Sickle cell disease and the risk of malignant haemopathies

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Drépanocytose et risque d'hémopathies malignes

sickle cell disease, haematological malignancies, busulfan, hydroxyurea, gene therapy, gene addition therapy, gene editing

Abstract

The occurrence of haematological malignancies years after allograft rejection or gene therapy for sickle cell disease requires further delineation of the potential role of the disease and its treatments.

Résumé

La survenue de cas d'hémopathies malignes, des années après rejet d'allogreffe ou thérapie génique de la drépanocytose impose une réflexion sur le rôle potentiel respectif de la maladie et de ses traitements.

Over the past 30 years, the treatment of sickle cell disease has changed dramatically. Neonatal screening, antibiotic prophylaxis and vaccinations, especially against pneumococcal disease, have significantly reduced infant mortality. Transfusion programmes for children at risk of stroke, detected by transcranial Doppler, have reduced the risk of stroke from 11% to 1% before the age of 18 [1, 2]. In the majority of patients treated long-term with hydroxyurea (or hydroxycarbamide), the frequency of vaso-occlusive crises (VOCs) and acute chest syndromes (ACS), as well as the need for transfusions, were very significantly reduced [3]. Although some observational studies suggest improved survival in adults on hydroxyurea [4, 5], there is still some doubt as to the efficacy of hydroxyurea in reducing adult mortality and preventing organ failure, in particular pulmonary hypertension [5]. Infant mortality has been significantly reduced, with 98% of homozygous S children reaching adulthood in Europe and the US. Adult survival, however, has not improved significantly following the use of hydroxyurea as a treatment for sickle cell disease [6-9]. However, progress has been made with allogeneic transplantation and gene therapy, and this progress is ongoing, encouraging doctors and patients to turn to these radical biotherapies. Allogeneic transplantation from a family HLA-identical donor has, since the 2000s, offered a 98% chance of cure to young sickle cell patients and adults under 30 years of age who can withstand myeloablative conditioning [10], while a new reduced-intensity non-myeloablative conditioning regimen, developed at the National Institute of Health (NIH), now allows adults, even those with organ damage, to be transplanted with an 87% chance of sickle cell-free survival and no risk of graft-versus-host disease (GVHD). In such cases, there is, however, a 13% risk of rejection and consequent recurrence of the sickle cell disease [11-13].

The problem for the majority of patients without a family HLA-identical donor has persisted. To date, "unrelated transplantation", used successfully for leukaemia patients, has been accompanied by an unacceptable incidence of extensive GVHD [14] in sickle cell disease. Furthermore, in developed countries, the chances of having a "good

To cite this article: Bernaudin F, Kuentz M, Beuzard Y. Sickle cell disease and the risk of malignant haemopathies. *Hématologie* 2021; xx(x): 1-14. doi: 10.1684/hma.2021.1703

doi: 10.1684/hma.2021.1703

unrelated donor" are very low for patients of African origin. However, two new types of transplants offer great hope for all patients:

– haploidentical transplantation through the use of post-transplant cyclophosphamide [15] with approximately 70–80% chance of cure [16, 17]. This can apply to almost all patients but has a significant risk of GVHD.
– gene therapy, i.e., the autotransplantation of genetically modified stem cells. This does not expose the patient to the risk of GVHD, which is feared in this non-malignant disease, and can be used to treat all sickle cell patients. In terms of haemoglobinopathies, the first successes of gene therapy were achieved in β-transfusion-dependent thalassaemia [18] and then in sickle cell disease [19]. Forty-seven sickle cell patients have benefited from it with remarkable results regarding the prevention of VOCs and ACS and an almost pancellular expression of therapeutic haemoglobin, correcting the anaemia, in some cases completely [20].

However, in February 2021, the company Bluebirdbio announced the provisional suspension of inclusions in gene therapy trials for haemoglobinopathies after discovering a case of myeloblastic leukaemia in a sickle cell patient treated five years previously [21]. This announcement has raised concerns in the sickle cell community and among the healthcare teams involved [22, 23]. This is especially true since another adult with sickle cell disease had already developed acute myeloblastic leukaemia (AML) with myelodysplasia (MDS) three years after inclusion [24], thus establishing the rate of this complication at 2/47 or approximately 4% (CI: 1.1–14.3%), while the follow-up time is still only eight years for the oldest sickle cell patients treated with therapeutic gene therapy (gene addition therapy [GAT]).
The most precise analysis possible to elucidate the mechanisms of these "serious adverse events" is necessary to determine their origin and, if possible, to reduce the oncogenic risks of this potential treatment to a minimum. The hypotheses to be considered are:

– The roles of the globin transgene and the vector?
– The toxicity of busulfan conditioning?
– The possible role of pre-transplant hydroxyurea treatment?
– The role of sickle cell disease itself?

These different hypotheses are not mutually exclusive, and a multi-factorial mechanism is perfectly possible.

Role of the transgene and the vector?

The globin transgene, expressed specifically and late in erythroid differentiation, is not known to be oncogenic and does not induce cell proliferation or transformation. It is only expressed in erythroblasts and not in other blood lines due to an erythroid-specific promoter. Its very high level of expression is due to the addition of regulatory sequences from the locus control region of β-type globin genes [25].
Oncogenicity through "insertional mutagenesis" associated with a GAT vector has unfortunately occurred frequently in early therapeutic trials for severe combined immunodeficiency in children. This risk of leukaemia (20% of children within a few years) was the result of the type of vector used to transfer the therapeutic gene into the haematopoietic cells *ex vivo.* The vector was derived from a non-inactivated γ-retroviral murine oncogenic virus. The viral promoter activated not only the expression of the therapeutic gene, but also the expression of a proto-oncogene, most often *LMO2* (for *LIM-only* 2), the promotor of which proved to be a preferred site for insertion of this vector into cellular DNA [26]. Fortunately, this risk was considerably reduced after inactivation of the viral promoter (self-inactivated

γ-retroviral vector), relayed by a cellular promoter. However, this dramatic period of gene therapy-induced leukaemia has left a lasting mark. Based on experimental, cell-based and animal studies, and clinical trials, the oncogenic risk of the vector is further reduced with the self-inactivated lentiviral vector used in GAT for haemoglobinopathies [27], but this risk is theoretically not zero. To date, there have been no cases of malignant myeloproliferative syndrome due to lentiviral vector insertion in any of the gene therapy trials with a cellular promoter [28, 29].

Haematological malignancies and gene therapy for sickle cell disease (homozygous S)

In the first case of haematological malignancy in the Bluebirdbio sickle cell gene therapy trial, myelodysplasia was discovered in a sickle cell patient three years after GAT [30]. The patient was 42 years old and had been treated for eight years with hydroxyurea. He showed aplasia on D17 and D29 for neutrophils and platelets, respectively. Since then, he had had a low transgene copy number (TCN) of 0.08–0.15 copies/blood nucleated cells *in vivo* over the three years of follow-up and persistent anaemia which warranted the resumption of hydroxyurea one year after GAT and treatment with erythropoietin after two years. The trial was not suspended at the time because the dysplastic cells did not have lentiviral vector insertions, and the presence of monosomy 7 and typical somatic mutations suggested neoplasia secondary to alkylating conditioning (busulfan).

However, the hypothesis of the potential genotoxicity of GAT lentiviral vectors hit the headlines again very recently, with the discovery of MDS/AML in an adult with sickle cell disease, five years after gene therapy. We do not have data on sickle cell disease prior to GAT and possible pre-GAT hydroxyurea treatment, nor on post-GAT haematological follow-up. In this case, the vector was present in the patient's leukaemia cells, leading to the suspension of ongoing clinical trials while its origins were investigated. Within a few weeks it was shown that the vector insertion was most likely not oncogenic. Indeed, it had integrated into the *VAMP4* (vesicle-associated membrane protein 4) gene. This gene is not known to play a role in AML, nor in any process related to other cancer, increased cell proliferation or genome instability. There was also no deregulation of this gene or the genes surrounding the vector insertion in this patient. The blasts from this patient, like those from the previous case, exhibited monosomy 7 and somatic mutations in the *RUNX1* (runt-related transcription factor 1) and *PTN11* (protein tyrosine phosphatase non-receptor type 11) genes, which are considered to be typical of alkylator-induced leukaemias, even though the patient's genetically modified and reinjected cells were clearly not exposed to busulfan. The absence of proto-oncogenes or oncogene suppressor genes in the vicinity of the vector insertion site, and the lack of a long-range effect on the genome, make it highly unlikely that the vector insertion in the *VAMP4* gene played a damaging role in the induction of the leukaemic clone or in its proliferation. Thus, it is highly unlikely that the integrated BB305 vector played a role in this case of AML (Bluebirdbio, press releases of 20 February, 10 March and 20 April 2021). For these reasons, the resumption of clinical trials for GAT of haemoglobinopathies with the BB305 vector was authorised in June 2021 by the FDA (press release of 7 June 2021).

Absence of post-gene therapy haematological malignancy in patients with β-thalassaemia

GAT trials involving 64 transfusion-dependent β-thalassaemic (TDT) patients (ClinicalTrials.gov: NCT02151526, NCT01745120, NCT03207009, NCT02906202) (or LG001, HGB-205, 207 and 212) have not resulted in any oncogenic complications to date, with a long follow-up period and a large number of patients (220 patient years). A single case of a myeloid clone, very partially

dominant for two years (<4% of nucleated blood cells), was detected in 2008 in the first β-thalassaemic patient successfully treated with GAT at the age of 18, in 2007, some 14 years ago. This clone was due to activation of the expression of HMGA2 protein (high-mobility group AT-hook 2) by insertion of the transgene within an intron, promoting cell proliferation [18, 31]. The initial trial of GAT for haemoglobinopathies (LG001) was suspended. The first-generation lentiviral vector also proved to be unstable after integration. It was replaced by an improved and stabilised vector (BB305) for a new Phase I/II trial in Paris (HGB-205), authorised in 2012. This vector is also used to this day in other GAT trials for haemoglobinopathies (Bluebirdbio).

In total, there were no cases of malignant haematopoietic proliferation, confirmed myelodysplasia or dominant cell clones due to the BB305 vector in the more than 100 sickle cell or β-thalassaemic patients treated with this vector and in all other trials using a lentiviral vector. All patients with haemoglobinopathy treated with GAT were followed for 15 years (NCT02633943, NCT04628585) to determine the actual risk of insertional oncogenesis and the long-term efficacy of this single-dose GAT. The marketing of GAT for β-thalassaemic patients using the same BB305 vector (Zinteglo®) was authorised in Europe in 2019 but temporarily suspended in 2021 at the request of Bluebirdbio following two cases of leukaemia in the sickle cell disease cohort. Marketing was then resumed on 9th July following the agreement of the European Medicines Agency (EMA) Pharmacovigilance Risk Assessment Committee (PRAC).

Role of busulfan conditioning?

The occurrence of secondary MDS/AML related to exposure to alkylating agents after autotransplantation for lymphoma or myeloma is, unfortunately, a well-known problem. Their frequency was estimated at 3.7% (335/9,028) in a recent review [32] of the International Bone Marrow Transplantation Registry (IBMTR). Several publications highlight the presence of clonal preleukaemic abnormalities detectable in bone marrow cells before conditioning due to the chemotherapies received previously by these patients [33]. The autograft setting does not allow us to determine whether the disease developed on the cells that were exposed to pre-transplant conditioning or on those of the graft.

Allografting makes it easy to distinguish between graft and host cells, and the persistence of detectable host haematopoiesis after transplantation or "mixed chimerism" is very common in sickle cell disease, even after so-called "myeloablative" conditioning, especially if the patient has not developed significant GVHD. In the French series published in 2020 [10], out of 234 genoidentical transplants performed between 1988 and 2012, 44% of patients had mixed chimerism, defined here as the persistence of at least 5% of the recipient's cells, detectable based on blood DNA, even though they had received conditioning with cyclophosphamide and high-dose busulfan. Of these patients, after a median follow-up of 7.9 years, no secondary cancers were observed, including the eight patients with autologous reconstitution due to no engraftment (n = 2) or rejection (n = 6).

In the recently published US experiment [34] on 910 sickle cell patients transplanted between 2008 and 2017 (25% of whom were adults, including genoidentical, unrelated and haplo-identical transplants, with various conditioning, etc.), six secondary cancers, including four MDS/AML, were observed (0.7%) in patients who rejected the graft. Three out of four of these patients were adults and had received a haploidentical transplant with post-transplant cyclophosphamide. All had undergone "non-myeloablative" conditioning based on low-dose total body irradiation. Again, no secondary cancer was observed after high-dose "myeloablative" conditioning.

An NIH study of 76 adult sickle cell patients treated between 2004 and April 2018 with haematopoietic allograft transplantation showed that three (3/76, 4%) of the 19 patients who rejected the transplant (genoidentical or haploidentical) developed myelodysplasia or AML two to five years after transplantation [35]. These three patients, who were aged 37–44 years at the time of transplantation, underwent non-myeloablative conditioning, as did all patients in this protocol. In two of these patients, the neoplastic clonal expansion carried a pre-transplant mutation in the p53 protein. This mutation promotes cellular resistance to conditioning. This study also shows that the 57 sickle cell patients who were transplanted with long-term success, did not have any alterations suggestive of neoplastic haematopoiesis. The haematopoietic clone carrying a p53 mutation was not observed in six other patients; four who had rejected the transplant and two who were successfully transplanted but with stable long-term mixed chimerism. p53 mutations are also observed in other cases of post-transplant secondary myeloblastic haemopathies in non-sickle cell patients [36].

The same is true for some other genomic alterations common in clonal haematopoiesis of undetermined potential (CHIP) [37], myelodysplasias and myeloblastic leukaemias, especially those occurring after transplant rejection or partial chimerism. New global molecular biological methods, next-generation sequencing (NGS), transcriptomics and bioinformatics will make it possible to address this fundamental question of the secondary oncological risk of allogeneic or autologous transplantation [38, 39].

In addition to this well-identified risk of secondary leukaemia developing on residual host haematopoiesis after allogeneic transplant rejection, there are also rare cases of post-transplant secondary leukaemia developing on donor cells that have not been exposed to conditioning (similar to the Bluebirdbio GAT case). A recent systematic review [40] of 137 cases in the literature estimates the frequency to be about 0.1%, and in 70% of cases it is MDS/AML. The age of the donor does not appear to be a significant factor and, among the cytogenetic abnormalities, monosomy 7 is noted with great frequency. The mechanisms of leukaemogenesis mentioned are numerous: existence of pre-leukaemic lesions in the graft–although in the donor they do not evolve into leukaemia–environmental lesions caused by conditioning, shortening of telomeres due to the expansion of the graft, but also failure of immunosurveillance linked to immunosuppressive treatments.

Treatment of sickle cell disease with hydroxyurea?

The efficacy of hydroxyurea (H_2N-CO-NH-OH) in reducing the frequency of crises via increasing foetal haemoglobin (HbF) has been clearly demonstrated. Not only does it reduce haemolysis and improve anaemia but, through its myelosuppressive effect, it reduces the number of neutrophilic leucocytes and platelets, and improves blood rheology [3]. However, its effectiveness varies greatly from patient to patient and its effect on survival and the prevention of organ damage is not well proven. In addition, the risk of chromosomal mutations raises concerns about potential oncogenic toxicity with prolonged treatment.

Hydroxyurea inhibits the nucleotide reductase enzyme which forms deoxynucleotides from ribonucleotides. As a result, this antimetabolic agent inhibits DNA synthesis by depleting deoxynucleotides and slowing down the S-phase of the cell cycle and repair mechanisms. It has a short half-life in the body (5.5 hours) and its immediate myelosuppressive effect is rapidly reversible. In contrast, DNA repair defects can lead to the accumulation of somatic mutations and chromosomal abnormalities [41, 42].

A study of acquired DNA mutations [43] showed a significantly higher number (albeit within a “normal” range) of illegitimate V(D)J recombination in children exposed to hydroxyurea over a median of 30 months. Moreover, a study of DNA

damage in sickle cell patients showed a significant positive correlation between the damage index and dose and duration of hydroxyurea treatment [44]. Hydroxyurea has also been shown to alter telomere replication and shorten telomeres [45].Since the 1960s, hydroxyurea has been used for myeloproliferative syndromes, known as "pre-leukaemic states", such as polycythaemia vera [46, 47] and essential thrombocythemia [48], and has been associated with an increased risk of leukaemia. In one randomised trial, hydroxyurea significantly reduced the dose of P^{32} received by increasing the duration of P^{32}-induced remission, however, this was associated with a significant increase in the risk of leukaemia and cancer and a 15% reduction in life expectancy [46, 47]. In contrast, in non-haematological diseases involving children, such as cyanotic congenital heart disease, hydroxyurea prescribed for an average of five years was not associated with increased malignancy [49].

In sickle cell disease, the potential role of hydroxyurea in oncogenesis in sickle cell patients has been feared [50] and isolated cases of haematological malignancies in sickle cell patients treated with hydroxyurea have been reported [51-59]. One recent publication [60] reporting four cases, including two patients treated with hydroxyurea, as well as 11 other cases reported in the literature demonstrate the presence of complex chromosomal abnormalities including total or partial 5q and 7q deletions, usually observed in MDS/AML secondary to treatment (alkylating agents or radiation). Another publication reports a case of AML with 5q deletion, trisomy 8 and deletion and numerous somatic mutations of the *p53* gene as a result of hydroxyurea treatment received over the course of 26 months [61]. In contrast, the study of haematopoiesis in sickle cell patients suggests that hydroxyurea may have an anti-stress haematopoietic effect and slow down the ageing of haematopoietic stem cells (HSCs), which seems to be accelerated in these patients [62]. However, to date, in large series of hydroxyurea treatments, but with limited follow-up, no increase in leukaemic risk has been noted [63]. Indeed, the MSH protocol which randomised adults to hydroxyurea versus placebo in 1992–1995 was followed by an observational period (1996–2001) during which patients were given the choice of discontinuing hydroxyurea, maintaining it or introducing it. Among patients who received hydroxyurea for at least one year, no increase in cancer incidence was detected: two cancers were detected out of 1,731 patient years (incidence: 0.12%) and no secondary leukaemia was detected. However, the follow-up period remains limited [64]. The British study [65] did not investigate the impact of sickle cell disease treatments on oncogenic risk. The Californian study by Brunson *et al.,* published in 2017, compared the incidence of cases between 1988 and 1999 to those between 2000 and 2014 [66] and revealed no increased leukaemic risk since the Food and Drug Administration (FDA) approved hydroxyurea on 21st December 1997 as a treatment for sickle cell disease in adults. However, an increase in leukaemic risk was reported for the most severely affected patients, with at least three events per year, warranting admission to the emergency room or hospitalisation.

The role of sickle cell disease itself

Initially, the genetic disease was suspected of having a protective effect, similar to the well-known protective effect against severe forms of malaria. This apparent effect was later attributed to the shortened life span of sickle cell patients. In 1972, in the United States [67], it was first reported that the incidence of leukaemia among sickle cell patients was identical to that of the African-American population (among 58 leukaemia patients, seven were AS and one was SS). In 1984, Johnson [68] reported the first case of AML cured by genoidentical transplantation in a sickle cell patient. In 1986, Stryker [69] reported four cases of haematological malignancy with a review of the literature mentioning 14 other cases in 13

homozygous S patients and one S/βThal. Interestingly, in this publication, long before the era of hydroxyurea treatments for sickle cell disease, two patients had chromosome 5 deletions, known to be associated with exposure to mutagens. Thus, the authors already suggested the harmful role played by the high medullary mitotic power of these patients, increasing the risk of chromosomal breaks. It is understandable that hydroxyurea, which is responsible for a deficit in DNA replication and repair, may increase this risk, as shown in the study by Maia Filho (2019) [70] who reported a higher chromosomal rearrangement index in sickle cell patients than in normal subjects with a further increase in this index under hydroxyurea. However, this observation has not been confirmed in a paediatric study [71].

A recent UK study, using administrative hospitalisation data collected between 1999 and 2011, compared 7,512 sickle cell patients with a control population of 118,821, stratified by age, gender and ethnicity. A 2.7-fold increase in the frequency of cancers in general was reported, and in particular a 5.1-fold increase in the risk of renal cancers. With regard to haematological malignancies, patients with sickle cell disease have a 2.6-fold increase in the risk of lymphoma, a 5.46-fold increase in the risk of myeloma and a 10-fold increase in risk of AML [65]. This study therefore suggests an increased risk related to sickle cell disease and not ethnicity. It should be noted that this British study did not have data on treatments for sickle cell disease and that the haematological malignancies observed concerned a small number of haematological diseases (14 myeloma, 14 AML, 13 lymphoma).

A Californian study [66] which followed 6,423 sickle cell patients for an average of 22 years, compared to the general population, reported a two-fold increase in the risk of leukaemia and a 3.59-fold increase in the risk of AML. Although statistically significant, this increase in the risk of AML remains modest in absolute terms: from 0.3/1,000 expected cases to 1.2/1,000 observed cases. The risk is greater in severe forms, but the treatments received (hydroxyurea) are not specified.

Longer life expectancy and "accelerated ageing" of the haematopoiesis of sickle cell patients would increase the incidence of dysmyelopoiesis and haematological malignancies, occurring at an age earlier than in the general population [72]. In the course of ageing [73] genomic, epigenetic and functional changes in haematopoietic cells are increased. These lead to deficits in self-renewal and activation of HSC differentiation, clonal haematopoiesis (CH) which is often clinically irrelevant, an increased frequency of MDS, and various malignant proliferations, in particular AML.

Chronic inflammation, overwhelmed defences against intravascular haemolysis, free radicals induced by "free" haemoglobin or haem or iron overload, chronic haematopoietic stress from accelerated blood cell turnover, and functional alterations in haematopoiesis or immunity could be involved in these patients [74].

Discussion

Is the oncogenic risk lower after gene editing therapy?

The first gene therapy trials for haemoglobinopathies by gene editing are progressing rapidly [74]. The principle of this "molecular surgery" is initially to select a target sequence in a gene and modify it by an endonuclease: ZFN (zinc-finger nucleases), TALEN (transcription activator-like effector nucleases) or CRISPR-Cas9 (clustered regularly interspaced short palindromic repeats [CRISPR]-associated protein 9). In cases of severe β-haemoglobinopathy, the most comprehensive approach is to limit repression of the four γ-globin genes in order to restimulate the synthesis of foetal haemoglobin, which is known to prevent haemoglobin S polymerization in sickle cell patients or to compensate for the deficit in synthesis of the β chain in β-thalassaemic patients.

Initial clinical results show that the therapeutic efficacy of gene editing is good and similar to that obtained by adding a globin gene for the prevention of vaso-occlusive crises in sickle cell disease and the correction of anaemia in β-thalassaemic patients [75]. The target of editing is the erythroid enhancer specific to the *BCL11A* gene, which encodes a potent repressor of foetal haemoglobin expression after birth, creating "acquired persistence" of HbF. An initial risk associated with this approach would be alteration of the expression of *BCL11A* required in blood lines other than red blood cells. This risk seems to have been ruled out in preclinical studies and during follow-up for the first patients who have so far been investigated for only one year [76].

The genotoxic risks of conditioning in gene editing therapy are identical to those of GAT, due to the use of busulfan at the same doses.

Specific genotoxic risks of undesirable collateral alterations of the genome, linked to the editing approach, are caused by the enzyme used at the precise target site or outside of the target, for example in regions of sequence homology: double-stranded DNA cuts, insertions and deletions (indels), chromosomal rearrangements, p53 activation, etc.

A novel editing approach was achieved in 2021 by David Liu's team, who established specific and effective correction of the mutation responsible for HbS, by editing adenine (ABE gene therapy) without cutting the double-stranded DNA. The substitution of adenine at an A/T base pair thus changes the sixth mutated codon of the globin β^S globin gene (GTG [valine] → GCG [alanine]) inducing a new mutation, the "asymptomatic" Makassar haemoglobin mutation and without p53 activation (activated by double-stranded DNA nicking) [77]. Based on preclinical studies performed on haematopoietic cells from sickle cell patients and the relevant mouse model of sickle cell disease (Townes knock-in mice), this remarkable achievement of molecular engineering demonstrates effective long-term stem cell correction (LT-CSH) *in vivo* and is associated with a very low risk of adverse side effects.

Can the risk of conditioning be reduced?

Reducing the dose of busulfan or substituting by another agent known to be less genotoxic (treosulfan, melphalan, etc.) to introduce partial chimerism has been proposed [78, 79]. Indeed, experiments with allograft transplantation with reduced conditioning intensity have established that due to the life span of corrected cells, partial donor chimerism of 20% may be sufficient for the prevention of vaso-occlusive crises [10, 80]. However, this reduced conditioning, which reduces organ toxicity and fertility impairment, gives rise to residual cells that have received conditioning, thus increasing the risk of secondary neoplasia. In particular, this reduced conditioning leads to cells that are more resistant to apoptosis, for example, those with a mutation or deficiency in p53 or other alterations may provide potentially leukaemogenic cells with a selective advantage to proliferate. The process of post-transplant stress can be an accelerator of all these mechanisms of haematopoietic alteration.

The development of alkylating agent-free conditioning would therefore be the preferred research pathway to limit the risk of induced or accelerated neoplasia. For this purpose, the use of antibodies recognising antigens on the surface of HSCs is being investigated. For example, the anti-CD117 antibody targeting c-KIT is being tested as a conditioning agent for allograft transplantation in children with severe combined immunodeficiency (SCID) [81] and other conditions. Studies in animal models are also under way with immunotoxins or radioisotopes linked to anti-CD117 or anti-CD45 [82, 83].

Finally, it is possible that in the future, genome editing for HSC *in vivo* may allow gene therapy to dispense with the need for conditioning by providing the corrected

cells with a proliferative advantage over the unmodified cells responsible for the haemoglobinopathy [84]. However, it must be concluded that, despite this promising ongoing research, alkylating myeloablative conditioning with its genotoxic risk will remain the rule for gene therapy in the coming years.

Should haploidentical allografts be preferred to gene addition therapy?

The advantage of haploidentical allografting is that it is now available to virtually all patients. This was made possible by the use of cyclophosphamide at D3/D4 post-transplant, which destroys the alloreactive cells of the graft, thus reducing the risk of GVHD. Initial results reported a very high rejection rate of 50% but no GVHD [15], with total donor chimerism in successfully transplanted patients. The risk of rejection was then reduced by making the conditioning heavier (addition of thiotepa [16] or increasing the total body irradiation dose [17]). It is possible, however, that this is associated with an increased risk of GVHD.

In two recent editorials [85, 86] the authors suggest that haploidentical transplantation should be preferred, considering that the risk of GAT is too high to offer it to children whose life expectancy is high despite their sickle cell disease. However, it should be noted that the post-transplant leukaemias observed occurred following graft rejection, the risk of which is high after haploidentical transplantation. In fact, of the four leukaemias that occurred in post-transplantation, three occurred after rejection of haploidentical transplants.

In contrast to gene therapy, haploidentical allografting has the advantage that, if successful, the patient's haematopoiesis is completely replaced. On the other hand, the increased conditioning, exposure of the graft to cyclophosphamide and the residual risk of rejection result in a risk of secondary oncogenesis that is probably no lower than with GAT.

Differences in the risk of leukaemia between thalassaemia and sickle cell disease?

In the GAT trials involving 64 β-thalassaemic patients (HGB-205, 207 and 212) and the long-term follow-up protocol, there have been no oncogenic complications to date, despite the longer follow-up. Is there therefore a lower risk of post-GAT haematological malignancy in thalassaemic patients than in sickle cell patients treated with the same vector (BB305) and the same protocol?

The literature reports an increased overall oncogenic risk in β-thalassaemic patients. A longitudinal cohort study conducted in Taiwan on 2,655 thalassaemic patients [86] (1998–010) showed an oncogenic risk of 3.96/1,000 patient years, which is 1.54 times higher than that of a population of 10,620 non-thalassaemic subjects; 2.60/1,000 person years. In particular, the risk of haematological malignancies was 5.32 times that of the general population, but the details were not reported. Solid cancers occur in 3.6% of thalassaemic patients, constituting the fifth leading cause of death in a prospective study of more than 1,000 patients in Italy [87, 88]. Iron overload, transfusion-related viral infections, and immunosuppression may contribute to this increased risk of solid cancers in β-thalassaemic patients.

After transplantation, very long-term follow-up of β-thalassaemic patients shows that cancers secondary to transplantation occur late, with a median of 18 years. The risk of solid tumours is four to six times greater than in transplant donors or in non-transplant patients [89]. A French paediatric study of 99 β-thalassaemic patients found no secondary cancer during the study period (median age at transplantation: 5.9 years [3.1–11.2 years]; median follow-up 11 years [7–19.3 years]) [90]. Thus, in this study, no particular risk of myeloid oncogenesis was detected, either spontaneously or after allograft or solid cancer in the short term.

Transfusion-dependent thalassaemia and severe sickle cell disease share the same problems associated with anaemia, transfusion-related viral risk (which has become minimal in recent decades), iron overload and ineffective erythropoiesis which is more severe in patients with thalassaemia than sickle cell disease. In contrast, intravascular haemolysis is more pronounced in sickle cell disease, and transfusion-dependent thalassaemic patients who are maintained above 9 g/dL haemoglobin are not exposed to vaso-occlusive crises, sometimes with deglobulation, nor to ischaemia-reperfusion phenomena. In addition, transfusion-dependent thalassaemic patients proposed for gene therapy were not treated with long-term hydroxyurea as adult sickle cell patients were. It should be recalled that in sickle cell disease, no post-transplant cancer has been observed after a median follow-up of 7.9 years in the French series of 234 patients transplanted before the age of 30. However, the main indication for transplantation was the presence of cerebral vasculopathy treated prior to transplantation with a transfusion programme and not with hydroxyurea, whereas 93% of adults transplanted at the NIH with non-myeloablative conditioning had been treated with hydroxyurea.

How can the risk of genetic mutations in sickle cell patients be reduced?

It is concluded that sickle cell disease in its severe form is associated with a small but significant increase in the risk of haematological malignancies, primarily myeloid (MDS/AML) and an early risk of clonality (indeterminate, premalignant or leukaemic clones). These increased risks may be related to the "accelerated ageing" of the haematopoiesis of sickle cell patients. The mechanisms generating these alterations are complex: increased blood cell turnover, chronic stimulation of haematopoiesis accentuated by more acute episodes of cell production, long underestimated dyserythropoiesis and tissue alterations related to hypoxaemia, increased oxidations, local or systemic inflammation, ischaemia/reperfusion, vaso-occlusions, microthromboses, areas of haematopoietic marrow destruction, necrosis and ossification.

Hydroxyurea, through the deficits in DNA replication and repair it causes, theoretically increases this risk, however, this has not been objectified in series so far, with an admittedly limited follow-up. It is conceivable that, if effective, hydroxyurea limits haematopoietic stress and the risk of somatic mutations. On the other hand, in the event of total or partial ineffectiveness, added oncogenic risk is highly likely. When ineffective, hydroxyurea should be stopped and replaced by a transfusion programme or the new, non-oncogenic, targeted drugs and by allogeneic transplantation–or, in the future, by gene therapy, as soon as this is authorised. Indeed, new anti-sickle cell drugs have appeared on the market. Voxelotor [91], administered *per os,* alters the affinity of haemoglobin for oxygen and significantly improves anaemia and haemolysis, but does not reduce the frequency of vaso-occlusive crises. Crizanlizumab [92], an anti-P-selectin antibody, can space out crises but does not improve the degree of haemolytic anaemia, and its use as a monthly IV infusion limits its value.

Conclusion

The absence of cases of leukaemia induced after transplantation or gene therapy in patients with β-thalassaemia therefore leads to a greater suspicion of sickle cell disease itself and its pre-transplant treatment with hydroxyurea, as cofactors of oncogenesis associated with the conditioning common to allografts and gene therapy. The insertional oncogenesis and the therapeutic globin gene cannot, at this stage, be implicated.

Severe sickle cell disease, due to its intravascular haemolysis, vaso-occlusive crises, haematopoietic stress, ischaemia-reperfusion phenomena, tissue hypoxia and

inflammation, can promote accelerated ageing of haematopoiesis and the occurrence of potentially oncogenic somatic mutations, the risk of which is very low in childhood but increases with increased lifespan in adult patients.
Hydroxyurea has a clear oncogenic potential, but its effect may be balanced by the positive role it plays in reducing the risk of VOCs and reducing intravascular haemolysis and chronic haematopoietic stress. In contrast, however, genotoxicity may increase with dose, altered pharmacokinetics (renal failure) and duration of treatment, and the risk may outweigh the benefit if hydroxyurea proves to be ineffective.
A prospective and comparative study between the different treatments of sickle cell disease and transplantation modalities with prior investigation into clonality, mutations associated with risk of myeloid haemopathies and, in particular, in p53 could improve knowledge of the different mechanisms involved and modify therapeutic indications.
These considerations lead us to currently recommend the following:

– early prescription of hydroxyurea in order to minimise the frequency of vaso-occlusive crises,
– systematic family HLA typing and offer of transplantation to children with an HLA-identical donor,
– awareness regarding when to stop hydroxyurea in the absence of sufficient efficacy beyond six months of treatment, replacing it with a transfusion programme with constant efficacy. Radical biotherapies (GAT or haploidentical transplantation) at an early stage should be proposed before the appearance of molecular or cellular markers which are the precursors of severe acquired haemopathies: significant somatic mutations, chromosomal alterations or clonality.

Gene therapy is being added to the radical therapeutic arsenal, in the very short term, for sickle cell disease as it is already used as treatment for β-thalassaemic patients. In the latest cohort, the occurrence of two cases of leukaemia unrelated to the vector among 47 sickle cell patients treated with GAT should not obscure the considerable benefits observed, with disappearance of VOCs and ACS and resolution of anaemia in 32 patients [20]. The above suggestions for patient management should limit the risks in the future.

Conflict of interest: Declared interests: FB is a consultant for Bluebirdbio, Vertex and GBT and receives grants from AddMedica.]

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