INSTITUTO POLITÉCNICO DE LISBOA

Instituto Superior de Engenharia de Lisboa
Escola Superior de Tecnologia da Saúde de Lisboa

Natural compounds with the capacity to reactivate the gene of *gamma-globin* and induction of fetal hemoglobin - *In vitro* test

Elisabete Cristina Conceição de Matos

Thesis to obtain the Master degree in
Biomedical Engineering

Definitive version

Supervisors
Dr. Rui Miguel Duque de Brito (ESTeSL)
Dr. Edna Soraia Gregório Ribeiro (ESTeSL)

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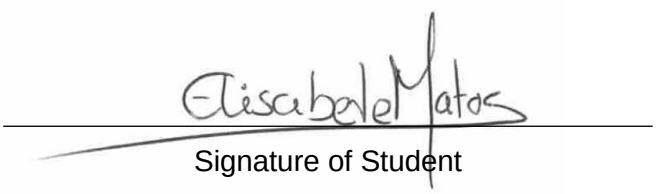
Statement of Original Authorship

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.



Signature of Student

Statement of Copyright

Dedication

I dedicate this work to all individuals with hemoglobinopathies who regardless of the clinical complications with which they live, see in researchers and in their research reasons for trust and hope.

Acknowledgments

Firstly, I would like to thank my mentors: Professor Doctor Miguel Brito and Professor Doctor Edna Ribeiro. The door (and email) was always open for my many questions, doubts and problems. I appreciate your willingness and generosity to guide me in this odyssey that was the realization of this thesis.

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Abstract

β-hemoglobinopathies are the most common recessive genetic diseases worldwide. Currently available treatments for these disorders are expensive and associated with severe side effects.

Pharmacological reactivation of Fetal Hemoglobin (HbF) is seen as a promising therapeutic strategy. Hydroxyurea (HU), a potent ribonucleotide reductase inhibitor, is the only Food and Drug Administration (FDA) approved HbF inducing agent. However, its cytotoxicity, potential carcinogenicity and variable clinical effects limit severely its use.

Identification of novel agents, with higher HbF inducing activity, lower cytotoxicity and available in low-income countries, such as natural compounds, has become a great challenge today.

Here, we performed an assessment of potential transcriptional effects induced by two natural bioactive compounds, namely Genistein (GN) a naturally occurring flavonoid found in soybean and soy derivates and Epigallocatechin-3-gallate (EGCG) the major polyphenol component of green tea, in *globin* and HbF regulators/silencer genes.

K562 cell line was exposed for 72 and 96 hours to three different concentrations of GN and EGCG that mimic cellular exposure *in vivo* after supplementation (100 ηg/ml, 250 ηg/ml and 500 ηg/ml), and 25 μg/ml HU was used as a positive control. Cell viability and proliferation were measured and the transcriptional effects of GN and EGCG on *α*, *β* and *γ-globin* genes, as well as the HbF regulators genes *BCL11A* and *KLF1*, were evaluated by qRT-PCR using specific primers.

Our results demonstrated that both compounds impact cellular metabolism and proliferation with no cytotoxic effects and have a potential specific molecular target for *BCL11A* with associated downregulation, without altering *KLF1* levels. GN and EGCG also affected *globin* expression levels in a dose and time-dependent manner. Interestingly, transcriptional analysis of both compounds revealed non-monotonic dose-responses (NMDRs).

Overall, our preliminary study sustains the potential of these compounds for *γ-globin* reactivation and consequently HbF induction, which indicates that GN and EGCG may be potential candidates for new therapeutic strategies. Further research must be performed in order to assess the effectiveness of HbF induction and the underlying molecular mechanisms.

Keywords: β-hemoglobinopathies; Fetal Hemoglobin reactivation; Genistein; Epigallocatechin-3-gallate; *globin* expression; *BCL11A*; *KLF1*

Resumo

As β-hemoglobinopatias são as doenças genéticas recessivas mais comuns no mundo. No entanto, os tratamentos atualmente disponíveis são dispendiosos e apresentam graves efeitos secundários.

A reativação farmacológica da Hemoglobina Fetal (HbF) é vista como uma estratégia terapêutica promissora. A Hidroxiureia (HU), um potente inibidor da ribonucleotídeo redutase, é atualmente o único fármaco indutor de HbF aprovado pela FDA. No entanto, a sua citotoxicidade, potencial carcinogénico e efeitos clínicos variáveis, limitam severamente o seu uso.

A identificação de novos agentes, com alta atividade indutora de HbF, baixa citotoxicidade e disponíveis nos países em desenvolvimento, como os compostos naturais, é um dos grandes desafios da atualidade.

Neste projeto avaliámos os efeitos transcricionais induzidos por dois compostos naturais, a Genisteína (GN), um flavonoide natural encontrado na soja e derivados da soja e a Epigalocatequina-3-galato (EGCG), o principal componente polifenol do chá verde, nos genes reguladores/silenciadores da *globina* e HbF.

A linhagem celular K562 foi exposta por 72 e 96 horas a três concentrações diferentes de GN e EGCG (100 ηg/ml, 250 ηg/ml e 500 ηg/ml) que reproduzem a exposição celular *in vivo* após suplementação, e 25 μg/ml de HU foi utilizada como controlo positivo. A viabilidade e proliferação celular foram medidas e os efeitos transcricionais da GN e EGCG nos genes da *α*, *β* e *γ-globina*, bem como nos genes reguladores da HbF, *BCL11A* e *KLF1*, foram avaliados por qRT-PCR utilizando *primers* específicos.

Os nossos resultados demonstraram que ambos os compostos afetam o metabolismo e proliferação celular sem efeitos citotóxicos observáveis, e têm como potencial alvo molecular o *BCL11A*, associado à regulação negativa deste gene, sem alterar os níveis do *KLF1*. A GN e EGCG também afetaram a expressão das *globinas* em função da dose e do tempo. A análise transcricional de ambos os compostos, revelou que ambos apresentam respostas não monotónicas em relação à dose (NMDRs).

Este estudo sustenta o potencial desses compostos para reativação da *γ-globina* e, consequentemente, indução da HbF, o que indica que a GN e EGCG podem ser potenciais candidatos a novas estratégias terapêuticas. Mais pesquisas devem ser realizadas para avaliar a eficácia na indução da HbF e os mecanismos moleculares subjacentes.

Palavras-chave: β-hemoglobinopatias; reativação da Hemoglobina Fetal; Genisteína; Epigalocatequina-3-galato; expressão da *globina*; *BCL11A*; *KLF1*

Relevant publications in the field

Conference-papers

Matos, E; Sousa, D; Delgadinho, M; Mateus, V; Silva, I; Ribeiro, E; Brito, M. (R33.) Potencial da genisteína na reativação da expressão do gene da *γ-globina* e indução da hemoglobina fetal. III Congresso Nacional de Ciências Biomédicas Laboratoriais. Lisbon, October 2019. Available from: https://iii-cncbl.site123.me/resumo-das-comunicações-livres

Delgadinho, M; **Matos, E**; Sousa, D; Ribeiro, E; Brito, M. The potential of natural compounds in HBG gene reactivation and fetal hemoglobin induction. Abstract submitted to 4th Global Congress on Sickle Cell Disease. Paris, April 7-9, 2020 (postponed to an undetermined date)

Poster

Sousa, D; **Matos, E**; Delgadinho, M; Ribeiro, E; Mateus, V; Silva, I; Brito, M. (R15.) O potencial da epigalocatequina-3-galato na reativação da expressão do gene da *γ-globina* e indução da hemoglobina fetal. III Congresso Nacional de Ciências Biomédicas Laboratoriais. Lisbon, Available from: October 2019. https://iii-cncbl.site123.me/resumo-dos-posteres

Journal article - manuscript submitted

Ribeiro, E; Delgadinho, M; **Matos, E**; Sousa, D; Galante, H; Brito, M. Epigallocatechin-3-gallate and Genistein potential HbF inducers? An *in vitro* and *in vivo* approach. Manuscript submitted to *Molecular and Cellular Biochemistry*

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List of Abbreviations

A, C, G, T	Adenine, cytosine, guanine, thymine (organic bases that constitute the nucleotides)
ACS	Acute chest syndrome
ATP	Adenosine triphosphate
BCL11A	B Cell Lymphoma 11A
BME	β-mercaptoethanol
cAMP	Cyclic adenosine monophosphate
Cas9	CRISPR associated protein 9
cDNA	Complementary DNA
CE	Capillary electrophoresis
cGMP	Cyclic guanosine monophosphate
CML	Chronic myelogenous leukemia
CO	Carbon monoxide
CO_2	Carbon dioxide
CpG	Cytosine-phosphate-guanosine
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CT	Cycle threshold
DHFR	Dihydrofolate reductase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
dNTP	Deoxynucleotide
ECACC	European Collection of Cell Cultures
EDCs	Endocrine-disrupting chemicals
EFSA	European Food Safety Authority
EGCG	Epigallocatechin-3-gallate
EKLF	Erythroid krüppel-like factor
EMA	European Medicines Agency
EPO	Erythropoietin
ER-α	Estrogen receptor alpha
ER-β	Estrogen receptor beta
ERK	Extracellular-signal-regulated kinase
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GATA1	GATA binding protein 1
GN	Genistein
GTC	Guanidine thiocyanate
GWA	Genome-wide association
HATs	Histone acetyltransferases
Hb	Hemoglobin
HbA	Adult hemoglobin
HbAS	Sickle cell trait
HBB	*β-globin* gene
HbF	Fetal hemoglobin
HBG	*γ-globin* gene

HCC	Hepatocellular carcinoma
HbS	Sickle hemoglobin
HbSS	Homozygous for HbS - Sickle cell anemia
HDACs	Histone deacetylases
HEPES	Hydroxyethyl piperazine ethanesulfonic acid
HLA	Human leukocyte antigen
HPFH	Hereditary persistence of fetal hemoglobin
HPLC	High-performance liquid chromatography
HSCT	Hematopoietic stem cell transplantation
H&TRC	Health & Technology Research Center
HU	Hydroxyurea
IC50	Half maximal inhibitory concentration
IL-1	Interleukin-1
IEF	Isoelectric focusing
JNK	Jun N-terminal kinase
KLF1	Kruppel-like factor 1
LCR	Locus control region
LD	Linkage disequilibrium
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MKPs	MAPK phosphatases
miRNAs	MicroRNAs
mRNA	Messenger ribonucleic acid
NMDR	Non-monotonic dose-response
NO	Nitric oxide
NTDTs	Non-transfusion dependent thalassemias
p38 MAPK	P38 mitogen-activated protein kinase
PALOP	From Portuguese “Países Africanos de Língua Oficial Portuguesa”
PBS	Phosphate-buffered saline
PfEMP1	Erythrocytic Protein-1 encoded by P. falciparum
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PPARγ	Peroxisome proliferator-activated receptor gamma
PTK	Protein tyrosine kinases
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RAR-beta	Beta retinoic acid beta receptor
RBC	Red blood cell
RLA	RNA Lysis Buffer
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
rRNA	Ribosomal ribonucleic acid
SAR1	Secretion Associated Ras Related *GTPase 1*
SCA	Sickle cell anemia
SCD	Sickle cell disease
SDS	Sodium dodecyl sulfate
TDTs	Transfusion-dependent thalassemias

TNF	Tumor necrosis factor
TSGs	Transcription of tumor-suppressor genes
VOC	Vaso-occlusive pain crises
WHO	World Health Organization

“The journey of a thousand miles begins with one step”

Lao Tzu

Chapter 1: Introduction

1.1 Hemoglobinopathies

Hemoglobinopathies are the most common monogenic diseases worldwide. According to the World Health Organization (WHO) 7% of the population carries with these disorders, recent researches suggest that between 300.000 and 400.000 newborns are affected each year with this problem and their prevalence is predicted to increase in the future (Cavazzana & Mavilio, 2018; Diepstraten & Hart, 2019; Goonasekera, Paththinige, & Dissanayake, 2018; Williams & Weatherall, 2012). Hemoglobinopathies are hemoglobin (Hb) disorders resulting from changes in genes responsible for the synthesis of globin chains. They can be characterized as qualitative or structural changes (synthesis of mutated globin variants, e.g. sickle syndromes, hemoglobin C, D, and E disease) and quantitative changes (decrease or abolition of globin chains production called the thalassemia syndromes) (Cavazzana & Mavilio, 2018; Modell & Darlison, 2008). To date, more than 1.000 hemoglobin disorders have been identified and characterized (Forget & Bunn, 2013).

It is thought that the high prevalence of hemoglobinopathies is due to a selective advantage of its carriers against malaria. As a consequence of this positive selection, these pathologies have a high frequency in the geographic areas of the tropics and subtropics (sub-Saharan Africa, the Mediterranean, and Southeast Asia) where malaria was endemic. However, due to population migrations, they have also become increasingly prevalent in non-endemic regions, making it a global health problem (Goonasekera et al., 2018; Modell & Darlison, 2008).

In Portugal, only three studies were carried out until the present to determine the prevalence of hemoglobinopathies. These studies demonstrated the existence of a significant incidence in the southern districts, explained by the occupation of these regions in the past by Arabs and African slaves (Bento et al., 2006; Inez, Sequeira, Santos, Santos, & Nunes, 1993; Martins, Olim, Melo, Magalhaes, & Rodrigues, 1993). Nowadays it is probable that prevalence has increased dramatically due to the migration phenomenon verified in recent years coming from Brazil, from the Portuguese-speaking African countries (PALOP) and East Europe (S. Costa, Madeira, Sobral, & Delgadinho, 2016; Miranda et al., 2013).

Hemoglobinopathies were the first diseases to be analyzed using molecular biology and helped to establish many of the general principles that nowadays support our understanding of human molecular genetics (Higgs, Engel, & Stamatoyannopoulos, 2012).

Modifications in the *β-globin* gene are the most prevalent. Sickle cell disease (SCD) and β-thalassemia are considered the most common hemoglobinopathies, with high rates of morbidity and mortality (Sankaran, 2011; Thein, 2017).

1.1.1 Motivation

Despite the high prevalence of β-hemoglobinopathies, available treatments continue to have many limitations and are poorly available in low-income countries where affected patients are frequently unable to sustain the high costs of clinical management (Dreuzy, Bhukhai, Leboulch, & Payen, 2016).

Novel therapeutic approaches are currently being developed, in an effort to move beyond the palliative management and the complications of the treatment (Dreuzy et al., 2016).

Pharmacological reactivation of Fetal hemoglobin (HbF) is seen as a promising therapeutic strategy. Hydroxyurea (HU) is the only HbF inducing agent approved for the Food and Drug Administration (FDA) for sickle cell disease treatment (Field & Nathan, 2014). However, its cytotoxicity, potential carcinogenicity and the moderate effects obtained after administration have limited their clinical use (Stamatoyannopoulos, 2005).

The inability of populations in the majority of highly endemic developing countries to access proper medication and sustain the high costs lead to the necessity to develop new therapeutic approaches with more accessible compounds at very low costs. That way, identification of novel agents with higher HbF inducing activity, lower cytotoxicity and available to all populations as natural compounds is one of today's greatest challenges (Theodorou et al., 2016).

In this context, this project aims to evaluate the potential of two naturally occurring compounds with high biological activity, namely: the genistein (GN) (a naturally occurring flavonoid found in soybean and soy derivates) (Nagaraju, Zafar, & El-Rayes, 2013) and epigallocatechin-3-gallate (EGCG) (the major polyphenol component of green tea) (Thichanpiang & Wongprasert, 2015), in the induction of *γ-globin* gene reactivation and HbF expression, as a possible therapy for β-hemoglobinopathies.

1.1.2 Thesis overview

This project was developed at the Health & Technology Research Center (H&TRC), located in Escola Superior de Tecnologia da Saúde de Lisboa, and funded by Instituto Politécnico de Lisboa: project IDI&CA-IPL/2019/HemoFet/ESTeSL.

For a better reading experience, this section aims to describe the structure and relevance of each chapter of this thesis.

The thesis is divided into 5 chapters, and the general organization is schematically represented in **Fig. 1**.

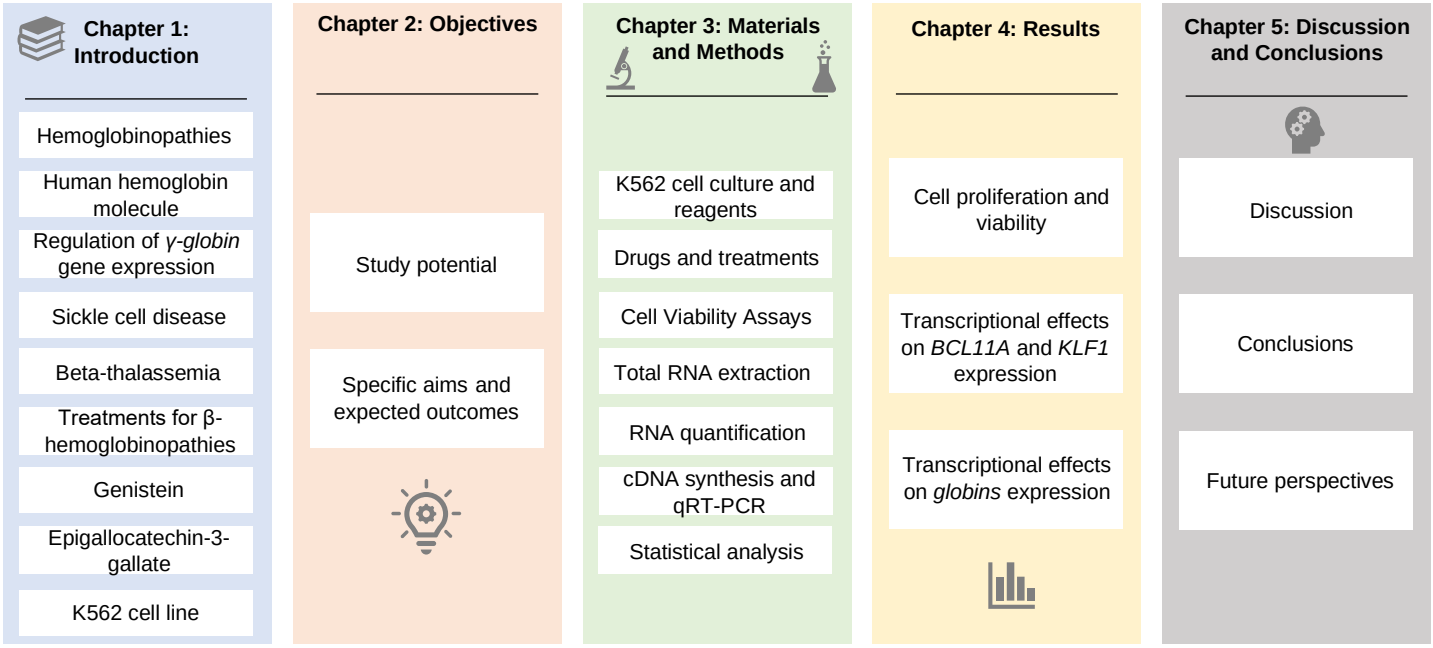


Fig. 1 Representative diagram of the general structure of the dissertation

The general introduction (Chapter 1) presents the theme in order to contextualize the reader with the project, describing the motivation for the study as well as the importance of the work presented here. In the first block of the thesis, the main theoretical concepts that support the study are presented. A general introduction to hemoglobinopathies is made, followed by a description of the structure of the human hemoglobin molecule and the main mechanisms associated with the expression of the *γ-globin* gene. The following is a general description encompassing the theoretical description and pathophysiology of SCD and β-thalassemia (general contexts and prevalences are described). This chapter also describes the main treatment methods available for β-hemoglobinopathies, with the main emphasis on HbF induction, and a general description of the two compounds to be studied is made: genistein and epigallocatechin-3-gallate. Lastly, a brief description/characterization of the K562 cell line is made.

Chapter 2 describes the specific objectives of this investigation, the theoretical context and the expected results. Materials and methodologies are described sequentially in Chapter 3.

In Chapter 4, the experimental results are listed simultaneously by demonstrating the cell proliferation and viability, the transcriptional effects on *BCL11A* and *KLF1* expression and the transcriptional effects on *globins* gene expression.

Finally, in Chapter 5, the results are discussed: interpreting the results and comparing them with the bibliography already published. The conclusions obtained in this experience are presented, as well as some of the limitations and future perspectives in relation to the continuation of the project.

1.2 Human hemoglobin molecule

Hemoglobin is a multiple subunits molecule, essential to the life of higher organisms (Forget & Bunn, 2013).

Present in large quantities in erythrocytes and responsible for oxygen-transporting from the lungs to all body in mammals, also interacts with three other gases such as carbon dioxide (CO_2), carbon monoxide (CO) and nitric oxide (NO). Hemoglobin thus plays a crucial role in the maintenance of homeostasis and buffering of acidic metabolic waste products (Forget & Bunn, 2013; Schechter, 2008). Each red blood cell (RBC) contains approximately 300 million molecules of hemoglobin, totaling about 30 picograms in weight per cell (Cappellini, Cohen, Porter, Taher, & Viprakasit, 2014).

Adult human hemoglobin is a tetrameric protein composed of two pairs of polypeptide chains (two *α-globins* and two *β-globins* - α2β2) folded into a compact globule. Each globin chain contains a central oxygen-binding heme group responsible for the oxygen transport capacity (**Fig. 2**) (Forget & Bunn, 2013; Goonasekera et al., 2018; Sankaran & Orkin, 2013; Schechter, 2008).

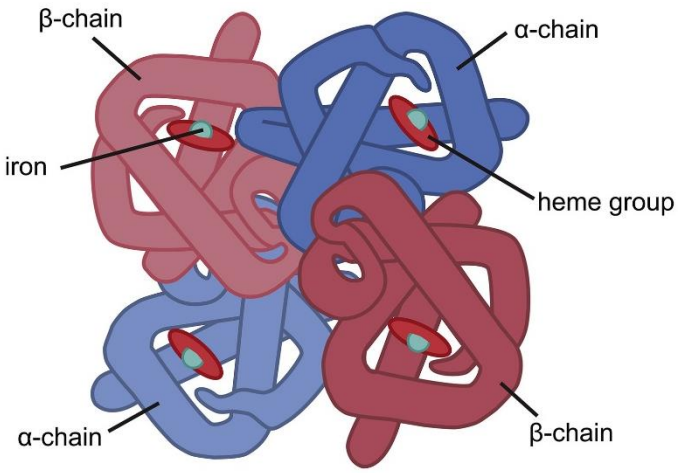


Fig. 2 The composition of human hemoglobin, adapted from (Diepstraten & Hart, 2019). Hemoglobin is a tetramer of two *α-like* and two *β-like* globin subunits, each globin subunit contains a central heme group with one iron atom.

In humans, hemoglobin synthesis is controlled by two similar multigenic clusters: the *α-globin* gene cluster on the short arm of chromosome 16 (16p13.3) in a region near the telomere and *β-globin* gene cluster on the short arm of chromosome 11 (11p15.5) (Schechter, 2008).

The *α-globin* cluster comprises about 30 kbp and contains an embryonic (*ζ*) gene and two identical adult (*α*) genes differing only in their expression. The α_2*-globin* gene encodes nearly two to three times much of its protein in comparison with the α_1*-globin* gene (Cui & Engel, 2017).

β-globin agglomerate comprises approximately 1.6 kbp and is composed of five functional genes: *ε*, *γ1*, *γ2*, *δ*, and *β*. The *ε* gene encodes an embryonic globin, *γ1* and *γ2* are fetal globin genes expressed at the end of gestation and differ only by a single amino acid (glycine/alanine at position 136), *δ* and *β* are two adult *globin* genes. *β-like globin* locus also contains the locus control region (LCR), located 50 kb upstream from the gene, made up of five DNase I hypersensitive sites responsible for high levels of *β-like globin* gene expression (Cui & Engel, 2017).

In both groups, there are also several *globin* pseudogenes with unknown function (Cui & Engel, 2017).

Hemoglobin genes are arranged along the chromosome in the order which they are expressed during the various development cycles to produce different tetramers (Cui & Engel, 2017; Goonasekera et al., 2018) in response to shifting needs for oxygen during these stages (Diepstraten & Hart, 2019).

Two sequential changes occur from embryonic hemoglobin (Hb Gower-I [ζ2ε2], Hb Gower-II [α2ε2] and Hb Portland [ζ2γ2]) to fetal hemoglobin (α2γ2) and from fetal hemoglobin to adult hemoglobin (HbA [α2β2] and HbA2 [α2δ2]) by coordinate switching of gene expression. Initial change occurs in the first trimester of pregnancy, from embryonic hemoglobin to fetal hemoglobin (HbF) and coincides with the transition from embryonic (yolk sac) hematopoiesis to definitive (fetal liver). The second alteration from HbF to adult Hb (HbA and HbA2) occurs near birth, here there is a decrease in *γ-globin* gene expression and a marked increase in *β-globin* expression as well as of the *δ-globin* (**Fig. 3-A**) (Goonasekera et al., 2018; Stamatoyannopoulos, 2005; Taher, Weatherall, & Cappellini, 2018).

The gene expression changes from *ε-globin* to *γ-globin* and from *γ-globin* to *β-globin* are controlled exclusively at the transcriptional level (Stamatoyannopoulos, 2005).

β-globin expression occurs via the interaction of the LCR with TATA, CCAAT, and CACCC boxes, which are the binding sites for transcription factors such as erythroid Kruppel-like factor 1 (*KLF1*) and GATA-1 (**Fig. 3-B**) (Goonasekera et al., 2018).

Unlike humans, most species have only one hemoglobin change in the development cycle (from embryonic to adult). Expression of *γ* genes during the fetal period is a relatively recent event that occurred during the evolution of primates (Stamatoyannopoulos, 2005).

Adult hemoglobin phenotype is generally established up to the end of the first year of life and consists approximately in HbA (α2β2, 97%), HbA2 (α2δ2, 2%) and HbF (α2γ2, 1%) (Goonasekera et al., 2018).

However, in some cases, *γ-globin* expression persists in the adults, this condition is known as hereditary persistence of fetal hemoglobin (HPFH) (Schechter, 2008). This condition may be due to point mutations in the upstream promoter region of the *γ-globin* gene or to different deletions affecting the *β-globin* gene cluster. Individuals with β-thalassemia or SCD who present

with concomitant HPFH have milder clinical manifestations, in contrast to the serious illness in homozygous patients for these disorders (Ye et al., 2016).

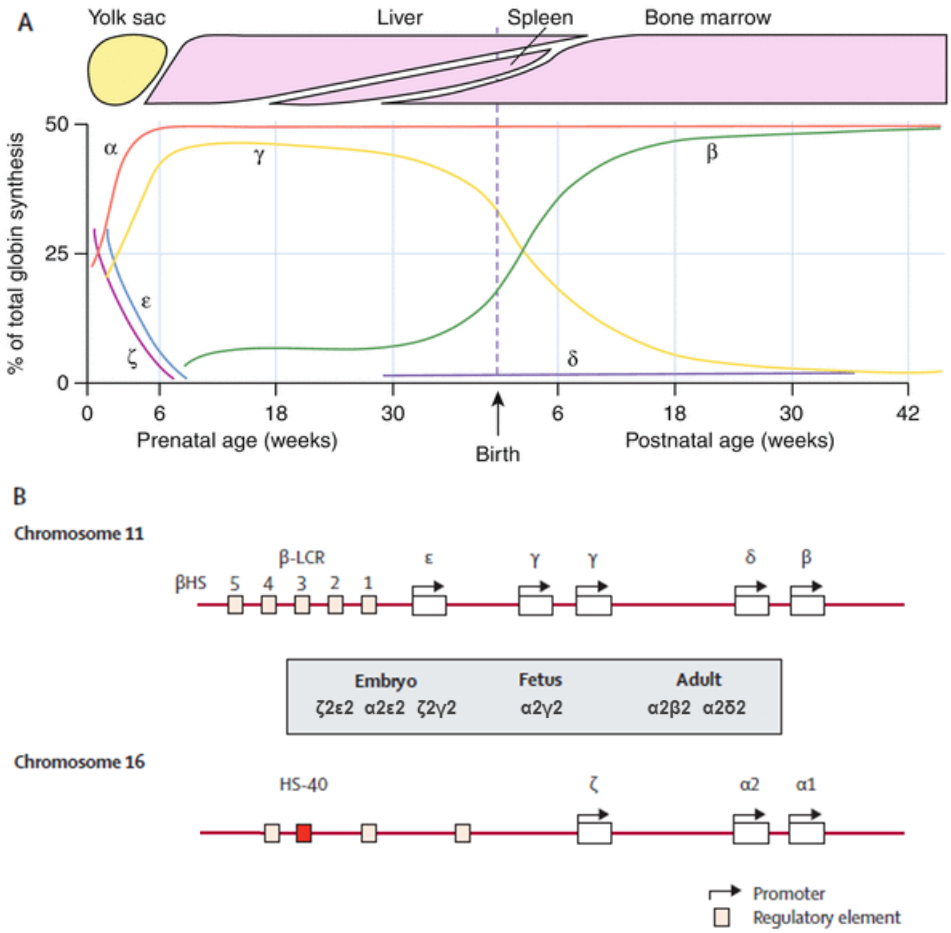


Fig. 3 Normal developmental switches of globin expression. (A) Levels of globin expression in the different stages of development, adapted from (Richard, Haley, & Recht, 2012). (B) Structure of the *α-like* and *β-like* globin gene clusters are shown together with the types of hemoglobin produced at each developmental stage, adapted from (Taher et al., 2018).

1.3 Regulation of *γ-globin* gene expression

A better understanding of the molecular processes of globin switching is the primary focus of many researcher's intents on developing therapeutic approaches for HbF induction. Regulation of *γ-globin* gene expression is complex and can be influenced by different regulatory pathways, genetic and environmental factors (Pourfarzad et al., 2013).

Multiple transcription factors involve the genetics that regulates the switch from fetal to adult hemoglobin, these factors interact with each other and with the promoters and enhancers of the

β-globin gene as well as with the epigenetic factors namely related to chromatin (Sankaran & Orkin, 2013).

Modulation of HbF gene expression involves the interaction of multiple proteins, such as transcription factors *KLF1*, *BCL11A* and *SOX6* and hematopoietic regulatory factor *MYB*, as well as other genetic elements such as *GATA 1* (Wilber, Nienhuis, & Persons, 2011).

Although numerous regulators of erythropoiesis and *globin* gene regulation have been identified, none of the molecules examined appeared to be a specific regulator of the exchange of HbF to HbA. However, relatively recent information on this process has come from studies using human genetic approaches, notably genome-wide association (GWA) studies, to find common genetic variants associated with variations in HbF levels. Initially, these studies were performed in healthy subjects and showed the association of variants in three genomic loci related to 45% variation in HbF levels, the *β-globin* locus on chromosome 11 (the *XmnI* polymorphism [rs7482144], in the *γ-globin* gene promoter region, which predisposes an increase in HbF expression), a region between the *HBS1L* and *MYB* genes on chromosome 6 and a region within the *BCL11A* gene on chromosome 2. These findings have been replicated in patients with SCD and β-thalassemia, and the intensifying effect of these sites on these diseases has also been demonstrated in several other studies (**Fig. 4**) (Sankaran, 2011).

Erythropoiesis expanded by stress is also another key factor described for maximal HbF expression (Ngo & Steinberg, 2015).

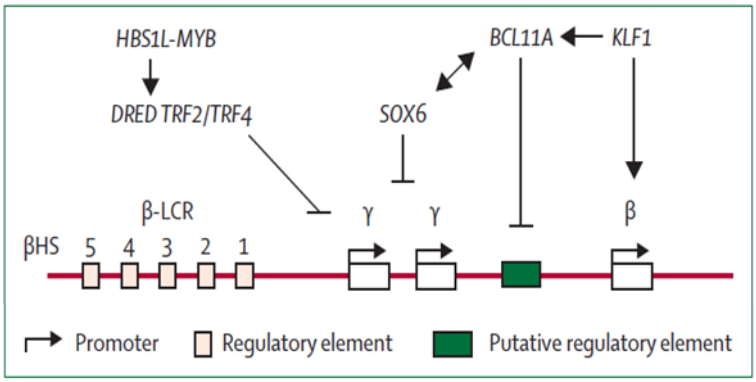


Fig. 4 Summary of pathways regulating the switch from *γ-globin* to *β-globin*, adapted from (Higgs et al., 2012). Many factors are involved in the regulation of *γ-globin* gene expression, but two pathways are the most described. *HBS1L-MYB* pathway increases the expression of *MYB* a proto-oncogene whose expression is crucial for erythropoiesis. The reduction of *MYB* reduces the synthesis of two nuclear receptors (TRF2 and TRF4) that repress the *γ-globins* by binding to their promoters. *BCL11A* pathway, that normally cooperates with other repressors (e.g., Sox6), silence the *γ-globin* genes, when connecting to the *β-globin* locus lying between the fetal and adult genes. The expression of *BCL11A* is also regulated by the transcription factor *KLF1* (a *β-globin* gene promoter).

More than three decades have passed from cloning the *β-globin* gene to the current understanding of hemoglobin exchange. It is hoped that in the future progress in this field may proceed more rapidly for the benefit of patients with β-hemoglobinopathy disorders (Sankaran, Xu, & Orkin, 2010).

1.3.1 *BCL11A*

B Cell Lymphoma 11A (*BCL11A*) is a zinc finger protein gene, that was first associated with lymphoid malignancies in humans. Subsequently, studies have evaluated *BCL11A* as a dominant regulator of developmental *globin* gene silencing. The reduced expression of *BCL11A* mRNA is associated with higher HbF levels. In erythroid progenitors, *BCL11A* physically interacts with the NuRD chromatin remodeling complex and the erythroid transcription factors *GATA1* and FOG1. The reduction of *BCL11A* in cultured human erythroid progenitors leads to robust HbF expression, consistent with the role of *BCL11A* as a repressor of *γ-globin* and as a key regulator of HbF to HbA in humans (Sankaran et al., 2010).

These findings suggest that *BCL11A* or its partner proteins may serve as excellent clues to targeted therapeutic approaches to reactivate HbF in patients with β-hemoglobinopathies, modified the clinical features of both diseases. Negative regulation of *BCL11A* expression or impairment of *BCL11A* function may be promising strategies (Akinsheye et al., 2011; Sankaran et al., 2010).

1.3.2 *KLF1*

Krüppel-Like Factor 1 (*KLF1*), originally known as Erythroid krüppel-like factor (*EKLF*), was initially shown to be critical for adult *β-globin* gene transcription and to increase the ability of the *β-globin* promoter to compete with the *γ-globin* promoter for the enhancer function of the *β-globin*, through a highly conserved CACCC motif that was known to be mutated in human β-thalassaemias (Ginder, 2015; Sankaran et al., 2010).

That way *KLF1* was initially thought to be a specific factor that facilitates HbF to HbA switching. However, besides regulating *β-globin* expression, studies demonstrated the crucial role of *KLF1* in regulating both definitive and primitive erythropoiesis. *KLF1* also functions as a transcriptional repressor by recruiting Sin3A and HDAC1. A more direct role of *KLF1* in *γ-globin* gene silencing occurs through its stimulation of *BCL11A* expression by binding to its promoter so, *KLF1* has a dual effect switching by directly on activating *β-globin* gene while repressing *γ-globin* gene indirectly by activating *BCL11A* (Ginder, 2015). Knockdown of *KLF1* in adult erythroid progenitors reactivates *γ-globin* gene expression by decreasing *BCL11A* levels and

by the concentration-dependent formation of *KLF1* complexes that favor binding to the *γ-globin* promoter (D. Zhou, Liu, Sun, Pawlik, & Townes, 2010).

Whether these effects of *KLF1* on hemoglobin gene expression are directly mediated at the locus or are due to indirect effects on erythroid maturation remains to be determined in future studies. The role of *KLF1* as a regulator of hemoglobin switching in human cells has yet to be assessed directly. Further studies probably will provide critical insight into the mechanisms of hemoglobin switching in humans through *KLF1* (Sankaran et al., 2010).

1.3.3 *SOX6*

SOX6 is another important protein involved in *γ-globin* gene expression that cooperates in silencing the *γ-globin* gene when interacting with *BCL11A*, but it also plays an important role in cell protection against apoptosis by positively regulating *Bcl-xL* (an anti-apoptotic protein) in the final stages of erythropoiesis (Sankaran et al., 2010).

If the effects of erythropoietin (EPO) on stimulating *Bcl-xL* expression decrease, *SOX6* acts as a compensatory factor activating the *BCL-xL* gene, protecting the cells from apoptosis (Mahdavi, Pourfarzad, Kosaryan, & Akbari, 2017).

1.3.4 *HBS1L-MYB* (HMIP)

HMIP is an intergenic region between the *HBSL1* and *MYB* genes on long arm of chromosome 6 (6q23), the polymorphisms in the HMIP intergenic region, distributed in 3 linkage disequilibrium (LD) blocks (HMIP blocks 1, 2, and 3), are associated with high HbF levels among SCD patients (Akinsheye et al., 2011).

The HMIP blocks exhibit marks of active chromatin characteristics namely the histone acetylation and RNA polymerase II binding sites (Wilber et al., 2011).

Recent data suggest that HMIP intergenic area that is highly associated with fetal hemoglobin expression has properties like that of a regulatory element (Wilber et al., 2011).

The most significant polymorphism in HMIP responsible for HbF modulation is a 3 bp deletion polymorphism [SNP rs9399137], located in the HMIP 2 block, 42.6 kb upstream of *HBS1L* and 83.8 kb upstream of *MYB*, common in European and Asian populations, although less frequent in African populations. Near this polymorphism, there is a binding of 4 erythropoiesis-related transcription factors, TAL1, E47, GATA2, and RUNX1 (Akinsheye et al., 2011).

HBS1L is described as a housekeeping gene, while *MYB* has a more restrictive expression pattern and is crucial for hematopoiesis and erythropoiesis. Studies show that high levels of *MYB* inhibit *γ-globin* gene expression in human erythroleukemia cells and low levels are

speculated to result in fewer cell cycle events at the onset of erythropoiesis and early maturation of erythroblasts produces red blood cells that contain higher levels of HbF (Wilber et al., 2011).

MYB expression is also reduced by GATA-1, and miRNA 15a and 16-1 (Sankaran, 2011). The increased HbF effect is mediated, at least in part, through down-modulation of *MYB* via targeting of its 3′ UTR by the microRNAs (Pule, Mowla, Novitzky, & Wonkam, 2016).

It's evident that *MYB* plays an important role in erythropoiesis, and more recent studies indicate that it acts in part by transactivating *KLF1* expression and other repressors (e.g., nuclear receptors TRF2/TRF4) of *γ-globin* genes (Akinsheye et al., 2011; Thein, 2017).

1.3.5 GATA1

GATA binding protein 1 (GATA1) is a zinc-finger transcription factor that both activates and represses target genes containing a consensus GATA binding motif (Sankaran et al., 2010).

Binding sites with this motif are present in many positions in the erythroid-expressed genes namely in *β-globin* loci. GATA1 was first discovered as a *β-globin* locus-binding protein and is essential for erythroid cell maturation (Sankaran et al., 2010).

GATA1 appears to participate in hemoglobin switch by facilitating chromatin loop formation at the *β-globin* sites and has been shown that GATA1 binds to a region upstream of the promoter (required for HbF silencing) of *y1-globin* and *y2-globin* in a FOG1-dependent manner, leading to recruitment of the repressive NuRD complex (Sankaran et al., 2010).

1.3.6 Epigenetic regulation of fetal globin gene expression

The ideal target for the treatment of β-hemoglobin disorders would be one that mimics and boosts the effect of the genetic variants that regulate HbF levels at loci such as *MYB* and *BCL11A* without affecting other biological pathways. However, *MYB* has a pleiotropic role in hematopoiesis and *BCL11A* plays key roles in neuronal and B-lymphocyte development (Thein, 2013).

Thus, in addition to the role of transcription factors in regulating *γ-globin* expression, in recent years there has been increasing awareness of the role of epigenetic mechanisms in gene regulation, in particular, gene silencing (Ginder, 2015).

The term epigenetics refers to post-synthetic modifications of chromosomal DNA and histone proteins that affect gene expression and can be inherited through somatic replication. A better understanding of the molecular mechanisms associated with epigenetic silencing of HbF expression may facilitate the development of more effective β-hemoglobinopathies treatments

and several researchers have focused their studies on the epigenetic induction of HbF expression in clinical and laboratory conditions (Ginder, 2015).

Proposed epigenetic mechanisms of HbF regulation include methylation, histone deacetylation, and chromosomal looping (Sankaran et al., 2010; Stamatoyannopoulos, 2005). In the last years, some studies have focused on microRNAs (miRNAs) a family of small non-coding RNAs of 19–25 nucleotides that regulate the gene expression by binding sequence-specific of mRNAs and that could lead to HbF induction (D. Costa, Capuano, Sommese, & Napoli, 2015).

The interplay between DNA methylation and histone modifications in regulating gene expression is well recognized (Ginder, 2015) and numerous studies have demonstrated that DNA hypomethylation and histone acetylation are effective in inducing *γ-globin* expression (Im, Grass, Christensen, Perkins, & Bresnick, 2002), these studies also suggest that DNA methylation and histone modification are dependent and each other reinforce. Any mark seems able to establish the other by recruiting epigenetic enzymes, resulting in a cycle that reinforces gene silencing (Ginder, 2015).

Additionally, histone modifications within *γ-globin* gene promoter regions, via activation of the MAPK signaling pathways (such as p38 MAPK) were proven to be crucial for the induction of *γ-globin* gene expression (Qian, Chen, Zhao, Guo, & Qian, 2013). The ability of several drugs and compounds presently utilized for induction of *γ-globin* expression and consequently promotion of HbF levels have been associated with epigenetic mechanisms and related signaling pathways (DeSimone, Heller, Hall, & Zwiers, 1982).

1.3.6.1 DNA methylation

DNA methylation represents the most important epigenetic mechanism of transcriptional repression through the binding of a methyl group in the 5′ position of cytosine residues in a cytosine-phosphate-guanosine dinucleotide (CpG) by the DNA methyltransferases (DNMTs). Generally, genes that contain the sequences with hypermethylated CpG islands present an inactive state, those with the hypomethylated CpG islands are in an active state. In accordance with this theory, during the fetal life *γ-globin* promoter is hypo-methylated while *β-globin* hyper-methylated. In contrast, in adult life, *γ-globin* promoter becomes hyper-methylated with low expression of HbF while *β-globin* becomes hypo-methylated with an increase of HbA (D. Costa et al., 2015).

1.3.6.2 Histone acetylation/methylation

Histone modification is another epigenetic mechanism. The N-terminal tails of histones are enriched of lysine residues that are subject to several post-transcriptional modifications including acetylation and methylation that can influence the chromatin structure. Usually, the acetylation of lysine residues regulated by histone acetyltransferases (HATs) promotes an open conformation of chromatin with activation of gene expression while the deacetylation promotes a closed conformation with repression of gene expression by histone deacetylases (HDACs) (D. Costa et al., 2015).

Recent large scale genetic studies identified the HDAC1 and HDAC2 inhibitors as inducers of *γ-globin* gene expression (Ginder, 2015).

1.3.6.3 microRNAs

The microRNAs (miRNAs) are included in the category of epigenetic regulatory mechanisms. These small RNAs are capable of well-characterized post-transcriptional gene silencing and have been shown to direct epigenetic modifications, by binding to transcription factors that inhibit their action (Ginder, 2015).

Several miRNAs have been implicated in the regulation of *γ-globin* gene expression, like miRNA-486-3p that is regulated during fetal to adult erythroid development (Ginder, 2015).

Enforced expression of miRNA-486-3p result in increased *γ-globin* gene expression, part of this effect was attributed to the effect on the expression of *BCL11A*. miRNA-486-3p was shown to bind to the *BCL11A* mRNA 3′UTR region and downregulate its expression concomitant with the upregulation of *γ-globin* gene expression (Ginder, 2015).

Thus, the regulation of miRNA-486-3p might contributes to HbF modulation among β-hemoglobinopathies patients (Ginder, 2015).

The miRNA-15a/16-1,miRNA-96, miRNA-221/222, miRNA-451 and miRNA-26b are also described as might be considered important therapeutic targets for increasing HbF levels to the treatment of SCD and β-thalassemia (D. Costa et al., 2015; Ginder, 2015).

1.3.7 Signaling pathways - p38 mitogen-activated protein kinase (p38 MAPK)

Recent evidence demonstrates the importance of the role of signaling pathways in mediating the reactivation of drug-induced *γ-globin* gene expression (Pace et al., 2003).

Implicit signaling pathways include those involving cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), nitric oxide (NO), ROS and p38 MAPK (Mabaera et al., 2008).

p38 MAPK signaling pathway has been implicated in the actions of several *γ-globin* expression inducing agents (Fung et al., 2011). Studies have shown that p38 MAPK is involved in erythroid differentiation and is necessary for hypoxia-induced erythropoiesis and erythropoietin production (Pace et al., 2003). Hydroxyurea (the only drug approved by the FDA for HbF induction) acts by mediating erythroid differentiation and *γ-globin* expression by inhibiting ERK and activating p38 MAPK signal transduction pathways (Fung et al., 2011).

The p38 MAPK is a member of the MAPK family that is essential for the regulation of many cellular processes, including inflammation, cell differentiation, cell cycle disruption, and apoptosis. The cells respond to changes in the physical and chemical properties of the environment. In response to these changes, mammalian cells activate four well-characterized subfamilies of mitogen-activated protein kinases (MAPKs): ERK1/2, ERK5, JNKs, and p38 MAPK (Cuenda & Rousseau, 2007). Inflammatory stimuli, such as lipopolysaccharide (LPS), tumor necrosis factor (TNF) and interleukin-1 (IL-1) are the main inducers of p38 MAPK activation, but p38 MAPK is also activated by heat stress, osmotic shock, light ultraviolet, and cytotoxic chemicals. All p38 MAPKs are activated by double phosphorylation of tyrosine and threonine residues, strongly regulated by phosphatases, such as MAPK phosphatases (MKPs) (Dörner & Lipsky, 2010).

As most of the *γ-globin* inducing agents described so far are cytotoxic and many activate the cell stress signaling pathway p38 MAPK, it is possible that the activation of the p38 MAPK pathway may be the target of new therapeutic approaches, and this and others stress-related pathways may be the keys to understanding *γ-globin* expression (Mabaera et al., 2008). Therefore, compounds that also target the same signaling pathway are of extreme interest in the study of new *γ-globin* inducing agents.

1.4 Sickle cell disease

The term sickle cell disease refers to a set of hematologic disorders that have in common the presence of sickle hemoglobin (HbS), a variant of normal adult hemoglobin (HbA) (Lettre & Bauer, 2016).

HbS (α2βs2) is caused by a missense mutation in the *β-globin* gene (HBB). In the 17th nucleotide of this gene occurs a replacement of thymine by an adenine (G**T**G for G**A**G). This substitution results in alteration of glutamic acid to valine in sixth amino acid of the *β-globin* protein, originating the synthesis of abnormal hemoglobin with altered physical and biochemical

characteristics (**Fig. 5**) (Fard, Hosseini, Shahjahani, Salari, & Jaseb, 2013; Rees, Williams, & Gladwin, 2010; Sankaran, 2011).

Presence of HbS under hypoxia conditions predisposes the red blood cells (RBCs) to change from biconcave discs into a sickle shape. When deoxygenated the HbS polymerizes resulting in a semisolid crystalline polymer structure that modifies the shape of RBCs (Lettre & Bauer, 2016; Piel, Steinberg, & Rees, 2017; Telen, Malik, & Vercellotti, 2019). Amino acid substitution causes a loss of negative charge and a gain in hydrophobicity in *β-globin* protein that foment the binding between *β1* and *β2* chains of two adjacent hemoglobin molecules (**Fig. 5**). HbS polymerization is proportional to the duration of hemoglobin deoxygenation, the intracellular HbS concentration and the presence of fetal hemoglobin in the RBCs (Rees et al., 2010; Telen et al., 2019).

The severity of the illness is mainly determined by the extent and rate of HbS polymerization (Akinsheye et al., 2011).

Sickling reaction is reversible following reoxygenation of the hemoglobin. However, repeated cycles of polymerization damage the RBCs membrane and cytoskeleton causing the premature destruction of cells leading to chronic hemolytic anemia (sickled RBCs have a reduced life span between 10 to 20 days and irreversibly sickled cells can be removed within a few hours (Field & Nathan, 2014)). Epidemiological studies suggest that several complications are associated with an increased rate of intravascular hemolysis like the cholelithiasis, cutaneous leg ulceration, priapism, and pulmonary hypertension (Forget & Bunn, 2013; Lobitz et al., 2018; Rees et al., 2010). Anemia is not the primary morbidity source in patients with SCD (Ashley-Koch, Yang, & Olney, 2000).

Sickled RBCs are rigid, increase blood viscosity and vaso-occlusion of the capillaries and postcapillary venules, causing tissue hypoxia and consequently cell death and tissue necrosis. The vaso-occlusion is the leading cause of morbidity. Acute episodes are characterized by vaso-occlusive pain crises (VOC), acute chest syndrome (ACS), cerebrovascular accidents, splenic and renal dysfunction (Field & Nathan, 2014; Forget & Bunn, 2013; Rees et al., 2010). The acute episodes can start early as 6-9 months of age and continue throughout adulthood. Bacterial infections are also common and extremely serious (Ware, de Montalembert, Tshilolo, & Abboud, 2017).

Individuals heterozygous with one copy of HbS and one copy of HbA (**Fig. 5**) are typically asymptomatic. A high hypoxia level is necessary for them to experiences the disease manifestations. This condition is called sickle cell trait or HbAS. Persons with this condition have typically 40% HbS and 56% to 58% HbA (Forget & Bunn, 2013).

Individuals homozygous have two copies of HbS variant (HbSS) inherited from both parents (**Fig. 5**). This is the most common and severe form of SCD (responsible for 70% of the cases of SCD in African origin populations) called of sickle cell anemia which leads to acute and chronic complications (Rees et al., 2010; Ware et al., 2017).

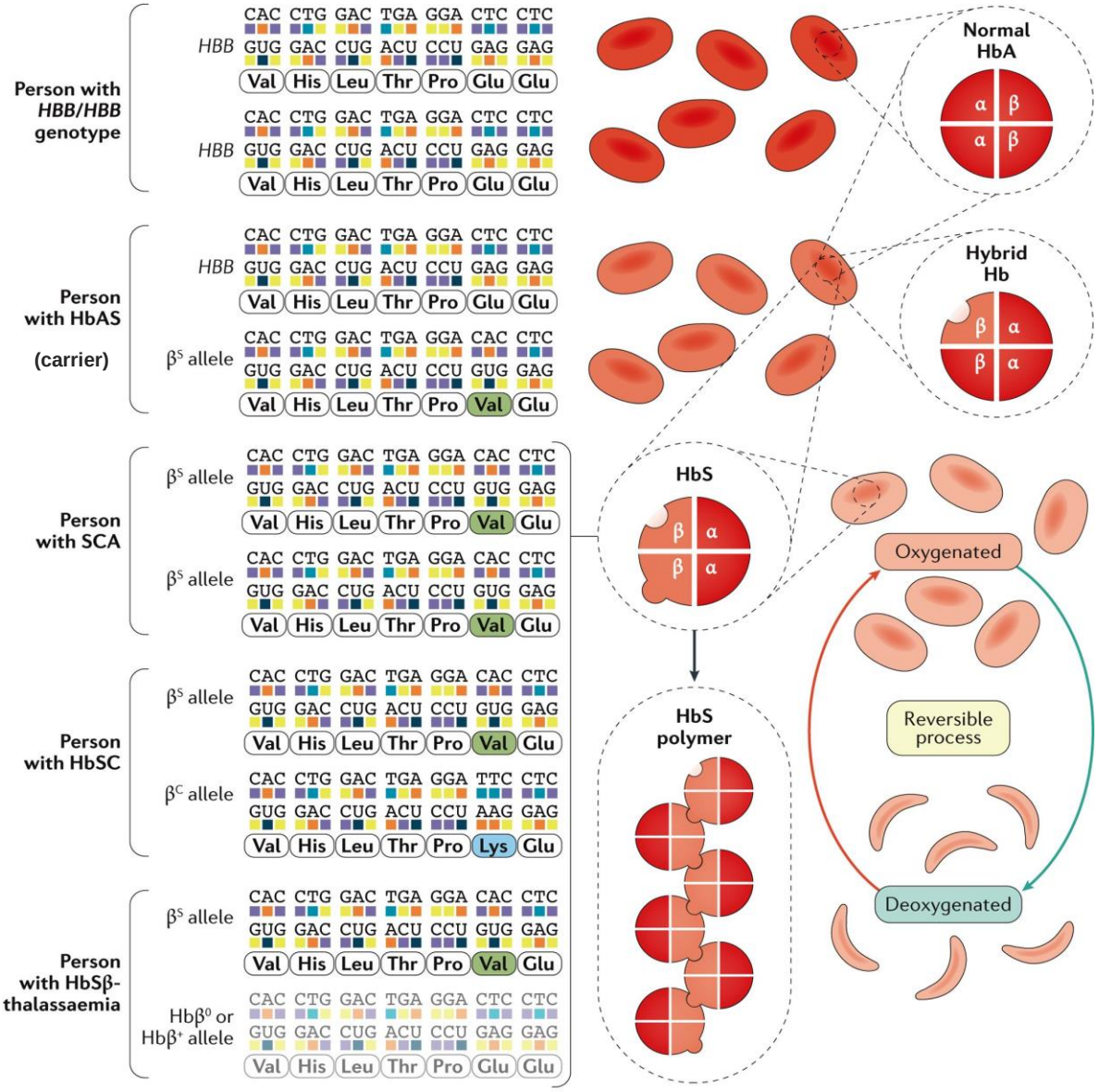


Fig. 5 Pathogenesis of sickle cell disease: Induction of sickling RBCs by polymerization of the HbS, adapted from (Kato et al., 2018). Normal adult hemoglobin (HbA) is formed by two *α-globin* subunits and two *β-globin* subunits. The sickle Hb (HbS) is an HBB allele in which a thymine-to-adenine substitution results in the replacement of glutamic acid with valine at position 6 in the mature *β-globin* chain. Deoxygenated HbS can polymerize, as deoxyhemoglobin S polymerizes, the fibers align and the red cell is distorted into an elongated banana or "sickle" shape. Individuals with one βS allele have the sickle cell trait (HbAS), and individuals with sickle cell anemia (SCA), the most common SCD genotype, have two βS alleles (βS/βS).

Others heterozygous genotypes can cause clinical syndrome of the disease, where HbS is express enough to cause intracellular sickling. These genotypes possess one copy of the HbS variant plus one other copy of Hb, such as HbS with hemoglobin C (HbSC). The third major type of SCD occurs when HbS is inherited with the β-thalassemia allele (HbS/β0-thalassemia or

HbS/β+-thalassemia). Ten other genotypes that cause SCD have been described, although most are rare like HbSD or $HbSO_{Arab}$ (Ashley-Koch et al., 2000; Rees et al., 2010; Ware et al., 2017).

To date, five haplotypes of the HbS gene (4 African and 1 Asian) have been defined, indicating that the HbS mutation initially occurred at least twice and possibly on different occasions (Rees et al., 2010).

The first observation of SCD was described in 1910 by Dr. James Herrick. He observed on a microscope "peculiar, elongated and sickle-shaped" cells in the blood of a 20 years old student with repeated episodes of pain and hemolysis. In the next 15 years were described several similar cases, however, only in 1945 Linus Pauling hypothesizes that the disease might originate from an abnormality in the hemoglobin. This hypothesis was validated in 1949 and SCD was heralded as the first "molecular disease" and with an autosomal recessive inheritance. In 1977 the genetic basis for sickle cell mutation was identified to be a missense mutation in the *β-globin* gene (Field & Nathan, 2014; Frenette & Atweh, 2007; Wienert, Martyn, Funnell, Quinlan, & Crossley, 2018). Since then, hemoglobin biophysics and the underlying genetics of SCD have been extensively studied and have helped to understand other molecular diseases (Rees et al., 2010).

Today is very simple to diagnose sickle cell disease, there are numerous techniques that can identify HbS and others hemoglobins variants. However, the most common technique is still the electrophoresis of hemoglobins where the normal hemoglobins are separated from the abnormal ones by using an alkaline gel, isoelectric focusing, capillary electrophoresis or high-performance liquid chromatography (Ware et al., 2017).

Currently, DNA or antibody-based tests offer more accurate diagnoses possibility (Ware et al., 2017).

Sickle-Cell disease presents a global distribution and it is considered the most common and severe monogenic disorder in the world (Rees et al., 2010).

The prevalence of the disease it's more prominent in malaria-endemic regions like sub-Saharan Africa, Mediterranean, Middle East and India (Piel et al., 2017). However, due to the human migration phenomena that occurred over the centuries, HbS allele has spread from the high malaria regions to other non-endemic regions like western Europe and the eastern coast of the Americas (Piel et al., 2013).

A demographic study conducted by Piel *et al*. (2013) presents global estimates for the annual number of HbAS and HbSS neonates in 2010. This study states that more than 5.000.000 babies are born with sickle cell trait and more than 300.000 with sickle cell anemia.

About 64.4% of the HbAS and 75.5% of HbSS neonates were born in sub-Saharan Africa.

The vast majority of SCD births (about 50%) occur in only three countries: Nigeria (1.223.330), India (1.038.579) and Democratic Republic of the Congo (527.963) (**Fig. 6**) (Piel et al., 2013).

Expected that these numbers increase in all world, with estimated births with sickle cell anemia being to exceed 400.000 by 2050 (Piel et al., 2017).

Although the information on SCD distribution and frequency is still limited it is clear that these are remarkably high in many tropical countries (Williams & Weatherall, 2012).

These frequencies appear to reflect a natural selection whereby SCD carriers have protection against severe forms of malaria (Williams & Weatherall, 2012).

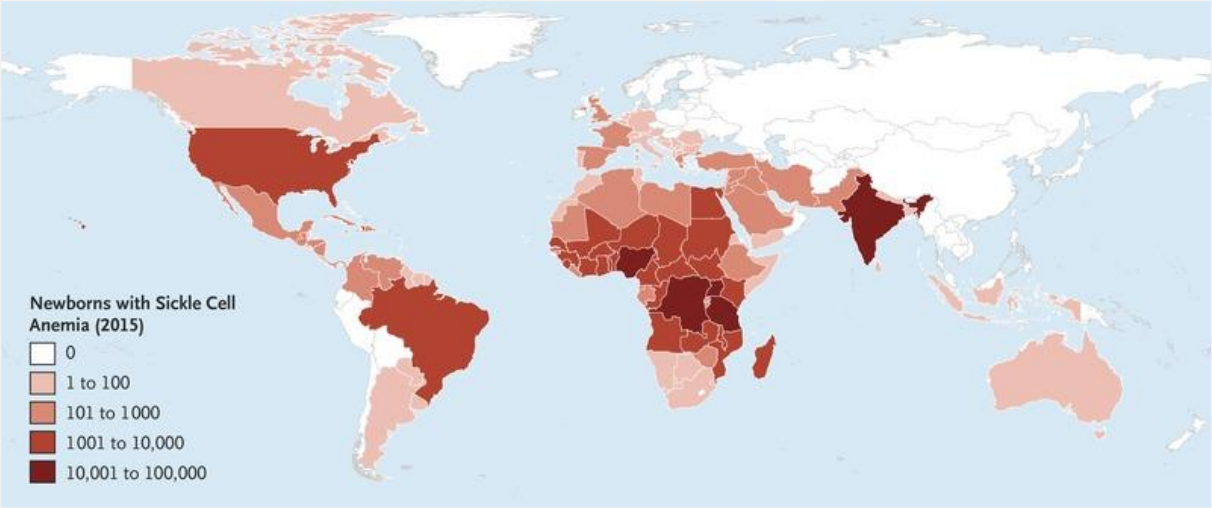


Fig. 6 Number of newborns (based on estimates) with sickle cell anemia in 2015, adapted from (Piel et al., 2017)

For more than 60 years the close similarity between the geographical distribution of SCD and malaria has been documented by several investigators and has given the first clues on possible protection against malaria by the SCD (Williams & Weatherall, 2012). Allison confirmed this hypothesis in 1954. He found that individuals with sickled RBCs were significantly less likely to be infected by malaria parasites. It also demonstrated that when inoculated intravenously with large volumes of blood infected by parasites SCD patients have a lower probability of developing a clinical malaria infection (Allison, 1954).

Over the past 30 years, several other studies have been conducted to prove this hypothesis. In general, these studies have shown that HbAS heterozygotes exhibit 50% protection against uncomplicated malaria and over 80% protection against severe malaria caused by *P. falciparum* (Williams & Weatherall, 2012).

HbAS was also associated with a 45% protection against malaria mortality in children between 2 and 16 months of age in a cohort study in Kenya (Aidoo et al., 2002).

All studies seem to prove the protective effect of HbAS against malaria indicating that in endemic environments genetic factors such as HbAS may confer an additional benefit (Williams & Weatherall, 2012).

From the above, it is clear that HbAS is associated with reliable protection against all forms of malaria. However, the mechanism that leads to this remains speculated (Williams & Weatherall, 2012).

Some studies have proposed different hypotheses, such as reduction of invasion and erythrocyte growth with *P. falciparum* under conditions of low oxygen tension; faster eradication of infected erythrocytes; reduced pathogenicity of infected red blood cells due to reduced expression of PfEMP1 (Erythrocytic Protein-1 encoded by *P. falciparum* parasites); and better acquisition of specific immunity against malaria (the mechanism is not only innate but also includes an acquired immunological component) (Williams & Weatherall, 2012).

However, there is a possibility that there is no single mechanism for the protective effect of HbAS but that the protection results from the set of all mechanisms described or from a different one (Williams & Weatherall, 2012).

The protective effect of HbAS against malaria is apparent; however, the effect of the HbSS homozygote is more controversial (Williams & Weatherall, 2012).

People with HbSS appear to be less susceptible than heterozygotes to the development of malaria infections, but if they are infected are highly vulnerable to the consequences, particularly severe anemia. Malaria is undoubtedly one of the leading causes of premature mortality in children born with HbSS in endemic areas. The early detection of SCD associated with active malaria prevention plays a crucial role in mortality rates (Williams & Weatherall, 2012).

Studies in Kenya and Ghana have shown that when inherited in isolation both HbS and thalassemia are strongly protective against malaria caused by *P. falciparum*, but the protective effects of each are lost when both conditions are inherited together (Williams et al., 2005).

The high frequencies described in malaria-endemic areas are not only due to natural protective malaria selection but are also attributed to a high rate of consanguineous marriages (among family-related), as well as improvements in public health conditions in many of these countries (Williams & Weatherall, 2012).

1.5 Beta-thalassemia

Beta-thalassemia is characterized by reduced (β^+-thalassaemia) or absent (β^0-thalassaemia) of *β-globin* subunits synthesis, resulting in imbalances of globins chains numbers cause hemolysis and impair erythropoiesis (Cao & Galanello, 2010; Galenello & Origa, 2010; Higgs et al., 2012; Taher et al., 2018; Thein, 2005).

The clinical manifestations of β-thalassemia are extremely diverse, broad from severe anemia with transfusion-dependency, where the patients inherit from both parents' deleterious mutations in both *β-globin* genes to the asymptomatic state with a mutation affecting only one nucleotide substitutions, deletions, or insertions *β-globin* gene (β-thalassemia trait) (Thein, 2005).

Molecular basis of the β-thalassemias is very heterogeneous, more than 300 β-thalassemia alleles have been described until today in the database of human hemoglobin variants and thalassaemias (http://globin.cse.psu.edu), which that causes a vast variety of phenotypes resulting from this allelic heterogeneity at the *β-globin* locus (Thein, 2017).

Most mutations are single leading to frameshift. The different mutations that act in this way may interfere with the action of the *β-globin* gene at the transcriptional level, in the processing of the primary transcript, in the translation of *β-globin* messenger RNA, or the post-translational stability of the *β-globin* gene product. Rarely, β-thalassemia results from large gene deletion (only a 619 bp deletion at the 3' end of the *β-globin* gene is common however restricted to the Sind and Punjab populations of India and Pakistan). Large deletions of the *β-globin* gene are responsible for the emergence of some rare forms like the δβ-thalassemias, the εγδβ-thalassemias, and the hereditary persistence of fetal hemoglobin (HPFH) syndromes (**Fig. 7**) (Bunn, 1997; Forget & Bunn, 2013; Olivieri, 1999; Stamatoyannopoulos, 2005; Ware et al., 2017).

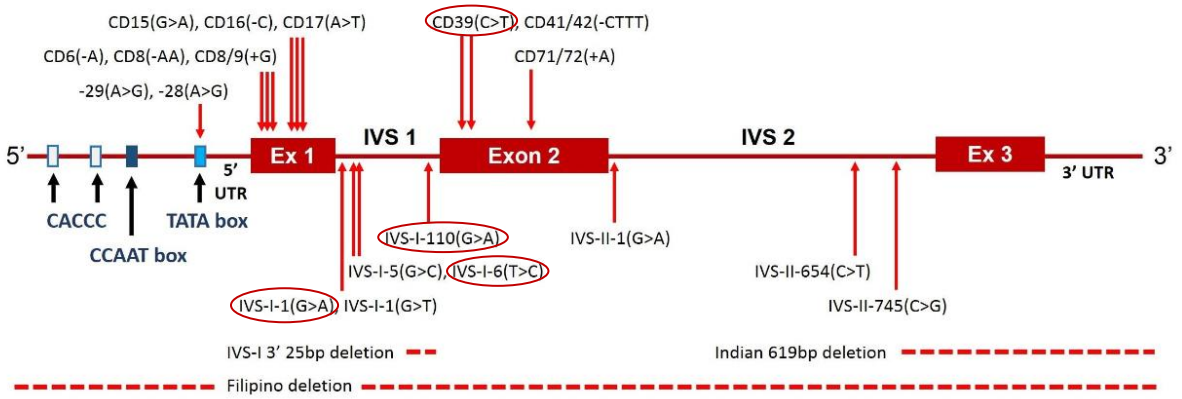


Fig. 7 Molecular localizations and types of mutations in *β-globin* gene resulting in common β-thalassemia worldwide. The *β-globin* gene contains three exons and two introns between codons 30 and 31 and 104 and 105, respectively. In β-thalassemia, the primary action of all the mutations is to abolish the output of *β-globin* chains (β^0-thalassemia) or reduce the output (β^+-thalassemia). The different mutations that act in this way may interfere with the action of the *β-globin* gene at the transcriptional level, in the processing of the primary transcript, in the translation *of β-globin* messenger RNA, or in the post-translational stability of the *β-globin* gene product. Adapted from (Goonasekera et al., 2018; Olivieri, 1999). The four most prevalent mutations in the Portuguese population are highlighted by red circles. Adapted from (Faustino et al., 1992).

The severity of β-thalassemia is related to the extent of imbalance between the *α-globin*/non-*α* chains, result from the insufficient synthesis of *β* chains to partner with the *α-globin* chains to generate adult hemoglobin (HBA-α2β2) (Cao & Galanello, 2010). Excess *α-globin* precipitate in red-cell precursors, causing defective maturation and ineffective erythropoiesis, leading to chronic hemolytic anemia, splenomegaly, marrow expansion (by stimulation the synthesis of erythropoietin), bone deformities (of the skull and face) and a variety of growth and metabolic abnormalities, hypermetabolic state, and iron accumulation (Higgs et al., 2012; Olivieri, 1999; Schrier & Angelucci, 2005).

In β-thalassemia often occurs a compensatory increase of HbA2 (α2δ2) and HbF (α2γ2) (Goonasekera et al., 2018).

Diagnosis of β-thalassemia can be based on hematologic and molecular genetic testing, however, the increased presence of HbA2 (> 3.5%) associated with decreased erythrocyte indices is the first factor in considering (S. Costa et al., 2016).

Three main forms of β-thalassemia have been described: β-thalassemia major, intermedia and minor (Galenello & Origa, 2010; Higgs et al., 2012).

Individuals with β-thalassemia major (also known as Mediterranean anemia or Cooley's Anemia) usually present within the first two years of life with severe anemia, requiring regular red blood cell (RBC) transfusions to survive (Galenello & Origa, 2010).

Patients with β-thalassemia intermedia present later in life with moderate anemia and do not require regular transfusions. β-thalassemia intermedia comprehend a clinically and genotypically very heterogeneous group, ranging in severity from the asymptomatic carrier state to the severe transfusion-dependent type (Galenello & Origa, 2010). β-thalassemia intermedia is the less common clinical phenotype (Forget & Bunn, 2013).

Both β-thalassemia major and intermedia can result from the homozygous or compound-heterozygous inheritance of mutations in the *β-globin* gene (Cao & Galanello, 2010).

β-thalassemia minor (trait or carrier), which results from heterozygosity for β-thalassemia, is clinically asymptomatic, but some subjects may have moderate microcytic-anemia (Taher et al., 2018).

β-thalassemia syndromes can also be classified phenotypically into two groups: transfusion-dependent thalassemias (TDTs) and non-transfusion dependent thalassemias (NTDTs) (Cappellini et al., 2014).

When β-thalassemia is co-inherited with the structural variant hemoglobin E (HbE/β-thalassemia) results in a clinical state closely resembling β-thalassemia major with severe anemia and transfusion-dependency (Higgs et al., 2012; Martin & Thompson, 2013; Taher et al., 2018; Thein, 2005). 50% of seriously affected patients with β-thalassemia have the βE/βT genotype (Higgs et al., 2012).

Other rare thalassemias like, HbC/β-thalassemia also exhibit a great range in terms of diversity of phenotypes and spectrum of severity (Cao & Galanello, 2010; Galenello & Origa, 2010). HbS/β-thalassemia leads to a clinical condition more similar to sickle cell disease than to thalassemia major or intermedia (Galenello & Origa, 2010).

β-thalassemias can be detected using qualitative or quantitative tests: including acid and alkaline Hb electrophoresis, capillary electrophoresis (CE), isoelectric focusing (IEF), and high-performance liquid chromatography (HPLC) (Goonasekera et al., 2018).

WHO estimates that about 60.000 severely β-thalassemia affected infants are born every year and about 1.5% (80 to 90 million people) of the world's population might be carriers, although the actual number of patients worldwide is probably underestimated (Higgs et al., 2012).

The annual incidence of symptomatic individuals with β-thalassemias is estimated at 1 in 100.000 throughout the world and 1 in 10.000 in the European Union (Galenello & Origa, 2010).

The epidemiology of the various clinical forms of β-thalassemia is still poorly understood, although the disease is known to be highly prevalent in Mediterranean countries, the Middle East, Central Asia, India, Southern China, and the Far East as well as the coast of Africa north countries and in South America (Galenello & Origa, 2010). That is, more than 90% of people with β-thalassemia live in low or middle-income countries, and this number is expected to increase in the coming years as infant mortality from infectious and nutritional causes decreases due to improved treatments and public health (Taher et al., 2018).

About 50 years ago fewer than 2% of patients with thalassemia were older than 25 years, nowadays, that number has risen to 36% (Martin & Thompson, 2013).

There is strong evidence that the high frequency of β-thalassemia throughout the tropics reflects an advantage of heterozygotes against *Plasmodium falciparum* malaria, as has already been demonstrated in SCD (Cao & Galanello, 2010; Olivieri, 1999).

Some of the proposed mechanisms of action for malaria resistance polymorphisms involving β-thalassemia are: faster removal of parasite-infected red blood cells; difficulties in invasion and growth of *P. falciparum* parasites in the RBCs of people with β-thalassemia; reduced pathogenicity by reducing cytoadherence or resetting (Williams & Weatherall, 2012).

Population migration and intermarriage between different ethnic groups have introduced thalassemia in almost every country of the world (Goonasekera et al., 2018; Thein, 2005).

According to the Thalassemia International Federation (TIF), only about 200.000 patients with thalassemia major are registered as receiving regular treatment around the world (Galenello & Origa, 2010; Higgs et al., 2012).

1.6 Treatments for β-hemoglobinopathies

Currently available treatments for β-hemoglobinopathies still very limited mostly in low-income countries (Ware et al., 2017). There are differences in the pathophysiology of these diseases. Vasoclusion is a feature of sickle cell disease, while iron loading is a more dominant problem in β-thalassemia (Dreuzy et al., 2016; Matte et al., 2019). However, treatment methodology mostly follows the same methods. Most treatments prevent or manage complications and help people live longer. Options vary according to what symptoms a person has and how severe their symptoms are. Health maintenance starts with early diagnosis, preferably in the newborn period and includes penicillin prophylaxis, vaccination against pneumococcus bacteria and folic acid supplementation (Kato et al., 2018).

At present, the only curative treatment is allogeneic bone marrow or stem cell transplantation. However, this procedure remains risky, and histocompatible donors are identified for only a small fraction of patients, remaining like this inaccessible for most patients (Dreuzy et al., 2016).

Most patients with severe forms of β-hemoglobinopathies have to settle for long-term is palliative management, namely, life-long red blood cell transfusion (which does not correct ineffective erythropoiesis and exacerbates systemic iron accumulation), iron chelation and splenectomy (removal of the spleen) (Martin & Thompson, 2013; Schrier & Angelucci, 2005; Ware et al., 2017).

Nowadays the two more powerful and promising strategies for the treatment of β-hemoglobinopathies are the pharmacological reactivation of Fetal hemoglobin (a tremendous promise to improve the clinical symptoms of sickle cell disease and β-thalassemia) and gene therapy (still in the experimental phase) (**Fig.8**) (Dreuzy et al., 2016; Lettre & Bauer, 2016; Sankaran & Orkin, 2013).

To date, few therapies have been approved by the US Food and Drug Administration, specifically for the treatment of SCD: Hydroxyurea (a pharmacological reactivator of HbF) and L-glutamine (an antioxidant agent) (Telen et al., 2019). Crizanlizumab and voxelotor are two new therapies that received FDA approval recently. Crizanlizumab is a humanized monoclonal antibody that interrupts activation and adherence of leukocytes that may be drivers of VOC. Voxelotor is a modulator and stabilizer of HbS that may improve oxygen-binding ability, prevent polymerization, and subsequent RBC sickling and damage (Cieri-Hutcherson, Hutcherson, Conway-Habes, Burns, & White, 2019).

Studies are also currently directed to anti-sickle cell agents, anti-adhesion agents, ischemia-reperfusion and oxidative stress modulators, agents that combat free hemoglobin, anti-inflammatory agents, antithrombotic agents and antiplatelet agents for the treatment of SCD (Matte et al., 2019; Telen et al., 2019).

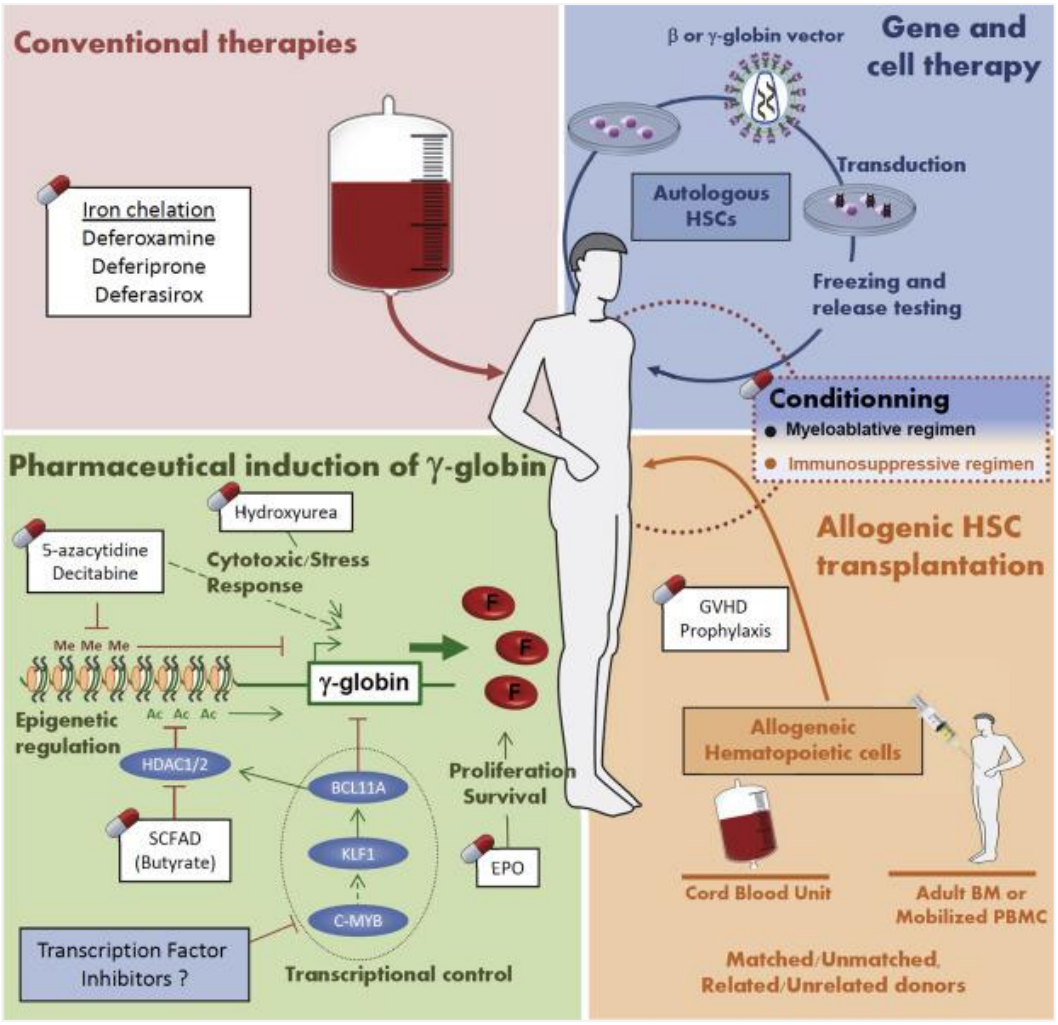


Fig. 8 Current and future therapies for β-hemoglobinopathies, adapted from (Dreuzy et al., 2016). Top left: Conventional therapy that includes regular red blood cell transfusions and iron chelation. Bottom left: *γ-globin* chain inducers. Bottom right: Allogeneic transplantation with related or unrelated donor cells from cord blood units or bone marrow. Top right: Gene therapy a promising single-dose therapy.

1.6.1 Stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) derived from bone marrow, or other sources (e. g. cord blood stem cells) currently offers the only hope of a definitive cure in patients with β-hemoglobinopathies (Dreuzy et al., 2016).

HSCT is a total hematopoietic cell replacement therapy that eliminates ineffective erythropoiesis and enables the body to produce new effective erythropoiesis (Dreuzy et al., 2016).

One of the main barriers to HSCT with cells from HLA identical siblings is the limited number of donors (Dreuzy et al., 2016), only 10-20% of patients have sibling donors, and many concerns

remain about transplant-related mortality and long-term toxicities (Galenello & Origa, 2010; Ware et al., 2017).

The positive outcome of treatment is influenced by the patient's pre-transplant clinical conditions, specifically the presence of hepatomegaly, the extent of hepatic fibrosis, and the magnitude of iron accumulation (Dreuzy et al., 2016). Children usually do not have the above risk factors, so disease-free survival is greater than 90% after transplantation. Individuals with all three risk factors have a reported survival rate of approximately 60%. Chronic graft versus host disease can occur in 5-8% of transplant patients (Cao & Galanello, 2010).

Bone marrow transplantation from unrelated donors has been performed in a limited number of individuals. However, as long as donor selection is based on strict HLA compatibility criteria and individuals have no iron overload, the results are similar to those obtained when the donor is a compatible sibling (Cao & Galanello, 2010; Galenello & Origa, 2010; Olivieri, 1999).

Hematopoietic stem cell transplantation is now widely applied with disease-free survival of over 80% with HLA-compatible sibling donor transplants (HLA-A, B, C, eDRB1 and DQB1) (Dreuzy et al., 2016; Taher et al., 2018).

The HSCT was successfully performed for the first time for the treatment of thalassemia 30 years ago by the Pesaro Group in Italy (Dreuzy et al., 2016; Martin & Thompson, 2013). Between 1981 and 2003, over 1.000 consecutive patients aged 1 to 35 years were transplanted in Pesaro. The Kaplan Meier probability >20 years of thalassemia-free survival was 68% (Schrier & Angelucci, 2005).

Concerning sickle cell disease, stem cell transplantation in adults allowed to report 87% of event-free survival (Ware et al., 2017).

1.6.2 Blood transfusion therapy

The main treatment for severe forms of β-hemoglobinopathies remains red blood cell transfusion, where transfusions begin before 1 year of age in β-thalassemia major and 90% of SCD patients receive at least one transfusion during their lifetime (Martin & Thompson, 2013; Ware et al., 2017).

The decision to initiate a transfusion regimen is based on clinical evaluation, notably on the impact of chronic anemia on the patient's life. Factors such as impaired growth, bone deformities, and fatigue are taken into consideration (Martin & Thompson, 2013; Olivieri, 1999).

Transfusions bring as immediate benefit the increased capacity of oxygen transport (increasing the hemoglobin level to 9-12 g/dl) and improvement of blood volume and flow. It allows normal growth and development and prevents congestive heart failure, severe bone deformities, and endocrinopathies (Schrier & Angelucci, 2005; Taher et al., 2018).

Despite proven benefits, transfusions have complications that may limit long-term use, regularly transfused patients develop hemosiderosis (patients receiving 2 to 4 blood units/month have an iron accumulation of 0.3 to 0.6 mg/kg per day) with deposition of parenchymal iron and may suffer fatal complications. Iron chelation by oral or parenteral drug administration is therefore essential in these cases to prevent accumulation in the various organs (Galenello & Origa, 2010; Martin & Thompson, 2013; Olivieri, 1999; Schrier & Angelucci, 2005; Taher et al., 2018; Ware et al., 2017).

Recurrent transfusions expose the patient to many samples of foreign blood, thus also increasing the risk of red blood cell alloimmunization and infection (Ware et al., 2017).

Access to blood for transfusion therapy remains a challenge in resource-poor countries and represents a substantial for public health burden (Taher et al., 2018).

1.6.3 Gene therapy

One of the most promising therapeutic strategies for the treatment of β-hemoglobinopathies is to attempt to correct the mutated *β-globin* gene (Telen et al., 2019).

β-thalassemia may have several genetic causes with more than 300 different mutations, while a single genetic mutation causes SCD. Repairing the disease-causing mutation in the *β-globin* gene is an attractive strategy that could be applied to all patients with SCD (Wienert et al., 2018), although it is a challenge to predict the correction of all the hundreds of mutations that cause β-thalassemia. Stopping factors that silence *γ-globin* genes, such as *BCL11A*, may allow more immediate treatment of β-thalassemia (Taher et al., 2018).

The concept of gene therapy involves the expression of a normal *β-globin* replacement gene using a virus-introduced expression vector. Hematopoietic stem cells from a patient with β-hemoglobinopathies are isolated and then incubated (*ex vivo*) with the virus containing the additional gene. Following treatment with myeloablative chemotherapy, the patient receives reinfusion of the modified autologous stem cells, which repopulate the marrow and express the new gene (Ware et al., 2017).

Gene therapy is still at an early stage of development, but clinical trials have provided evidence of sustained clinical efficacy. The most promising results were obtained with lentiviral vectors (Dreuzy et al., 2016).

Gene therapy was included as a viable plan for the treatment of hemoglobinopathies in 1978 at the University of California, Los Angeles. However, it took more than 25 years to achieve these goals, which were only possible by characterizing the regulatory elements of the *β-globin* locus and the advent of lentiviral vectors, which can transfer complex sequences to hematopoietic stem cells (Dreuzy et al., 2016).

Lentivirus-mediated gene transfer can correct hematological defects and organ damage in mice with sickle cell disease, and a clinical trial has been started in France (Rees et al., 2010).

The first clinical trial using a lentiviral *β-globin* construct to treat patients with β-thalassemia was started in 2007. Two patients received gene therapy. The second patient had a hemoglobin concentration of 90 to 100 g/L, due to the predicted increase in *β-globin* expression and also due to an unexplained rise in fetal hemoglobin, remaining well and not transfusion dependent for almost two years. However, a detailed analysis showed that the viral vector integrated with a proto-oncogene, which could be a precursor of a leukemic transformation, as previously seen in retrovirus gene therapy studies (Higgs et al., 2012).

A lentivirus vector containing HS2, 3, and 4 of LCR associated with a *β-globin* gene led to partial correction of anemia in a β-thalassemia mouse. As for the alternative approach, in a mouse model with sickle cell anemia, it was possible to correct the molecular defect by homologous recombination by transfecting the embryonic stem cells of the affected mice with a DNA fragment containing the normal *β-globin* gene. Hematopoietic stem cells, derived from *ex vivo* corrected embryonic stem cells, could produce HbA and HbS, leading to a phenotype similar to the human sickle cell trait. Similar experiments demonstrate the possibility of a cure for hemoglobinopathies by homologous recombination in embryonic stem cells (Cao & Galanello, 2010).

However, there are still many remaining problems that include identifying all sequences necessary for stable, high-level gene expression and the development of safer and more effective vectors (Olivieri, 1999). Further studies on new non-viral method technologies for the introduction of the therapeutic gene into stem cells that can help standardize gene therapies and make them more available and safer are also crucial (Cavazzana & Mavilio, 2018).

Instead of gene therapy, recent approaches have been developed to directly correct genetic mutations in the cell's endogenous DNA or to disrupt specific DNA sequences in the genome. This approach is known as genome editing and has been facilitated by the identification of several enzymes, including CRISPR/Cas9, that can introduce DNA breaks into specific regions of the genome (Taher et al., 2018). At this moment on *clinicaltrials.gov* there is information from three clinical trials for the treatment of β-thalassemia using CRISPR/Cas9 technology. The European Medicines Agency (EMA) approved in June 2019 a first gene therapy for β-thalassemia, an orphan drug ZYNTEGLO™ (autologous CD34+ cells encoding βA-T87Q-*globin* gene), previously known as *LentiGlobin*, for patients 12 years and older with TDT who do not have a β0/β0 genotype. Developer Bluebird Bio, Inc. has priced the therapy at €1.58 million ($1.8 million) (Stanton, 2019).

The imminent explosion of gene therapies raises many accessibility issues for all health systems, not just for low-income countries, the practical challenges of bringing these therapies to patients who need them are not just a hypothetical issue for global health (F. Xu & Guo, 2018),

these approaches will not be at the forefront of treatment options for some time, mainly as their technical demands and cost may limit their application in countries with the highest numbers of people with β-hemoglobinopathies (Telen et al., 2019).

1.6.4 Reactivation of fetal hemoglobin

In 1948, Janet Watson found that newborns had no sickle cell disease complications until they were about six months old, predicting that red blood cells in sickle cell infants are relatively protected from sickle cell disease compared with adults or older babies with the disease. These infants were few symptoms and that their deoxygenated erythrocytes took longer to sickle and did not deform as extensively. It was first hypothesized that this protection in infants would be due to the high concentration of fetal hemoglobin (Akinsheye et al., 2011).

A few years later, reports of asymptomatic SCD patients who co-inherited a hereditary persistence of the fetal hemoglobin (HPFH) phenotype were described. HPFH is typically caused by large deletions in the *β-globin* gene or by point mutations in the promoters of the *γ-globin* genes and is characterized by high lifetime fetal hemoglobin concentrations (Lettre & Bauer, 2016).

More recently, some sickle cell populations in Saudi Arabia and India have been noted to have higher concentrations of fetal hemoglobin and a milder form of sickle cell disease than patients of African descent. Several studies have reported that these populations have a relatively low prevalence and late-onset of many of the complications attributed to high fetal hemoglobin production in adulthood (Lettre & Bauer, 2016).

Such evidence led to the initial hypothesis that HbF would have a potentiating effect on SCD and, therefore, in recent decades, several studies based on epidemiological, clinical and laboratory observations have attempted to identify an effective way to induce HbF synthesis as a potential therapy to the SCD (Sankaran & Orkin, 2013).

In relation to β-thalassemia, increasing synthesis of *γ-globin* chains compensates for the deficit of *β-globin* by improving the imbalance between *α* and non-*α globin* chains. The *γ* chains combine with the excess of unmatched accumulative of *α-globin* chains. The combination produces useful hemoglobin (HbF), but more importantly, reduces the burden of *α* chains that produce most of the pathophysiology associated with β-thalassemia (Akinsheye et al., 2011; Dreuzy et al., 2016).

Several compounds have been tested, including cytotoxic compounds and epigenetic regulators (Akinsheye et al., 2011).

The first drug that was shown to *increase γ-globin* expression was 5-azacytidine via epigenetic silencing. It has also been shown that small chain fatty acid derivatives increase the expression of *γ-globin*, by inhibiting histone deacetylation. Erythropoietin (EPO) is also

described as a potentiality for its proliferative and anti-apoptotic properties (Sankaran & Orkin, 2013).

HU is the only drug currently approved for inducing HbF in SCD. It works through multiple mechanisms, but its cytotoxic activity is believed to accelerate the differentiation process and stimulate cell stress response pathways, leading to a general increase in HbF numbers (Dreuzy et al., 2016).

Currently, the new targets for HbF induction strategies are transcriptional *γ-globin* repressors such as *BCL11A*, *MYB*, *KLF1* and additional repressors belonging to the same family. As well as regulators like *Mi2β*, which binds directly and regulates the *KLF1* and *BCL11*A genes, making these promising genes targets for HbF inducing therapy. Although there is currently no therapy to target these molecules or related pathways, further work motivated by the findings already reported may lead to the discovery of promising candidates for clinical HbF inducers in the near future (Finotti et al., 2016).

1.6.4.1 DNMT Inhibitors: 5-azacytidine and decitabine

5-azacytidine is an antineoplastic drug that inhibits maintenance methylation of DNA (adult *β-globin* genes are hypomethylated and the non-transcribed fetal *γ-globin* genes are hypermethylated in adult life), caused a marked increase in HbF. These results prompted clinical trials that demonstrated a significant, albeit less dramatic, induction of HbF production in patients with sickle cell disease and β-thalassemia. In spite of those promising results, this drug was never tested in large-scale clinical trials as a result of considerations concerning potential carcinogenicity (Bunn, 1997; Frenette & Atweh, 2007).

Decitabine (5-aza-2'-deoxycytidine) is an analog of 5-azacytidine that does not incorporate into RNA, with a safer side effect profile (Cappellini et al., 2014), resulting in a renewed interest in the use of DNA hypomethylation therapy for the induction of HbF production. In recent clinical trials in patients with SCD, treatment with decitabine resulted in significant increases in the synthesis of HbF (Frenette & Atweh, 2007). In patients with β-thalassemia, in a single pilot study, subcutaneous decitabine given at 0.2 mg/kg two times per week for 12 weeks increased total hemoglobin from 78.8 to 90.4 g/l and absolute fetal hemoglobin from 36.4 to 42.9 g/l in five patients (Cappellini et al., 2014).

The increase of HbF with decitabine was associated with significant improvement in several factors that are important in the pathophysiology of SCD and β-thalassemia's: as the RBC adhesion, endothelial damage, and activation of the coagulation pathway. However, larger and longer-term studies are needed to confirm the efficacy and safety of decitabine in the treatment of SCD and its effectiveness in β-thalassemia (Frenette & Atweh, 2007).

1.6.4.2 HDAC Inhibitor: butyrate

Observations that infants of diabetic mothers had a more delayed switch from fetal to adult hemoglobin led to the hypothesis that short-chain fatty acids could act as inducers of HbF (Cappellini et al., 2014).

Butyrate is a short-chain fatty acid that inhibits histone deacetylase (HDAC). It has been shown to stimulate embryonic and fetal *globin* gene expression in chickens, mice, and baboons. When it was administered intravenously to patients with SCD, it resulted in sustained induction of HbF production in the majority, however, a pulsed, or intermittent, dosing regimen was necessary to avoid cytotoxicity (Akinsheye et al., 2011; Frenette & Atweh, 2007).

In β-thalassemia patients, an initial study involving a 2-3-week butyrate infusion (at a dose of 500 mg/kg/day) in three β-thalassemia patients showed a decrease in globin chain imbalance. However, a follow-up study extending this therapy to 13 weeks at a dose of 2000 mg/kg/day for six days a week failed to achieve a primary hematological outcome of a rise in hemoglobin (Cappellini et al., 2014).

Despite the considerable promise of this agent in the treatment of SCD, the difficulty of administering large volumes of this drug through venous catheters presents a major therapeutic challenge. The full potential of butyrate is unlikely to be realized until an oral compound that has the same efficacy as butyrate is identified (Dreuzy et al., 2016; Frenette & Atweh, 2007).

1.6.4.3 Erythropoietin

Administration of pharmacological amounts of erythropoietin has been shown to cause a reversal of fetal programming in the maturation of erythroid precursors, which involves an increase in *γ-globin* synthesis (Schrier & Angelucci, 2005).

Some studies have demonstrated this benefit in patients with β-thalassemia. The combination of HU and erythropoietin has been shown to be associated with higher increases in total hemoglobin levels than HU alone (17 g/l versus 2 g/l after 6 months of therapy) in patients with intermediate β-thalassemia. Limited further studies of these combined therapies have been performed (Cappellini et al., 2014).

1.6.4.4 Hydroxyurea

Hydroxyurea (HU), alternatively known in some countries as Hydroxycarbamide, is the only HbF inducer drug approved by the US FDA in 1998 and by the European Medicines Agency (EMA) in 2007 for the treatment adults with SCD, and its use has led to a reduction of

complications of SCD in adults, children and even infants, as well as prolonged survival (Kato et al., 2018; Telen et al., 2019).

HU is a cytotoxic, antimetabolic, and an antineoplastic agent is known for its use in the management of patients with myeloproliferative disorders (myeloid tumors) and human immunodeficiency virus infection, where the drug acts as a potent inhibitor of ribonucleotide reductase, an enzyme required for DNA synthesis and repair (Musallam, Taher, Cappellini, & Sankaran, 2013). In addition to all these physiological effects, HU increases HbF expression by inducing *the γ-globin* gene and reduce the expression of *β-globin* gene (in most individuals with SCD), which decreases the total leukocyte count and prevents their activation and thereby reduces the incidence of vaso-occlusive crises. HU thus reduces the number of hospitalizations and mortality with an excellent safety profile, although some patients do not have a beneficial response, usually due to limitations of treatment adherence, but also for pharmacogenomic reasons (Kato et al., 2018).

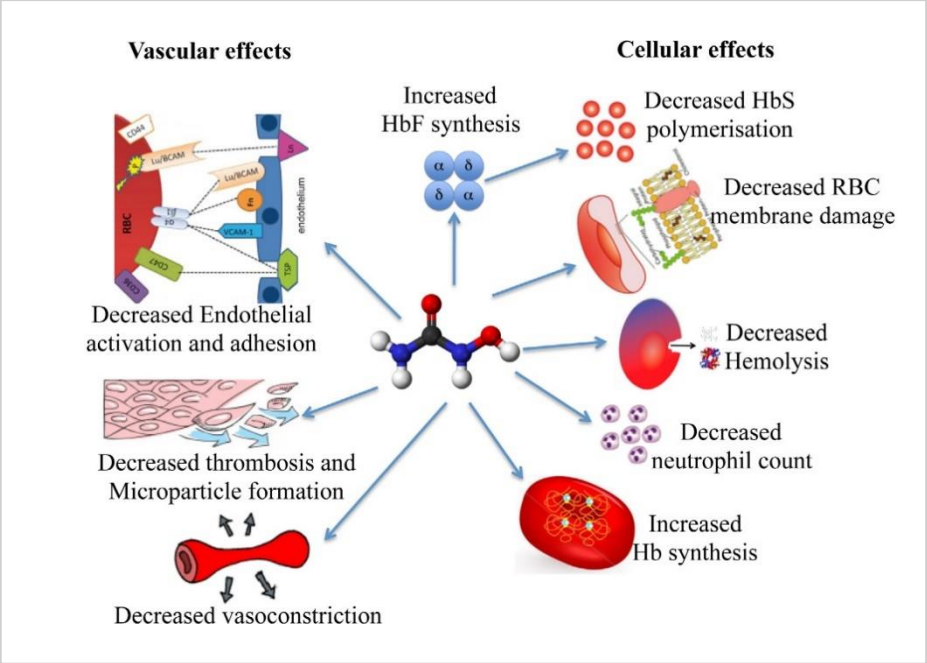


Fig. 9 Multiple effects of hydroxyurea administration in sickle cell disease patients, adapted from (Verma, Lakkakula, & Lakkakula, 2018). HU has been used for the treatment of SCD for some years. The main justification behind its use is its ability to induce HbF. Several lines of evidence suggest that HU induces HbF induction and offers clinical benefits to patients with SCD through a wide range of possible mechanisms, with reported cellular and vascular effects.

Studies in SCD show a multiple action of HU, which increases HbF production, resulting in late HbS polymerization, reducing hemolysis and increasing nitric oxide (NO) availability for cyclic guanosine monophosphate (cGMP) production, modulating endothelial activation and

reducing neutrophil count, contributing to the reduction of chronic inflammation (**Fig. 9**) (Matte et al., 2019).

That way, HU interrupts SCD pathophysiology, the higher induction of HbF correlates continuously with less renal damage, less pulmonary hypertension, fewer strokes, and longer survival (Lavelle, Engel, & Saunthararajah, 2018).

HU is also widely used for β-thalassemia though the results are not been as consistent as in SCD. A number of studies have shown a clear benefit from the use of this agent in some patients, includes a decreased need for transfusion, an increase in hemoglobin levels (1-3 g/dl), decreased markers of ineffective erythropoiesis, and decreased morbidities (Cappellini et al., 2014; Schrier & Angelucci, 2005).

The exact mechanism by which HU increases HbF is unclear, however, its cytotoxic activity is believed to accelerate the differentiation process and stimulate cell stress response pathways, leading to an overall increase in F cell numbers (Dreuzy et al., 2016).

These response pathways include rapid erythroid regeneration, increased erythropoietin (EPO) production, apoptosis, NO production, increased guanylate cyclase and SAR1 (a guanosine triphosphate binding protein) activity, and via p38 MAPK (Pourfarzad et al., 2013).

It was demonstrated that HU increases the total intracellular hemoglobin in the human K562 erythroleukemia cell line and preferentially increases the *γ-globin* mRNA levels in these cells. In two-phase mixed cultures of peripheral blood erythroid progenitor cells from normal donors, HU significantly increases *γ-globin* and HbF mRNA levels and also has a comparatively small stimulating effect on *β-globin* mRNA expression (Cokic et al., 2003).

Although HU has not been identified as a hypomethylating agent, an early study suggested an association between HU exposure and decreased methylation within the *γ-globin* gene accompanies increased in the expression of the gene (Platt et al., 1984). However, the cytotoxicity, the potential carcinogenicity and the moderate effects obtained after the administration of these agents have limited their clinical use (Cui & Engel, 2017).

HU does not improve HbF production in all patients, about half of patients do not improve with this treatment, which requires regular monitoring of blood counts with intolerable side effects observed in some patients (Field & Nathan, 2014; Saunthararajah, Lavelle, & DeSimone, 2004).

Myelosuppression is the most common and known side effect. Among the cutaneous effects were observed: hyperpigmentation of nails, palms, and soles, besides the development of ulcers in the lower limbs in patients with myeloproliferative syndromes. Nausea and gastrointestinal symptoms have been associated with the use of HU. It should be borne in mind that due to renal excretion, dose reduction is required in patients with renal impairment. To date is described a low risk of developing cancer associated with HU. However, *In vitro* studies have indicated that HU can induce mutations and chromosome instability and due to the concerns about the long-

term administration of an antitumor drug in patients with a nonmalignant congenital disorder, there is considerable interest in identifying safe alternatives to induce HbF production, which has spurred the search for new compounds (Figueiredo, 2007; Saunthararajah et al., 2004).

1.6.4.5 Natural inducers of fetal hemoglobin

Several inducers of HbF were tested in clinical trials, but only HU received FDA approval, and the effects of this therapeutic approach have stimulated the interest in identifying further molecules capable of inducing HbF (Feriotto et al., 2018).

Vegetables are used largely in traditional medicine and represented a natural reservoir for future therapeutic drugs (Feriotto et al., 2018). Recently, many efforts have been focused on natural strategies for the treatment of β-hemoglobinopathies. Hoping that natural inducers can increase the level of HbF and can also reduce iron overload, but most important of all: to make them available to all populations (Kukreja, Wadhwa, & Tiwari, 2013).

Induction of HbF using natural agents is an effective approach already described in some studies. Various natural agents like *Angelicin*, *Rapamycin*, *Bergamot*, *Fructus trichosanthis*, *Romidepsin*, *Wheat grass*, *Curcuma comosa*, *Astragalus*, *Apicidin*, *Curcuminoid*, *Piceatannol*, and *Resveratrol* (**Fig.10**) have been reported to induce HbF level. However, more research is still needed in their biological activity. There is a need to find out the most promising natural therapeutic agent which could effectively induce HbF production and reduce iron overload, thereby improving the life span of diseased patients. More data are needed on the bioavailability of these natural compounds and their effects on humans (Kukreja et al., 2013).

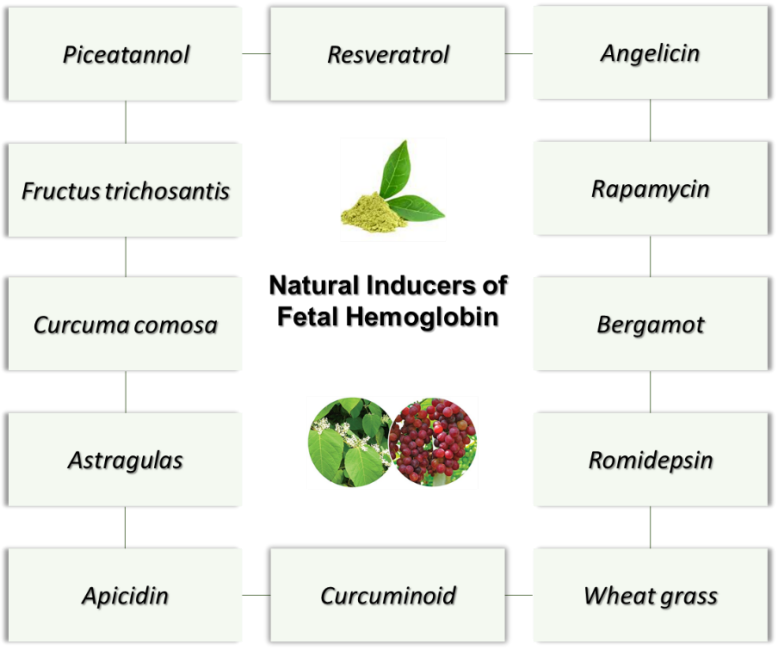


Fig. 10 Natural inducers of fetal hemoglobin already reported, adapted from (Kukreja et al., 2013)

1.6.4.6 Potential new natural inducers of fetal hemoglobin

1.6.4.6.1 Genistein

Genistein (chemically known as 4', 5, 7-trihydroxyisoflavone) is a non-steroidal polyphenol and is the major soy isoflavones, described with multiple beneficial effects including anti-inflammatory, anti-oxidative, and protective properties (Mirahmadi et al., 2017; Yang, Kulkarni, Zhu, & Hu, 2012).

Its many biological activities have made it a target for the many studies published in recent years. Most of these studies have focused on the pharmacological activities of GN as a tyrosine kinase inhibitor, its chemoprotection activities against cancers and cardiovascular disease, and its phytoestrogen activity (Dixon & Ferreira, 2002).

GN is structurally similar to estrogen 17β-estradiol, particularly the phenolic ring and the distance between its 4'- and 7'- hydroxyl groups (**Fig. 11**). This feature confers ability to bind estrogen receptors and sex hormone-binding proteins, GN can thus exert both estrogenic and antiestrogenic activity, the latter by competing for receptor binding by estradiol (Dixon & Ferreira, 2002) The binding affinity of GN for estrogen receptor α is 4% and for the estrogen receptor β is 87% (Nagaraju et al., 2013).

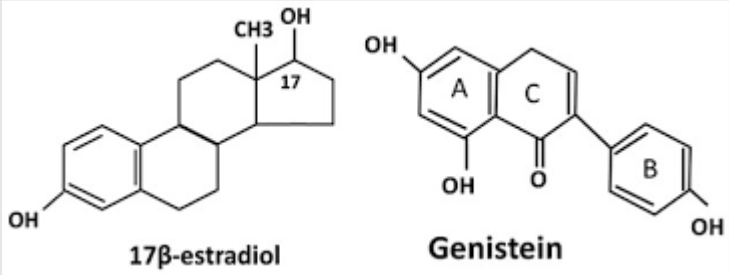


Fig. 11 The generic structure of 17β-estradiol and genistein. (Nagaraju et al., 2013). Genistein it was shown to possess a structural similarity with estrogen. Two benzene rings, linked through a heterocyclic pyran ring, form the basis of its flavone nucleus. The C4 and C7 phenols of genistein are structurally and functionally similar to the active -OH groups on 17b-estradiol.

GN modulates many nuclear events, including G2/M cell cycle arrest, inhibits several adenosine triphosphates (ATP) binding enzymes *in vitro*, such as protein tyrosine kinases (PTK) (Frey & Singletary, 2003).

GN has also been reported has a capacity to influence various cell-signaling pathways such as mitogen-activated protein kinase (MAPK) superfamily that is composed of several signaling pathways involved in growth regulation, GN rapidly and strongly activates p38 MAPK pathway and inactivates the ERK1/ERK2. These data suggest that the interaction between the p38

pathway and the G2 cell cycle checkpoint control may provide information on the possible mechanisms by which this isoflavone may inhibit early events in carcinogenesis (Frey & Singletary, 2003), and make it a good candidate as a chemopreventive agent, namely in breast and prostate cancers (Dixon & Ferreira, 2002; Yang et al., 2012).

GN has been shown to play an important role in epigenetic modulation of the transcription of genes involved in various stages of carcinogenesis. In prostate cancer GN has been shown to alter chromatin configuration by increasing the acetylation of the H3 and H4 histones around the p21 and p16 transcription sites, thereby upregulating the expression in the cancer cells. GN also inhibits DNA methyltransferase (DNMT), leading to demethylation of CpG islands in the promoter regions and causing reactivation of important tumor suppressor genes, such as beta retinoic acid beta receptor (RAR-beta), p16 and O^6-methylguanine methyltransferase in squamous cancer of the esophagus and prostate (Nagaraju et al., 2013).

High intake of GN in the diet is described as having major health-promoting benefits, such as antidiabetic, hepatoprotective, renoprotective, neuroprotective, and anti-inflammatory effects, and improved learning and memory under various conditions (Mirahmadi et al., 2017).

Take in to account the presented results we consider that GN can be a candidate for new natural inducers of fetal hemoglobin

1.6.4.6.2 Epigallocatechin-3-gallate

Green tea (*Camellia sinensis*) has been widely consumed (is next to the water in habitual consumption) for more than 4000 years, and its medicinal applications associated with many benefits have been actively studied with scientific methods in the last decades (Yamashita et al., 2014; Q. Zhou et al., 2002).

(−)-epicatechin-3-gallate (EGCG) (**Fig. 12**) a polyphenolic compound is the major catechins in green tea and accounting for 50–80% of a cup of green tea (Philips, Coyle, Morrisroe, Chanchellor, & Yoshimura, 2009; Thichanpiang & Wongprasert, 2015).

EGCG is described as a powerful antioxidant, that prevents oxidative damage in healthy cells, but also as an antiangiogenic and antitumor agent and as a modulator of tumor cell response to chemotherapy (B. Singh, Shankar, & Srivastava, 2011).

Various clinical studies have revealed that treatment by EGCG inhibits tumor incidence and multiplicity in different organ sites such as liver, stomach, skin, lung, mammary gland and colon, and its effect on slowing the onset and progress of common chronic diseases such as cardiovascular disease (possibly via their anti-inflammatory effects) is also reported (Shukla, 2007).

In vitro and in animal models studies have demonstrated that EGCG blocks carcinogenesis by affecting a wide array of signal transduction pathways including JAK/STAT, MAPK,

PI3K/AKT, Wnt and Notch (Hussain, & Ashafaq, 2018). EGCG triggers p38 MAPK signaling pathway (Shu et al., 2018) as HU (Chou et al., 2015).

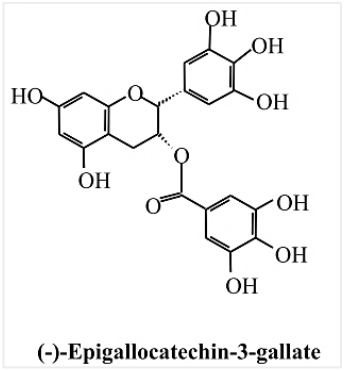


Fig. 12 Structure of Epigallocatechin-3-gallate, adapted from (B. Singh et al., 2011).

EGCG also stimulates telomere fragmentation through inhibiting telomerase activity. Recent studies also demonstrated that EGCG acts as an epigenetic agent by reducing DNMTs, proteases, and Dihydrofolate reductase (DHFR) activities, which would affect transcription of tumor-suppressor genes (TSGs) and protein synthesis (B. Singh et al., 2011).

Another mechanism associated with catechins is the potential to induce beneficial gene expression profiles (Muzolf-Panek et al., 2008).

EGCG thus has great potential in preventing various pathologies because it is clearly palatable, inexpensive and widely available, and tea exhibits little or no toxic effects in normal, healthy cells (Baek et al., 2004).

However, the high concentration of catechins employed in most studies might not be relevant to those obtained by tea drinkers (Baek et al., 2004).

At present, medical specialty proof of the protecting result of tea consumption against the event of human cancers isn't conclusive. This might be attributed to variables associated with individual variations in tea preparation and consumption patterns and seasonal and geographic variations in tea production (Chow et al., 2003).

Take in to account the presented results we consider that EGCG can be a candidate for new natural inducers of fetal hemoglobin.

1.7 K562 cell line

K562 cell line is a transformed human erythroleukemic cell line isolated from a 53-year-old patient with chronic myelogenous leukemia (CML) in 1975 by Lozzio & Lozzio and is positive for the oncogenic *Bcr-Abl* fusion gene (ATCC, 2016).

Erythroleukaemic cell lines have been used to study blood for decades due to their unlimited differentiation potential, and therefore the ease with which they can be obtained and cultured (Diepstraten & Hart, 2019).

K562 cells share phenotypical characteristics of embryonic erythroid progenitors and have the potential to differentiate into cells from the erythroid, macrophage and megakaryocytic lineages, and as a human cell line no require genetic modification to study the human globin locus (Diepstraten & Hart, 2019).

Erythroid differentiation of K562 cells is associated with an increase of expression of embryonic-fetal *globin* genes, such as the *ζ*-, *ε*- and *γ-globin* genes (Bianchi et al., 2001).

A number of agents have been shown to activate or suppress *globin* genes expression in these cells (Fathallah, Portnoy, & Atweh, 2008). Published observations demonstrate that hormones, cytotoxic agents (hydroxyurea), and agents associated with epigenetic mechanisms (butyrate, 5-azacytidine and decitabine) are capable of inducing HbF levels (*γ-globin* mRNA accumulation) in K562 cells (Bianchi et al., 2001; Fathallah, Weinberg, Galperin, Sutton, & Atweh, 2007).

That makes the K562 line a popular choice to study the molecular activators of fetal hemoglobin expression which is of the fundamental importance for the understanding of disease mechanisms such as β-thalassemia and sickle cell anemia (Diepstraten & Hart, 2019).

K562 cells contain two different *β-globin* alleles, both inactive or poorly transcribed during the erythroid differentiation of these cells (Fordis, Anagnou, Dean, Nienhuis, & Schechter, 1984).

Studies of the epigenetic configuration of the *β-globin* cluster suggest that DNA methylation and histones acetylation are important for the regulation of developmental stage-specific expression of the *β-like globin* genes. The augmenting fetal hemoglobin levels in the K562 cell line can be associated with increased histone acetylation and decreased DNA methylation of the *γ-globin* genes, with opposite changes in the *β-globin* gene (Fathallah et al., 2008, 2007).

K562 cells have CpG sites at *β-globin* promoter hypermethylated. This effect can be changed after exposure to epigenetic compounds (X. F. Li et al., 2012).

The methylation level at the *γ-globin* promoters is very low in K562 cells (< 5%) and your expression has been demonstrated to be inversely correlated with the methylation level at the promoter (Y. Li et al., 2013). There existed several binding sites for transcription factors in the *γ-globin* promoter and these findings suggested that *γ-globin* promoter with hypomethylation might become accessible to these transcription factors (X. F. Li et al., 2012).

The levels of acetylation of histone H3 at the *β-globin* cluster correlate with the level of expression of the different genes of that cluster in the embryonic-fetal environment of K562 cells. In other words, the high level of histone H3 acetylation at the promoters of the embryonic *ε*- and fetal *γ-globin* genes is consistent with the high-level expression of these genes in K562 cells.

Similarly, the low level of acetylation at the promoter of the β-globin gene is consistent with its low but detectable level of mRNA expression (Fathallah et al., 2008, 2007).

In recent years, several studies have used the K562 cell line as an in vitro biological model to reproduce the characteristics of erythroid or megakaryocytic cells, helping to elucidate the mechanisms not yet known in these cell types. However, as a cancer line cannot 100% represent a normal erythroid population (Diepstraten & Hart, 2019).

K562 cells have a diameter of approximately 20 µm, have a basophilic cytoplasm without granules, and two or more prominent nucleoli, these cells are rounded, non-adherent and are able to grow in suspension (**Fig. 13**) (Koeffler & Golde, 1980).

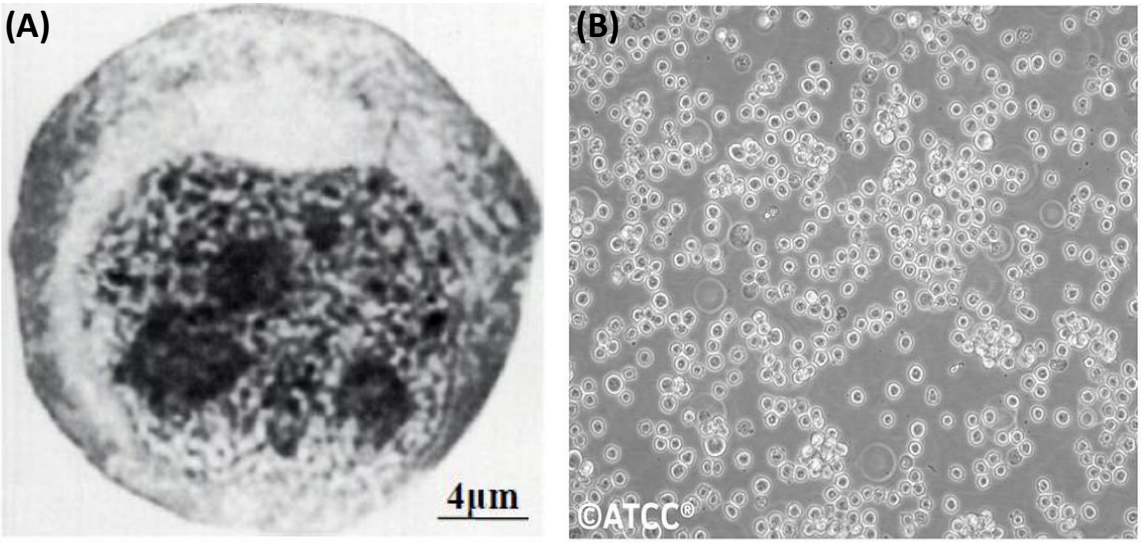


Fig. 13 General morphology of a K562 cell. (A) Where its prominent nucleoli and absence of granules can be observed, adapted from (Koeffler & Golde, 1980). (B) K562 cells in suspension, adapted from (ATCC, 2016).

Chapter 2: Objectives

2.1 Study potential, specific aims and expected outcomes

Research in the area of the hemoglobinopathies aims to develop curative therapies that correct the pathogenic mechanisms underlying the disease. One approach to achieve this goal is to look for new pharmacological agents that have HbF inducing properties (Mabaera et al., 2008).

To date, over fifty HbF inducing compounds have been described in various experimental systems, however, the exact mechanism of action of most of these remains elusive. Currently, only HU is in clinical use, not showing positive results in all patients and often not available in the most prevalent areas (Mabaera et al., 2008).

These considerations suggest that the most direct route for identifying new HbF inducing compounds is to understand/identify which specific molecular targets that we intend to study in relation to the mechanisms that act on cellular decisions that regulate globin exchange and prevent programmed suppression in the development of HbF expression (Thein, 2013).

A current related model of the developmental switch of fetal to adult hemoglobin suggests that *KLF1* regulates HbF production through positive regulation of *BCL11A*, identified as the dominant regulator of the *γ-globin* gene silencing. Therefore, the ideal target for the treatment of β-hemoglobin disorders would be one that mimics and boosts the effect of the genetic variants that regulate HbF levels at loci such as *BCL11A* and *KLF1* without affecting other biological pathways (Thein, 2013).

Other promising targets include epigenetic mechanisms that are also involved in the developmental regulation of human *β-globin* gene expression and hence HbF silencing (Ginder, 2015). The two main proposed epigenetic mechanisms of HbF regulation include methylation of DNA and histone deacetylation (Sankaran et al., 2010; Stamatoyannopoulos, 2005). The interplay between DNA methylation and histone modifications in regulating gene expression is well recognized (Ginder, 2015) and numerous studies have demonstrated that DNA hypomethylation and histone acetylation are effective in inducing *γ-globin* expression (Im et al., 2002). Additionally, histone modifications within *γ-globin* gene promoter regions, via activation of the MAPK signaling pathways were proven to be crucial for the induction of *γ-globin* gene expression (Qian et al., 2013). The ability of several drugs and compounds presently used for induction of *γ-globin* expression and consequently promotion of HbF levels have been associated with epigenetic mechanisms and related signaling pathways (DeSimone et al., 1982; Ley et al., 1983).

Presently it is acknowledged that epigenetic mechanisms can be influenced by diet, as nutrient-dependent epigenetic variations can significantly affect genome stability, mRNA and protein expression (Vahid, Zand, Nosrat-Mirshekarlou, Najafi, & Hekmatdoost, 2015).

Genistein (GN) a naturally occurring flavonoid found in soybean and soy derivates constitutes an important component in the majority of people's daily diet and is one of the most studied isoflavones associated to a variety of biological activities (Akiyama et al., 1987; Akiyama & Ogawara, 1991; Nagaraju et al., 2013). Another main component of the human daily diet is green tea (Camellia sinensis), epigallocatechin-3-gallate (EGCG) is the major polyphenol component of green tea with potent antioxidant and anti-inflammatory properties (Thephinlap et al., 2007; Thichanpiang & Wongprasert, 2015). EGCG was reported to effectively inhibit *in vitro* dehydration of sickle red blood cells (Ohnishi, Ohnishi, & Ogunmola, 2001) and decrease oxidative stress in iron-treated erythrocytes (Thephinlap et al., 2007).

Relevantly, GN and EGCG health benefits have been associated to its epigenetic effects as these compounds are able to target both histone acetyltransferases (HATs) and histone deacetylases (HDACs), regulate acetylation of histones and non-histone chromatin proteins (Vahid et al., 2015) and affect DNA methylation (Mirza et al., 2013; D. Wu et al., 2013).

The development of new compounds capable of HbF induction with high efficiency and lower toxicity than the currently available therapeutic agents are the focus of an intense investigation (Fard et al., 2013), particularly due to the high prevalence and incidence of these diseases worldwide. Furthermore, the inability of populations in the majority of highly endemic developing countries to access proper medication and sustain the high costs of clinical management leads to the need to develop new therapeutic approaches with exceedingly accessible compounds at very low costs. In this context, naturally occurring antioxidant substances, such as polyphenols and flavonoids, derived from herbs, fruits, vegetables, and medicinal plants, provide new insight into the prevention and therapy of numerous pathologies.

This project aims to evaluate the GN and EGCG *in vitro* potential, two naturally occurring compounds with high biological activity that triggers p38 Mitogen-activated protein kinase (p38 MAPK) signaling pathway (Adler, Pellizzer, Paparella, Hartung, & Bremer, 2006; Shu et al., 2018) as HU (Chou et al., 2015), in the induction of *γ-globin* gene reactivation and HbF expression. Hypothesizing, in a first phase, whether if these induce HbF expression by modulating of hemoglobin switching factors *BCL11A* and *KLF1* activity, with consequent influence on the *globin* genes, seeking to evaluate possible signaling pathways and epigenetic mechanisms associated.

Specific aims: Evaluate the potential of GN and EGCG in the induction of *γ-globin* mRNA and HbF expression in K562 cell line.

Expected Outcomes: This study will yield important endpoints, namely: discover the effect of distinct concentrations of GN and EGCG on the transcriptional response of *α-globin, β-*

globin, and γ-globin genes in the K562 cell line, and correlate the effects with the transcriptional levels of key regulators of HbF expression (*BCL11A* and *KLF1*).

The data obtained will allow evaluating the potential involvement of signaling pathways in reactivation *of γ-globin* and defining these two compounds as possible ways forward for a pharmacological reactivation of HbF.

This project will undoubtedly generate important results with a significant impact in various areas. It will allow doing to determine the potential of natural compounds that are highly accessible and with affordable prices in the reactivation of human HbF.

Chapter 3: Materials and Methods

3.1 Cell culture and reagents

K562 cell line (ECACC NO: 89121407) was purchased from the European Collection of Cell Cultures (ECACC, UK).

Under sterile conditions (**Fig.14-A**), according to the ECACC Laboratory Handbook 4th Edition protocol, cells were thawed. Thawing was performed quickly in order to minimize cellular damage caused by dimethyl sulfoxide (DMSO) cryoprotectant.

Cells were pipetted into a 15 ml Falcon™ centrifuge tube and added to a 10ml of RPMI 1640 (Sigma-Aldrich) media containing GlutaMAX-I, 25 mM HEPES (Invitrogen, USA) pre-warmed at 37 °C and supplemented with 10% (*v/v*) fetal bovine serum (FBS), 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (G6784, Sigma-Aldrich). A 5 minutes centrifugation at 1.500 rpm was performed in order to remove the cryoprotectant. Cell pellet was resuspended in 5 ml of fresh media and transferred to a 75 cm^2 culture flask (T-75) with 10 ml of fresh media (**Fig.14-C**). Cellular culture was maintained in suspension in a 5% (*v/v*) CO_2 humidified atmosphere at 37 °C for 3 days.

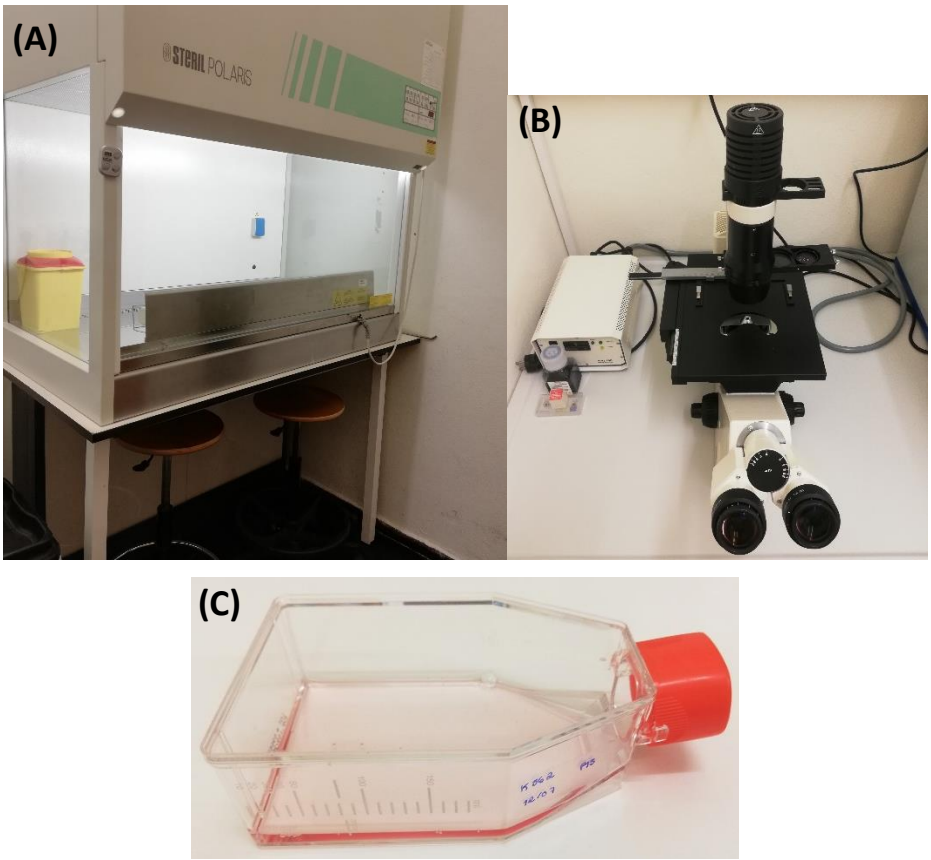


Fig. 14 Photos taken during the project: (A) Polaris vertical laminar airflow cabinet, class 100. (B) Inverted phase-contrast microscope. (C) Cell culture in T-75 flask.

Cells were stabilized between the density of 1×10^5 and 1×10^6 cells/ml, performing every three days subcultures and examining microscopically (phase contrast) (**Fig.14-B**) regarding their confluence, number, morphology and viability.

For subculture procedures, cells were washed by centrifugation with PBS (Sigma-Aldrich) and the pellet was resuspended in fresh media.

For cellular cryopreservation, 2×10^6 cells/ml were added to 1.5 ml EmbryoMax® 2X Cell Culture Freezing Medium (ES-002-D; Sigma-Aldrich) formulated with 20% DMSO + FBS and then frozen at - 80 °C.

For treatments and experiments, K562 cells were used between passages 1 and 14.

3.2 Drugs and treatments

Epigallocatechin-3-gallate (EGCG) (CAS number 989-51-5; Sigma-Aldrich) and Genistein (GN) (CAS No 446-72-0; Sigma-Aldrich) were purchased as a crystalline solid of 50 mg and 5 mg, respectively. Two 5 mg/ml stock solutions were prepared by dissolving the EGCG in ultra-pure water and GN was dissolved in DMSO because GN is soluble in organic solvents such as DMSO and is practically insoluble in water. A volume of 50 µl was used as a working solution and diluted in a 1:100 in RPMI 1640 media to yield the EGCG and GN final concentrations of 100 ηg/ml, 250 ηg/ml and 500 ηg/ml.

Human clinical pharmacokinetic studies show that the effective plasma level of total GN is in the micromolar range while the GN aglycone is in hundred nanomolar range *in vivo* (Yang et al., 2012). However after supplementation of purified isoflavones, blood concentration may increase (Raschke, Rowland, Magee, & Pool-Zobel, 2006) and steady-state plasma concentration would be more readily maintained by repeated ingestions throughout the day (Setchell et al., 2003). Previous human clinical trials demonstrated that 400 mg and 800 mg of EGCG intake, results in peak serum concentrations in the range of 100 ηg/ml to 400 ηg/ml (Chow et al., 2003) with no reported severe secondary effects. Therefore, the concentrations of the compounds in this study are considered safe and mimic the *in vivo* cellular exposure of GN and EGCG after supplementation.

For experiments, K562 cells at a density of 1×10^4 cells/ml were exposed to EGCG and GN at the three final concentrations of 100 ηg/ml, 250 ηg/ml and 500 ηg/ml and seeded in 6-well plates (3 ml/well) (**Fig.15-A**). For assessment of potential cytotoxic effects, K562 cells were exposed to EGCG and GN and seeded in 96-well plates (100 µl/well) (**Fig.15-B**).

In this study, six biological replicates for each compound and concentration were performed.

Furthermore, K562 cells were exposed to 25 µg/ml Hydroxyurea (HYDREA®, USP) a known HbF inductor compound was used as a positive control. A 500 mg HU capsule was diluted (in

biological safety cabinet) in 100 ml of ultra-pure water, obtaining a concentration of 5 mg/ml, which was distributed in aliquots and frozen at -20 ºC. One of the aliquots was used to make a 0.5 mg/ml stock solution by performing a 1:10 dilution in RPMI 1640 media.

As negative control cells were grown in the standard culture medium and in culture medium 500 ηg/ml DMSO which corresponds to the final concentration of the vehicle solution in GN exposed cells.

Exposed cells and controls were incubated at a humidified atmosphere of 5% CO_2, at 37°C for 72h and 96h, and harvested for post-treatment assays and analysis.

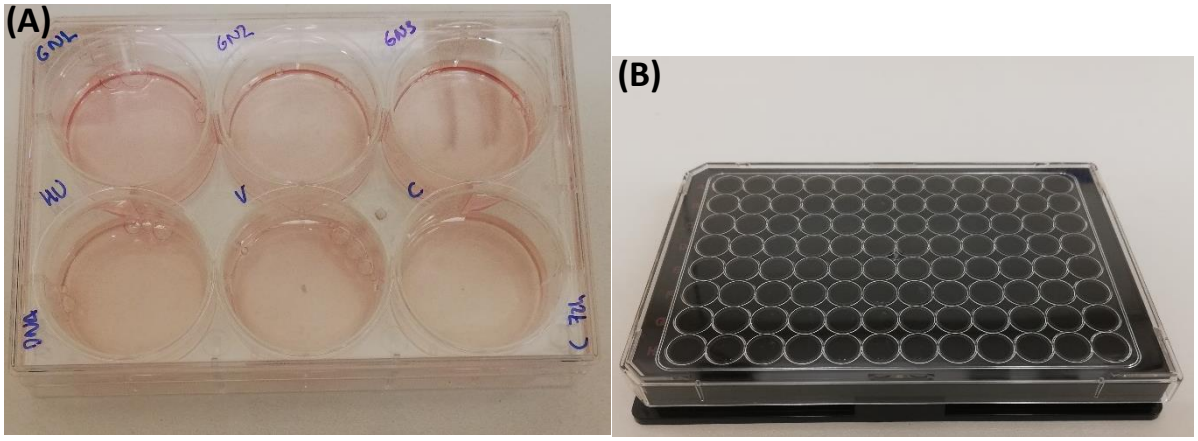


Fig. 15 Photos taken during the project: (A) Treatment in 6-well plates. (B) Treatment in 96-well plates.

3.3 Cell viability assays

Cellular proliferation was assessed trough trypan blue exclusion assay (Sigma, St. Louis, USA) after 72h and 96h of exposure.

Under sterile conditions, 100 µl K562 cell suspension was removed from each well an equal volume of trypan blue (1:1 dilution) added and incubated at room temperature for a few minutes.

One side of hemocytometer (**Fig.16**) was filled with cell diluted samples (approximately 5 - 10 µl) and counted under an inverted-phase contrast microscope using magnification x20.

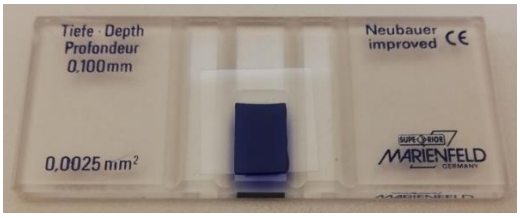


Fig. 16 Photos taken during the project: Trypan blue exclusion assay in a Neubauer improved hemocytometer.

The number of viable cells (seen as bright and colorless) and not viable (blue-stained) were counted. Concentration of viable and non-viable cells and the percentage of cell viability were calculated using the equations below:

$$\text{Viable Cell Count (live cells per millilitre)} = \frac{\text{Number Live Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) x 10,000}$$

$$\text{Non-viable Cell Count (dead cells per millilitre)} = \frac{\text{Number Dead Cells Counted}}{\text{Number of large corner Squares counted}} \times \text{Dilution factor (in this example 2) x 10,000}$$

$$\text{Percentage Viability} = \frac{\text{No of Viable Cells}}{\text{Total No. of Cells}} \times 100$$

Cellular viability was also evaluated through the CellTiter-Blue® Cell Viability Assay (Promega). This assay is a method used to assess cellular viability by testing whether metabolic processes remain active. Viable cells carry out metabolic reactions to generate the energy required to maintain homeostatic processes and nonviable cells lose membrane integrity and their ability to metabolic. This method provides a procedure for measuring the cell viability by fluorescence or absorbance quantitation. The assay is based on the ability of the living cells to convert the resazurin, a redox dye of blue color and no fluorescence, into resorufin of pink color and intensely fluorescent (CellTiter-Blue® Cell Viability Assay protocol) (**Fig. 17**).

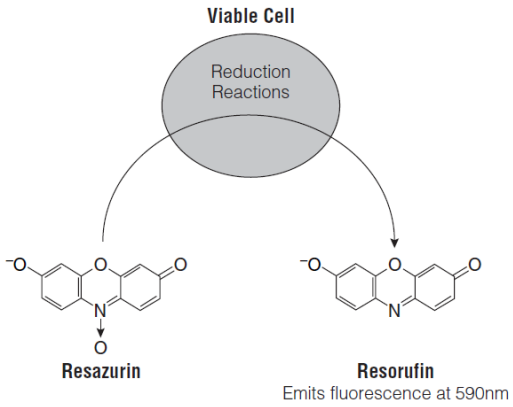


Fig. 17 Normal conversion of resazurin to resorufin by metabolically active cells, adapted from CellTiter-Blue® Cell Viability Assay protocol.

Nonviable cells do not reduce the dye for losing their metabolic capacity so do not generate a fluorescent signal and the solution stays blue.

According to the manufacturer's instructions, CellTiter-Blue® Reagent (20 µl/well) was added directly to cells seeded in 96-well plates and incubated at 37 °C for 4h.

Evaluation of standard culture media and reagent was used as a "white's medium" to compare the results as controls.

Viability was determined by measuring absorbance using a microplate reader spectrophotometer (Optic Ivyman; System 2100C) with 580 ηm and 620 ηm filters. Absorbance was used to monitor results by comparing the values of treatment with the blank wells. The absorption is proportional to the number of viable cells.

Experiments were performed in the three replicates *per* treatment.

3.4 Total RNA extraction

Total RNA was extracted by centrifugation in a silica spin column using the SV Total RNA Isolation System (Promega), according to the manufacturer's instructions.

EGCG and GN treated cells, as well as the positive and negative controls, were collected for 15 ml Falcon™ tubes and centrifuged at 1.500 rpm for 5 minutes. The cell pellets were washed by centrifugation twice with 10 ml of PBS, the supernatants were discarded and 175 µl of RNA Lysis Buffer (RLA) was adding.

RLA contains 4M of guanidine thiocyanate (GTC), 0.01M of Tris buffer (pH 7.5) and 0.97% of β-mercaptoethanol (BME). The GTC and BME have disruptive and protective properties that inactivate the ribonucleases present in cell extracts. The GTC in association with sodium dodecyl sulfate solution (SDS) acts disrupted the nucleoproteins complexes allowing the RNA to be released into solution and isolated free of proteins. Dilution of cell extracts in the presence of high concentrations of GTC causes selective precipitation of cellular proteins to occur, while the RNA remains in solution.

Cellular lysates were transferred into 1.5 ml tubes and 350 µl of RNA Dilution Buffer was added. The tubes were incubated in the heating block at 70 °C for 3 minutes and centrifuged at 11.000 rpm for 10 minutes. Lysates were pipetted into new microcentrifuge tubes and 200 µl of 95% ethanol was added. Each blend was transferred to Spin Column Assembly and centrifuged for one minute.

Ethanol clears the lysate of proteins and cellular debris, precipitating the RNA into the silica surface in Spin Basket due to the disruption of water molecules by the chaotropic salts favoring adsorption of nucleic acids to the silica.

Furthermore, 600µl of Wash Solution RNA was added and centrifuged at 11.000 rpm for 1 minute**.**

DNase incubation mix solution was prepared, *per* each sample, by combining 40 µl Yellow Core Buffer, 5 µl 0.09M $MnCl_2$ and 5 µl of DNase I enzyme. 50 µl of this preparation was added directly to the membrane inside of Spin Basket and incubated for 15 minutes at room temperature. RNase-Free DNase I solution digests the contaminating genomic DNA.

200 µl of DNase Stop Solution was added to the Spin Basket and centrifuged, then, 600 µl RNA Wash Solution was added and centrifuged for one more minute.

The Collection Tube was emptied, and a new wash was performed and centrifuged for two minutes.

Washing steps purified the RNA from contaminating salts, proteins and cellular impurities still present.

The Spin Basket was transferred from the Collection Tube to the Elution Tube and 100 µl Nuclease-Free Water was added to the membrane and centrifuged.

This procedure yielded a substantially pure fraction of total RNA after only one round of purification without organic extractions or precipitations.

3.5 RNA quantification

The concentrations of all RNA samples were determined by a fluorescence-based assay, the Qubit™ RNA HS Assay Kit in Qubit™ 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

This assay is a highly selective method for low-abundance RNA samples and will not quantitate DNA, proteins, free nucleotides and the common contaminants that are well tolerated.

A Qubit™ working solution was prepared by a 1:200 dilution of Qubit™ RNA HS Reagent in Qubit™ RNA HS Buffer. 190 µL working solution was added to two Qubit™ assay tubes used as standards and 195 µL to individual Qubit™ assay tubes. 10 µL of the two Qubit™ standard and 5 µL of each sample were added to the appropriate tube, mixed by vortexing 2 to 3 seconds and incubated at room temperature for 2 minutes.

The first standard was read and then the second, they are high-quality RNA calibration standards that run a calibration curve used to generate the quantification result. Each sample was read, and the output sample concentration was expressed in ηg/ml **(Appendix 1)**.

3.6 cDNA synthesis and real-time quantitative PCR (qRT-PCR)

After verifying the concentration, 2.0 µg of total RNA from each sample were reverse transcribed into cDNA using the Applied Biosystems™ TaqMan™ Reverse Transcription

Reagents (Invitrogen™, Thermo Fisher Scientific Inc.), with random hexamers as primers, in a scaled-down (20 µL) RT reaction, according to the directions provided by the manufacturer.

Each reaction was comprised of 2.0 µL 10X Reverse-Transcription Buffer, 1.4 µL of $MgCl_2$ Solution (1.75 mM), 4.0 µL of dNTP Mixture (2.5 mM each dNTP), 1.0 µL of DTT (5.0 mM), 1.0 µL of RNase Inhibitor (1.0 U/µL), 1.0 µL of MultiScribe™ Reverse Transcriptase (2.5 U/µL) and 1.0 µL of Random hexamers (2.5 µM). These components were prepared as a master mix and then dispensed into 0.5 PCR reaction tubes. Total RNA was added in the amount needed to make a final volume of 20 µl in each tube.

The Bio-Rad iCycler® Thermal Cycler was used to carry out the RT reactions using the following conditions: 25°C for 10 min, 37°C for 30 min, 95°C for 5 min, hold at 4°C.

cDNA was used for the hemoglobin quantification using primers for *α-globin*, *β-globin* and *γ-globin* mRNAs, as well as for the HbF regulators genes *KLF1* and *BCL11A*.

Transcriptional analysis was performed by quantitative real-time PCR (qRT-PCR) on CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad), with the specific primers listed in **Table 1**. The housekeeping gene, human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), was used for qRT-PCR normalization.

iTaq™ Universal SYBR® Green Supermix is a mix optimized for dye-based quantitative PCR (qPCR). It contains iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR Green I dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein).

SYBR Green I is a fluorophore that binds to double-stranded DNA as they are synthesized. The SYBR Green I fluorescence is measured at the end of each elongation cycle and is an indicator of the amount of DNA synthesized during that cycle.

Reactions were performed in a 20 µL volume containing: 10 µl of iTaq™ Universal SYBR® Green supermix (2x); 0.6 µl of forward and reverse primer (300 nM each); 1 µl cDNA template and nuclease-free water added until obtaining the final volume.

The reaction parameters used were: 95˚C for 2 minutes; 95˚C for 30 seconds, 55˚C for 30 seconds, 72˚C for 1 minute, this was repeated for 45 cycles (**Table 2**).

The cycles were followed by the acquisition of a melting curve, in order to check for primer-dimer formation and contaminations.

qRT-PCR analysis of the results was obtained as threshold cycle (C_t) values. The C_t value is the number of cycles required for the fluorescent signal to cross the threshold. The threshold is defined as the noise, or background, fluorescence level in the absence of any amplicons.

Table 1: Primer sequences and sizes of the real-time qRT-PCR

Primer		Accession Number*		Sequence (5'→3')	Tm (ºC)	Size (bp)
Housekeeping	***GAPDH***	NM_002046	Forward	GAGTCAACGGATTTGGTCGTA	56,2	245
			Reverse	GCAGAGATGATGACCCTTTTG	55,1	
Globins	***α-globin***	NM_000558.5	Forward	TCCCCACCACCAAGACCTAC	64	63
			Reverse	CCTTAACCTGGGCAGAGCC	62	
	β-globin	NM_000518.5	Forward	GCACGTGGATCCTGAGAACT	62	117
			Reverse	GCCACCACTTTCTGATAGGC	62	
	γ- globin	NM_000559.3	Forward	TGGATGATCTCAAGGGCAC	58	258
			Reverse	TTGCAGAATAAAGCCTATCCTTGA	47,2	
Signaling	***KLF1***	NM_006563.5	Forward	GGTGTGATAGCCGAGAC	52,22	164
			Reverse	GCGTATGGCTTCTCCC	52,83	
	BCL11A	NM_022893.4	Forward	ATTCGGCGTAGTACCC	50,96	191
			Reverse	CAACGGCTTCTTGGAG	50,36	

*GenBank accession numbers (National Center for Biotechnology)

Table 2: qRT-PCR amplification conditions

Step (nº of cycles)		Amplification Conditions
Initial Denaturation (1x)		95˚C, 2 minutes
Amplification (45x)	Denaturation	95˚C, 30 seconds
	Annealing	55˚C, 30 seconds
	Elongation	72˚C, 1 minute
Final elongation (1x)		72˚C, 15 minutes
Hold (∞)		until analysis

The data obtained (C_t values) in the qRT-PCR was analyzed by relative quantification. The relative quantification method shows the difference in the target gene expression relative to some reference group. This reference group includes endogenous housekeeping genes such as *GAPDH* that is expressed at a constant level (Sinhadri, 2009).

In this study, levels of mRNA were expressed as the relative fold/percentage using the $2^{-\Delta\Delta Ct}$ method to calculate the relative gene expression change. This method is summarized below:

$$\Delta C_{t\ (sample)} = C_{t\ (target\ gene)} - C_{t\ (GAPDH)}$$

$$\Delta\Delta C_t = \Delta C_{t\ (sample)} - C_{t\ (reference)}$$

$$\text{Relative quantification} = 2^{-\Delta\Delta C_t}$$

The $C_{t\ (reference)}$ is the C_t value of the calibrator, which is also normalized to the *GAPDH*. This normalization is done by subtracting the C_t value of the housekeeping gene from the C_t value of the calibrator.

In the treatment with GN, we used the vehicle solution (DMSO) as a calibrator, and for the remaining treatments, the calibrator was the negative control (untreated cells).

Each reaction was performed in triplicate and a control PCRs were also performed for all primer combinations without a template. Obtained values allowed to calculate the mean ± standard deviation for the three independent experiments, and the control samples always had a value of 0.

3.7 Statistical analysis

Statistical analysis was performed using IBM® SPSS® Statistics 25 software (Armonk, NY, USA).

The significant differences between the two treatments, cells grown in standard medium (to the EGCG) and the cells exposed to vehicle DMSO (to the GN) were assessed using Student's t-test followed by one-way analysis of variance (ANOVA). The results are presented as means ± standard deviation. P-Values < 0.05 were considered significant.

All graphs were created and evaluated with Microsoft Excel 2016.

Chapter 4: Results

With this study, we hypothesized that genistein and epigallocatechin-3-gallate may be capable of modulating HbF expression *in vitro*, through modulation of *BCL11A* and *KLF1* genes, with consequent effects on *globin* genes expression, as possible strategies for future treatment of SCD and β-thalassemia patients.

To test these hypotheses the GN and EGCG effects were investigated in human erythroleukemia cells (K562).

Prior to this dissertation, no studies have examined the molecular mechanism and *in vitro* activity of GN and EGCG in HbF induction.

4.1 Cell proliferation and viability

Human K562 cell line accumulates HbF if appropriately stimulated, and it is this innate ability to produce hemoglobin, their immortality and accessibility that renders K562 cells one of the most used models as a first-line screen of potential agents (Theodorou et al., 2016). In this study, K562 cell line was used to study the time-dependent and dose-dependent effects of GN and EGCG on HbF induction.

To assess the effect of GN and EGCG on the proliferation of K562 cells, 1×10^4 K562 cells/ml were cultured in the absence or presence of GN and EGCG at concentrations of 100 ηg/ml, 250 ηg/ml and 500 ηg/ml, or in the presence of 25 μg/ml HU as positive control and 500 ηg/ml DMSO as vehicle solution (GN dilution agent), for a period of up to 4 days. At 72h and 96h, 100 μl K562 cell suspensions were removed and potential cytotoxic and genotoxic effects of exposure were evaluated by analyzing cell viability and proliferation using the Trypan-Blue exclusion assay, that exploits the loss of membrane integrity, and CellTiter-Blue assay, that assesses metabolic activity.

4.1.1 GN affects proliferation and cellular metabolism with no effects on K562 cell viability

Viable cell counting was studied by the trypan-blue method. From the result of the assay, a dose and time-dependent bar chart was drafted (**Fig. 18**).

Results show no significant differences in dose-dependent proliferation (with increasing concentrations) to GN exposure ($p > 0.05$). However, significant results ($p = 0.002$) regarding time-dependent exposure were observed, for each of the three concentrations studied, cell proliferation increased from 72h to 96h: after 96h of culture, the proliferation rates for 100 ηg/ml, 250 ηg/ml and 500 ηg/ml GN were 8.64×10^5cells/ml ± 11.96%, 7.75×10^5cells/ml ± 9.4%,

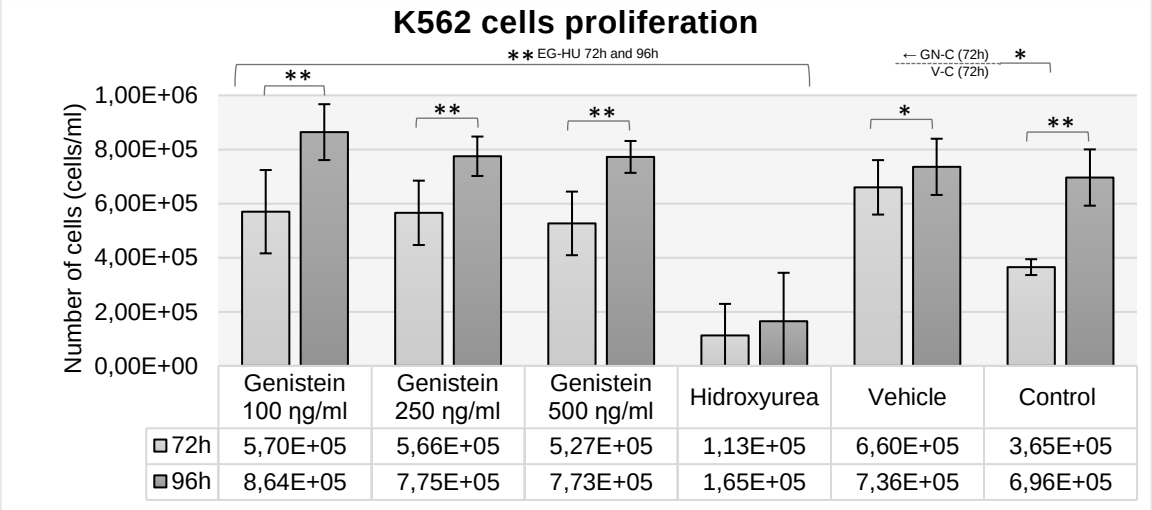
and 7.73×10^5cells/ml ± 7.63%, respectively. Maximal stimulation of K562 was observed at a GN concentration of 100 ηg/ml after 96h of exposure: 8.64×10^5cells/ml ± 11.96%.

High cell proliferation and a time-dependent response were also observed in the untreated control and vehicle solution.

GN was shown to have the same pattern of cell growth as the negative control, however, showing a significantly higher proliferation rate at 72h (p = 0.020).

Proliferation of vehicle solution showed no significant differences in relation to GN (72h [p = 0.610] and 96h [p = 0.371]).

HU inhibited almost 50% of cell proliferation (both times) compared to the other compounds studied, presenting a very significant difference in relation to GN (72h [p = 0.004] and 96h [p = 0.009]). This was the only compound with possible observable cytostatic effects at the concentration studied.



	Genistein 100 ηg/ml	Genistein 250 ηg/ml	Genistein 500 ηg/ml	Hidroxyurea	Vehicle	Control
72h	5,70E+05	5,66E+05	5,27E+05	1,13E+05	6,60E+05	3,65E+05
96h	8,64E+05	7,75E+05	7,73E+05	1,65E+05	7,36E+05	6,96E+05

Fig. 18 Effect of GN on the proliferation of K562 cells. Proliferation of K562 cells after 72h and 96h culture in control (control medium), vehicle (control media supplemented with 500 ηg/ml DMSO), 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of GN. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as the number of viable cells ± standard deviation of three independent experiments. Student's t-test significant differences in mean are shown as **p < 0.01 and *p < 0.05.

Monitoring the viability of cells (cytotoxicity of test compounds) is one of the most important steps when conducting drug trials. All the effectiveness of drugs is monitored by survival outcomes. This emphasizes the crucial importance of having a viability technique that can be as accurate and precise as possible.

Potential cytotoxic and genotoxic effects on K562 cells using Trypan-Blue exclusion assay (**Fig.19**) show no significant differences (p > 0.05) in cellular viability associated with dose and

time exposure (98% of viability for 100 ηg/ml, 250 ηg/ml and 500 ηg/ml at 72h and 96h). There are also no significant differences between GN and negative control. The negative control at 96h presented the highest cell viability rate (99% ± 1,5%).

Vehicle solution also demonstrates not to affect cellular viability for the period of study. HU concentration optimization results showed that 25 µg/ml HU had worse results than GN, toxic effects were minimal and not significant, but more cells exposed to HU died, mainly at 72h (96% ± 2.6%).

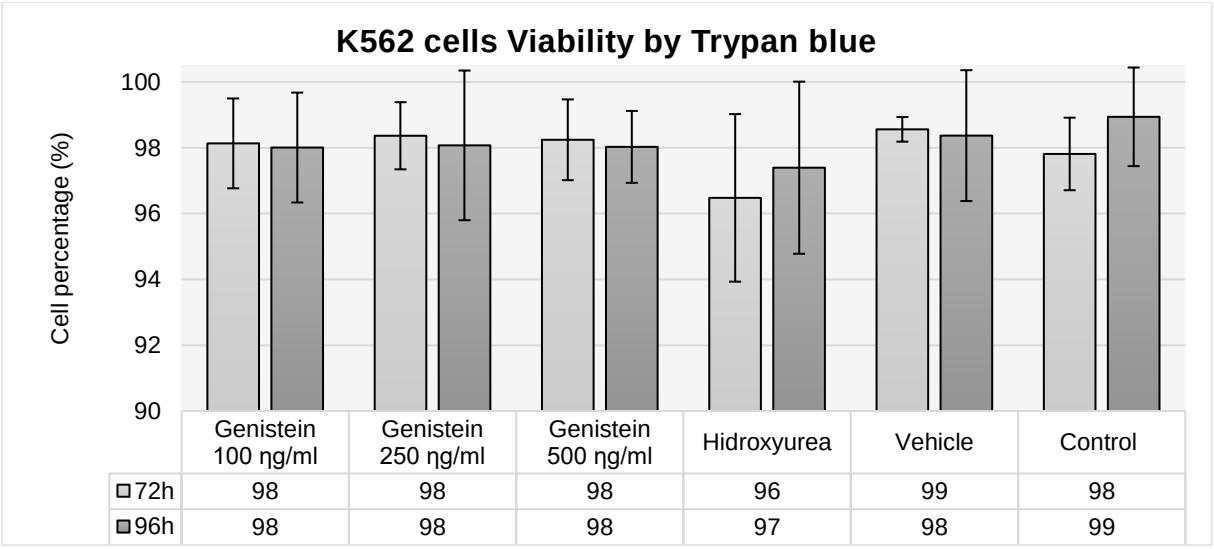


Fig. 19 Effect of GN on K562 cells viability by Trypan-Blue exclusion assay. Viability assay of K562 cells after 72h and 96h culture in control (control medium), vehicle (control media supplemented with 500 ηg/ml DMSO), 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of GN. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as the percentage of viable cells ± standard deviation of three independent experiments, calculated by dividing the number of viable cells by the number of total cells and multiplying by 100.

Cellular viability measurements based on cellular metabolic capacity through the Cell-Titer Blue assay method demonstrated that GN had no effects after 72h of exposure (**Fig. 20**). Interestingly at 96h of 100 ηg/ml GN exposure resulted in a decrease in the parameter in relation to control. Vehicle solution did not affect cellular metabolic activity at any time point whereas HU effects exposure resulted in altered cellular metabolism with time divergent effects (72h [54,8% ± 16%] and 96h [-22,7% ± 11,5%]).

The experiment was performed simultaneously for all growing conditions using three replicates for each condition tested and was repeated at least twice. The absorbance value for each well was discounted from the result obtained in "white's medium", and the results are presented as percentage of variation over control, for an absorbance intensity of 590 ηm (using 560 ηm for excitation).

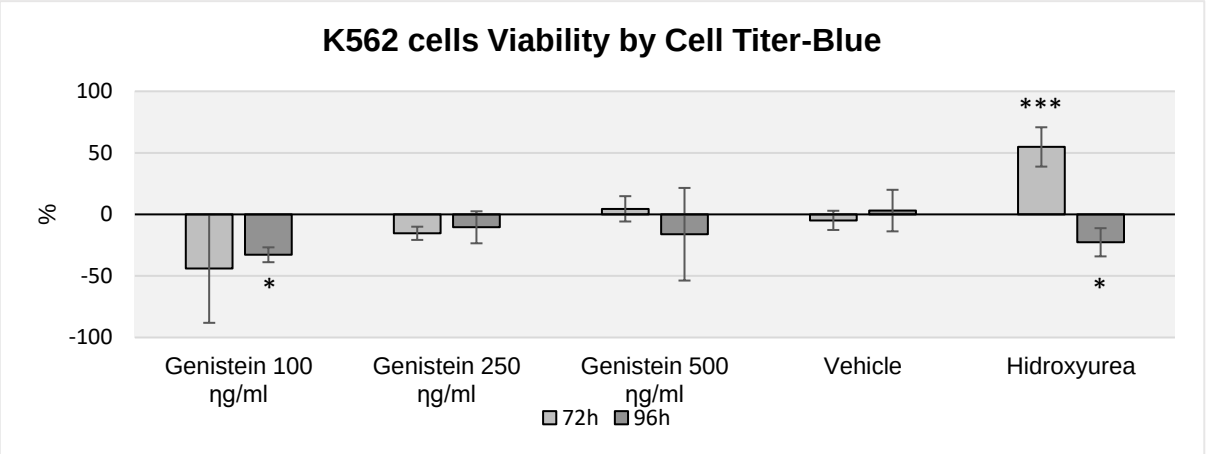


Fig. 20 Effect of GN on K562 cells viability by CellTiter-Blue assay. Viability assay of K562 cells after 72h and 96h culture in the vehicle (control media supplemented with 500 ηg/ml DMSO), 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of GN. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as percentage of variation over control of three independent experiments. ***$p < 0.001$ and *$p < 0.05$ for Student's t-test in relation to control.

4.1.2 EGCG affects proliferation and cellular metabolism with no effects on K562 cell viability

EGCG potential impact on cell proliferation was also evaluated through trypan blue exclusion assay, after 72h and 96h for EGCG concentrations of 100 ηg/ml, 250 ηg/ml and 500 ηg/ml (**Fig. 21**).

Just like GN, EGCG has not demonstrated significant dose-dependent differences (for equal exposure times) between each treatment, however the time-dependent difference (for each dosage) was quite significant ($p \leq 0.000$), with an increased cell concentration at 96h compared to 72h: after 96h of culture, the proliferation rates for 100 ηg/ml, 250 ηg/ml and 500 ηg/ml EGCG were 5.18×10^5cells/ml ± 19.24%, 5.24×10^5cells/ml ± 2.38%, and 4.92×10^5cells/ml ± 5.58%, respectively. High cell proliferation and a time-dependent response were also observed in the negative control.

EGCG had a lower proliferation rate than the negative control (72h [$p = 0.002$] and 96h [$p = 0.011$]), however, cell proliferation obtained for EGCG was superior to that obtained for the positive control (HU) (72h [$p = 0.012$] and 96h [$p = 0.003$]), which seems to have a cytostatic effect.

Because EGCG follows the same growth pattern as the negative control and the proliferation values are higher than HU, we can consider the result obtained for EGCG as not negatively affecting cell proliferation.

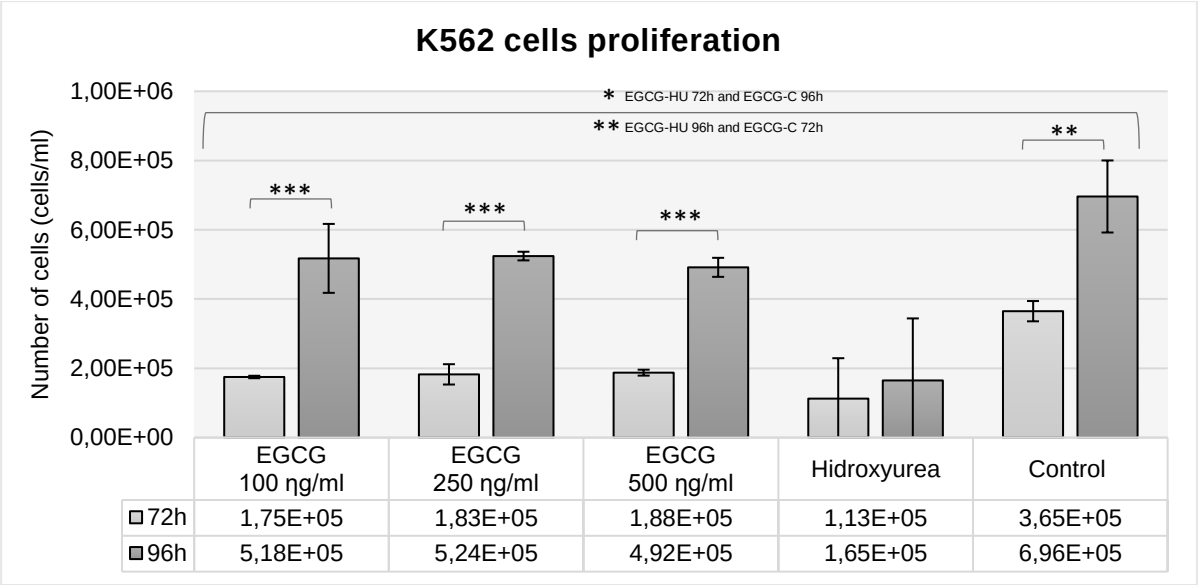


Fig. 21 Effect of EGCG on the proliferation of K562 cells. Proliferation of K562 cells after 72h and 96h culture in control (control medium), 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of EGCG. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as the number of viable cells ± standard deviation of three independent experiments. Student's t-test significant differences in mean are shown as ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

When comparing the results of cell proliferation of GN and EGCG, it appears that treatment with GN had a significantly higher value (72h [$p = 0.012$] and 96h [$p = 0.003$]) of cell number than treatment with EGCG. The EGCG proliferation values are closer to those found for negative control, than in relation to GN.

EGCG does not show significant differences between the different doses of study in the cell viability evaluated by Trypan blue assay (**Fig. 22**). Viability observed was greater at 96h (72h ≥ 97% and 96h 100%). Differences between EGCG, HU and negative control (dose and time-dependent) were not significant ($p > 0.05$).

However, the differences in EGCG treatment were significant ($p = 0.020$) when compared to the same study concentrations to both exposure times (time-dependent difference).

EGCG was also shown not to affect cell metabolism at the three concentrations studied with the CellTiter-Blue assay after 72h, whereas after 96h of 250 ηg/ml and 500 ηg/ml EGCG exposure increased cell metabolic activity was reported (**Fig. 23**).

The results obtained for EGCG at 72h present quite significant differences ($p \leq 0.000$) in relation to the HU (similar result to the obtained to the GN and HU at 72h).

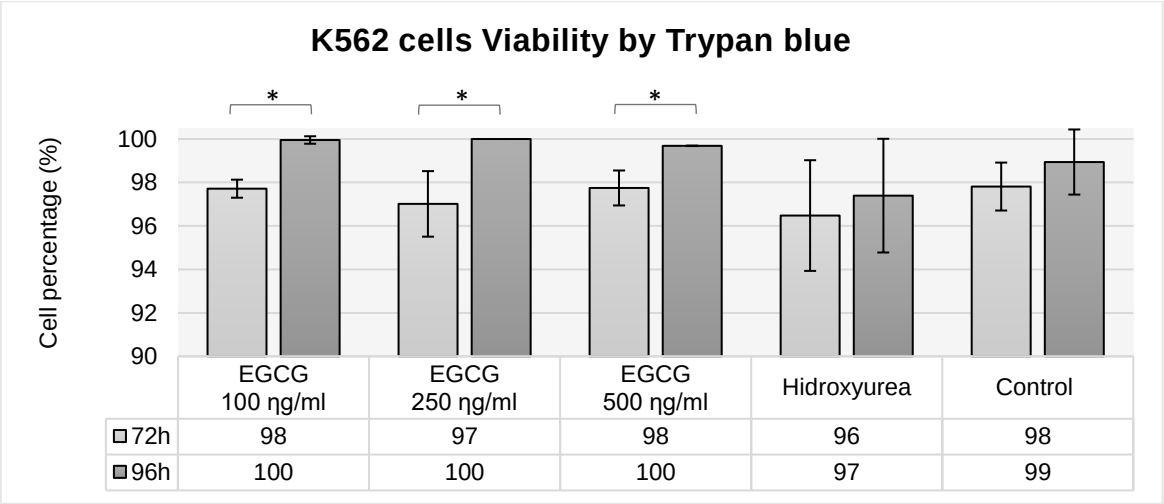


Fig. 22 Effect of EGCG on the viability of K562 cells by Trypan-Blue exclusion assay. Viability assay of K562 cells after 72h and 96h culture in control (control medium), 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of EGCG. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as the percentage of viable cells ± standard deviation of three independent experiments, calculated by dividing the number of viable cells by the number of total cells and multiplying by 100. Student's t-test significant differences in mean are shown as *p < 0.05.

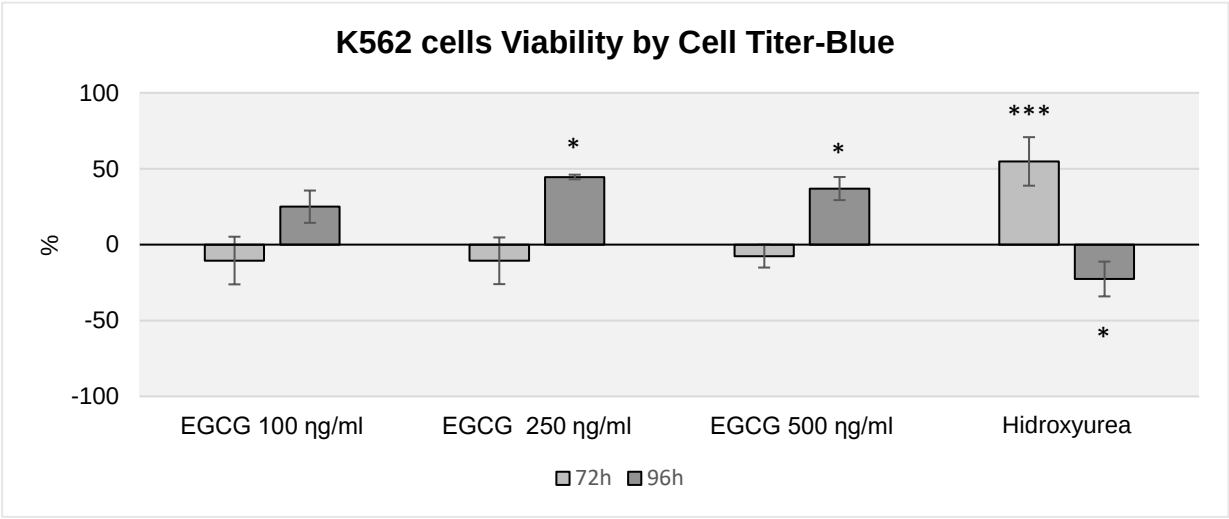


Fig. 23 Effect of EGCG on the viability of K562 cells by CellTiter-Blue assay. Viability assay of K562 cells after 72h and 96h culture in 25 µl/ml HU (positive control) and 100 ηg/ml, 250 ηg/ml and 500 ηg/ml of EGCG. Cultivation and viability assays were performed simultaneously for all growth conditions. Results are presented as percentage of variation over control of three independent experiments. ***p < 0.001 and *p < 0.05 for Student's t-test in relation to control.

4.2 Transcriptional effects on *BCL11A* and *KLF1* gene expression

The activity of *BCL11A* and *KLF1* are critical to the silencing of HbF during development (Ginder, 2015). To determine whether GN and EGCG modulate these two key factors involved in the repression of HbF, we assayed by qRT-PCR the inducing effects on *KLF1* and *BCL11A* mRNA expression, using the *GAPDH* mRNA as normalizing reference.

Results represent the mean of three independent experiments (n = 3), the group means of GN treated cells were compared to vehicle (DMSO) as calibrator, EGCG and HU treated cells were compared to the negative control (untreated cells), using the statistical tests previously described (Chapter 3) through the SPSS statistical software.

4.2.1 GN differentially affects mRNA of the genes related with silencing of HbF in K562 cells

The transcriptional effects on *BCL11A* and *KLF1* expression after GN administration at 72h and 96h, relative to the vehicle solution, were expressed as Log_2Fold Change (Log_2FC) ± standard deviation.

Relative expression of HbF main repressor (*BCL11A*) was analyzed in K562 cells, through qRT-PCR, after exposure to different and relevant GN concentrations (100 ηg/ml, 250 ηg/ml and 500 ηg/ml).

We observed that all GN concentrations evaluated at both exposure times induced the downregulation of *BCL11A* mRNA levels (**Fig. 24**). The downregulation was significant at 72h for the lowest (100 ηg/ml $p = 0.038$) and highest (500 ηg/ml $p = 0.003$) concentrations of study, this result was higher than that observed for the positive control which did not obtain a significant result at 72h.

In intermediate GN concentration (250 ηg/ml) with increasing exposure time (96h) a significant response ($p = 0.003$) was also obtained for the downregulation of *BCL11A* ($Log_2FC = -0,500 \pm 0,155$), non-observable at the concentrations of 100 ηg/ml and 500 ηg/ml.

The most significant result for repression of *BCL11A* was obtained by HU at 96h ($p \leq 0.000$).

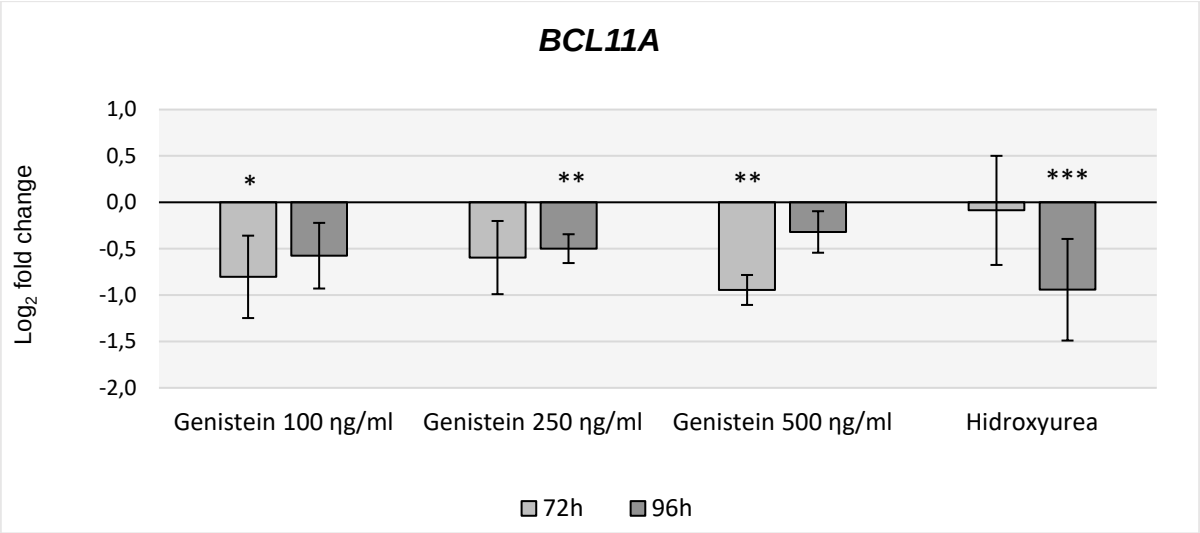


Fig. 24 GN effects on expression of *BCL11A* gene. Graphic representation showing quantitative real-time PCR analysis of *BCL11A* gene transcription after exposure to GN and HU. Log_2Fold changes [$Log2(2^{-\Delta\Delta Ct})$] ± standard deviation of three independent experiments, in gene expression after 72h and 96h of GN exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml. *GAPDH* was utilized as a reference gene and Student's t-test significant differences in average mean $Log2$fold changes are shown as ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

The relative expression of *KLF1*, described as a silencer of the *γ-globin* gene by stimulating *BCL11A* expression (Ginder, 2015), was analyzed using the same conditions as described for *BCL11A*.

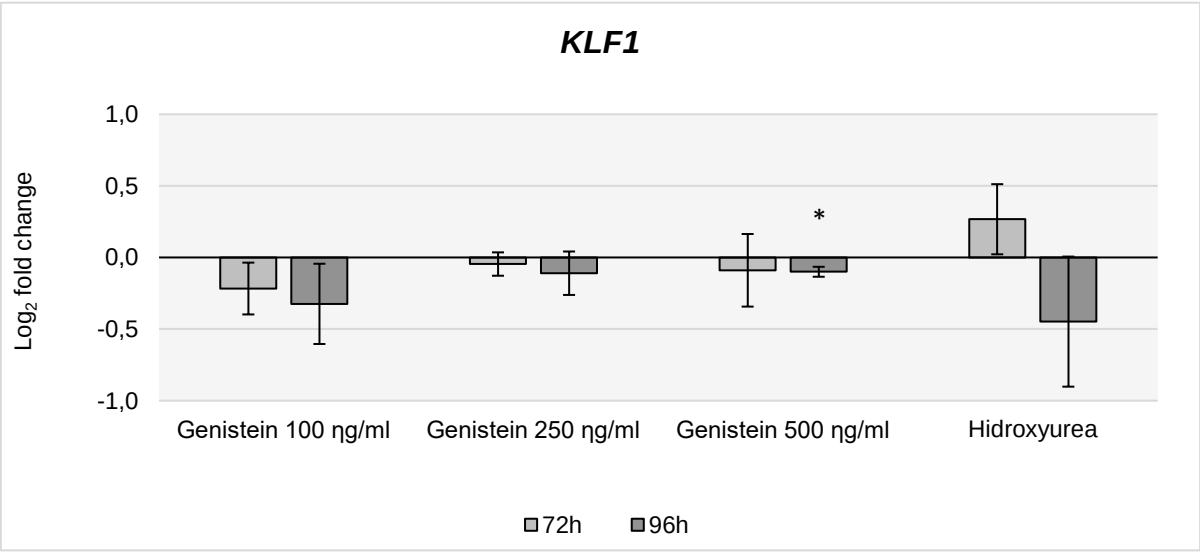


Fig. 25 GN effects on expression of *KLF1* gene. Graphic representation showing quantitative real-time PCR analysis of *KLF1* gene transcription after exposure to GN and HU. Log_2Fold changes [$Log2(2^{-\Delta\Delta Ct})$] ± standard deviation of for three independent experiments, in gene expression after 72h and 96h of GN exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml. *GAPDH* was utilized as a reference gene and Student's t-test significant differences in average mean Log_2fold changes are shown as *$p < 0.05$.

Although the three GN concentrations of the study showed a slight downregulation of *KLF1*, we only obtained a significant response (p = 0.028) of GN for the higher dose with increased exposure time (500 ηg/ml at 96h). No significant response of HU on *KLF1* mRNA was obtained in the exposure periods (**Fig. 25**).

4.2.2 EGCG induces downregulation in *BCL11A* but does not affect *KLF1*

Results of the EGCG proved to be promising in relation to the suppression of the *BCL11A* mRNA (**Fig. 26**).

For exposure to 100 ηg/ml EGCG at 72h a significant response (p ≤ 0.000) was obtained regarding to *BCL11A* downregulation, this result was identical to that obtained for the 96h positive control (HU) and appeared to have until a repressive effect greater than HU 96h of treatment (Log_2FC EGCG [100 ηg/ml at 72h] = -1.093 ± 0.053 and Log_2FC HU [25 µ/ml at 96h] = -0.943 ± 0.548). EGCG at 250 ηg/ml at 96h also shows a downregulation in *BCL11A* (p = 0.041) not observed at the lowest and highest concentration with increasing exposure time.

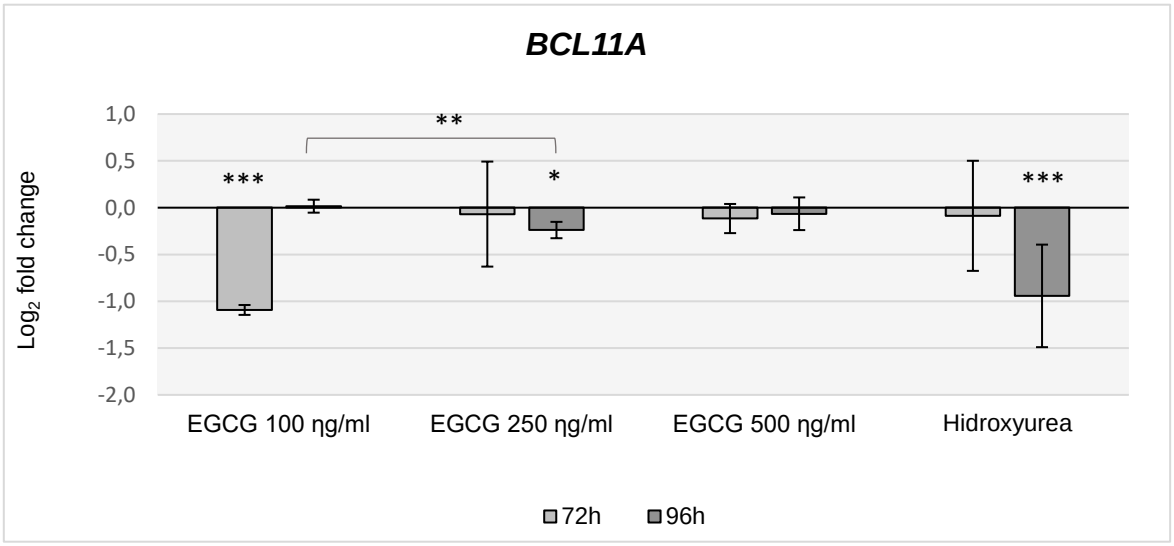


Fig. 26 EGCG effects on expression of *BCL11A* gene. Graphic representation showing quantitative real-time PCR analysis of *BCL11A* gene transcription after exposure to EGCG and HU. Log2Fold changes [$Log2(2^{-\Delta\Delta Ct})$] ± standard deviation of three independent experiments, in gene expression after 72h and 96h of EGCG exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml. *GAPDH* was utilized as a reference gene and Student's t-test significant differences in average mean Log2fold changes are shown as ***p < 0.001, **p < 0.01 and *p < 0.05.

Although the effects of EGCG at the two lowest concentrations tended to suppress *KLF1* mRNA, the results were not significant for this gene (**Fig. 27**).

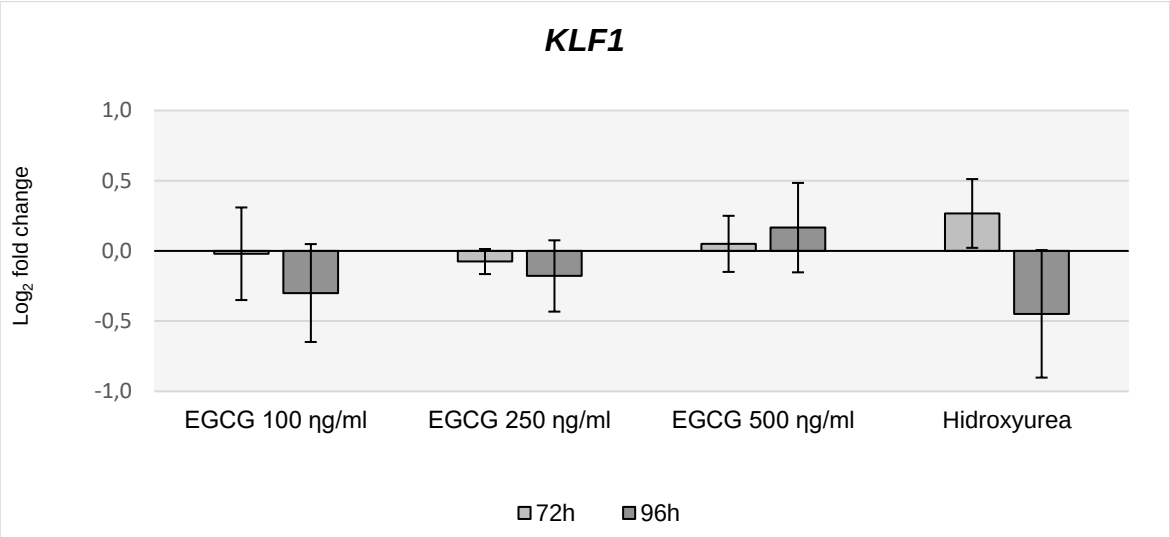


Fig. 27 EGCG effects on expression of *KLF1* gene. Graphic representation showing quantitative real-time PCR analysis of *KLf1* gene transcription after exposure to EGCG and HU. Log2Fold changes [Log2($2^{-\Delta\Delta Ct}$)] ± standard deviation of three independent experiments, in gene expression after 72h and 96h of EGCG exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml.

4.3 Transcriptional effects on *globins* gene expression

Possible HbF expression in K562 cells by GN and EGCG will be associated with the positive regulation of *γ-globin* gene expression (Chou et al., 2015). To verify this condition, the expression of *globin* genes (*α*, *β* and *γ-globin*) were analyzed by qRT-PCR using the expression of *GAPDH* mRNA as an internal control.

4.3.1 GN down-regulates *α-globin* mRNA

Representative results of *globins* genes analysis of GN treatment are shown in **Fig. 28**. GN exposure exclusively affected *α-globin* with downregulation of mRNA levels at 250 ηg/ml ($p = 0.006$) after 72h and 100 ηg/ml ($p \leq 0.000$) after 96h of exposure (**Fig. 28-A**).

For the second exposure time (96h), there was a significant dose-response ($p = 0.001$) between the concentrations of 100 ηg/ml and 250 ηg/ml, and between 100 ηg/ml and 500 ηg/ml for the *α-globin* gene.

Cells treated with HU did not show significant results for the expression of *α-globin*.

The expression of *β-globin* mRNA (**Fig. 28-B**) did not show significant results in relation to the repression of this gene by GN. However, at 72h exposure, a dose-dependent effect is observed between 100 ηg/ml and 250 ηg/ml concentrations ($p = 0.008$) and between 100 ηg/ml and 500 ηg/ml ($p = 0.030$), as 100 ηg/ml appears to have a repression effect while 250 ηg/ml

and 500 ηg/ml appears to have an inducing effect on the *β-globin* gene at the lowest exposure time. Positive control has not been shown to have significant results for the *β-globin* gene.

At study concentrations and exposure times, GN and HU have not yet been shown to have a significant effect on the expression of *γ-globin* mRNA (**Fig. 28-C**).

4.3.2 EGCG affects the expression of *β* and *γ-globin* genes.

Study of the EGCG effect on *globin* mRNA expression is shown in **Fig. 29**.

The three EGCG concentrations (100 ηg/ml, 250 ηg/ml and 500 ηg/ml) as well as the 25 μg/ml HU, at 72h and 96h, showed no significant effects on *α-globin* expression ($p > 0.05$) (**Fig. 29-A**).

EGCG has been shown to have a significant effect on *β-globin* repression (**Fig. 29-B**). A significant downregulation of *β-globin* levels was reported at 72h time point for lower tested concentrations, namely 100 ηg/ml ($p = 0.003$) and 250 ηg/ml ($p = 0.015$). The repression of *β-globin* at 72h for 500 ηg/ml apparently will also be significant, due to its pattern similar to the lowest concentrations, however, the high standard deviation may have influenced the statistical result.

On the other hand, in relation to *γ-globin* mRNA expression, a downregulation was observed for 100 ηg/ml exposure after 72h ($p = 0.027$) and 250 ηg/ml after 96h ($p = 0.036$) (**Fig. 29-C**)

Positive control had no significant effect on *γ-globin* expression ($p > 0.05$).

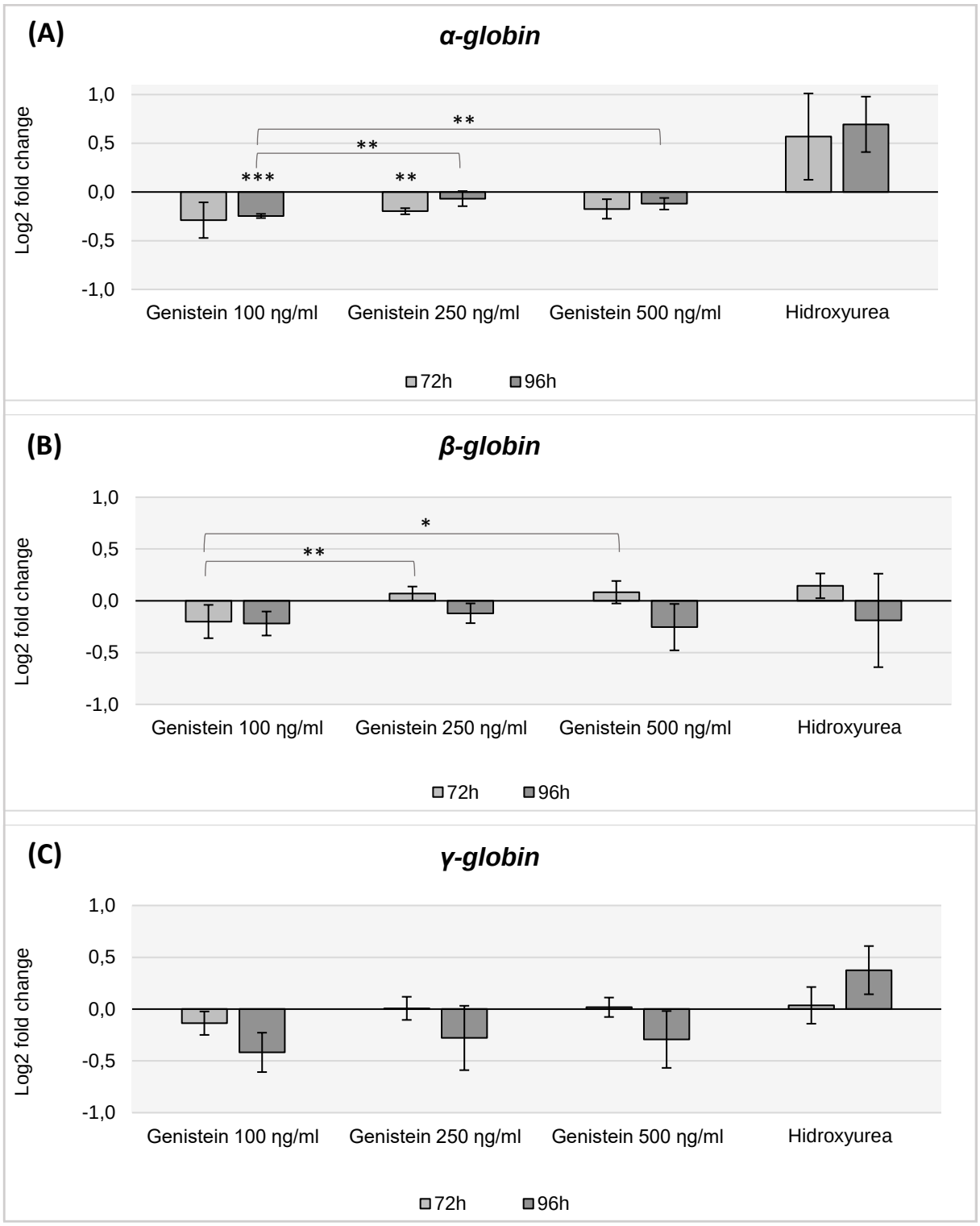


Fig. 28 GN effects on the expression of the *globin* genes. GN effects on the expression of the *globin* genes. Graphic representation showing quantitative real-time PCR analysis of *globins* genes transcription after exposure to GN and HU. Log2Fold changes [$Log2(2^{-\Delta\Delta Ct})$] ± standard deviation of for three independent experiments, in gene expression after 72h and 96h of GN exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml are shown for (A*) α-globin*, (B) *β-globin* and (C*) γ-globin*. *GAPDH* was utilized as a reference gene and Student's t-test significant differences in average mean Log2fold changes are shown as ***p < 0.001, **p < 0.01 and *p < 0.05.

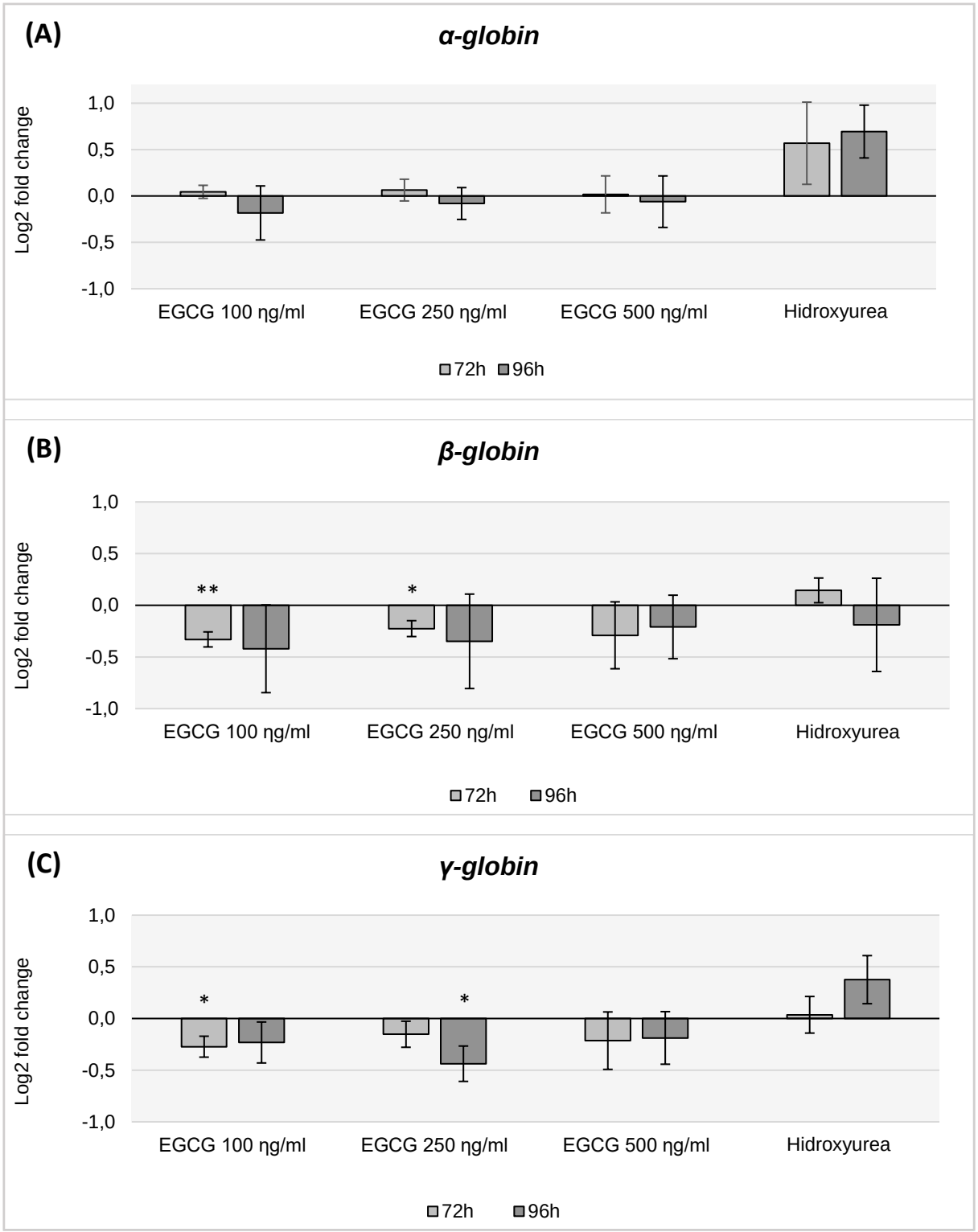


Fig. 29 EGCG effects on the expression of the *globin* genes. EGCG effects on the expression of the hemoglobin genes. Graphic representation showing quantitative real-time PCR analysis of *globins* genes transcription after exposure to EGCG and HU. Log2Fold changes [$Log2(2^{-\Delta\Delta Ct})$] ± standard deviation of three independent experiments, in gene expression after 72h and 96h of EGCG exposure at 100 ηg/ml, 250 ηg/ml and 500 ηg/ml are shown for (A) *α-globin*, (B) *β-globin* and (C) *γ-globin*. *GAPDH* was utilized as a reference gene and Student's t-test significant differences in average mean Log2fold changes are shown as **p < 0.01 and *p < 0.05.

Chapter 5: Discussion and Conclusions

5.1 Discussion

The benefits of increased HbF in individuals with β-hemoglobinopathies are well established and acknowledged by the scientific community. Currently, pharmacologic compounds with capacity for HbF induction both *in vitro* and *in vivo* are available, however, these therapies have major hazardous effects (there are concerns over their cytotoxicity and carcinogenic potential in humans) and mechanisms by which expression of HbF occurs in response to these agents are not established (Theodorou et al., 2016).

Cytostatic drugs, such as HU, induce HbF by "slowing" the process of erythroid differentiation and creating a pool of more immature erythroid progenitors, a milieu that favors HbF production (Lavelle et al., 2018). More recently researchers have begun to examine the effects of HbF inducing agents on specific signaling molecules and signal transduction pathways. Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP), nitric oxide (NO), p38 MAPK, reactive oxygen species (ROS) and cytokine signaling have all been implicated in drug induced HbF production. A comparison of the modes of action of known HbF inducers might provide clues for more effective therapy (Mabaera et al., 2008).

Several *in vivo* and *in vitro* studies have indicated that a variety of naturally occurring flavonoids have been found to have beneficial effects in health. These compounds have drawn attention because of their relative safe-ness in animals and humans (Panche, Diwan, & Chandra, 2016), and also activate p38 MAPK signaling pathway (Adler et al., 2006; Shu et al., 2018) as HU (Chou et al., 2015). Flavonoids are polyphenolic compounds that are present in fruits, vegetables, and beverages derived from plants (tea and wine), as well as many dietary supplements or herbal remedies, and are categorized, according to chemical structure, as flavanols, flavones, flavanones, isoflavones, anthocyanidins, and chalcones (Lv, Shao, Chen, Ho, & Sang, 2011).

The purpose of this dissertation was to analyze the potential of epigallocatechin 3-gallate a dietary flavonoid from green tea, and genistein (5,7,4'-trihydroxyisoflavone) one of the major dietary isoflavones present at a relatively high level in soybeans and plant-derived foods, in fetal hemoglobin reactivation and its possible therapeutic potential for β-hemoglobinopathies, in K562 leukemic cells line. It was hypothesized that GN and EGCG may endorse HbF expression mediated through suppression of *KLF1* and *BCL11A* genes, two signaling molecules involved in the silencing of the duplicated *γ-globin* genes (Sankaran, 2011).

In humans, plasma or serum levels of GN from soy food ingestion range from less than 1 μM to about 5 μM. Since much of the “free” GN in serum may become bound to serum proteins, the actual concentration of biologically active GN in serum is more likely to be in the nanomolar range. Plasma levels of GN have been reported at 2-5 ηg/ml (7-18 ηM) in individuals

consuming standard western diets, with measurably higher levels of GN in individuals consuming vegetarian diets (0.04-0.09 μM) or high-soy diets (0.27-1.2 μM) (Klein & King, 2007).

In a study where single oral doses of 30 mg, 60 mg, 150 mg and 300 mg of GN were administered to 40 healthy volunteers, were rapidly absorbed and the kinetic profiles revealed a one-peak plasma concentration-time course. The mean maximum concentration values were 241 ηg/mL, 550 ηg/mL,1484 ηg/mL and 1773 ηg/mL, respectively in the 30 mg, 60 mg, 150 mg and 300 mg dose groups, these values were reached after 4h to 6h, and after 48h GN was nearly completely eliminated from the blood (Ullmann, Metzner, Frank, Cohn, & Riegger, 2005).

In consideration of human dietary intake of soy foods and resulting serum/plasma levels of GN, we defined for the study three “low” concentrations of GN (100 ηg/ml [~0.37 μM], 250 ηg/ml [~0.93 μM] and 500 ηg/ml [~1.85 μM]), since it is reasonably certain that the physiological serum levels of GN in humans are unlikely to be achieved by diet or supplementation in concentrations above these.

A study of Chow *et al*. (2003) was observed that EGCG intake at the doses of 400 mg and 800 mg, established peak serum concentrations of both free and total EGCG at the nanomolar range (lower than 1 μM) (Chow et al., 2003). Maximum plasma concentrations of EGCG are reached in 1-2 hours in healthy individuals and these levels gradually decrease to undetectable levels at 24 h. The EGCG elimination half-life occurs at 3.4 ± 0.3h. Most of the EGCG ingested apparently does not enter the blood, since absorption occurs in the small intestine and substantial amounts pass into the large intestine, where it undergoes further degradation by the action of the local microbiota. Therefore, the bioavailability of EGCG needs to be considered when extrapolating the results obtained *in vivo* to *in vitro* situations (Mereles & Hunstein, 2011). The consumption of 400 mL green tea per day equals approximately to ~247 mg of EGCG and in ~692 mg of total catechins (Wolfram, 2007). Relevant plasma concentrations of EGCG must be in the high nanomolar range. Therefore, high micromolar concentrations are unlikely to be established in the bloodstream of individuals that simply drink green tea or ingest only two to three capsules of green tea extract each day (Davis, Singh, Bhuiyan, & Sarkar, 1998). The most *in vitro* studies use EGCG concentrations in the range of 5–100 μM. Therefore, studies are needed using lower *in vitro* doses, which can still be effective to exert biological activities and which could be more readily achieved *in vivo* in humans (F. Wu et al., 2012).

Thus, based on bibliographic data that suggest serum levels after drinking green tea in the nanomolar order, we selected the concentrations of (100 ηg/ml [~0.22 μM], 250 ηg/ml [~0.55 μM] and 500 ηg/ml [~1.09 μM]) of EGCG for our study.

This project was part of a pilot study, who's observed effects, if promising, will allow the continuation of the investigation, in relation to specific mechanisms (epigenetic) and signaling pathways that regulate the fetal-to-adult hemoglobin switch.

To better understand the effectiveness of any compound that is a candidate for a type of treatment, it is essential to determine the effectiveness of that compound *in vitro* as accurately as possible. A variety of indicators are generally assessed as a starting point for assessing the effectiveness of the compound, including the impact on cell viability, cytotoxicity, and cell proliferation. Generally, when cells are exposed to compounds with different concentrations, they can react through many different pathways and at different rates that depend on exposure dosage (Riss & Moravec, 2004). Therefore, the evaluation of cell proliferation and viability constitutes the first significant step in the toxicological studies necessary to determine the activity of the compound and the pharmacological safety at the cellular and even molecular level, of any candidate to a therapeutic drug (Horváth, 1980). Cell viability represents the number of healthy cells present in the culture and cell proliferation represents the ability of healthy cells to divide and create "daughter cells" (Gordon, Brown, & Reynolds, 2018).

GN and EGCG impact on cell proliferation and viability were measured trough two methods (Trypan blue assay and CellTiter-Blue assay) to quantify the number of healthy K562 cells and their growth rate after exposure to the two compounds.

Our data demonstrated that after 72h of exposure GN and EGCG do not affect cellular metabolic capacity, however, after 96h of exposure we reported divergent effects, whereas EGCG exposure resulted in increased metabolic capacity in a dose-dependent manner, and GN at the lowest concentration tested results were similar to HU. Nevertheless, no effect on cellular proliferation or viability was reported.

Previous studies have demonstrated that high GN and EGCG concentrations (greater than 5 μM) are genotoxic, and resulted in decreased cellular proliferation as well as viability (associated with its anti-tumor properties) (Klein & King, 2007; F. Wu et al., 2012). On the other hand, GN biological effects diverge in relation to doses applied (Russo et al., 2016) including metabolic alterations and decreased cellular proliferation (Ganai & Farooqi, 2015).

Among the many properties of GN and EGCG described, chemopreventive and chemoprotective activities are the most reported, possessing the ability to inhibit the proliferation of several cell lines, however only in high concentrations (Ingram, Sanders, Kolybaba, & Lopez, 1997; Kurahashi et al., 2007; Lee et al., 2003).

GN has been investigated extensively for its chemopreventive potential, especially against tumors of breast and prostate origin (Park & Surh, 2004). *In vitro* studies often attribute to GN the inhibition of tyrosine kinase which inhibits cell growth (Klein & King, 2007). Davis *et al*. (1998) have shown that induction of apoptosis by GN is accompanied by G2/M arrest, up-

regulation of p21 and down-regulation of cyclin B in prostate carcinoma cells (Davis et al., 1998).

Hsu *et al*. (2001) demonstrate that EGCG affects a p57-mediated survival pathway in normal epithelial cells while inducing a proapoptotic pathway in oral carcinoma cells (Hsu et al., 2001). It has been suggested by Kong *et al*. (2000) that at low concentrations of EGCG, the expression of MAPKs (ERK2, JNK1, p38) and consequently survival genes can be activated, providing survival and protective mechanisms as a homeostatic response. At increased concentrations, both MAPK and caspase pathways are activated, leading to apoptosis (potential cytotoxicity), and EGCG at suprapharmacological doses predominantly causes cell death (Kong, Yu, Chen, Mandlekar, & Primiano, 2000). EGCG at concentrations between 100 and 250 μM strongly activated ERK, JNK, and p38, as well as induction of ARE-reporter gene activity, whereas potent cytotoxic effects were evident at 500 μM (Park & Surh, 2004). It is speculated that apoptotic cell death occurs with EGCG treatment through G0/G1 cell arrest (Ahmad, Adhami, Gupta, Cheng, & Mukhtar, 2002).

In this work, we selected concentrations of GN and EGCG considered safe (for cell survival) and that *in vitro* mimic the plasma concentrations obtained after ingestion of these two compounds (Klein & King, 2007; F. Wu et al., 2012), Thus we predicted that they would not affect cell viability, which was confirmed by the results obtained.

There were no significant differences in cell proliferation between the three studied concentrations of GN and EGCG under the study; however, it was possible to verify time-dependent differences with the increase in proliferation being significant at 96h compared to 72h. Doses between 100 ηg/ml and 500 ηg/ml of GN and EGCG, had no apparent cell growth negative inhibitory effect. Our data demonstrated that after 72h of exposure EGCG and GN do not affect cellular metabolic capacity, however, after 96h of exposure we reported divergent effects, whereas EGCG exposure resulted in increased metabolic capacity in a dose-dependent manner, and GN at the lowest concentration tested results in decreased cell metabolism. Interestingly the higher proliferation level of K562 was observed at a GN concentration of 100 ηg/ml after 96h of exposure inversely to cellular metabolic activity.

Our results are consistent with previously published studies that prove that at concentrations up to 5 μM these two naturally occurring flavonoids have a significant effect on cell proliferation and metabolism (Chung et al., 2003; Fu et al., 2010; Pan, Han, Zhong, & Geng, 2012).

It was demonstrated that GN induced the proliferation of INS1cells and human clonal β-cells after 24h of incubation, with 0.1 μM, 1 μM and 5 μM, being suggested that the GN stimulated β-cell proliferation is dependent of protein kinase A (PKA) and ERK1/2 (Fu et al., 2010). 20 ηM to 2000 ηM of GN significantly promoted H19-7/IGF-IR neural cell line viability

and proliferation, inducing an increase in the percentage of cells in the S phase. These effects might be mediated by the BDNF-Trk pathway (Pan et al., 2012).

Treatment of cultured keratinocytes with EGCG for 5 days significantly increased their proliferation in a dose-dependent manner up to 1.0 μM (0.01 μM, 0.1 μM, 0.5 μM, and 1.0 μM). However, concentrations of EGCG >100 μM significantly inhibited the proliferation of cultured keratinocytes (Chung et al., 2003).

In addition to studying the cell viability/proliferation of the two compounds in the study, it was also important to determine the level of proliferation and cytotoxicity of the positive control (HU), the negative control (untreated cells) and the vehicle solution (DMSO).

The negative control showed positive results regarding the proliferation and cytotoxicity of K562 cells coinciding with the cell counts of the cells in the maintenance RPMI 1640 media.

DMSO is commonly used as a cryoprotective agent during the process of freezing cells for storage. DMSO has also been widely used as a vehicle for several drugs (Hebling et al., 2015). In this study, DMSO was used as a vehicle solution for GN, at a concentration of 500 ηg/ml (6.4 μM). Our study showed that DMSO alone did not affect cell viability and proliferation (at 96h, however at 72h a higher proliferation is observed) compared to untreated control.

Our results indicate that low-concentration DMSO may have an effect similar to other studies in which the low concentration of DMSO is shown to have no cytotoxic activity (Adler et al., 2006; Hebling et al., 2015). One study showed that 0.05 mM to 1 mM DMSO did not affect the number, adherence and cell death of odontoblast cells. An increase in cell viability was observed with concentrations of 0.3 and 0.5 mM DMSO (Hebling et al., 2015). Adler *et al*. (2006), using different embryonic stem cell lines, demonstrated that there were no changes in cell viability up to 0.5% of the DMSO concentration, with other cell lines, the viability remained unchanged up to 1% of the DMSO concentration (Adler et al., 2006).

It should be noted that all of these studies tested DMSO at even higher concentrations than those used in this study, so the fact that the vehicle solution does not alter cell viability, is in line with expectations for concentrations below 0.05 mM (50 μM). Although studies suggest that DMSO in low concentrations can be used as a vehicle solution without causing significant damage to cells (Adler et al., 2006; Hebling et al., 2015), it is always important to study the possible effects of this compound when used as a vehicle.

In our experiments, HU showed a substantially high growth-inhibitory effect towards K562 cells at an effective concentration of 25.0 μg/mL (~329 μM), probably due to its potent ribonucleotide reductase inhibitory activity (Musallam et al., 2013). Ribonucleotide reductase catalyzes rate limitation in the biosynthesis of all precursors for DNA replication, and its activity is tightly regulated during the cell cycle, which generates a periodic fluctuation in the concentration of dNTP in proliferating cells. In the presence of HU, proliferating cells are

stopped in the S-phase due to decreased levels of dNTPs, which decreases the movement of DNA polymerase (A. Singh & Xu, 2016).

The results obtained with the positive control (HU) are in accordance with what has already been demonstrated for the concentration of study in relation to its effect on K562 cells (K. Liu, Xing, Zhang, Liu, & Fung, 2010). HU at a concentration of 25.0 µg/mL has been shown to inhibit nearly 75% of K562 cell proliferation ($p < 0.001$) after 6 days of incubation. In this study, the IC50 of the HU was calculated at 63.1 µg/mL (K. Liu et al., 2010). After 5 days of exposure to 150 µM HU, the percentage of K562 cell survival was 38.9% (Theodorou et al., 2016).

If we compare the results of cell proliferation of GN and EGCG treatments, we found that treatment with GN had a significantly higher value (72h [$p = 0.012$] and 96h [$p = 0.003$]) of cell number than treatment with EGCG. However, other studies that analyzed simultaneously the effects of GN and EGCG in cell cultures, demonstrate that for the same concentrations, in cells incubated with lower concentrations, EGCG shows to have a lower effect on cell proliferation compared to GN (Bhatia & Agarwal, 2001; Zhang & Zhuo, 2006), which is in accordance with our result. The divergence may be related to the activation of different signaling pathways, between the two compounds at lower concentrations.

In a study where GN and EGCG effects were assessed on hepatic stellate cells, T6/GFAP-lacZ cells (rat HSC-T6 cell line transfected with a GFAP-lacZ reporter transgene) were treated with increasing concentrations of GN and EGCG (0 to 80 µM) and cell proliferation was evaluated after 48h. It was possible to verify that up to 5 µM (lower concentrations) GN has a proliferative effect superior (± 20%) to EGCG (which demonstrates inhibitory activity from the beginning). At concentrations above 5 µM the effect of GN and EGCG on cell proliferation was similar, showing both a strong inhibitory effect. IC50 was 31 µM for EGCG and 25 µM for GN respectively (Zhang & Zhuo, 2006). In DU145 cells treated with similar doses (100–200 µM) of GN and EGCG, higher doses of GN showed a cytotoxic effect causing 30–40% cell death. A more profound cytotoxic effect was observed with EGCG accounting for 50% cell death at lower doses and complete loss of viability at higher doses (Bhatia & Agarwal, 2001).

From the *in vitro* analysis of cell viability and proliferation, we can discuss that GN and EGCG (with the study concentrations) have the potential to be used as possible pharmacological agents, showing no cytostatic effects. It is very important that the cellular induction is not associated with inhibition or excessive proliferation. For this reason, these two compounds have a significant advantage over other known inducers, such HU, which exerts a greater inhibitory effect on cell proliferation.

The activity of *BCL11A* and *KLF1* are critical to the silencing of HbF during development (Sankaran, 2011). This study investigated a possible induction of HbF by GN and EGCG exposures and if it could operate through transcriptional regulation of *BCL11A* and *KLF1*, by analyzing the expression mRNA in the K562 cell line.

In vivo, the possible efficacy of EGCG in increasing HbF concentrations has already been demonstrated through the ingestion of green tea extract.

In a simultaneously ongoing study at H&TRC, researchers demonstrated that prolonged human exposure (90 days) to EGCG significantly increase HbF levels *in vivo*. Healthy individuals ingested green tea extract capsules for 90 days (containing 225 mg of EGCG per capsule), and hematological parameters were tested at time 0 and 90 (days). Only the hemoglobin associated parameters from the performed complete blood count were significantly affected after EGCG oral exposure, particularly increase in HbF (manuscript currently submitted for publication).

The promising results observed *in vivo* stimulate us to understand the mechanisms involved in these responses, namely if *BCL11A* and *KLF1*, the main repressors of the *γ-globin* gene (Sankaran, 2011), it would have a central role in this result and if consequently, the same is observed in the expression of the *globin* genes.

GN, in the three studied concentrations and in the two periods of exposure, significantly suppressed the *BCL11A* mRNA. This effect was observed at 72h for the concentrations of 100 ηg/ml and 500 ηg/ml and at 96h for the concentration of 250 ηg/ml.

Our results clearly demonstrated that GN effects on K562 cells follow a non-monotonic dose-response (NMDR) and time divergent response, which is common in hormones as well as endocrine-disrupting chemicals (EDCs) such as phytoestrogens (Vandenberg et al., 2012).

In classical risk assessment, adverse responses are proportional to the exposure, which implies that higher doses of chemicals should cause greater effects than low doses, mainly based on the theories of “harmfulness from dosage” and “the dose makes the poison” (Z. Xu, Liu, Wu, Huang, & Pan, 2017), where assumes that there is no adverse effect below a certain exposure level. However, there is a class of toxins and EDCs, for which there is evidence that they do not obey the principles of toxicology (Beausoleil et al., 2013). There are several generic nonlinear dose-response curves that no linear relationship over the range from low to high doses, described as NMDRs (Z. Xu et al., 2017). In these cases, it is impossible to predict the potential harmfulness of low doses using data from high-exposure situations, so the entire dose-response curve must be investigated. Unfortunately, the mechanisms involved in the nonmonotonic response are complex and time-consuming to elucidate. A few researchers have hypothesized that the NMDRs reflect various mechanisms including genomic and nongenomic pathways, as well as the time frame of the response (Z. Xu et al., 2017). Phytoestrogens exert estrogenic effects at low concentrations and antiestrogenic effects at high concentrations. Therefore, it could be that high doses of GN may weaken the beneficial effects of GN through its actions on the estrogen pathway (Kohara, Kuwahara, Kawaguchi, Jojima, & Yamashita, 2014). GN is described as acts as an estrogen and inhibits lipogenesis by ER-β signaling at low concentrations, but it promotes lipogenesis at high concentrations

through the peroxisome proliferator-activated receptor-gamma (PPARγ) molecular pathway (Penza et al., 2006). PPARγ is a class of ligand-activated cytokine of the steroid hormone receptor, its most important function is the mediation of gene transcription and subsequent regulation on its activation after combining with its ligands (S. Xu & Xu, 2018).

The downregulation of *BCL11A* by GN has already been reported in a study in which the exposure of LNCaP cells to 50 µM of treatment with GN resulted in the negative regulation of this gene (Merchant et al., 2012).

In addition to *BCL11A*, GN is described as capable of influencing the expression of other genes (Sundaram et al., 2019).

The EGCG in our experiment also demonstrated a strongly downregulated *BCL11A* mRNA levels after 72h of EGCG exposure at the lowest concentration and at 96h time point, a more subtle but significant effect was observed for the intermediate dosage (250 ng/ml), which is also suggestive of a NMDR effect. This effect has not yet been reported previously for this compound. However, there is evidence in very recent previous studies which demonstrate that EGCG also targets estrogen receptors (ER-α and ERα36) (Chen et al., 2019; Shu et al., 2018). It suggests that estrogen receptors might be efficient targets of EGCG, so perhaps it can reproduce NMDR effects like those reported for GN.

EGCG exerts an anti-tumoral (anti-proliferation and pro-apoptosis) effect in HCC (hepatocellular carcinoma) cell lines by inhibiting ERα36 (a new isoform of estrogen receptor alpha [ER-α]). Inhibiting ERα36-EGFR-Her-2 feedback and PI3K/Akt, MAPK/ERK pathway, activating caspase 3, and accumulating p-ERK in the cytoplasm. It suggests that ERα36 and other estrogen receptors might be efficient targets of EGCG (Chen et al., 2019).

EGCG has also been described as having the potential to inhibit the human aortic smooth muscle cell (HASMC) proliferation through the PGC-1α/ERR-α/Mfn-2 pathway (Shu et al., 2018).

HU at 96h exposure also induced the downregulation of the *BCL11A* gene, emphasized that this was the only significant effect that we obtained for the positive control throughout the experiment. This result is quite similar to obtain in the treatment with 100 ηg/ml and 500 ηg/ml of GN at 72h, and lower than that obtained for EGCG 100 ηg/ml at 72h.

The repression of *BCL11A* by HU is already described in the literature, for example: after 6h and 12h of 100 µM HU treatment existed a down-regulation of *BCL11A* that was associated with a seven-fold increase in *γ-globin* expression in both primary and K562 cells (Pule et al., 2016). However, the mechanism of the molecular action of HU in inducing *γ-globin* is not completely understood. Only two transcription factors the *BCL11A* and *SOX6* have been shown to decrease in response to HU *in vivo* in sickle cell reticulocytes (Grieco, Billett, Green, Driscoll, & Bouhassira, 2015).

Current models suggest that *KLF1* positively regulates the expression of the *γ-globin* gene repressor *BCL11A* by interacting with the *BCL11A* promoter and other regions of control in the *β-globin-like* cluster (Ginder, 2015). In this way we expected that the downregulation that we observed in *BCL11A* would be, at least in part, explained by a reduction in the levels of intracellular proteins of *KLF1*, that is, the depletion of *KLF1* would secondarily decrease the levels of *BCL11A* and thus reduce its ability to repress the expression of *γ-globin* genes. However, we only observed the downregulation of *KLF1* in the highest concentration of GN (500 ηg/ml) at 96h, which indicates that the downregulation observed in *BCL11A* is not caused by the reduction in *KLF1*.

These results suggest that both GN and EGCG may have a specific molecular target for *BCL11A* but not *KLF1*.

The repression of *BCL11A*, without altering *KLF1* levels, has already been described as capable of strongly inducing HbF, by other compounds such as e.g. Pomalidomide. Pomalidomide is an FDA-approved third-generation immunomodulatory drug, and originally developed for the treatment of myeloma. However, further studies demonstrated that pomalidomide induces HbF production by promoting erythropoiesis both *in vitro* and *in vivo*, nevertheless, the molecular pathway by which modulates erythropoiesis, leading to HbF induction, is unknown (Dulmovits et al., 2016). Appiah-Kubi *et al*. (2013), presented evidences that pomalidomide significant decrease in *BCL11A* expression levels, but it does not affect the expression of the *KLF1*. Pomalidomide appears to target the erythroid-specific *BCL11A* but not the more pleiotropic transcription regulator *KLF1* (Appiah-Kubi et al., 2013).

Despite erythroid-specific *BCL11A* downregulation, no significant effects were observed regarding *γ-globin* induction. However, considering that exposure to HU which is currently utilized for treatment of SCD patients (Field & Nathan, 2014) due to *γ-globin* gene induction and reduce *β-globin* gene expression (Chou et al., 2015), also did not result in augmented *γ-globin* transcription or diminished *β-globin* gene expression we postulated that exposure time should be increased in further studies.

On the other hand, the treatment with GN in the lowest concentration (100 ηg/ml) at 96h caused very significant repression of the expression of *α-globin* mRNA. This effect was also observed in the concentration of 250 ηg/ml but was suppressed with the increase in concentration and exposure time, this result is again characteristic of an NMDR effect.

The EGCG did not influence the expression of *α-globin* mRNA, however, it significantly repressed the expression of *β-globin*, however, this repression was lost with the increase in the time of exposure and concentration (a possible feature of its also NMDR).

Despite the erythroid differentiation of K562 cells is only associated with an increase of expression of embryonic-fetal *globin* genes (Bianchi et al., 2001), it is known that the

transcription of the *β-globin* gene in K562 cell line can be influenced by epigenetic mechanisms (Fathallah et al., 2007).

In a study using K562 cells, were analyzed the effect of catechol (found in fruits and vegetables) on erythroid differentiation and DNA methylation levels in specific genes of the erythroid lineage. After K562 cells were treated with catechol, the mRNA expression of the *α*, *β*, and *γ-globin* genes showed a significant concentration-dependent increase, associated with a decrease in DNA methylation levels (X. F. Li et al., 2012).

For this reason, we also chose to analyze the transcriptional levels of the *β-globin* gene, associated with possible epigenetic mechanisms by GN and EGCG.

The fact that our results have not induced the expression of *γ-globin* suggest that probably the *BCL11A* protein level may not yet have decreased with the reduction of its mRNA, so, the result regarding the induction of *γ-globin* is not yet visible, as protein synthesis takes time, and changes in transcription affect protein levels with a delay of time. Variations in mRNAs, as well as the availability of resources for protein biosynthesis, can strongly influence the relationship between protein levels and transcripts. During highly dynamic phases, such as cell differentiation or stress response (as in the case of cell treatments), post-transcriptional processes can lead to stronger deviations from an ideal correlation, and transcription levels alone are not sufficient to predict levels of proteins in many scenarios (Y. Liu, Beyer, & Aebersold, 2016).

Another justification may be due to the fact that the studied concentrations are quite low.

In a study by Naciff et al. (2016), with the objective of evaluating the transcriptional response of human endometrial cancer cells to treatment with GN (10 ρM, 1 ηM, 100 ηM and 10 μM), the response in the gene expression to exposure to GN was time and dose-dependent. Although the response in genetic expression to low doses (10 ρM - 100 ηM) of GN was extremely limited. The authors also determined that the response of Ishikawa cells to exposure to GN is progressive and results in changes in specific pathways at different times (Naciff et al., 2016).

Taking our results into account, we consider that by increasing the dose and exposure time of the two compounds, to levels still considered non-cytotoxic and representative of food intake or by supplementing soy and green tea, we can probably obtain more robust results and the decrease in *BCL11A* mRNA may have time to influence the decrease in the level of its protein with effects on the expression of *γ-globin* and consequent induction of HbF.

5.2 Conclusions

In summary, we have demonstrated that GN and EGCG, at concentrations physiologically attainable by diet or supplementation, can downregulate the expression of *BCL11A*, which is considered the main repressor of HbF, more strongly than HU which is currently the only FDA-approved HbF inducer.

The conclusions obtained are summarized below:

GN and EGCG are not cytotoxic (> 95% viability), do not negatively influence cell proliferation, and have an influence on cell metabolism; the two compounds were shown to have a non-monotonic dose-response (NMDR) effects; GN and EGCG strongly and significantly downregulate the transcription of the *BCL11A* gene, an effect greater than that observed for HU.

This study presents a possible new potential for GN and EGCG to induce fetal hemoglobin expression. Our data encourage further study of the effects of GN and EGCG and structurally related analogs not only in immortalized cell lines but also in erythroid progenitor cells (with the potential to express embryonic, fetal and adult globin genes), to conclusively determine the potential use of such agents in the treatment of β-hemoglobinopathies.

This project was carried out to support the concept that, among the compounds identified from nature, some of them may exhibit the ability to increase HbF expression. We firmly believe that this field is exciting from a scientific point of view, but it also represents hope for several patients, whose survival will depend on the possible use of drugs that do not require blood transfusion and consequent chelation therapy, which are easily accessible.

5.3 Future perspectives

The promising results obtained in relation to the suppression of the *BCL11A* gene support the continuation of this study.

As mentioned earlier, we obtained results compatible with NMDR for both GN and EGCG, that is, these two compounds probably interact and activate their receptors in a non-linear manner. As most of the significant effects that we obtained were observed at the lowest concentrations, we can predict that the dose-response curve will be in the form of a "U", in which the maximum responses will be observed at the lowest and highest doses, respectively. Thus, we predict that, by increasing the concentration and the exposure time (so that the decrease in the *BCL11A* transcript causes observable effects), the results may be more robust

and, possibly, we will obtain a significant response in relation to the induction of *γ-globin*. In this way, we will also better understand GN and EGCG effectiveness and safety.

The conditions of exposure to GN and EGCG with effects demonstrated in the repression of *BCL11A* gene identified in this project will be selected for further analysis, with the addition of a higher concentration of 800 ηg/ml (~3 μM of GN and ~1,7 μM of EGCG), increasing the exposure time to 120h.

These doses are still considered safe (< 5 μM) and representative of the plasma concentration after alimentation or supplementation. For example, when taken as food supplements, catechin doses at or above 800 mg/day, corresponding to a plasma peak of 1682.1 ηg/ml (Ullmann et al., 2004), and plasma concentrations of GN in the range from 50 to 800 ηg/ml have been reported, in adults after consumption of soy foods in Japanese traditional diet (Cassidy, Hanley, & Lamuela-Raventos, 2000).

On the other hand, given to the all complexity of factors involved in the *γ-globin* gene expression, the mechanism by which GN and EGCG acts need to be elucidated. Further studies regarding mediated epigenetic effects and p38 MAPK signaling pathway activation, must be performed in order to better understand the molecular mechanisms underlying HbF induction and its potential use as a new therapy approach.

The continuation of the study is considered promising with the following conditions:

- Increase the exposure time to 120h.
- Increase the dose of GN and EGCG to 800 ηg/ml.
- Evaluate the expression of the *MYB* regulatory gene.
- Treatment in human erythroid progenitor cells.
- Evaluate protein expression.
- Study the involvement of MAPK signaling pathways.
- Study the epigenetic mechanisms of GN and EGCG possibly associated with the reactivation of *γ-globin* (DNMTs and HDACs).

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Appendices

Appendix 1

- RNA quantification of genistein treatments

Genistein 72h		[mRNA] ηg/μl
Plate A	GN 100 ηg/ml	9.68
	GN 250 ηg/ml	37.80
	GN 500 ηg/ml	6.48
	HU 25 μg/ml	72.60
	DMSO 500 ηg/ml	5.40
	Control	2.60
Plate B	GN 100 ηg/ml	22.40
	GN 250 ηg/ml	7.64
	GN 500 ηg/ml	9.08
	HU 25 μg/ml	44.00
	DMSO 500 ηg/ml	2.74
	Control	6.64
Plate C	GN 100 ηg/ml	8.00
	GN 250 ηg/ml	8.96
	GN 500 ηg/ml	26.40
	HU 25 μg/ml	60.20
	DMSO 500 ηg/ml	5.20
	Control	11.10

Genistein 96h		[mRNA] ηg/μl
Plate D	GN 100 ηg/ml	153
	GN 250 ηg/ml	34.4
	GN 500 ηg/ml	16.4
	HU 25 μg/ml	26.6
	DMSO 500 ηg/ml	19.9
	Control	20.4
Plate E	GN 100 ηg/ml	36
	GN 250 ηg/ml	11
	GN 500 ηg/ml	14
	HU 25 μg/ml	17.9
	DMSO 500 ηg/ml	66
	Control	88.8
Plate F	GN 100 ηg/ml	200
	GN 250 ηg/ml	112
	GN 500 ηg/ml	64.4
	HU 25 μg/ml	18.2
	DMSO 500 ηg/ml	112
	Control	36

- RNA quantification of epigallocatechin-3-gallate treatments

Epigallocatechin-3-gallate 72h		[mRNA] ηg/μl
Plate A	EGCG 100 ηg/ml	43.20
	EGCG 250 ηg/ml	47.60
	EGCG 500 ηg/ml	30.80
	HU 25 μg/ml	24.50
	Control	28.60
Plate B	EGCG 100 ηg/ml	37.40
	EGCG 250 ηg/ml	53.20
	EGCG 500 ηg/ml	77.20
	HU 25 μg/ml	29.10
	Control	91.20
Plate C	EGCG 100 ηg/ml	44.40
	EGCG 250 ηg/ml	64.00
	EGCG 500 ηg/ml	46.40
	HU 25 μg/ml	2.55
	Control	57.60

Epigallocatechin-3-gallate 96h		[mRNA] ηg/μl
Plate D	EGCG 100 ηg/ml	162.00
	EGCG 250 ηg/ml	12.80
	EGCG 500 ηg/ml	16.70
	HU 25 μg/ml	1.33
	Control	121.00
Plate E	EGCG 100 ηg/ml	56.80
	EGCG 250 ηg/ml	169.00
	EGCG 500 ηg/ml	141.00
	HU 25 μg/ml	3.65
	Control	134.00
Plate F	EGCG 100 ηg/ml	32.70
	EGCG 250 ηg/ml	33.70
	EGCG 500 ηg/ml	49.60
	HU 25 μg/ml	6.57
	Control	59.70

Elisabete Cristina Conceição de Matos

2020