Haemoglobin and haemoglobinopathies at the forefront of the 21st century

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Conflicts on interest: The author declares no conflict of interest concerning this article.

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Hemoglobin, sickle cell disease, thalassemia

Abstract

This review aims to take stock of recent advances and perspectives in various fields (physiology, physiopathology, and therapy) related to haemoglobin and haemoglobin disorders. This includes gaseous signalling molecules (NO, CO, H_2S), blood flow changes in microcirculatory units, metabolic regulation of red blood cells and other systems, the regulatory stages that determine the expression and intracellular concentration of a model protein, control of differentiation and cell mass of tissues or organs, gene switching during development and its therapeutic reversion, comprehensive care of patients with sickle cell disease or β-thalassaemia, targeted therapies, allo-transplantation, and gene therapy (gene addition or gene editing).

Résumé

Cette revue a pour but de faire le point des avancées récentes et des perspectives dans différents domaines (physiologiques, physiopathologiques et thérapeutiques) de l'hémoglobine et des hémoglobinopathies. A savoir: les gaz transmetteurs de signaux (NO, CO, H2S); l'ajustement du débit sanguin des « unités microcirculatoires »; l'orchestration métabolique du globule rouge et au-delà; la régulation « étagée » de l'expression et de la concentration intracellulaire d'une protéine modèle; le contrôle de la différenciation et de la masse cellulaire d'un tissu ou organe; la commutation de gènes lors du développement et sa réversion thérapeutique; « la prise en charge globale » des patients drépanocytaires ou β-thalassémiques; les agents thérapeutiques ciblés, les allogreffes et la thérapie génique par addition ou édition de gènes.

Context

Haemoglobin

Haemoglobin (Hb) has become known as the protein of the 20th century, due to key discoveries that made it a model for the three-dimensional structure of biological macromolecules, complex physiological functions and "molecular diseases". It remains at the forefront of medicine to this day, particularly with regards to innovative biotherapies.
Experimental reconstruction of the evolution of Hb [1] shows that "modern Hbs" derive from a common molecular ancestor formed by a single globin chain associated with haem, which has a very high affinity for oxygen (O_2). This is exemplified, in particular, by myoglobin. The "historical missing links" only required a small number of mutations to give rise to homodimers, and then to allosteric heterotetramers, such as HbA ($\alpha_2\beta_2$). Some artificial mutations have

*Pour citer cet article : Beuzard Y. Hémoglobine et hémoglobinopathies à l'avant-garde au xxie siecle. *Hématologie* 2021 ; 27 (2) : 55-77. doi: 10.1684/hma.2021.1647

To cite this article: Beuzard Y. Haemoglobin and haemoglobinopathies at the forefront of the 21st century. *Hématologie* 2021; xx(x): 1-21. doi: 10.1684/hma.2021.1687

doi: 10.1684/hma.2021.1687

created polymers of Hb, as well as new functions. In total, 1,820 naturally occurring mutations in Hb, of which 230 have been recently discovered, have been identified which have implications on the structure, function, and molecular pathology of Hb, secondary alterations of red blood cells (RBCs) and their clinical consequences [2]. Other members of the globin superfamily (cytoglobin, neuroglobin, androglobin, *etc.*) play multiple roles in the anti-stress homeostasis of all cells [3]. Through its interactions, Hb orchestrates energetic (anaerobic glycolysis), redox and nucleotide (pentose phosphate pathway) metabolism [4]. Hb ensures the buffering capacity of the blood and helps to adjust blood flow in the "microcirculatory units", according to local oxygen requirements [5]. Its 60 properties and functions are listed in *Table 1*.

Erythron

The erythron, a kind of diffuse organ, combines two parts of equivalent mass. RBCs, derived from circulating reticulocytes, make up almost half the volume of blood. The other part is the erythropoietic mass, which is dispersed in the red marrow of the bones, where the progenitors proliferate and differentiate into erythroblasts. This long lineage is initiated by rare, very long-term self-renewing haematopoietic stem cells (LT-HSCs). The erythroblasts surround a particular macrophage forming the erythroblastic island (EBI), described by Marcel Bessis. The EBI macrophage, which also carries the erythropoietin receptor (EPO-R), nourishes the erythroblasts and degrades their expelled nuclei at the end of maturation *(Figure 1*). In mice, basic erythropoiesis is localised in the bone marrow, but *stress erythropoiesis* occurs in the spleen, where short-term repopulating stem cells (ST-HSCs) proliferate and differentiate directly into *stress erythroid progenitors* (SEP), inducing splenic hypertrophy. In humans, both basic and stress types of erythropoiesis coexist in the bone marrow [6].

Haemoglobinopathies

Major haemoglobinopathies (sickle cell disease and thalassaemia) are a growing public health problem due to their severity, with millions of patients facing reduced life expectancy, except in developed regions, due to complex and costly "global" management.

Haemoglobin and blood gases

Binding, transport and delivery of oxygen

One gram of oxygen-saturated Hb in arterial blood carries 1.34 mL of oxygen. Thus, oxygen is 70 times more concentrated in RBCs than in plasma. The affinity of deoxygenated Hb (T state) for oxygen is low. The binding of a first molecule of O_2 induces the cooperative binding of three other molecules of O_2 (haem-haem cooperativity), allowing the molecules of Hb to be saturated within a fraction of a second in the pulmonary capillaries [8]. Conversely, in the microcirculation of other tissues, the release of oxygen is facilitated by a decrease in tissue concentration of oxygen and by 2,3-diphosphoglycerate (2,3-DPG) in RBCs which promotes the T-state of Hb and the release of O_2. The concentration of 2,3-DPG is normally close to that of the Hb, in the order of 5 mmol. Its formation is derived from glycolysis (1,3-DPG → 2,3-DPG) by means of the GR-specific enzyme, DPG mutase. The concentration of 2,3-DPG is increased in the presence of anaemia and increased plasma adenosine [9]. 2,3-DPG is required for tissue oxygenation, but promotes polymerisation of deoxygenated HbS, responsible for sickle cell disease. 2,3-DPG deficiency induces increased Hb affinity for oxygen and erythrocythaemia–in the case of DPG mutase deficiency or as part of the increased glycolysis associated with certain mutations in PIEZO1 [10, 11].

Table 1

Haemoglobin: properties and functions.

1) Gas transport: O_2, CO_2, CO
2) NO scavenging (by $HbFe^{2+}$ + O_2) converted to nitrate (NO_3H^-) eliminated in the urine
3) Transport of NO bound to the haem Fe^{3+} or nitrosothiol (SNO-Hb); local production of NO from nitrite
4) Capture and release of H^+ protons regulating blood pH (buffering capacity of Hb)
5) Assembly of four identical chains in pairs, forming two dimers connected together
6) Hb chain = globin chain + haem
7) Unpaired chains stabilised by a chaperone protein (AHSP for a and HSP-90 for β and γ)
8) Allostery promoting cooperative binding of O_2 (lungs) and its release (other tissues)
9) Binding of 2,3-DPG between the two β chains of Hb, the allosteric effector of Hb
10) ROS production: oxidation, then instability and denaturation of Hb which releases the oxidised haem (haemin)
11) Binding of HSP-70 by excess free α-chains → inefficient erythropoiesis in β-thalassaemia
12) Embryonic Hb: Gower I, Gower II, Portland
13) HbF ($\alpha_2\gamma_2$); the chains γ ($^{G}\gamma$ and $^{A}\gamma$) differ by one amino acid (Gly or Ala at position 136)
14) HbA ($\alpha_2\beta_2$ and HbA2, $\alpha_2\delta_2$). The α-chain is common, the non-α chain is specific to Hb
15) Adaptation of tissue oxygenation according to altitude, blood pressure, fever, local pH (acidosis)
16) Methaemoglobin (metHb) (Fe^{3+}), oxidised form, physiologically reduced by metHb reductase
17) Ferryl Hb, hemichromes and other toxic oxidised derivatives of Hb
18) Sulfhaemoglobin: Hb binding physiologically produced or exogenous hydrogen sulphide gas (H_2S)
19) Haem regulation of globin chain synthesis in erythroblasts and reticulocytes
20) Interactions of the H2b (interactome), in particular, with the membrane band 3 ion exchanger (AE)
21) Regulation of RBC energy metabolism by Hb: anaerobic glycolysis
22) Regulation of RBC reducing metabolism by Hb: pentose phosphate pathway
23) Hb forms a mixed disulphide with glutathione (GSSG + $Hb\beta^{93SH}$ ↔ GS-Hb)
24) Hb is a major antioxidant of RBC; it is protective against oxidations of blood and vessels
25) Glucose binding → glycated Hb (HbA1c)
26) HbA1c → AGE (for advanced glycated end products: toxic by-products, especially in diabetics
27) HbA1c: a marker of diabetes, the level of HbA1c is crucial for monitoring and adapting the treatment of diabetics
28) Blood HbA1c level: predictive factor of cardiovascular disease
29) Other derivatives of Hb: acetylated, phosphorylated, *etc.*
30) Capture and transport of heavy metals (lead, mercury, *etc.*) eliminated directly by the kidneys
31) Regulation of the normal concentration of Hb in RBCs (identical HCC in mammals)
32) Depth-dependent adaptation of bony fish to water density: degassing in the swim bladder

33) Oxygen reserve in prolonged apnoea (Hb and/or myoglobin: crocodiles, marine mammals)

34) Various "catalytic" activities of Hb (peroxidase, nitrite reductase, *etc.*)

35) Derivatives with biological and chemical activity (haem, iron, peptides, *etc.*)

36) Inflammation, oxidation and production of oxidants (ROS) in haematoma or hyperhaemolysis

37) Hb: iron and amino acid reserve, (prolonged fasting, starvation, hunger strike)

38) Hb: nutrient for parasites of RBCs (plasmodium, babesia, *etc.*) or extracellular *(e.g.* trypanosome)

39) "Aged" or altered Hb, bound to the membrane → "Eryptosis" of RBCs, captured by macrophages

40) Anaemia: decrease in the concentration of Hb in the blood

41) Intravascular haemolysis: the Hb released into the plasma is trapped by haptoglobin (Hp-Hb complex)

42) Toxicity of excess "free" haem in plasma after saturation with haemopexin (haem-Hx complex)

43) Cell protection by induction of the key antioxidant and anti-inflammatory enzyme haem oxygenase

44) Degradation of haem (haem + haem oxygenase → Fe + CO + biliverdin → bilirubin)

45) Human food (black pudding = 75% Hb = iron + protein)

46) Source of iron from "blood-drinking animals" (mammals, worms, insects, parasites, microorganisms, etc.)

47) Complete "bio" fertiliser (dried blood containing 90% haemoglobin)

48) Diversity of the 1,820 natural mutations in human globin genes

49) Malaria resistance or tolerance factors: haemoglobins S, C, E, thalassaemia minor

50) "Cyanosis" due to methaemoglobinaemia or sulphaemoglobinaemia

51) Major sickle cell syndromes (homozygous S or composite S/C, S/D, S/β-thal, *etc.*)

52) Thalassaemic syndromes (α, β), major, intermediate, minor, silent

53) Unstable Hb (intravascular haemolysis, dominant haemoglobinopathies)

54) Polycythaemia (erythrocythaemia) due to increased affinity of Hb for O_2 or 2,3-DPG deficiency

55) Hbs during development: Embryonic Hb → HbF ($\alpha_2\gamma_2$) → HbA ($\alpha_2\beta_2$) + HbA_2 ($\alpha_2\delta_2$) Hb Switch = repression of HbF by BCL11A, ZBTB7A/LRF, *etc.*

56) Hereditary persistence of HbF: PHHF (pan-or heterocellular)

57) Pharmacological and therapeutic re-expression of HbF (hydroxyurea = hydroxycarbamate)

57) Gene therapy by gene addition: production of therapeutic Hb

58) Gene therapy by editing of globin or *BCL11A* repressor genes

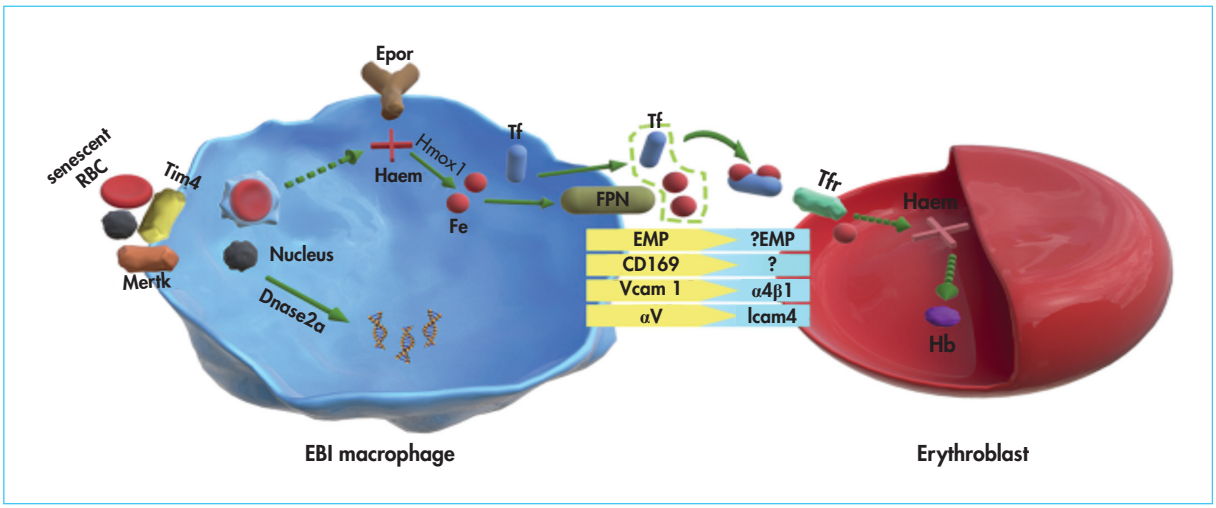
59) Therapeutic inhibition of HbS polymers: voxelotor

60) Substitute for blood transfusions: Bovine PEG-Hb (Sanguinate®); human PEG-Hb

Transport of carbon dioxide

Deoxygenated Hb transports some of the carbon dioxide (CO_2) produced in the tissues. CO_2 is bound to the N-terminal end of each α chain (*Figure 2*). The rest of the CO_2 produced in the tissues is hydrated to carbonic acid (CO_3H_2) by carbonic anhydrase which is particularly active in RBCs. The carbonic acid formed

Figure 1



Macrophage of the erythroblastic island and iron recycling [7].

dissociates into bicarbonate (CO_3H^-) and a proton (H^+). Deoxygenated Hb binds protons. In the lungs, the release of CO_2 and protons is facilitated by the allosteric conformational change of Hb (T → R state) upon oxygen uptake. The two pairs, oxygenated Hb/deoxygenated Hb and CO_3H^-/CO_2, form the main buffer systems in the blood to maintain a constant pH.

Nitrous oxide or nitric oxide

Hb plays three roles in the metabolism of nitric oxide (NO), produced locally from arginine by NO synthase, mainly in endothelial cells:

– NO is a powerful local vascular relaxant, activating guanylate cyclase in smooth muscle cells. Hb (HbO_2) neutralises and limits the intravascular diffusion of NO by converting it to nitrate (NO_3H^-) which is eliminated in the urine,
– Hb can also transport NO, bound either to the haem of deoxygenated Hb (Hb-NO) or to the thiol (SH) group of β^{93} cysteine (HbS-NO). Transported NO is released into hypoxic tissues, inducing local vasodilation and consequently increased blood flow for improved tissue oxygenation in hypoxic conditions [5],
– Hb can also generate NO from nitrite, due to the nitroreductase activity of Hb [12].

Carbon monoxide

Carbon monoxide (CO) is, like oxygen, bound to haem and transported by Hb in the form of carboxyhaemoglobin (Hb-CO) (stabilising the R-state) which normally constitutes less than 1% of the total Hb in the blood. It is produced physiologically during the degradation of haem by haem oxygenase (HO), mainly in macrophages. This degradation also releases an iron atom and biliverdin, the precursor of bilirubin. The high concentration of oxygen in the pulmonary capillaries displaces the CO that is exhaled. CO is the ultimate “silent poison”; it is odourless, colourless and tasteless, which abounds in tobacco smoke. Smoking a pack of 20 cigarettes a day increases the Hb-CO to 5–8%. It is more abundant in exhaust gases and may be present in the case of a faulty stove, fire, occupational risk or death by suicide

involving gas. CO binds to the haem of Hb, instead of the oxygen. Cells, particularly those in the brain, are protected from excess CO by the high affinity of Hb for carbon monoxide, about 240 times that of oxygen. However, above a certain amount of CO trapped by Hb (Hb-CO >20%), the same proportion of transported oxygen is reduced, causing headache, nausea, altered consciousness and potentially fatal or life-threatening coma, due to the binding of CO to the cytochrome C oxidase enzyme in neuronal mitochondria.
At low doses, briefly inhaled CO (100–200 ppm) has an anti-cellular stress, anti-inflammatory and vascular relaxant effect for a few hours. Increased endogenous CO production in the case of increased haemolysis or inflammation is generally beneficial for cell defence and survival [14]. Carbon monoxide is also said to induce a calming effect from smoking the peace pipe, to "bury the hatchet", and contribute to the anti-stress effect sought by smokers.
The potential medical benefit of CO is increasing as an anti-inflammatory, antioxidant and against anti-cellular stress. It stabilises Hb released by haemolysis in plasma by preventing NO scavenging and neutralisation by plasma Hb, as well as oxidative stress from excess free haem. In addition, CO, administered as a concentrated solution per os or inhaled inhibits vaso-occlusion in sickle cell mice [15, 16]. In humans, a Phase I clinical trial is ongoing (NCT03926819).
In addition, when inhaled in small doses, CO has been shown to improve physical performance and reduce oxygen consumption in athletes (high-level footballers) in China [17], and could become an "invisible" doping agent.

Hydrogen sulphide

Hydrogen sulphide (H_2S), well known for its rotten egg smell, is also produced in small amounts physiologically by two evolutionarily conserved cellular metabolic pathways. Like NO and CO, it dilates blood vessels [18], and also protects them against ageing [19]. As is the case for NO and CO, the role of Hb is to neutralise excess H_2S, limit its diffusion, transport it, and transform it into other sulphur compounds, the physiological functions of which are currently being explored, in particular in the fields of ageing and prevention of cardiovascular diseases [20].
Overall, Hb plays multiple roles in the "safe" transport of oxygen, the transport of CO_2 in the opposite direction and the physiological metabolism of "signalling" gases (NO, CO, H_2S), enhancing the instantaneous adjustment of blood flow in "microcirculatory units" and the neutralisation of gases to limit their diffusion or when inhaled at toxic doses.

Stepwise regulation of the expression of haemoglobin

Hb is a remarkable model of the coordinated expression of the components of a heteroprotein (globin and haem chains) and its life cycle [21]. The large amount and concentration of Hb in RBCs depends on successive molecular and cellular adjustments:

– extensive transcription of various genes, specifically in erythroblasts,
– highly active translation of long-lived messenger RNAs (mRNAs),
– assembly of Hb chains guided by chaperone proteins resulting in functional Hb,
– adjustment of cellular hydration.

The haem molecule

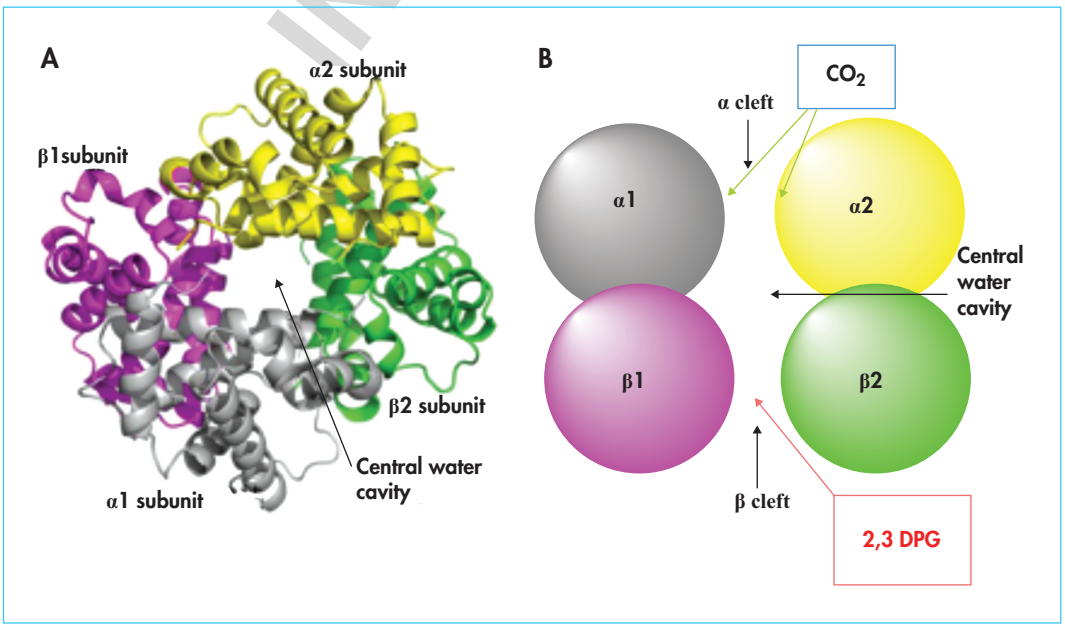
Haem is produced in eukaryotes by a series of enzymatic reactions, the first and last of which are intramitochondrial. During erythropoiesis, haem increases the number of ribosomes by activating transcription of ribosomal proteins and ribosomal RNAs.

In addition, haem very strongly stimulates the initiation of translation of globin mRNAs. At the end of erythroblast maturation, free haem reduces the expression of the key transcriptional factor, GATA1, slowing the transcription of globin genes [22] and cell division genes [23]. Subsequently, the reticulocyte gradually loses its mitochondria and thus its ability to synthesise haem and globins. At the end of reticulocyte maturation, the kinase, HRI (*haem regulated inhibitor*), becomes active in the absence of "free haem", which blocks translational initiation of globin mRNA [24].

Iron deficiency induces an early deficiency of haem, and *haem-regulated inhibitor* (HRI), once activated, reduces globin synthesis. Hypochromic and microcytic anaemia results. At the opposite end of the spectrum, an abnormal excess of "free haem" becomes toxic to the erythroblast, generating oxidative stress and inefficient erythropoiesis. This is the case for β-thalassemia major, Blackfan-Diamond anaemia and, to a lesser degree, homozygous S sickle cell patients [25, 26].

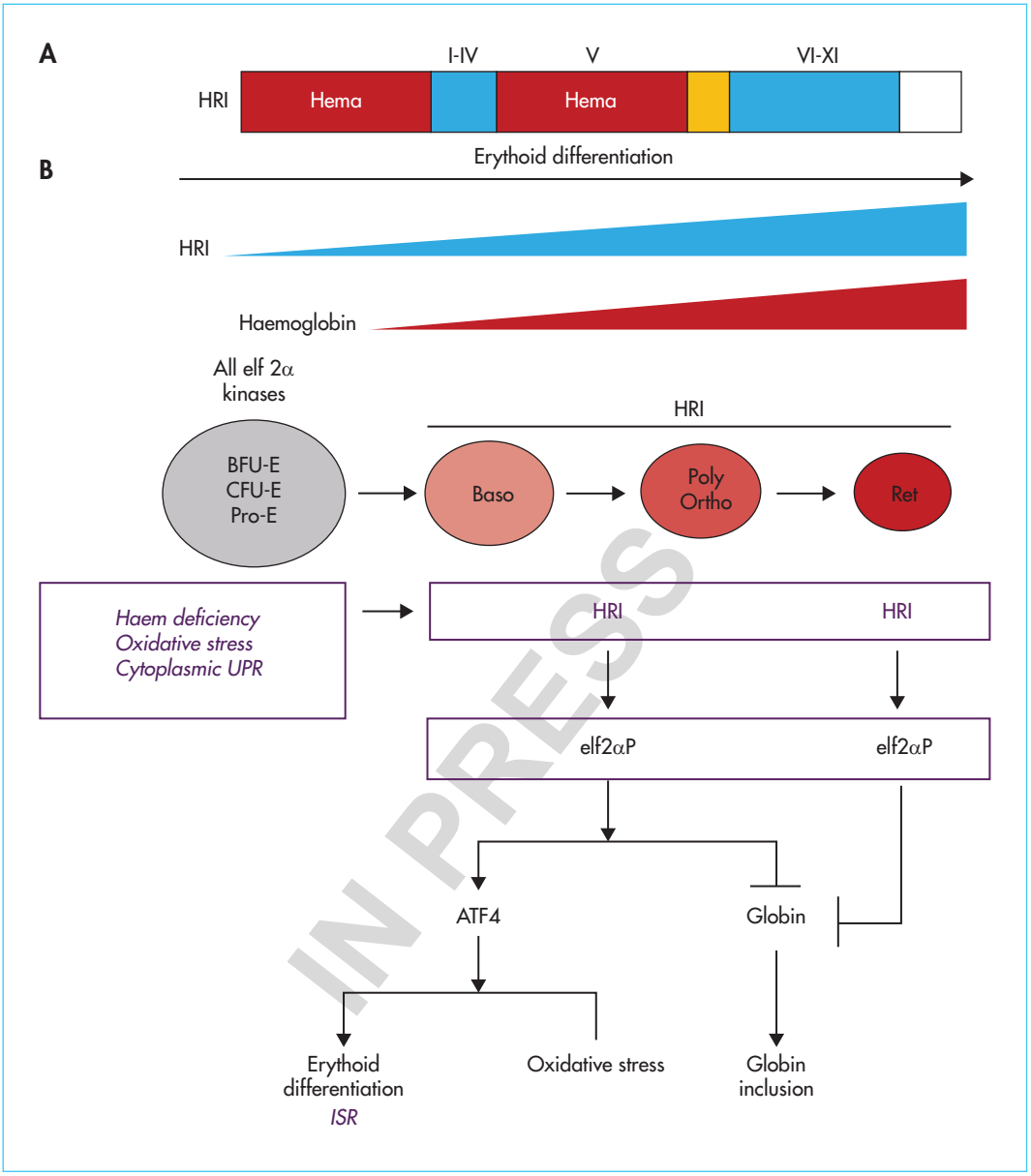
HRI is part of an integrated intracellular stress signalling network, *the integrated stress response* (ISR). It involves adaptation to variations in the environment as well as cellular safeguarding, which is conserved in all living cells [27]. It maintains protein homeostasis (cellular proteostasis) under various stresses: nutrient (amino acid) deficiencies, oxidative stress, protein instability or alterations in protein production (UPR [*unfolded stress response)*, or viral infection. The sensors of these different stresses include HRI kinases, GCN2 (*general control non-derepressible* 2), PKR (*protein kinase R)* and PERK (PKR-like *endoplasmic reticulum kinase).* These two kinases converge by specifically phosphorylating the initiation factor, e1F2a (serine 51), thereby blocking its production which is required to renew translational initiation of the majority of mRNAs, those with a classic initiation codon, such as globin mRNAs. In the presence of phosphorylated e1F2a, other mRNAs are translated, in particular, those encoding for transcriptional factor ATF4 (*activating transcription factor* 4), as well as other anti-stress proteins. In turn, these proteins activate transcription of genes that safeguard the cell *(figure* 3). In haem deficiency, the increased amount of ATF4 counteracts the stimulatory

FIGURE 2



A) HbA is formed by four haemoglobin chains (two α and two β subunits) surrounding a central cavity. B) Scheme of two α/β dimers artificially separated to show the two parts of the central water cavity (α and β clefts) where 2,3-DPG and carbon dioxide (CO_2) are located [13].

FIGURE 3



Haem-regulated inhibitor (HRI) of the integrated stress response (ISR) in erythroblastic differentiation. A) Haem binding domains (red) and kinase domains (blue) of the HRI protein. B) HRI activation, elf2 α inactivation (phosphorylation by HRI) and inhibition of globin synthesis in the case of the integrated stress response. BFU-E: burst-forming unit; CFU-E colony-forming unit erythroid; Pro-E: pro erythroblast; Baso: basophilic erythroblast; Poly Ortho: polychromatophilic erythroblast; Ortho: acidophilic erythroblast; UPR unfolded stress response [24].

effect of haem-stimulated erythropoiesis by the increased amount of erythropoietin secreted in renal hypoxia. ATF4 inhibits one of the activation pathways of the erythropoietin receptor (EPO-R → P-AKT → mTORCl) [24].

Haemoglobin chain assembly and allosteric cooperativity

The high level of "native" globin chain synthesis is supported by chaperone proteins: α *Hb-stabilising protein* (αHSP) for the α globin chain and *heat-shock*

protein 90 (HSP90) for β and γ globins, which stabilises the chains and promotes the binding of haem [28]. αHSP accelerates the assembly of the two types of Hb chains, forming a free αβ dimer [29]. Physiologically, there is a small amount of free globin chains in the RBC, especially α, which is rapidly degraded [30]. Free unpaired α chains, present at excessive levels in β-thalassemia major, exceed the intracellular capacity for proteolysis and precipitate in cells, leading to the formation of inclusions (Heinz bodies), which generates abnormal oxidation, dyserythropoiesis and haemolysis.

The concentration of haemoglobin in RBCs

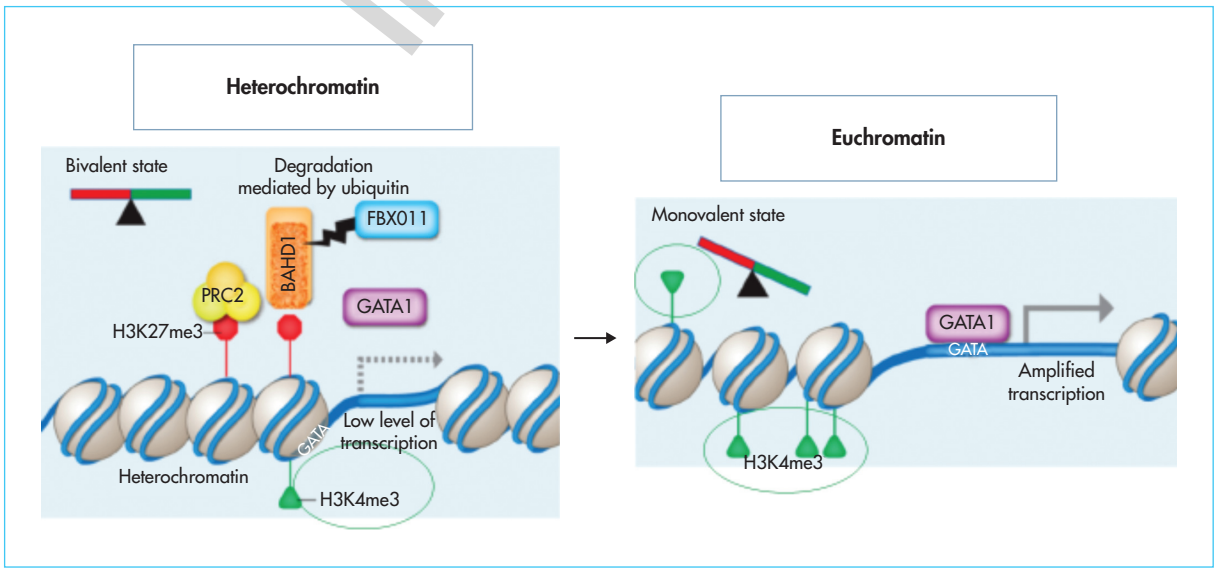
This is adjusted by a progressive decrease in cell volume during erythropoiesis by reticulocytes. This RBC Hb concentration (mean corpuscular Hb concentration [MCHC]) is the same in all mammals (32 g Hb/dL). In contrast, Hb content (HCT) and mean cell volume (MCV) are highly variable between species. The loss of reticulocyte volume and water (20%) results from activation of the KCl cotransporter. Reticulocyte dehydration may continue in the RBC due to the presence of abnormal Hb (HbS, HbC, *etc.*), inducing an increase in Hb concentration, which may exceed its solubility limit leading to the formation of Hb crystals in vivo (HbC) or polymers of deoxygenated HbS molecules which may initiate the intra-erythrocytic pathophysiological mechanism of sickle cell disease.

Selection, transcription and switching of globin genes

Epigenetic modifications of chromatin

These modifications are essential in the repression or activation of genes in general, and globin genes in particular, specifically in erythroblasts. These modifications include cytosine methylation at CpG pairs of the DNA of repressed genes as well as modifications of chromatin histones (methylation, acetylation, ubiquitination, *etc.*) at specific sites constituting the histone code (*figure* 4).

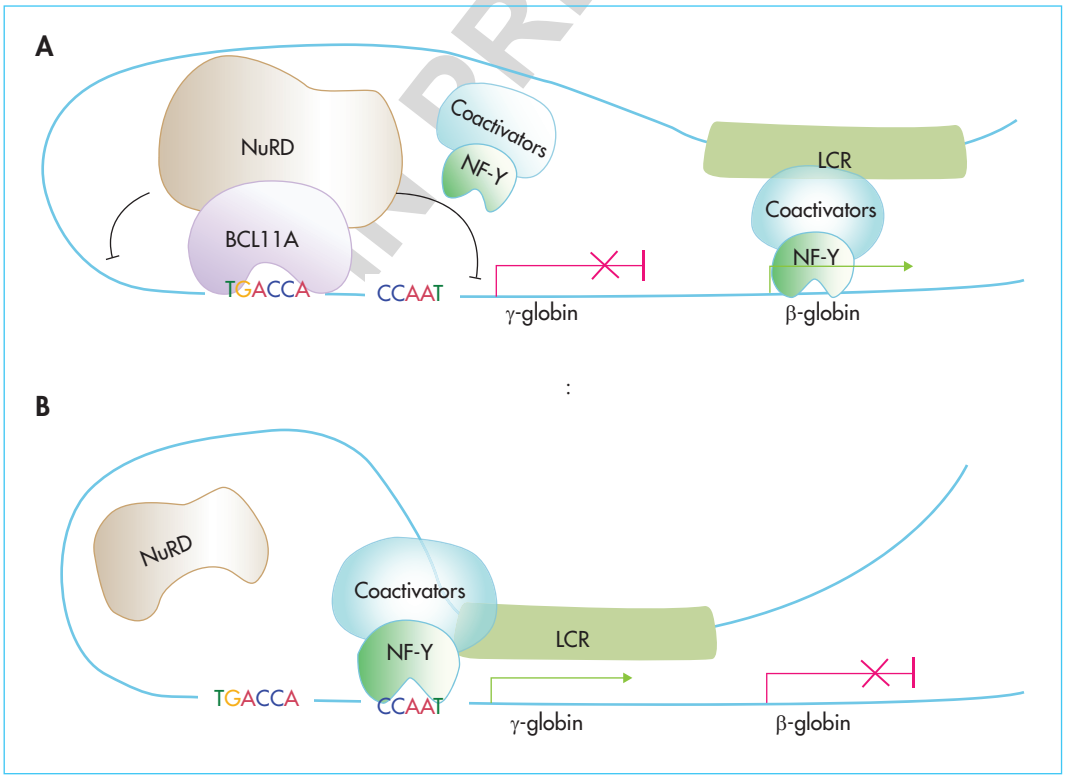
Figure 4



Inactivation (bivalent state of heterochromatin) and activation (monovalent state of euchromatin) of erythroid genes by histone H3 modifications: FBXO11 of the ubiquitin ligase E3 complex induces proteolysis of BADH1 which associates with another co-repressor (PCR2) localised to H3-histone, trimethylated on lysine 27 (H3K27me3); the demethylation of this lysine and the trimethylation of H3 lysine 4 (H3K4me3) induces the binding of the activating transcription factor GATA1 and the expression of erythroid genes [31].

The `master' factor of globin gene transcription, GATA1, forms complexes with other factors during erythroblast differentiation that include: LDB1 (*LIM domain binding 1),* TAL1 (*T-cell acute lymphocytic leukaemia protein* 1), NF-Y (*nuclear factor Y)*, FOG1 (*friend of GATA-1),* E2A, EKLF1 (*erythroid Kruppel-like factor* 1), LMO2 (*LIM-domain-only* 2), *etc*.. Among the target genes, the globin genes comprise eight pairs of alleles in humans, divided into two groups on different chromosomes: type α (ζ, α_2, α_1), on chromosome 16 and type non-α (ε, Gγ, Aγ, δ, β) on chromosome 11. These genes are transcribed sequentially, depending on the stage of development: embryonic (ζ and ε), then foetal (α_2, α_1 and Gγ, Aγ) and finally after birth (α_2, α_1 and δ, β) due to transcriptional "super-controllers" that are rich in transcriptional cofactor binding sites located mainly upstream of the non-α type globin genes (*locus control region [LCR])* or α type globin genes (R1-4). The second switch of globin genes during development is foetal Hb (HbF) → HbA + HbA_2, which occurs only for non-α type genes. Starting at 20 weeks of development, this *switch* lasts for about a year. It occurs in the same cell type, but the production of RBCs shifts from foetal liver to bone marrow. At birth, RBCs contain both HbF (80–85%) and HbA (15–20%). Progressive repression of foetal γ genes *(*HBG1 and HBG2*)* is perfectly compensated by the expression of "adult" (δ and β) genes, which are physically linked (in *cis*). After the age of one, adult Hb (HbA or HbA1) normally constitutes 97% of total RBC Hb, HbA2 2-3% and HbF less than 1%. HbF is present in significant amounts in less than 5% of RBCs; the F cells in which HbA remains the most abundant.

FIGURE 5



A) HbF -> HbA after birth. B) Expression of HbF (γ genes) in the foetus or in the case of "hereditary persistence of foetal haemoglobin" (HPFH) after birth. Other co-activators are not detailed [33].

After birth, the two contiguous γ genes (HBG1 and HBG2) of HbF are repressed by repressor proteins, such as BCL11A (*B-cell lymphoma/leukaemia 11A*), through binding upstream of each of the γ genes (*figure* 5). This repression prevents the formation of an activation loop for these γ genes in cis. During the *switch,* the CSF complex shifts and activates downstream expression of the "adult" globin genes on the same chromosome, in particular the β (*HBB*) gene. Expression of the β gene is also directly stimulated by KLF1 (*Kruppel-like factor 1)* which further promotes the binding of BCL11A to repress γ genes [32]. Finally, HRI inhibits the expression of HbF by increasing the transcription of BCL11A via ATF4.

Hereditary persistence of foetal haemoglobin

Hereditary persistence of foetal Hb (HPFH) is not uncommon. The following two types exist:

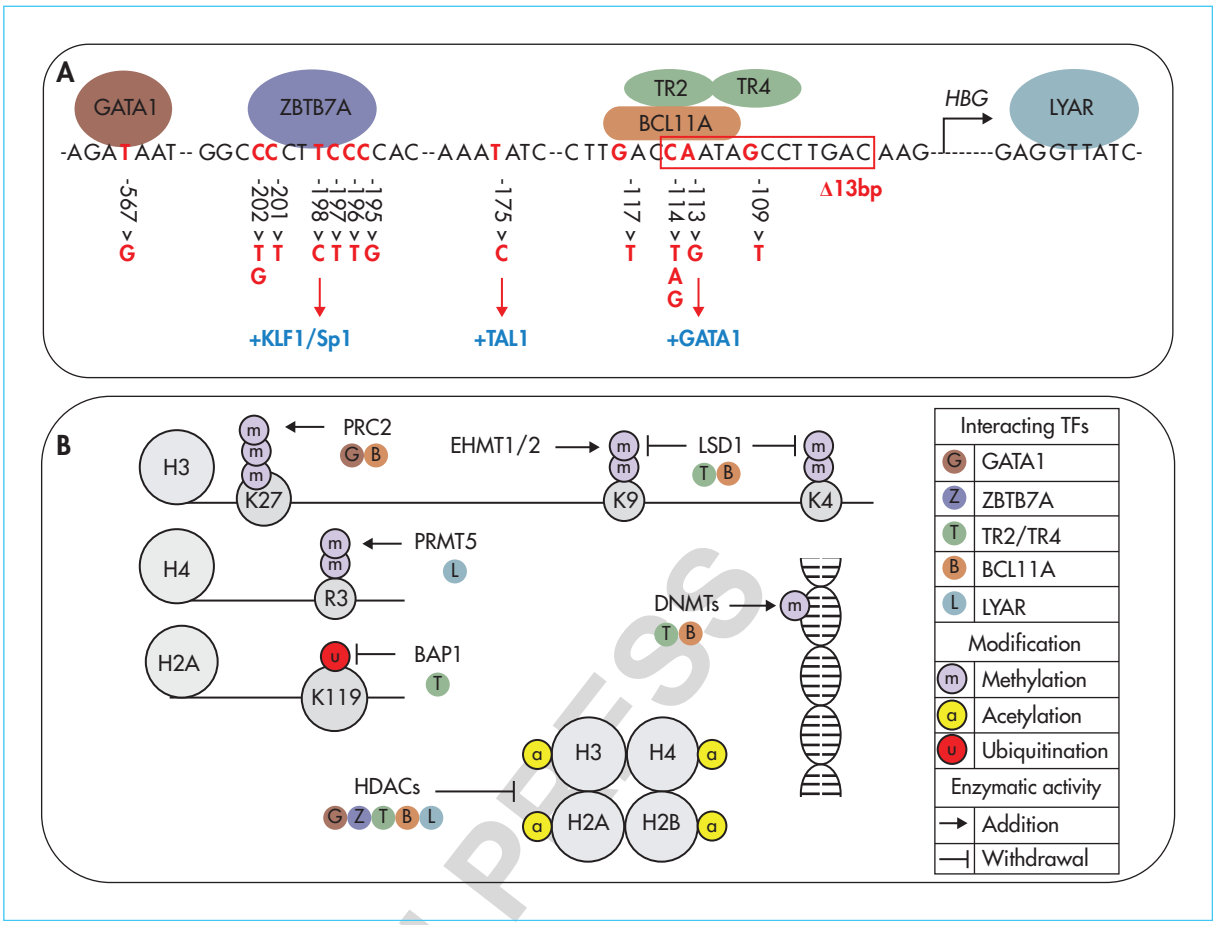
– "Pancellular" HPFH: HbF, as 25–35% of total Hb in the heterozygote, is equally distributed in all RBCs. HbF completely replaces the expression of adult Hbs (HbA and HbA2) in the homozygous state, without inducing anaemia. This persistence results from large gene deletions of β and δ genes,
– "Heterocellular" HPFH: the increase in HbF is often less and heterogeneously distributed in more than 5% of RBCs. This type of HPFH can be caused by up to 15 different point mutations or a small deletion near the promoter of one of the two γ genes. These mutations alter the binding of a transcriptional repressor -BCL11A, ZBTB7A (*zinc finger and BTB domain containing* 7A)/LRF (*lymphoma/leukaemia related factor)* or TR2/TR4 (*testicular nuclear receptors 2/4)- on* γ genes or creates a new binding site for a factor that activates these γ genes (GATA1, KLF1/SP1, TAL1 [*T-cell acute lymphocytic leukaemia* 1]) (*Figures 5, 6A*)

Some forms of heterocellular HPFH, not genetically linked to globin genes, are due to mutations of γ gene repressors such as BCL11A. The expression of HbF and the increase in the percentage of RBC-F are amplified by the stimulation of erythropoiesis by EPO and by certain cytokines (IL-3, SCF, *etc.*).
Normally, the transcription of BCL11A is elevated in erythroblasts after birth by binding of the transcriptional activating factor, GATA1, to an enhancer sequence *(erythroid-specific enhancer*) within an intron of BCL11A. However, this sequence is also the target for inhibition of *BCL11A*, consequently reactivating the expression of HbF by gene editing in order to compensate for the loss of HbA expression (β-thalassaemia) or inhibit polymerisation of HbS responsible for sickle cell disease.

Production, metabolism, senescence and eryptosis of red blood cells

All RBCs combined consume 10% of the body's basic energy expenditure, exclusively in the form of glucose, through the insulin-independent membrane transporter, Glut1. In the absence of mitochondria, RBC energy metabolism is thus limited to anaerobic glycolysis, with no oxygen consumption and production of lactate which is recycled to glucose by the liver. The energy yield is low, producing only two molecules of ATP per molecule of glucose consumed. Other cell types, equipped with mitochondria, produce 36 molecules of ATP per molecule of glucose in the basal state. However, this is no longer the case for nucleated cells in a hypoxic state that are actively multiplying. Energy metabolism is switched in these cells, especially T cells and tumour cells, in poorly oxygenated areas. In this case, their greatly increased energy metabolism is very similar to that of RBCs [35]. This switch saves oxygen and supports the pentose phosphate pathway to enhance antioxidant defence (nicotinamide adenine dinucleotide phosphate [NADPH], reduced glutathione, *etc.*) and the synthesis of ribose 5-phosphate and deoxynucleotides required for DNA replication. Thus, the metabolism of RBCs and rapidly multiplying cells ensures a high level of production of adenosine

FIGURE 6



Hereditary persistence of foetal hemoglobin of the heterocellular type induced by point mutations or small deletions. A) Location of mutations at binding sites of various factors. B) Epigenetic modifications of chromatin histones (H3, H4, H2A, and H2B) interacting with factors (activating or repressing) for γ globin genes after birth. DNMT: DNA methyltransferase; HDACS (histone deacetylase) [41].

triphosphate (ATP) and increased reducing power as well as export of lactate and protons to reduce intracellular acidification. In addition, the Hb in RBCs is protective in tissues against toxicity due to heavy metals (lead, mercury, *etc.*) that may be trapped with Hb.

Hb also plays a role in RBC senescence and premature eryptosis in haemoglobinopathies (sickle cell disease and thalassemias, unstable Hb) or glucose-6-phosphate dehydrogenase (G6PD) deficiency with increased oxidation. RBCs export appreciable amounts of glutathione in the form of disulphide (GSSG) and GSH taken up in tissues by other cells. Degradation of Hb haem by macrophage-induced haem oxygenase 1 increases the recycling of iron exported by ferroportin and transported by plasma transferrin.

The "global" management of patients with severe haemoglobinopathy

Mass neonatal screening for severe haemoglobinopathies, initiated in the French West Indies and then extended worldwide, enables very early management of children with severe haemoglobinopathies, reducing early morbidity and mortality of children with sickle cell disease. The first few months are the prime time to

educate parents, before the onset of symptoms and any risk of certain dreaded complications such as stroke, in order to mitigate the mortality associated with acute splenic sequestration and prevent bacterial infections by preventive antibiotic therapy. Early and comprehensive care for patients is provided by a network of 72 dedicated centres in France, including seven overseas sites (filiere-mcgre.fr). The prevention of these severe haemoglobinopathies, through pre-implantation diagnosis or early prenatal diagnosis, is rapidly increasing [36].

Targeted therapies for sickle cell disease

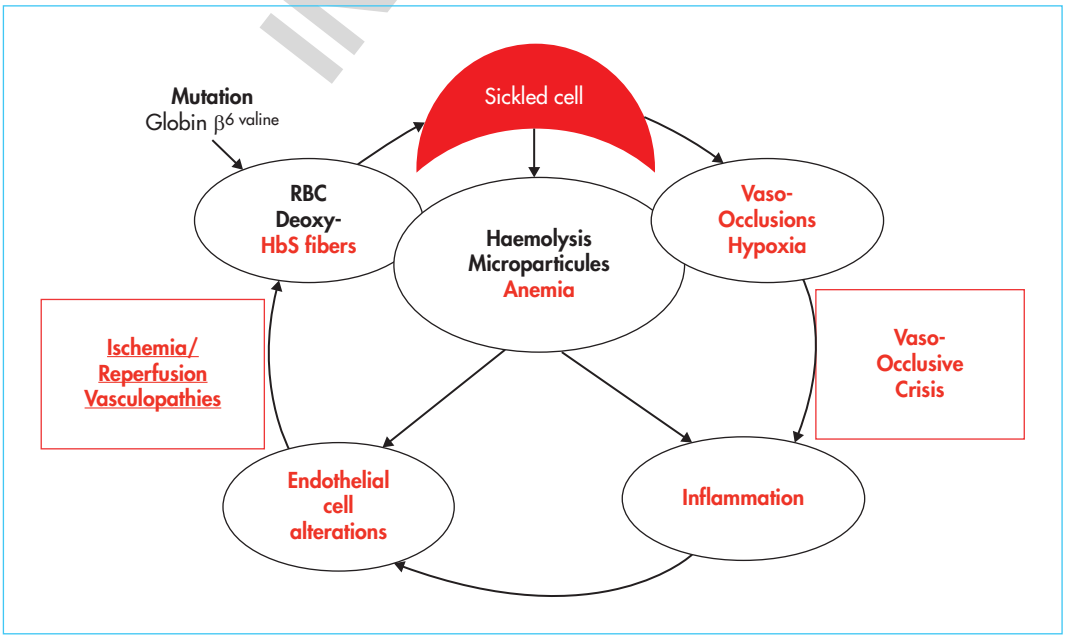
They aim of treatment is to break the vicious cycle of sickle cell disease, from a point mutation that causes polymerisation of abnormal HbS upon deoxygenation, to the accumulation of polymers, and manifestation of the disease. In the absence of a direct, non-toxic inhibitor of deoxygenated HbS polymer fibres, other targeted approaches have had to be sought [34], involving:

- either an increase in the affinity of HbS for oxygen, as the oxygenated R-form of HbS which does not polymerise,
- or prevention of secondary alterations associated with the "cyclic pathophysiology" of sickle cell disease: RBC dehydration, reduced deformability, increased adhesion, oxidation (reactive oxygen species [ROS]), intravascular haemolysis as a source of NO deficiency and alterations of the endothelium, vaso-occlusion, ischaemia/reperfusion, local or systemic inflammation, a prethrombotic state, neutrophil, platelet and monocyte/macrophage activation, *etc.* (*figure 7*).

The first approach to a specific therapy, of which the clinical efficacy was demonstrated in 1985, involved hydroxycarbamide (hydroxyurea [HU]), which was shown to have various beneficial biological effects:

– increased expression of HbF, partially replacing HbS and directly inhibiting the formation of the HbS polymers
– decrease in the proportion of dense and dehydrated RBCs
– decrease in haemolysis

FIGURE 7



– decrease in the number of abnormally increased and activated neutrophils
– generation of NO by hydroxyurea

This preventative treatment reduces the frequency of painful vaso-occlusive crises and certain acute or chronic complications. It also improves patients' life expectancy. However, the amount of induced HbF and the proportion of F-cells are highly variable from patient to patient [38]. This long-term preventative treatment remains under-used because of short- to medium-term adverse side effects, in particular, amenorrhoea, temporary reduction of spermatogenesis and potential mutagenic and teratogenic risk, not yet demonstrated in humans.
Recently, three other targeted therapies have been conditionally approved in the US: voxelotor (Oxbryta®), crizanlizumab and L-glutamine. Their clinical or biological benefits remain modest. They may be used in combination or with hydroxycarbamide due to complementary actions.
The increase in glycolysis by allosteric activation of erythrocyte pyruvate kinase (PKR) by mitapivat (Phase III trial to treat PK deficiencies) slightly increases blood Hb and ATP, NADPH and GSH in RBCs and decreases 2,3-DPG in sickle cell patients, which increases the affinity of Hb for oxygen and ultimately reduces polymerisation of HbS and haemolysis [31, 39].
Iron depletion by repeated bleeding, as evidenced by decreased transferrin saturation in S/C or S homozygous patients with iron accumulation, reduces haemolysis and increases blood Hb and RBC life span [40].
Other targeted therapeutic approaches are the subject of ongoing therapeutic trials (clinicaltrials.gov.).

Targeted therapies for β-thalassemia

In addition to the improved efficacy and tolerability of oral chelators for transfusion-induced iron overload in particular, recent advances show that slowing down iron-deficient Hb synthesis (HRI activation) or improving dyserythropoiesis and reducing transfusions by luspatercept (Reblozil, ACE 536), which also stimulates the production of erythropoietin, are beneficial [42]. The same applies to three other therapeutic approaches that will be authorised in the short term concerning:

– mitapivat (AG-348), an oral treatment that increases ATP (which is insufficient in thalassaemic RBCs), thereby reducing haemolysis (clinical trial: NCT03692052),
– TMPRSS6-LRx, an antisense oligonucleotide (ASO), which inhibits the synthesis of TMPRSS6, a metalloprotease that plays a key role in the regulation of the expression of hepcidin. Increased expression of hepcidin reduces iron uptake and recirculation due to hyperhaemolysis, improving RBC survival, the efficiency of erythropoiesis and reducing hepatic iron overload (clinical trial: NCT04059406),
– VIT-2763, a ferroportin inhibitor, which reduces the absorption and circulation of iron. This improves Hb levels and iron regulation in β-thalassaemic mice (VITHAL trial: NCT04364269).

Haematopoietic allografts

Thalassemia

Over the past 20 years, advances in the efficacy and tolerability of allografts have made these the reference treatment for children with β-thalassemia major. A few thousand patients have been successfully treated worldwide, more in developing countries than in developed regions. The transplant is best tolerated when performed in young children, in the absence of liver enlargement and severe iron overload. Under these conditions, graft-related mortality and early graft rejection

are reduced (<5%). This is no longer the case for older children or those with a large liver associated with iron overload, adolescents and even less so for adults. The planned birth of a disease-free and HLA-identical child can be achieved by in vitro fertilisation followed by selection of embryos for implantation. Several dozen β−thalassaemic children have benefited from this approach. However, the likelihood of obtaining an HLA-identical stem cell donor remains low and more than one attempt is often required.

Sickle cell disease

For patients with sickle cell disease, transplantation is increasingly indicated for children at risk of stroke or other severe complications [43, 44]. Mortality and severe transplant complications are less common before the recipient reaches the age of five and in cases with a familial genotype-matched donor. For more than 10 years, 98% of these transplanted sickle cell children have had long-term survival without sickle cell disease [45]. There is no increased risk of vaso-occlusive crises when the donor myeloid cell chimerism is ≥30% in the case of a HbA/A donor. However, haemolysis remains excessive if this chimerism is < 50% for both types of donors (HbA/A and heterozygous A/S). The blood Hb is then > 10 g/dL in the case of an HbA/A donor but remains < 10 g/dL when the donor is A/S. A vaso-occlusive crisis was even reported to occur in a patient transplanted by an A/S donor, retaining 68% HbS in the blood, *i.e.* with 30% A/S RBCs [46].

Unrelated donor banks are another source of grafts. However, the probability of finding a highly compatible donor (HLA: 10/10) is low, especially if the patient is from an ethnic minority in developed countries or if the donor population is very heterogeneous.

The use of stem cells from "haplo-identical" family donors could solve the problem of the small number of HLA identical family donors [47]. A simple and robust predictive score for allograft complication-free survival was determined from 1,425 sickle cell transplant patients. It is important to consider the age of the recipient (under or over 12 years) and the type of donor [48].

Ultimately, targeted *myeloablation* using a cytotoxic agent coupled to a specific ligand could replace conventional myeloablative conditioning and reduce its iatrogenic effects [49, 50].

Gene therapy by gene addition

β-thalassaemic patients

After an initial encouraging result obtained for a first β−thalassaemic patient treated at the Saint-Louis Hospital [51], a new generation of the LentiGlobin vector (BB305) [52] was used in other Phase I/II and Phase III trials, including 60 thalassaemic patients, adults and children, requiring chronic transfusions. Improvements were also made to the protocol: mobilisation in blood and increased enrichment of long repopulating stem cells (CD34 + + cells), increased vector transfer using two additional small molecules, and increasing the number of copies of the therapeutic gene in treated cells ex vivo *and* the number and proportion of modified stem cells of the reinjected therapeutic product.

Preliminary results of the trials [53] indicate:

– rapid uptake of the genetically modified stem cell autologous transplant in all cases (median neutrophil reappearance by 20 days);

– total Hb and therapeutic Hb rise rapidly and usually reach a stable plateau between six and nine months after gene therapy (GT). Permanent cessation of transfusions (total Hb > 9 g/dL) is achieved in 10/12 adults (83%) and 19/22 children (86%) with a stable therapeutic Hb level of 5.1 to 10.9 g/dL;

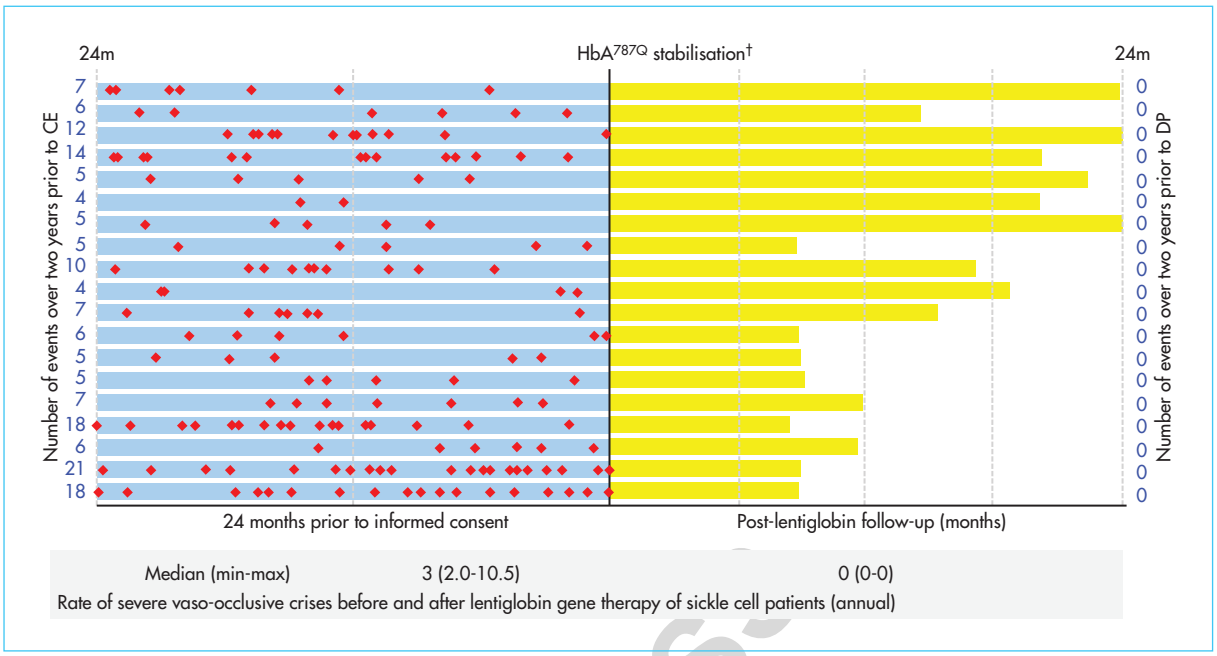
– an absence of adverse effects related to the vector and the transfer of the therapeutic gene, in the short or medium term;
– a high number of several thousand vector integration sites documented in all patients, as well as a lack of a dominant cell clone.

The best results were obtained for non-β° thalassaemic patients (β^{E}/β°thal, β^+thal/β^+thal, β°thal/β^+thal), of whom 91% (20/22) became transfusion-independent. The median total Hb was 11.5 g/dL of blood, and that for Hbβ^{T87Q} was 9.5 g/dL.
In June 2019, following a favourable opinion from the European Medicines Agency (EMA), the European Commission conditionally authorised marketing of the GT product, Zynteglo® (CD34+ cells modified with the BB305 vector), for patients in the European Union with a non-β° form of transfusion-dependent thalassaemia major, aged 12 years or older and without a familial genotype-matched transplant donor.
After two years, patients were included in a long-term follow-up protocol (LTF-303) for 13 years to evaluate the long-term efficacy and oncogenic safety of the therapeutic vector. For the past 11 years, all thalassaemic patients who were free of transfusion remained so. To date, there has been no clinical toxicity associated with the vector or a dominant and stable clone.
Other clinical trials in which a globin gene has been added in β−thalassaemic patients receiving regular transfusions are underway around the world. Preliminary results from a trial conducted in Italy indicate rapid graft uptake (median: 16 days) and good tolerance of GT in patients treated after reduced intensity conditioning (treosulfan, thiotepa) [54]. Transfusion independence was achieved in the three youngest children treated, one of whom was β°/β° thalassaemic. A decrease in the frequency of transfusions was achieved in the remaining patients (three adults and one child). Another trial, using attenuated conditioning, was suspended in New York due to ineffectiveness. Two other trials are on-going in China, with no published results to date.

Sickle cell patients

In order to perform GT for sickle cell disease using the same vector, the globin β^A gene was slightly modified by changing the codon for the amino acid at position 87; threonine being replaced by glutamine ($\beta^{A\text{-}T87Q}$) [52]. Thus, therapeutic Hb specifically inhibits the formation of HbS fibres, in a manner analogous to HbF, as has been clearly demonstrated in preclinical studies with sickle cell transgenic mice [55, 56].
A first sickle cell patient, treated in 2014 at the Necker Hospital in Paris, was 13 years old (Bluebird-bio trial HGB-205, protocol B; principal investigator M. Cavazanna and scientific director P. Leboulch). This patient has maintained with 12 g/dL total Hb in the blood for six years. The proportion of HbS was in the order of 50%. He is no longer anaemic and no longer has abnormally shaped or abnormally dense RBCs in the blood [57]. His condition, both clinically and biologically, has dramatically improved. Another patient in this trial (genotype: S/β°thal) no longer has a vaso-occlusion. Therapeutic Hbs (Hb$\beta^{A\text{-}T87Q}$ = 2.7 g/dL and HbF = 1.6 g/dL) constitute 40% of a total of 10.3 g/dL of Hb in the blood. A third patient, S homozygous, had a lower level of expression of therapeutic Hb (Hb$\beta^{A\text{-}T87Q}$: 1.4 g/dL) and HbF (1.1 g/dL) with total blood Hb of 7.8 g/dL. In this case, there was no real clinical benefit. The long-term follow-up of these three patients is scheduled for publication in 2021. Since then, improvements have been made to the protocol (protocol C): haemapheresis of plerixafor-mobilised stem cells, and further selection and increased number of more efficiently modified CD34 + + cells, containing more than one copy of the vector per cell. The result, more than six months after GT for the first 19 patients treated with this protocol C, is an

Figure 8



Complete resolution of VOEs (vaso-occlusive events: painfull sickle cell crises and acute chest syndrome), following gene therapy by lentiglobin vector in HGB-206 Group C sickle cell disease patients (bluebird bio, September 2021).

increased amount of total Hb, averaging 11.5 g/dL of blood (9.6–16.2 g/dL) and including 2.7–9.4 g/dL of therapeutic Hb in about 90% of RBCs ($Hb\beta^{A\text{-}T87Q}$: 12–20 pg/GR). Finally, haemolysis is reduced, as reticulocytes, bilirubin and lactate dehydrogenase are decreased. No painful episodes occurred more than three months after the GT (*figure* 8). The decrease in intracellular HbS by at least 40% is due to competition of the expression of the β globin genes; the therapeutic transgene (> one copy per cell) and the two β^S endogenous genes, as the concentration of RBC Hb is not increased. Thus, some of the HbS is substituted by therapeutic Hb. The limiting factor that may account for this competition is probably transcriptional, however, this has not yet been characterised.

The clinical and biological assessment of each sickle cell patient will be performed two years after GT for the 40 patients included in this Phase I/II trial. Patients then entered a 13-year long-term follow-up study (LTF-303 trial). A Phase III trial is underway for a further 35 sickle cell patients aged 2–50 years (HBG-210 trial: NCT04293185).

Risks of oncogenesis

Two severe adverse events were reported in February 2021 by the company, Bluebird-bio, for two sickle cell patients treated with GT (protocol C): a case with suspected myelodysplastic syndrome and another with acute myeloblastic leukaemia (AML), the latter was detected five years after GT. Could these myeloid pathologies be the result of either insertional mutagenesis induced by the therapeutic vector, the risk associated with myeloablative conditioning, or the spontaneous risk of myeloid oncogenesis due to the haemoglobinopathy? The spontaneous risk of AML is 10-fold higher in sickle cell patients than in the comparable general population [58]. The risk of myeloid haemopathies in these patients is further increased in the case of myeloablation prior to allogeneic transplantation [59]. Analysis of the GT vector insertion site showed that GT vector insertion was most likely not involved in AML (Bluebird-bio, press release,

20 April 2021). Myelodysplasia was suspected due to prolonged anaemia after TG and the presence of trisomy 8 in a small percentage of marrow cells, but in the absence of blasts or morphological dysplasia. These findings do not support a diagnosis of myelodysplasia.
These results allow the resumption of GT trials for sickle cell patients and β thalassaemic patients, for whom treatment was also suspended. To date, there have been no cases of haematological oncogenesis in the many other GT trials using lentiviral vectors.
In 2010, insertion of the first-generation lentiglobin vector into HMGA2 (*high-mobility group AT-hook 2)*, that promoted the proliferation of a partially dominant clone, in a β-thalassaemic patient treated in 2007 was reported [51]. This clone spontaneously reduced in size over time and, to date, has proven to be benign; it has not been shown to be dominant for 10 years [60]. The first-generation vector, which proved to be unstable, was modified to carry out the trials conducted since 2012.

Organisation and cost of gene therapy

Negotiations are underway between Bluebird-bio and the health authorities of each country regarding operational organisation, price and payment for this marketed therapy for β-thalassaemic patients. Due to the complexity and cost of this unique therapeutic agreement, Bluebird-bio proposes a payment spread over five years, taking into account the therapeutic efficacy according to the *pay-for-performance principle*, *i.e.* according to the level of efficacy achieved (editorial of the journal Nature Biotechnology July 2019 and Bluebird-bio "Recode", 2021).
The first GT products, marketed from 2012 in Europe for single-gene diseases were commercial failures because they were too expensive [61].
New rules should also be based on a "societal value" for these therapies. This would be a form of *benefit corporate certification* to establish a compromise between the various partners, including associations representing families and patients, in order to allow access to this therapy for as many patients as possible [62].
By way of comparison, it should be noted that the global care of sickle cell patients (S/S or S/β°thal genotype) with a severe form (at least one severe vaso-occlusive crisis per year) is particularly expensive in developed countries, if one includes cumulative acute or chronic complications and induced comorbidities, which increase with the age of the patient. The overall cost up to the age of 50 was close to $9 million per sickle cell patient with a *severe form* in Philadelphia, not including chronic transfusions and iron chelation required in 10–20% of these patients [63].

Prospects for gene therapy

GT for severe haemoglobinopathies at a lower cost and with reduced oncogenic risk would be possible in the short term based on the following [64]:

– reduction in the dose of vectors required per patient. Based on dose/kilogramme of weight, this amount is reduced in younger children. This dose can also be reduced through increased transduction efficiency of the targeted cells;
– HSC mobilisation in the blood may be increased [65];
– improvement of modified HSCs in vitro [66] or in vivo;
– simultaneous reinjection of short-lived progenitors, separated before CD34+ cell transduction, to compensate for the transient myeloablative conditioned aplasia, which should prevent the patient from being isolated in a confined transplant unit;
– use of reduced intensity conditioning [54];

– replacement of conventional chemical myeloablation with a targeted method under development [50, 67].

Thus, the potential for optimisation, cost reduction and improved safety of GT, regarding therapeutic gene addition, is considerable in the short term. The aim is to establish a mass-market therapy, accessible to as many patients as possible, including those in developing countries and, if possible, ultimately on an outpatient basis.

Gene therapy by gene editing

A recent approach in GT is developing at great speed: *gene editing*. This involves modifying the sequence of a target gene by "molecular surgery", derived from the CRISPR-Cas system, a bacterial system consisting of an endonuclease and a small guide RNA (sgRNA). This extraordinarily precise system was devised by Emmanuelle Charpentier and Jennifer Doudna, who won a Nobel Prize in 2020. This molecular engineering can be used to correct a mutation or, on the contrary, induce mutations (indels: substitutions, insertions or deletions) in a precisely targeted region of the genome. This targeted mutagenesis has been shown to induce the re-expression of γ genes of foetal Hb, and this reactivation of HbF constitutes an "acquired persistence of foetal Hb". Two types of target exist:

– *direct activation* of γ genes of HbF by mutation (indels) of γ gene repression sites [60, 68].
– *indirect activation* of γ genes of HbF, due to transcriptional inhibition by a physiological γ gene repressor, such as BCL11A [69].

Phase I/II therapeutic trials are underway for 90 adults with β-thalassaemia or sickle cell disease: "Modified autologous CD34+ cells ex vivo by CRISPR-Cas9 (CTX001) inactivating the gene-specific erythroid enhancer, BCL11A" by the company CRISPR Therapeutics, founded by Emmanuelle Charpentier (ClinicalTrials.gov NCT03655678 and NCT03745287; ASH 2020). Preliminary results from the first two patients treated with CTX001, have been published [70]. The first fifteen patients with β-thalassemia major, treated by BCL11A editing, and followed for three to 18 months, rapidly became transfusion-independent with 9.7 to 14.1 g/dL blood HbF. Seven sickle cell patients (S/S genotype), followed for 3 to 15 months, expressed 11-15.9 g total Hb, with 39.6-49.6% of HbF distributed in nearly 100% of RBCs. These patients did not have a vaso-occlusive episode after gene editing (CRISPR Therapeutics, 2021). It will be essential to determine the risk associated with in vivo genotoxicity due to gene editing and to conduct long-term monitoring for the efficacy and safety of this therapeutic approach.
Two other Phase I/II trials for hemoglobinopathies involving ex vivo gene editing are currently underway, based on a *zinc finger protein* targeting BCL11A (Sangamo Therapeutics, NCT03432364 and NCT03653247). Other gene editing clinical trials are in preparation [64].

Base editing

New artificial variants of the CAS protein have been shown to induce single base editing without cutting double-strand DNA. Such editing approaches should eventually be able to correct 90% of the mutations responsible for single-gene disorders [71, 72].

In vivo gene therapy for haemoglobinopathies: what does the future hold?

The preclinical results in animals provide proof of principle that in vivo GT targeting HSCs mobilised from the blood can effectively correct the thalassaemic or sickle cell phenotype without aplasia [73].

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