNA, which gives rise to the formation of a stop codon resulting in an arrest of translation of the messenger. The most common mutation is the $\beta^0$39 (or cod39).
3) *Frame shift mutations*: it comes to insertions or deletions in the DNA of one or more nucleotides that cause a shift of the reading frame of the RNA messenger and hence a more or less early arrest of translation. If the shift starts in a codon away from the stop codon, the synthetized chain will have an amino acid sequence altered and will be shorter than normal. If the mutation is located in a region closer to the end of the translation, this will continue until it comes across a new stop codon producing a globin chain stretched. In any case, there is a lack in normal globin chains, thus constituting type defects β^0.

- **Mutations that cause instability of the beta globin chain**: they are nonsense mutations, frame shift or deletions within the exon 3. They generate elongated chains (rarely truncate) highly unstable that precipitate before forming tetramers with the alpha chains. These mutations are considered dominant in that, even if in heterozygosis, they cause a relevant clinical condition (thalassemia intermedia). [21]

The thalassemias associated to this genotype β^0/β^0 or β^0/β^+ are defined **Major,** since they present a clinical condition much more severe than the one more heterogeneous and milder observed in thalassemia **Intermedia**.
The description and characterization of the various forms of thalassemia, however, is based more on the clinical severity than the type of mutation present. The phenotypic expression of β-thalassemia varies from a clinical condition almost normal with a minimally imbalanced globin (thalassemia minor) to situations of severe anaemia associated with a genotype β^0/β^0 or β^0/β^+ (thalassemia major).

The individuals homozygous β^0 as well as the double heterozygous β^0/β^+ and many homozygous β^+ develop a clinical picture of thalassemia major which is manifested in early childhood with severe transfusion-dependent anaemia. With the term "thalassemia intermedia" are defined cases instead genotypically heterogeneous, characterized by a clinical condition more moderate, late-onset and with the absence of transfusion requirements at least for long periods. [22]

2.2 Thalassemia Major

The clinical manifestations of thalassemia major (TM) typically occur in the first year of life.

The genotype is characterized by the lack of presence or the total absence of beta globin chains, while the production of alpha chains remains unchanged. In patients with thalassemia, erythropoiesis is severely altered because the red blood cells will never come to fruition due to the accumulation of alpha globin chains, which are complexed to heme forming the Heinz bodies. From the maturational point of view, we are at the intermediate step of erythrocyte differentiation (day 7), where there is an abundance of polychromatic erythroblasts that will be degraded by a phenomenon called intramedullary haemolysis: the Heinz bodies precipitate at the membrane level of these erythroblasts devoid of haemoglobin and cause serious alterations incompatible with the survival of the cell, which is then eliminated.

Phenotypically TM subjects have chronic severe anaemia, accompanied by tissue hypoxia, which can be cured by transfusion only, so much so that thalassemia major is also described as transfusion dependent.

The continuous transfusions are used to compensate for the lack of red blood cells in the periphery and erythropoiesis in these patients is almost absent. [23] [24] (Figure 2.1)

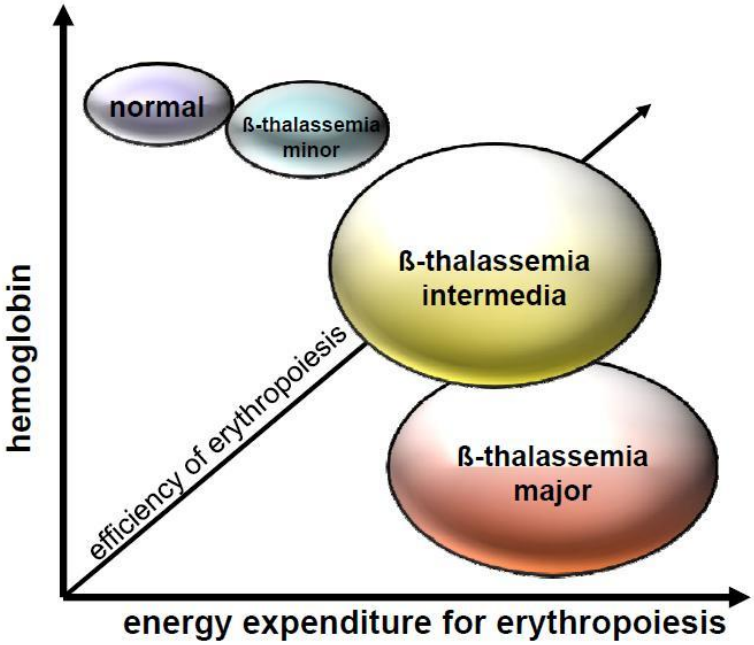
The problem of anaemia, however, is much more complex, it is not solved with the transfusions. In situations of anaemia is observed the stimulation of the production of erythropoietin (EPO) in an attempt to increase erythropoiesis in the bone marrow (up to 25-30 times the normal levels) without succeed. In fact, it is impossible to restore a physiological situation, as there are anomalies at the globin chain, causing the absence or a decreased production of haemoglobin in red blood cells, which makes it impossible to hold optimal erythropoiesis.

Unfortunately, the strategy of transfusion may be a solution for chronic anaemia, but it leads to the development of an additional problem: the iron overload.

Echoing what has been said in the first chapter, it was observed that hepcidin is silenced by hypoxia and ineffective erythropoiesis, this inhibition of hepatic hormone is found in those thalassemia intermedia conditions; so there is an iron overload due to the increase in intestinal absorption and ineffective erythropoiesis. Regarding the thalassemia major, the levels of hepcidin are not affected by the contribution of iron given from transfusions as this iron is exogenous.

This overload leads to a total transferrin saturation and the appearance of free non-transferrin-bound iron (NTBI), which accumulates in the tissues of the heart and endocrine organs causing severe toxicity if not treated pharmacologically. [24]

Figure 2.1: Erythropoiesis and Thalassemia



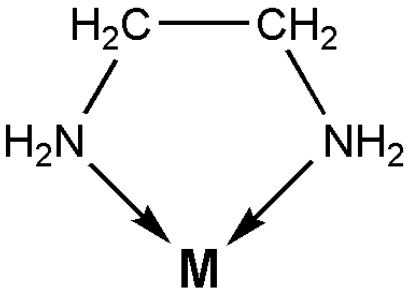
Source: Yelena Ginzburg, Stefano Rivella: "β-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism". Blood, 2011.

2.2.1 β – Thalassemia Therapy

The mainstay of therapy in patients with thalassemia treated with blood transfusion is therefore represented by the iron chelation therapy with deferoxamine or with the newer oral chelators: deferiprone and deferasirox. The term **chelation** refers to a chemical reaction in which usually a metal atom, behaving as a Lewis acid, binds a reagent called chelator through more than a coordinative bond. The structure of the resulting compound is a particular complex very stable (Figure 2.2). It shows that the central atom is surrounded by a chelating pincer, as if caught between the claws of a crab (hence the term chelation). In the biological field, haemoglobin binds iron through the chelation. In medicine chelation is exploited in the chelation therapy for the treatment of some forms of intoxication due to the

accumulation of metals in the body: once chelated, the metal loses its characteristics (and therefore it loses its toxicity) and then be eliminated tied together the chelating through the urinary tract.

Figure 2.2 : Example of a Metal Chelation



The most common chelating agents in the treatment of thalassemia are: desferoxamine (trade name of the drug: Desferal) which is administered intravenously, or the oral chelators such as deferiprone and deferasirox.

Deferoxamine (DFO) forms complexes with $Fe3^{+}$. The affinity of DFO for divalent ions such as $Fe2^{+}$ is substantially smaller. The chelation occurs with molar ratio 1: 1, (i.e.1 g of DFO can theoretically bind 85 mg of trivalent iron). Thanks to its chelating properties, DFO can bind free iron both in plasma and cells, forming the complex ferrioxamine (FO). The urinary excretion of FO is derived mainly from the turnover of plasmatic iron, while the iron present in the feces mainly reflects the intrahepatic chelation. The iron can be chelated by ferritin and by hemosiderin, but is relatively low at clinically relevant concentrations of DFO. The DFO does not mobilize the iron content in transferrin, hemoglobin or other substances containing the heme group.

Other alternative therapies for the β - thalassemia include the bone marrow transplantation from HLA-identical donor family that made possible to achieve a cure in 80% of cases, but only for subject at the paediatric age. [21] Although transplantation may allow the resolution of the disease, it may

not be successful and may lead to complications (GvHD, abnormal growth or neurological complications). To date, the transplant is considered appropriate only for patients with an HLA-identical donor, hence, for 30-40% of all β-thalassemia patients. [25]

New experimental therapies include gene therapy and induction of fetal hemoglobin (HbF).

The autologous transplant of hematopoietic stem cells corrected by vectors containing the β-globin gene is the basis of gene therapy of β-thalassemia. In recent years there have been numerous studies on in vitro cultures of $CD34^+$ stem cells derived from patients with thalassemia major treated with lentiviral vectors and on mouse models of β-thalassemia; all gave promising results thus provide a solid rationale for clinical application. [26] Despite these early observations, many studies are still needed to make it possible to consider gene therapy as a definitive solution of β-thalassemia.

2.3 Thalassemia Intermedia

The clinical phenotype of thalassemia intermedia (TI) is extremely heterogeneous and it is intermediate between thalassemia major and minor, although there are many features in common between the three conditions. Some patients are completely asymptomatic until adult life, and have only a moderate anaemia; these patients require only occasional transfusions as needed. Other patients have a severe anaemia that occurs between 2 and 6 years, and although they are able to survive without a regular transfusion regimen, they present alteration in growth and development. [26] [27]

Three factors are responsible for the clinical sequelae of TI: ineffective erythropoiesis, chronic anaemia and iron overload. Unlike TM, thalassemia intermedia has an accumulation of iron due to intestinal absorption. [28] Do not forget that the severity of the clinical condition mainly depends on the

underlying molecular defects that cause ineffective erythropoiesis and iron overload.

2.3.1 The Ineffective Erythropoiesis

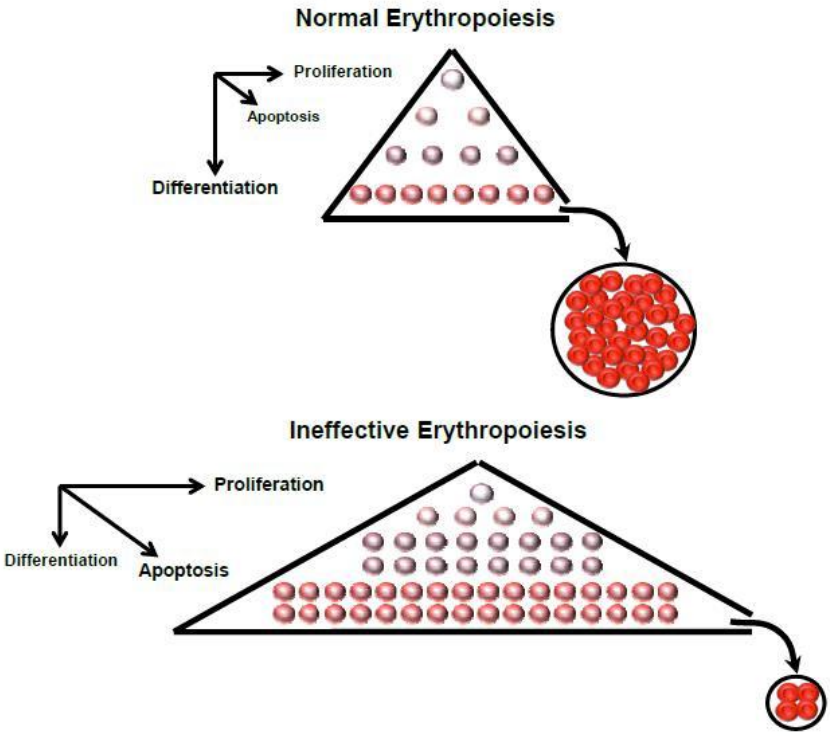
Erythropoiesis is a physiological process by which, in the bone marrow, mature erythrocytes are generated starting from their $CD34^+$ erythroid precursors. Erythropoietin (EPO) is the major regulator of this process and its release by the kidney is determined by situations such as hypoxia and iron depletion. Here it is possible to find a first correlation between erythropoiesis and hepcidin: the synthesis of red blood cells occurs if the hepatic hormone is not working.

In pathological conditions such as thalassemias it is possible to see the phenomenon of ineffective erythropoiesis. (Figure 2.3)

Ineffective erythropoiesis in β thalassemia is characterized by the expansion, poor differentiation and premature death of erythroid precursor cells through a process mediated by factors involved in the cell cycle, iron intake and heme synthesis. [29]

In TM there is a supply of red blood cells by blood transfusions that cause an almost complete silencing of the erythropoietic apparatus of the patient, for this reason thalassemia major is not a good model for the study of the ineffective erythropoiesis. It is instead thalassemia intermedia, being transfusion independent, allows a better study of the phenomenon.

Figure 2.3 : Ineffective and Normal Erythropoiesis



Source: Yelena Ginzburg, Stefano Rivella: "β-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism". Blood, 2011.

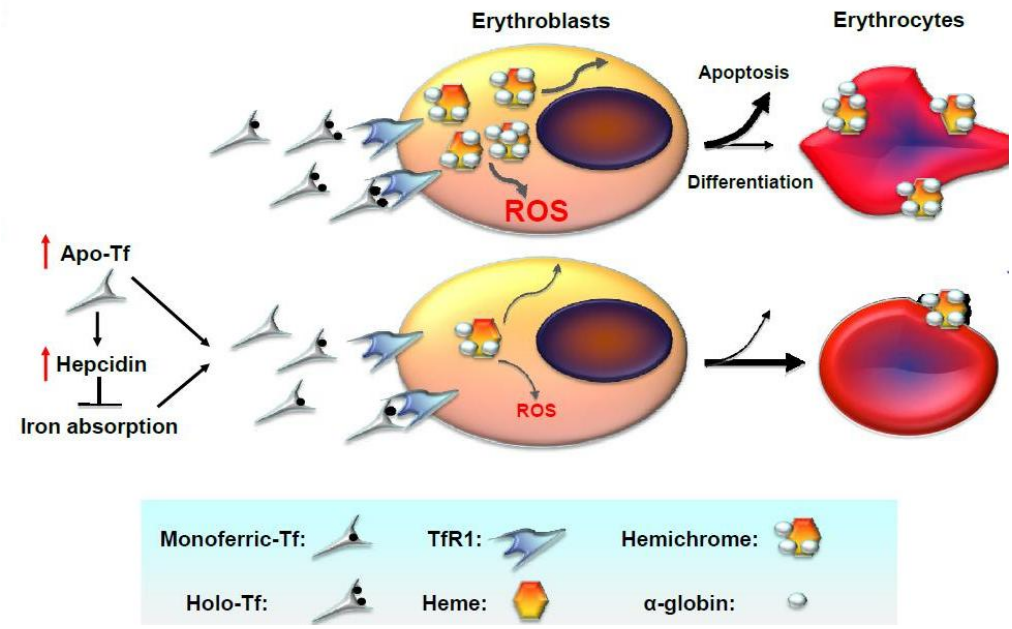
The erythropoietic apparatus of TI is affected by alterations in the production of beta globin chains; this leads to an incomplete maturation of erythrocytes: most of them undergoes an intramedullary hemolysis, while the red blood cells that are able to reach the bloodstream, are eliminated due to the deposition of Heinz bodies at the level of the membranes that alter their structure. (Figure 2.4)

Intramedullary and peripheral haemolysis generate a state of hypoxia that ensures the production of erythropoietin, which will stimulate erythropoiesis but, as it has been seen, is incomplete. This establishes a vicious circle also characterized by a continuous iron overload. The iron overload in TI,

unlike the TM, is not due to transfusions, but to the continuous intestinal absorption and to the continued destruction of the erythrocytes by the reticuloendothelial apparatus (RE) that releases iron into the bloodstream; iron that was supposed to compose the heme group.

Iron overload in thalassemia is due to the fact that, at the molecular level, there is an incorrect regulation of hepcidin.

Figure 2.4 : The Iron Increase in to Erythroid Progenitors Causes Damage and Apoptosis



Source: Yelena Ginzburg, Stefano Rivella: "β-thalassemia: a model for elucidating the dynamic regulation of ineffective erythropoiesis and iron metabolism". Blood, 2011.

2.4 Hepcidin / Ferroportin and Thalassemic Syndromes

This chapter has highlighted the procedure of transfusion TM in order to cope with chronic anemia; transfusions induce an increase in heminic iron circulating in the patient. If hepcidin was regulated primarily by iron, it should be observed an increase in its expression; well, in the sera of

patients affected by beta thalassemia very low levels of hepcidin have been reported compared to healthy controls**.** [30] This leads to the conclusion that in the thalassemias hepcidin is not expressed due to altered regulations upstream of the gene.

Unluckily there is very few information about the expression of Ferroportin into the erythroblast cells in healthy and thalassemic subject. For this reason is important to further investigate in order to see how this protein involved in the iron metabolism is expressed and regulated in the thalassemia syndromes.

CHAPTER 3: THE ERYTHROID DIFFERENTIATION

3.1. The Haemopoiesis

The blood cells can be classified into two main categories: lymphoid cells (B cells, T and natural killer) and myeloid cells (granulocytes, monocytes, erythrocytes and megakaryocytes); since they all have a limited life expectancy (several hours for granulocytes, few weeks for erythrocytes and few years for the memory T cells), they must be continually renewed or replaced. [31] [32]

The mature cells all originate from a hematopoietic multipotent stem cell (HSC); from this common precursor derive the pluripotent stem cells lymphoid and myeloid; from the latter (CFU-GEMM) arise at least three types of progenitor cells commissioned, that are oriented towards the differentiation into erythroid lineage, megakaryocytic, eosinophilic or granulocytic - macrophage. From the different commissioned stem cells, take origin the precursors, morphologically recognizable, of differentiated cell lines: proerythroblasts, myeloblasts, megakaryoblasts, monoblasts and eosinophiloblasts. From these derives the mature progeny.

Hematopoiesis is therefore a highly regulated process consisting of several stages:

i) Commitment to a specific chain differentiation,

ii) Proliferation and differentiation of progenitors,

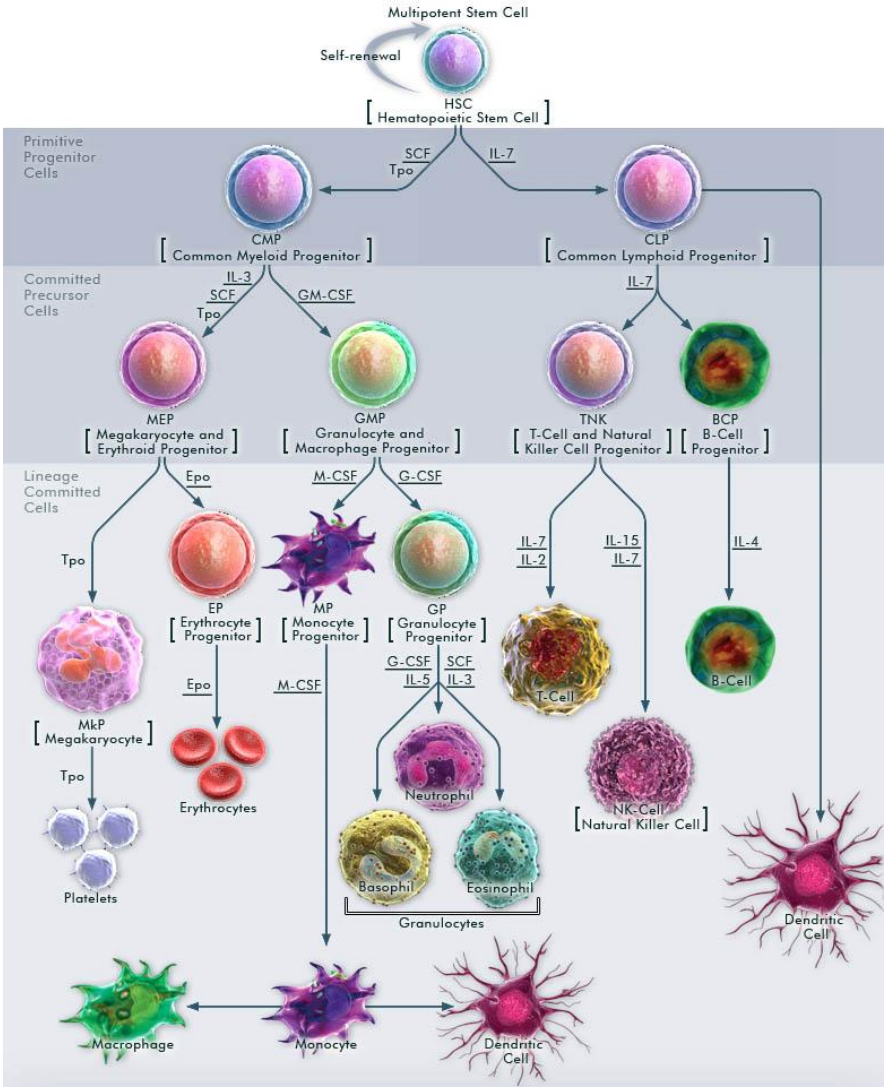
iii) Maturation of precursors

In adult life, the bone marrow is the only site of production of the HSC, which constitute a very small percentage, estimated to be between 0.05% and 0.5% of all bone marrow cells.

Multipotent stem cells HSC can self-renew or differentiate into progenitors commissioned, which lose their ability to self-renewal and give rise to mature cells having a particular function.

The choice between the two possible fates (self-renewal or commitment), is highly regulated by both intrinsic signals (transcription factors, signal transduction molecules, factors that regulate the cell cycle) and the external microenvironment.

Figure 3.1 : The Haematopoiesis



Source: eBioscience 2012

The bone marrow microenvironment plays a critical role in the regulation of haematopoiesis; it includes stromal cells, the extracellular matrix molecules and soluble cytokines that act as growth factors and differentiation by binding to specific receptors expressed on the surface of hematopoietic progenitors.

The various growth factors may act at different stages of hematopoietic; these factors are known and well characterized; they are essential for the recruitment of progenitor stem cells and guarantee their entry into the cell cycle, which is normally at G0 phase.

Among the factors that act at the level of primitive progenitors, has particularly importance the factor SCF (stem cell factor or c-kit ligand) produced by stromal cells and essential for the self-renewal of pluripotent stem cell. [33]

The commitment to a specific lineage of differentiation is determined by the nature and concentration of the factors acting on the progenitor cells.

While precursors and mature cells can be recognized morphologically, stem cells and commissioned progenitors can be only identified through in vitro functional assays or with immunophenotypic analysis.

The functional assays are based on the ability of the hematopoietic progenitor cells to generate colonies of mature cells in appropriate culture conditions and with the appropriate growth factors.

From the immunophenotypic point of view, stem cells are characterized by the presence of surface antigens: CD34 and AC133. [34]

The $CD34^+$ cell fraction is heterogeneous, including both primitive cells (stem cells HSC) and commissioned cells (progenitors); the concomitant expression of CD34 and immunological markers associated to a specific lineage, such as CD38 and HLA-DR, however, allows distinguishing the population into subpopulations of progenitors more or less primitive.

The $CD34^+$ cells can also be found in the umbilical cord blood and peripheral blood. Under physiological conditions, 1-3% of the mononuclear fraction of the bone marrow express the CD34 antigen, compared with 0.1-0.2% of peripheral blood and 0.8-1.2% of umbilical cord blood.

3.2 The Erythropoiesis

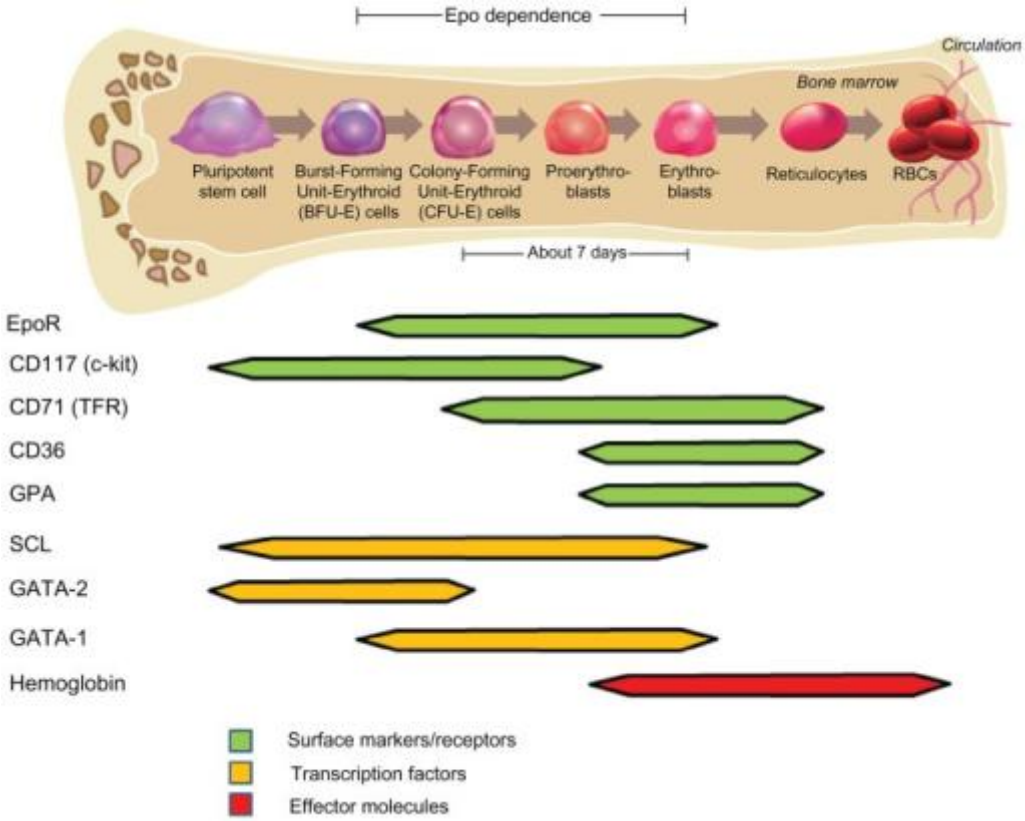
The process of formation of mature erythrocytes from the circulating hematopoietic stem cell (HSC) is called erythropoiesis. It consists of several stages: HSC commitment in a erythroid way, proliferation and differentiation of progenitors (BFU-E and CFU-E) and maturation of erythroid precursors or erythroblasts.

Erythropoiesis is regulated by several cytokines and growth factors, which by binding to specific receptors located on the membrane of erythroid progenitors or precursors, induce the activation of transcription factors; these by binding to specific DNA sequences, allow the expression of genes necessary for proliferation, differentiation and maturation.

Going through the various stages of erythropoiesis cells lose their ability to differentiate into other cell types (lymphocytes, monocytes, etc...) and become more oriented in erythroid sense.

Before the formation of polychromatic erythroblasts, the number of cells in each stage is higher than the previous one; this is due to the intense activity of cell division. Besides this step, the erythroid cells do not divide but they undergo a process of maturation that leads to the formation of erythrocytes, cell characterized by the absence of nucleus and organelles, with a haemoglobin content of more than 30% of the total volume. [35]

Figure 3.2 : The Erythroid Differentiation



Source: Elliott S., Sinclair AM. : "The effect of erythropoietin on normal and neoplastic cells." Biologics, 2012

The erythroid precursors are morphologically recognizable as the ancestors of mononuclear cells are identifiable only on the basis of functional tests.

The BFU-E (burst-forming unit erythroid) and CFU-E (colony forming unit erythroid) constitute the first commissioned elements capable of forming colonies when stimulated with appropriate growth factors and, in particular, with erythropoietin.

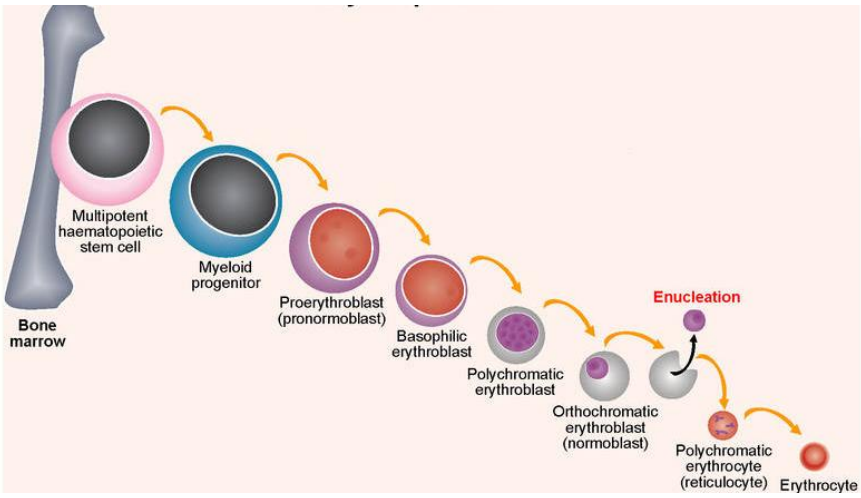
The BFU-E after 14-16 days of culture in semisolid medium give rise to macro colonies (burst) of polychromatic orthochromatic erythroblasts

containing haemoglobin. The BFU-E cells are mostly quiescent (only 10-20% is in the cycle) and are also present in peripheral blood (10-20 BFU-E per 105 of total cells). They express on the cell surface antigens such as CD34 and HLA class 2; only the late BFU-E also have a small amount of receptors for erythropoietin.

The CFU-E progenitors are considered to be more mature than the BFU-E, for this reason, when placed in culture, it only takes 5-6 days to form erythroid colonies. The CFU-E have an intense proliferative activity: most of them are located in the S phase of the cell cycle; these cells have lost the ability to self-renew and are not present in the periphery. The CFU-E no longer express CD34 on their surface nor HLA 2, while they acquire surface proteins specific to the erythroid lineage such as glycophorin A and antigen of the Rh system, in addition to erythropoietin receptors: these reach a maximum amount of 1.1×10^3 per cell. [35]

The population of erythroid precursors is composed by pro-erythroblasts, basophilic erythroblast, polychromatic erythroblast, orthochromatic erythroblast, reticulocyte and erythrocyte finally.

Figure 3.3 : The Erythroid Precursors



Source: Immunopaedia.org. Website

In the process of red blood cell maturation there is a progressive decrease in cell size (20-25µm for the pro-erythroblast, 7µm for the erythrocyte) and in the ability of protein synthesis.

At the stage of reticulocyte there is the complete ejection of the nucleus and the subsequent degradation of cytoplasmic organelles; this appears to be mediated at least in part by the 15-lipoxygenase, which would enhance the enzymatic oxidation of the phospholipids and the permeabilization of the membrane organelles.

During the final stages of erythropoiesis there is then a gradual increase in the expression of anti-apoptotic gene Bcl-XL, which allows cell survival.

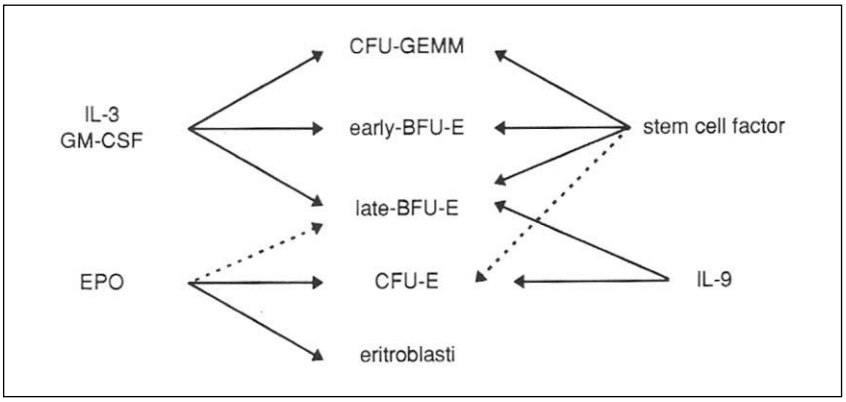
With the elimination of mitochondria and lysosomes, the erythrocyte can be considered mature. The main function of red blood cell is to carry oxygen from the lungs to peripheral tissues and carbon dioxide from the tissues to the lungs by using haemoglobin. The erythrocyte is able to perform these functions thanks to its digestive and metabolic enzyme and the structure of its membrane.

With the disappearance of ribosomes, the decline of the red blood cell begins and ends with the complete elimination in the spleen. [35] [36]

3.2.1 Growth Factors Regulating the Erythropoiesis

Proliferation, maturation and differentiation of erythroid progenitors are regulated by the interaction of several hematopoietic growth factors (SCF, EPO, TPO, IL-3, IL-6, GM-CSF, G-CSF) with specific membrane receptors and with the consequent activation of the appropriate signal transduction pathways.

Figure 3.4 : Growth Factors Regulating Erythropoiesis



Source: Stamatoyannopoulos G., "The molecular basis of blood diseases", 2001

Growth factors can be classified in several ways:

- depending on the cell type on which they act (primitive progenitors or already commissioned in a specific differentiation pattern)
- according to the type of receptor to which they bind (with intrinsic tyrosine kinase activity or not)
- depending on the mode of presentation to the target cells (such as soluble factors, molecules requiring a direct cell-to-cell or as part of the bone marrow microenvironment). [37]

The various hematopoietic cytokines have a common tertiary base structure; the specificity of binding to the receptor is guaranteed by the presence of a particular surface set of amino acid residues that are different from cytokine to cytokine.

Even hematopoietic receptors have a common basic structure; their segment contains a cytoplasmic domain with tyrosine kinase activity, which, however, is inactive in the absence of phosphorylation. The binding of the receptor with the growth factor induces the activation of this kinase activity through a mechanism based on dimerization.

Cytokines are dimeric and allow the interaction of two receptor subunits; the juxtaposition of the two catalytic domains promotes the mutual activation and phosphorylation (trans-phosphorylation). The tyrosine phosphorylated form the binding site for the molecules capable of transduce the signal [39].

The erythropoietic factors are able to activate different signal transduction pathways, among which there are p42 MAP kinase/44 (Mitogen Activated Protein Kinase), JAK-STAT kinase and PI-3K-AKT axis (phosphoinositide 3-kinase). In particular, cell proliferation is linked to the simultaneous activation of the axis p42 MAPK/44 and JAK-STAT, which induce the expression of the mitogen genes c-myb, c-myc and c-sis. Cell survival instead requires the activation of PI-3K-Akt axis which allows the expression of the genes for erythroid differentiation, and probably of the anti-apoptotic gene Bcl-XL. [35] [38]

- **Erythropoietin**

Erythropoietin is the primary regulator of erythropoiesis; it acts both at the level of primitive progenitors (BFU-E) in combination with other growth factors (c-kit ligand, TPO, IL-3), and of late progenitors (CFU-E) allowing the final differentiation.

Erythropoietin is a sialoglycoprotein composed of 165 amino acids and with a molecular weight of about 34 kD. In the foetus it is produced mainly in the liver, while in the adult the organ responsible for the synthesis of more than 80% of the circulating hormone is the kidney, in particular, the cells of the internal cortical and those adjacent to the proximal convoluted tubules; only a minor part (about 10%) is synthesized in the liver. [39]

The main physiological factor that regulates the production of erythropoietin is the supply of oxygen to the tissues, a condition of hypoxia induces an increase in serum levels of EPO. The increased production of EPO is

mainly due to increased gene transcription: is demonstrated the presence of a hypoxia-inducible element at the level of the promoter and at the flanking region in 3' end of the gene for EPO, located on chromosome 7. It is also possible a regulation at the mRNA level: a protein has been identified, (whose levels are increased in hypoxia) which binds to the untranslated region 3' (UTR) of EPO mRNA, prolonging its stability.

Erythropoietin allows the body to face the condition of hypoxia in three ways:

- Stimulates the release of normoblasts in the bone marrow
- Increases the amount of hemoglobin synthesized by each erythrocyte
- Stimulates the growth of BFU-E and CFU-E differentiation into mature red blood cells.

The increased number of circulating erythrocytes thus obtained is probably not due to an increased cell proliferation, but rather to the survival of a higher proportion of erythroid progenitors. It was shown, in an *in vitro* culture systems, that EPO prevents programmed cell death (apoptosis) at the level above the CFU-E and erythroblasts stage, expanding thereby erythropoiesis. [37] Erythropoietin therefore acts more as a survival factor that is not as mitogen.

According to the model proposed by DeMaria, the presence of EPO allows the activation of the transcription factor GATA1 and the subsequent expression of the anti-apoptotic gene Bcl-XL. In the absence of EPO there is instead the binding of Fas-Ligand to its receptor: this leads to the activation of caspases, cleavage of GATA1 and then the cell death by apoptosis. [40]

EPO plays its role by binding to a specific receptor (EPO-R) on the cell surface of erythroid precursors; the gene for this receptor is localized on chromosome 19p and encodes a protein of about 55kDa. The number of receptors per cell varies in relation to the stage of differentiation and maturation, being maximum at the CFU-E/erythroblasts stage, as well as in

relation to the serum levels of erythropoietin: a decrease of these induces an increase in the number of receptors.

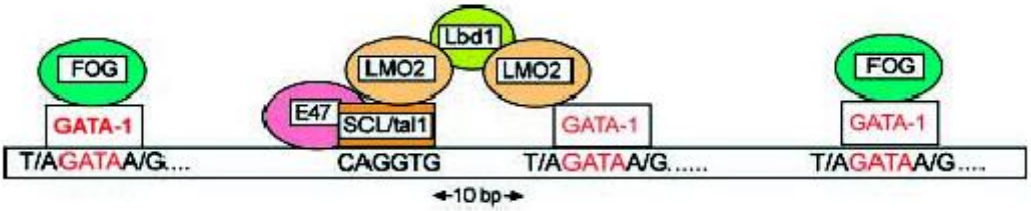
The expression of EPO-R depends mainly on the transcription factor GATA1; also are important the binding sites for Sp1 and CACCC consensus sequence located in the promoter, in addition to DNase hypersensitive sites of the LCR (locus control region) upstream of the starting site of transcription. [37]

3.2.2 Transcription Factors Regulating Erythropoiesis

The differentiation and proliferative potential of progenitors to a different cell line (commitment) is regulated by factors that allow the transcription of specific genes for that cell lineage; this occurs through binding to the consensus sequences present within promoters, enhancers and in the DNase 1-hypersensitive sites of the LCR.

The transcription factors can be divided into two categories: the firsts act at the level of pluripotent progenitors determining the erythroid commitment (TAL1, LMO2, GATA2), the seconds induce the differentiation and maturation of commissioned precursors (NF-E2, EKLF, GATA1). [41]

Figure 3.5 : Transcription Factors Regulating Erythropoiesis



Source : Oncogene, vol. 21, p. 3372, 2002

TAL1: it belongs to the family of transcription factors helix-loop-helix (bHLH) and is believed to be involved in the erythroid commitment. In the

cells of murine erythroleukemia TAL1 acts as a transcriptional repressor being associated with the corepressor mSin3A and the histone deacetylase HDAC1; thereby inhibits erythroid differentiation.

GATA: the GATA family consists of seven zinc-finger proteins including GATA1 and GATA2 which seem to have a fundamental role in erythropoiesis; knock-out experiments for these two transcription factors show an arrest of erythroid differentiation and maturation. [42] [43]
GATA2 is involved in proliferation, differentiation and survival of early hematopoietic cells, where it is highly expressed. The amount of this factor decreases during maturation of circulating cells. It seems that the mechanism of action of GATA2 is to increase the responsiveness to various growth factors and in particular to the c-kit ligand (SCF). [44]
GATA1 is especially important for erythroid differentiation and maturation: its absence causes an arrest at the pro-erythroblast stage. The central motif of the binding site, containing the consensus sequence (A/T) GATA (A/G), is present in the promoters and enhancers of many erythroid-specific genes: globin genes, the receptor for erythropoietin and of the same GATA. [45] It is indeed likely that GATA1 negatively regulates GATA2 as the latter decreases with the increase of GATA1 during differentiation.

EKLF (Erythroid Kruppel-Like Factor): it is an erythroid-specific transcription factor that allows the expression of the β globin gene by binding to the CACCC consensus sequence present in its promoter. This factor could also facilitate the interaction of the β globin gene promoter with the LCR, thereby contributing to the reduction of the expression of the γ globin gene during the globin switching. It was also recently shown that knockdown of EKLF1 reduces the levels of BCL11A and increases the γ/β globin ratio. It was then proposed that EKLF1 controls the globin switching

by directly activating the β globin gene and by indirectly repressing the expression of the γ globin gene. [46]

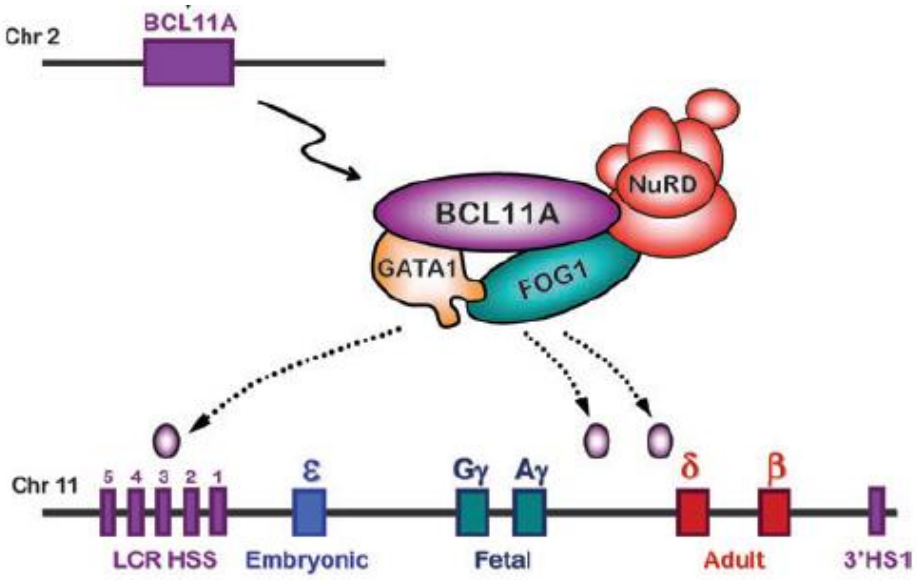
SP1: it is a factor involved in the regulation of erythroid maturation. It has been hypotised that, by interacting with the GATA1 and binding to the LCR or to the promoter region of the γ globin gene, it could activate the transcription or interfere with the globin switching. [47]

SOX6: it is a member of the transcription factors Sox (Sry-type HMG box) family, characterized by the presence of an HMG domain that recognizes the minor helix of the DNA. The ability of Sox proteins to bind to other transcription factors and to alter the DNA sequence, suggesting that they act as "architectural protein" probably by promoting the assembly of multiprotein complexes. Sox6 seems to act both as an activator and as a transcriptional repressor depending on the interaction with specific target sequences. [48] Recent studies indicate that the complete ablation of Sox6 causes an alteration of erythropoiesis with the consequent release into the circulation of an increased number of fetal nucleated cells and leads to an increased expression of embryonic globin gene ε. In particular, it was suggested that Sox6 decreases the expression of the εγ globin gene during the murine erythropoiesis and it cooperates with BCL11A in the γ globin gene downregulation in adult erythroid cells. [49] Finally, it was recently demonstrated that the expression of Sox6 decreases during the final stages of erythropoiesis and how Sox6 is able to regulate its downregulation through a double bond within its own promoter. [48]

BCL11A: it is transcription factor that acts as a repressor of γ globin gene expression. In fact, recent studies have shown that the knockdown of BCL11A results in an increased levels of γ globin. [50] The expression of

BCL11A varies during erythroid differentiation and during ontogeny. It is expressed at high levels and as a full-length active form in the adult stages when the γ globin gene is silent, while in the embryo and foetus its expression is lower and its shorter less active isoforms predominate. [51]

Figure 3.6 : Mechanism of Action of BCL11A



Source: Sankaran, "Transcriptional silencing of fetal hemoglobin", Ann N Y Acad Sci, p. 65, 2010

BCL11A appears to regulate specifically the expression of the γ globin gene. In fact, it binds at chromatin level of the β globin locus interacting with the third hypersensitive site of the LCR and with distinct regions located between the Aγ and δ globin genes, and does not seem to tie the γ globin promoter. [50]

Thanks to proteomic studies, it was shown how BCL11A is able to interact with different proteins: with NuRD, a complex of transcriptional repressors containing two histone deacetylases (HDAC1 and HDAC2), with the

transcription factor GATA1 and FOG by zinc –fingers bound, [52] and with the component of the nuclear matrix Matrin-3 (Figure 3.6.). It seems that through these interactions, BCL11A has a key role in the regulation of the switch from o to adult haemoglobin, as well as in the γ globin gene silencing in mature erythroid cells. For this reason, BCL11A constitutes an important therapeutic target for the activation of foetal haemoglobin (HbF) in patients with β thalassemia.

3.2.3. The Globin Swithcing

The proliferation and erythroid maturation are characterized by a progressive increase in the production of haemoglobin (Hb). The structure of human haemoglobin changes during the development in order to satisfy the different needs of oxygen by the body. [53]

The synthesis of haemoglobin is characterized by two main switches: the first, from embryonic to foetal Hb coincides with the transition from primitive haematopoiesis (which occurs in the yolk sac) to definitive haematopoiesis (in the foetal liver from the fourth month of pregnancy); the second, from foetal to adult Hb, that occurs in the perinatal period.

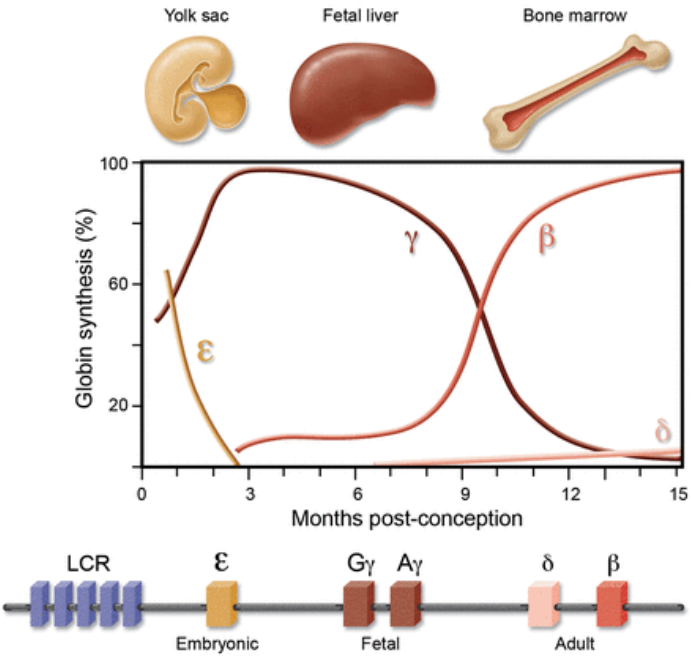
The embryonic haemoglobin is formed by two ζ chains and two ε chains ($\zeta_2\varepsilon_2$); foetal hemoglobin is composed by two γ chains and two β chains ($\gamma_2\beta_2$); finally, adult haemoglobin (HbA) is composed by two α chains and two β chains ($\alpha_2\beta_2$). In the adult, in addition to HbA, which is the main form of haemoglobin (98%), there is also has a smaller portion of HbA2 ($\alpha_2\delta_2$, 2%) and HbF (in trace amounts).

The genes for α and β globin chains are located in clusters and arranged from 5’ to 3' end in the order in which they are expressed during development.

The α globinic cluster is located on chromosome 16 and is composed by the ζ embryonic gene, two copies of the gene α and two non-functional pseudogenes as well. The β globinic cluster is located on chromosome 11 and is constituted by the embryonic gene ε, and by two copies of the gene γ that differ for one amino acid in position 136 (G or A) and by the δ and β genes that are expressed in adult.

Upstream of the two globinic clusters there is a regulatory region called LCR (locus control region) for the cluster β and HS-40 for the cluster α. This region allows the transcriptional activation in sequence of the globin genes of each clusters.

Figure 3.7: the Globin Switching



Source : Bauer D.E. et all "Reawakening fetal hemoglobin: prospects for new therapies for the β-globin disorders" Blood, October 11, 2012, 120 (15)

The adjustment of the globin switch occurs primarily at the transcriptional level but its mechanisms are not yet fully known. Molecular studies have so

far shown that the transcription of the globin genes is regulated by protein-DNA and protein-protein interactions. At the level of the promoter of each globin gene are present consensus sequences, such as TATA box, CCAAT box and CACCC box, which interacts with the LCR region and with the trans-activating factors to promote gene transcription.

Studies of genome-wide association and linkage disequilibrium have shown that the variability of HbF levels, in β thalassemia patients and in healthy subjects, is at least in part genetically determined and these studies have allowed us to identify three main polymorphisms (QTL: quantitative trait loci) that explain 20-50% of the variability. Xmn1-HBG2, the HBS1L-MYB intergenic region on chromosome 6q23 and BCL11A on chromosome 2p16. [54] In particular, for example, the polymorphism rs4671393 GG in place of the gene BCL11A is found to be associated with low levels of HbF and high levels of BCL11A, while the variant AA is associated with high levels of HbF and low levels of BCL11A. [52]

A key role in the regulation of the globin switching has been assigned:
- To BCL11A, a potent silencer of HbF
- To KLF1, the transcription factor that activates BCL11A
- The epigenetic modifications of the cluster □.

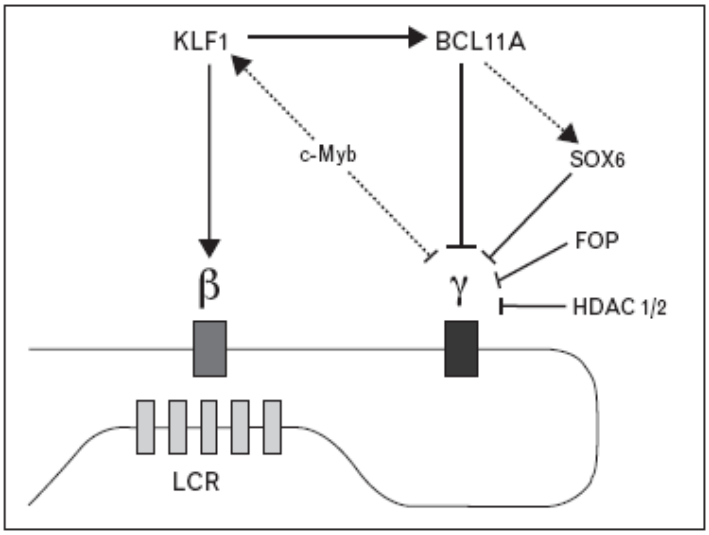
As previously reported, BCL11A is associated with different transcription factors in order to compose a multiprotein complex, including NuRD, GATA1 and SOX6, important for the suppression of the γ globin gene [50] (Figure 3.6).

KLF1 seems to have a dual role: it diminishes the γ globin gene expression by stimulating the transcription of the β globin gene by activating the repressor BCL11A with further silencing of the γ globin expression. [55]

cMyb could repress the expression of the γ globin through the activation of BCL11A mediated KLF1. [55] [56] [57]
Finally multiprotein complexes containing the PRMT5 arginine methyltransferase, the enzyme demethylating DNMT3A, the serine/threonine kinase CK2alpha and components of NuRD, occupy the γ globin promoter in order to methylate and make it transcriptionally inactive.

In Figure 3.8 it is shown a possible mechanism of interaction between the various transcription factors cooperating to decrease the expression of the gene γ globin. All of these factors represent potential new therapeutic targets to reactivate the γ globin gene and increase the levels of HbF in patients with β thalassemia. [56]

Figure 3.8 : A Possible Model of Globin Genes Regulation



Source: Bauer D., Orkin S., "Update on fetal hemoglobin gene regulation in hemoglobinopathies", Curr Opin Pediatrics, p.5, 2011

AIMS

A new perspective to amplify the knowledge of the molecular β-thalassemia is based on the possibility of studying different molecules participating in the huge pathway that is the iron metabolism.

One of these molecules is the FERROPORTIN.

In the literature, there are many works mostly conducted in vivo, cell lines, or not using the beta thalassemia as models of ineffective erythropoiesis and iron overload.

It is our interest to check what has been said in the literature, not through the cell lines, but using an *ex vivo* model, which consists in cultured erythroid differentiation obtained from $CD34^+$ precursors collected from the peripheral blood of healthy subjects and non – transfusion dependent thalassemia patients.

To check how the ferroportin is regulated, for each framework (physiological and thalassemia), are set up six conditions: standard, iron depletion (using the chelator Desferal), iron saturation (by Iron Ammonium Citrate), with heme bound iron (by adding Hemin), with heme iron free (by using SnPP IX). Plus, for the control cell culture is set another condition in order to simulate the oxidative stress by adding Hydrogen Peroxide.

These components were added to cell cultures at day 0 and 7 and it has been evaluated their effect on:

- growth and cell viability;
- the trend of the expression of FPN isoforms and their effect on the intra and extracellular iron concentration, measured respectively by RealTime - PCR and an Iron Assay kit.

MATERIALS AND METHODS

1. Cell Cultures

1.1. CD34$^+$ cell cultures from peripheral blood

Mononuclear cells derived from peripheral blood are enriched in CD34$^+$ cells with an immunomagnetic method by using magnetic beads binding the antibody anti-CD34$^+$ (*Mini & Midi MACS Separation System, Miltenyi Biotech*).

Separation of mononuclear cells

The peripheral blood of healthy subjects, provided by the Blood Transfusion Centre after routine inspections, is diluted 1:1 with PBS 1X (*phosphate buffered saline, GIBCO BRL*), layered on a gradient of Limphoprep (*Nycomed - Pharma*) in the ratio 2:1 and centrifuged for 20 minutes at 1980xg at room temperature. The ring of mononuclear cells thus formed, is carefully collected, transferred into 50 ml tubes and centrifuged at 300xg for 10 minutes. To eliminate the mature erythrocytes possibly present, the pellet is resuspended in 50 ml of lysis buffer (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA), incubated for 5-7 minutes at room temperature and centrifuged at 300xg for 10 minutes. There are two washes at 300xg for 10 minutes each, by resuspending the pellet in 50 ml of PBS 1X; in order to eliminate the platelets. At the conclusion of the second wash, the pellet is diluted in 10 ml of PBS 1X for cell counting.

Immunomagnetic separation of CD34 +

The cells are resuspended in Buffer B 1X (1x PBS, BSA 0.5%, EDTA 2mM, H2O) (300µl / 100x10^6 cells) and incubated for 30 minutes at 4°C with magnetic beads anti-CD34$^+$ (100µL / 100x10^6 cells) (*MACS Micro Beads*)

and FcR blocking reagent that inhibits the non-specific binding between cells and beads (100µL / $100x10^6$ cells).

The cell suspension is then passed through a column, previously hydrated with Buffer B 1X (1 mL for the MiniMACS column, 5 mL for the MidiMACS column), containing a magnetisable matrix and, in turn, inserted into a permanent magnet (*MiniMACS Separation unit* for cell concentrations up to $2x10^8$ cells; *Midi MACS Separation unit* for cell concentrations up to $2x10^9$). In this way, the $CD34^+$ cells bound to the beads are retained in the column and separated from the other non-labelled cells, which exceed the matrix. The column is then washed three times with Buffer B 1X (3x500 µl for the MiniMACS column, 3x3 ml for MidiMACS column), in order to completely remove the $CD34^-$ cells. Detaching the column from the magnet, the $CD34^+$ are eluted with buffer B 1X (1 ml for MiniMACS column, 5 ml for MidiMACS column) with the aid of a plunger and recovered in a 15 ml conical tube, ready for the cell count.

After cell counting, the $CD34^+$ cells are washed and seeded at a concentration of 10^5 cells / ml in a medium consisting of:

70% Mem-Alpha Medium (*GIBCO, BRL*)

30% foetal bovine serum (*GIBCO, BRL*)

1 U / ml recombinant human erythropoietin (*r-HuEPO Euroclone*)

25 ng / ml stem cell factor (*Peprotech*)

10 ng / ml interleukin-3 (*Peprotech*).

The cells are incubated at 37°C in a humidified atmosphere of 5% CO_2 for 14 days. [58]

1.2. Cellular Morphology Evaluation

At the end of the cell culture, or at predetermined time intervals, the morphology is evaluated, and thus the degree of cell differentiation, by centrifuge $0.2x10^6$ cells for 5 minutes at 350 rpm on a slide (*Cytospin Shadon Elliot*). For each cell culture two slides are prepared, one is stained with May-Grunwald mixture (eosin methylene blue solution containing more than 50% of methanol, SIGMA) - Giemsa (solution azure eosin methylene blue, *SIGMA*), the other with benzidine and then with May-Grunwald - Giemsa.

<u>Benzidine staining</u>

This coloration allows highlighting with a greenish colour in the presence of iron bound to heme in the cells examined.

It's a great method to identify the stage of maturation and cell differentiation.

Figure 1 : Mature Erythrocyte

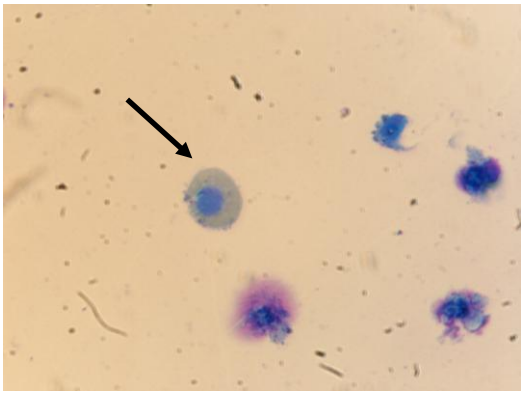


Figure 1: indicated by the arrow is possible to see a mature erythrocyte at day 14 of differentiation. Are highlighted in blue the nucleus and in green the cytoplasm rich of iron bound to heme

Staining with May-Grunwald - Giemsa

This staining method permits the evaluation of the sterility of the culture and to analyse the morphology, through the binding of the dyes to the acid component (methylene blue) and basic (eosin) cells. The slides are covered with May-Grunwald filtered for 7 minutes (the methyl alcohol present in the solution fixing the spot), rinsed in water and then covered with Giemsa filtrate and diluted 1:16 (v / v) for 17 minutes. Finally, the slides are washed in water, dried and read under the immersion microscope. The nuclei appear in a more intense purple compared to the cytoplasm.

1.3. Analysis by flow cytometry

The analysis by flow cytometry is used to determine the presence of membrane markers (CD34, CD45, glycophorin) by labelling with specific antibodies and to assess cell viability by incubation with propidium iodide, which penetrates the dead cells.

The cells are harvested at days 0, 7 and 14 of culture, washed with PBS 1X (*phosphate-buffered saline PBS; GIBCO*) and resuspended at a concentration of 10^5 cells/100 uL. For the analysis of the membrane markers, cells are incubated for 25 minutes at 4°C with anti-CD71 (10µl, *BectonDickinson*), anti-CD45 (10µl, *BectonDickinson*) and anti-glycophorin (5uL, *BectonDickinson*). After incubation, the cells are centrifuged at 460 rpm for 10 minutes and resuspended in PBS. For the assessment of cell viability, cells are incubated for two minutes with propidium iodide.

The analysis is performed with a flow cytometer with 3 fluorescences (FACS, Becton Dickinson) at a wavelength of 480 nm using the Cell Quest software.

● *Cell count*

The cell count is commonly performed with the Burker's chamber. This consists of a microscope slide comprising a series of lines that form squares (hemocytometer). It is covered with a coverslip, resulting in a capillary chamber.

The set of orthogonal lines define a number of areas and thus of volumes. The room is in fact divided into 9 quadrants in turn divided into smaller squares inside them.

Applies a cell suspension of about 10µl in the chamber, and, looking through a microscope, count the cells of at least three quadrants. Usually is preferable choosing three fields on the diagonal in order to have a good statistical variability.

It is then calculated the number of cells according to the following formula:

$$n = \frac{nc}{nq} \cdot F \cdot V \cdot 10^4$$

Where: *nc* = viable cell counted; *nq* = number of squares counted; *F* = dilution factor, and *V* = volume in which the cells are resuspended

Cell viability test

The Trypan blue is a dye used in tests determining the cell viability. This dye allows to discriminate between viable cells and dead ones as it is a dye capable of stain dead cells selectively. The reason why this dye does not stain the live cells is found in the extreme selectivity of the cell membrane. Viable cells, having the membrane intact, do not allow the penetration of this dye in the cytoplasm; on the contrary, in the dead cells the stain penetrates easily, making them indistinguishable from the alive ones with a rapid microscopic analysis. The trypan blue is not able to distinguish apoptotic cells from necrotic cells. For the cell count with the Burker's chamber, the cells are diluted with the dye in order to have a dilution factor

equal to 10 according to the following preparation: [70 µL of α - MEM; 20µL of trypan blue solution and 10µl of cell suspension].
Then all the cells are counted (the bright and the blue ones).
The percentage of viability is obtained by the ratio between the number of viable cells and the total number of cells:
Viability = No. of live cell x 100 / number of total cells.

2. Genetic expression analysis

2.1. RNA extraction

Total RNA is extracted from the cells using a mixture of guanidine isothiocyanate -phenols - chloroform, according to the method described by Chomczynski - Sacchi. The protocol is divided into several phases: lysis of the sample cell, the organic phase separation, precipitation, washing and solubilisation of RNA.

Lysis of the cell sample

The cells are washed three times with PBS 1X. To lyse them, the pellet is resuspended in a denaturing solution (solD, 500 µl/10^7 cells) consisting of: 4M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% laurilsarcosil, 0.1M β-mercaptoethanol. The guanidine thiocyanate inhibits the ribonucleases and then maintains the integrity of the RNA; as an added precaution, all the solutions are kept on ice.

Separation of organic phases

Sodium acetate 2M, pH 4.5 (50µl/500µl solD), phenol saturated in water (400µl/500µl solD) and CIAA (chloroform - isoamyl alcohol 24:1 v/v) (100 uL/500µl solD) are added to the suspension obtained after solubilization with the solD. The solution is then mixed by inversion and incubated on ice for 15 minutes. After incubation, the sample is centrifuged for 10 minutes at

10.000 rpm at 4°C. In this way two phases are formed: an upper aqueous and lower organic one; RNA is contained exclusively in the aqueous phase, while DNA and proteins are present in the interphase and organic phase.

Precipitation of RNA

The aqueous phase is recovered and transferred into a new eppendorf; RNA is then precipitated by adding a volume of isopropanol equal to that recovered (about 600µL) and incubating at -20°C for one hour. The sample is then centrifuged for 10 minutes at 13.000 rpm at 4°C.

Washing and solubilization of RNA

The pellet is resuspended in 500µL of 70% ethanol (diluted in water treated with diethylpyrocarbonate 7mM for the inhibition of ribonuclease [DEPC-H2O]) and centrifuged for 10 minutes at 13.000 rpm at 4°C. Finally the pellet is left to air dry, and then resuspended in 20µl of DEPC-H2O and stored at -80 ° C.

2.2. Determination of RNA concentration and its purity evaluation

The purity and integrity of the extracted RNA are evaluated by electrophoresis in 0.7% agarose gel (agarose dissolved in TBE 1X) or by analysis of the ratio of the absorbance at a wavelength of 260 nm and 280 nm. The RNA is considered sufficiently pure when is possible to see three sharp bands corresponding respectively ribosomal RNA 28S, 18S and 5S by analysing the gel at the ultraviolet light; excessive background noise is an indication of the high level of RNA degradation. Similarly, the preparation is considered pure if the ratio 260/280 nm has a value between 1.6 and 1.8; a lower value indicates the presence of contaminating proteins or phenol.

The concentration of the RNA sample is determined by spectrophotometric analysis by measuring the absorbance at a wavelength of 260 nm. Considering that an optical density of 1 corresponds to 40 µg/ml of RNA, the concentration of the sample is calculated according to the formula:

C (µg/µl) = OD x F x 40/1000

2.3. RNA retrotranscription

The messenger RNA (mRNA) present in the solution of total RNA extracted from the cells, are reverse transcribed into cDNA using as primers the random examers and as enzyme the reverse transcriptase of Moloney murine leukemia virus (MMLV) produced by a recombinant strain of E. coli.

To perform the reverse transcription reaction, the protocol *High Capacity cDNA Reverse Trascription kit from Applied Biosystems* is used. To 1µg of total RNA the reagents are added to the final concentrations indicated in Table 1, in a final volume of 20µl.

Table 1. Retrotranscription reagents

Reagents	**Final concentration**
Tampon (10X)	50 mM KCl, 10 mM Tris-HCl pH 8.3
dNTP (x 4)	1 mM (x 4)
Random examers	3 µM
RNase Inhibitor	1 U
Reverse Transcriptase	2.5 U
Final volume	**20µL**

All the reagents and the 1µg of RNA are added into the Eppendorf tubes and put into the thermocycler following this temperature protocol:

- 70°C for 10 minutes with the aim to denature any secondary structures of RNA that may interfere with reverse transcription.
- 25°C for 10 minutes
- 37°C for 120 minutes
- 85°C for 5 minutes.
- 10°C overnight

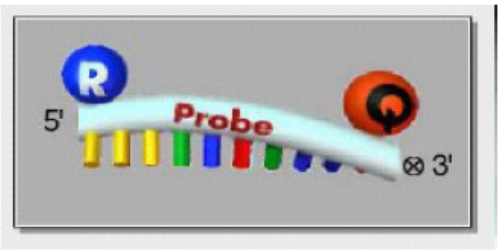
2.4. Real-time PCR

This technique is very sensitive and can be achieved by the use of nonspecific intercalating dyes (SYBR Green), or with probes labelled with fluorescent molecules (reporter) which bind specifically our RNA Target. In both cases, the Real-Time PCR quantifies the fluorescence emitted during each PCR cycle.

In this study, the Real-Time PCR was performed by using TaqMan probes.

The TaqMan probe is an oligonucleotide which, as the two PCR primers, is designed to be complementary to the target sequence. Presents at the 5' end a fluorophore called REPORTER (FAM in this case) and at the 3' end a molecule called QUENCER (MGB in this case) (Figure 2.1):

Figure 2.1: TaqMan probe



In a configuration of this type, the molecule QUENCHER prevents the fluorescence emission by the REPORTER. During each PCR cycle, however, in the phase of extension of the DNA strand complementary to the target sequence, the enzyme Taq polymerase encounters the 5' end of the probe and performs, at first, an undermining of the latter, followed by the detachment of the REPORTER thanks to the exonuclease activity (5' > 3') of the Taq. In this way the fluorophore in solution does not undergo the inhibition of the QUENCER anymore, and it is excited by the halogen lamp thus, emits fluorescence. According to this mechanism, the intensity of fluorescence increases with the concentration of the specific amplified for each reaction. All the pairs of primers used in this study have an annealing temperature of 60°C while for the probes is 10°C higher. The first probes produced, usually 30-35pb, the quencer absorbed and emitted fluorescence at the same time, in the last generation probes, such as those used in this study, the quencer has been replaced by the MGB (minor groove binder) that does not emit fluorescence and that is still to the minor groove of DNA and it increases the Melting temperature and therefore allowing the use of smaller probes (15-20bp) that allow to reduce the length of the amplicon.

The Real Time PCR Plate Preparation

Each plate for Real-Time PCR consists of 96 wells in which are deposited 22.5µL of MIX and 2.5µL of cDNA (50 ng) diluted in water by a factor of 1.5. The MIX is thus prepared for each sample:

• 12.5µL of Universal PCR Master Mix (2X) (Applied Biosystems) containing: buffer, Taq polymerase, nucleotides

• 8.75µL of RNase free H_2O

• 1.25µL of PRIMER AND PROBE (20X)

The reaction mixture has a final volume of 25µl. For each gene analysed a specific MIX is prepared.

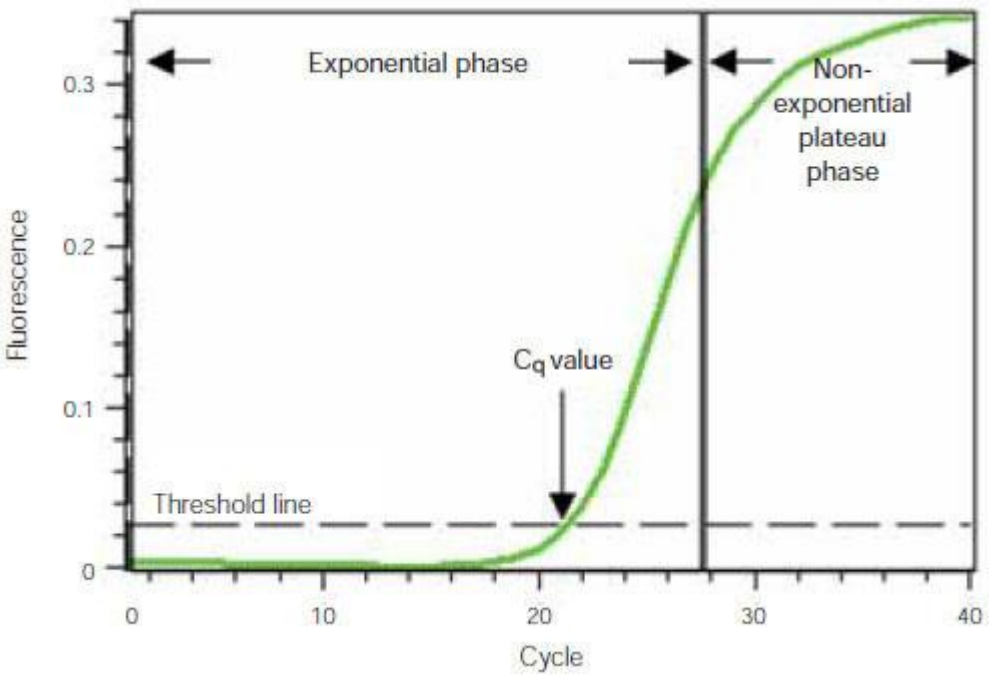
The plate thus prepared, is covered with an optical adhesive that lets the light from the lamp in order to excite the fluorophore but prevents the evaporation of samples during PCR. The plate is then centrifuged at 900 rpm for 5 min to remove any bubbles formed during preparation. Meanwhile, the program is set for the analysis.

Each sample was analyzed in triplicate. For the relative quantification of gene expression we use the mathematical model based on the calculation of ΔΔCt using as internal standard the normalizer gene GAPDH and as calibrator the level of expression of the gene in the control culture not treated with the various drugs.

Protocol for Real Time PCR

- 1 cycle: 95°C for 10' initial denaturation
- 40 cycles: 95 C for 15 " denaturation
- 60°C for 1' extension and annealing

Figure 2.2 : PCR amplification curve



The fluorescence is recorded during the extension phase.

The molecular analysis in vitro was performed for the genes FPN1A and 1B starting from $CD34^+$ cells obtained from liquid cultures treated with iron chelator, FeAMCit, H_2O_2, SnPP IX and Hemin. All molecular analyses were conducted on the samples treated with the various concentrations of compounds and their respective controls at days of culture 7 and14.

All the operations relating to measurements and analysis are performed under the control of a software (SYSTEM SOFTWARE SDS) managed by a personal computer.

The program automatically calculates:

1. the baseline fluorescence indicating the number of cycles in which the instrument does not record any fluorescence;

2. the threshold line, fluorescence value below which do not consider truthful data;

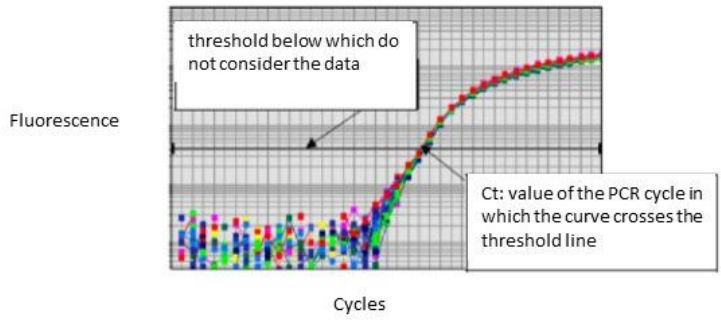
3. the threshold cycle (Ct), specific for each sample that identifies the value of the PCR cycle in which the curve intersects the line in the exponential phase threshold. Over the sample reaches the threshold to a lesser cycles of PCR is more concentrated and therefore expressed.

Moreover, the software normalizes the data of target genes (FPN) compared to endogenous genes (β-actin or GAPDH) and calibrates them to a reference value (for our particular case it is the untreated control) and calculates:

● **The ΔΔCt**, ie the difference between the ΔCt (= Ct target - Ct housekeeping), respectively, of the analysed samples and the calibrator (control).

● **The RQ** (= $2^{-\Delta\Delta Ct}$), which indicates how many times the target gene is more or less expressed than the calibrator (Figure 2.3).

Figure 2.3: Real Time – PCR Amplification Plot



3. Iron Assay Kit

The kit used for the intra and extracellular iron detection is the *Iron Colorimetric Assay Kit (Biovision, San Francisco, CA)*

<u>Procedure:</u>

For the intracellular iron

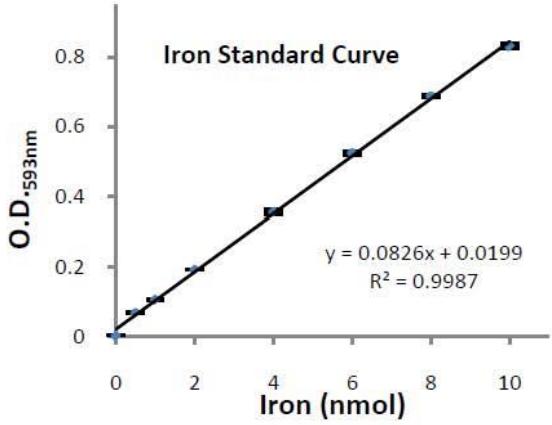
1) $1x10^6$ cells are lysed in 20µL of Potassium Phosphate buffer (KP), incubate for 10' at -20°C and then centrifuged at 2000rpm for 10'
2) Take the supernatant and the remaining pellet is lysed in 20µL of IRON BUFFER and centrifuged at 16000rpm for 10'
3) Take all the supernatant and put 10µL of each in a 96 well plate in order to have $0.5x10^6$/10µL of lysed cells in each well (each samples is studied in duplicate)
4) Prepare the standards using the standard solution from the kit (0 – 2 – 4 – 6 – 8 – 10)
5) Each well is brought to the final volume of 50µL with the IRON BUFFER
6) Prepare the blank for the KP (10µL of KP plus 40µL of IRON BUFFER)
7) Add to each samples and to the standards as well, 2,5µL of IRON REDUCER
8) Leave 30' in the dark at room temperature
9) Add to each samples and to the standards as well 50µL of IRON PROBE
10) Leave 3 hours in the dark at room temperature
11) Read the plate at 540nm

For the extracellular iron

1) The standards preparation is the same of the intracellular iron
2) Thaw the supernatants and put 50µL of each samples in a 96 well plate
3) Prepare the blank (in this case is 50µL of alpha – MEM)

   At this point, follow the procedure written above, starting from the point 7

Figure 3.1 Example of the Iron standard curve



All the results should be normalized on the cell number in order to have the right value of iron concentration.

4. Compounds Used

- DESFERAL (deferoxamine mesylate, DFO; Biofutura Pharma SpA)
  Are dissolved 66 mg of compound in 2.5mL PBS 1X to obtain a concentration of 40mM, this is then diluted to a 20 mM concentration. This solution is added to the cells at a final concentration of 10μM and 4 μM.

  Deferoxamine (DFO) forms complexes with the trivalent ions of Fe and Al: the formation constants of the complexes are, respectively, 1031 and 1025. The affinity of DFO for divalent ions such as Fe^{++}, Cu^{++}, Zn^{++}, Ca^{++} is substantially smaller (formation constant of the complex is 1014 or lower). The chelation occurs with molar ratio 1:

1, it means that 1 g of DFO can theoretically bind 85 mg of trivalent iron or 41 mg of trivalent aluminium.

Thanks to its chelating properties, DFO can bind free iron both into plasma and into the cells, forming the complex ferrioxamine (FO). The urinary excretion of FO is derived mainly from the turnover of plasma iron, while the iron present in the feces mainly reflects the intrahepatic chelation. The iron can be chelated by ferritin and by the hemosiderin, but is relatively low at clinically relevant concentrations of DFO.

The DFO does not mobilize, however, the iron content in transferrin, haemoglobin or other substances containing a heme group.

- FERRIC AMONIUM CITRATE (FeAmCit / FAC; Sigma)
  650 mg of compound are dissolved in 5mL of PBS 1X to obtain a concentration of 0.4M, this is subsequently diluted to a concentration of 20 mM. This solution is added to the cells at a final concentration of 100 µM.

- HEMIN CLORIDE (Sigma)
  2.2 mg of powder are dissolved in a solution composed of 100µL of NaOH and 200µL of media, resulting in a concentration of 11mM, this is subsequently diluted with PBS 1X to the concentration of 5,55mM. This solution is added to the cells at final concentrations of 20 and 10µM.

- PROTOPORFIRIN IX DICLORIDE (SnPP IX; Frontier Scientific)

20 mg of powder are dissolved in a solution composed of 1064µL of NaOH 1M, resulting in a concentration of 25mM, this is subsequently diluted with PBS 1X to the concentration of 10mM and 4mM. These solutions are added to the cells at final concentrations of 50 and 20µM.

- HYDROGEN PEROXYGEN (H_2O_2; J.T. Baker)
  Diluted the 33M stock solution with water in order to obtain the 20mM. Add this one to the cell culture in order to have a final concentration of 0.1mM

5. Statistical Analysis

All the significances are calculated by using the T test

RESULTS

1. FERROPORTIN 1A and 1B analysis in control cell cultures

CD34$^+$ cells from healthy donors were cultured under conditions that stimulate erythroid differentiation. The culture medium contains, in fact, cytokines such as stem cell factor (SCF), interleukin 3 (IL-3) and erythropoietin (Epo). The cells were cultured for 14 days.

The cells were analysed at different days of culture to assess growth and cell viability, morphology, gene expression and protein assay.

For each condition three independent cell cultures were arranged, the results shown are the average of these three.

1.1 Cell growth and viability analysis

Growth and viability were assessed at different days of cell culture (0, 7, and 14).

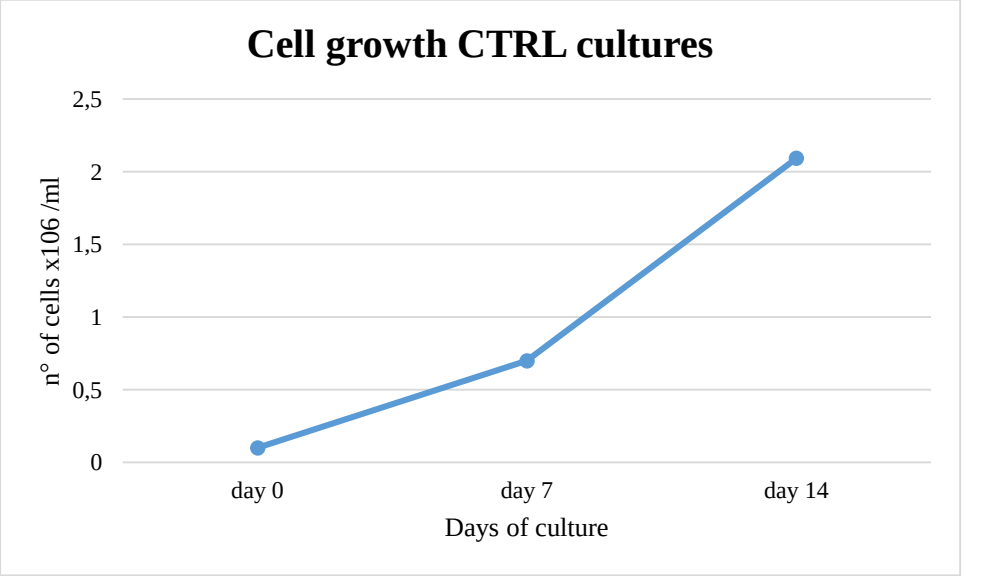


Chart 1: cell growth of the control culture analysed at days 0, 7, 14

From the chart is possible to see a neat cell growth from $0.1x10^6$ cells/mL at day 0, to $2.09x10^6$ cells/mL at day 14, obtaining an increase of the cell number 21 times superior.

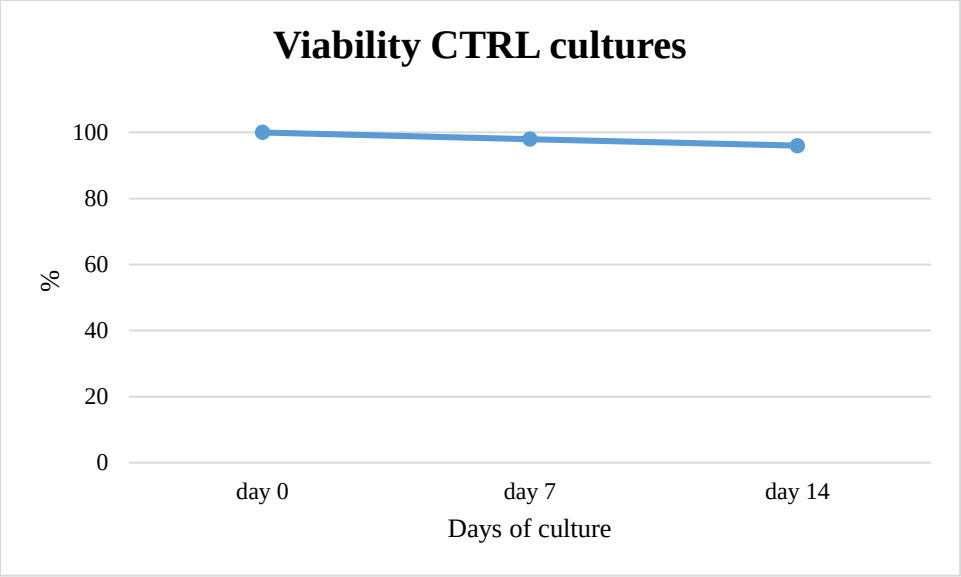


Chart 2: analysis of the cell viability at days 0, 7, 14 of the CTRL cell culture

The control cell cultures present an high viability during the entire erythroid differentiation process, in fact, the range of the viability percentage is between 100 and 96 %.

1.2 Morphology

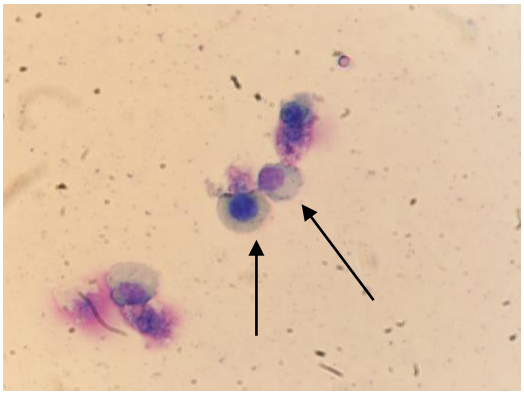


Figure 1: mature erythroblast at day 14 of the cell culture

The figure shows a mature erythroblast at the end of its differentiation process. It is possible to see the round and regular shape of the cell.

1.3 Analysis of the FERROPORTIN 1A and 1B expression during the erythroid differentiation

The gene expression of FERROPORTIN isoforms was assessed at days 0, 4, 7, 11 and 14 of the cell culture through the method of real - time PCR from cDNA obtained by reverse transcription of RNA extracted from cells of each condition.

The results of the analysis were converted to charts that explain when and in which quantities our genes of interest were expressed during the differentiation, compared to an endogenous gene called housekeeping, which was the GAPDH.

The values of expression of each ferroportin isoform are reported as the relative percentage on the 100% of the total ferroportin (the total ferroportin is calculated by summing the **RQ = $2^{-\Delta Ct}$** of each isoforms and then converted to a percentage of 100%).

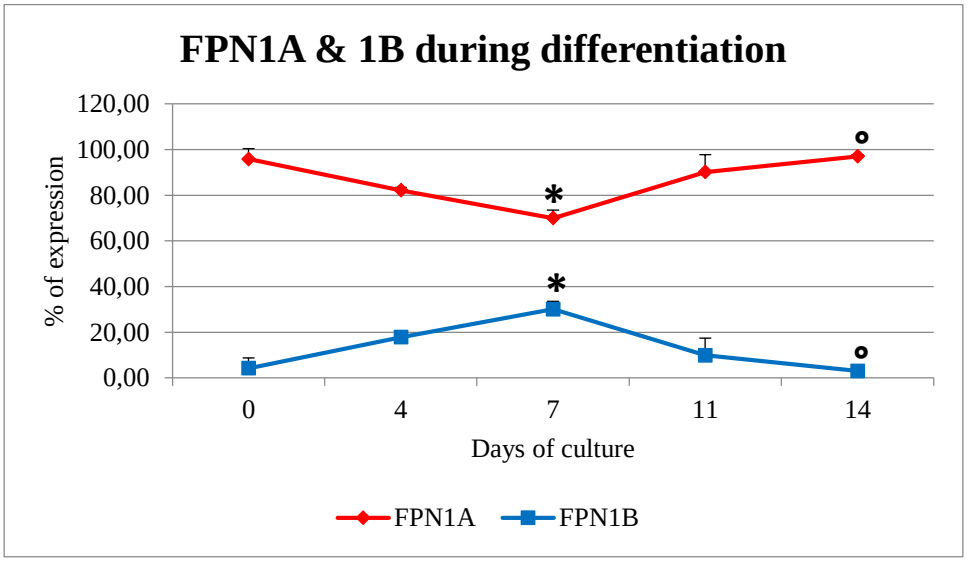


Chart 3: genic expression of FPN isoforms during erythroid differentiation

From the chart, it is clear that the expression of FPN1B is very low in the early days of culture and increases at day 7 (pro erythroblasts) and then decreases again at day 14 (mature erythroblasts).

The FPN1A has the opposite trend: is high at the precursors stage (day 0), then decreases at day 7 and increases again at day 14.

All the increasing and decreasing values are statistically significant:

***** $p < 0.05$ day 7 vs day 0

° $p < 0.05$ day 14 vs day 7

1.4 Intracellular and extracellular iron levels during differentiation

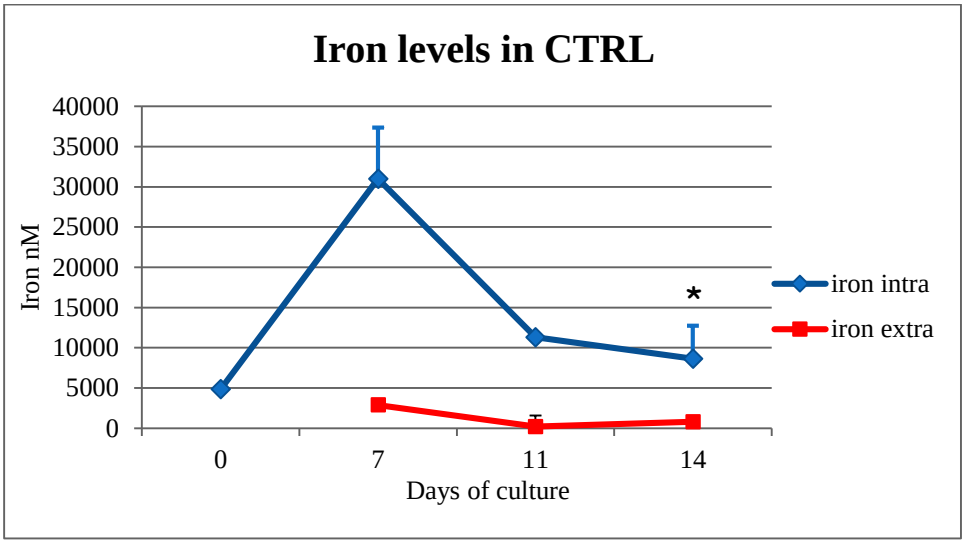


Chart 4: intra and extracellular iron levels at days 0, 7, 11, 14 in control cultures

The iron level measurement was obtained by using a colorimetric Iron Assay Kit at different days of culture. There is not the extracellular iron value at day 0 since there is not culture supernatant, but just plasma from the peripheral blood; thus, it can be difficult comparing the iron levels between plasma and cell culture supernatants because they are not the same materials.

The chart confirms what was observed at the genetic level: a decrease of the 1A iron related isoform at day 7 consist in an increase of intracellular iron

and, on the other way round, an increase of ferroportin 1A at day 14 is linked to a major iron export, thus, a decrease of intracellular iron.

The increase of the expression of FPN1B at day 7 can be justified by the fact that at this stage the erythroblast need iron for the haemoglobin synthesis, thus is important to have an isoform non - iron related in order to guarantee the presence of the intracellular iron without exporting it.

The decrease of intracellular iron from day 7 to day 14 is statistically significant: $p < 0.05$

2. FERROPORTIN 1A and 1B analysis in NTDT cell cultures

$CD34^+$ cells from NTDT patients were cultured under conditions that stimulate erythroid differentiation. The culture medium contains, in fact, cytokines such as stem cell factor (SCF), interleukin 3 (IL-3) and erythropoietin (Epo). The cells were cultured for 14 days.

The cells were analysed at different days of culture to assess growth and cell viability, morphology, gene expression and protein assay.

For each condition three independent cell cultures were arranged, the results shown are the average of these three.

2.1 Cell growth and viability analysis

Growth and viability were assessed at different days of cell culture (0, 7, 14).

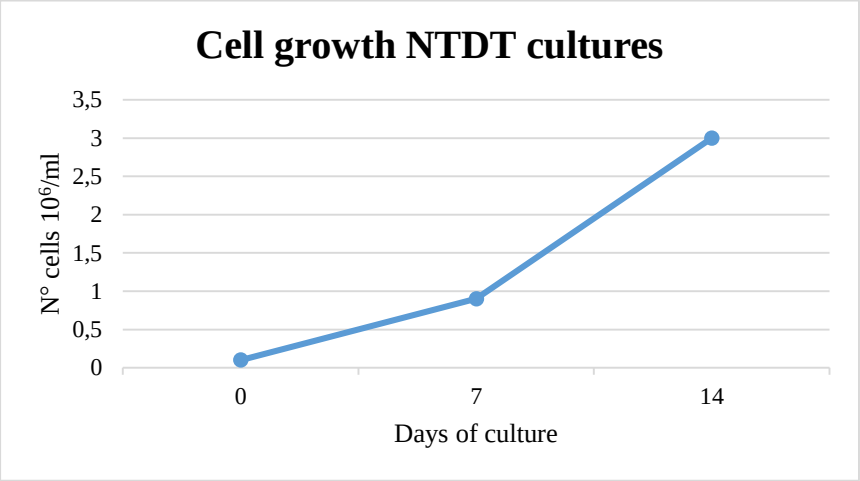


Chart 5: cell growth analysis at day 0,7,14 of NTDT cultures not treated

From the chart is possible to see a neat cell growth from $0.1x10^6$ cells/mL at day 0, to $3.0x10^6$ cells/mL at day 14, obtaining an increase of the cell number 30 times superior.

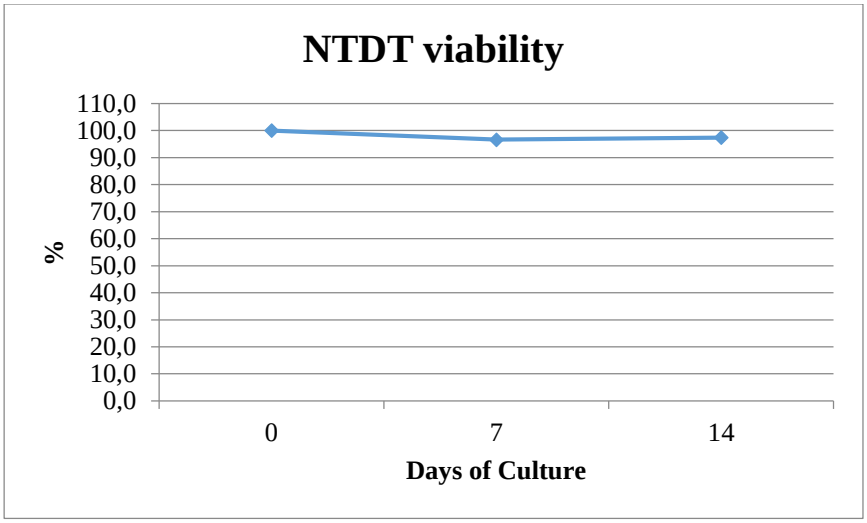


Chart 6: analysis of the cell viability at days 0, 7, 14 in NTDT cell cultures

The NTDT cell cultures present an high viability during the entire erythroid differentiation process; in fact, the range of the viability percentage is between 100 and 96,6 %.

2.2 Morphology

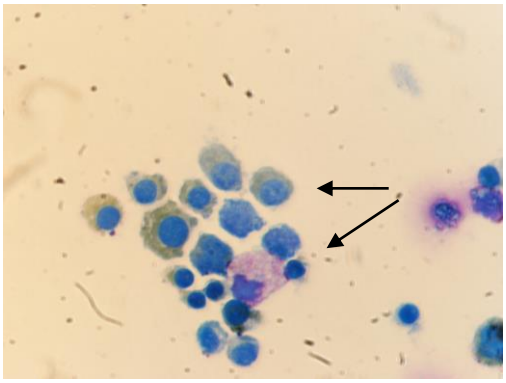


Figure 2: NTDT erythroid cells morphology at day 14 of culture

From this picture is possible to see erythroblast cells at day 14 of culture. These cells have irregular shapes and contours, despite the fact that all cells are at day 14, the benzidine staining indicates that not all of them are at the same stage of maturation and differentiation (see cells marked with arrows).

2.3 Analysis of the FERROPORTIN 1A and 1B expression during the NTDT erythroid differentiation

The genetic expression of FPN 1A and 1B was evaluated at day 0, 7 and 14 of the cell culture. The values of each isoform expression are reported as the relative percentage on the 100% of the total ferroportin (the total ferroportin is calculated by summing the **RQ = $2^{-\Delta Ct}$** of each isoforms and then converted to a percentage of 100%).

The results represented the mean of three independent cell cultures.

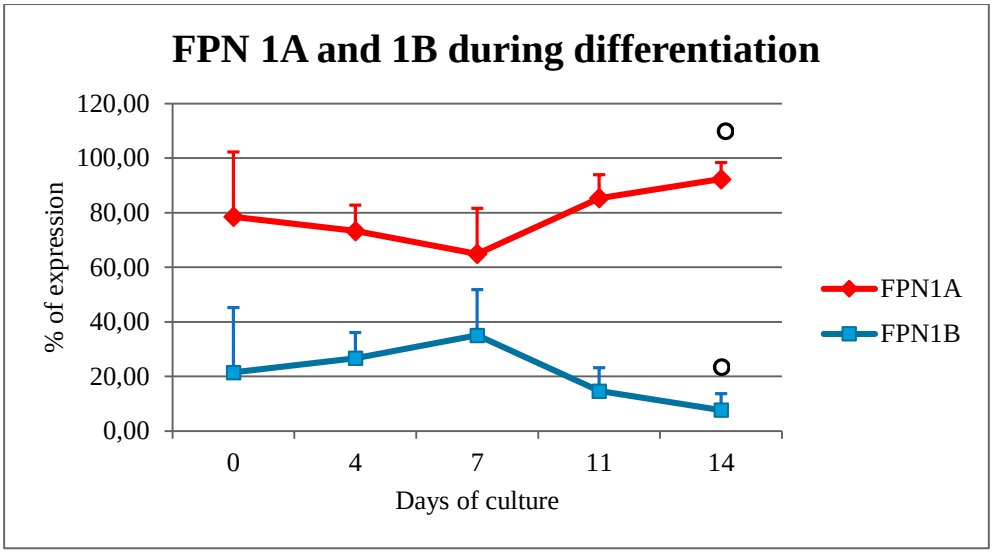


Chart 7: FPN 1A and 1B expression during the NTDT erythroid differentiation

From the graph, it is clear that the expression of FPN1B is low in the early days of culture (only 20%) and increases at day 7 (pro erythroblasts) and then decreases again at day 14 (mature erythroblasts).

The FPN1A has the opposite trend: is high at the precursors stage (day 0), then decreases at day 7 and increases again at day 14.

All the increasing and decreasing values are statistically significant:

° $p < 0.05$ day 14 vs day 7

2.4 Intracellular and extracellular iron levels during NTDT differentiation

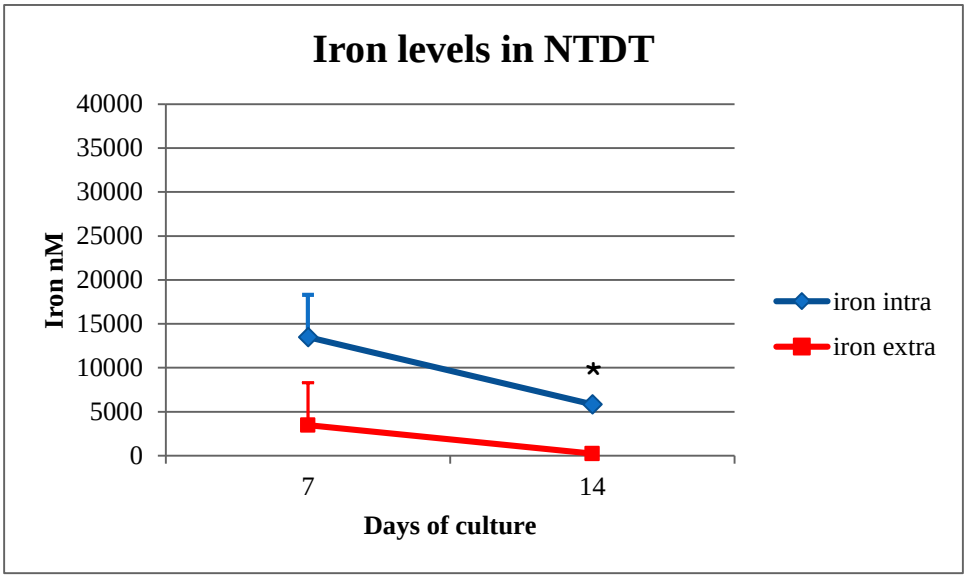


Chart 8: intra and extracellular iron levels at days 7 and 14 in NTDT cultures

The iron level measurement was obtained by using a colorimetric Iron Assay Kit at different days of culture

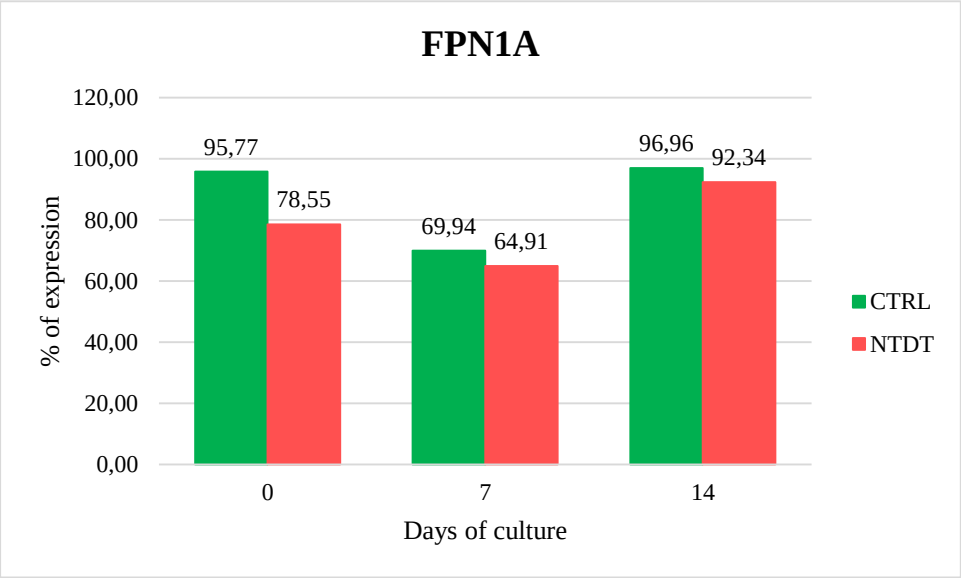
The chart confirms what was observed at the genetic level: the increase of the FPN1A expression at day 14 causes a decreasing of intracellular iron.

The decrease of intracellular iron from day 7 to day 14 is statistically significant: $p < 0.05$

3. Comparison between CTRL and NTDT

3.1 Ferroportin 1A and 1B expression in CTRL and NTDT cell cultures

A



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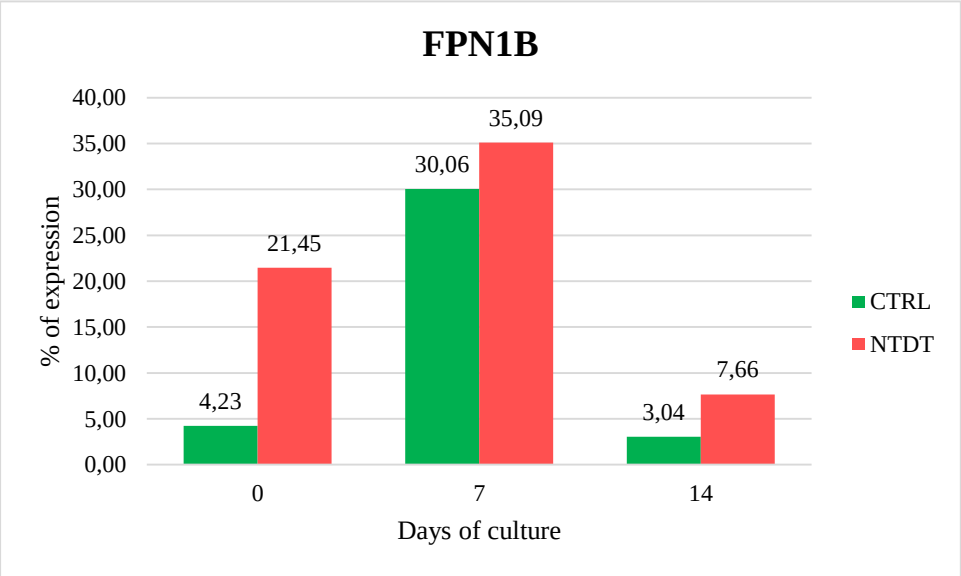


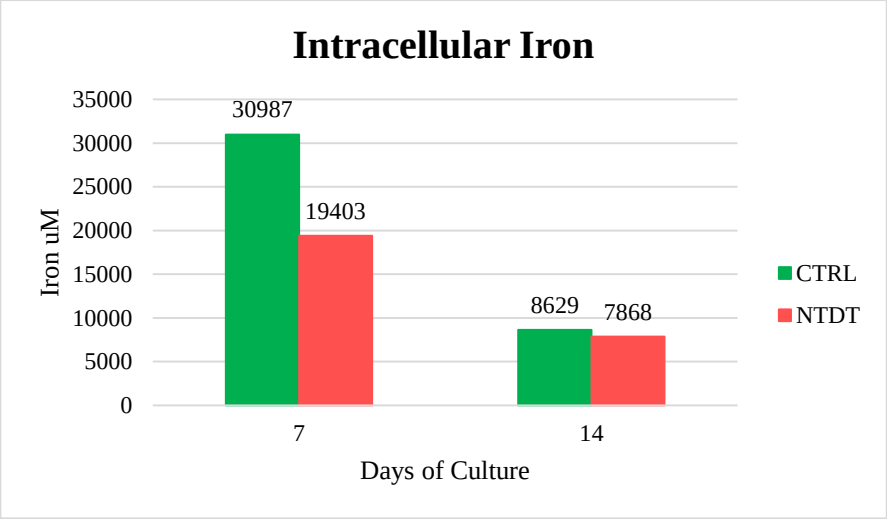
Chart 9 : comparison of the two FPN isoforms between CTRL and NTDT conditions during the erythroid differentiation

In these charts are compared the values of FPN1A and 1B expression during the erythroid differentiation in CTRL and NTDT cell cultures.

It is possible to see that the FPN1B is the more expressed isoform in NTDT condition, especially at the beginning and at the end of the erythropoietic process (see chart 9B). While the ferroportin 1A is the predominant isoform in the CTRL cell cultures, even if the difference of expression between the isoform 1B is not so high (see chart 9A).

3.2 Intracellular and extracellular iron levels during differentiation in CTRL and NTDT cell cultures

A



B

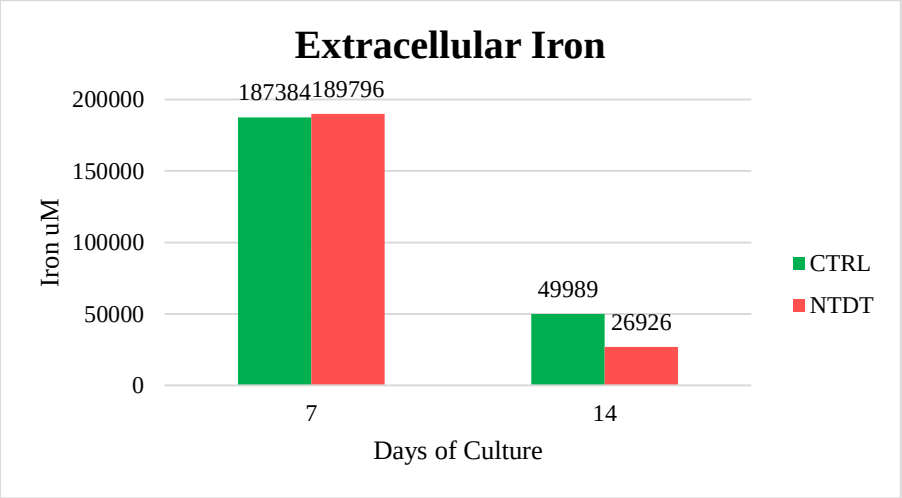


Chart 10 : comparison of intra and extracellular iron levels between CTRL and NTDT cell cultures

The charts represent the intra and extracellular iron levels at two different stages of cell culture (day 7 / pro erythroblasts, and day 14 / orthochromatic cells) in CTRL and NTDT conditions.

The chart 10A shows that, at day 7, the intracellular iron levels are higher in CTRL cultures compared to the NTDT ones, while at the end of the erythropoiesis, the intracellular iron levels are the same in both conditions.
The chart 10B shows the extracellular iron levels, and it is possible to see an inverted trade compared to the intracellular ones. In fact, at day 7, the iron levels are the same in both conditions, while at day 14 the extracellular iron levels are higher in the CTRL cell cultures.

4. Analysis of the FPN1A and 1B in different cell culture conditions

*4.1. Analysis of the FPN1A and 1B in **CTRL** and **NTDT** cell cultures in condition of iron depletion and iron overload*

CTRL cell cultures

4.1.1 Cell growth and viability

$CD34^+$ cells are cultured under conditions previously described.
At day 0 of the cell culture, Ferric Ammonium Citrate (FAC) with a concentration of 100µM and the iron chelator (Desferal DFO) at concentration of 4mM were added.
To observe if the differentiation took place according to the protocol, different evaluations of growth and viability at day 0, 4, 7, 11 and 14 were taken.
When drugs or chemicals compounds such as iron chelator and FAC are added to the cells culture media, the evaluation of the growth and viability is important, especially when these treated condition are compared to the untreated control, allowing assessing the effect of the drugs in question.
The data represent the average of three independent cultures.

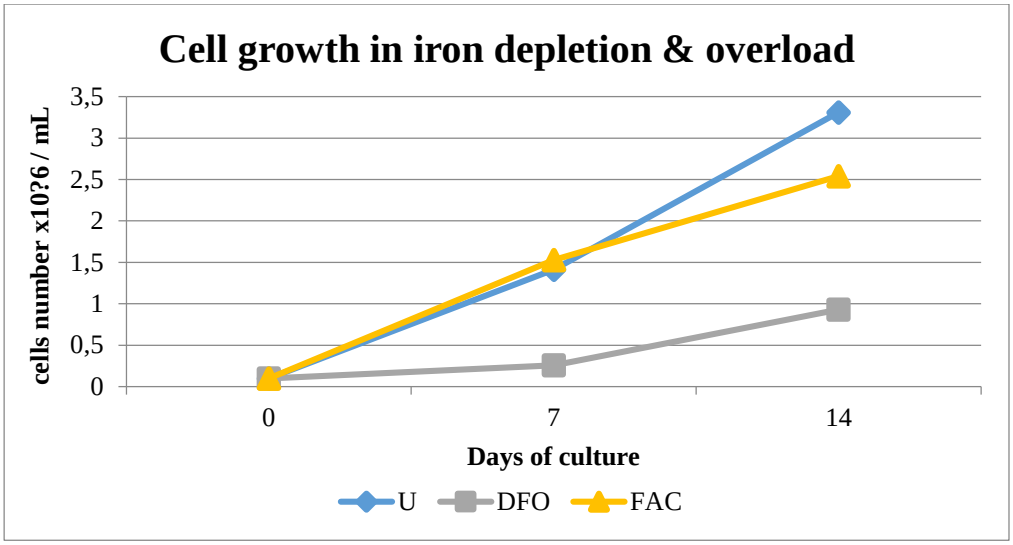


Chart 11: evaluation of the cellular growth in cell cultures treated with DFO 4µM and FAC compared to the untreated condition during the differentiation

From the chart, it can be observed that, until day 7, all conditions have an increasing trend; only the condition with the addition of FAC has a growth higher than the control, while the addition of DFO reduces growth compared to the control.

At day 14 the FAC condition has a greater growth than the control one, while the DFO condition presents, compared to the untreated, a lower growth.

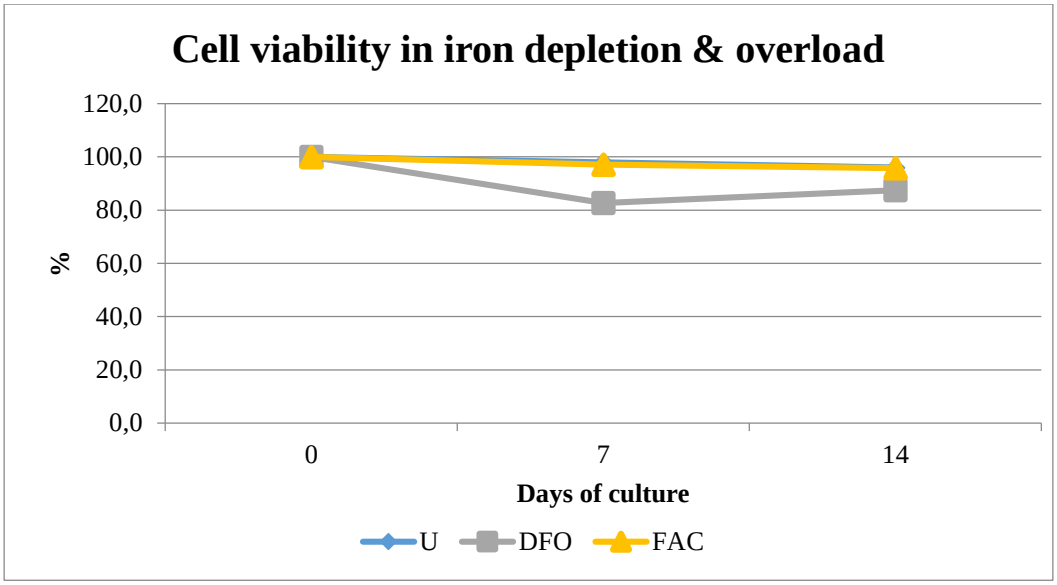


Chart 12: evaluation of the viability of the cell culture treated with DFO and FAC during the differentiation

The cell viability of the culture is in the range between 100 and 80% and the viability of the FAC condition totally overlap with the untreated one.

4.1.2 Cell morphology of DFO and FAC conditions

A **B**

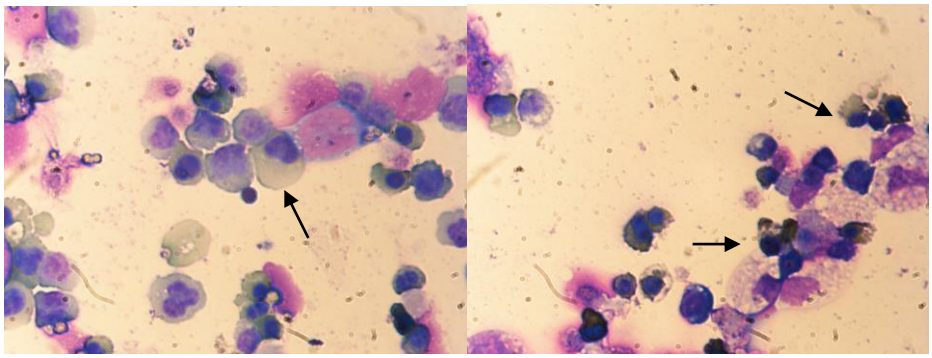


Figure 3: morphology of erythroblasts treated with DFO (A) and FAC (B)

From the pictures, it is possible to see that the culture treated with the chelating agent (Figure 3A) has the cells larger, less differentiated. The

culture treated with iron (Figure 3B) shows the cells more similar morphologically to the mature erythroblasts of the untreated control; plus, the benzidine staining indicates a significant increase of intracellular heme iron bound.

4.1.3 Analysis of the FPN1A and 1B expression in iron depletion and iron overload cell cultures

The expression of both ferroportin isoforms has been evaluated at day 14 of cell cultures at three different conditions: untreated, DFO, FAC.

The results are the means of three independent cell cultures.

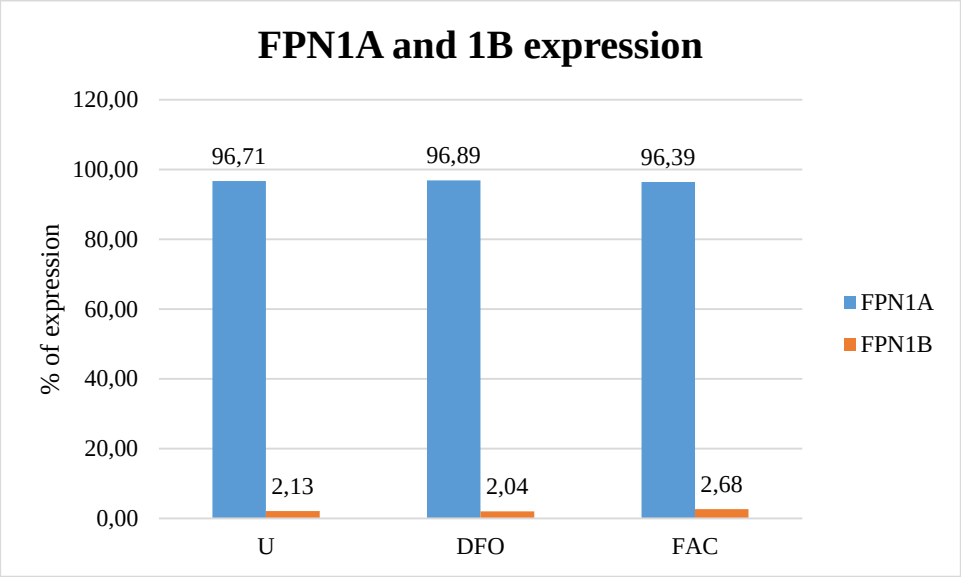


Chart 13: FPN1A and 1B expression at day 14 of cell culture, in untreated, DFO and FAC conditions

From the chart is clear that the ferroportin isoforms do not change their expression if the cells are treated with DFO or FAC in comparison to the untreated condition.

4.1.4 Analysis of the intra and extracellular iron levels in iron depletion and iron overload cell cultures

The iron level measurements was obtained by using a colorimetric Iron Assay Kit

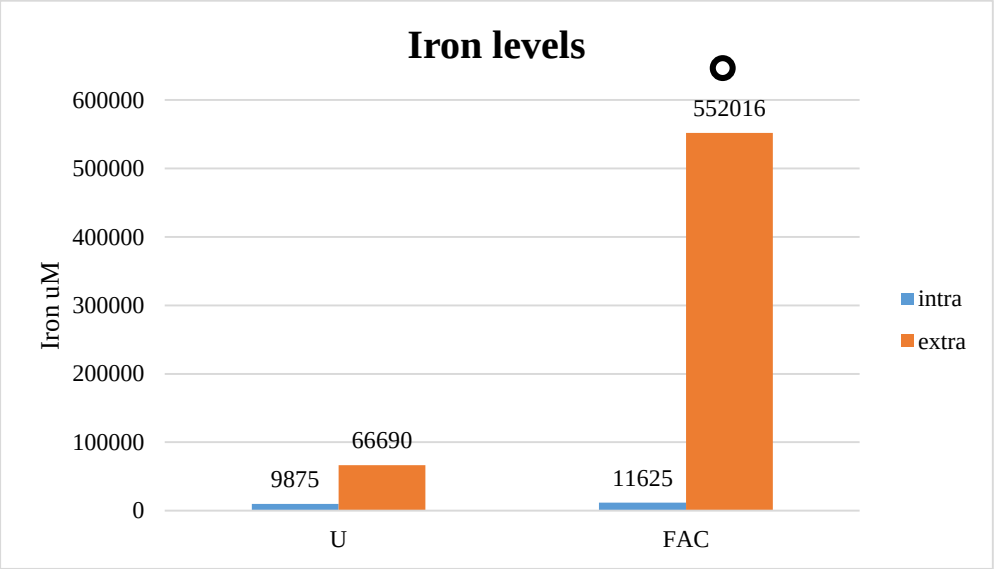


Chart 14: intra and extracellular iron levels in CTRL cell cultures treated with FAC

The intracellular iron level does not change between the FAC and untreated conditions. There is a consistent increase of extracellular iron in the FAC condition compared to the untreated ($p < 0.05$). This it might be related with the possibility that not all the FAC is absorbed by the cells, causing a high presence of iron into the supernatant.

NTDT cell cultures

4.1.5 Cell growth and viability

$CD34^+$ cells are cultured under conditions previously described.

At day 0 of the cell culture, Ferric Ammonium Citrate (FAC) with a concentration of 100µM or the iron chelator (Desferal DFO) at concentration of 4mM were added.

To observe if the differentiation took place according to the protocol, different evaluations of growth and viability at day 0, 7, and 14 were taken.
When drugs or chemicals compounds such as iron chelator and FAC are added to the cells culture media, the evaluation of the growth and viability is important, especially when these treated conditions are compared to the untreated control, allowing assessing the effect of the drugs in question.
The data represent the average of three independent cultures.

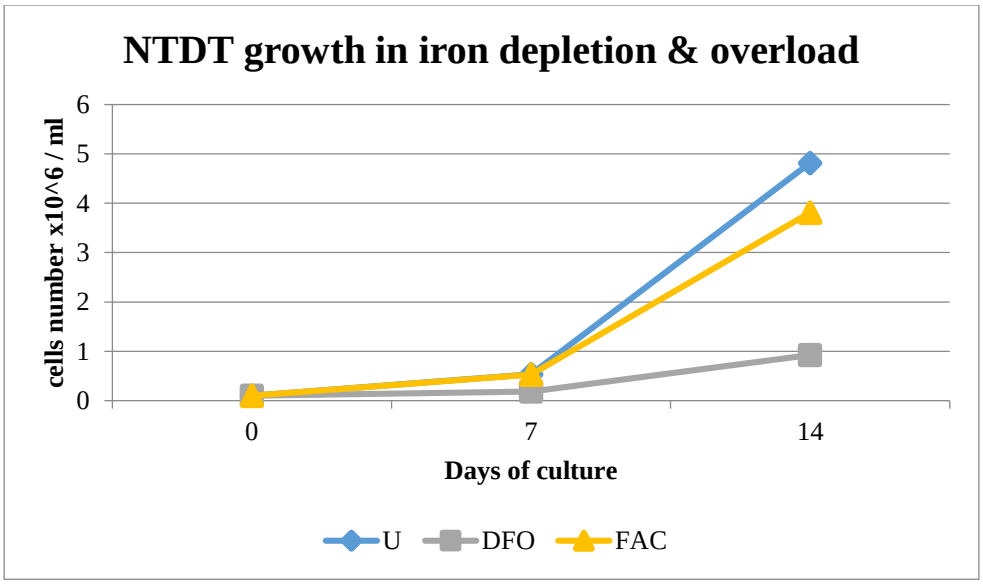


Chart 15: cell growth evaluation during cell differentiation in NTDT cell cultures treated with DFO 4µM and FAC compared to the untreated condition

The graph shows that at day 7 and 14 the cell growth of the treated conditions is lower compared to the control one. The growth value of the DFO condition is significantly lower, while the condition with FAC shows a growth value intermediate between the control and the Desferal.

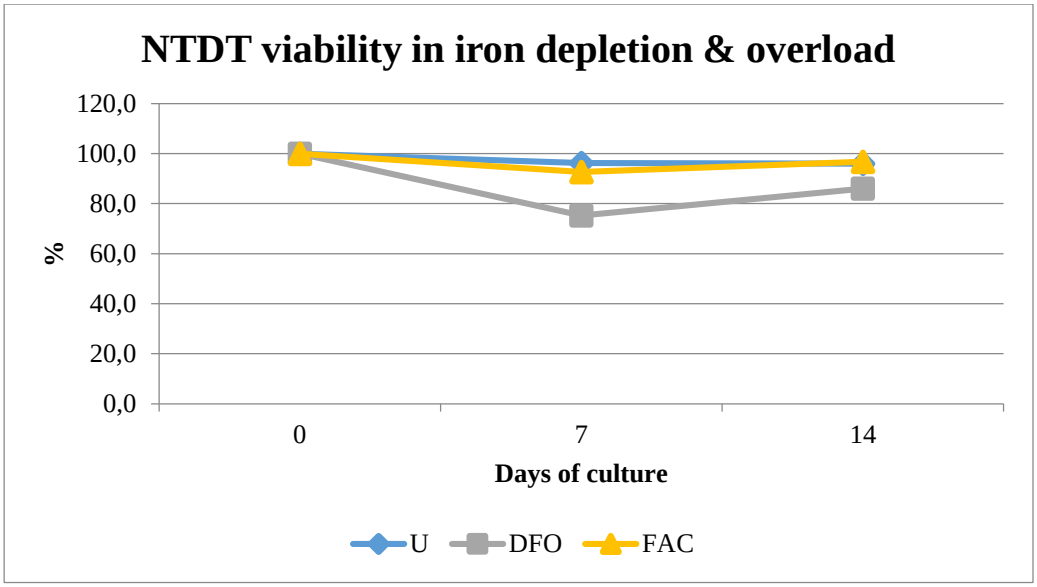


Chart 16: NTDT cell viability evaluation during cell differentiation in cell cultures treated with DFO 4µM and FAC compared to the untreated condition

The FAC condition shows a completely overlap with the untreated one and the range of vitality is between 100 and 92; while the DFO condition shows a little of cellular sufferance with a range of percentage of viability between 100 and 75.

4.1.6 Morphology of NTDT cells treated with FAC

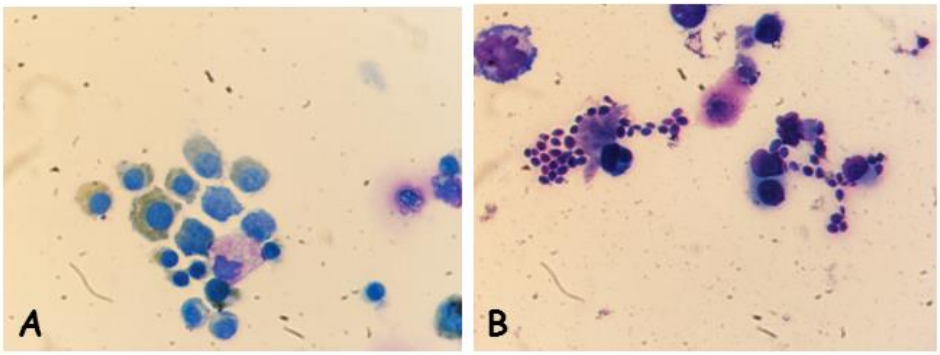


Figure 4: NTDT cells at day 14 untreated and treated with FAC

The picture shows that the erythroblast cells treated with FAC (figure 4B) are more little compared the untreated ones (figure 4A). The fact of seeing agglomerations of minute cells leads to the conclusion that these ones have entered into apoptosis due to the iron overload simulated by adding FAC.

4.1.7 Analysis of the FPN1A and 1B expression in iron depletion and iron overload cell cultures

The expression of both ferroportin isoforms has been evaluated at day 14 of cell cultures at three different conditions: untreated, DFO, FAC.

The values of the genetic expression of the ferroportin isoforms are shown as percentage.

The results are the means of three independent cell cultures.

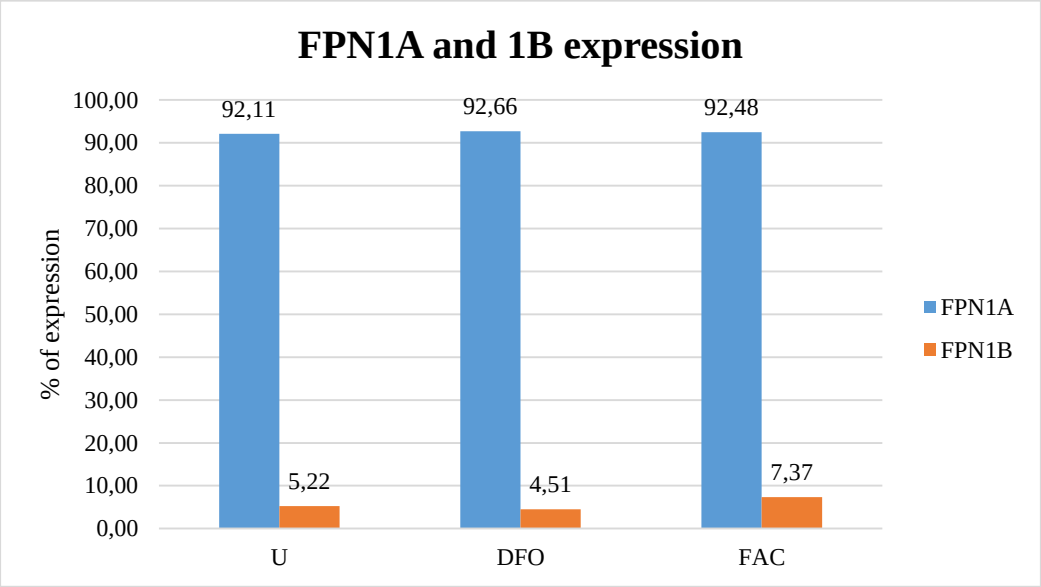


Chart 17: FPN1A and 1B expression in NTDT at day 14 of cell culture, in untreated, DFO and FAC conditions

From the chart is clear that the ferroportin isoforms do not change their expression if the cell cultures are treated with DFO and FAC in comparison to the untreated condition.

4.1.8 Analysis of the intra and extracellular iron levels in iron depletion and iron overload cell cultures

The iron level measurements was obtained by using a colorimetric Iron Assay Kit

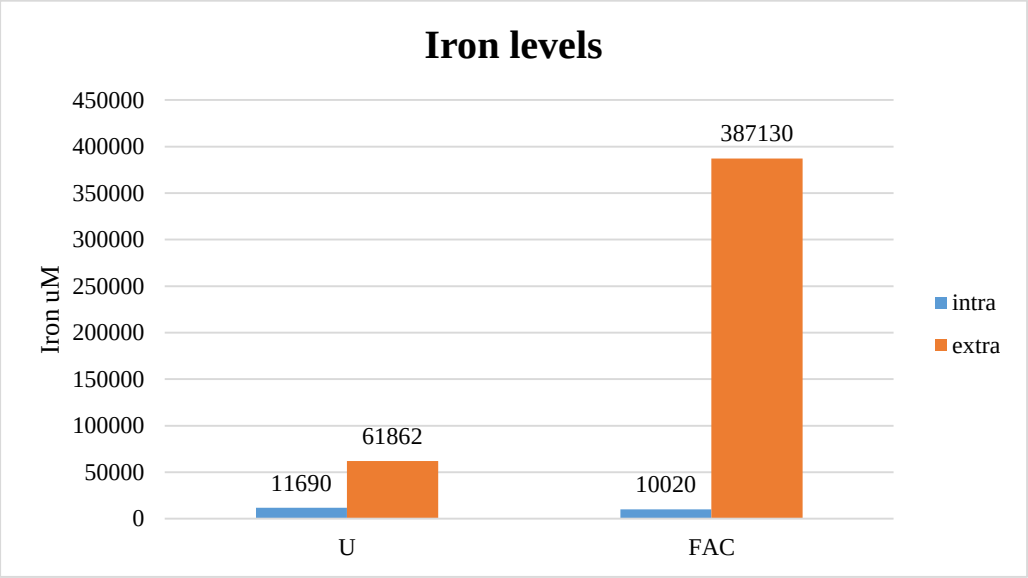


Chart 18: intra and extracellular iron levels in CTRL cell cultures treated with FAC

The intracellular iron level does not change between the FAC and untreated conditions. There is a consistent increase of extracellular iron in the FAC condition compared to the untreated one (fold increase: 6.25). As it has been already said for the CTRL cell cultures, the increase of extracellular iron concentration in the FAC condition could be related with the possibility that not all the FAC is absorbed by the cells, causing a high presence of iron into the supernatant.

*4.2. Analysis of the FPN1A and 1B in **CTRL** and **NTDT** cell cultures at the presence of free iron heme and heme iron bound*

CTRL cell cultures

4.2.1 Cell growth and viability

CD34$^+$ cells are cultured under conditions previously described.

At day 0 of the cell culture, the compounds Protoporfirin IX (SnPPIX, iron free heme) at the concentration of 50 and 20µM and the Hemin (iron bound to heme) at the concentration of 20 and 10µM were added.

To observe if the differentiation took place according to the protocol, different evaluations of growth and viability at day 0, 7 and 14 were taken.

When drugs or chemicals compounds such as protoporfirins and hemin are added to the cells culture media, the evaluation of the cell growth and viability is important, especially when these treated conditions are compared to the untreated control, allowing assessing the effect of the drugs in question.

The data represent the average of three independent cultures.

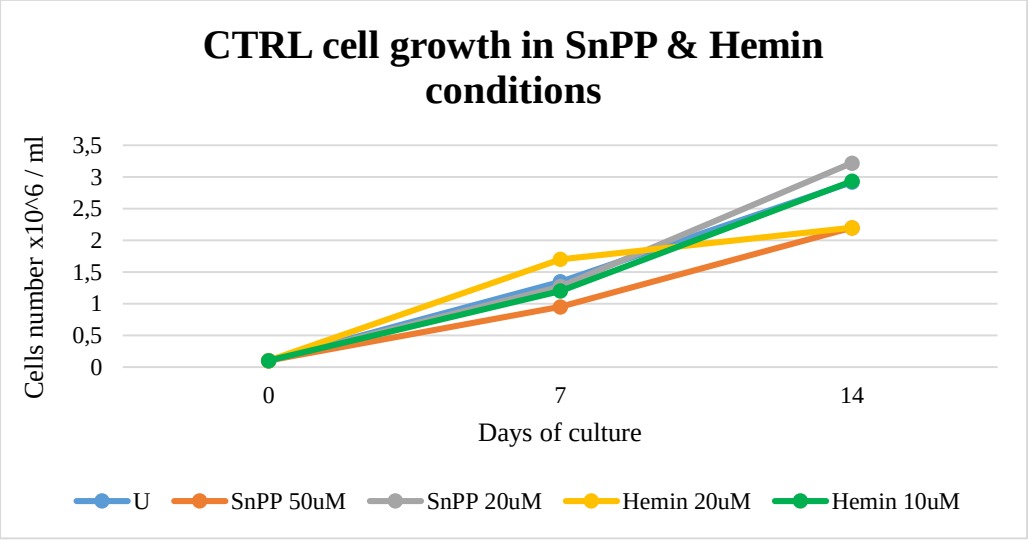


Chart 19: evaluation of the cellular growth in cell cultures treated with SnPPIX and Hemin compared to the untreated condition during the differentiation

All the conditions show an increase of cell growth during the erythroid differentiation. At day 14 is possible to see that the conditions with the high concentration of SnPP IX and Hemin show a cell increase less than the untreated condition.

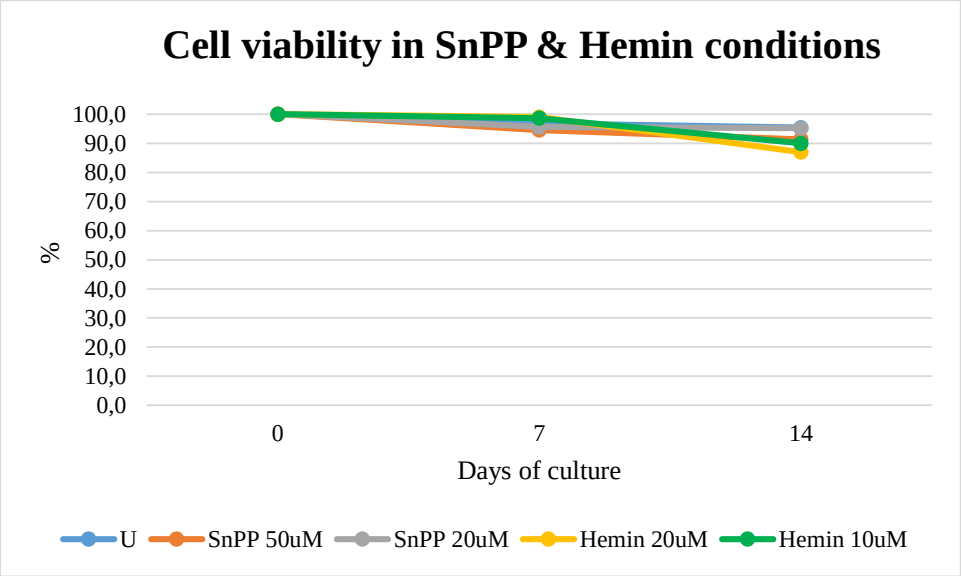


Chart 20: evaluation of the viability of the cell culture treated with SnPPIX and Hemin during the differentiation

All the compounds show a cell viability similar to the untreated condition; all the values are between 100 and 94,6.

4.2.2 Cell morphology of SnPPIX and Hemin conditions

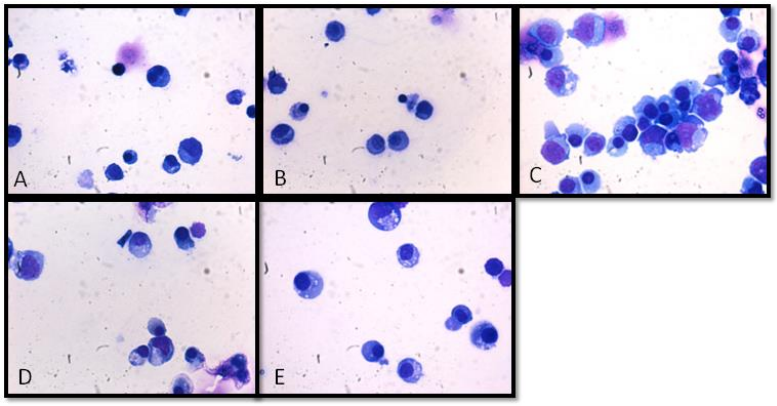


Figure 5: Cell morphology of day 14 of CTRL cell culture treated with SnPP IX and Hemin

The picture 5 shows five panels in which are represented the Untreated, SnPP IX 50µM, 20µM, the Hemin 20µM and 10µM conditions (5A, 5B, 5C, 5D and 5E respectively).

All the treated conditions show the same type of mature and differentiate cells as the untreated one; it means that the adding of those compounds to the cell cultures does not affect the erythroid differentiation process.

4.2.3 Analysis of the FPN1A and 1B expression at the presence of free iron heme and heme iron bound

The expression of both ferroportin isoforms has been evaluated at day 14 of cell cultures at five different conditions: untreated, SnPPIX 50 and 20µM and Hemin 20 and 10µM.

The results are the means of three independent cell cultures.

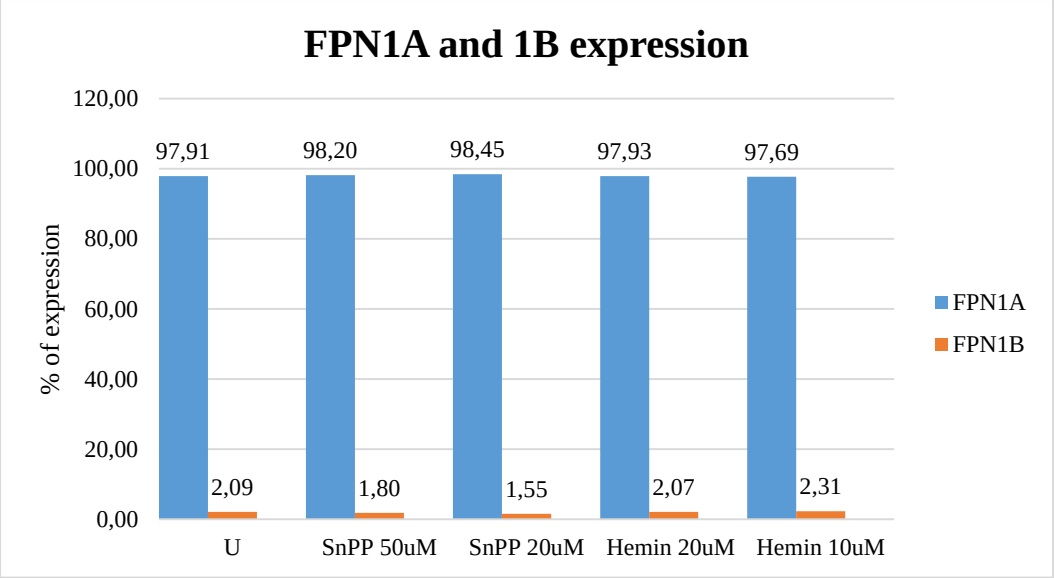


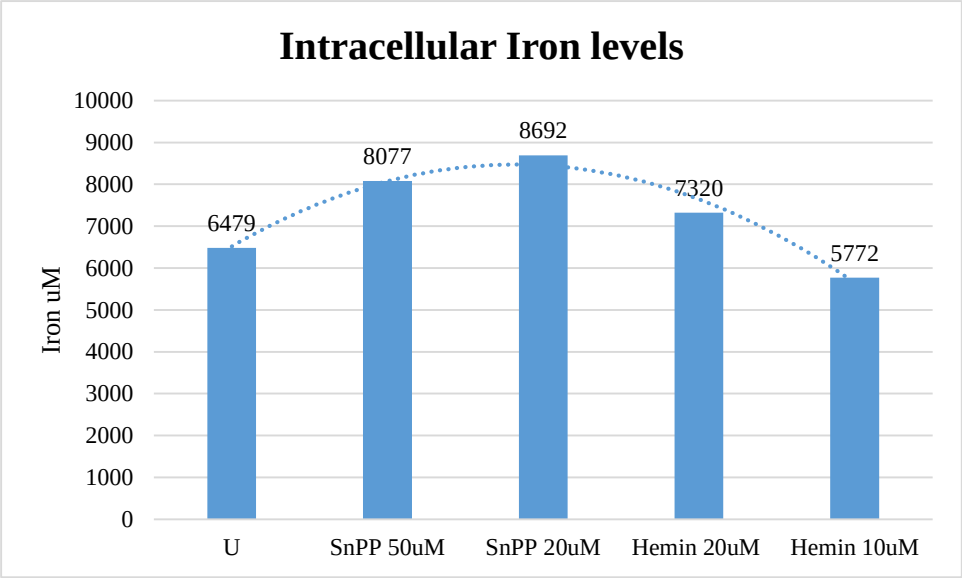
Chart 20: FPN1A and 1B expression at day 14 of CTRL cell culture, in untreated, SnPP 50, 20µM and Hemin 20, 10µM conditions

From the chart is clear that the ferroportin isoforms do not change their expression if the cell cultures are treated with both Protoporfirin IX and Hemin at different concentrations, compared to the untreated condition.

4.2.4 Analysis of the intra and extracellular iron levels at the presence of free iron heme and heme iron bound

The iron level measurements was obtained by using a colorimetric Iron Assay Kit

A



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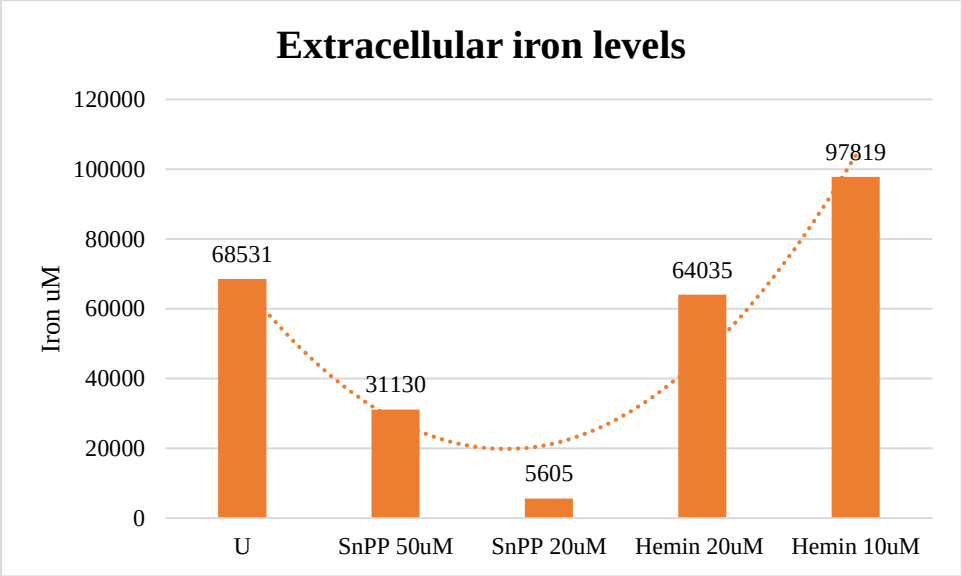


Chart 21: intra and extracellular iron levels at day 14 of differentiation, in CTRL cell cultures treated with SnPP 50, 20µM and Hemin 20, 10µM conditions

Regarding the intracellular iron levels (chart 21A) there are not significant differences between the treated and the untreated conditions. It is possible

to notice a slight increase of iron intracellular in the SnPP 20µM condition (8692 µM of intracellular iron vs 6479 µM for the untreated).

The chart 21B shows the extracellular iron levels. Observing the SnPP IX concentrations, it is possible to see a decrease of the martial levels in a dose – dependent manner; vice versa, there is an increasing of iron concentration into the supernatant at the condition treated with hemin, in a dose – dependent manner as well.

NTDT cell cultures

4.2.5 Cell growth and viability

$CD34^+$ cells are cultured under conditions previously described.

At day 0 of the cell culture, the compounds Protoporfirin IX (SnPPIX, iron free heme) at the concentration of 50 and 20µM and the Hemin (iron bound to heme) at the concentration of 20 and 10µM were added.

To observe if the differentiation took place according to the protocol, different evaluations of growth and viability at day 0, 7 and 14 were taken.

When drugs or chemicals compounds such as protoporfirins and hemin are added to the cells culture media, the evaluation of the growth and viability is important, especially when these treated conditions are compared to the untreated control, allowing assessing the effect of the drugs in question.

The data represent the average of three independent cultures.

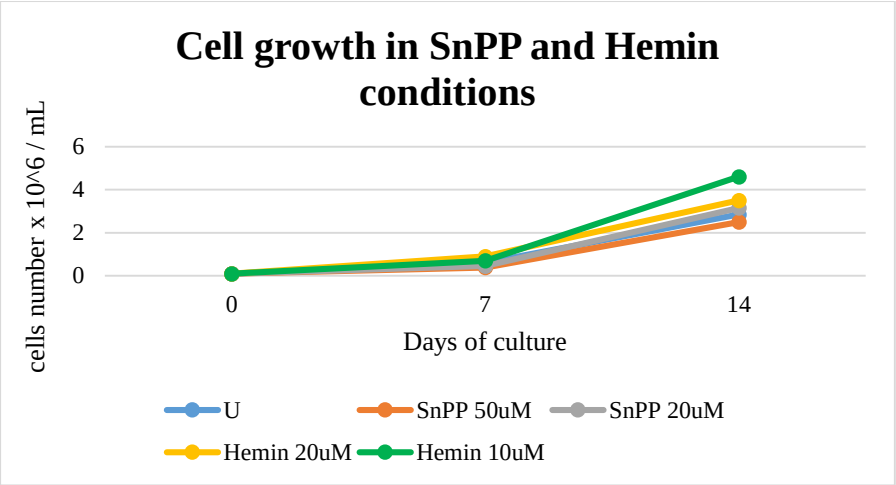


Chart 23: evaluation of the cellular growth in cell cultures treated with SnPPIX and Hemin compared to the untreated condition during the differentiation

All the conditions show an increase of cell growth during the erythroid differentiation. At day 14 is possible to see that both the Hemin and the SnPP IX 20µM shows values of cell growth major than the untreated, while the SnPP IX 50µM shows a cellular increase minor than the untreated.

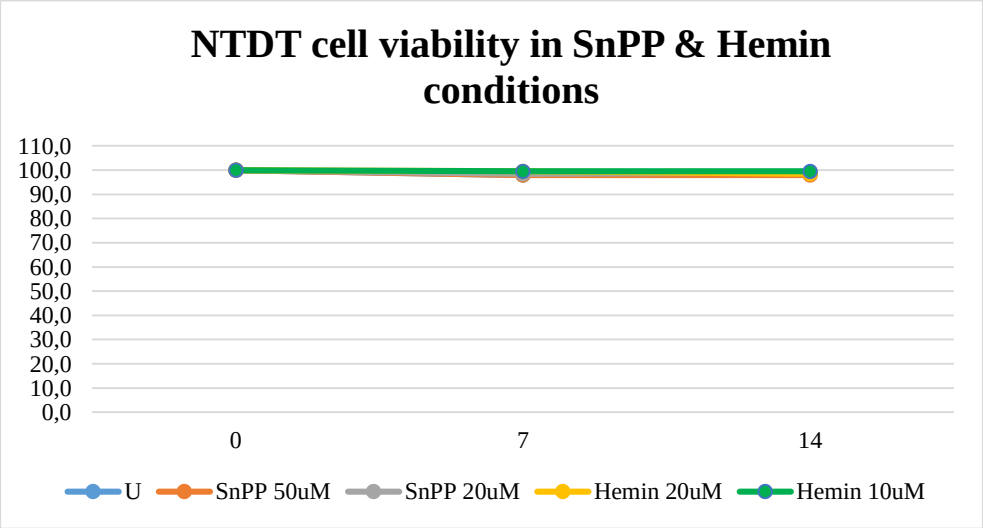


Chart 24: evaluation of the viability of the NTDT cell culture treated with SnPPIX and Hemin during the differentiation

From the chart is possible to see that all the compounds do not show cellular toxicity since all their viability values are overlapping the untreated ones (viability values range between 100 and 98).

4.2.6 Morphology of NTDT cells treated with SnPP and Hemin

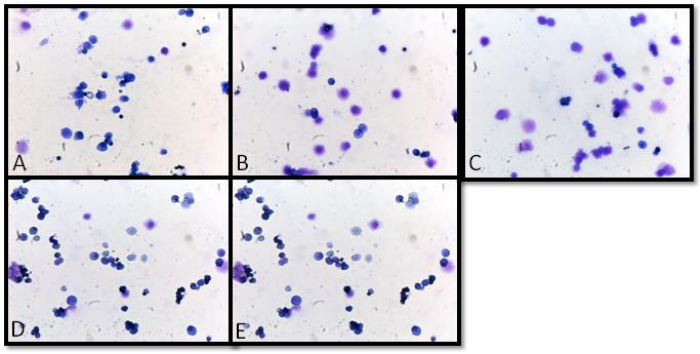


Figure 6: NTDT cells at day 14 treated with SnPP and Hemin

The picture 6 shows five panels in which are represented the Untreated, SnPP IX 50µM, 20µM, the Hemin 20µM and 10µM conditions in the NTDT cell culture (5A, 5B, 5C, 5D and 5E respectively).

All the treated conditions show the same type of erythroid cells as the untreated one; it means that the adding of those compounds to the cell cultures does not affect the erythroid differentiation process as it has been already seen for the CTRL cell cultures.

4.2.7 Analysis of the FPN1A and 1B expression at the presence of free iron heme and heme iron bound

The expression of both ferroportin isoforms has been evaluated at day 14 of cell cultures at five different conditions: untreated, SnPPIX 50 and 20µM and Hemin 20 and 10µM.

The values of genetic expression are shown as percentage.

The results are the means of three independent cell cultures.

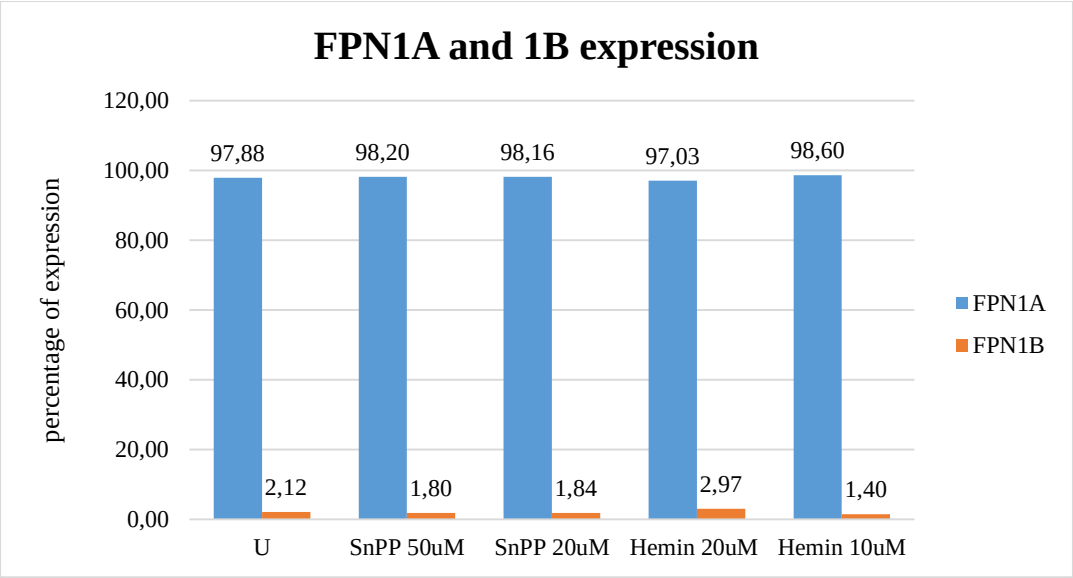


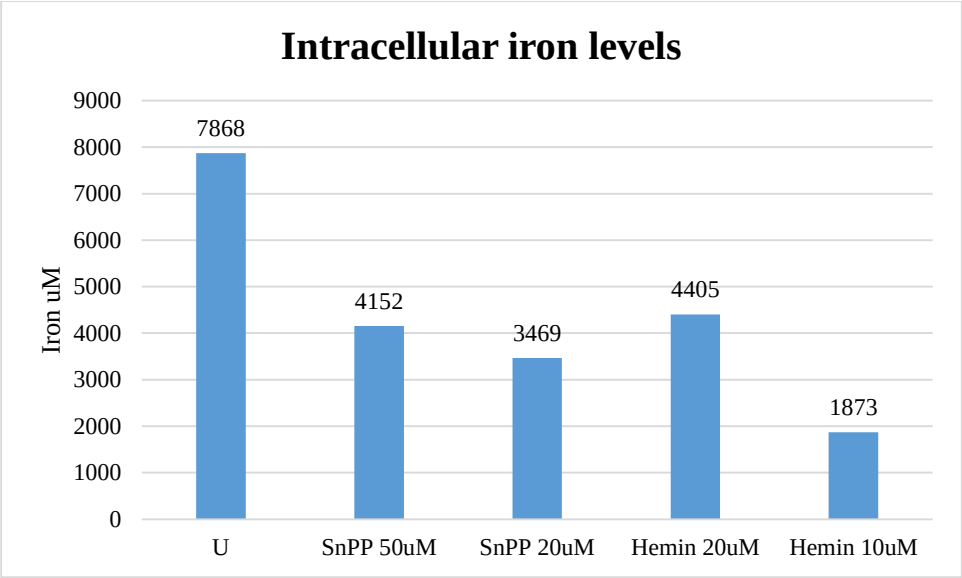
Chart 25: FPN1A and 1B expression in NTDT at day 14 of cell culture in untreated, SnPP 50, 20µM and Hemin 20, 10µM conditions

From the chart is clear that the ferroportin isoforms do not change their expression if the cell cultures are treated with both Protoporfirin IX and Hemin at different concentrations, compared to the untreated condition

4.2.8 Analysis of the intra and extracellular iron levels at the presence of free iron heme and heme iron bound

The iron level measurements was obtained by using a colorimetric Iron Assay Kit

A



B

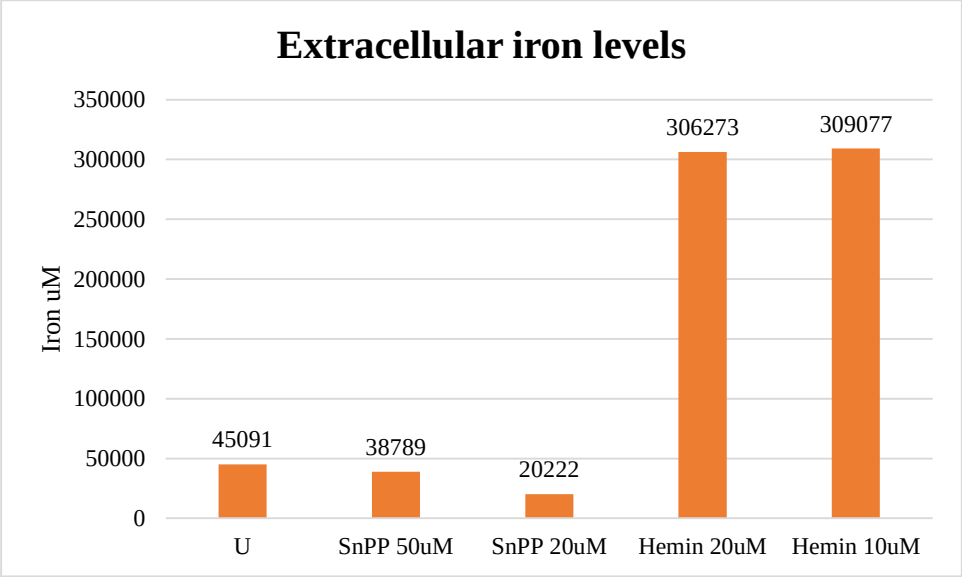


Chart 26: intra and extracellular iron levels in NTDT cell cultures treated with SnPP IX 50, 20µM and Hemin 20, 10µM

The intracellular iron level (chart 24A) decreases in the treated conditions compared with the untreated one (2 fold decrease regarding all the SnPP IX and the Hemin 20µM conditions, 7 fold decrease regarding the Hemin 10µM condition).

The chart 24B represent the extracellular iron level: no much difference is observed between the untreated and the SnPP IX conditions, while a big change is notable between the control and the Hemin conditions where is possible to see a marked increase of iron present into the cell culture supernatant. The situation is different from the FAC condition previously described since the kit cannot measure the iron bound to different proteins such as heme.

*4.3. Analysis of the FPN1A and 1B in **CTRL** cell cultures under oxidative stress*

4.3.1 Cell growth and viability

$CD34^+$ cells are cultured under conditions previously described.
To simulate the oxidative stress condition, hydrogen peroxide 0.1mM (H_2O_2) was added at every other days from day 0 to day 14 of the cell cultures.
To observe if the differentiation took place according to the protocol, different evaluations of growth and viability at day 0, 7 and 14 were taken.
When drugs or chemicals compounds such as the hydrogen peroxide are added to the cells culture media, the evaluation of the growth and viability is important, especially when these treated conditions are compared to the untreated control, allowing assessing the effect of the compounds.
The data represent the average of three independent cell cultures.

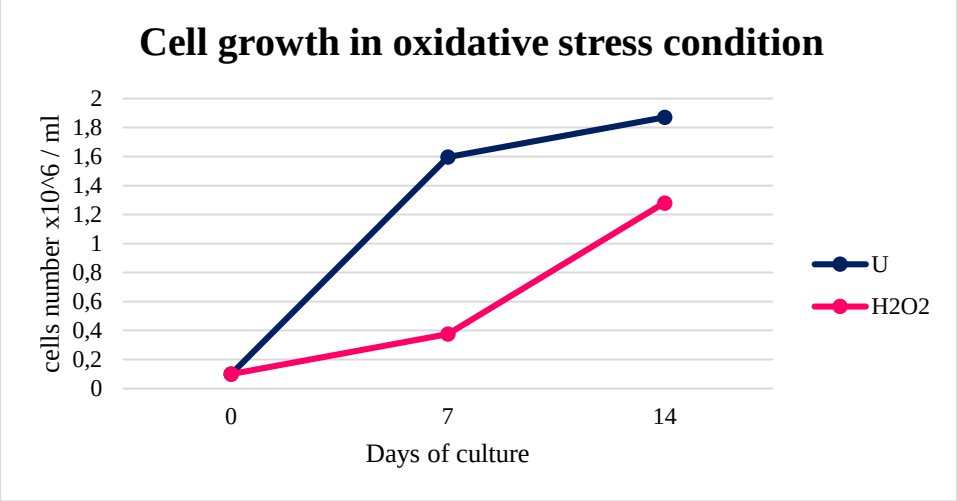


Chart 27: evaluation of the cellular growth in cell cultures treated with H_2O_2 0.1mM compared to the untreated condition during the differentiation

From the chart is possible to see that, even if there is an increase of cell number during the entire process of erythroid differentiation in both conditions, the adding of H_2O_2 causes a minor cell growth compared to the CTRL condition.

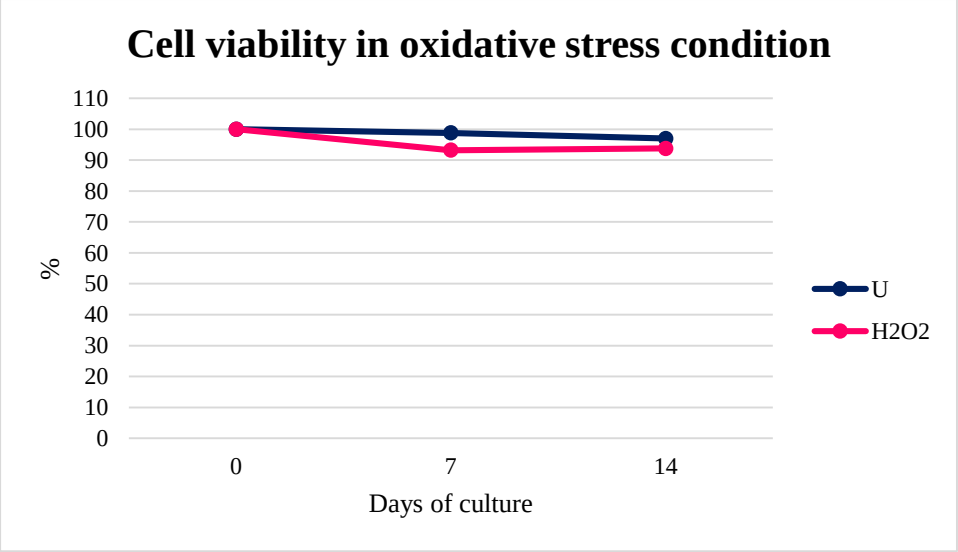


Chart 28: evaluation of the viability of the cell culture treated with H_2O_2 0.1mM during the differentiation

From the chart is possible to see that the adding of H_2O_2 0.1mM does not cause cell toxicity. In fact, the cell growth in the condition under oxidative stress is almost the same of the untreated condition. The percentage values are between 100 and 93.7 %.

4.3.2 Cell morphology of H_2O_2 0.1mM condition

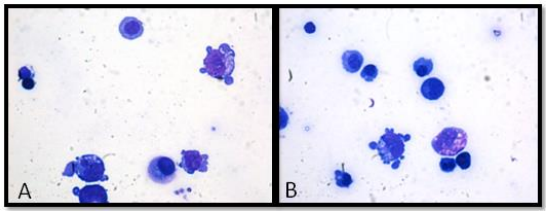


Figure 7: cell morphology at day 14 of CTRL cell culture treated with H_2O_2

The figure 7A and 7B shows the erythroblasts morphology at day 14 of the untreated and the H_2O_2 0.1mM conditions. As we can see, there are no differences between the two panels; it means that the adding of hydrogen peroxide does not affect the erythroid differentiation.

4.3.3 Analysis of the FPN1A and 1B expression under oxidative stress

The expression of both ferroportin isoforms has been evaluated at day 14 of cell cultures.

The results are the mean of three independent cell cultures.

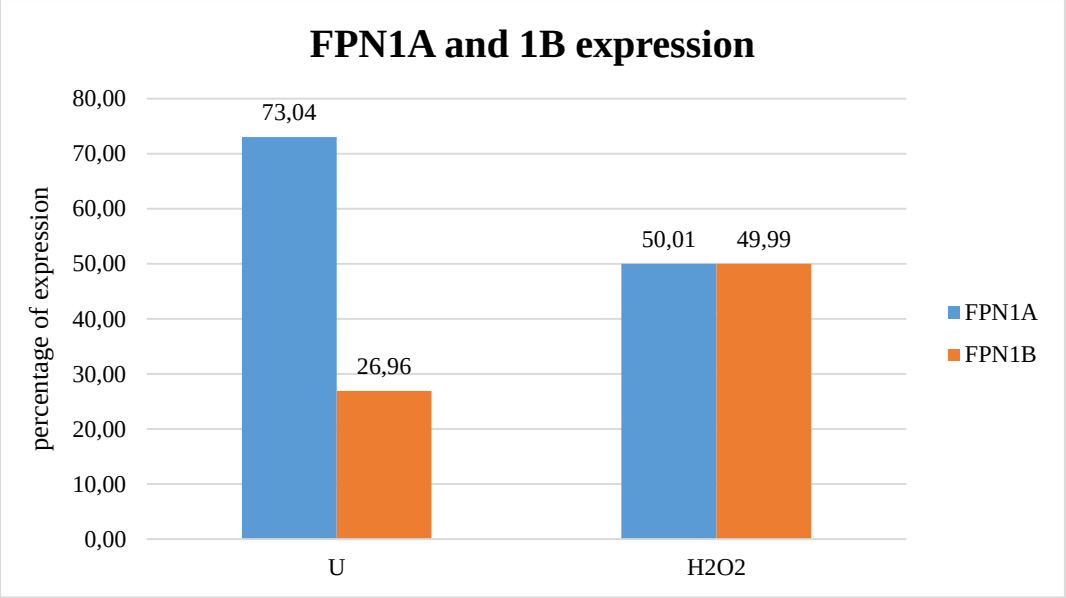


Chart 29: FPN1A and 1B expression at day 14 of CTRL cell culture, in untreated and H_2O_2 0.1mM conditions

This chart shows a change in the FPN1A and 1B expression in the condition treated with H_2O_2 0.1mM compared to the untreated one. In fact, the ratio of the two isoforms goes from 2,7 : 1 for the control to 1 : 1 for the H_2O_2 0.1mM condition suggesting a possible role of the oxidative stress in the modulation of the FPN1B expression.

4.3.4 Analysis of the intra and extracellular iron levels under oxidative stress

The iron level measurements was obtained by using a colorimetric Iron Assay Kit

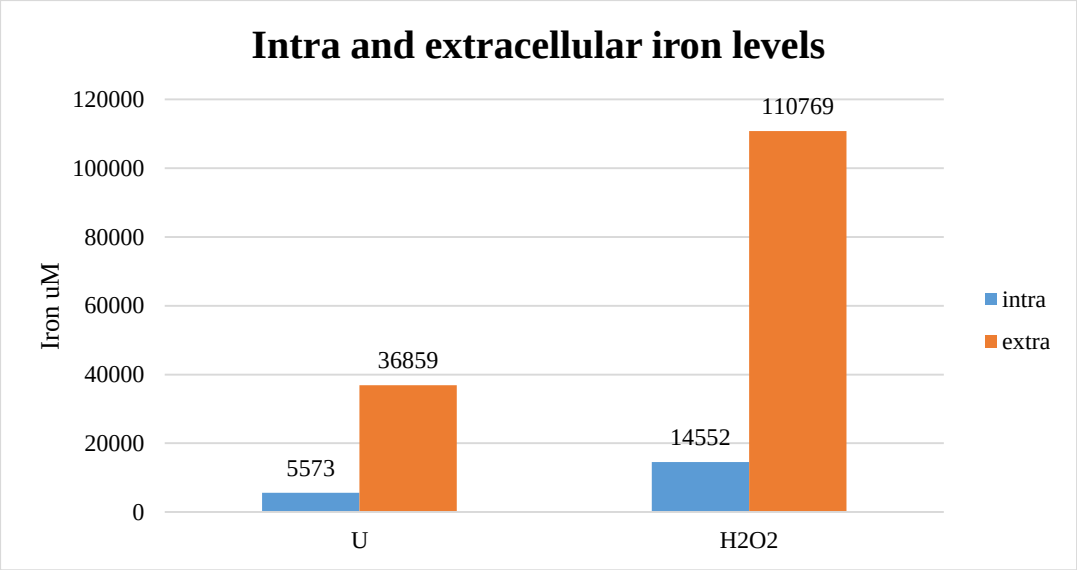


Chart 30: intra and extracellular iron levels at day 14 of differentiation, in CTRL cell cultures treated with H_2O_2 0.1mM

Regarding the intracellular iron levels there is a slight increase of the ion concentration in the treated condition compared to the untreated one. It is possible to see a marked increase of extracellular iron levels into the H_2O_2 0.1mM condition compared to the control.

These results, taken together with the expression profiling of the ferroportin, suggest that under oxidative stress, there is a change of predominant ferroportin isoform expression (from 1A to 1B) suggesting a possible role of the ROS in the regulation of FPN1B expression.

DISCUSSION

Thalassemia intermedia (TI) is a heterogeneous group of syndromes caused by a defect in the synthesis of β-globin chains and characterized by ineffective erythropoiesis and decreased production of red blood cells. The clinical manifestations of TI are the result of three key factors: ineffective erythropoiesis, chronic anaemia and iron overload. Unlike patients with thalassemia major (TM), in which iron overload is mainly due to the transfusion therapy, patients with TI accumulate iron mainly due to an increased intestinal absorption and to the ineffective erythropoiesis. [60] The pathophysiology of iron overload in TI and its toxicity are still not completely known.

In recent years there have been major advances in our knowledge about the regulation of iron metabolism that have had implications for understanding the pathophysiology of some human disorders like β-thalassemia and other iron overload diseases. However, little is known about the relationship among ineffective erythropoiesis, the role of iron-regulatory genes, and tissue iron distribution in β-thalassemia.

Regarding these last two points, there are many studies about the role of hepcidin for the regulation of iron metabolism in pathologic diseases such as the thalassemias, but less has been published about its ligand, the ferroportin.

Ferroportin can be considered as a new protein since it has been almost fully described only in these last few years (by Cianetti and Zhang groups); for the first time in 2010 it has been demonstrated that the FPN is expressed even in the erythroblast cells [3] and not only in the macrophages, enterocytes and hepatocytes.

From this new information it has been formulated the hypothesis that the erythroid ferroportin could participate in a pathway in which the red blood

cell communicates the iron need in order to expand the erythroid compartment ignoring the systemic iron levels. [3]

Moreover, another one flanked this discovery: the ferroportin has two different isoforms; one iron responsive (FPN1A) and the other one that is not (FPN1B).

It meant that even if the ferroportin had an important role in the iron metabolism; it could be regulated by other mechanisms as well and not only by iron.

From this assessment, different lab groups tried to find which was the regulatory mechanism for the expression of the ferroportin and, in particular, of its isoforms.

In the literature, it is possible to find many works on the study of the FPN1A in different cell types and cell lines.

Cianetti and Marro hypothesized the role of heme in the regulation of the ferroportin 1A (but nothing has been said about the isoform 1B) [3][6]. Although those studies were conducted on macrophages and not on erythroid cells, they lead to an important conclusion: the iron recycling from heme involves a single transcription control mechanism that regulates heme catabolism, iron storage and detoxification as well as iron export in a coordinated manner.

Zhang was the first to study the role of the isoform 1B in mice. These studies confirmed the expression of this isoform at the erythroid level; plus, the FPN1B was not repressed in situation of iron depletion suggesting a possible role of the FPN1B: this protein could make the erythroblast cells more responsive to the changes of the systemic iron levels compared to the intracellular ones. [5]

In 2013 Chiabrando found another factor that could regulate the ferroportin expression: the Hypoxia Inducible Factor 2α (HIF2α). This study was

conducted in macrophages and enterocytes, but not in erythroid precursors. [61]

From the time of its discovery to the present day, a lot of information about the ferroportin has been collected. In fact, now it is known that the expression of FPN is controlled by multiple mechanisms underlining the importance of precise regulation of iron export: 1) transcriptional control by haemoglobin; 2) alternative promoter usage in duodenal enterocytes and erythroid precursor cells; 3) translational control by the IRE/IRP system in response to iron levels; as well as 4) post-translational control by hepcidin and systemic iron requirements. How the regulation of FPN by the different stimuli is prioritized will be the subject of further investigation.

Unluckily less is known about how the ferroportin is expressed and regulated in the thalassemia syndromes, more specifically, in non-transfusion dependent thalassemia (NTDT).

The principal aim of our work is to try to describe the role of ferroportin during human normal and pathological erythroid differentiation. Particular attention will be given to the NTDT in order to collocate the ferroportin in the huge puzzle such as the iron metabolism process and try to find a possible role in an environment where the iron metabolism is altered.

In the literature the majority of the studies are conducted on cell lines and mouse models, hence, it is of relevant importance try to work out a study based on *ex vivo* culture systems in order to study erythroid differentiation in both physiological and thalassemia conditions.

A method for the preparation of cell cultures erythroid is the "**method of Fibach**". It is divided into 2 phases: the first, which consists in the cultivation of mononuclear cells, obtained by layering with limphoprep and centrifugation, in the presence of various growth factors that eliminates the

lymphocytic component; in this way the primitive erythroid progenitors (BFUe) proliferate and differentiate, while other cells such as lymphocytes and monocytes are eliminated. In the second phase, the progenitors recovered from the phase in suspension and cultured in a medium containing erythropoietin for fourteen days, continue to proliferate and mature into orthochromatic normoblasts and then in erythrocytes enucleated. It remains, however, a significant component of lymphocytes (about 38% of $CD45^+$ cells) that interferes with the analysis of erythroid cells. [62] In our laboratory has been optimized then a new method of cultivation that allows to have a pure erythroid population. To do this, we start from CD34 positive cells separated from monocytes and lymphocytes by using immune magnetic beads binding the antibody anti - CD34. Using a combination of growth factors and cytokines (SCF, Epo, IL-3), the progenitors are induced to proliferate and then differentiate. With the latter method is possible to reproduce all stages of erythropoiesis in 14 days, to obtain a large number of cells even starting from a low amount of peripheral blood. [58] For these characteristics, the culture protocol developed by us is the best method to be applied to the study of genes involved in iron metabolism.

Our work proposes the study of **ferroportin** in erythroid cell cultures:

A. <u>in physiological conditions (or control), in cell cultures set with erythroid precursor from peripheral blood of healthy donors:</u>

During the differentiation our study confirmed what has been said in the literature (by Cianetti), the expression of FPN1B is very low in the early days of culture and increases at day 7 (pro erythroblasts) and then decreases again at day 14 (mature erythroblasts).

The FPN1A has the opposite trend: is high at the precursors stage (day 0), then decreases at day 7 and increases again at day 14. From these results

is possible to speculate that the erythroid cells need a major expression of the isoform non-iron related at day 7, because at this stage the cells need iron for the haemoglobin synthesis and it is important to have an iron transporter sensitive to the systemic iron, not to the intracellular one. On the other hand, if at this stage the FPN1A would be the major isoform expressed, the cells could not have enough iron for the haemoglobin synthesis, since this isoform allows the exit of all the surplus of iron necessary for the heme synthesis.

The intra and extracellular iron levels confirm the expression trade of the ferroportin mRNA (see Appendix, chart 1A): from day 7 to day 14 of the cell culture, there is a decrease of intracellular and a slight increase of extracellular iron.

That is explained by the fact that the erythroid cells do not need iron anymore at the end of the differentiation process and there is less iron into the supernatant as what we would expect because the abundant part of the ion was used for all the biosynthetic process at the pro-erythroblast stage (around day 7 of differentiation).

B. <u>In thalassemic conditions in cell cultures set with erythroid precursor from peripheral blood of NTDT patients</u>.

During the thalassemic differentiation, the expression of both FPN isoforms follows the same trade of the control cell cultures: the FPN1B is low in the early days of culture and increases at day 7 (pro erythroblasts) and then decreases again at day 14 (mature erythroblasts).

The FPN1A has the opposite trend: is high at the precursors stage (day 0), then decreases at day 7 and increases again at day 14.

If we compared the expression of the ferroportin isoforms between the CTRL and NTDT differentiation, is possible to see that the FPN1B is the major expressed isoform in NTDT compared to the physiologic condition.

The intra and extracellular iron levels confirm the expression trade of the ferroportin mRNA (see Appendix, chart 1B): from day 7 to day 14 of the cell culture, there is a decrease of intracellular and a slight increase of extracellular iron.

This has the same explanation given for the Control cell cultures. The erythroid cells do not need iron anymore at the end of the differentiation process; there is less iron in the supernatant as we would expect because the abundant part of the ion was used for all the biosynthetic process at the pro-erythroblast stage (around day 7 of differentiation).

Comparing the intra and extracellular iron levels between the CTRL and NTDT cell cultures during the erythroid differentiation process, it is possible to see that both the conditions have roughly the same amount of iron concentration at both the intra and extracellular levels.

C. <u>In condition of iron depletion and iron overload by using DFO and FAC</u>.

<u>In CONTROL cell cultures</u>

The results show that the conditions of iron depletion and iron overload do not cause a change of mRNA expression in both the FPN isoforms. Regarding the FAC condition, this is a confirm of what has been illustrated by Marro [6] In fact, they treated cultures of macrophages with FAC and the FPN did not show any changes in its mRNA expression; here we had the same result in the erythroblast cell cultures.

The genetic analysis are confirmed by the intra and extracellular iron levels, if the adding of FAC does not change the FPN isoforms expression, there are not differences at the intracellular iron levels between the untreated and the FAC condition.

The only change is in the iron levels in the supernatant, but this could be surely related to the amount of Ferric Ammonium Citrated that has not been

imported into the cells and this amount has been measured by our Iron assay Kit.

In NTDT cell cultures

The results show that the conditions of iron depletion and iron overload do not cause a change of mRNA expression in both the FPN isoforms as it has been already seen in the CTRL cell cultures.

The genetic analysis are confirmed by the intra and extracellular iron levels, if the adding of FAC does not change the FPN isoforms expression, there are not differences of the intracellular iron concentration between the untreated and the FAC condition.

The only change is in the iron levels in the supernatant, but this could be surely related to the amount of Ferric Ammonium Citrated that has not been imported into the cells and this amount has been measured by our Iron assay Kit.

So even for the NTDT cell cultures, the iron depletion and overload do not influence the FPN isoforms expression nor the cellular iron content.

D. In condition of free iron heme and heme iron bound by adding SnPP IX and hemin to the cell cultures.

In CONTROL cell cultures

As it has already seen for the iron depletion and overload conditions, the ferroportin isoforms mRNA do not show any changes under free heme and heme treatment.

That is not what has been explained by Marro in mice and macrophages treated with SnPP IX and Hemin, since they discovered some MARE/ARE sequences on the FPN promoter (they did not distinguish between the two isoforms) responsive for the heme. [6]

From their results, the FPN mRNA increased under heme exposure, in our erythroblasts cell cultures the amount of FPN mRNA (both isoforms) is the same in conditions treated with Hemin or SnPP IX.

The only explanation could be that the MARE/ARE sequences (MAF Recognition Elements / Antioxidant Response Elements) are tissue – specific, that means they are present only on the macrophages promoters; or we need to improve the number of our experiments and see what happens next.

As there is no difference in the FPN expression, even the intracellular iron levels remain the same compared to the untreated condition. The only change can be observed in the extracellular iron: the SnPP IX conditions show a decrease in iron concentration that could be related to the fact the SnPP IX is heme without iron and when this compound enters into the cells in order to participate to the heme biosynthesis, the cells need iron to bound to the protoporfirins and make heme. To do that, the cells have to take iron from the extracellular environment, that is the reason why the amount of iron into the supernatant of SnPP IX conditions is lower compared to the untreated one.

In NTDT cell cultures

The ferroportin isoforms do not change their expression if the cell cultures are treated with both Protoporfirin IX and Hemin at different concentrations, compared to the untreated condition. Those are similar result as the CONTROL cell cultures.

Regarding the iron concentrations, the intracellular iron level decreases in the treated conditions compared with the untreated one. This is not reflecting the FPN expression, so it is important to repeat the experiment in order to have more data and try to see if there is some modulation of the intracellular iron content by adding those compounds or not in the NTDT

cell cultures. The extracellular iron levels show no much difference between the untreated and the SnPP IX conditions, while a big change is notable between the control and the Hemin conditions where is possible to see a marked increase of iron present into the cell culture supernatant. The situation is different from the FAC condition previously described since the kit cannot measure the iron bound to different proteins such as heme. That could be related to a heme degradation in the extracellular environment, releasing the free iron and this one could be measured by the kit.

E. <u>In condition of oxidative stress by adding hydrogen peroxide 0.1mM at the **control** cell cultures.</u>

There are many papers explaining the high presence of ROS production in the beta thalassemia due to the Fenton's reaction caused by the iron overload. [63][64][65]

This lead us to further investigate the role of the oxidative stress in the erythropoietic process and if the ROS could be the responsive element for the ferroportin isoform 1B expression.

We tried to simulate a situation of oxidative stress in the control cultures by adding hydrogen peroxide and then we studied the mRNA of both isoforms to see if something has changed in their expression.

Something did change, in fact, the condition treated with H_2O_2 shows an increase of the FPN1B expression and a decrease of the FPN1A isoform compared with the untreated condition. Even the intra and extracellular iron levels follow the genetic expression of the protein: an increase of the FPN1B is related with an increase of iron export from the cell to the supernatant, showing an increase of extracellular iron in condition under oxidative stress compared to the untreated.

CONCLUSIONS

In conclusion, it can be said that the method defined by our lab was optimal for the study carried out and new results unknown up to now have been obtained.

To have a complete view of all the work will be necessary to define some macrophage cultures in which the expression and production of Ferroportin 1A and 1B in the same condition of the erythroid cultures will be studied.

It will also be of great importance alongside the results obtained in this work, with an analysis of the Ferroportin by the technique of Western – Blot in order to study in deep if the oxidative stress could influence the amount of translate protein and not only the mRNA expression. The problem of this technique is that the ferroportin isoforms are the same once translated, so the western blot could not distinguish between the FPN1A and 1B, however, it could be also useful to determine the amount of the protein produced. We have to define other techniques in order to find the proper one that could discriminated between the production of the two isoforms.

It could be also interesting moving from the *ex vitro* to an *in vivo* contest by using wild type and thalassemic mice models in order to be able to study the large pathway of iron and heme metabolism and have a major comprehension of all the mechanisms elapsing and the differences between the expression of the FPN1A and 1B isoforms in different cellular compartments.

The last, but not least, is try to confirm the results obtained in the control cultures by using the NTDT cells. We have to set a panel of minimum three NTDT cell cultures and treat them with hydrogen peroxide and see if the FPN1B will behave in the same way as the CTRL one. This will be the confirm that the major expression of the FPN1B during all stages of the erythroid differentiation in NTDT compared to the CTRL is due to the fact that the NTDT patients have a major level of ROS compared to the healthy

subjects and the oxidative stress could be the responsive factor of the FPN1B expression.

Discover how the ferroportin is regulated in the NTDT contest could help us to think about and define new therapy strategies not only oriented to the hepcidin itself, but involving the ferroportin as well.

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APPENDIX

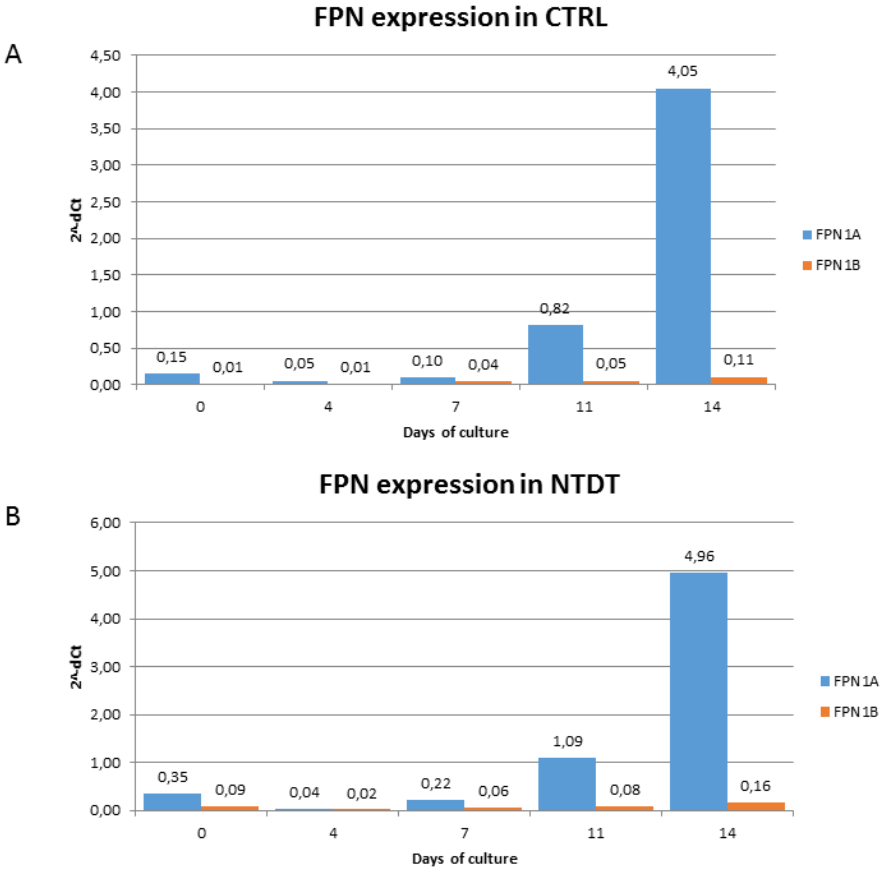

Chart 1: mRNA expression (2^{-dCt}) of both ferroportin isoforms in CTRL and NTDT during erythroid differentiation

From the chart is possible to see at day 7 of CTRL (1A) and NTDT (1B) cell differentiation a decrease of both FPN isoforms expression compared to day 0. At day 14, there is an increase of the FPN1A and 1B compared to day 7 for both CTRL and NTDT conditions.

SCIENTIFIC PRODUCTS

PAPERS

Ronzoni L., **Sonzogni L.,** Duca L., Graziadei G., Ferru E., Cappellini M.D., " Growth differentiation factor 15 expression and regulation during erythroid differentiation in non – transfusion dependent thalassemia", Blood Cells, Molecules and Diseases, 2014, in press

Ronzoni L., **Sonzogni L.**, Fossati G., Modena D., Trombetta E., Porretti L., Cappellini M.D., " Modulation of gamma globin genes expression by histone deacetylases inhibitors: an in vitro study", British Journal of Haematology, Vol. 165, no. 5, 2014, pp. 714 – 21

ABSTRACTS

II Research Day INGM – Policlinico.
November, 5th , Milano (IT)
"*In vitro Ferroportin expression in Non-transfusion Dependent Thalassemia during erythroid differentiation"*
Sonzogni L., Ronzoni L., Graziadei G., Marcon A., Cappellini M.D.
Oral presentation

XIII National Congress of Italian Society of Experimental Haematology. October, 15th -17th 2014, Rimini, (IT)
"*In vitro Ferroportin expression in Non-transfusion Dependent Thalassemia during erythroid differentiation"*
Sonzogni L., Ronzoni L., Graziadei G., Marcon A., Cappellini M.D.
Oral presentation

Fifth International BioIron meeting. April, 14th -18th 2013 University of London, London (UK)
"*In vitro Ferroportin expression in Non-transfusion Dependent Thalassemia during erythroid differentiation*"
Sonzogni L., Ronzoni L., Graziadei G., Marcon A., Gandolfi I., Cappellini M.D.
Poster Session

Fifth International BioIron meeting. April, 14th -18th 2013 University of London, London (UK)
"*Growth Differentiation Factor 15 expression and regulation during erythroid differentiation in non-transfusion dependent beta thalassemia syndromes*"

Ronzoni L., **Sonzogni L**., Graziadei G., Marcon A., Duca L., Cappellini M.D.
Poster Session

XII National Congress of Italian Society of Experimental Hematology. October, 17th -19th 2012, Rome (IT)
"*In vitro Ferroportin expression in Non-transfusion Dependent Thalassemia during erythroid differentiation"*
Sonzogni L., Ronzoni L., Graziadei G., Marcon A., Cappellini M.D.
Poster Session

XII National Congress of Italian Society of Experimental Hematology. October, 17th -19th 2012, Rome (IT)
"*Modulation of Gamma globin gene expression by a new histone deacetilase inhibitor"*
Ronzoni L., **Sonzogni L**., Cattaneo A., Fossati G., Cappellini M.D.
Oral presentation

17th congress EHA. June, 14th-17th 2012, Amsterdam (NL)
Growth Differentiation Factor 15 expression and regulation during erythroid differentiation in non – transfusion dependent thalassemia syndromes
L Ronzoni ,**L Sonzogni** ,L Duca ,A. Colancecco ,G Graziadei ,KM Musallam ,MD Cappellini
Poster Session

"In Vitro GDF15 Expression During Thalassemic Erythroid Differentiation and Maturation"
Alessandra Colancecco, Luisa Ronzoni, Lorena Duca, **Laura Sonzogni**, Isabella Nava, Giovanna Graziadei, and Maria Domenica Cappellini
Blood (ASH Annual Meeting Abstracts), Nov 2011; **118**: 5285.
Online publication

"In Vitro Ferroportin Expression in Thalassemia Intermedia During Erythroid Differentiation"
Luisa Ronzoni, Alessandra Colancecco, Laura Sonzogni, Giovanna Graziadei, and Maria Domenica Cappellini
Blood (ASH Annual Meeting Abstracts), Nov 2011; **118**: 5287
Online publication

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"Laura, science is never straight forward"

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