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Epigallocatechin-3-gallate and Genistein as potential Fetal Hemoglobin inductors

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Resumo

Sendo das doenças genéticas recessivas de maior impacto no mundo, as β-hemoglobinopatias representam uma ameaça real às pessoas. Os tratamentos disponíveis apresentam-se como caros e com efeitos colaterais associados prejudiciais à saúde dos pacientes. A reativação da hemoglobina fetal por meio de mecanismos de regulação epigenética aparece como um método eficaz para o tratamento de pacientes com essas patologias. Novos agentes facilmente acessíveis capazes de induzir a hemoglobina fetal são desejáveis, com baixos níveis de citotoxicidade, que podem alterar a expressão génica sem alterar o material genético.

Neste projeto, foram avaliados os efeitos induzidos por dois compostos naturais, numa linhagem celular precursora de eritroides e em linhagem celular imortalizada para avaliar os efeitos em genes reguladores epigenéticos, tais como ADN metiltransferases (DNMTs) e histonas deacetilases (HDACs).

A linha celular foi exposta por 72 horas a uma concentração de 100 ng/ml de genisteína, epigalocatequina-3-galato e ambos em co-exposição para replicar a exposição celular *in vivo* após a suplementação. A exposição a 25 µg/ml de um tratamento padrão de hidroxiureia foi usada como controle positivo. A viabilidade celular foi avaliada para os precursores eritroides e os efeitos transcricionais dos compostos nos genes DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 e HDAC3 na linha celular imortalizada K562.

Os resultados demonstraram maior viabilidade da genisteína e do epigalocatequina-3-galato na linha celular de precursores eritroides em comparação à HU que exibiu maior percentagem de células mortas. Estes compostos naturais presentes no chá verde demonstraram potencial na regulação epigenetica de DNMTs, com resultados dispares para HDACs em relação à literatura existente.

Este estudo pioneiro descreve o potencial desses compostos naturais individualmente ou em co-exposição para regulação epigenética em linhagem celular de precursores eritroides.

Palavras-chave: β-hemoglobinopatias, hemoglobina fetal, compostos naturais, genisteína, epigalocatequina-3-galato, precursores eritróides, DNMTs, HDACs

Abstract

As the most impacting recessive genetic diseases in the world, β-hemoglobinopathies represent a real threat to people. Available treatments present themselves as expensive with serious side effects associated. Fetal hemoglobin reactivation through epigenetic regulation mechanisms appears as one effective method for therapy of patients with these pathologies. New easily accessible agents capable of inducing fetal hemoglobin are desirable, coupled with low cytotoxicity levels, that can shift gene expression without altering the genetic material.

In this project, the effects induced by genistein and epigallocatechin-3-gallate were evaluated, in an erythroid precursor cell line as well as in an immortalized cell line to evaluate the effects on epigenetic regulatory genes, like DNA methyltranferases (DNMTs) and histone deacetylases (HDACs).

Cell lines were exposed for 72 hours to a concentration of 100 ng/ml of genistein, epigallocatechin-3-gallate and both in co-exposure, to replicate cellular exposure *in vivo* after supplementation. Exposure to 25 µg/ml of a standard treatment of hydroxyurea was used as a positive control. Cellular viability was measured for the erythroid precursors and the transcriptional effects of the compounds on DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 and HDAC3 genes cells were evaluated in K562 immortalized cell line.

Results showed greater viability of genistein and epigallocatechin-3-gallate in the erythroid precursor cell line compared to HU that displayed higher percentage of dead cells. These natural compounds present in green tea showed potential in the epigenetic regulation of DNMTs, with different results for HDACs in relation to the existing literature.

This pioneer study describes the potential of these natural compounds individually or in co-exposure for epigenetic regulation in a cell line of erythroid precursors.

Keywords: β-hemoglobinopathies, fetal hemoglobin, natural compounds, genistein, epigallocatechin-3-gallate, erythroid precursors, DNMTs, HDACs

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Abbreviations and acronyms

ACS - Acute chest syndrome

cDNA - Complementar DNA

CRISPR - Clustered regularly interspaced short palindromic repeats

Ct - Cycle threshold

DNA - Deoxyribonucleic acid

DMSO - Dimethylsulfoxide

DNMT - DNA methyltransferase

EGCG - Epigallocatechin-3-gallate

FDA - Food and Drug Administration

GAPDH - Glyceraldehyde-3-phosphate dehydrogenase

GN - Genistein

GR - Graft rejection

Hb - Hemoglobin

HbF - Fetal hemoglobin

HBG - Human β-globin

HbS - Hemoglobin S

HDAC - Histone deacetylase

HSC - Hematopoietic stem cell

HU - Hydroxyurea

qRT-PCR - Quantitative real-time polymerase chain reaction

mRNA - Messenger ribonucleic acid

PE - Pulmonary embolism

RBC - Red blood cell

RNA - Ribonucleic acid

RNases - Ribonucleases

SCD - Sickle cell disease

1. Introduction

With an expected worldwide growth in the following decades, hemoglobinopathies are some of the most common autosomal recessive diseases, that spreads great concern and challenges to combat its symptoms, especially disorders in β-hemoglobin. [1]

Currently, the available treatment options are severely limited, and pharmacological reactivation of fetal hemoglobin (HbF) reveals itself as a promising therapeutic strategy as hereditary persistence of HbF minimizes the severity of associated symptoms, while delivering increased cell survivability rates. [2]

Considering the limited treatments available, the high economic cost and the associated hazardous effects, the identification of novel agents with high HbF inducing activity and lower cytotoxicity is a major challenge and a desirable goal to achieve in this field. [3]

Silenced by epigenetic mechanisms, human β-globin genes, responsible for oxygen transport properties of hemoglobin (Hb), are imperial for HbF induction. Appling epigenetic modifiers can stimulate an inhibitory action on the activity of those gene silencer factors, thus reactivating the epigenetically silenced target genes. [4]

The interplay between DNA methylation and histone modifications in regulating gene expression is well recognized and several epigenetic modulators have been utilized as HbF inducers (including hypomethylating agents and histone deacetylase inhibitors). Epigenetic mechanisms can be influenced by diet given that some natural compounds are potent epigenetic modulators. [4]

1.1. Human Blood

Blood is a body fluid that runs through veins both humans and animals. It delivers essential oxygen and nutrients to cells, while also transporting metabolic waste products away, such as carbon dioxide. It is pumped by the heart to all body parts, after which it is returned to the heart to repeat the process. Blood flow must never cease, with death occurring consequently. The explanation resides in the effects of an unfavourable environment on highly susceptible cells.

Human blood is an opaque red colour imparted by Hb, denser and more viscous than water. Hb is a protein molecule with four polypeptide chains. Each one comprises more than 140 amino acids. Around 95 percent of red blood cells RBCs dry weight consists of Hb, allowing oxygen transfer through the blood. Hb lightens in colour when saturated with oxygen (oxyhemoglobin) and dims when oxygen is separated from it (deoxyhemoglobin). Therefore, deoxygenated blood extracted from a regular vein is darker than oxygenated blood obtained from an artery.

RBCs, also referred to as erythrocytes, comprise around 45 percent of the total volume of blood, while the remaining cells correspond to about 1 percent or less. Plasma, the fluid portion, can be characterized as clear, sticky, and yellowish liquid. Blood is permanently fluid, and turbulent flow assures that cells and plasma are homogeneously mixed within the human body. [5]

Hematopoiesis consists in the construction of all blood cellular elements and plasma. It happens within the hematopoietic system, which includes organs and tissues such as the bone marrow, liver, and others. This process starts during the embryonic development first weeks. [88]

1.1.1. Reticulocytes

Reticulocytes are immature RBCs, absent of a cell nucleus. In erythropoiesis, reticulocytes grow and mature in the bone marrow, before developing into fully mature RBCs. These were named reticulocytes based of a ribosomal RNA reticular network that becomes visible under microscopic observation with certain stains, such as methylene blue. Immature RBCs can be recognized from other cells, as they radiate neither a strong signal (lymphocytes), nor weak (RBCs). [6]

The quantity of reticulocytes represents a valuable indicator of bone marrow activity since it represents recent production and allows for the determination of a reticulocyte production index to determine whether a production problem is contributing to the existence of hemoglobinopathies. [6]

Increased production of RBCs tends to happen to overcome chronic and/or severe loss of mature RBCs in anaemia cases with high percentage of reticulocytes in the blood (reticulocytosis). [6]

Patients affected by β-hemoglobinopathies, affecting the production or structure of adult Hb, suffer serious health difficulties and damage. The production of reticulocytes developing and maturing in the bone marrow, before developing into mature RBCs in the blood stream, becomes of great importance to allow these pathologies symptoms to be ceased. [89]

Reticulocytes are a valuable tool in protein translation studies because they are unusual among cells as they contain all the machinery necessary to translate proteins but lack a nucleus. Since a cell's nucleus contains many components that make studying translation difficult, these cells are quite useful. Scientists can collect reticulocytes from animals such as rabbits and extract the mRNA and translation enzymes to study protein translation in a cell-free, *in vitro* system, allowing greater control over the environment in which proteins are being synthesized. [7]

1.2. Hemoglobinopathies

Hemoglobinopathies are the most common monogenic blood disorders and diseases that affect RBCs. [8] These genetic defects result from structure abnormalities of one of the globin proteins chains in Hb, proteins that should carry oxygen through the body. Several cases appear to be inherited as autosomal co-dominant traits. [9] In contrast, thalassemia, which are also inherited blood disorders, affect globin protein's synthesis, often through mutation in regulatory genes.

From these, β-hemoglobinopathies are the most common and are caused by mutations affecting the production and/or structure of adult Hb. Patients impacted may endure anemia, impaired oxygen delivery to the tissues, and organ damage. In the absence of a compatible donor for allogeneic bone marrow transplantation, the lifelong therapeutic options are symptomatic care, red blood cell transfusions and pharmacological treatments. [89]

1.2.1. Sickle Cell Disease

From all hemoglobinopathies, sickle cell disease (SCD) is the most prominent group of inherited RBC disorders. [10] Patients with SCD present

abnormal sticky sickle shaped Hb. Typically, RBC are disc shaped and flexible, allowing for ease of movement through the blood vessels, but SCD patient cells tend not to bend and move easily, blocking the normal blood flow through the human body. [11-12]

SCD patients represent various challenges due to the complexity of their condition and need for frequent medical attention. Some complications that might be common for these patients are acute chest syndrome (ACS), pulmonary embolism (PE) and pneumonia. [13] These are highly considerable mortality threats that evidence a greater susceptibility to respiratory infections, making these highly vulnerable patients. There are no concrete evidence and/or scientific results about the risks associated with the current SARS-CoV 2 infectious virus pandemic. [14] More concrete information regarding this threat is vital for patients' risk management.

1.2.1.1. Epidemiological context

As mentioned as introduction, the number of new-borns with SCD worldwide is expected to increase by a third by 2050. Regionally, Sub-Saharan Africa accounted for 79% of new-borns with SCD in 2010, a proportion that could rise to about 88% by 2050. [15]

Worldwide, more than 1% of couples are at risk of having child suffering from diseases related to Hb malformation. While the under-5 mortality rate in West Africa is 18.4%, the rate is 16.5% for children born to couples who are not at risk, compared to 40% for children born in couples who are at risk. [16]

Based on literature, the geographic distribution of the βS allele is largely driven by two key factors: malaria's endemic distribution and movements at the population level. [17] The overlap between the geographic distribution of the β allele and the endemic distribution of malaria in Sub-Saharan Africa led in the 1950s to the hypothesis that individuals with hemoglobin S (HbS) possess remarkable protection against *Plasmodium falciparum* induced malaria, latter being confirmed that those individuals are 90% less likely to experience severe malaria than normal Hb individuals. [18]

Population movements, including slave trade in the 16th, 17th, and 18th centuries, led to a widespread distribution of the βS allele, particularly in North

America and Western Europe. Detailed mapping of the frequency of the βS allele has highlighted those geographic heterogeneities of inherited Hb disorders can occur over short distances. [17]

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1.3. HbF

During human globin's synthesis, two switches occur: the transition from embryonic Hb to HbF (around 6 weeks gestation), and HbF to adult Hb, after childbirth. [19]

During human erythroid development, there is a sequential shift in expression from the embryonic ε-globin gene to the fetal γ-globin gene in utero. After partum, the γ-globin gene is silenced as the β-globin gene expresses itself with predominance. [20] More specifically, the primitive erythroid lineage originates in the extraembryonic yolk sac, corresponding to the first cells with Hb, and undergoes final maturation in the bloodstream and some reticuloendothelial organs. This transitory population is later replaced by a definitive lineage that generates smaller erythrocytes absent of nuclei. These play the predominant role in oxygen transport throughout gestation and postnatal development.

HbF switch begins to occur from the HBG1 and HBG2 genes (γ-globin) to the adult HBB gene (β-globin). [21] These structural changes are typically completed during childhood and last roughly the first half year of age. [19] Healthy adults continue to express a low level of HbF, which is largely concentrated in a small percentage of erythrocytes referred to as F cells. [22]

Knowledge about the mechanisms underlying HbF pharmacological induction remain unclear, being complex and susceptible to several pathways. These include chromatin structure's modifying to increase transcription factor accessibility to promoters of the globin genes of interest, such as the inhibition of histone deacetylase activity, hypomethylation of HbF gene promoters and their

transcriptional activation, as well as acceleration of erythroid cell differentiation. [23-24]

According to prior studies, HbF induced by epigenetic strategies was distributed in the erythrocyte population and, despite a smaller gradient in the rates of increase percentage of HbF, the rates of increase of F cells was higher in patients with a lower baseline. This means that, after starting therapy, at a given period, the increase in F cells is potentiated and maintained. Thus, F cells entering the circulation will combine with a similar number of F cells leaving circulation, producing plateaus in the percentages of HbF and F cells. [25]

1.4. SCD current treatments and new approaches

SCD therapeutic choices have been generally limited with amplified knowledge of the disease's molecular underpinnings, plus regulatory authorities backing, has led to the growth of freshly studied therapies. These primarily address the disease from the proximal HbS polymerization to more distal pathologic processes, such as RBC adhesion to the vascular endothelium. Also, initial studies imply that gene and/or stem cell therapies may possibly have the potential to allow curative treatment. [32]

1.4.1. Hydroxyurea

Hydroxyurea (HU) is a ribonucleoside diphosphate reductase inhibitor chemical compound and was firstly synthesized by Dresler and Stein (1869) in a succession of experiments trying to extract urea derivatives. [33] It was only first introduced as a clinical agent a century later, mainly for chemotherapy in the treatment of a variety of solid tumours and leukaemia. [34] It was later approved by the Food and Drug Administration (FDA) in 1998 to reduce the frequency of pain crises and, also, to avoid heavy needs for blood transfusions in adults with homozygous SCD. [35] It is considered the state-of-the-art therapy for SCD treatment.

In SCD, HU may be described as multifactorial mechanism of action, however, it primarily increases production of HbF. HbF induction represents an effective therapy approach for SCD, as the mutated β chain absence in these

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proteins implies that it is not affected by the sickle mutation and while the HbF properties are slightly dissimilar than those of normal adult Hb, his incorporation into normal adult RBCs has not been associated with functional impairment. [36] Some patients might naturally produce higher levels of HbF, which results in milder symptoms of SCD. [37][38]

Despite the benefits, HU still represents does not represent a harmful-free solution, as there are concerns regarding long-term safety and significant toxicity reports. Evidence contradicting these fears as also been documented, which leaves us in this grey area regarding HU's safety, resulting in underusage in SCD treatment and the pursuit for alternatives, due to safety concerns expressed by both the providers and diseased. [38] Also, a very considerable number of patients display poor or no response at all to HU treatment. [39]

1.4.2. Cellular and biomolecular therapies

Substantial developments in cellular and biomolecular approaches have led to powerful therapies for SCD with allogenic hematopoietic stem cell (HSC) transplantation appears to be the most efficient therapeutic alternative for patients with severe symptoms. [40] Regardless of its high success rate, a considerable applicant's percentage does not have a proper matched sibling donor with remaining posterior risk factors, such as graft rejection (GR) and transplant related mortality, keeping this approach also limited as of right now. [41][42]

1.4.3. Gene therapies

As for other alternatives, genetic strategies aiming for HSCs, either gene addiction or editing, remains as a possible alternative with high potential. Genetically modified therapeutic cells are originated from the host cells, substantially decreasing the danger of GR and transplant rejection, supressing posterior need for immunosuppression as part of the treatment. [43] Primary conclusions from scientific research studies with clinical trials associated with genetically customized autologous HSCs expressing potential therapeutic genes for immunodeficiency disorders. [44][45][46]

More focused onto gene editing methodologies, targeted nucleases represent very powerful and innovative instruments for mediating genome modification with incredibly high accuracy. [47] RNA-guided Cas9 nuclease from the microbial clustered regularly interspaced short palindromic repeats (CRISPR) adaptive immune system, a Nobel Prize in Chemistry award winning technique developed by Emmanuelle Charpentier and Jennifer Doudna (2020), promises to enable highly efficient and precise genome engineering by specifying a targeting sequence within its guide RNA. [48]

Induction of HbF synthesis by CRISPR/Cas9-mediated editing appears to be possible with promising results as a future treatment for SCD. [49] Meanwhile, correction of the sickle mutation in human CD34+ cells through this method provides another strategy which could be followed as a new state-of-the-art gene therapy and inexpensive. [50]

There are, however, some critical concerns involving possible long-term repercussions about gene editing technologies. Since CRISPR is a very recent discovery, it might take some time to study these issues and validate its safety. Using this approach is also an extensively time-consuming process, especially optimizing the protocol can be particularly challenging, demanding skill, perseverance and even some luck at times. [51][52]

1.4.4. Natural compounds

Ever since ancient times, like natural compounds have been utilized as medication for many disease and illness therapies, with many of these gone to become treatment candidates. [53][54] These products continue to deliver distinctive structural variety in comparison to traditional combinatorial chemistry, presenting opportunities to uncover novel low molecular weight lead compounds. [55]

For instance, the usage of medicinal plants has been demonstrated through records in the form of traditional remedies with many of their bioactive natural products still being unknown. The predominant source of knowledge regarding natural product from this source is the consequence of trialling and error through the millenniums. [56][57]

1.5. Epigenetic regulation

Epigenetics refers to DNA and histones post-translational alterations that affect gene expression and can be inherited through somatic cell replication. [20] In other words, the ability to control gene expression without altering a DNA sequence. It encompasses the subsequent epigenetic traits: DNA methylation, histone covalent modifications, non-coding RNA mechanisms, and chromatin remodelling complexes. Although all epigenome components seem to regulate gene expression, DNA methylation is believed to be crucial for stabilizing signalled changes in gene expression, while inability to propagate epigenetic information results in deviations from the normal pattern of gene expression. [26-27]

A cellular epigenetic process should meet the following criteria: demonstrate noticeable contrast in expression between two cells that share the same genotype; this difference must persist in the absence of an initial signal and must be passed through mitotic division. Some epigenetic marks can also be inherited through meiosis. [27]

In the field of epigenomics, nutrigenomics explores and defines the effect of diet and its interactions with the genome. Lifestyle and diet significantly influence epigenetic mechanisms (and life in general), which cause inheritable changes in gene expression without changing DNA sequences. Nutrient-dependent epigenetic variations can meaningfully impact genome constancy, protein expression, and metabolic changes, which will affect food absorption and the activity of its components. [21]

Epigenetic regulation of eukaryotic genomes has become progressively clear as a participant in the stability of DNA sequencing and for maintaining the integrity genomic information. Regardless of their heritability, histone modelling provides crucial information for genomic stability and proper DNA methylation, all key to establish epigenetic DNA methylation tags which in turn increase epigenetic stability. [28]

1.5.1.   Epigenetic and transcriptional modulators

Ideally, pharmacological agents should modify the epigenetic configuration of γ-globin genes, providing a viable therapeutic approach for HbF induction. DNA methyltransferases (DNMTs) represent an important group of epigenetic writers, as DNA methylation was the first well-described epigenetic regulatory process. [29] The most characterized are the *de novo* methylases, DNMT 3A and 3B, which symmetrically methylate the cytosines in the CpG dinucleotides. There is also DNMT1, a maintenance methylase that adds a methyl group to CpG and becomes a target to reverse the silencing of globin genes. [20][26][30]

Cytotoxicity must be prevented and some epigenetic targets, like histones deacetylase (HDACs), hold pleiotropic functions in cells (regulate different phenotypes). Thus, even HDAC inhibition on the target can induce cytotoxicity, potentially limiting the role of these epigenetic targets and their drugs in inducing HbF. [25]

It is also known that the selective elimination of an individual HDAC can induce HbF without changes in global histone acetylation, with less extensive effects on global gene expression and without inhibiting the cell cycle. [31] Increased histone acetylation has long been associated with decondensed chromatin and hence active gene expression. [20][21]

1.5.2.   Epigallocatechin-3-gallate and Genistein

Tea is the most consumed drink in the world after water, with one fourth of its sales specifically being accounted by green tea. Growing evidence continues to emerge revealing a variety of potential health benefits derived from the consumption of green tea and its constituents. [58][59][60] Epigallocatechin-3-gallate (EGCG) and Genistein (GN) are two found in green tea that have shown incredible therapeutic potential and epigenetic mechanisms.

It has been demonstrated through studies and research that EGCG has inhibitory effects, such as antioxidant, anti-inflammatory and/or antifibrosis effects. [61] To some extent, there is speculation that EGCG possess protecting effects for organs/tissues. Scientific evidence also indicates that EGCG may prevent tumorigenesis by constraining carcinogen activity. [62] On the other

hand, GN, mostly found in soy-based foods, also appears to contribute favourably to the treatment of hormone-related cancers, like liver, gastric, lung, colorectal and breast cancer. [63][64][65]

With all the characteristics and effects revealed by previous research, plus epigenetic mechanisms, there is hope that the compounds of green tea may show promising arguments against the development and health endangerment from SCD through HbF regulation.

2. Objectives

The research for alternative treatments for hemoglobinopathies has inspired greater comprehension about epigenetic mechanisms inherent to the regulation of the human β globin genes expression and HbF silencing mechanisms [20], since they display great potential for rectifying pathogenic mechanisms underlying the disease. Presently, about half a hundred HbF-inducing compounds have been identified, but their action mechanisms are still mostly a mystery. [66][67][68] So far, hydroxyurea is the most used pharmacological agent for HbF induction in hemoglobinopathies therapy. However, this medication has the drawback that it does not produce the desired effects in a significant percentage of patients, is not easily available in areas where these pathologies tend to be more frequent and display harmful side effects.

The ideal target for an alternative therapy would be one that mirrors and enhances the genetic variants effect that regulate HbF levels at loci, such as B-cell lymphoma/leukemia 11A (BCL11A) and Krueppel-like factor 1 (KLF1), without impacting other biological pathways or causing severe side effects. [69] The proposed epigenetic mechanisms for the regulation of HbF are based on DNA methylation and histone deacetylation. [22][70] Several studies provide scientific evidence that deoxyribonucleic acid (DNA) hypomethylation and histone acetylation allow the induction of γ-globin expression effectively through naturally available compounds [70][71][72][73], which can significantly affect genome stability, messenger RNA (mRNA), and protein expression. [21][26][68]

GN, a natural flavonoid compound that can be found in soy derivatives, is present in the daily diet of several individuals. It is also one of the most studied compounds and associated with a variety of biological events. [74] Green tea possesses EGCG as its main polyphenolic component, which holds antioxidant and anti-inflammatory properties. [75] With the consumption of green tea, effective inhibition of dehydration of sickled red blood cells *in vitro* was observed [76] and reduction of oxidative stress in iron-treated erythrocytes.

Where the disease prevalence is higher, the populations inability to access suitable medicine and sustain the high costs of clinical management leads to the

need of new approaches at lower costs without lacking in therapeutic efficacy. In this context, natural antioxidant substances, such as polyphenols and flavonoids, present in diet, capable of inducing γ-globin become crucial. This project aims to evaluate the potential GN and EGCG *in vitro*, in the expression of HbF, compared to HU.

Main objective: study the potential HbF induction in erythroid precursor cells from GN and EGCG and their effects in cellular viability.

Specific objectives: To evaluate the potential of GN and EGCG in the induction of HbF in erythroid precursors, compared to HU, and to evaluate the transcriptional response of epigenetic regulators, DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 and HDAC3, to these compounds. Hopefully, this project will allow to determine the potential of highly accessible natural compounds in HbF reactivation, without major hazard to cells.

3. Materials and methods

3.1. Blood samples collection and cell lines isolation

Erythroid precursors were isolated from peripheral blood samples collected from 2 volunteer researchers. They are 2 healthy adults of opposite sex who have declared their informed consent about the collection and treatment of the samples. Ethics Committees from all involved research institutions also gave positive feedback. Blood samples were used to isolate cells according to the Erythroid Cell Differentiation Protocol. [77]

Peripheral blood was diluted 1:1 (v/v) with PBS (Sigma-Aldrich). A smooth layer was placed on top of a layer of Ficoll-Paque® (Eppendorf AG), in such way that it constituted one third of the total volume. The sample was then centrifuged for 20 min at 400 x g (all centrifuge from this protocol was performed at room temperature). The upper layer, mainly consisting of plasma and platelets, was gently removed, while the mononuclear cells middle layer was subsequently collected with a pipette into a 50 ml tube. 40 ml of PBS were added, and the solution was centrifuged for 5 min at 160 × g. Pellet was resuspended in residual buffer, to which 40 ml of PBS were added with the solution being centrifuged for 5 min at 100 x g. and the supernatant was aspirated. This last step was repeated one more time.
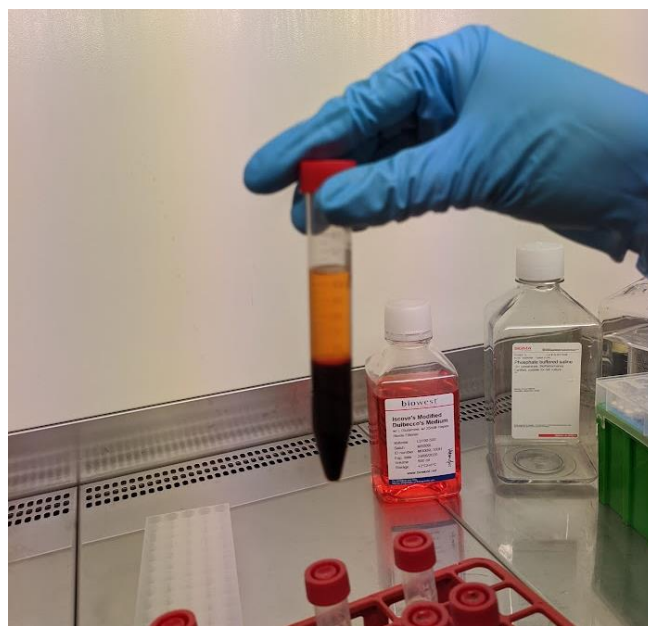

Figure 1 - Blood sample after first centrifugation (from author)

Table 1 - Erythroid Cell Differentiation Protocol reagents and volumes

Reagents	Volumes (mL)
Peripheral blood	45,0
PBS (pH 7,4)	15,0
Ficoll-Paque® (ρ = 1,077 g/mL)	10,0

3.2. Cell culture

3.2.1. Erythroid cell culture

Reticulocytes are approximately 20% larger than differentiated erythrocytes, have a lifespan of about 3 days (time required for enucleation to occur) and exhibits trace RNA attached. [78] It spends two of these days in the bone marrow and on the third it appears in the peripheral blood, ending the maturation process to become the erythrocyte. [78][79] Most erythroid cells in the bone marrow have a distinct morphology, but the differentiation that occurs during erythroid maturation is observed even in cells that do not display morphological features normally associated with the erythroid lineage. These immature red blood cells can perform oxygen transfer. These cells, however, have not yet adopted the characteristic biconcave shape of mature erythrocytes that guarantees their stability and flexibility necessary to support blood flow stress. [78]

Phase I medium was prepared by adding FBS (Sigma-Aldrich), cyclosporine A (Sandoz) and 10% (v/v) conditioned medium from 5637 human bladder carcinoma lines in the stipulated volumes (Table 2) to complete the complete MEM culture medium (Sigma-Aldrich).

Once the cells were obtained from peripheral blood original samples, mononuclear cells were washed in phase I medium and plated in 25 cm^2 tissue culture flasks (T-25), in a volume equivalent to the original blood (per donor). Cell suspension cultures were incubated at 37°C in a humidified oven, 5% CO_2 for 5 days. After the incubation period in phase I medium, the cultures were shaken and the non-adherent cells were collected for centrifugation for 5 min at 160 × g (room temperature), to extract the supernatant and complete the Falcon™ (15 mL) with complete MEM culture medium. Two additional 5-minute centrifugations

at 160 x g with aspiration of the supernatant and addition of the same volume of complete MEM culture medium were performed.

Phase II medium was prepared by adding the following reagents in the stipulated volumes (Table 2): 30% FBS (v/v), 1% (c/v) BSA, 10^{-5}M β-mercaptoethanol, 10^{-6}M dexamethasone, 0.3 mg/ml human holo-transferrin, 10 ng/ml human recombinant stem cell factor (SCF) and 1 U/ml human erythropoietin (EPO). Cells were grown in their original volume in phase II medium and incubated for 7 days at 37°C in a humidified, 5% CO_2 oven, until the experimental exposure was performed. All these steps were performed in a sterile environment, in a Polaris vertical laminar flow chamber.

Each cell grows as an individual, being non-adherent to the culture flask and therefore cultured in suspension. Methylene blue dye was used, which bonded with the RNA. The accuracy of this assay is compromised by its subjective nature, and the limited number of cells (200-1000) that can be counted in a reasonable period. [80]

Table 2 - Basic Protocol 2 Reagents and Volumes

Reagents	Volumes
Complete MEM culture medium	32 mL
FBS, 10% (v/v)	4 mL
50 mg/mL ciclosporin A	0.8 µL
Medium from human bladder carcinoma 5637 cell line (see recipe)	4 mL
Human erythropoietin	2.47 µL
10% (c/v) deionized BSA (Sigma-Aldrich)	400 µL
β-mercaptoethanol (Sigma-Aldrich)	2.8 µL
4 mg/ml dexamethasone sodium phosphate (Sigma-Aldrich)	0.6 µL
Human Holo-Transferrin (Sigma-Aldrich)	336 µL
Recombinant factor from human stem cells (Sigma-Aldrich)	200 µL
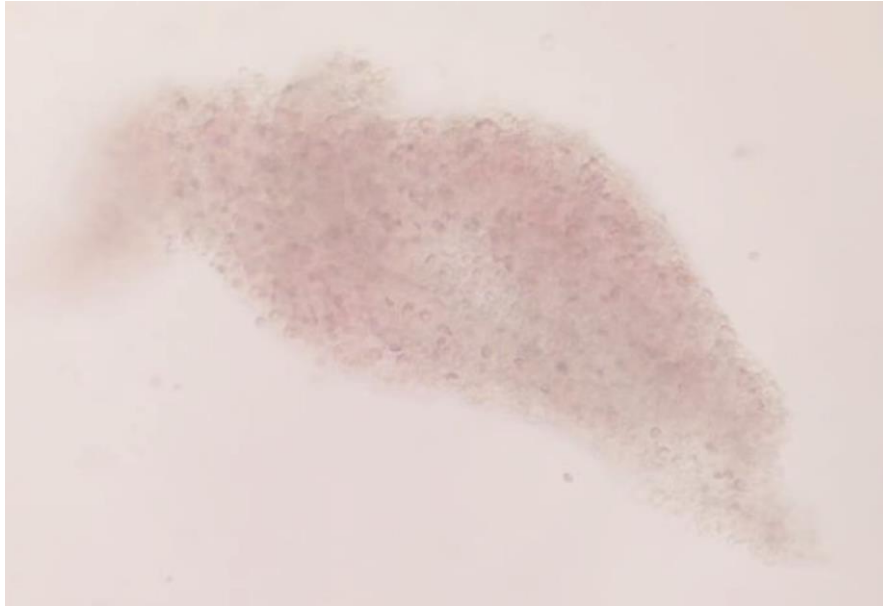
Figure 2 - Erythroid cells captured with a phase reversed contrast microscope (20x) after the first culture (from author)

3.2.2. **K562 Cell Culture**

K562 cells (Figure 3) represent erythroleukemic cell lines and are crucial in hematological experiments, as they show great differentiation potential, thus being easily acquired, and maintained in culture. [81][82] These cells share phenotypic traits of embryonic erythroid progenitors and can originate erythroid cells or macrophages, as well as megakaryocyte lineages. As a human line, it is perfect for studying human globins, manifesting specificity for changes in HbF globins. [81][82] Therefore, activators and regulators of HbF gene expression, theme of fundamental significance for understanding the sickle cell disease mechanisms of diseases, becomes friendlier to study. Generally, their approximately 20 µm in diameter, have a basophilic cytoplasm without granules, and two or more prominent nuclei. Present a rounded shape, are non-adherent, and capable of growing in suspension. [81][83]
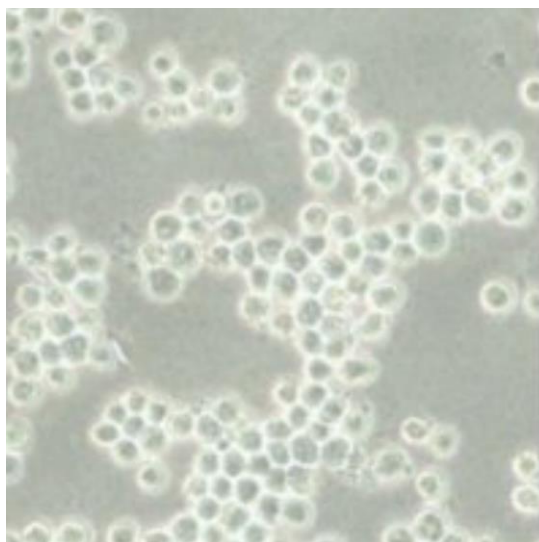
Figure 3 - Phase contrast microscope image of cells K562 (x400) (adapted from Xu et al., 2015)

Firstly, in a sterile environment, commercially acquired cells were defrosted quickly in an oven at 37ºC, to minimize cellular damage caused by the cryopreservative agent dimethylsulfoxide (DMSO). [84] Culture medium was also heated.

Subsequently, K562 cells were transferred to a 15 mL Falcon™ centrifuge tube and 10 ml of RPMI 1640 culture medium (Sigma-Aldrich) containing GlutaMAX-1 were added, 25 mM HEPES (Invitrogen, USA) preheated to 37 °C and supplemented with 10%(v/v) FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (Sigma-Aldrich). A 5-minute centrifugation at 100 x g was performed to guarantee DMSO deletion. Cell pellet was resuspended in 5 ml of RPMI 1640 medium and transferred to a T-25 culture flask with 10 mL of medium. The cell culture was maintained in suspension in a humidified atmosphere of 5%(v/v) CO_2 at 37°C for a total period of 3 days.

After being cultured in suspension, the cells were washed through centrifugation with PBS and the resulting pellet was resuspended in fresh RPMI 1640 media. The cells were cryopreserved for further use by adding the cell amount corresponding to 2 x cells/mL to 1.5mL of Cell Freezing Medium (C6164, Sigma-Aldrich) supplemented with 10%(v/v) DMSO and 10%(v/v) FBS, 10% and frost at -80°C.

3.3. Natural compounds exposure

GN (CAS number 446-72-0; Sigma-Aldrich) and EGCG (CAS number 989-51-5; Sigma-Aldrich) were purchased in crystal format of 50 mg and 5 mg, respectively. Two 5 mg/mL solutions were prepared by dissolving EGCG in highly purified water and GN in DMSO. A volume of 50 µL was used as study solution, later diluted (1:100) in the complete MEM culture medium for the erythroid precursors, achieving a final concentration of ECGC and GN of 100 ŋg/mL.

Human clinical trials reviewed within the state-of-the-art about this subject, have proved that 400 mg and 800 mg of EGCG ingestion yield peak serum concentrations at levels of 100 ŋg/ml to 400 ŋg/ml with no serious side effects identified [132]. Thus, the chosen concentration of 100 ŋg/ml for the experiments was based on this scientific evidence, as well as previous research experimental studies. [86] This way, compound concentrations are ensured to be safe and mimic the *in vivo* cellular exposure of GN and EGCG, as it is desired. A 500 mL capsule of hydroxyurea was diluted in a biological safety cabinet. 100 mL of ultrapure water were added, obtaining a final concentration of 5 mg/mL, spread in aliquot part and frozen at -20ºC. Take note that one of the aliquots was utilised to make a 0.5 mg/mL stock solution (1:10 dilution in complete MEM medium).

As negative controls, standard culture medium of each cell line for EGCG was used, as well as the same culture media diluted with 500 µg/mL of DMSO, to correspond to the final concentration of vehicle solution in cells exposed to GN.

For the exposure, the erythroid precursors were exposed to EGCG and GN, individually and in a co-exposure regime, at a concentration of 100 ŋg/ml, 25 µg/ml for hydroxyurea (HYDREA®, USP), to the negative controls and were sown in 6-well plates. This was followed by incubation in humidified atmosphere of 5% CO_2 at 37°C for 72 hours for assays and post-treatment analyses.

3.4. Cell viability assays

The trypan blue exclusion assay (Sigma, St. Louis, USA) followed the protocol described by the European Collection of Cell Cultures (ECACC). [84]

This assay required the use of a Newbauer chamber, under an inverted microscope to determine the quantity of viable cells present at cell culture in

suspension. It was assumed on a theoretical foundation that living cells have an undamaged cell membrane, resistant to trypan blue dye, while non-viable cells allow their passage through the membrane.

Under sterile environment, 100-200µL of the cell suspended medium is withdrawn from culture, adding 0.4% total solution volume of trypan blue solution (dilution factor equal to 2). After a few minutes of incubation, one side of the chamber is filled with 10µL cell suspension, placing at the microscope (20x magnification). Viable (translucent) and non-viable (blue cytoplasmic staining) cells were then counted to calculate the total and percentage of viable and non-viable cells.

3.4.1. Cell quantification

Cell counting was performed under a traditional method. In the traditional method, cell counts were performed if they are under the upper limits and the extreme left of the squares, excluding cells that fall within the limits upper and far right (Figure 4).
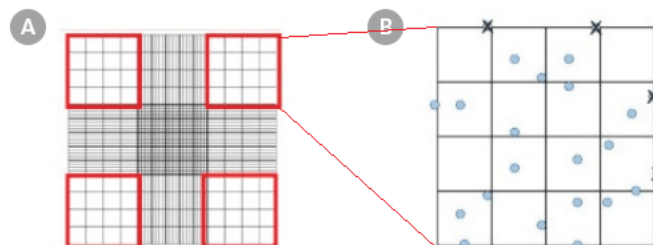
Figure 4 - Cell count according to the traditional method; Cells are counted if they are not within the limits upper and far right
(From https://www.stemcell.com/how-to-count-cells-with-a-hemocytometer.html)

After counting, the number of total cells was determined through the following equation:

Number of total cells (cells/mL)
$$= \text{mean of cells in the quadrants} \times \text{Dilution factor (2)} \times 10^4$$

10^4 corresponds to the factor founded by the 9 squares by which the Newbauer chamber is divided. Each with an area of $0.1mm^2$ and the depth of

which is 0.1mm. That means each one of the squares where the cells are counted will be 0.1mm^3 of volume so the conversion factor to mL is 10^4.

Then, the number of viable cells was obtained through the following formula:

Number of viable cells (cells/mL)

$$= \frac{number\ of\ viable\ cells\ per\ quadrant}{number\ of\ quandrats\ counted} X\ Dilution\ factor\ (2)\ X\ 10^4$$

Finally, the percentage of total viable cells was achieved through the subsequent calculation:

Percentage of viable cells (%)

$$= \frac{number\ of\ viable\ cells\ per\ quadrant}{number\ of\ total\ cells} X\ 100$$

Concentration and viability percentage were obtained according to the calculation methods performed through this traditional method.

3.5. Total RNA extraction

Total RNA was extracted by microcentrifugation on a silica column using the SV Total RNA Isolation system (No. Z3101; Promega), according to the procedure specified by the manufacturer. Total RNA extraction purifies, isolates, and extracts small amounts of total RNA present in a cell culture. 4 vital steps for this process are the rupture of the cell membrane, nuclear proteins denaturation, inactivation of endogenous ribonucleases (RNases) and the removal of contaminants (DNA and proteins).

Both cell lines were previously centrifuged to detach the pellets. These were then centrifuged twice with 10mL of PBS, discarding the supernatant. The extraction process started with the addition of 175μL of RNA lysis buffer to the washed cells, becoming homogenized. Homogenization is carried out with a reagent containing sodium dodecylsulphate, guanine thiocyanate and β-mercaptoethanol. Sodium dodecylsulphate acted in the disruption of cell

membranes and, together with guanine thiocyanate, denatures nuclear proteins that involve the RNA. β-mercaptoethanol inactivated RNases, thus preventing RNA digestion, by reducing disulphide bonds of the protein structure, with irreversible denaturation of these enzymes.

Subsequently, 350µL of RNA dilution buffer was added to 175µL of the lysate, mixing by performing inversion of the tube. It was then placed in a 70°C water bath for 3 min and centrifuged at 12,000-14,000 x g for 10 min at room temperature. The RNA dilution buffer precipitates the proteins, keeping the isolated RNA in solution. When this mixture is centrifuged, the cell debris and precipitated proteins removal is assured.

The supernatant was then transferred to the spin column, where 200 µL of 95% ethanol were added, selectively precipitating the RNA that was deposited and adsorbed on the silica membrane present in the spin column fibers, during 1 min centrifugation (12,000-14,000 x g, room temperature).

After, liquid was discarded from the collector tube, mainly containing impurities and cellular debris, and the spin column was placed in it again. 600µL of the RNA wash solution was added to the spin column assembly, centrifuged again under the same conditions mentioned above. The RNA wash solution allowed to remove present salts and impurities.

The protocol proceeds by applying a treatment with the mixture of DNase enzyme (previously prepared for each sample with 40µL of Yellow Core buffer, 5µL of 0.09M $MnCl_2$ and 5µL of the DNase I enzyme), for digestion of the genomic DNA, reducing possible contamination and interference in the future realization of the molecular amplification technique. 50 µL of the DNAse mixture was added directly to the spin column of each sample and incubated for 15-20 minutes at room temperature.

After this incubation, 200 µL of DNase Stop Solution was added to the spin column and centrifuged at 12,000–14,000 x g for 1 min (room temperature) to stop enzyme action and prevent cell damage. A first wash was performed with 600 µL of RNA washing solution and a second with 250 µL of this same sample and centrifuged at high speed for 2 min at room temperature. The last two washes ensure removal of impurities and total RNA will be eluted with 100 µL of RNA-free water into a 1.5 mL tube, centrifuged at 12,000–14,000 x g for 1 min at room

temperature. The resulting total RNA was promptly stored at -70ºC for future usage.

3.6. Quantification of total RNA and conversion to cDNA

The concentration of total RNA samples for K562 cells was determined by a fluorescence-based assay with a Qubit™ RNA HS assay kit (Q32852, Thermo Fisher Scientific Inc.) on a Qubit™ 3.0 fluorometer (Q33216, Invitrogen). The Qubit® RNA HS reagent contains a fluorescent compound that binds to RNA, increases its fluorescence, which was analysed using a fluorometer and increased depending on the concentration of RNA present in the sample.

To start, a 1 µL Qubit® RNA HS dilution was prepared with 199 µL of Qubit® RNA buffer. From the resulting product, 190µL were taken into two Qubit® test tubes, to have a standard solution. 10µL of each Qubit® standard was then added to the respective tube and 1 µL of sample to the respective test tube. All tubes were vortexed for 2-3 seconds and incubated for a period of 2 minutes at room temperature.

The recording of concentrations was carried out up to 3 hours after incubation. Since the equipment allows for defining the initial amount of each sample in the tube (1µL), the desired concentration unit (ng/mL, for this case) can be chosen and that calculation of concentration of total RNA present in each sample can be performed automatically.

From each sample, was taken a cellular amount corresponding to 2.0µg of total RNA, for conversion to complementar DNA (cDNA), using the Applied Biosystems™ TaqMan™ Reverse Transcriptase reagents (No. 4304134, Invitrogen™, Thermo Fisher Scientific Inc.), which is the reverse transcription (RT) enzyme required for conversion, with random hexamer primers, into a small-scale real-time reaction (20µL), following the manufacturer's specific instructions.

Each reaction is prepared with – 2.0 µL of 10x buffer for RT; 1.4 µL of 25 mM MgCl2; 4.0 µL of 10mM dNTP mix (each 2.5mM dNTP); 1.0 µL dithiothreitol (100mM stabilizer); 1.0 µL RNase inhibitor (20 U/µL); 1.0 µL of RT MultiScribe™ (50 U/µL) and 1.0 µL of random hexamer primers (50 µM). All these components were prepared as a main solution for all conversion reactions and later dispensed

into 0.5mL tubes. The calculated amount of RNA was added to each tube to result in a final volume of 20 µL. For this reaction, a Bio-Rad iCyler® thermocycler was used, at room temperature for 10 minutes, 37°C for 30 minutes, 95°C for 5 minutes and stopped at 4°C.

3.7. Quantitative real-time PCR (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed on a CFX Connect™ Real-time PCR Detection System (Bio-rad), using the Supermix iTaq™ Universal SYBR® Green (Bio-Rad) kit and primers specified (Table 3). Final volume was 20µL and instructions specified by the manufacturer were followed, performing this procedure. Supermix iTaq™ Universal SYBR® Green is a commercial ready solution that incorporates an iTaq DNA polymerase enzyme, MgCl2, SYBR Green I dye, dNTPS (deoxyribonucleotide triphosphate) and a mixture of passive reference dyes. For each reaction, Supermix iTaq™ Universal SYBR®, the DNA template that was amplified and specific primers were added, to detect the genes of interest. SYBR Green I is a fluorochrome that binds to double stranded DNA as it is synthesized. With a greater number of bonds formed, fluorescence intensifies, as higher concentrations of amplified DNA are noticed.

A PCR reaction can be partitioned into 4 stages: linear, exponential, log-linear and plateau phases. During the linear phase, corresponding to the first 10-15 cycles, the emission of fluorescence begins, which should not exceed the background level, namely base fluorescence level. In the exponential phase, it reaches a threshold, where fluorescence is significantly higher than the baselevels. The PCR cycle in which this phenomenon occurs is then called the cycle threshold (Ct). The amount of DNA is inversely proportional to this value. This means that the greater the amount of DNA in the initial sample, the faster the fluorescent signal increases and lower the Ct value will be. During the log-linear phase, a period of linear amplification is noted, with PCR product doubling at the end of each cycle. Finally, the plateau phase is reached when the reaction components become limited, and the fluorescence intensity is of no use. [86]

Table 3 - Specific primers used for the qRT-PCR

FUNCTION	GENE	GENBANK ACESS NUMBER	ALIGNMENT	PRIMER SEQUENCES	SIZE (PB)	MELTING TEMPERATURE (ºC)
HOUSEKEEPING	GAPDH[1]	NM_001 357943.2	Forward (5'→ 3') Reverse (3'→ 5')	GAGTCAACGGATTTGGTCGTA GCAGAGATGATGACCCTTTTG	295	54,7 53,7
EPIGENETIC MODULATORS	DNMT1[2]	NM_001 379.4	Forward (5'→ 3') Reverse (3'→ 5')	CCTCCAAAAACCCAGCCAAC TCCAGGACCCTGGGGATTTC	101	56,9 59
	DNMT3A[2]	NM_001 552.5	Forward (5'→ 3') Reverse (3'→ 5')	CCAACATCGAATCCATGAAA CTTGCGCTTGCTGATGTAGT	140	50,7 55,7
	DNMT3B[2]	NM_001 850.3	Forward (5'→ 3') Reverse (3'→ 5')	CGAATTTTACCACCTGCTGAATT AGAACGGCCGGTCATCAC	59	53,9 57,8
	HDAC1[3]	NM_001 964.3	Forward (5'→ 3') Reverse (3'→ 5')	GGAAATCTATCGCCCTCACA AACAGGCCATCGAATACTGG	168	54,3 54,8
	HDAC2[3]	NM_001 527.4	Forward (5'→ 3') Reverse (3'→ 5')	CTGTTAATTGGGCTGGAGGA AATTCAAGGATGGCAAGCAC	94	54,6 53,9
	HDAC3[3]	NM_001 355040.2	Forward (5'→ 3') Reverse (3'→ 5')	GGACCAGATCCTCCAGACAA CAGCCTCATCAGTCCTGTCA	116	56,1 56,5

(1) Obtained from Matos, 2020

(2) Obtained from D. Zhang, An, Li, & Zhang, 2019

(3) Obtained from software Primer3 and designed specifically for this study

qRT-PCR was used to determine the level of gene expression of epigenetic modulators (DNMT1, DNMT3a, DNMT3b, HDAC1, HDAC2 and HDAC3), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene to normalize gene expression. The individual and synergistic

epigenetic modulation mechanism of EGCG and GN was assessed through changes in DNMTs and HDACs gene expression in all treatments performed.

Conditions in each cycle in the Bio-Rad iCyler® thermocycler were as follows: polymerase activation at 95˚C for 2 minutes, denaturation at 95˚C for 30 seconds, annealing at 55˚C for 30 seconds, elongation at 72˚C for 1 minute and final elongation 72ºC for 15 min. After a total of 45 repeated cycles, denaturation curves were analysed to discard the hypothesis of primer dimers formation and possible contamination had occurred.

Relative quantification, through Ct values, is a method for analysis of the PCR, that demonstrates the difference between the expression of genes of interest in relation to a reference gene (GAPDH). It is obtained by normalizing Cts mean value with the mean Ct of the reference gene. Transcript or mRNA levels were expressed as a relative percentage and the calculation of the relative difference in gene expression was performed using the 2ΔΔCt method.

In the treatment with GN or in the co-exposure with EGCG and GN, the vehicle solution (DMSO) was used as calibrator and in the remaining treatments, the calibrator was the negative control (untreated cells). The reactions were performed in triplicate to ensure a smaller intra-assay variation, allowing for greater statistical power in the representativeness of the Ct value obtained for each sample. The values obtained from the three replicates per sample made it possible to calculate the mean and standard deviation, excluding values whose standard deviation was greater than 0.04.

3.8. Statistical methods

For the statistical analysis, the statistical computing software R, version 4.2.1 (2022 release) was used. Results for erythroid cell quantification were analysed through the Shapiro test to ensure that the data followed an approximately normal distribution. Variance was evaluated with the Levene test for normal populations and the Bartlett test for those that did not check for normality.

Significant differences between treatments were assessed applying the mean values against control (for EGCG), against vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control, since HU

exposure had to be repeated. These were evaluated through T-test, followed by ANOVA for all groups. P values lower than 0.05 were considered significant. All charts were designed with Microsoft Excel.

4. Results

4.1. Erythroid precursors exposure

Three assays were performed in total, with the first two ending before cDNA extraction, due to obstacles faced by the absence of holo-transferrin to produce phase II medium as intended by the cell culture protocol.

In the first assay, the cells were grown only in phase I medium. Immediately after blood harvesting, the first count was performed, inducing an amount slightly superior to those recorded after 7 days of incubation in phase I medium. However, as dead cells were not counted, it is not possible to compare in terms of cell viability.

In the second trial, an apo-transferrin conversion to holo-transferrin was executed. Unfortunately, severe cell contamination was detected before exposure to the compound and, therefore, cells were not counted.

In the third and final test, commercial holo-transferrin was bought and used, obtaining full results after 72 hours of incubation (in Annex I).

Normal distribution for alive cells can be assumed (Shapiro test), while for dead cell populations, in both donors, no statistically significant evidence exists to allow a normality assumption, since p-value is lower than 0,05. For both viable cells (Bartlett test) and dead cells (Levene test), it was assessed the absence of significant statistical evidence to suggest that the standard deviation is different for distinct treatment groups, within the different donors' populations.

According to collected data, it is possible to infer that, regardless of the individual's sex, after exposure, an increase in alive and total erythroid cells was observed with EGCG and GN, individually and in co-exposure (Figures 5 and 6). Additionally, analysis based on the donor's sex was performed. (Figure 7).

Cellular viability results were analysed for all treatments and compared between both donors. Results show higher percentage of dead cells when exposed to a standard treatment of HU (Figure 8) and, therefore, lower viability when compared to exposure to natural compounds (Figure 9). With viabilities over 10 to 15% higher when the erythroid cell line was exposed to natural compounds, as opposed to HU, it shows promise in lower cytotoxicity and greater safety in its ingestion in the human organism.

As explained previously, the quantification of extracted RNA was performed according to an assay with the Qubit™ reagent. It was not possible to detect RNA concentrations, as the values were inferior to the equipment's detectable limit. Thus, transcriptional analysis of the epigenetic regulatory genes was not carried out by qRT-PCR for the erythroid cell line.

Cells concentrations for different exposures (donor 1)

	Pre-exposure	GN	EGCG	EGCG + GN	HU	Vehicle
Dead	2,00E+04	7,20E+04	6,00E+04	3,60E+04	8,40E+04	4,80E+04
Alive	6,80E+04	2,36E+05	2,24E+05	2,20E+05	1,68E+05	1,48E+05

Figure 5 - Erythroid cell concentration from donor 1 (adult female)
Values at pre-exposure state and after exposure to the compounds (72h). Blue corresponds to alive cells and orange to the one's dead, confirmed using trypan blue. Both combined, correspond to the mean concentration of total cells observed per mL
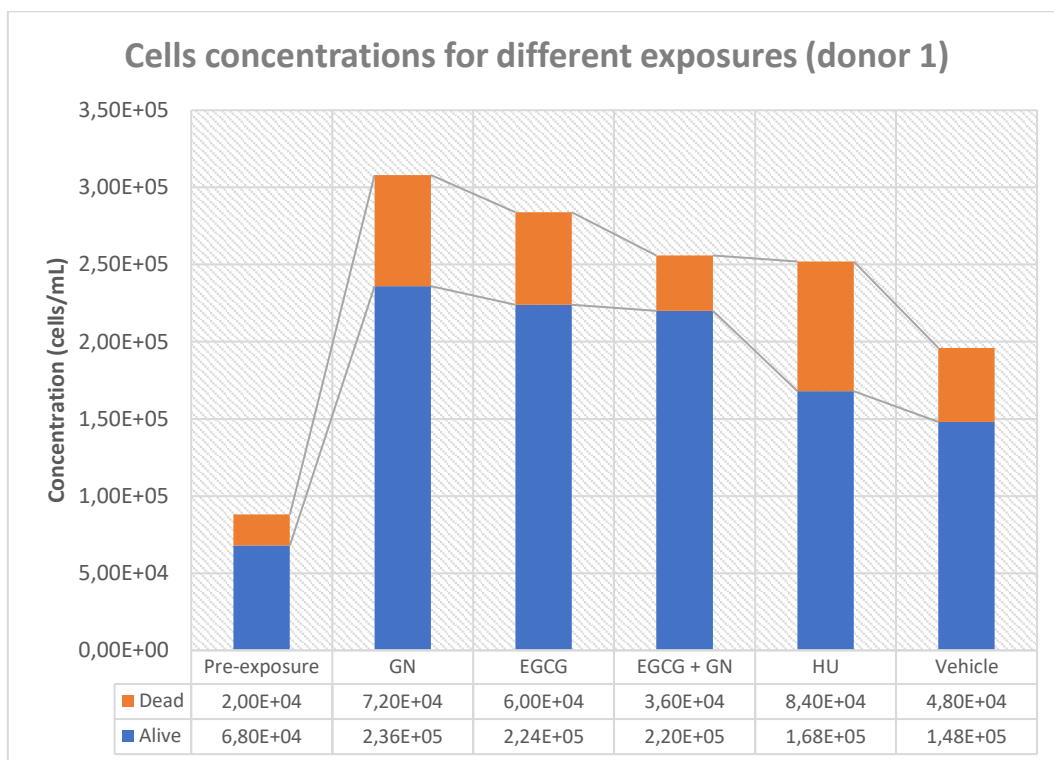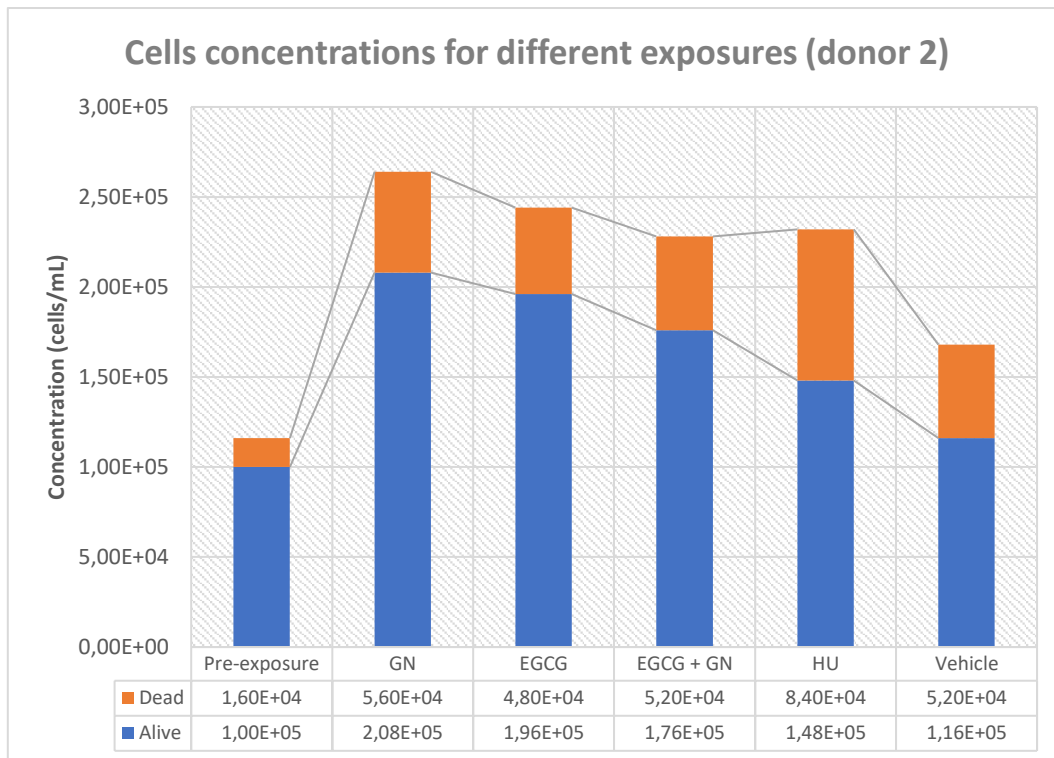
Figure 6 - Erythroid cell concentration from donor 2 (adult male)

Values at pre-exposure state and after exposure to the compounds (72h). Blue corresponds to alive cells and orange to the one's dead, confirmed using trypan blue. Both combined, correspond to the mean concentration of total cells observed per mL

	Pre-exposure	GN	EGCG	EGCG + GN	HU	Vehicle
Dead	1,60E+04	5,60E+04	4,80E+04	5,20E+04	8,40E+04	5,20E+04
Alive	1,00E+05	2,08E+05	1,96E+05	1,76E+05	1,48E+05	1,16E+05
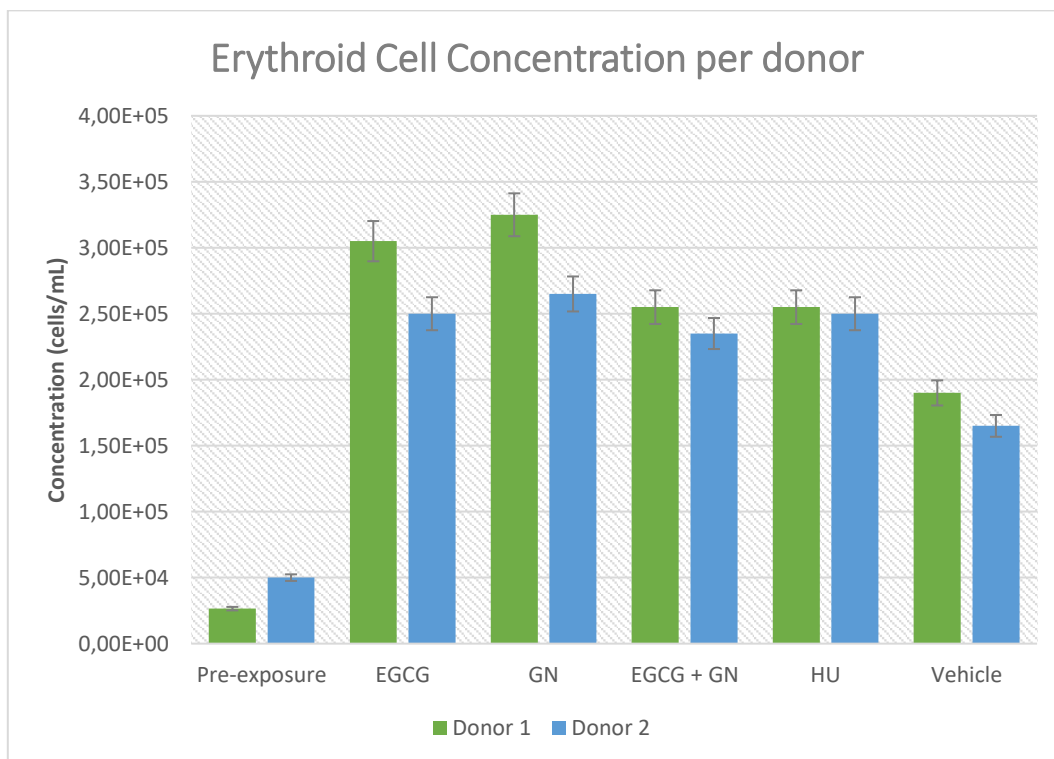
Figure 7 - Cell concentration (cells/mL) per donor per compound (72h); calculated by the traditional counting method. Donor 1 is the adult female and donor 2 is the adult male
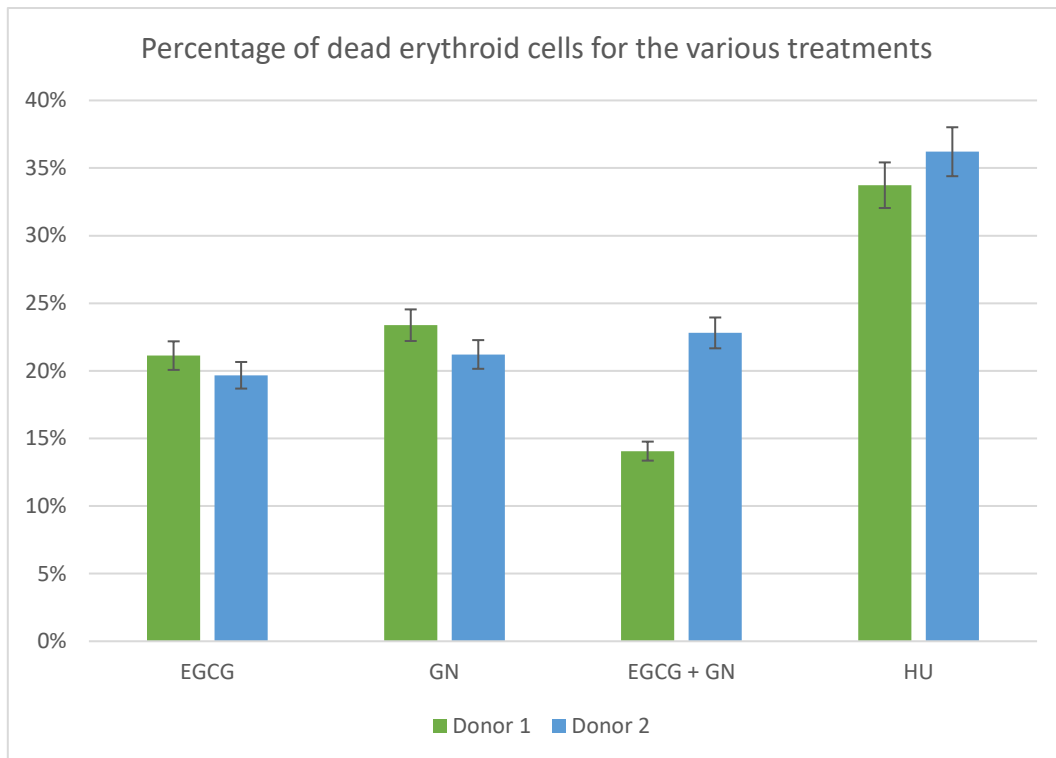
Figure 8 - Comparison of cellular viability per donor and per compound (72h); Donor 1 is the adult female and donor 2 is the adult male
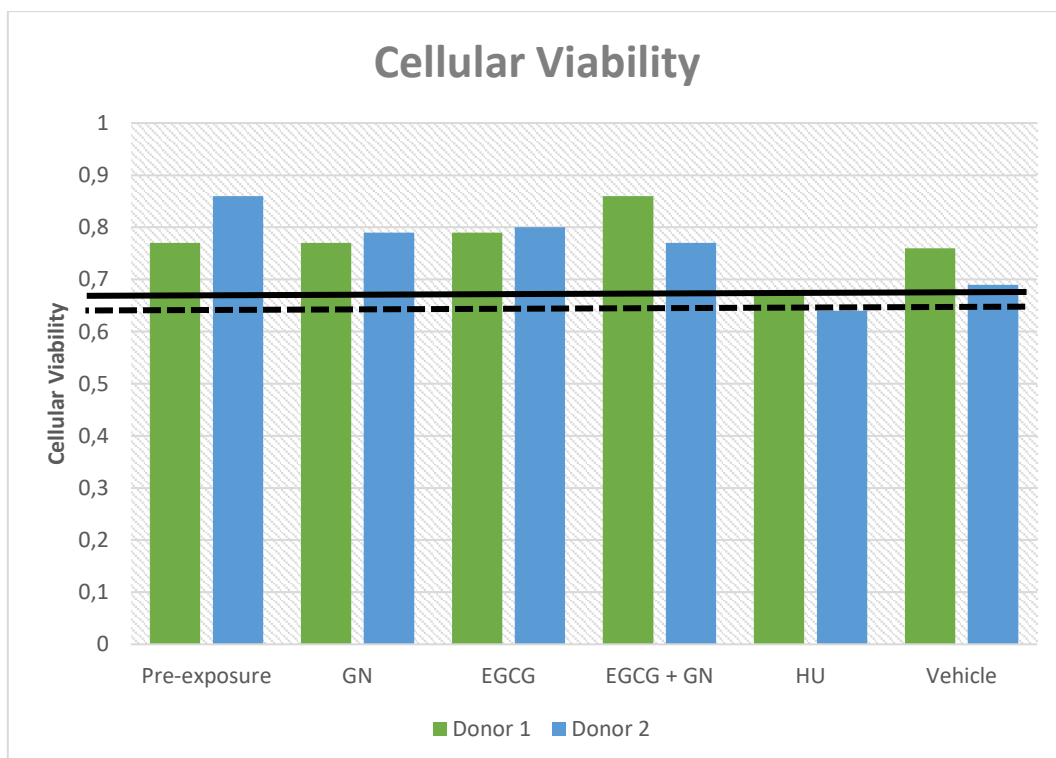
Figure 9 - Comparison of cellular viability per compound exposure (72h) and donor
A straight line (donor 1, adult female) and a dashed line (donor 2, adult male) for the maximum viability value for HU was placed to validate the viability for the other compounds

4.2. K562 immortalized cells exposure

For the K562 immortalized cell line, the same cellular counting and viability assessment methods were used (full results in Annex II). The absence of significant statistical evidence to suggest that the standard deviation was different for distinct treatment groups was verified and confirmed through the Bartlett test (p=0,1516). Normal distribution for alive and dead cells can was assumed (Shapiro test, p=0,0638).

Total K562 line cell concentrations (figure 10) and their cellular viability after exposure to the different studied compounds (figure 11) results were evaluated. K562 viability after 72 hours was around 90% for all compounds with minor dead K562 cells concentration to be noted. Higher total k562 cell concentration was registered for the exposure to EGCG, with co-exposure to both EGCG and GN demonstrating lower total K562 cell concentration from all exposures. Experiences with GN and HU shown total concentrations between those seen for EGCG and co-exposure to both EGCG and GN.

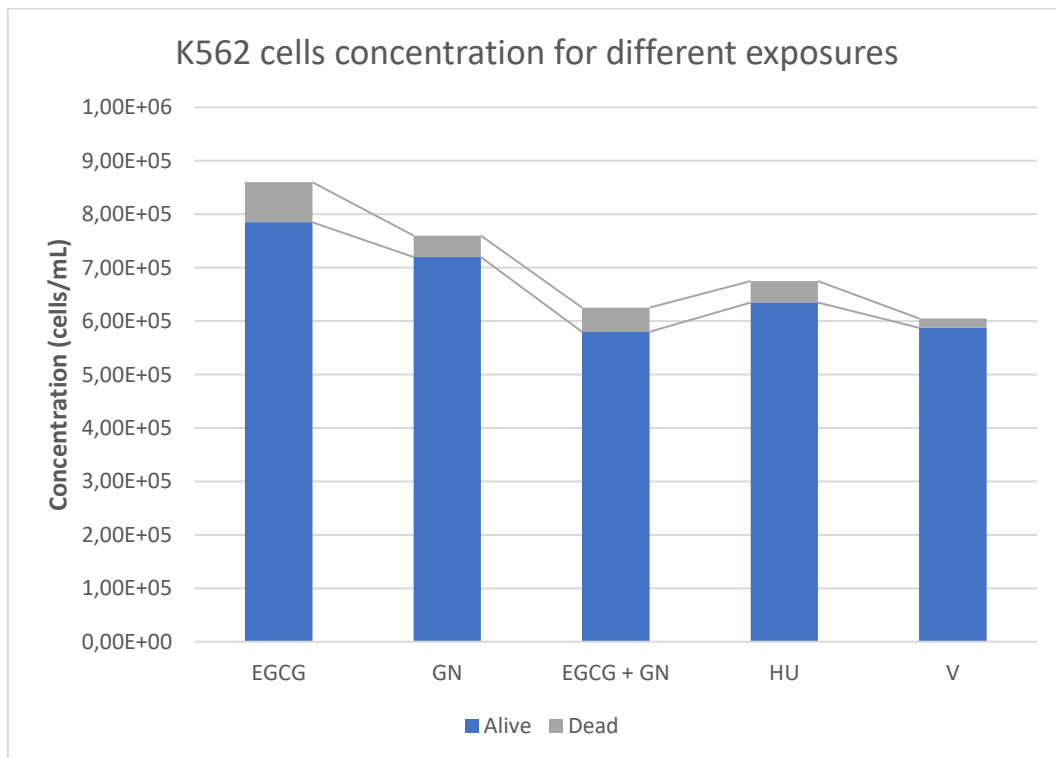RNA concentrations were obtained according to an assay with the Qubit™ reagent (Annex III).

Figure 10 - K562 cell concentration comparison between different treatments (72h)
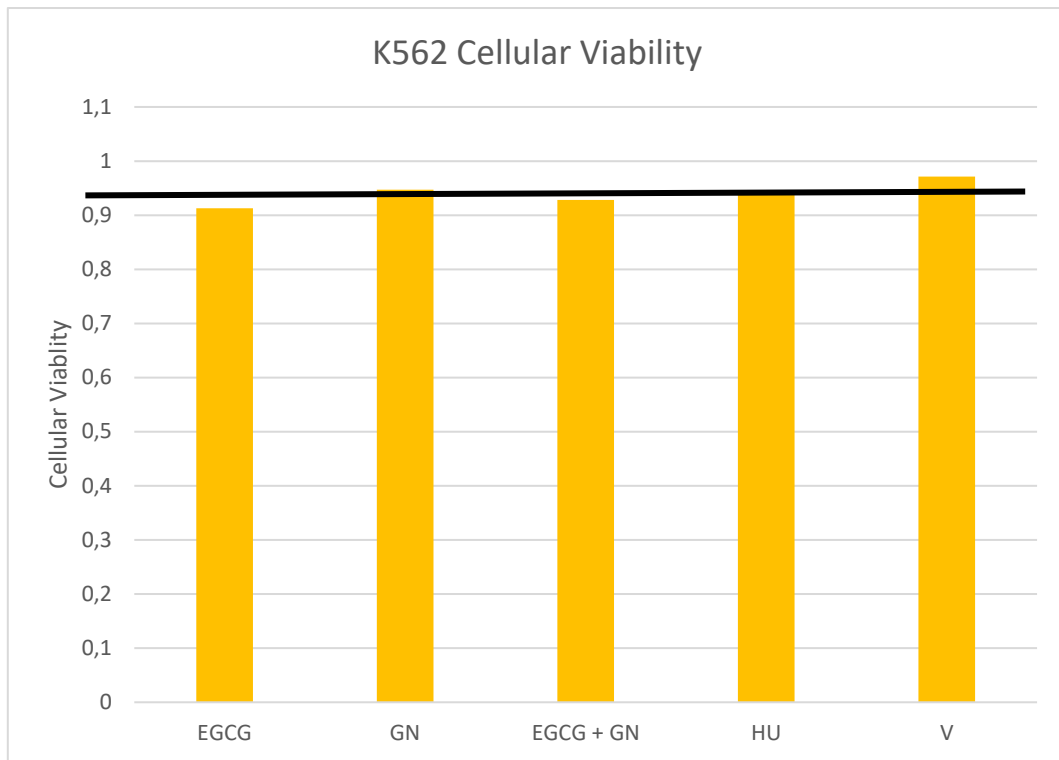
Figure 11 - Comparison of K562 cellular viability per compound exposure (72h)
A straight line for the maximum viability value for HU was placed to validate the viability for the other compounds

4.3. K562 epigenetics qRT-PCR results

In this section, results were plotted for the values of Log2fold change, based on the 2ΔΔCt method, as mentioned previously. Statistically significant results were signed with asterisk (*) for the respective treatment bar.

4.3.1. DNMT1 gene

Related to the expression of the DNMT1 gene for a 72h period (Figure 12), statistically significant differences were noted for an increase in this gene's expression after exposure to EGCG (p=0.014) and to HU (p=0.028). Oppositely, a decrease in DNMT1 regulation was observed when exposed to GN (p=0.041).

Figure 12 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
*Data represent relative expression for the DNMT1 gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control. T-test values are illustrated as: *p<0.05*

4.3.2. DNMT3a gene

For DNMT3a (Figure 13), exposure to a standard treatment (HU) for a 72h period, outcomes significant differences in the shape of a decrease in expression for EGCG (p=0,035), GN (p=0,046), co-exposure to both EGCG and GN (p=0,039) and HU (p=0,004) in comparison to their respective controls.

Figure 13 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
Data represent relative expression for the DNMT3a gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control. T-test values are illustrated as: *p<0.05 and **p<0.01.

4.3.3. DNMT3b gene

Presented in Figure 14, the graph displays a considerable increase in DNMT3b gene expression for EGCG (p=0,021), co-exposure to both EGCG and GN (p=0,026) and HU (p=0,033) in comparison to their respective controls. On the opposite, GN (p=0,032) demonstrated a decrease in gene expression.
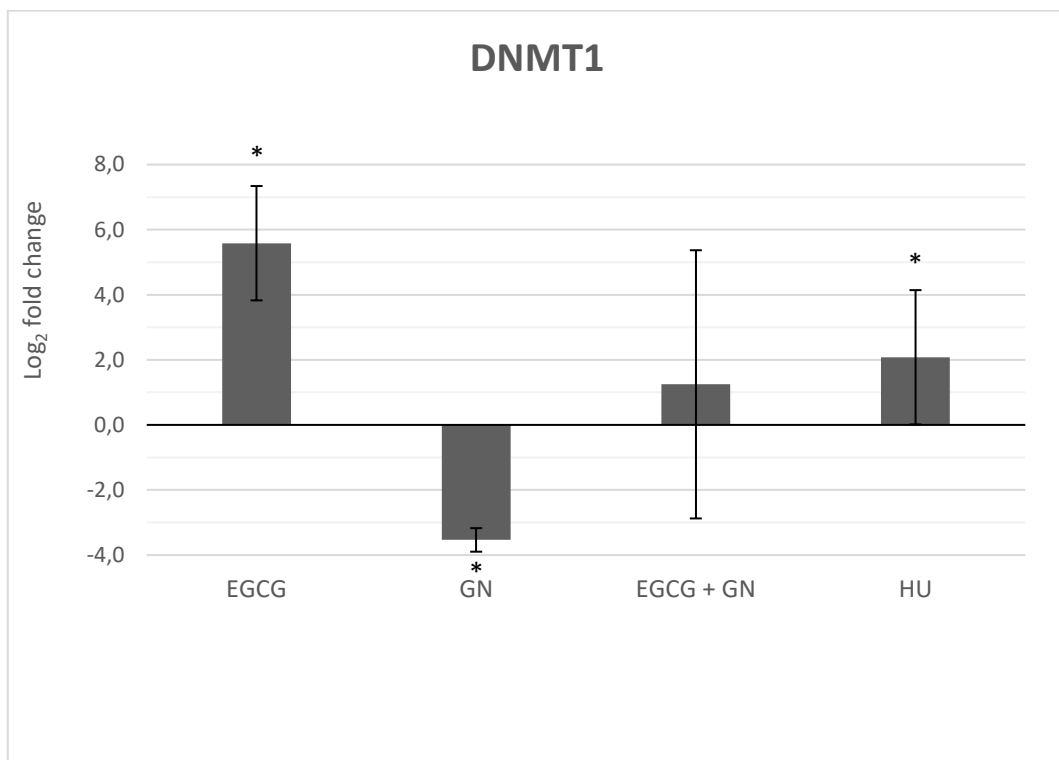

Figure 14 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
*Data represent relative expression for the DNMT3b gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control T-test values are illustrated as: *p<0.05.*

4.3.4. HDAC1 gene

As for the qRT-PCR results performed for the HDAC1 gene (figure 15), different responses were obtained for the different compounds of exposure. Exposure to HU resulted in a considerable decrease in gene expression at 72H (p=0.019) when compared to the control sample, while co-exposure to both EGCG and GN (p=0,049) also revealed a decrease. An increase with statistical significance was observed for the exposure of K562 cells to EGCG (p=0,011) and GN (p=0.02).
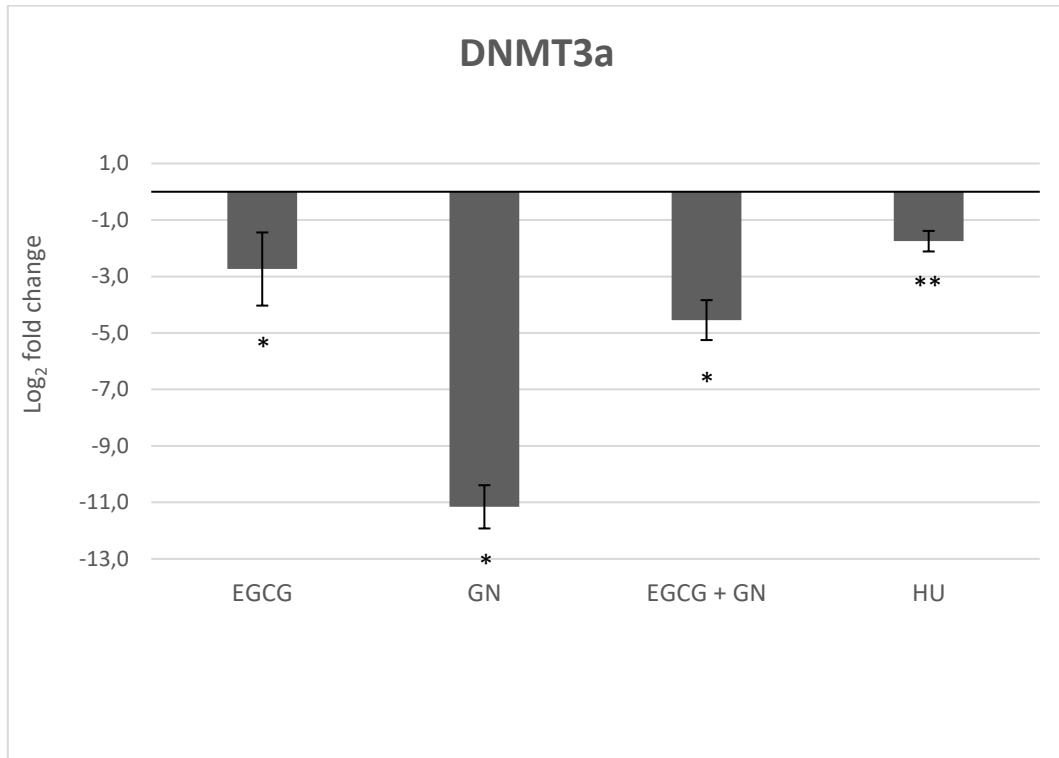

Figure 15 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
*Data represent relative expression for the HDAC1 gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control. T-test values are illustrated as: *p<0.05.*

4.3.5. HDAC2 gene

Linked to HDAC2's gene expression (Figure 16), a significant decrease in expression when cells were co-exposed to EGCG with GN (p=0.021) was observed in relation to its control (vehicle). The same reaction was demonstrated by HU (p=0,042) and individual exposure to GN (p=0,047). On the other hand, EGCG (p=0,033) demonstrated upregulation.
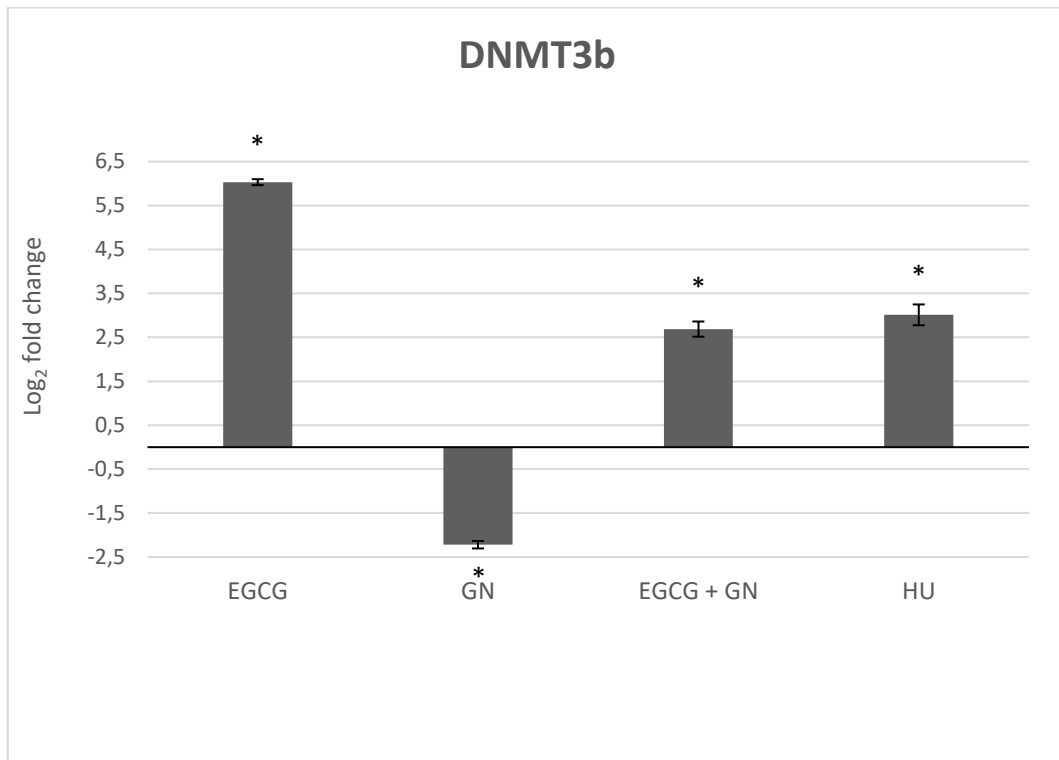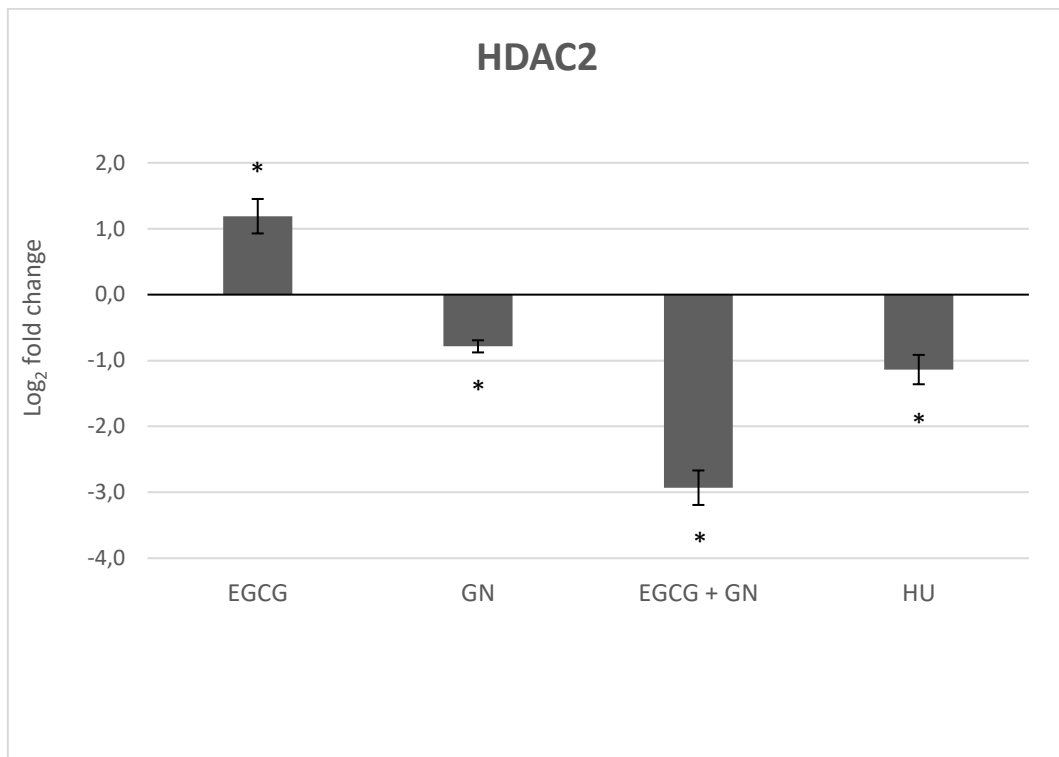

Figure 16 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
*Data represent relative expression for the HDAC2 gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control. T-test values are illustrated as: *p<0.05.*

4.3.6. HDAC3 gene

In the analysis of histone 3 deacetylases expression (figure 17), it can be observed in the graph a significant increase in expression from GN (p=0.017) in comparison to the vehicle. Other statistically substantial variation of notice was a decrease in expression of the HDAC3 gene when K562 cells were exposed to HU (p=0.03).
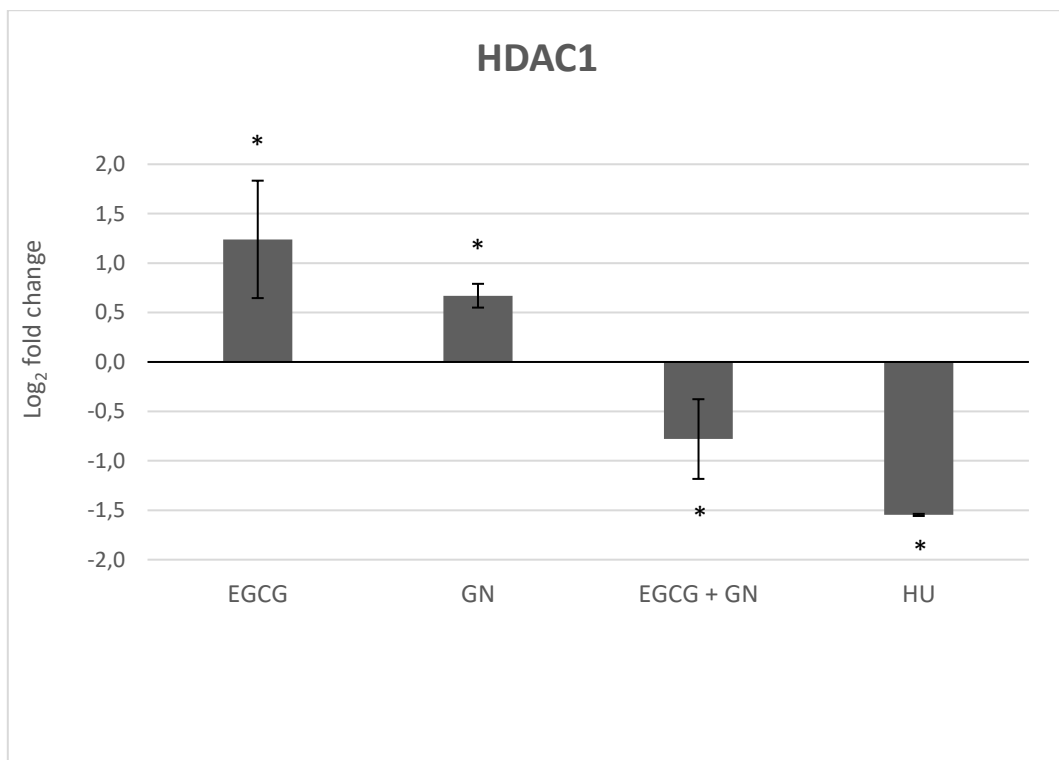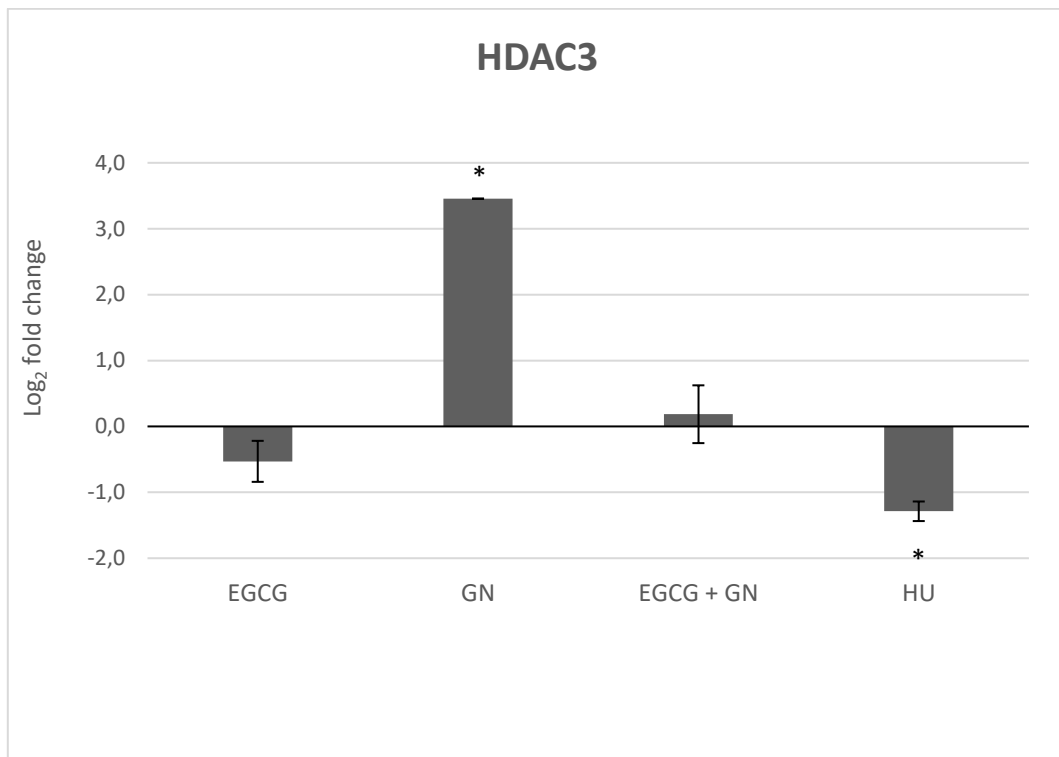

Figure 17 - PCR in K562 cells after exposure to GN, EGCG, co-exposure (GN+EGCG) and HU for 72h
Data represent relative expression for the HDAC3 gene. GAPDH was used for normalization. Error bars represent the standard deviation between two treatments. independent and three qRT-PCR replicates. Significant differences between treatments were assessed against control (for EGCG), vehicle (for GN and co-exposure of EGCG with GN) and, for HU, against a specific control. T-test values are illustrated as: *p<0.05.

5. Discussion

5.1. EGCG and GN demonstrated potentially lower cytotoxicity and higher cellular viability compared to HU

It can be stated that the treatments did not affect the analysed cell line viability, not showing themselves as majorly cytotoxicity compared to HU, preserving a larger cell population. The lower percentage of dead erythroid cells, underlying higher cell viability when compared to their exposure to HU, provide a high degree of confidence in the application of EGCG and GN, either standalone or in co-exposure, to patients with SCD. [100]

Exposure to GN, EGCG and both combined shown lower percentage of dead cells (Figure 8) and, consequently, higher cellular viability when compared to the standard treatment of HU (Figure 9). Given such a one-dimensionality for the results obtained for the exposure to all treatments under study in this cell line, there are no great correlations compared to the results previously described for the erythroid cell line, where it can be highlighted a greater viability of EGCG and GN compared to the exposure of this cell line to HU.

It would be expected cytotoxicity from EGCG, although it presents behavioural duality, acting as pro-oxidant/carcinogenic and antioxidant/anti-carcinogenic [97], and GN, as it induces apoptosis in a variety of cell lines, but also autophagic cell death. [98] As already studied and discussed, the standard treatment with HU also shows levels of cytotoxicity *in vitro* as demonstrated by other authors [25][99][100]. These results reveal less cytotoxicity using natural compounds, establishing them as more viable options when it comes to this parameter. [101][102]

According to the graphs from figures 5 and 6, for both donors, the number of alive cells is higher than those counted dead, which represents a cellular viability superior to 50%. A substantial increase of total cells after exposure to the different treatments has been verified for both donors' erythroid cell cultures. However, that increase was less substantial when cells were exposed to a standard treatment (HU). Reticulocytes synthesis can increase by two tens in patients with SCD, resulting in the alteration of erythrocytes circulation patterns

in the blood. [93] Therefore, in healthy individuals, at the time of collection, the number of available precursors is lower, since they are produced in the bone marrow and subsequently allocated to where their maturation is necessary and tends to decrease after the harvesting. [94] Therefore, observed variations may be related to the period when erythroid precursors were counted after blood collection, thus no negative impact from the exposure to EGCG and GN to assign to these changes between pre and post exposure to these compounds. [92]

Concentration of total cells, achieved through the traditional cell counting method, were displayed in the graph from figure 7, with apparent higher concentration of cells in donor 1 after exposure to all treatments. These results differ from literature, where differences between erythroid precursor counts depending on sex are known. In other studies, reticulocyte counts were, on average, 20% higher in men compared to women, where adult female rats injected with testosterone increased their number of peripheral blood reticulocytes. [91] It is expected that viability is higher in male donors, as they have a higher number of erythroid precursor cells and, therefore, viability is expected to be higher [95][96]

Results presented for erythroid precursor counting's were performed after two culture phases as recommended, with all reagents commercially available acquired to avoid contamination, thus under as close to ideal conditions as possible. To establish an erythrocyte precursor cell culture, several components are essential to maintain these cell lines growth, to promote synthesis of Hb. Moreover, cell culture in contaminated media would result in cell viability decrease, thus negatively influencing results, therefore achieving the presented results was rather challenging, but crucial to further validate EGCG and GN as save compounds for cells. [90]

For the K562 immortalized cell lines (Figure 10), it appears that exposure to both EGCG and GN combined, resulted in a lower concentration of K562 cells when compared to their individual exposure and to HU for the same period (72h). Viability (Figure 11) was around 90% for all treatments, with no major differences to be noted. The effects of exposure to HU in K562 cell lines have been studied in the past, with decreased the percentage of viable and overall cells, as well as a reduction of Hb generation. [103][104]

As for green tea constituents (EGCG and GN), research on K562 cell lines exhibited that catechins like EGCG may play a role in protecting cellular damage, preventing disease and being most effective cancer chemopreventive polyphenol in green tea. [103] On the other hand, studies related to GN in the same cell lines, demonstrated that soy consumption in diet inhibited cell proliferation and enhanced its apoptosis. [104]

Overall, EGCG and GN did not exhibit major cytotoxicity in comparison to a standard treatment. These results attribute these natural compounds a high level of trust in their application to SCD patients, without majorly affecting cells. [100]

5.2. Influence of natural compounds on epigenetic regulators

5.2.1. Natural compounds on DNMTs

Since there is no evidence of these two natural compounds studied in co-exposure to both DNMTs and HDACs as genes of interest, the results obtained are somewhat pioneering in this field of study. Limitations in obtaining the indicated volume of blood specified in the erythroid precursor cell culture protocol, followed with a very complex experimental procedure, prevented the extraction of amounts of RNA that would allow the study of epigenetic regulation by the effect of the constituent compounds of green tea, specifically EGCG and GN, in erythroid precursors as intended. Results were obtained for K562 immortalized cell lines.

For DNMTs, according to the graphs from figures 12, 13 and 14, results demonstrate consistent decrease in expression at 72h when cells are exposed to GN, compared to its respective control (vehicle). Scientific literature on this matter, describe a downregulation of DNMT1 by exposure to the compound at 72h, stating minimal gene expression and maximum inhibition with GN exposure in DNMT1. [107] The same study states that exposure for longer periods can restore some expression potential of DNMT1, DNMT3a and DNMT3b genes. The lack of results for other time periods other than 72h excludes the possibility of direct comparison of quantitative analysis between different states of exposure.

Future validations on this matter would be beneficial and would substantially validate the results obtained with previously validated literature and to further evaluate the response in time for other treated samples. [107]

For the DNMT3a gene in particular, significant differences to their respective treatments were detected for 72h exposure to treatments of EGCG, GN (very substantial) and HU. In certain cell lines, it has been stated that DNMT1 and DNMT3b have comparatively higher transcription levels to the DNMT3a gene. Such behaviour has never been confirmed for K562 cell lines. [108] With that in mind, this may explain significant decreases in DNMT3a gene expression, when compared to DNMT1 and DNMT3b, independent of exposure to natural green tea compounds (EGCG and/or GN) or a standard treatment of HU. [108] Graphs for DNMTs gene expression demonstrate these results, with DNMT1 and DNMT3b having similar results. EGCG demonstrating higher expression, followed by HU and co-exposure to both EGCG and GN, demonstrating this natural compound's potential for epigenetic regulation in DNMTs. [109]

Co-exposure to both EGCG and GN in DNMTs demonstrated lower quantitative gene expression when compared to EGCG on its own. These results, aligned with those from exposure to GN in DNMTs (decrease in expression at 72h), establish a link to various other scientific reports and literature, affirming that cellular exposure to GN significantly decreases the enzymatic activity of DNMTs, thus slightly decreasing the effects of co-exposure when compared to solo exposure of EGCG. [110]

As for EGCG, several other studies revealed that this polyphenol present in green tea decreased overall levels of DNA methylation and showed a protective effect by inhibiting promoter hypermethylation of specific genes. These effects were attributed to decreased mRNA activity and expression of DNMT1 and EGCG proteins, inducing the binding domain of DNMT1 to the promoter of specific genes. [111]

Overall, results confirmed most of the statements presented in scientific literature thus far and co-exposure to both green tea polyphenols exhibited increased potential for the activity of DNMTs in varied gene regulatory roles, such as transcriptional silencing, transcriptional activation, and post-transcriptional regulation, making DNMTs a versatile toolkit for epigenetic regulation. [112]

These results boost the prospect of EGCG and GN as effective tools in HbF induction, either standalone (particularly EGCG) or in co-exposure, as they have shown upregulation in DNMTs, potentially reversing the silencing of globin genes, and allowing the production of HbF in patients with SCD, like a standard treatment of HU, the only approved therapy by the FDA for SCD associated with HbF induction to relieve symptomatic crises. [109][112]

5.2.2.   Natural compounds on HDACs

Like DNMTs, HDACs are known in the scientific community for their epigenetic regulatory behaviour. They are responsible for the histone tail regulation, protein-DNA interaction, Their even act as transcription and post transcription modifiers. [113] Studies shown the great potential of these mechanisms in different pathologies, like cancers or cardiovascular diseases. [109][114][115] Therefore, evaluating the two natural compounds studied in co-exposure to the following genes of interest was also relevant.

For HDAC1 (figure 15), EGCG (p=0,011) and GN (p=0,02) significantly increased the expression of deacetylases at 72h, while HU (p=0,019) significantly decreased it. Co-exposure to both EGCG and GN (p=0,049) combined demonstrated downregulation in the HDAC1 gene. GN proved HDAC1 inhibitory effect, that is dose-dependent and DNMT1 activity-dependent, also decreasing the activity of DNMTs in KYSE cells. [111] These compounds effectiveness and influence in epigenetic regulators is susceptible to several factors, such as the concentration and levels of HDAC1 translated in the cells. [116]

As for EGCG, results obtained for our essays challenge those from other research literature, where EGCG-treated HeLa cells showed no significant changes in HDAC1 expression compared to untreated cells, [117] while in breast cancer cells, a decline in HDAC1 protein was detected. [118][119][120] It also reduced the enzymatic activity of DNMTs and HDACs in HeLa cells, which resulted in the reactivation of tumour suppressor genes. [118]

As for HDAC2 (figure 16), statistically significant changes were found for the co-exposure EGCG and GN (p=0,021), as well as HU (p=0,042), delighting considerable downregulation. EGCG (p=0,033) demonstrated upregulation,

which opposes literature, that claims EGCG action on HDAC2 to reduce the biochemical HDAC activity. [121][122]

GN (p=0,047) showed genomic down-regulation, as normally described (although not statistically significant compared to its "vehicle control"). [123] The absence of results for other time periods, precludes the afference of a biphasic behaviour disposed by this compound. [107] It was expected a weaker response to GN exposure since it is stated to cause weaker responses than EGCG. However, co-exposure shown lower levels compared to even GN, which indicates fault in these results. [111]

Regarding HDAC3 (figure 17), GN (p=0,017) exposure caused significant up-regulation, with HU (p=0.03) causing the inverse effect. Even though not yet verified and lacking more scientific validation, EGCG can act as an inhibitor in post-translational histone modifications. One of the proposed mechanisms identified in colon cancer cells suggests that catechin may contribute to the degradation of both DNMT1 and HDAC3. [124]

Interactions between EGCG to DNMT1, DNMT3B and HDAC1 are backed by scientific research. [119][125][126] These are expected to cause the reduction of DNA hypermethylation, reducing the expression levels of these genes, re-establishing the expression of repressed genes, [119] as intended with HbF for SCD patients. In myeloid cell lines (closer to K562 used in our essays), EGCG downregulated the expression of DNMT1, HDAC1 and HDAC2. [123][127][128]

Green tea polyphenols target HDACs for inhibition, leading to increased histone acetylation that regulates gene expression. [128] This causes chromatin to reach a more open configuration that promotes replication, repair, and transcription. [123][129]. Supplementation through polyphenols and flavonoids allows the suppression of HDACs, thus becoming great targets of interest for future studies in terms of epigenetic regulation. [128]

Throughout this research, these effects were always evaluated against a standard treatment of HU, the only inducer currently approved by the FDA. This therapeutic agent can induce HbF production and increase its levels. [130][131]

Results for EGCG and HU, as standalone or in co-exposure, demonstrated mixed results that did not correlate with literature. [121][122] HDAC inhibition on the target can induce cytotoxicity, potentially limiting the role of these epigenetic targets and of referred compounds in inducing HbF, which may be the reason

affecting our ability to take major conclusions for the effects of the natural compounds on HDAC regulation for potential HbF induction in SDC patients. [25][128]

6. Conclusions

Obtained results demonstrated the potential EGCG and GN in the epigenetic regulation in DNMTs in the K562 cell line, with mixed results in HDACs, prospecting the possible induction of HbF in patients with SCD, with concentrations of these compounds physiologically reachable by diet or supplementation and, apparently, less cytotoxic to cell than HU, thus displaying higher cellular viability when exposed to these treatments.

Most previous studies depict responses of the influence of these compounds about their anti-carcinogenic effects. A new potential for EGCG and GN in relation to the ability to induce HbF expression through epigenetic regulation presents itself as as exciting possibility for SCD and overall β-hemoglobinopathies alike. The results obtained require a more in-depth study of the effects of these natural compounds and structural analogues in a well-established erythroid progenitor cell line, to assess their effect on epigenetic regulators as well as whether this regulation will promote the induction HbF and become an addiction to well established therapy for β-hemoglobinopathies.

To recognize and classify easily accessible natural compounds, like green tea polyphenol which, as individual or in co-exposure, could affect epigenic regulators to relate to an increase in HbF expression was the main aim of this research study. Future investigations that establish these compounds into therapeutic agents in the induction of HbF, easily accessible to patients with hemoglobinopathies, may further confirm their high capacity.

7.Future perspectives

The promising results and conclusions obtained throughout this study encourage a future extension in this research topic.

Mainly, by increasing the amount of blood collected from donors and improving cell culture conditions, it is expected to better establish a lineage of erythroid precursor cells, to further determine on HbF induction through the examined compounds, although it would be more desirable to collect samples from SCD donors, which possess higher quantity of reticulocytes and, therefore, lower blood volumes are required. With increased concentrations of compounds in co-exposure, the results may reveal themselves more robust, while a substantial and uniform response in relation to epigenetic regulators may occur, thus better understanding the efficacy and safety of GN and EGCG in HbF induction. Future comparation for different time periods before and after 72h (48 and 96h, for instance), would be highly desirable for determine and confirm how an exposure affects regulation over time for the compounds in co-exposure.

On the other hand, there should be an effort to interpret compounds factors and action mechanisms involved in γ-globin gene expression. Complementary studies using other compounds would also be a plus.

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Annexes

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Annex I

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Table I-1 - Erythroid cellular quantification at pre-exposure state

	Pre-exposure					
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	3,4	1	4,4	5	0,8	5,8
Nº Cells /mL (Traditional)	6,8E+04	2E+04	8,8E+04	1E+05	1,6E+04	1,16E+05
Viability	0,77			0,86		

Table I-2 - Erythroid cellular quantification after exposure to GN

	Post exposure to GN					
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	11,8	3,6	15,4	10,4	2,8	13,2
Nº Cells /mL (Traditional)	2,36E+05	7,2E+04	3,08E+05	2,08E+05	5,6E+04	2,64E+05
Viability	0,77			0,79		

Table I-3 - Erythroid cellular quantification after exposure to EGCG

	Post exposure to EGCG					
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	11,2	3	14,2	9,8	2,4	12,2
Nº Cells /mL (Traditional)	2,24E+05	6E+04	2,84E+05	1,96E+05	4,8E+04	2,44E+05
Viability	0,79			0,8		

Table I-4 - Erythroid cellular quantification after exposure to EGCG + GN

Post exposure to EGCG + GN						
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	11	1,8	12,8	8,8	2,6	11,4
Nº Cells /mL (Traditional)	2,2E+05	3,6E+04	2,56E+05	1,76E+05	5,2E+04	2,28E+05
Viability	0,86			0,76		

Table I-5 - Erythroid cellular quantification after exposure to HU

Post exposure to HU						
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	8,4	4,2	12,6	7,4	4,2	11,6
Nº Cells /mL (Traditional)	1,68E+05	8,4E+04	2,52E+05	1,48E+05	8,4E+04	2,32E+05
Viability	0,67			0,64		

Table I-6 - Erythroid cellular quantification after exposure to DMSO (vehicle)

Post exposure to DMSO						
	Donor 1			Donor 2		
Cells	Alive	Dead	Total	Alive	Dead	Total
Mean	7,4	2,4	9,8	5,8	2,6	8,4
Nº Cells /mL (Traditional)	1,48E+05	4,8E+04	1,96E+05	1,16E+05	5,2E+04	1,68E+05
Viability	0,76			0,69		

Table II-1 - K562 cellular quantification after exposure to GN

Post exposure to GN			
Cells	Alive	Dead	Total
Mean	39,25	3,75	43
Nº Cells /mL (Traditional)	7,20E+05	4,00E+04	7,60E+05
Viability	0,95		

Table II-2 - K562 cellular quantification after exposure to EGCG

Post exposure to EGCG			
Cells	Alive	Dead	Total
Mean	36	2	38
Nº Cells /mL (Traditional)	7,85E+05	7,50E+04	8,60E+05
Viability	0,91		

Table II-3 - K562 cellular quantification after exposure to EGCG + GN

Post exposure to EGCG + GN			
Cells	Alive	Dead	Total
Mean	29	2,25	31,25
Nº Cells /mL (Traditional)	5,80E+05	4,50E+04	6,25E+05
Viability	0,93		

Table II-4 - K562 cellular quantification after exposure to HU

Post exposure to HU			
Cells	Alive	Dead	Total
Mean	31,75	2	33,75
Nº Cells /mL (Traditional)	6,35E+05	4,00E+04	6,75E+05
Viability	0,94		

Table II-5 - K562 cellular quantification after exposure to DMSO (vehicle)

Post exposure to DMSO			
Cells	Alive	Dead	Total
Mean	29,375	0,875	30,25
Nº Cells /mL (Traditional)	5,88E+05	1,75E+04	6,05E+05
Viability	0,97		

Table II-6 - RNA quantification from K562 cell lines

Assay		Concentration (ng/uL)
72h - A	EGCG	49,5
	GN	43,3
	E + G	38,7
	V	8,6
	CN	22,2
	HU	291,1
	CN HU	113,2
72h - B	EGCG	23,1
	GN	12,7
	E + G	17
	V	7,7
	CN	62
	HU	184,4
	CN HU	168,8

(Note: CN HU represents a specific control since exposure to HU had to be repeated)