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Constructing new motifs in hematology

Edited by
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Constructing new motifs in hematology

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Editorial: Constructing new motifs in hematology

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KEYWORDS

anemia, Myelodysplastic Syndrome, lymphoma, granulocyte infusion, coagulation

Editorial on the Research Topic
Constructing new motifs in hematology

The word "hematology" was referred to first in 1748 with the publication of Thomas Schwenke's book, Haematologia, a "treatise as complete as it could then be, upon the blood considered in its state of health and of disease" (1). From then on, tremendous efforts were put forth to evolve hematology with the influences of the application of novel diagnostic technologies, genetic revolution, and molecular mechanisms to lighten the challenges in the pathogenesis of diseases. In the current Research Topic, we aimed to update the community on the new era in hematology, with state-of-the-art knowledge in all fields of hematology including recent diagnostic technologies, novel treatment approaches of malign/benign hematological diseases, and unsolved issues.

Anemia, the reduction in hemoglobin (Hb) or hematocrit (HCT) or red blood cells, is the most common blood disorder. Research is being undertaken for methods of detection, genetic and molecular aspects in pathophysiology as well as treatment approaches. Voxelotor is an HBs polymerization inhibitor approved by the FDA that has demonstrated an improved Hb level, and reduced hemolysis, with an improved rate of vaso-occlusive crises (2). Alkindi et al. defined the abnormal HbD variant on high-performance liquid chromatography (HPLC) in patients under Voxelotor, which will provide data on patient compliance. β-thalassemia is another inherited hemoglobin disorder mainly affecting the Mediterranean area, North and Central Africa, Southeast Asia, and the Middle East (3). Sanchez-Villalobos et al. reviewed the latest advances in the pathophysiology of β-thalassemia by categorizing it into three categories: correction of the globin chain imbalance, reverse ineffective erythropoiesis, and improving iron overload. As potential targets for future use, inflammasomes and HSP70 nuclei regulation were demonstrated (4, 5).

Myelodysplastic Syndrome (MDS) is a heterogeneous group of diseases in which ineffective hematopoiesis and cytopenia are predominant. Toprak published a detailed review of Low-Risk MDS including an updated definition, classification, pathogenesis, clinical presentation, risk stratification, prognostic assessment, and treatment. The presence of somatic mutations has been demonstrated, with TP53, ASXL1, EZH2, ETH2, ETV6, and RUNX1 showing a poor clinical course, whereas SF3B1 leads to good clinical outcomes defined with next-generation sequencing (NGS) (6). Interestingly, Zou et al. reported a case of MDS with an abnormal karyotype- t(11;22)(q23;q11) and the presence of an MLL-SEPT5 fusion transcript that is uncommon in MDS and more likely presents in adult and pediatric leukemia (7). For differential diagnosis between MDS, aplastic anemia (AA), and megaloblastic anemia (MA), Zhao et al. demonstrated a rapid and efficient method by identifying the area of red blood cells in peripheral blood smears based on the image processing technology, which might be an alternative in low-resource settings.

In one of the manuscripts included in this Research Topic, Chang et al. described a novel phenotype of the Factor V Gene Mutation (Homozygote Met1736Val and Heterozygote Asp68His) in the Asian population. Although the risk of bleeding in moderate Factor V deficiency is low (8), one of the cases presented a massive postpartum hemorrhage, which should be considered for future cases. In the next article, He et al. conducted a retrospective study among 121 chronic active Epstein-Barr virus infection (CAEBV) disease that progressed to hemophagocytic lymphohistiocytosis (HLH), which is characterized by multi-organ dysfunction due to excessive immune activation (9). They explored the risk factors and generated a nomogram to predict the risk of progression by plasma EBV-DNA load, platelet count, elevated alanine aminotransferase, and $\geq$2 of 3 lineages of cytopenia.

Granulocyte transfusions (GCs) are a potential therapy for neutropenic patients with infections resistant to antibiotics and anti-fungal drugs (10). Murru et al. investigated unknown points in various manufacturing strategies of granulocyte transfusions by evaluating leukocyte composition, neutrophil viability, calcium mobilization, chemotaxis, phagocytosis, reactive oxygen species, cytokine production, and metabolites. G-CSF GCs contained more neutrophils than prednisone GCs. Prednisone GC neutrophils showed enhanced phagocytosis and G-CSF GC neutrophils exhibited decreased chemotaxis but increased IL-8 production. G-CSF neutrophils seemed to be more sensitive to storage. A review by Castagna et al. lightens up a controversial issue in the care of relapsed/refractory aggressive lymphomas (large B-cell lymphoma and MCL) by results obtained after allo-HSCT and CAR T cell therapies.

In summary, today, excessive data have been accumulated in malign and benign hematology. Novel diagnostic methods sculpt the concept of "personalized medicine" rather than the conventional approach.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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ORIGINAL RESEARCH
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MLL-SEPT5 Fusion Transcript in Myelodysplastic Syndrome Patient With t(11;22)(q23;q11)

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Objectives: This study aimed to identify unknown mixed lineage leukemia (MLL) translocation partner genes in a *de novo* patient with myelodysplastic syndrome (MDS) with t(11;22)(q23;q11) and investigate the clinical and molecular features of this patient.

Methods: Bone marrow cells were assessed by karyotype analysis to reveal chromosomal abnormalities. Fluorescence *in situ* hybridization (FISH) was performed to detect MLL gene rearrangement using an MLL-specific break-apart probe. LDI-PCR and RT-PCR were performed, and the PCR products were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The sequence data of the PCR products were analyzed using bioinformatics tools. Meanwhile, clinical data were collected to evaluate the prognosis of the patient.

Results: Chromosomal karyotype analysis showed that the karyotype of the patient was 46, XX, t(11;22)(q23;q11)[10]/46, XX[1]. Subsequently, FISH data confirmed MLL gene rearrangement in the patient. LDI-PCR precisely showed that SEPT5 was the MLL translocation partner gene. RT-PCR and sequencing analysis disclosed the presence of MLL-SEPT5 fusion transcript and confirmed the fusion between MLL exon 8 and SEPT5 exon 3. Moreover, the patient had a recurrence shortly after allogeneic hematopoietic stem cell transplantation.

Conclusion: Although the MLL-SEPT5 fusion transcript was occasionally described in acute myeloid leukemia, it was first identified in MDS. Patients with MLL-SEPT5 fusion gene exhibited a poor prognosis even with an aggressive treatment.

Keywords: myelodysplastic syndrome, MLL rearrangement, septin, SEPT5, t(11;22)(q23;q11)

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INTRODUCTION

Myelodysplastic syndromes (MDS) are clonal myeloid malignancies characterized by ineffective hematopoiesis, refractory cytopenia, and an increased risk of progression to acute leukemia (1). Chromosomal translocations involving the mixed lineage leukemia (MLL) gene are the most frequent genetic alteration in adult and pediatric leukemia (2), especially in pediatric leukemia, which is reported to have an incident rate of up to 70% (3). In contrast to acute leukemia, MLL rearrangement occurs rarely in patients with MDS. Multiple studies have demonstrated that most patients with acute leukemia with MLL rearrangements are characterized by a high degree of

malignancy, low rates of remission, insensitive to chemotherapy, and poor prognosis. Accordingly, unambiguous identification of MLL rearrangements is useful not only for diagnosis at an early stage but also for prognostic evaluation.

Balanced chromosomal translocations are the most common rearrangements for MLL. Because of the promiscuous features of MLL, the fusion partner genes diversity was determined. MLL fusion partner genes are divided into 4 classes: nuclear proteins (such as AF9, AF10, and ENL), cytoplasmic proteins (such as EPS15, SH3GL1, and GAS7), histone acetyltransferases (such as EP300 and CREBBP), and septin gene family members (such as SEPT5, SEPT6, and SEPT9). So far, over 100 different MLL reciprocal translocations have been described and approximately 80 partner genes for MLL have been identified at the molecular level (4). Among them, AF4, AF9, and ENL were the most frequent fusion partner genes with MLL. However, many uncommon MLL rearrangements are easily overlooked during the diagnostic encounter. Our study elucidated that the MLL gene at band 11q23 was involved in chromosome translocations with the 22q11-22q13 region of chromosome 22. Existing literature has found that SEPT5 or EP300 is located at 22q11-22q13 and acts as the translocation partner of MLL (5, 6). As expected, SEPT5 has been precisely identified as a partner of the MLL gene in our patient using LDI-PCR. Patients with MLL gene rearrangement are classified as a distinct subcategory in acute leukemia according to the classification of 2008 WHO (7). However, patients with MDS with *MLL* gene rearrangement, especially MLL-SEPT fusion, have not been well defined.

Here, we reported a case of MDS with t(11;22)(q23;q11) abnormal karyotype in a 46-year-old woman and confirmed the presence of an MLL-SEPT5 fusion transcript. To our knowledge, the MLL-SEPT5 fusion transcript has not yet been reported in MDS.

PATIENT AND METHODS

Patient

In June 2020, a 46-year-old, previously healthy woman was evaluated at a local hospital for leukopenia. After symptomatic support treatment for 1 month, no improvement in leukocyte numbers was observed, then the patient was admitted to our hospital (Ningbo First Hospital, Ningbo, China) for further examinations. She denied any symptom of fever, respiratory disorder or other symptoms such as skin rashes and joint pain before the onset. Moreover, she did not have any history of exposure to ionizing radiation, chemotherapy or toxic substances, and again no history of malignancy, alcohol, tobacco, or drug addiction. A complete blood count was carried out using routine automated analyzers and showed a decrease in the erythroid and myeloid lineage (hemoglobin of 9.7 g/dl, white blood cell count of 1.65×10^9/L, absolute neutrophil count of 0.4×10^9/L, and a platelet count of 132×10^9/L) in the peripheral blood. Bone marrow smears showed granulocytic-lineage dysplasia, containing 1% myeloblast, 15% monoblasts, and promonocytes. Flow cytometry data showed that the blasts were positive for CD45, CD15, CD13, CD33, and CD38 and negative for lymphoid markers. A diagnosis of MDS-EB was established according to classification of the 2016 WHO. The patient with MDS was classified as very high IPSS-R risk (score: 6.5) and high WPSS risk (score: 4) at the time of diagnosis.

After a detailed assessment by hematologists, she received allogenic hematopoietic stem cell transplantation (allo-HSCT) on August 26, 2020. Before transplantation, the patient received myeloablative conditioning regimens (azacitidine plus modified busulfan/cyclophosphamide plus ATG). The patient underwent allo-HSCT from unrelated cord blood, peripheral blood stem cells and bone marrow (HLA6/10) of her daughter, followed by cyclosporine A, mycophenolate mofetil, and short-term methotrexate for prophylaxis of graft vs. host disease (GVHD). The patient received supportive care, including red blood cell transfusions, platelet transfusions, and treatment with recombinant human granulocyte colony stimulating factor (rhG-CSF) and thrombopoietin (TPO). In March 2021, the patient presented with chills, sputum expectoration, and cough under no obvious inducement. In total, 1 month later, the patient successively developed fever (38°C), pulmonary fungal infections, and extensive skin chronic GVHD. Thus, cefoperazone combined micafungin was prescribed. Unfortunately, she experienced a relapse 10 months after transplantation. The patient received 100 mg of azacitidine on day 1–7, and was subsequently treated with a PD-1 inhibitor combined with peripheral blood stem cells. In October 2021, the disease progressed to AML, and the evaluation of bone marrow showed 27% of blasts. The patient subsequently received decitabine in combination with cytarabine.

Cytogenetic Analysis

The moderate numbers of BM cells were cultured in a commercial cell culture medium (Tianjin Reagent Biotech Corporation Ltd., Tianjin, China) at 37°C for 24 h. The cells were processed with colchicine, centrifuged, and treated with hypotonic solution (0.075 M KCl) at 37°C for 40 min. Then, the cell suspension was prefixed and finally fixed using methanol and acetic acid. Finally, the metaphase chromosomes were R-banded, stained with Giemsa, and scanned on a fully automated GSL-120 Leica microscope (Leica, Germany) for karyotype analysis. The chromosomes were described according to the International System for Human Cytogenetics Nomenclature (ISCN 2016).

Fluorescence *in situ* Hybridization

After centrifugation, BM cells were harvested and resuspended in hypotonic KCl solution at 37°C for 40 min. The cells were pre-fixed, fixed, and stored at 4°C until the fluorescence *in situ* hybridization (FISH). FISH was performed on blasts from the BM samples according to product instructions using the MLL break-apart probe (Guangzhou LBP, China). Separation of the green and red fluorescence signals indicates the chromosomal rearrangements in MLL/11q23. At the same time, BCR/ABL fusion analysis was conducted as a control using BCR/ABL dual-color, dual-fusion probe (Guangzhou LBP, China).

Long Distance Inverse-Polymerase Chain Reaction Analysis

Deoxyribonucleic acid was extracted from cell suspensions that were used for karyotype analysis. DNA samples were digested by the restriction enzyme Bam HI (Takara, Japan). Subsequently, phenol-chloroform was used to remove the residual Bam HI enzymatic activity. After purification, the digested linear DNA was religated into a circular DNA molecule at 16°C overnight using T4 DNA ligase (Takara, Japan). The religated DNA samples were terminated for 10 min at 65°C, then stored at −80°C until the LDI-PCR. PCR amplifications were conducted with TaKaRa LA Taq DNA Polymerase Kit (Takara, Japan) and following the instructions of the manufacturer. MLL gene-specific oligonucleotides sequences (**Supplementary Table 1**) were obtained from Prof Rolf Marschalek, and were used according to their experimental method in a previously published article (8). In total, 5 μl of religated DNA samples were used for LDI-PCR in a total volume of 50 with 5 μl of LA Taq Buffer, 5 μl of $MgCl_2$ (25 mM), 8 μl of dNTP Mixture (2.5 mM each), 0.5 μl of TaKaRa LA Taq, 2 μl of forward primer, 2 μl of reverse primer, and 22.5 μl of RNase-free H_2O. PCR amplification was carried out at 94°C for 1 min, followed by 30 cycles at 98°C for 10 s, 68°C for 10 min, 72°C for 10 min using the Bio-Rad PCR instrument (Bio-Rad, CA, USA). The LDI-PCR products were electrophoresed on a 0.8% agarose gel, and were then sequenced by Shanghai Sangong Biotech Corporation (Shanghai, China). The sequencing results were performed by alignment with BLAST searches of NCBI databases to determine the fusion site of MLL and MLL partner gene.

RT-PCR and Sequencing of PCR Product

Blast cells were isolated from the samples of BM using Ficoll-Paque Plus (GE Healthcare, Sweden). RNA was extracted using RNAiso Plus reagent (Takara, Japan), and reverse transcription was performed with a cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the instructions. Primers were synthesized by Tsingke Biotechnology (Hangzhou, China). Sequences of the primers are as follows: MLL-E7, 5′-TACAGGACCGCCAAGAA-3′ from exon 7 of MLL; SEPT5-E3, 5′-CAAAGCCTTTCTTCACCGAC-3′ from exon 3 of SEPT5; SEPT5-E5, 5′-TGTCCACGATGGTGAGCTTC-3′ from exon 5 of SEPT5. About 1 μl cDNA was used for amplification in a total volume of 20 with 0.8 μl of forward primer, 0.8 μl of reverse primer, 10 μl of SYBR Green (Takara, Japan), 0.4 μl of ROX, and 7 μl of RNase-free H_2O. PCR procedures were carried out as described previously (9). The products were analyzed by electrophoresis with a 1% agarose gel and were then sequenced by the Shanghai Sangong Biotech Corporation (Shanghai, China). The product sequencing results were analyzed using Splign and BLAST searches of NCBI databases.

RESULTS

Cytogenetic analysis by R-banding stain showed an abnormal karyotype: 46, XX, t(11;22)(q23;q11)[10]/46, XX[1]. The result is shown in **Figure 1A**. FISH analysis was performed to validate the existence of MLL rearrangements. The break-apart probe to 11q23 shows 1 normal fusion signal and dissociation of the 5′ MLL green and 3′ MLL red components of the other signal (arrows), suggesting that a translocation had occurred involving the MLL gene (**Figure 1B**). In order to exclude BCR (chromosome region 22q11) variants, FISH analysis for BCR-ABL was performed using dual-fusion probe, and BCR rearrangement was negative (**Figure 1C**).

To identify the involved partner gene in the current patients with MDS, LDI-PCR was performed using MLL gene-specific oligonucleotides. The amplification PCR product was separated by horizontal electrophoresis and obtained an 815 bp product in length (**Figure 2A**). Sequencing analysis indicated that intron 2 of SEPT5 is fused to intron 8 of MLL on the DNA molecule (**Figure 2B**). Furthermore, RT-PCR and direct sequencing were performed using MLL and SEPT5 specific primers to detect MLL-SEPT5 fusion transcript in the BM cells. As expected, sequencing and bioinformatics analysis revealed that exon 8 of MLL is fused to exon 3 of SEPT5 (**Figure 3B**), and a 416 bp and 215 bp product was observed when amplified using 2 different sets of primers, respectively (**Figure 3A**).

To better understand the possible roles of leukemogenesis of MLL-SEPT5 fusion, the main clinical and molecular features of MLL-SEPT5 fusion-positive patients are summarized in **Table 1**. Of the 10 MLL-SEPT5 fusion patients, 3 were infants, and seven were adults. The blast cells of these patients in the bone marrow showed myeloid phenotype. Impressively, our patient was diagnosed with MDS, while others were all diagnosed with AML. Furthermore, the prognosis for these patients with MLL-SEPT5 fusion seems poor, even if some of them had undergone stem cell transplantation.

DISCUSSION

Many common fusion gene detections, including MLL fusion, have already been applied as routine examinations for the patients with hematologic malignancies in clinical practice. However, some rare fusion genes are still challenging to be found in patients. Here, we have reported a patient with MDS with t(11;22)(q23;q11), which resulted in an MLL-SEPT5 fusion.

Septins are GTP-binding proteins associated with crucial biological processes such as cytokinesis, membrane dynamics, and cytoskeletal reorganization (10). Increasing reports link septins to cancer in humans, including hematological malignancies (11). However, the exact function of septins in hematologic tumors remains incompletely known. In total, 5 septin genes (SEPT2, SEPT5, SEPT6, SEPT9, and SEPT11) are involved in chromosome translocations with MLL in patients with hematological malignancies, producing oncogenic MLL-fusion protein (12). As the MLL fusion partner, SEPT5 has been reported to be aberrantly expressed in acute myeloid leukemia (AML) (13). MLL-SEPT5 fusion transcript was the first reported in AML of infant twins with t(11;22)(q23;q11.2) (5). RT-PCR and subsequent products sequencing demonstrated that exon 7 of MLL is fused to exon 3 of SEPT5. Previous research has also indicated that the breakpoints in the MLL

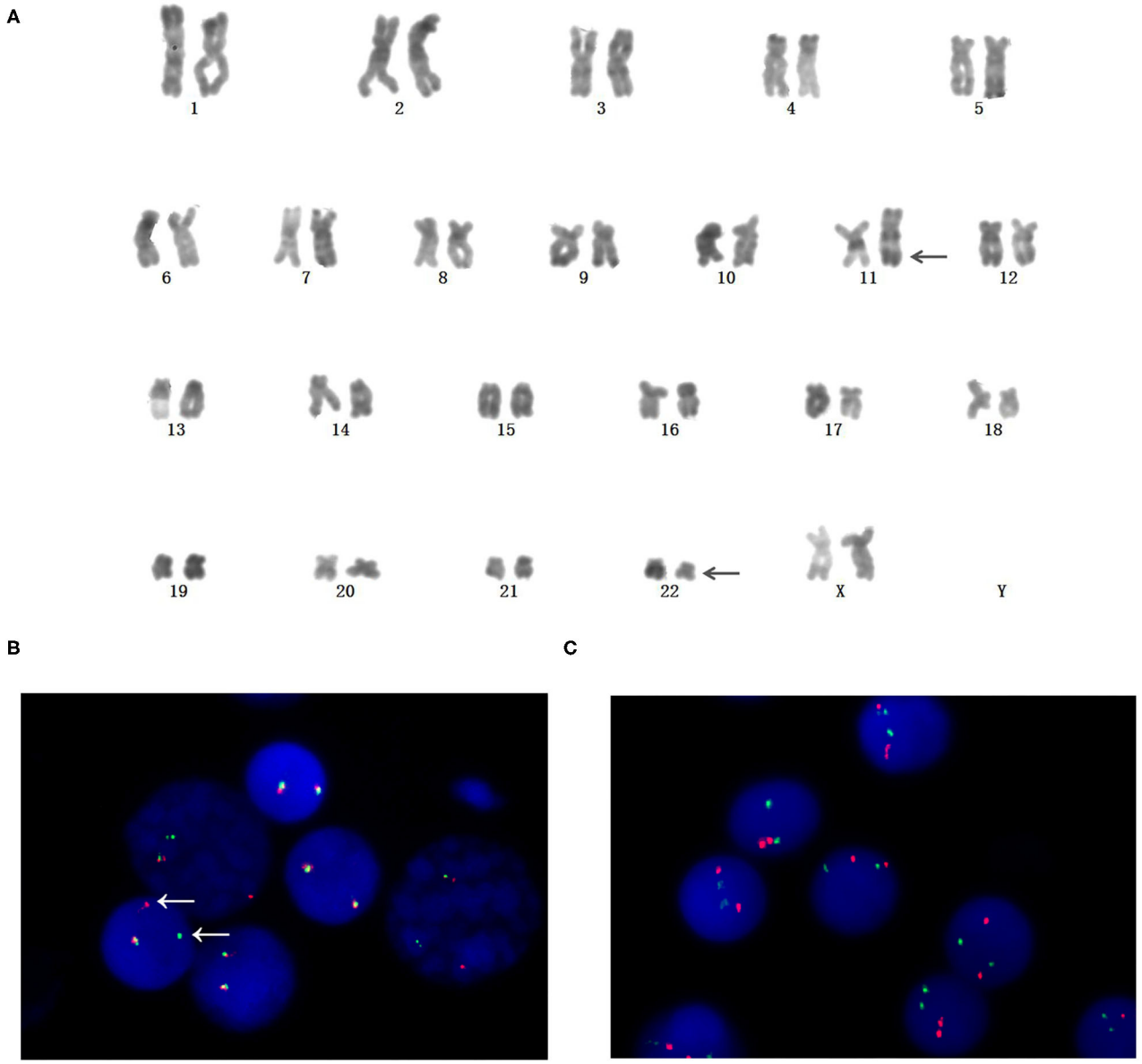

FIGURE 1 | Identifification of chromosome rearrangements involving mixed lineage leukemia (MLL)/11q23. **(A)** Karyotype (R-banded) on bone marrow cells. Arrowheads indicate der(11) and der(22). **(B)** MLL rearrangement was confifirmed by Fluorescence *in situ* hybridization (FISH). Dissociation of a green and a red signal indicated the rearrangement of MLL. **(C)** BCR rearrangement was negative by FISH analysis.

gene always occurs in the 6–11 exons (12), and as a partner gene of MLL, the SEPT5 gene easier occurs in the 5′ region (exons 1–3), resulting in the fusion that contains the entire open reading frame of the SEPT5 gene. Tatsumi et al. (14) reported that the SEPT5 gene fused to MLL in an adult patient with *de novo* AML and also found that SEPT5 expression in AML was markedly higher than that in ALL cell lines. Almost a decade later, the fourth patient with MLL-SEPT5 fusion transcript was reported by Wang et al. (15). We believe that some cases with MLL-SEPT5 fusion are easily ignored during that time due to its rarity. With more attention in the clinic, a growing amount of MLL-SEPT5 fusion has been discovered and identified in the recent years (16–19). The breakpoint of the MLL gene in 2 cases was same as that in our patient, while the breakpoint of the SEPT5 gene in 6 cases. Although the splice of the MLL-SEPT5 fusion gene in different ways in these patients, it is worth noting that involvement of SEPT5 exon 3 occurred with high frequency.

In addition, a high proportion (16%) of marrow blast cells was observed in our case. It is unclear whether our

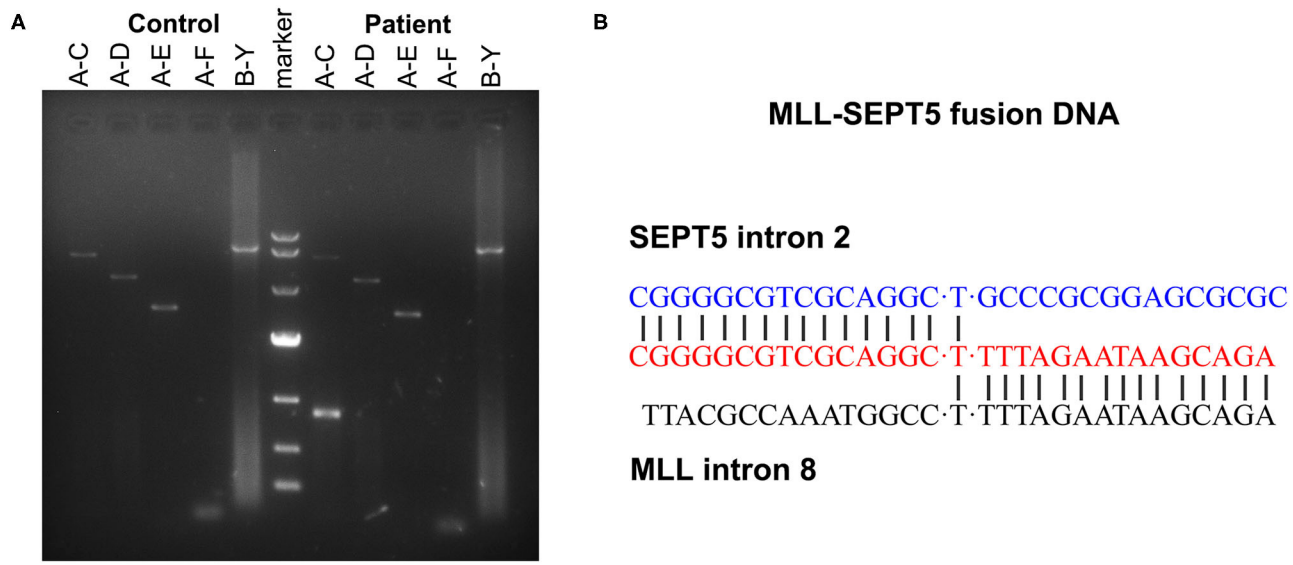

FIGURE 2 | LDI-PCR analyses of MLL translocation partner gene. **(A)** Genomic DNA of healthy individual and the patient were tested with five different primer combinations (A–C, A–D, A–E, A–F and B–Y). (Left) control DNA (healthy individual peripheral blood). (Middle) DNA marker (TaKaRa, 3584A). (Right) the patient. **(B)** Sequence analysis of the SEPT5-MLL fusion gene showing juxtaposition of SEPT5 intron 2 and MLL intron 8. "T"was used to connect the two broken chromosomes.

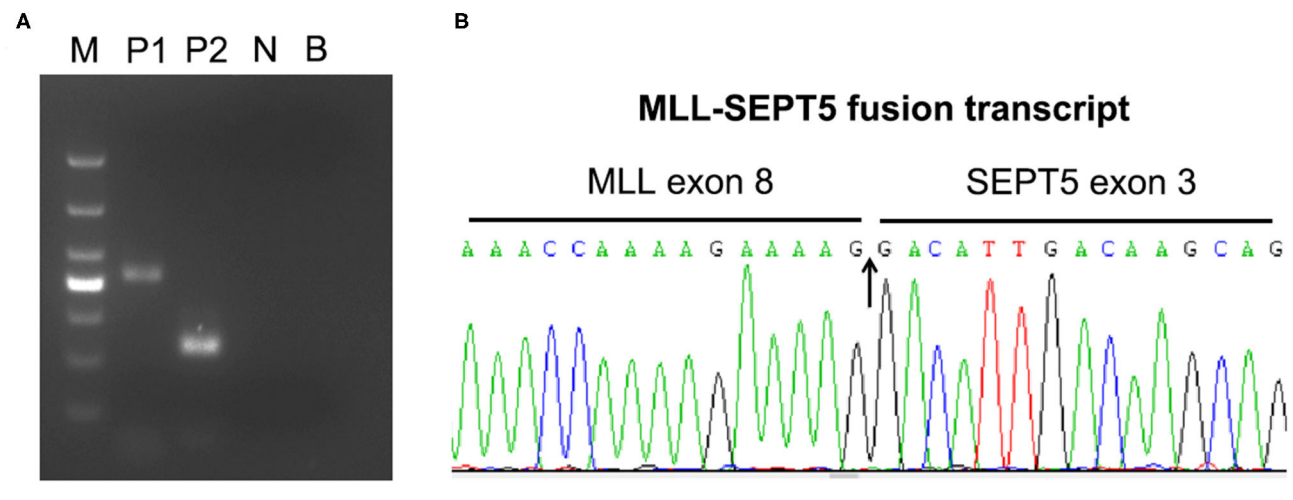

FIGURE 3 | RT-PCR analyses of MLL-SEPT5 fusion transcript. **(A)** The products were detected with 2% agarose gel electrogenesis after amplification. M, DNA marker (TaKaRa, 3591A); P1, patient sample (primer combination: MLL-E7 and SEPT5-E5); P2, patient sample (primer combination: MLL-E7 and SEPT5-E3); N, PCR control; B, blank control. **(B)** Sequence analysis of the SEPT5-MLL fusion transcript showing juxtaposition of MLL exon 8 and SEPT5 exon 3.

patient represented evolving AML or whether these patients with AML with MLL-SEPT5 fusion had a short history of MDS. In some unique entities (such as AML1-ETO, PML-RARa, and CBFβ-MYH11), the fusion gene is considered to be AML-defining according to the classification of 2008 WHO (7). Indeed, numerous clinical studies showed that almost all MLL-SEPT fusion genes had been identified in AML or therapy-related AML (20–22). Only 1 article reported that the identified fusion MLL-SEPT9 may be associated with *de novo* MDS until now. Interestingly, the authors also considered that the patient with MDS actually represented evolving AML that was discovered earlier than usual (23). For this reason, our patient was sufficient for the diagnosis of AML regardless of the bone marrow or peripheral blood blast cell proportion. If our view was true, it is time to classify MLL-SEPT, at least MLL-SEPT5, as a new provisional entity in AML.

Mixed lineage leukemia rearrangements are considered as an adverse risk factor and are often associated with adverse prognosis in acute leukemia (24). Currently, the effect of MLL rearrangements on the MDS prognosis remains poorly understood. In our study, the patient received an allogeneic

TABLE 1 | Clinical and molecular features of patients with MLL-SEPT5.

Patient	Age/sex	Diagnosis	Immunophenotype	MLL-SEPT5 fusion transcript	Prognosis	References
1	11.5months/F	AML	Not recorded	MLL exon 7-SEPT5 exon 3	Not recorded	Megonigal et al. (5)
2	13months/F	AML	Not recorded	MLL exon 7-SEPT5 exon 3	Not recorded	Megonigal et al. (5)
3	39years/M	AML	Not recorded	MLL exon 6-SEPT5 exon 4	OS: 12 months	Tatsumi et al. (14)
4	44years/F	AML	CD45(dim)/HLA-DR/CD38/CD15/CD33/CD13/CD11b/CD64/CD4	MLL exon 8-SEPT5 exon 3	CR by induction chemotherapy	Wang et al. (15)
5	32years/M	AML	CD33/CD64/CD117/HLA-DR/CD15/MPO/CD13	MLL exon 9-SEPT5 exon 3	OS: 7 months	Gao et al. (16)
6	32months/F	AML	CD45/MPO/CD15/CD33/CD116/HLA-DR	MLL exon 10-SEPT5 exon 3	Survived >2 years after transplantation	Launay et al. (17)
7	21years/M	AML	CD45/CD33/ CD117/CD15/HLA-DR	MLL exon 8-SEPT5 intron 2	Transplantation, in complete remission	Wang et al. (18)
8	22years/F	AML	CD45/CD14/CD13/CD33	MLL exon 10-SEPT5 exon 3	Not recorded	Wang et al. (18)
9	43years/F	AML	CD4/CD11b/CD15/CD33/HLA-DR/	MLL-SEPT5(FISH)	OS: 2 months	Elzamly et al. (19)
10	46years/F	MDS	CD45/CD15/CD13/CD33/CD38	MLL exon 8-SEPT5 exon 3	Relapsed 10 months after transplantation, progressed to AML	Present

hematopoietic stem cell transplantation. Unfortunately, she suffered a relapse of the disease after 10 months of transplantation. Most significantly, we found that MLL-SEPT5 fusion transcript is abundantly expressed in the bone marrow blast cells of the patient by RT-PCR. The patient eventually progresses to AML, as expected. Thus, MLL rearrangements involving the SEPT5 gene appear to be associated with a high risk for AML progression in MDS and an increased incidence of relapse. Because per leukemic cell carry only 1 copy of MLL-SEPT5 fusion on the DNA molecule, it is also suggested that detection of specific fusion sequences of patients may be helpful to monitor minimal residual disease and thus to take measures in time to prevent relapse.

In conclusion, although evidence from early studies (21, 25) suggested that MLL-SEPT fusion formation was likely due to this hypothesis that exposure to drugs targeting topoisomerase II can result in double-strand DNA breaks that trigger the error-prone non-homologous end joining pathway, the molecular mechanism that whether MLL-SEPT5 fusion can induce the transformation of MDS to AML require further investigations through experiments.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of Ningbo First Hospital. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

DZ performed most of the experiments. Experimental analysis was performed by YC and YZ. QM and GO conceived and designed the study. NW edited the manuscript. All authors contributed to the study and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.783229/full#supplementary-material

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Measurement for the Area of Red Blood Cells From Microscopic Images Based on Image Processing Technology and Its Applications in Aplastic Anemia, Megaloblastic Anemia, and Myelodysplastic Syndrome

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Background: Aplastic anemia (AA), megaloblastic anemia (MA), and myelodysplastic syndrome (MDS) were common anemic diseases. Sometimes it was difficult to distinguish patients with these diseases.

Methods: In this article, we proposed one measurement method for the area of red blood cells (RBCs) from microscopic images based on image processing technology and analyzed the differences of the area in 25 patients with AA, 64 patients with MA, and 68 patients with MDS.

Results: The area of RBCs was 44.19 ± 3.88, 42.09 ± 5.35, 52.87 ± 7.68, and 45.75 ± 8.07 μm^2 in normal subjects, patients with AA, MA, and MDS, respectively. The coefficients of variation were 8.78%, 10.05%, 14.53%, and 14.00%, respectively, in these groups. The area of RBCs in patients with MA was significantly higher than normal subjects ($p < 0.001$). Compared with patients with AA and MDS, the area of RBCs in patients with MA was also significantly higher ($p < 0.001$). The results of correlation analysis between the area of RBCs and mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), MCH concentration (MCHC), and red cell distribution width showed no significant correlations ($p > 0.05$). The area under the curve (AUC) results of the Receiver Operating Characteristic (ROC) curves of RBCs area were 0.421, 0.580, and 0.850, respectively, in patients with AA ($p = 0.337$), MDS ($p = 0.237$), and MA ($p < 0.001$).

Conclusion: Identifying the area of RBCs in peripheral blood smears based on the image processing technology could achieve rapid and efficient diagnostic

support for patients with MDS and MA, especially for patients with MA and in combination with MCV. However, a larger sample study is needed to find the cutoff area values.

Keywords: myelodysplastic syndrome, megaloblastic anemia, aplastic anemia, image processing technology, area of red blood cells

INTRODUCTION

Myelodysplastic syndrome (MDS) is a group of heterogeneous clonal diseases originated from hematopoietic stem cells, which is characterized by ineffective hematopoiesis, refractory hemocytopenia, and high-risk transformation to acute myeloid leukemia (1). Aplastic anemia (AA) is a group of diseases that result in the decrease of blood cells due to acquired bone marrow failure (2, 3). Megaloblastic anemia (MA) is an anemic disease caused by the disorder of DNA synthesis of blood cells, which is characterized by the megaloblastic metamorphosis of red blood cells (RBCs) and myeloid cells. Vitamin B12 and/or folate deficiency are the most common causes of MA (4). It is sometimes difficult to make differential diagnoses among these three diseases according to clinical manifestations and blood examinations because of similar findings shared by them. Anemia, bleeding, and infections due to cytopenia in one or more lineages can be seen in all these diseases mentioned above. Dysplasia in lineages of peripheral blood was shown not only in MDS, but also in MA. Especially, megaloblastic metamorphosis of erythrocytes in myelogram of patients with MDS needs to be differentiated with MA. Some patients with MDS who do not display prominent dysplasia are difficult to be made differential diagnoses with patients with AA. Sometimes, it was difficult to distinguish patients with MA from MDS of refractory anemia (RA) type (MDS-RA) (5) and multilineage dysplasia type (MDS-MLD), as well as difficult to distinguish patients with AA from hypoplastic MDS. The detection of folate and vitamin B12 is helpful to diagnose MA. Finding the clonal evidence of MDS is helpful to diagnose MDS. There were possibly 52% of patients who had one or more clonal chromosome abnormalities (6). The acquired molecular mutations were possibly found in 80–90% of patients with MDS (7, 8). However, the limitations of testing cost and laboratory test conditions in many low-resource settings make detections of abnormal chromosomes, molecular mutations, and concentrations of vitamin B12 and folate not available for some patients suspected of MDS, AA, and MA.

In this study, we described a measurement method based on image processing technology, which was developed to localize and extract RBCs from microscopic images and further calculated the area of RBCs for the first time. It can help for the diagnosis and identification of MDS, AA, and MA because the hematological analyzer can release the result within minutes.

METHODS

Patients and Diagnosis

Myelodysplastic syndrome was diagnosed according to the 2016 WHO classification (9). The diagnosis of AA was made according to the standard published by the British Society for Standards in Haematology in 2016 (10). Patients with vitamin B12 values below 150 pmol/l were diagnosed with MA. This study and all the procedures used were approved by the Institutional Review Board of the Wuhan University, China. All the patients were from Zhongnan Hospital of Wuhan University. There were 25 normal subjects, 25 patients with AA, 64 patients with MA, and 68 patients with MDS.

Measurement Method

We used a machine learning method based on image processing technology to obtain the area of RBCs in normal subjects or patients with anemia. The specific process included preparation of blood smears, magnified images of blood smears, and area calculation of RBCs (shown in **Figure 1**).

Preparation of Blood Smears

Blood smears at the time of initial diagnosis or entering the study were acquired from all patients or subjects. The following steps were observed to obtain blood smears. At first, 5–7 μl of Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulated peripheral blood or one drop of peripheral blood was directly collected from all subjects and patients, and the blood collected was dripped to 1 cm at one end of the slide or 3/4 end of the whole slide. Then we pushed the cover slide close to the blood drop, gently touched the blood drop, pressed it on the blood drop, and filled the width of the slide. Finally, Wright-Giemsa mixed staining was needed.

Magnified Images of Blood Smears

The magnified images of peripheral blood smears were obtained by microscope (magnified 1,000 times). The obtained magnified images were input into the computer system, which were Red Green Blue (RGB) images or hue, saturation, and value Hue Saturation Value (HSV) images.

Area Calculation of RBCs

Segmentation of Cell Region and Background

The first step was converting the RGB space to HSV space about images, which included HSV. Hue contains hue and hue information. Saturation contains saturation and color purity information. Value contains lightness information. The next step was image segmentation, which included image threshold segmentation and edge-based segmentation. Otsu threshold segmentation method was mainly used for image segmentation.

Location of the Single Cell in the Image

The outer contour of the image was extracted in the cell region by the findCounter method. After traversing all outer contours, the pending contour was the outer contour with an area less than the

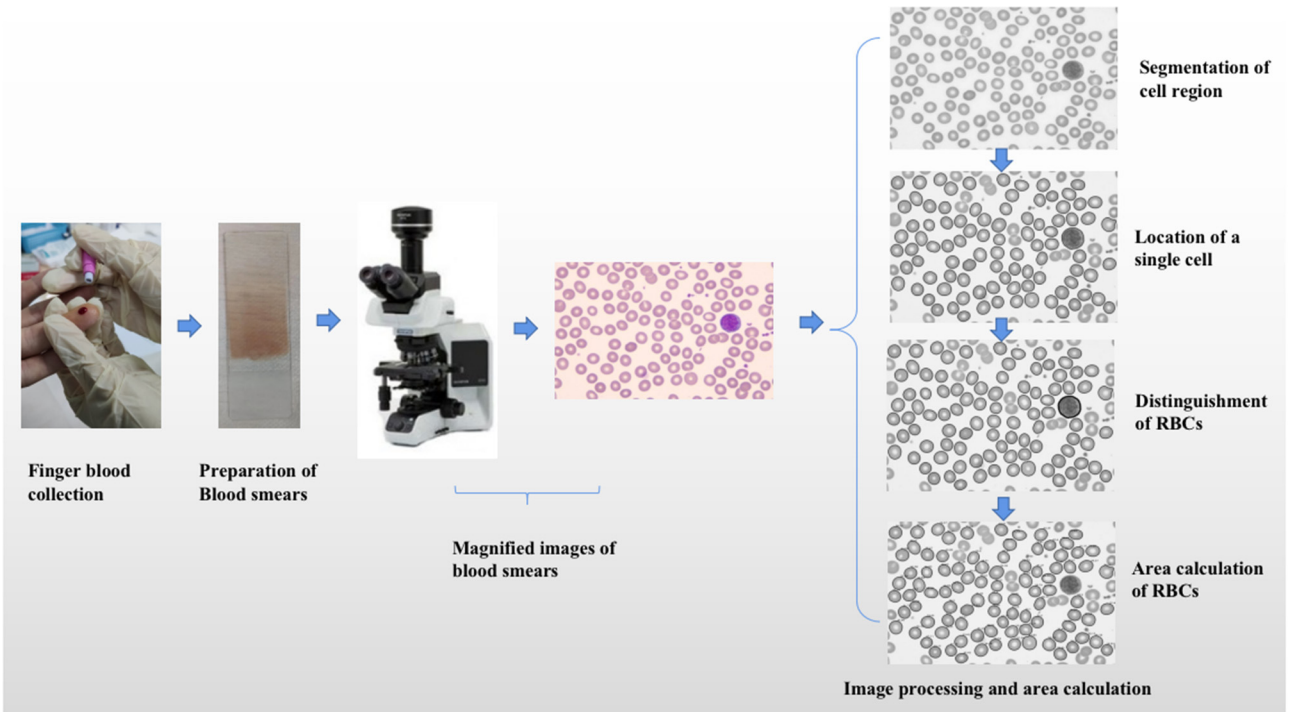

FIGURE 1 | The specific process of area calculation of red blood cells. The specific process included preparation of blood smears, magnified images of blood smears, and area calculation of red blood cells.

threshold (0.5–10 μm^2). S_{mean} was the average area of all outer contours except the pending contour.

The convex hull algorithm was used to identify the effective contour. The cell with the contour of which was convex edge shape was selected as a single cell. After traversing all outer contours except the pending contour, the outer contour with the area of which was in the specific range ($a \times S_{mean}$, $b \times S_{mean}$) was the effective contour. The value of a was less than 1, and the value of b was greater than 1. After many tests, the accuracy was best when a was 0.3 and b was 5. When the area of one outer contour was larger than $b \times S_{mean}$, it would be judged as multiple cells merging.

Distinguishment of RBCs From Nucleated Cells

Single cells include nucleated cells and RBCs. After the above location of single cells, they also should be divided into nucleated cells and RBCs. The mean gray value of all located single cells images was calculated and recorded as Mg. The single gray value of each single cell image was also calculated and recorded as G. If the ratio of G to Mg was greater than 0.8, the single cell would be selected as a RBC. If the ratio of G to Mg was less than 0.8, the single cell would be selected as a nucleated cell.

Area Calculation of RBCs

If the ratio of the distance from all points to the center of contour (r_i) to the radius (r) of the minimum enclosing circle was above 0.85, and the area of the minimum enclosing circle was in the special range ($a \times S_{mean}$, $b \times S_{mean}$), the cell would be labeled as a recall RBC. The average ratio of r_i to r was named as P_{mean}.

The area of the selected RBC was labeled as S_i, the area of recall RBC was labeled as S_r. The area of recall RBC was calculated based on the idea of calculus and according to the following formula. Finally, we calculated the average area of all RBCs in the blood smear in every subject and patient.

$$S_i = \pi \times r \times r$$
$$S_r = Si \times P_{mean} \times P_{mean}$$

Statistical Analysis

The normality of area values was carried by the Shapiro–Wilk test. The area was expressed by "mean ± SD" or "median ± quartile range." If the area values satisfied normal distribution and homogeneous variance, they would be analyzed by an independent sample *t*-test. If the distribution was normal but the variance was not uniform, the corrected *t*-test would be used. If the normal distribution could not be satisfied, the rank-sum test would be used for analysis. The coefficient of variation was calculated by the ratio of mean to standard deviation. Pearson or Spearman correlation analysis between the area and standard RBC complete blood count (CBC) indices were used according to the normality of data. The ROC curves and the values of the area under the curve (AUC) were used to compare the area of RBCs with MCV.

TABLE 1 | The characteristics of normal subjects and patients with AA, MDS, and MA.

	Normal subjects	AA	MDS	MA
Sex [(male,%)]	14(56%)	17(68%)	30(44%)	37(58%)
Age (years)	32.88 ± 9.13	47.36 ± 21.33	63.87 ± 13.51	60.73 ± 7.62
WBC (109/L)	5.88 ± 1.42	1.76 ± 0.65	2.62 ± 2.67	3.77 ± 3.37
Hemoglobin (g/L)	129.49 ± 9.58	66.29 ± 14.21	63.10 ± 17.53	61.30 ± 14.57
RBC (10^{12}/L)	4.80 ± 0.69	2.21 ± 0.65	2.49 ± 1.05	2.01 ± 0.98
PLT (10^9/L)	241.24 ± 58.30	33.64 ± 12.95	58.50 ± 65.50	64.50 ± 37.50
MCV(fL)	89.94 ± 5.87	95.82 ± 14.25	100.80 ± 13.33	123.26 ± 13.28
MCH(pg)	29.60 ± 2.33	33.22 ± 5.12	33.21 ± 4.92	41.91 ± 4.93
MCHC (g/L)	328.44 ± 9.93	347.05 ± 15.01	328.90 ± 14.59	340.84 ± 11.47
RDW(%)	13.76 ± 1.66	15.20 ± 3.91	18.90 ± 5.45	19.16 ± 4.84

WBC, white blood cell; RBC, red blood cell; PLT, platelet; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red cell distribution width; AA, aplastic anemia; MA, megaloblastic anemia; MDS, myelodysplastic syndrome.

TABLE 2 | The results of area in normal subjects and patients with AA, MA, and MDS.

	Mean (μm^2)	Median (μm^2)	Standard deviation (μm^2)	Quartile range (μm^2)	*P*-value (Shapiro-Wilk)
Normal subjects	44.19	43.01	3.88	5.47	0.561
AA patients	43.47	42.09	4.37	5.35	0.003
MA patients	52.87	51.72	7.68	10.84	0.637
MDS patients	45.86	45.75	6.42	8.07	0.006

AA, aplastic anemia; MA, megaloblastic anemia; MDS, myelodysplastic syndrome.

RESULTS

Characteristics of Normal Subjects and Patients With AA, MDS, and MA

In all the normal subjects, the average number of RBCs studied was 86.80. In patients with AA, MDS, and MA, the average numbers were 88.84, 113.97, and 106.73, respectively. **Table 1** shows the characteristics of normal subjects and all the patients included in this study. The average or median values of mean corpuscular volume (MCV) in patients with AA, MDS, and MA were 95.82, 100.80, and 123.26 fl, respectively. The average or median values of mean corpuscular hemoglobin (MCH) in patients with AA, MDS, and MA were 33.22, 33.21, and 41.91 pg, respectively. The average or median values of MCH concentration (MCHC) and red cell distribution width (RDW) were seen in **Table 1**.

Area of RBCs in Normal Subjects and Patients With AA, MDS, and MA

In 25 normal subjects, the mean area was 44.19 μm^2, and the standard deviation was 3.88 μm^2 (**Table 2**). The image result of the area calculation in one normal subject is shown in **Figure 2A**. The area values of RBCs in normal subjects satisfied normal distribution (**Figure 3A**). The coefficients of variation (CV) were 8.78%.

In 25 patients with AA, the median area was 42.09 μm^2, the quartile range was 5.35 μm^2. In 68 patients with MDS, the median area was 45.75 μm^2, the quartile range was 8.07 μm^2. In 64 patients with MA, the mean area was 52.87 μm^2, and the standard deviation was 7.68 μm^2 (**Table 2**). The image results of area calculation in one patient with AA, MA, and MDS are, respectively, shown in **Figures 2B–D**. The area values of RBCs in MA patients satisfied normal distribution, not patients with AA and MDS (**Figures 3B–D**). The CV were 10.05, 14.53, and 14.00%, respectively in patients with AA, MA, and MDS.

Differences Between the Area of Normal Subjects and Patients With AA, MDS, and MA

Compared with normal subjects, the area of RBCs in patients with MA showed significant differences (52.87 ± 7.68 vs. 44.19 ± 3.88 μm^2, $p < 0.001$). The area of patients with AA and MDS showed no significant differences compared with normal subjects (42.09 ± 5.35 vs. 44.19 ± 3.88 μm^2, $p = 0.337$) (45.75 ± 8.07 vs. 44.19 ± 3.88 μm^2, $p = 0.237$).

Differences Between the Area of Patients With AA, MDS, and MA

Compared with the area of RBCs in patients with MA, patients with MDS and AA showed significantly smaller area of RBCs, respectively (45.75 ± 8.07 vs. 52.87 ± 7.68 μm^2, $p < 0.001$) (42.09±5.35 vs. 52.87±7.68 μm^2, $p < 0.001$). There were also significant differences between patients with MDS and AA (45.75 ± 8.07 vs. 42.09 ± 5.35 μm^2, $p = 0.048$).

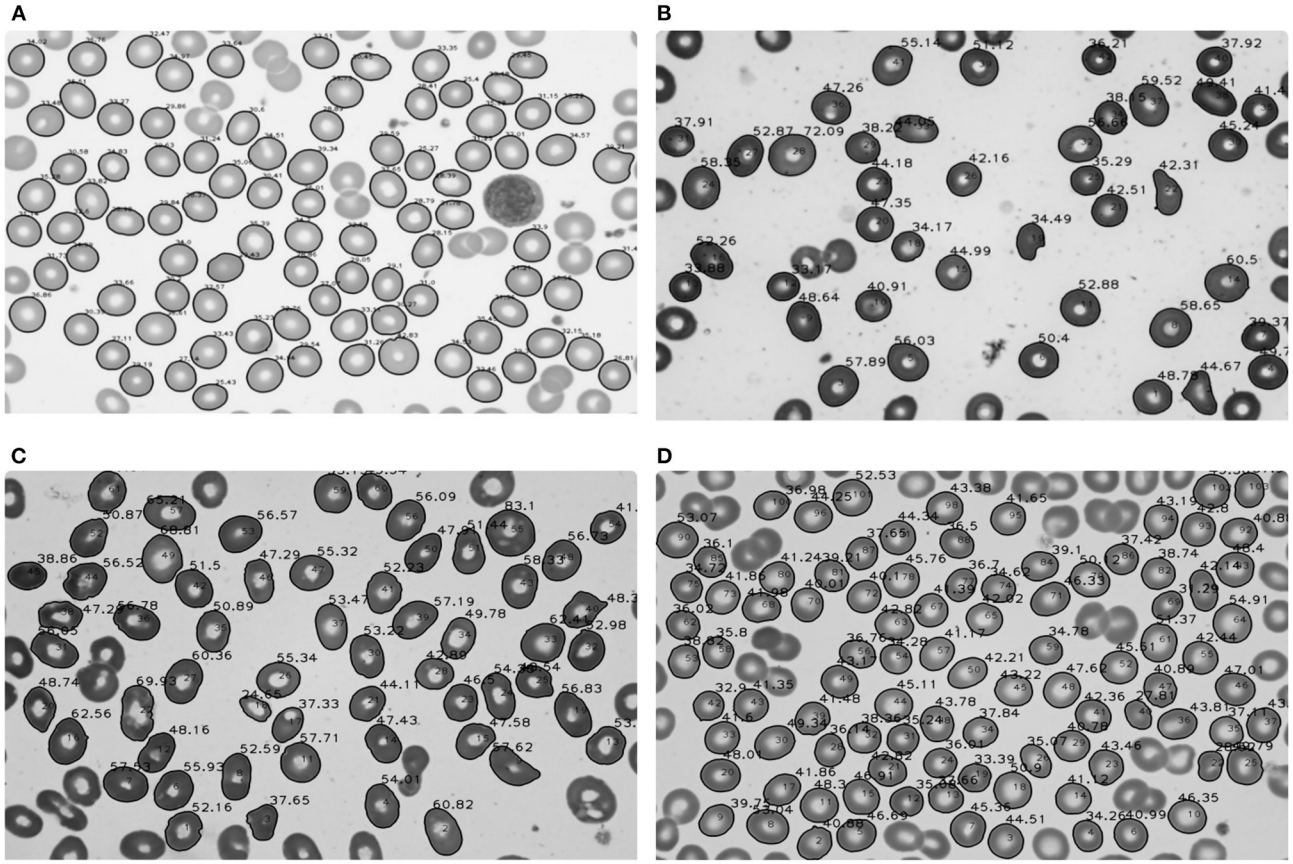

FIGURE 2 | Area calculation results of red blood cells in normal subjects and patients with aplastic anemia (AA), megaloblastic anemia (MA), and myelodysplastic syndrome (MDS). **(A)** Area calculation results in one normal subject; **(B)** Area calculation results in one patient with AA; **(C)** Area calculation results in one patient with MA; **(D)** Area calculation results in one patient with MDS.

Correlation Analysis Between the Area of RBCs and Standard Red Blood Cell CBC Indices

In all patients with AA, the results of correlation analysis between the area **of** RBCs and MCV showed no significant correlation ($r = -0.173$, $p = 0.409$). No significant correlations were also showed between area and MCH ($r = 0.065$, $p = 0.758$), area and MCHC ($r = 0.244$, $P = 0.240$), area and RDW ($r = -0.239$, $p = 0.250$). In all patients with MDS, the correlation analysis between the area **of** RBCs and MCV also showed no significant correlation ($r = -0.038$, $p = 0.760$). No significant correlations were also showed between area and MCH ($r = -0.010$, $p = 0.934$), area and MCHC ($r = 0.066$, $p = 0.595$), area and RDW ($r = -0.085$, $p = 0.490$). In all patients with MA, the results of correlation analysis between the area of RBCs and MCV ($r = -0.039$, $p = 0.760$), MCH ($r = -0.015$, $p = 0.906$), MCHC ($r = -0.030$, $p = 0.812$), RDW ($r = -0.049$, $p = 0.698$) showed no significant correlations (**Table 3**).

Comparison of the Area of RBCs With MCV

We compared the AUC results of ROC curves of RBCs area, MCV, and predicted probability of the two indicators in patients with AA, MA, and MDS. In patients with AA, the AUC results of RBCs area, MCV, and predicted probability were 0.421, 0.585, and 0.581, respectively. We found that MCV and RBCs area had little diagnostic significance in patients with AA. In patients with MDS, the AUC results of RBCs area, MCV, and predicted probability were 0.580, 0.763, and 0.784, respectively. The area of RBCs did not show obvious advantages, but the diagnostic value of the combination of the two indexes increased ($p = 0.048$). In patients with MA, the AUC results of RBCs area, MCV, and predicted probability were 0.850, 0.984, and 0.991, respectively. The area of RBCs showed a very good diagnostic value and the combined diagnostic value of the two indexes increased significantly ($p < 0.001$). These results are shown in **Figure 4** and **Table 4**.

DISCUSSION

Myelodysplastic syndrome, AA, and MA are three common hematologic diseases that cause pancytopenia. MDS and MA possibly share similar characteristics in bone marrow cells morphology, such as abnormal nuclear division and nuclear morphology of erythrocyte lines, which make doctors difficult to distinguish them. The diagnostic distinction of AA and

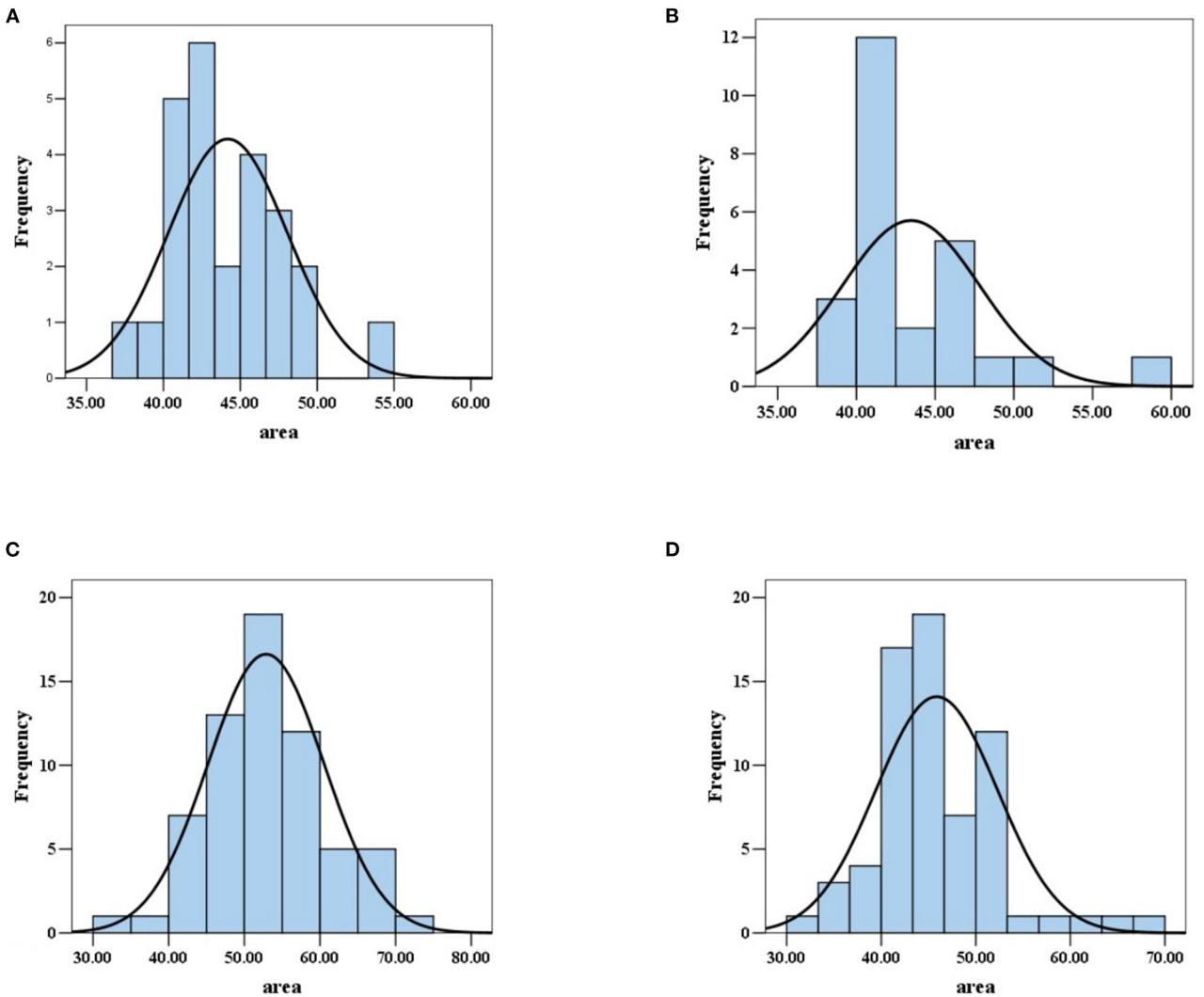

FIGURE 3 | Area values distribution of red blood cells in normal subjects and patients with AA, MA, and MDS. **(A)** The area values of red blood cells in normal subjects satisfied normal distribution. **(B)** The area values of red blood cells in patients with AA did not satisfy normal distribution. **(C)** The area values of red blood cells in patients with MA satisfied normal distribution. **(D)** The area values of red blood cells in MDS patients did not satisfy normal distribution.

TABLE 3 | Correlation analysis between the area of RBCs and standard red blood cell indices.

	r^a	r^b	r^c	r^d	P^a	P^b	P^c	P^d
Normal subjects	0.02	−0.113	−0.275	0.335	0.924	0.592	0.184	0.102
AA	−0.173	0.065	0.244	−0.239	0.409	0.758	0.240	0.250
MDS	−0.038	−0.01	0.066	−0.085	0.760	0.934	0.595	0.490
MA	−0.039	−0.015	−0.03	−0.049	0.760	0.906	0.812	0.698

[a]for MCV; [b]for MCH; [c]for MCHC; [d]for RDW.

AA, aplastic anemia; MA, megaloblastic anemia; MDS, myelodysplastic syndrome; RBCs, red blood cells.

hypocellular MDS is also difficult because of some shared clinical features such as bone marrow hypocellularity (11). Dysplasia in one or more hematopoietic cell lineages is a prerequisite for the diagnosis of MDS. However, dysplasia is not specific for MDS. A small number of patients with MDS display no dysplasia in the early stage of the disease (12, 13). The proper diagnostic distinction of these diseases with pancytopenia is challenging.

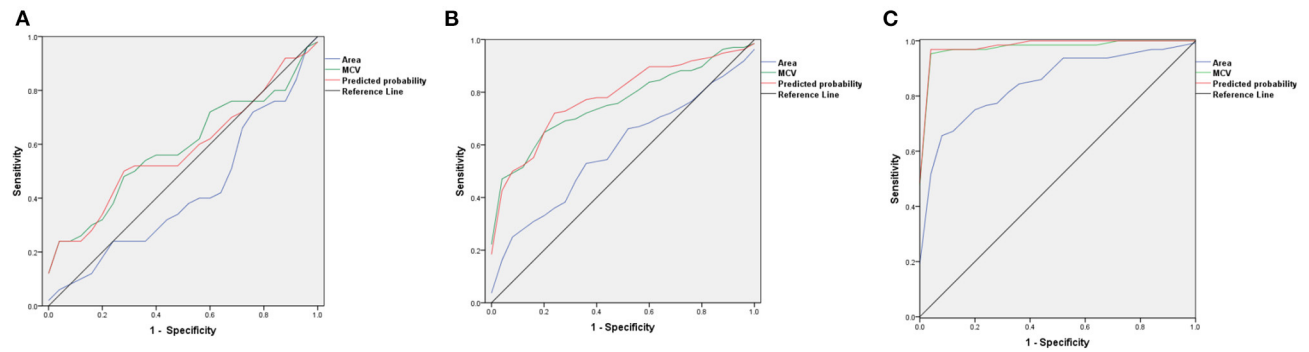

FIGURE 4 | The ROC curves of red blood cells (RBCs) area, mean corpuscular volume (MCV), and predicted probability in patients with AA, MDS, and MA. **(A)** The ROC curves in patients with AA; **(B)** The ROC curves in patients with MDS; **(C)** The ROC curves in patients with MA.

TABLE 4 | The AUC results of RBCs area, MCV, and predicted probability in patients with AA, MA, and MDS.

	AA		MDS		MA	
	AUC	***P***	**AUC**	***P***	**AUC**	***P***
Area	0.421	0.337	0.580	0.237	0.850	0.000
MCV	0.586	0.295	0.763	0.000	0.984	0.000
Predicted probability	0.581	0.327	0.784	0.000	0.991	0.000

AA, aplastic anemia; MA, megaloblastic anemia; MDS, myelodysplastic syndrome; AUC, area under the curve; MCV, mean corpuscular volume.

In bone marrow morphology, the number of blasts, pseudo-Pelger–Huet anomaly, and micro megakaryocytes are of great diagnostic value for MDS (14, 15). In bone marrow biopsy, reticular fibers, increased CD34+cells and more residual hematopoietic area in bone marrow biopsy specimens are helpful for the diagnosis of MDS (16, 17). Abnormal localization of immature precursor that appeared in the medullary cord can help confirm the diagnosis of MDS. In recent years, advances in the molecular pathogenesis of MDS have been greatly enriched by the systematic application of next-generation sequencing. Deep next-generation sequencing panel assays for detection of somatic mutations are now routinely available helpful in distinguishing AA from hypocellular MDS (18). Advances in novel sequencing techniques have led to the discovery of one or more gene mutations in more than 90% of all cases, and there are more than 60 genes involved (7, 8). These cytogenetic changes can help to diagnose MDS. Karyotypic abnormalities that can help doctors confirm an MDS diagnosis are present in about 50% of all cases (19). So, cytogenetic and molecular examination may not help for diagnostic value in some patients. Besides, early MDS may present no dysplasia, and the clinical symptoms are also not typical. MA and MDS both used to be classified into macrocytic anemia, and the similar manifestations of dysplasia in bone marrow increased the difficulty of differential diagnosis of the two diseases. One rapid method for assisting the diagnosis and identification of MDS, AA, and MA was very important.

Red blood cells are the most commonly and intensively studied type of blood cells in cell biology. At present, many hospitals and research institutes conducted RBCs examinations using conventional techniques which included the CBC and microscopic examinations. CBC, as the current standard technique for measuring RBCs properties, contains several important diagnostic parameters, such as the MCV, MCH, MCHC, and RBCs distribution width. Microscopic examinations classify and identify RBCs by artificially observing the morphology of cells under a microscope. Although the conventional measurement techniques can make a general view of RBCs and help identify shapes, roundness, and other information, they have difficulties in extracting high-dimensional information at the individual cell level (20). It has been challenging to establish new systems for morphological and classification analysis of erythrocytes based on the properties of individual RBCs.

In the recent years, recognition of RBCs from microscopic images using imaging processing technology has been proposed. In 2016, HA Elsalamony et al. presented algorithms capable of counting and detecting sickle and microcytic RBCs on a smear based on circular Hough transform. The neural network had been applied on their extracted data to evaluate the algorithm (21, 22). He also proposed an algorithm of assigning and counting normal, sickle RBCs and elliptocytosis (23). Other researches were conducted to screen for diseases and syndromes based on computer systems through the extraction of information of RBCs. Kim et al. (24) also demonstrated a rapid and label-free method by combining quantitative phase imaging-based single-RBC profiling with machine learning to screen for iron-deficiency anemia, reticulocytosis, hereditary spherocytosis (>98% accuracy). In Delgado-Font et al. (25) presented a high-accuracy neural network classifier for the support of sickle cell anemia by classifying RBC shape in peripheral blood images using the basic shape analysis descriptors which included circular shape factor and elliptical shape factor. The normal RBCs have a biconcave-disk shape rather than the spherical shape, with an average diameter of 7.2 μm and thickness of 2 μm. The specific shape of the RBC allows it to maximize the uptake of oxygen from its surroundings and release of carbon dioxide produced by

the body. Therefore, the surface area rather than volume of the RBC is more reflective of its capacity of oxygen transportation. The above-mentioned researches have achieved some progress in extracting morphological, chemical, and mechanical properties of individual RBCs based on computer systems and even offered diagnostic support for some anemic diseases, but none of them tried to measure the area of RBCs, which may help improve the accuracy of RBC detection.

We carried out this study on the area of RBCs in peripheral blood smears of patients with MDS, AA, MA, and normal subjects by conducting RBCs segmentation and morphological analysis based on image processing technology. The results of this study showed that the mean or median RBCs area was 44.19, 42.09, 45.75, and 52.87 μm^2, respectively in normal subjects, patients with AA, MDS, and MA. Compared with the normal subjects, the RBCs area in patients with MA was significantly higher. Compared with patients with AA and MDS, the RBCs area in patients with MA was also significantly higher. There were also significant differences between patients with AA and MDS. Therefore, we preliminarily verified the differences in the area of RBCs among patients with AA, MDS, and MA. We found no significant correlations between area of RBCs and MCV, MCH, MCHC, and RDW. Therefore, the results in this study can assist the diagnosis of patients with AA, MDS, and MA, possibly independent of standard RBC CBC indices. After comparing the diagnostic values of MCV and RBCs area in these three diseases, we found that the RBCs area showed very good diagnostic value as MCV in patients with MA. Moreover, the combined diagnostic value of MCV and RBCs area increased significantly, which could be significantly close to 1.

There are several advantages in this study. First, blood smears are easier to be obtained. Second, the area of RBCs and the differences of the area in patients with AA, MDS, and MA were preliminarily obtained. This study also has several limitations, which can be improved in the future work. First, the number of samples is not large enough. Second, cluster cells were not taken into account in this study. At last, we did not find the cutoff area values for screening patients with AA, MDS, and MA. If we get the cutoff area values to distinguish the three diseases and the methodology is mature and standardized, the detection cost and speed will be improved.

In conclusion, identifying the area of RBCs in peripheral blood smears based on the image processing technology could achieve rapid and efficient diagnostic support for patients with MDS and MA, especially for patients with MA and in combination with MCV. However, a larger sample study is needed to find the cutoff area values.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Wuhan University, China. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

YZ and XW wrote the manuscript and analyzed the results. TH and HS prepared blood smears and microscopic examination. QC processed the data. BX designed the project, provided professional guidance, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Development of a Nomogram to Predict the Risk of Chronic Active Epstein-Barr Virus Infection Progressing to Hemophagocytic Lymphohistiocytosis

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Background: Chronic active Epstein-Barr virus infection (CAEBV) disease is sometimes associated with an aggressive clinical course, such as hemophagocytic lymphohistiocytosis (HLH). To explore the risk factors and predict the risk of CAEBV infection progressing to HLH, a retrospective research study was conducted.

Methods: We retrospectively reviewed the medical records of 187 CAEBV-infected patients who were admitted to our center between January 2015 and December 2020. The patients were followed up until May 2021. The patients were divided into a progression-to-HLH group and a no-progression-to-HLH group. Demographic, clinical and laboratory data were collected for each patient.

Results: Among the 121 CAEBV-infected patients who fulfilled the study's inclusion criteria, 48 (30.7%) patients did not progress to HLH, and 73 (60.3%) patients progressed to HLH. The median time from CAEBV infection to progression to HLH was 14 months, and the cumulative incidence rate of HLH increased as the duration of follow up increased (24.9, 47.3 , 55.1, and 85.2% at 1, 3, 5, and 10 years, respectively). Multivariate analyses showed that the independent risk factors for CAEBV progression to HLH were plasma EBV-DNA load (OR = 3.239, 95% CI 1.219–8.603, $P = 0.018$), Platelet count (OR=0.991, 95%CI 0.985–0.998, $P = 0.010$), elevated alanine aminotransferase (OR=1.019, 95%CI 1.005–1.034, $P = 0.009$) and ≥2 of 3 lineages of cytopenia (OR=8.364, 95%CI 1.062–65.839, $P = 0.044$). The regression coefficients (β) from the multivariate logistic model were used to construct a model for estimating the risk of CAEBV infection progressing to HLH. The discriminatory ability of the model was good, and the area under the receiver operating characteristic (ROC) curve (AUC) was 0.925.

Conclusion: plasma EBV-DNA load, platelet count, elevated alanine aminotransferase and ≥ 2 of 3 lineages of cytopenia increase the risk of CAEBV infection progressing to HLH. A nomogram can be used to estimate the risk of CAEBV-infected patients progressing to HLH.

Keywords: CAEBV, HLH, thrombocytopenia, alanine aminotransferase, cytopenia, nomogram

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INTRODUCTION

Chronic active Epstein-Barr virus infection (CAEBV) disease is a lymphoproliferative disease associated with EBV infection that is characterized by chronic or recurrent infectious mononucleosis-like symptoms, including fever, lymphadenopathy, hepatitis, splenomegaly or pancytopenia (1, 2). The clinical course of CAEBV disease is heterogeneous: some patients may survive for more than 10 years without effective treatment, whereas others progress rapidly to hemophagocytic lymphohistiocytosis (HLH), multiple organ failure, or leukemia/lymphoma within a few years (3–6). HLH is a life-threatening syndrome involving excessive immune activation, and it is characterized by an inflammatory cytokine storm that causes multi-organ dysfunction. The clinical manifestations and laboratory findings characteristic of HLH include fever, hemocytopenia, splenomegaly, hypertriglyceridemia, hyperferritinemia, hypofibrinogenemia, and hemophagocytosis in the bone marrow, spleen, or lymph nodes (7, 8). The incidence of the progression of CAEBV infection to HLH is currently unknown. A Japanese study showed that 24.4% of CAEBV-infected patients progressed to HLH (9). In general, CAEBV infection is a fatal disease with high mortality and morbidity. Once CAEBV infection progresses to HLH, the prognosis worsens. Standard therapy regimens for CAEBV infection have not been established. Hematopoietic stem cell transplantation (HSCT) is currently the only regimen that can cure the disease (10, 11). However, HSCT presents significant risks and complications for patients. Although HSCT treatment of CAEBV infection may result in life-threatening complications, patients with poor prognosis require aggressive treatment to reduce or eliminate EBV-infected cells. Studies have shown that early HSCT for patients in relatively good clinical condition may improve the prognosis of HSCT (12); therefore, predicting the progression of CAEBV to HLH is particularly important for patients with a potential poor prognosis.

The purpose of this study was to explore the clinical and laboratory risk factors for the progression of CAEBV infection to HLH, which is extremely significant for enabling clinicians to adjust their treatment choices in a timely manner, thus prolonging the survival time and improving the prognosis of CAEBV-infected patients.

MATERIALS AND METHODS

Study Design

A retrospective analysis was performed on 187 patients with a definitive diagnosis of CAEBV infection who were admitted to our center from January 2015 to December 2020. The patients were followed up until May, 2021. The median follow-up time of this study was 51 months. **Inclusion criteria:** 1) meeting the recently revised diagnostic criteria for CAEBV disease, including persistent infectious mononucleosis like symptoms for more than 3 months, increased EBV-DNA in peripheral blood, histological evidence of organ disease, and EBV-RNA or viral protein in affected tissues (13); 2) meeting the diagnostic criteria of HLH (for patients for whom CAEBV infection progressed to HLH) (7); and 3) complete laboratory examination results and case data. **Exclusion criteria:** 1) Presence of autoimmune or immunodeficiency diseases; 2) progression of CAEBV to neoplastic diseases, such as lymphoma, including extranodal NK/T cell lymphoma, aggressive NK cell leukemia, and peripheral T cell lymphoma; 3) Acute EBV-associated HLH; and 4) CAEBV patients received immunotherapy or allogeneic hematopoietic stem cell transplantation therapy. The 121 patients who met the inclusion criteria were divided into a progression-to-HLH group (73 cases) and a no-progression-to-HLH group (48 cases).

For patients no-progression-to-HLH, the blood drawn for analysis in this study were 1 week prior or after the diagnosis of CAEBV. While, for progression-to-HLH patients, the time points of blood drawn for analysis in the study were 1 week prior or after the diagnosis of CAEBV and HLH.

Methods

The following data were collected for each patient: the age at onset, gender, clinical symptoms, interval time from clinical symptoms to diagnosis of CAEBV infection, EBV-infected lymphocyte subpopulations, EBV-DNA quantity in plasma, EBV-DNA quantity in peripheral blood mononuclear cells (PBMCs), splenomegaly, $\geq$2 of 3 lineages of cytopenia, platelet count (PLT), alanine aminotransferase (ALT), albumin (ALB), total bilirubin (TB), lactate dehydrogenase (LDH), decreased natural killer (NK) cell activity, hemophagocytosis in bone marrow, and presence of abnormal phenotypic cells in bone marrow. Real-time fluorescent quantitative PCR (qPCR) and TaqMan hydrolysis probes were used to detect EBV-DNA in plasma and PBMC. Intracellular EBV-DNA copies were quantified by qPCR in sorted B-, T-, and NK-cells. The clinical and laboratory data of the two groups were comparatively analyzed to explore the risk factors for the progression of CAEBV infection to HLH. The regression coefficients (β) from the multivariate logistic model were used to construct a model for estimating the risk of CAEBV infection progressing to HLH.

Statistical Analysis

Categorical variables were compared using the chi-square test, and the Wilcoxon rank sum test of two-side test was applied to all continuous variables because the distributions of most of these variables were skewed. The medians and 25^{th}-75^{th} percentiles of the continuous variables were presented. Categorical variables were presented as proportions. The cumulative incidence of HLH estimate using the Kaplan-Meier method. Logistic regression analysis was used for multivariate analysis. A value of $p < 0.05$ was considered statistically significant. The nomogram and time-dependent ROC curve were established with R (http://www.R-project.org) and EmpowerStats software (www.empowerstats.com, X&Y solutions, Inc. Boston MA). Other analyses were performed by IBM® SPSS® software, version 20.0 (IBM Corporation, Armonk, NY, USA).

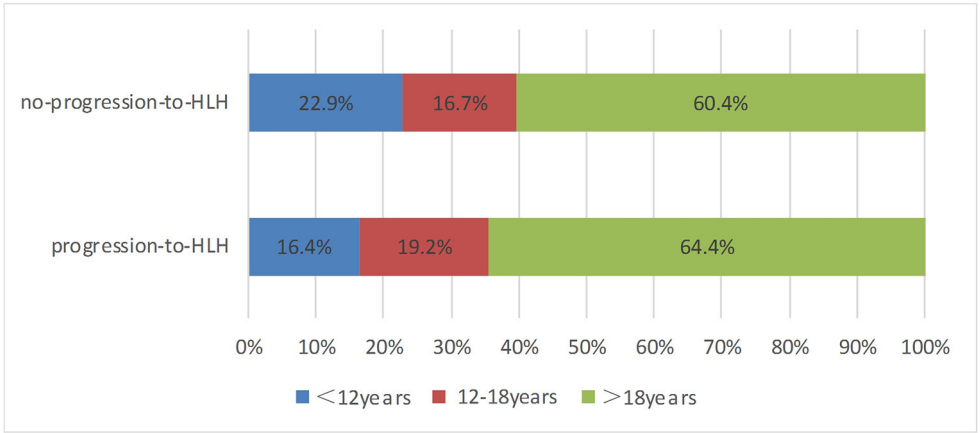

FIGURE 1 | The age distribution of CAEBV infection progression to HLH and not progression to HLH.

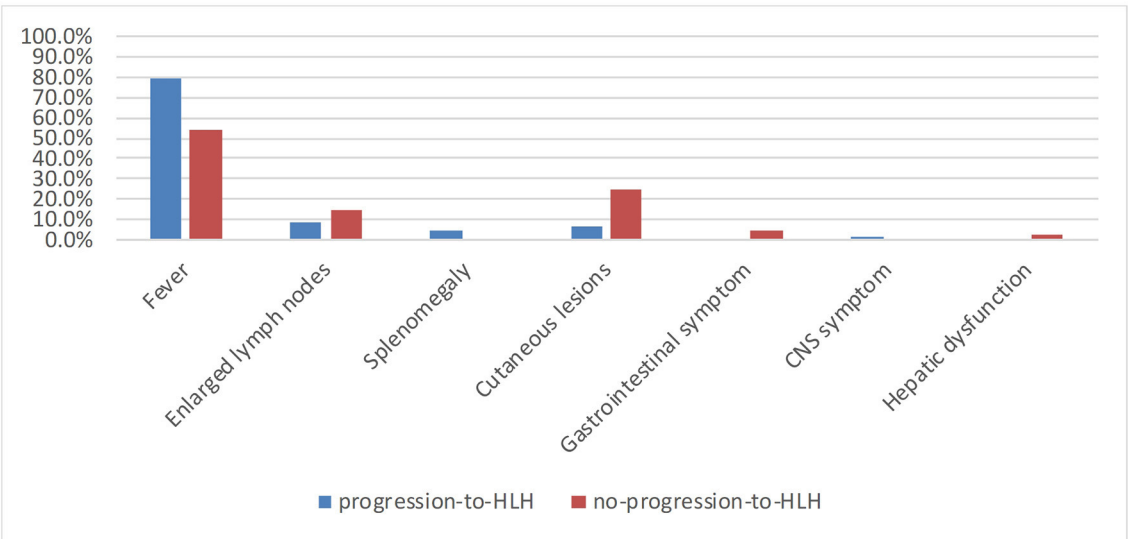

FIGURE 2 | Symptoms and signs at onset of patients with CAEBV infection progression to HLH or not.

RESULTS

Epidemiology

A total of 121 patients with CAEBV infection who meet the inclusion criteria were enrolled in this study, where 73 cases progressed to HLH and 48 cases did not progress to HLH. The age at the onset of disease ranged from 2 to 74 years (mean, 27 years), including 45 (37.2%) young people (under 18 years of age) and 76 (62.8%) adults. The age distribution of the two groups is shown in **Figure 1**. Among the 121 patients, 77 were male and 44 were female, with a male: female ratio of 1.75:1. **Figure 2** shows the signs and symptoms of the two groups at the onset of CAEBV infection as percentages. Most patients presented with high fever in both groups (progression-to-HLH, 79.5%; no-progression-to-HLH, 54.2%); enlarged lymph nodes were found in 8.2% of the patients who progressed to HLH compared to 14.6% of the patients who did not progress to HLH; cutaneous lesions were present in 6.8% of the progression-to-HLH group and 25% of the no-progression-to-HLH group. Splenomegaly and central nervous system (CNS) symptoms were only presented by CAEBV-infected patients who progressed to HLH and was found in 4.1 and 1.4%, respectively, of these patients. Gastrointestinal symptoms (4.2%) and hepatic dysfunction (2.1%) were observed in patients who did not progress to HLH.

Clinical and Laboratory Features

The 121 CAEBV-infected patients were divided into a progression-to-HLH group ($n = 73$) and no-progression-to-HLH group ($n = 48$), and the clinical and laboratory characteristics of the two groups were compared. **Table 1** is a comparison of the laboratory data obtained at diagnosis for the

TABLE 1 | Univariate analysis of factors related with chronic active Epstein-Barr virus (EBV) infection progress to HLH [n(%) or M(P25 P75)].

Factor		All patients	Whether CAEBV progressed to HLH or not		X^2/Z	*P*-value
			No ($n = 48$)	Yes ($n = 73$)		
Gender	Male	77 (63.6)	32 (66.7)	45 (61.6)	0.316	0.574
	Female	44 (36.4)	16 (33.3)	28 (38.4)		
Age (years)	< 12	23 (19.0)	11 (22.9)	12 (16.4)	0.812	0.666
	12–18	22 (18.2)	8 (16.7)	14 (19.2)		
	> 18	76 (62.8)	29 (60.4)	47 (64.4)		
Time intervals from onset symptoms to diagnosis of CAEBV (months)		10 (4.5∼24)	12 (6∼24)	9 (4∼14)	1.396	0.163
EBV-infected	T	13 (13.5)	7 (53.8)	6 (46.2)	7.387	0.057
lymphocyte	NK	17 (18.1)	3 (17.6)	14 (82.4)		
subpopulations	B	7 (7.45)	5 (71.4)	2 (28.6)		
	≥ 2 types	59 (62.8)	23 (39.0)	36 (61.0)		
EBV-DNA load in plasma (log copies/mL)		3.72 (2.70∼4.37)	2.82 (2.70∼3.93)	4.19 (3.52∼4.78)	4.421	<0.001
EBV-DNA load in PBMC (log copies/mL)		4.76 (3.67∼5.98)	3.93 (3.17∼5.45)	5.07 (3.81∼6.09)	2.270	0.023
Splenomegaly		90 (74.4)	28 (58.3)	62 (84.9)	10.751	<0.001
≥ 2 of 3 lineages cytopenia		82 (67.8)	21 (43.8)	61 (83.6)	21.013	<0.001
Platelet count (10^9/L)		155 (80∼236.5)	236.5 (189.25∼332.75)	96 (60∼163.5)	6.154	<0.001
Alanine aminotransferase (U/L)		53 (21∼123.5)	22 (16∼54.5)	78 (38∼158)	4.981	<0.001
Albumin (g/L)		34.2 (29.4∼39.93)	38.7 (33.8∼43)	31 (25.6∼36.8)	4.494	<0.001
Total bilirubin (umol/L)		16.01 (9.43∼26.38)	10.56 (7.22∼20.39)	18.09 (11.65∼45.02)	4.027	<0.001
Lactate dehydrogenase (U/L)		345 (207∼499)	226 (185∼394)	409.5 (287.5∼701.5)	4.341	<0.001
Absent NK-cell activity		48 (42.1)	18 (40.9)	30 (42.9)	0.042	0.838
Hemophagocytosis		26 (21.5)	4 (8.3)	22 (30.1)	6.919	0.009
Abnormal phenotypic cells		29 (24.0)	6 (12.5)	23 (31.5)	5.741	0.017
PT(s)		11.9 (11∼12.8)	11.8 (11.1∼12.4)	12.2 (10.9∼13.45)	1.134	0.257
APTT(s)		32.1 (27.95∼36.6)	31.3 (27.7∼34)	32.6 (27.9∼38.7)	1.401	0.161
D-dimer (mg/L)		0.9 (0.5∼2.07)	0.6 (0.4∼0.95)	1.7 (0.7∼3)	4.809	<0.001

two groups. Univariate analysis showed that the risk factors associated with the progression of CAEBV to HLH were the plasma EBV-DNA load ($P < 0.001$), the PBMC EBV-DNA load ($P = 0.023$), splenomegaly ($P < 0.001$), platelet count ($P < 0.001$), elevated alanine aminotransferase ($P < 0.001$), ≥2 of 3 lineages of cytopenia (hemoglobin<90 g/L, platelet count $< 100\times10^9$/L, neutrophils count $<1.0 \times 10^9$/L) ($P < 0.001$), hypoalbuminemia ($P < 0.001$), elevated total bilirubin ($P < 0.001$), increased LDH ($P < 0.001$), hemophagocytosis in bone marrow ($P < 0.001$), abnormal phenotypic cells in bone marrow ($P < 0.001$) and D-dimer levels ($P < 0.001$). Factors for which no significant differences were observed between the two groups included gender, age, time interval from the onset of symptoms to the diagnosis of CAEBV, EBV-infected lymphocyte subpopulations, APTT, PT and low or absent NK cell activity (all, $P > 0.05$) (**Table 1**). Multivariate analysis [including the plasma and PBMC EBV-DNA loads, splenomegaly, platelet count, elevated alanine aminotransferase, ≥2 of 3 lineages of cytopenia, hypoalbuminemia, elevated total bilirubin, hemophagocytosis in bone marrow, abnormal phenotypic cells in bone marrow and D-dimer] showed that the independent factors for progression of CAEBV to HLH were plasma EBV-DNA load (OR = 3.239, 95% CI 1.219–8.603, $P = 0.018$), platelet count (OR = 0.991, 95% CI 0.985–0.998, $P = 0.010$), elevated alanine aminotransferase (OR = 1.019, 95% CI 1.005–1.034, $P = 0.009$) and ≥2 of 3 lineages of cytopenia (OR = 8.364, 95% CI 1.062–65.839, $P = 0.044$) (**Table 2**). Additionally, we found the best cut-off value for plasma EBV-DNA obtained from the ROC curve was $10^{2.84}$ copies/mL (AUC was 0.775, 95% CI 0.673–0.858, $P < 0.001$, sensitivity 0.932, specificity 0.524%).

Treatment Protocols of the Two Groups After Diagnosis of CAEBV

Of the 121 patients diagnosed with CAEBV, 39 patients did not receive any treatment, and 18 of them progressed to HLH; 22 patients were treated with antiviral therapy, and 20 patients developed HLH; 31 patients were treated with methylprednisolone or dexamethasone, and 27 patients

TABLE 2 | The independent risk factors for CAEBV infection progression to HLH.

Factor	B	SE	Walds	df	*P*-value	OR	95% CI
EBV-DNA quantity in plasma (log copies/mL)	1.175	0.498	5.558	1	0.018	3.239	1.219∼8.603
Platelet count	−0.09	0.003	6.597	1	0.010	0.991	0.985∼0.998
Alanine aminotransferase	0.019	0.007	6.788	1	0.009	1.019	1.005∼1.034
≥ 2 of 3 lineages cytopenia	2.124	1.053	4.067	1	0.044	8.364	1.062∼65.893

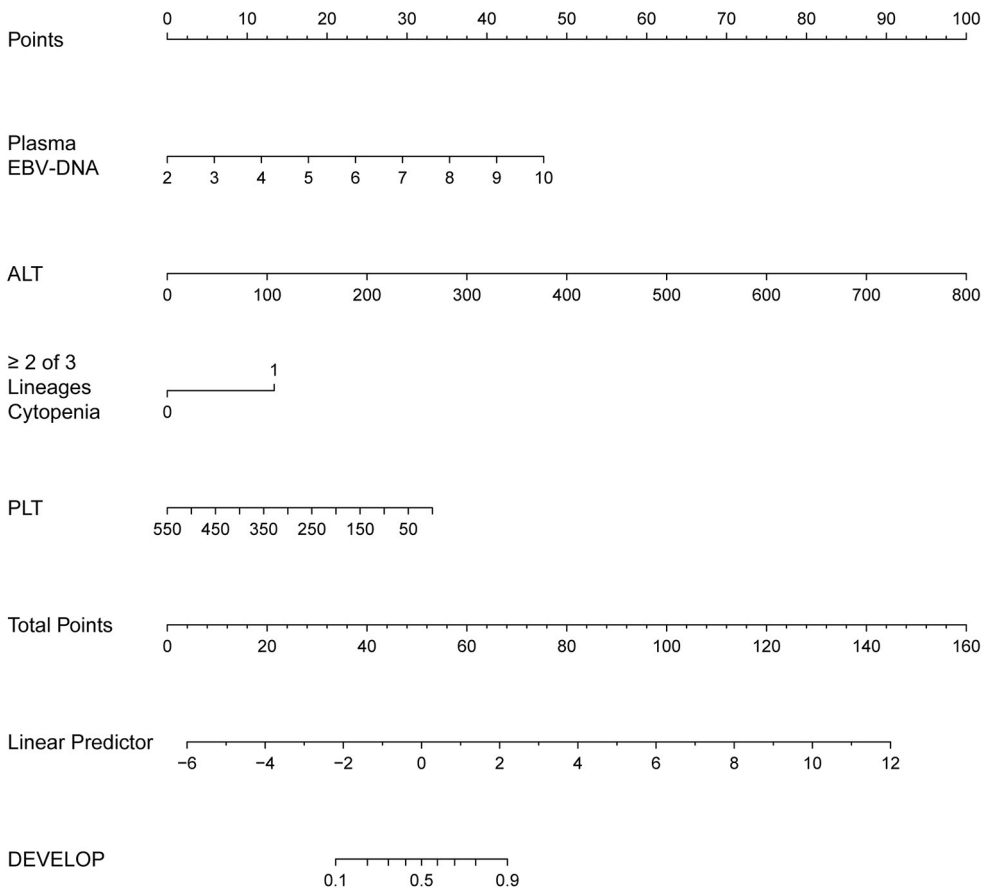

FIGURE 3 | The nomogram to estimate the risk of CAEBV infection progress to HLH. To use the nomogram, find the position of each variable on the corresponding axis, draw a line to the points axis for the number of points, add the points from all of the variables, and draw a line from the total points axis to determine the probabilities of CAEBV infection progressed to HLH at the lower line of the nomogram.

developed HLH; 2 patients were treated with ruxolitinib, and 1 patient progressed to HLH; 1 patient was treated with rituximab without progression to HLH by the end of follow-up; 3 patients were treated with asparaginase, and 2 patients progressed to HLH; 7 patients were treated with HLH-94 or HLH-2004 regimen, and 1 patient progressed to HLH; 16 patients were treated with DEP regimen (Liposomal doxorubicin + etoposide + methylprednisolone), ruxolitinib + DEP regimen, or asparaginase + DEP regimen, and 4 patients progressed to HLH.

Treatment Protocols After CAEBV Progression to HLH

There were 73 patients diagnosed with CAEBV progression to HLH. Fourty-six patients adopted DEP regimen, ruxolitinib + DEP regimen, or asparaginase + DEP regimen as initial induction treatment, of which 21 patients underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) followed DEP regimen and 10 patients died by the end of follow-up; 25 patients were not able to undergo allo-HSCT for various reasons,

and 7 patients died by the end of follow-up. Nine patients were treated with HLH-94 or HLH-2004 regimen initially, of which 5 patients were treated with DEP regimen as salvage therapy, and 3 patients underwent allo-HSCT were alive by the end of follow-up; 6 patients failed to undergo allo-HSCT, and 3 patients died by the end of follow-up. Four patients adopted E-CHOP like regimens (etoposide + cyclophosphamide + epriubicin + vincristine + glucocorticosteroid) or E-COP like regimens (etoposide + cyclophosphamide + vincristine + glucocorticosteroid) as initial treatment, 1 patient died after allo-HSCT, and 2 patients died without undergoing allo-HSCT. Six patients were treated with ruxolitinib or combined with methylprednisolone, and 1 patient died. There were 7 patients' treatment protocols unclear, and 3 patients died at the end of follow-up. One patient treated with FC therapy (fludarabine + cyclophosphamide) was still alive at the end of follow-up.

The Nomogram and Its Predictive Performance

The regression coefficients (β) obtained using the multivariate logistic model were used to construct a model for estimating the risk of CAEBV infection progressing to HLH (**Figure 3**). The scoring model was as follows:−3.77 + 0.75*EBV-DNA load + 0.02* elevated alanine aminotransferase + 1.71* (≥2 of 3 lineages of cytopenia) - 0.01* platelet count. The performance of the nomogram was measured by ROC curves, and the AUC for the model was found to be 0.925 (95% CI 0.867–0.984) using the observed data. The cut-off score was−0.271 with a sensitivity of 0.932 and a specificity of 0.833 (**Figure 4**).

Time Interval for CAEBV to Progress to HLH

The time from CAEBV infection progression to HLH was in the range of 1–120 months with a median progression time of 14 months. The cumulative incidence rate of HLH increased as the duration of follow up increased (24.9, 47.3, 55.1, and 85.2% at 1, 3,5, and 10 years, respectively) (**Figure 5**). Among patients under 18 years of age, 26 (21.5%) developed HLH, with a median progression time of 20 months. However, in patients over 18 years old, 47 cases (38.8%) progressed to HLH, with a median progression time of 13 months. The median progression times of the two groups were not significantly different ($P = 0.333$).

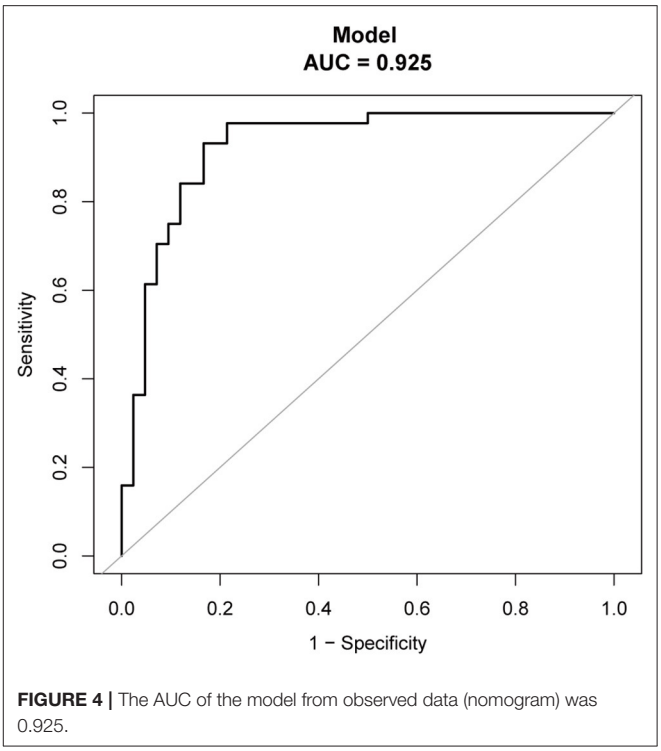

FIGURE 4 | The AUC of the model from observed data (nomogram) was 0.925.

DISCUSSION

CAEBV infection is considered to be a fatal disease. Approximately 50% of CAEBV-infected patients die within 5 years of diagnosis due to progression to lymphoma, HLH, heart or liver failure. Almost all patients who do not receive reasonable and effective treatment die within 15 years of diagnosis due to various complications, including HLH, multiple organ failure, or leukemia/lymphoma (14). The risk factors that predict CAEBV progression to HLH are significant for enabling clinicians to adjust therapies in a timely manner to improve prognosis.

This study is the first analysis of the risk factors for the progression of CAEBV infection to HLH. We retrospectively reviewed the medical records of 187 CAEBV-infected patients who were admitted to our hospital between January 2015 and December 2020, where 121 patients met the inclusion criteria for the study. Four factors were identified as independent risk indicators for the progression of CAEBV infection to HLH, including plasma EBV-DNA load, platelet count, elevated alanine aminotransferase and ≥2 of 3 lineages of cytopenia. Previous studies have found several of these variables to be risk factors for the prognosis of CAEBV infection. Hiroshi Kimura et al. found that platelet count, late onset of disease and T-cell infection were correlated with CAEBV patient mortality (9). LU Gen and his colleagues showed that platelet count and decreases in albumin are potential risk factors for a poor prognosis of CAEBV infection (15). Hiroshi Kimura et al. revealed that age at onset of disease (> 8 years) and liver dysfunction were risk factors for mortality, whereas transplant patients had a better prognosis (16). Interestingly, from our date, we find these risk factors related to the prognosis of CAEBV infection in previous studies were also indicators for CAEBV progression to HLH. These results indicate that CAEBV progress to HLH may has a worse prognosis.

It is noteworthy that CAEBV infection in patients with a persistent high EBV-DNA load and chronic unregulated active EBV replication are associated with poor prognosis. Hiroshi Kimura et al. studied 30 CAEBV-infected patients and found that all patients had high viral loads in their peripheral blood (more than $10^{2.5}$ copies/μg DNA) (9). LU Gen et al. studied 53 Chinese CAEBV pediatric patients and found a mean plasma EBV DNA level of $10^{3.7}$ copies/mL for 23 cases (15). Akihiko Maeda et al. examined the relationship between the clinical

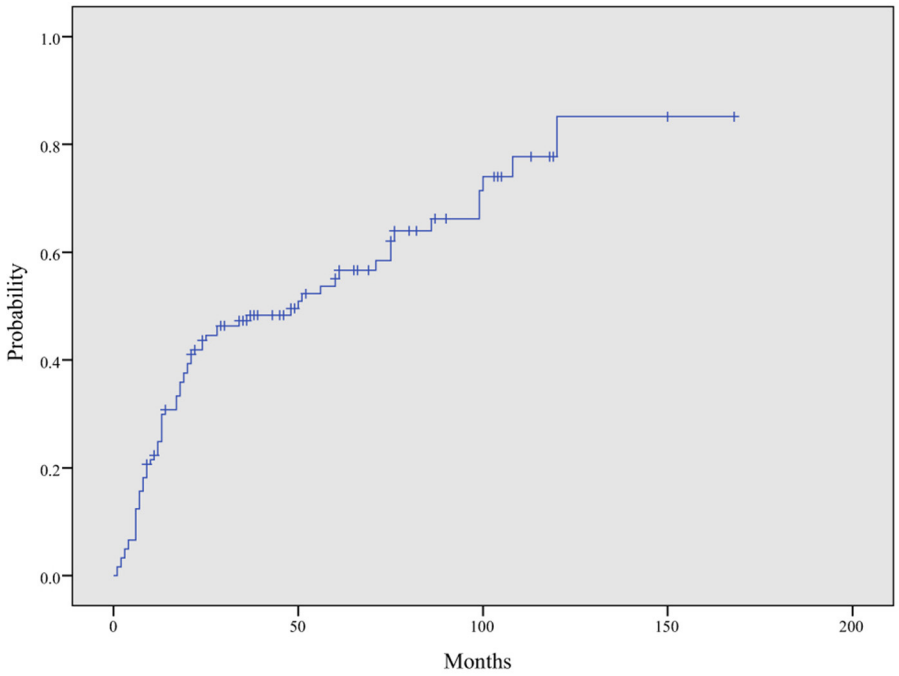

FIGURE 5 | Cumulative incidence curve of HLH in CAEBV patients. The cumulative incidence of HLH at 1, 3, 5, and 10 years was 24.9, 47.3, 55.1, and 85.2%, respectively, by Kaplan–Meier analysis.

manifestation of CAEBV and EBV-DNA load and found that fever was correlated with the virus load (12). In the present study, we also found that the plasma EBV-DNA load was an independent risk factor for the progression of CAEBV to HLH. A ROC curve analysis showed that the best cut-off value for plasma EBV-DNA was $10^{2.84}$ copies/ml, indicating that the patients with CAEBV infection are prone to HLH, even though plasma EBV-DNA is low. The EBV-DNA load in PBMCs, however, was found not the predictor of CAEBV progression to HLH. This maybe EBV in plasma had higher specificity and sensitivity for EBV infection related diseases compared with EBV in PBMCs (16).

In previous studies, EBV infection of T cells was found to be more likely to progress to multiple organ failure (MODS) and had a worse prognosis than EBV infection of other lymphocyte subpopulations (9, 17). However, in the present study, the number of EBV infection of T, B, NK lymphocyte subpopulations and ≥2 lineages lymphocyte subpopulations were 6 (46.2%), 2 (28.6%), 14 (82.4%), and 36 (61.0%), respectively, in the progression-to-HLH group and 7 (53.8%), 5 (71.4%), 3 (17.6), and 23 (39.0%), respectively, in the no-progression-to-HLH group. We did not find that EBV infection of T cell subpopulations was more likely to progress to HLH than EBV infection of other lymphocyte subpopulations. This result may have been obtained because some patients were infected with B and T/NK lymphocyte subpopulations at the same time, where the main infection was from B lymphocyte subpopulations. The clinical course of EBV infection of B lymphocyte subpopulations was not aggressive and associated with a better prognosis than for other lymphocyte subpopulations (18).

Previous reports showed a better prognosis for CAEBV infection for children than adults. Arai et al. showed more progressive and aggressive courses in adult-onset CAEBV patients than childhood-onset patients (19). A prospective study conducted by Hiroshi Kimura et al. showed that an older onset age (≥8 years) was associated with mortality in CAEBV patients (9, 17). However, a study of 53 Chinese pediatric patients with CAEBV infection conducted by LU Gen and his colleagues revealed a severe clinical course and poor prognosis (15). In the present study, we found no statistically significant association between the age of onset and the progression of CAEBV to HLH ($P = 0.666$). Additionally, the median progression times for patients under 18 years of age and over 18 years old were also not significantly different ($P = 0.333$). Ayako Arai et al. reviewed 23 adult-onset CAEBV infection patients, showed that the time during from the onset of disease to initiation treatment averaged 20 months and 7 patient died at an average of 8 months after initiation of treatment (19). Kimura et al. reviewed 30 Japanese CAEBV infection patients, found that young patients could have a time duration without treatment of 12–336 months (mean 71 months) and the 5 year survival rate was 0.68 ± 0.06 (20). In our study, the median time for CAEBV infection to progress to HLH was 14 months, and the cumulative incidence rate of HLH increased as the duration of follow up increased (24.9, 47.3, 55.1, and 85.2% at 1, 3, 5, and 10 years, respectively), which indicating that the clinical course of CAEBV is rapidly progressive and aggressive.

In addition, we developed a nomogram that is easy to use and integrates 4 predictors for the risk of CAEBV-infected patients progressing to HLH. The nomogram showed good predictive accuracy based on an AUC of 0.925 and can be easily used by clinicians. The four variables required for the nomogram are generally readily available at admission. These indicators can also be monitored during the clinical course of the disease to predict the risk of progression of CAEBV to HLH. To improve the therapeutic effect and prognosis of CAEBV, aggressive treatment, such as HSCT, could be considered once the prediction of progression to HLH is high. In general, this nomogram serves as a reference for clinicians to predict the risk of progression of CAEBV infection to HLH. The decision to undertake aggressive treatment also depends on a patient's condition, willingness, and finances.

In general, it is extremely important to explore the clinical and laboratory risk factors for CAEBV progressing to HLH. As this retrospective study was conducted in a single center using a small sample size, a prospective study should be conducted in multicenters on large populations of CAEBV-infected patients. Other parameters, such as lymphocyte subpopulations values, immunoglobulins levels, and soluble CD25 etc. should be discussed in the next prospective study.

CONCLUSION

In conclusion, 4 factors were identified in this study as independent risk indicators for the progression of CAEBV infection to HLH, including an increased plasma EBV-DNA load, platelet count, elevated alanine aminotransferase and $\geq$2 of 3 lineages of cytopenia. A nomogram can be used to estimate the risk of patients with CAEBV progressing to HLH.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Beijing Friendship Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

XH designed and performed the research and wrote the paper. ZW designed the research and supervised the report. JW provided clinical advice and supervised the report. DS contributed to the data collection and statistical analysis. All authors approved the final manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comparison of Neutrophil Function in Granulocyte Concentrates From Prednisone- and G-CSF-Treated Donors: Effect of Stimulant, Leukapheresis and Storage

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Transfusion of granulocyte concentrates (GC) is an alternative therapy for neutropenic patients with life-threatening infections. While neutrophils are the main source of antimicrobial activity, only neutrophil numbers are used to certify GCs. The objective of this study was thus to functionally characterize neutrophils in GCs prepared by leukapheresis from G-CSF-stimulated donors and compare to the less characterized prednisone GCs. GCs prepared from healthy donors stimulated with prednisone and then G-CSF after a 6-month washout period were analyzed prior to and after leukapheresis, and after storage. Leukocyte composition, neutrophil viability, calcium mobilization, chemotaxis, phagocytosis, reactive oxygen species, cytokine production and metabolites were determined. G-CSF GCs contained significantly more neutrophils than prednisone GCs of which 40% were immature. In comparison to non-stimulated healthy donor neutrophils, prednisone GC neutrophils exhibited enhanced phagocytosis and G-CSF GC neutrophils showed decreased chemotaxis but increased IL-8 production. Leukapheresis altered prednisone GC neutrophil responses. Storage had a significant, negative impact on G-CSF GC neutrophils compared to prednisone GC neutrophils. G-CSF and prednisone GC neutrophils thus differ in maturity and function, and G-CSF GC neutrophils are more sensitive to storage. Functional testing of GC neutrophils and better storage conditions would improve the quality of this blood product.

Keywords: granulocyte concentrates, neutrophils, leukapheresis, G-CSF, prednisone

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INTRODUCTION

Life-threatening infections are a major health concern due to growing resistance to antimicrobial and antifungal therapies (1). This is of particular concern for neutropenic patients as they are highly susceptible to infections due to a low number of neutrophils (2), the primary source of anti-microbial defenses (3–5). Moreover, the prevalence of neutropenic patients with antimicrobial resistant infections is increasing due to the growing use of aggressive chemotherapy and hematopoietic stem cell transplants (6). A potential lifesaving therapy for these patients is the transfusion of granulocytes.

Granulocyte transfusions (GTXs) temporarily increase neutrophil count until the bone marrow restores granulopoiesis (7). To harvest the minimal number of granulocytes for GTX (10^{10}/transfusion) healthy donors are stimulated with G-CSF and/or a corticosteroid. In Canada, all GCs are prepared from prednisone-stimulated donors by leukapheresis, whereas in the US GC donors are stimulated with G-CSF (8). In Europe, buffy-coat GCs are more routinely used.

Neutrophil anti-microbial activity encompasses several effector functions including chemotaxis, phagocytosis and pathogen destruction by antimicrobial peptides and reactive oxygen species (ROS). In addition, neutrophils sequester and destroy pathogens by releasing extracellular traps (NETs) composed of chromatin, histones and intracellular proteins (9, 10). Cytokines also play a role by further activating neutrophils and neighboring cells, and promoting the additional leukocyte recruitment of neutrophils and monocytes (11–13). Without these defenses, survival from an infection is limited to a few days in persons with absolute neutropenia (14–16).

While GCs are transfused to temporarily provide antimicrobial defenses, only the absolute neutrophil count (ANC) is used to certify GCs for transfusion (17). This is problematic as studies report functional differences between GCs and non-stimulated healthy donor neutrophils. Impaired phagocytosis was observed in G-CSF GC neutrophils (18) and elevated pro-inflammatory cytokine levels in GC supernatants, a likely cause of febrile reactions (19, 20). In contrast, other studies observed no differences in chemotactic activity (21, 22) or ROS production (22) in G-CSF GC neutrophils compared to neutrophils of non-stimulated healthy donors. Together, these findings underscore the need for a comprehensive characterization of GC neutrophils to ensure their optimal antimicrobial capacity prior to transfusion. Since GC neutrophils prepared from prednisone-stimulated donors are less well characterized and currently used for GTX, the objective of this study was to compare the functional responses of prednisone and G-CSF GC neutrophils prior to and after leukapheresis and during storage.

MATERIALS AND METHODS

Recruitment and GC Collection

Ten healthy donors were recruited by Héma-Québec for two GC donations (the first after prednisone stimulation and the second after G-CSF stimulation) separated by a 6-months wash out period prior to leukapheresis with SpectraOptia (Terumo) according to Héma-Québec guidelines. All donations were collected in blood collection bags containing sodium citrate (46.7%) and hydroxyethyl starch (HESPAN® 6% B. Braun Medical Inc.) to enhance red blood cell sedimentation at a ratio of 13:1 (product:anticoagulant solution). GCs were sent by the Globule Laval collection center to Québec City Héma-Québec for irradiation at 25 Gy prior to delivery to our laboratory for analysis on the day of collection (D1) as well as 24 h (D2) and 48 h (D3) post-collection. GCs were stored at room temperature without agitation. A pre-leukapheresis blood sample was drawn from donors in acid-citrate-dextrose (ACD) (BD Vacutainer) tubes. Blood sample and GC composition was determined at the Héma-Québec collection center with a cell counter (Ac•T 5diff hematology analyser, Beckman Coulter). One donation was excluded from the functional analysis because of a change in the sedimentation agent during the first GC donation. For comparison, blood donations from unstimulated frequency matched healthy donors was drawn in ACD tubes by the Clinical Research Platform at the CHU de Québec-Laval University and were used as control.

Material and Reagents

HES was purchased from Braun Medical Inc. (US). Fura-2-acetoxymethyl ester (Fura-2AM), CM-H2DCFDA, calcein-AM, the 7AAD viability staining solution and the pHRodo Red Zymosan A Bioparticles kit were obtained from Thermo Fisher (ON, Canada); dextran T500, cytochrome C, Hemacolor ® Rapid staining kit from Sigma-Aldrich (ON, Canada); lymphocyte separation medium, RPMI-1640, BSA (bovin serum albumin) and FBS (fetal bovine serum) from Wisent Bioproducts (QC, Canada); and the ChemoTx microplates (101–8) from NeuroProbe (MD, USA).

Neutrophils and Peripheral Blood Mononuclear Cell Isolation

Pre-leukapheresis circulating neutrophils were isolated by density gradient from peripheral blood at room temperature under sterile conditions as described in Fernandes et al. (23). Neutrophils were resuspended in Mg^{2+}-free HBSS containing 1.6 mM $CaCl_2$ at 10 x 10^6 ml^{-1}. Neutrophils isolated from GCs did not require dextran sedimentation as they already contain hydroxyethyl starch, a sedimenting agent. The mononuclear cell layer was harvested after density gradient separation, washed and resuspended in PBS at 10 x 10^6 ml^{-1}.

Flow Cytometry Analysis

A LSRII flow cytometer was used for immunophenotyping with a twelve antibody panel and for fluorescent non-opsonized zymosan phagocytosis analysis. A CantoII flow cytometer was used for viability and intracellular ROS production assays. Cells were always used at 10 x 10^6 ml^{-1}, stained at room temperature and kept on ice

TABLE 1 | Donor characteristics and collection regimens.

	Apheresis group ($n = 10$)		Non-stimulated control group ($n = 10$)
Sex (M:F)	M:5 F:5		M:5 F:5
Age, median year (range)	42 (23–58)		42 (21–61)
GC final volume (ml)	350		–
	Pred	**G-CSF**	
Leukapheresis duration, min (range)	96 (64–138)	80 (66–104)	–
Dose	50 mg (*per os*)	300 mcg (intravenous)	–
Time before apheresis	12 to 18 h	24 h	–
	Inclusion criteria Age 18–60 Men and women		
	Exclusion criteria Infectious diseases transmitted by blood (hepatitis B and C, syphilis, HIV 1 and 2 and HTLV I/I) Complications during apheresis procedure Recent infections that required treatments (antibiotics, antifungals)		

TABLE 2 | Composition of donor peripheral blood and granulocyte concentrates.

	Prednisone ($n = 9$)	G-CSF ($n = 10$)
Donors peripheral blood (pre-collection)		
Total leukocytes (x10^9/L) (%)	8.42	24.56
Neutrophils	6.30 (74.8)	20.55 (83.7)
Lymphocytes	1.52 (18.1)	2.01 (8.2)
Monocytes	0.49 (5.1)	1.16 (4.7)
Eosinophils	0.08 (1.0)	0.49 (2.0)
Basophils	0.03 (0.4)	0.35 (1.4)
Granulocyte concentrate		
Filtrated blood volume (ml)	4,964	4,832
Total leukocyte (x10^9/L) (%)	62.85 (100)	109.45 (100)
Neutrophils	35.70 (56.8)	82.12 (75.0)**
Lymphocytes	18.29 (29.1)	17.89 (16.3)
Monocytes	6.59 (10.5)	7.35 (6.7)
Eosinophils	0.75 (1.2)	1.58 (1.4)
Basophils	1.52 (2.4)	0.52 (0.5)
Dose ANC/GC unit (x10^{10})	1.28	2.85
Hemoglobin (g/L)	34.56	22.20*
Hematocrit (L/L)	0.108	0.071*
Platelets (x10^9/L)	297.6	261.0

*ANC, absolute neutrophil count. Comparison of prednisone vs. G-CSF group: t-test, *p-value < 0.05; **p-value < 0.01.*

until analysis. All assays are explained in detail in the **Supplementary Material**.

RESULTS

GC Collection

Ten healthy donors that met the exclusion and inclusion criteria in **Table 1** were recruited to donate GCs. Inter-donor variability was minimized by harvesting from all donors a prednisone GC followed by a G-CSF GC with a wash out period of 6 months between GC donations. The average blood volume filtrated from prednisone-stimulated donors during leukapheresis was 4,964 ml (4211-6001 ml) and 4,831 ml (3,611–6,039 ml) for G-CSF-stimulated donors (**Table 2**). Ten healthy, age and sex frequency-matched donors were also recruited for comparative purposes.

Comparison of Neutrophil Counts in Prednisone and G-CSF GCs

Since G-CSF mobilizes neutrophils more efficiently than glucocorticoids, we analyzed the leukocyte content in both types of GCs. Prednisone stimulation consistently generated GCs of a similar or significantly lower leukocyte concentration than G-CSF (62.9 x $10^9.L^{-1}$ and 109.5 x $10^9.L^{-1}$ leukocytes, respectively; **Table 2**, **Figure 1A**). The minimal ANC required *per* transfusion of 10^{10} neutrophils was thus observed in 6/9 (67%) of prednisone GCs compared to 10/10 G-CSF GCs (**Figure 1**). Neutrophils were 1.5 to 4-fold more abundant in G-CSF than prednisone GCs and comprised an average of 75.5% of all leukocytes in G-CSF GCs compared to 56.8% in prednisone GCs.

While neutrophils are the most abundant cells in GCs, other leukocytes are also present in this cellular product. Notably, lymphocytes represented 30% of all leukocytes in prednisone GCs and 16% in G-CSF GCs (**Table 2**). A substantial number of monocytes are also detected, 11% in prednisone GCs and 7% in G-CSF GCs. Minor leukocytes populations in these GCs included basophils and eosinophils. Together, these findings indicate that G-CSF is more efficient at mobilizing neutrophils for GCs than prednisone, and that GCs contain a significant proportion of lymphocytes and monocytes in addition to neutrophils.

Viability and Surface Marker Expression of Prednisone and G-CSF Mobilized Neutrophils

To characterize the effect of prednisone and G-CSF stimulation on neutrophils *in vivo*, we compared their viability, maturity and cell-surface marker expression prior to leukapheresis. Spontaneous apoptosis and necrosis was not significantly altered in G-CSF GC neutrophils compared to neutrophils of non-stimulated healthy donors (**Figure 2A**).

Since G-CSF induces the release of immature neutrophils from the bone marrow (24), we stained G-CSF mobilized neutrophils with the maturity marker CD10. G-CSF induced the

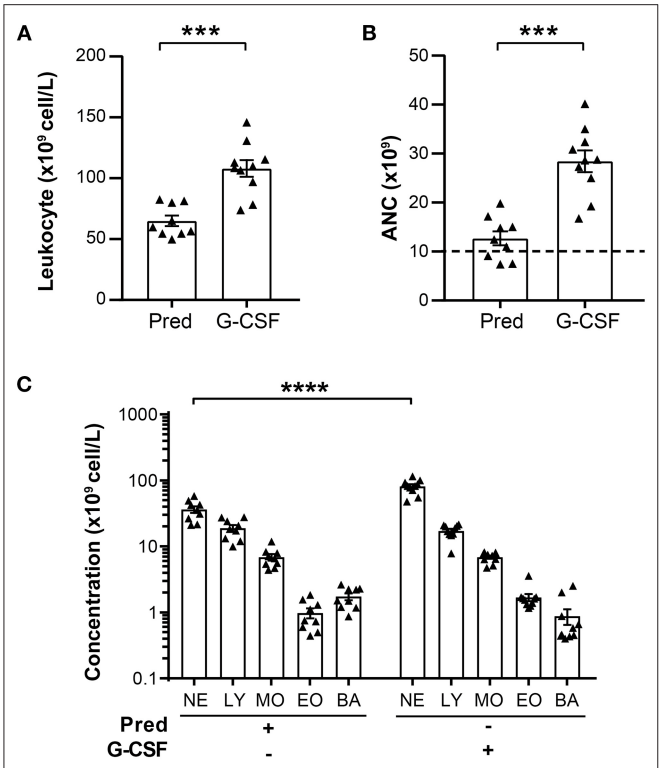

FIGURE 1 | Leukocyte composition in prednisone and G-CSF GCs. **(A)** Total leukocyte concentration in prednisone and G-CSF-derived GCs. **(B)** ANC calculated for a final GC volume of 350 ml. The dashed line indicates the minimal number of neutrophils (10^{10}) required for granulocyte transfusion. **(C)** Concentration of leukocyte populations (neutrophils, NE; lymphocytes, LY; monocyte, MO; eosinophils, EO; basophils, BA) in prednisone and G-CSF GCs expressed as cell number per liter. Each dot represents an individual GC donor. Statistical analysis: Mann-Whitney test performed to compare leukocyte concentration and ANC between prednisone and G-CSF GCs: ***p-value < 0.001. Sidak multiple comparisons test performed to compare the concentration of leukocyte populations between prednisone and G-CSF GCs: ****p-value < 0.0001. These data are compiled from the analysis of 9 prednisone and 10 G-CSF GCs.

release of both mature (60% of $CD10^{high}$) and immature (40% of $CD10^{low}$) neutrophils into the circulation (**Figures 2C,D**). The presence of neutrophils with a mature or immature nuclear morphology confirmed this observation (**Figure 2B**). Contrary to low-density, immature neutrophils that increase in numbers in some disease states, G-CSF GC immature neutrophils have a similar density as mature neutrophils as they pellet to the bottom of the density gradient. In contrast, prednisone only mobilized $CD10^{high}$ neutrophils with multi-lobular nuclei, a typical feature of mature neutrophils (**Figures 2B,C**) (25). An additional distinctive feature of G-CSF GC immature neutrophils is their significantly lower expression of CD16 ($CD16^{low}$) compared to their $CD16^{high}$ mature counterparts. The expression of the other cell-surface markers tested in prednisone and G-CSF-mobilized neutrophils was not altered (**Figure 2D**). Together, these data indicate that prednisone and G-CSF mobilized neutrophils had similar viability but differed in their maturation stage.

Neutrophil Antimicrobial Defenses in Prednisone and G-CSF GCs

Since neutrophils are the main source of antimicrobial activity in GCs, we assayed the key neutrophil functions required to fight infections in GC neutrophils 6 h post-leukapheresis. For comparative purposes, the assays were also performed on neutrophils from unstimulated, healthy donors. Of these neutrophil responses, the function that was significantly altered in prednisone GC neutrophils was the phagocytosis of non-opsonized zymosan. While considerable variability in the phagocytosis was observed amongst non-stimulated healthy donors, prednisone leveled this response to 90% phagocytosis in all donors (**Figure 3**). A diminution in fMLF-induced chemotaxis was also observed but did not reach significance. Likewise, no significant difference in the LPS-induced production of IL-8 and the fMLF and PMA-induced ROS production, or increase in cytoplasmic calcium was observed between prednisone or G-CSF GC and non-stimulated, healthy donor neutrophils (**Figure 3**).

G-CSF GC neutrophils also exhibited functional differences with non-stimulated, healthy donor neutrophils albeit for different functions (**Figure 3**). Chemotaxis induced by fMLF was significantly downregulated in G-CSF GC neutrophils whereas LPS-induced production of IL-8 was significantly enhanced (**Figure 3**). No significant differences in the other functional assays were observed. Together, these data reveal a mobilizing agent-dependent alteration in GC neutrophil function.

Effect of Leukapheresis on the Function of Prednisone and G-CSF GC Neutrophils

To determine whether leukapheresis contributes to the functional alterations observed in GC neutrophils, their responses were compared before and after leukapheresis. A significant diminution in the fMLF-induced increase in cytoplasmic calcium (**Figure 4A**) and an increase in PMA, but not fMLF, -induced intracellular ROS production in prednisone GC neutrophils was observed after leukapheresis (**Figures 4B,C**). In contrast, G-CSF GC neutrophils were unaffected by this procedure (**Supplementary Figure 1**). Together, these data indicate that prednisone GC neutrophils are more affected by leukapheresis than G-CSF GC neutrophils.

Effect of Storage on Neutrophil Viability, Cell-Surface Markers, pH, and Metabolite Concentration

While the negative effect of storage on G-CSF GC neutrophil viability and function was reported in several studies (18, 26, 27), the impact of storage on prednisone GC neutrophils remains unknown. Storage negatively affects neutrophils as we were not able to isolate the predicted quantity of neutrophils from these GCs based on the neutrophil concentration in the GC prior to isolation. The number of neutrophils isolated from prednisone GCs after 24 and 48 h of storage declined by 20 and 40%, respectively, compared to the neutrophil yield on the day of

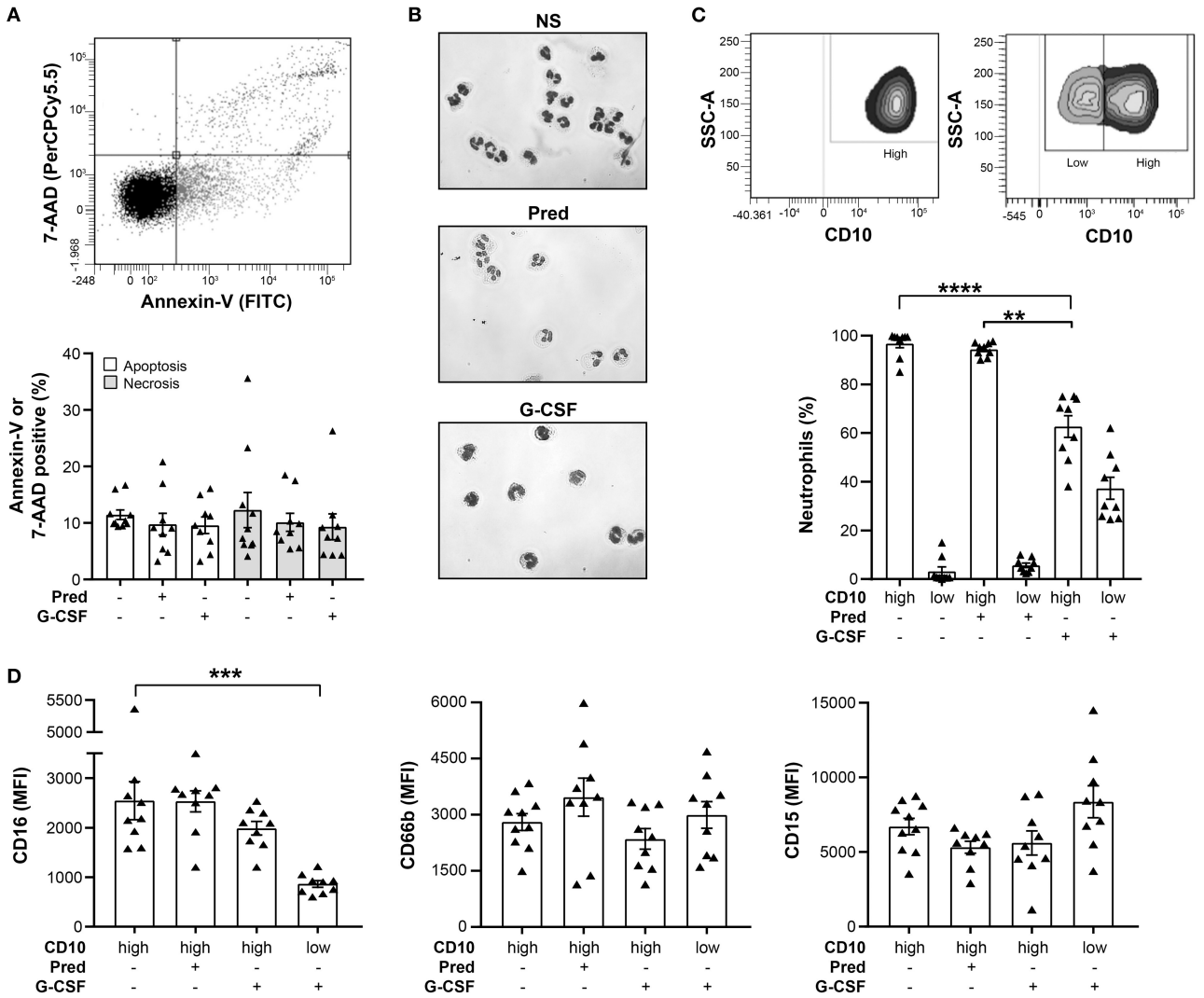

FIGURE 2 | Viability, morphology, and cell-surface marker expression of prednisone- and G-CSF-mobilized neutrophils. **(A)** The proportion of neutrophil, spontaneous apoptosis and necrosis was determined by 7-AAD/Annexin-V staining in prednisone and G-CSF GCs as well as neutrophils of non-stimulated, healthy donors. A representative two-dimensional plot of Annexin-V/7-AAD staining is shown *above* the graph. **(B)** Nuclear morphology of neutrophils from control, non-stimulated-donors **(NS)** *(top)*, prednisone- *(middle)* and G-CSF-mobilized neutrophils *(bottom)* after Pappenheim staining. **(C)** Proportion of circulating neutrophils that display high or low CD10 cell-surface expression determined by flow cytometry. A representative plot of CD10 staining in a $CD10^{high}$ *(left)* and a $CD10^{high}/CD10^{low}$ *(right)* sample is shown *above* the graph. **(D)** Cell-surface expression of CD16 *(left)*, CD66b *(middle)* and CD15 *(right)* determined by flow cytometry and expressed as mean fluorescence intensity **(MFI)**. Statistical analysis: Dunn's multiple comparison test was performed to compare cell-surface marker expression between prednisone- and G-CSF-mobilized neutrophils, $^{**}p$-value < 0.01; $^{***}p$-value < 0.001; $^{****}p$-value < 0.0001. These data are compiled from the analysis of 9 prednisone and 9 G-CSF GCs as well as neutrophils of 10 non-stimulated healthy donors.

collection (**Figure 5A**). Apoptosis of the isolated neutrophils harvested from prednisone GCs after 24 h of storage significantly increased by 8–10% in 4/9 donors (**Figure 6A**). As for cell-surface marker expression, CD66b expression significantly increased after 48 h of storage suggesting an increased degranulation (**Supplementary Figure 2**).

As observed for prednisone GC neutrophils, G-CSF GC neutrophils were also affected by storage. The most striking effect was the difficulty in isolating neutrophils from these GCs. The neutrophil yield dropped by 40% after 24 h storage and 80%, 48 h post-leukapheresis (**Figure 5A**). The functional data on these GCs is thus shown as supplementary data (**Supplementary Figures 3, 4**). Storage had no significant effect on cell-surface marker expression of G-CSF GC neutrophils *(data not shown)*.

A change in metabolite concentration and/or pH could explain the observed negative effects of storage on GCs. Data for two prednisone GC donations and for all G-CSF GCs was generated as the metabolite measurements began later in the study. We consistently observed a decrease in pH

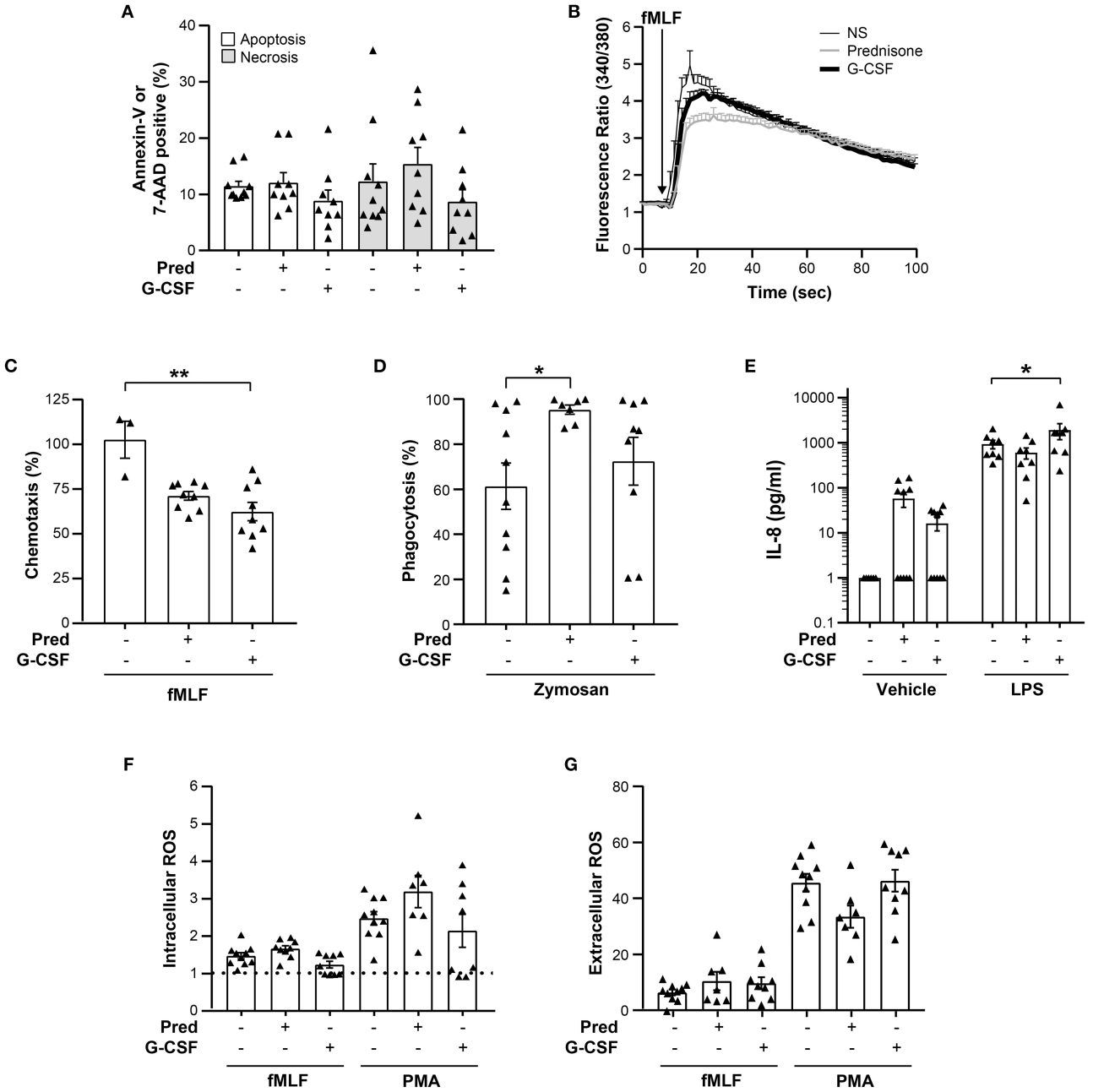

FIGURE 3 | Viability and function of prednisone and G-CSF GC neutrophils. **(A)** Proportion of apoptosis and necrosis determined by Annexin-V and 7-AAD staining of neutrophils isolated from non-stimulated, healthy donors *(-/-)*, and prednisone or G-CSF GCs after leukapheresis on the day of collection. **(B)** Intracellular calcium levels measured with a spectrofluorometer after fMLF stimulation as in "Materials and Methods." The kinetics of the change in intracellular calcium concentration is shown. For **(A,B)**, neutrophils of 10 non-stimulated, healthy donors, 9 prednisone and 9 G-CSF GCs were analyzed. **(C)** Chemotaxis toward fMLF expressed as the maximal proportion of migrated neutrophils [non-stimulated, healthy donors ($n = 3$), prednisone ($n = 9$) and G-CSF GC donors ($n = 9$)]. **(D)** Phagocytosis of non-opsonized pHRodo Red zymosan conjugated bioparticles is expressed as the proportion of phagocytic neutrophils [non-stimulated, healthy donors ($n = 10$), prednisone ($n = 7$) and G-CSF GC donors ($n = 9$)]. **(E)** LPS-induced IL-8 release by neutrophils determined by ELISA (n=8 for all experimental conditions). **(F)** Intracellular ROS production induced by fMLF [non-stimulated, healthy donors ($n = 10$), prednisone and G-CSF GC neutrophils ($n = 9$)] or PMA [non-stimulated. healthy donors ($n = 10$), prednisone ($n = 7$) and G-CSF GC neutrophils ($n = 8$)]. Data are expressed as a MFI ratio of stimulated cells/cells incubated in DMSO (diluent). A ratio of 1 corresponds to the absence of an increase in ROS production compared to the DMSO-treated neutrophils *(dotted line)*. **(G)** Extracellular ROS production measured with cytochrome *c* by neutrophils incubated with DMSO, fMLF [non-stimulated, healthy donors ($n = 10$), prednisone GCs ($n = 7$), G-CSF GCs ($n = 9$)] or PMA [non-stimulated, healthy donors ($n = 10$), prednisone ($n = 7$), G-CSF ($n = 8$)] expressed as the concentration of superoxide produced. Statistical analysis: Dunn's and Dunnett's multiple comparison test performed to compare neutrophils from non-stimulated, healthy donors with prednisone and G-CSF GC neutrophils, *p-value < 0.05; **p-value < 0.01.

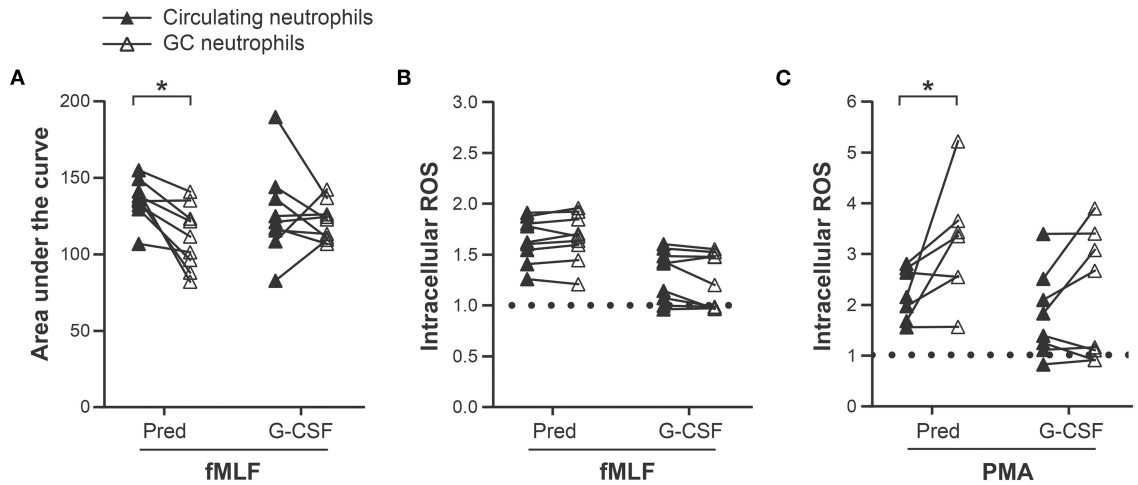

FIGURE 4 | Effect of leukapheresis on neutrophil responses. **(A)** Intracellular calcium levels measured with a spectrofluorometer after fMLF stimulation of circulating neutrophils (pre-leukapheresis) and prednisone and G-CSF GC neutrophils (post-leukapheresis) as described in "Materials and Methods." **(B)** fMLF and **(C)** PMA-induced intracellular ROS production measured with CM- H2DCFDA in circulating neutrophils and prednisone and G-CSF GC neutrophils. The results are expressed as a MFI ratio of stimulated cells/cells incubated in DMSO (diluent). The dotted line represents the absence of increase in ROS production (ratio = 1). Statistical analysis: Sidak multiple comparison test performed to compare circulating neutrophils with GC neutrophils from prednisone- and G-CSF-pre-treated donors: *p-value < 0.05. These data are compiled from the analysis of 9 donors for the fMLF stimulation and 7 prednisone and 8 G-CSF GCs for the PMA stimulation.

(7,25 to 6,82) 24 h post-collection in G-CSF GCs as well as glucose with a concomitant 5 to 8-fold increase in lactate concentration during storage (**Figure 5B**). Together, these data indicate that the decrease in GC neutrophil viability is highly likely due to rapid changes in pH and a significant change in metabolite concentration.

Effect of Storage on Neutrophil Function

A diminution in most neutrophil responses including calcium mobilization, fMLF-induced chemotaxis and the phagocytosis of non-opsonized zymosan by prednisone GC neutrophils was observed after 24 h storage but did not reach significance (**Figures 6B–D**). In contrast, a significant increase was observed in both PMA-induced superoxide production and spontaneous release of IL-8 but not in intracellular ROS production (**Figures 6E–G**). The increase in superoxide production is suggestive of neutrophil priming during storage. Since the number of neutrophils that could be isolated from the collection bag diminished considerably with storage, functional data on prednisone GC neutrophils after 48 h storage and G-CSF GC neutrophils after 24 and 48 h storage may not be representative of all neutrophils in GCs (**Supplementary Figures 3, 4**). Together, these data reveal that G-CSF GC neutrophils rapidly deteriorate with storage and that storage differentially affects prednisone GC neutrophil effector functions.

DISCUSSION

Comparison of neutrophil functional studies performed on GCs prepared by different blood centers is challenging due to differences in GC preparation and analysis. Moreover, there are few reports on prednisone GCs. To address these gaps in our knowledge of GCs, we compared prednisone and G-CSF GC neutrophil viability and function in a single center study and on the same donors to minimize differences in GC production and inter-donor variability. Major differences in ANC, neutrophil maturity, functional capacity and response to storage were observed between prednisone and G-CSF GCs. To our knowledge, this is the first comprehensive study that compares the antimicrobial defenses of prednisone- and G-CSF GC neutrophils on the day of collection and during storage.

Mobilizing a sufficient number of neutrophils is considered crucial for the therapeutic efficacy of GTX. We show that G-CSF was more efficient at mobilizing neutrophils into circulation in all donors than prednisone corroborating a similar observation by Hiemstra et al. (28). Worel et al. (29) also reported that a lower proportion of prednisolone GCs (56%) contained the minimal ANC *per* GC unit for transfusion (29). These findings underscore the importance of verifying ANC prior to transfusion, especially for prednisone GCs due to its variable efficacy in mobilizing neutrophils.

A striking difference between prednisone- and G-CSF- GC neutrophils was the presence of 30–40% immature neutrophils in the latter. These neutrophils are not low-density granulocytes as they are of a higher density than PBMCs. The release of immature, low-density neutrophils from the bone marrow seems to require more than one injection of G-CSF as Marini et al. (30) reported their presence in the circulation in donors stimulated with G-CSF for 5 days (10 μg/kg/day). The difference in maturity between prednisone and G-CSF GC neutrophils is thus highly likely due to the preferential mobilization of neutrophils from the marginated pool by prednisone and bone marrow by G-CSF (31).

Functional analysis revealed major differences between GC and non-stimulated healthy donor neutrophils. Most notably,

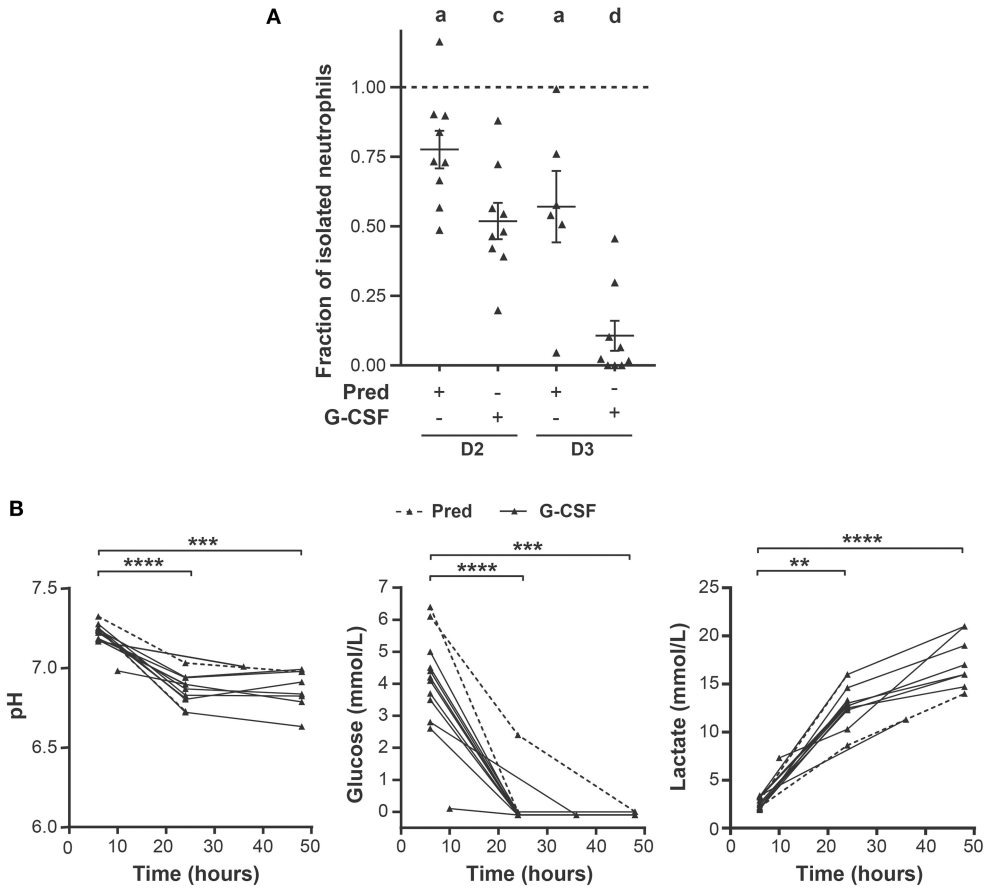

FIGURE 5 | Effect of storage on neutrophil isolation from GCs and GC metabolite composition. **(A)** Ratio of the number of neutrophils isolated with a density gradient 24 h (D2) or 48 h (D3) post-leukapheresis, and the number of neutrophils isolated from GCs right after leukapheresis (D1); (D2/D1 and D3/D1). Sample size: D1 ($n = 9$), D2 ($n = 9$), D3 ($n = 7$). Cell counting was performed with a hemacytometer after trypan blue staining. The dashed line corresponds to a ratio of 1. **(B)** The pH *(left),* glucose *(middle)* and lactate *(right)* concentrations were measured in GCs after the indicated times of storage. Prednisone, *dashed line* ($n = 2$), G-CSF *solid line*, ($n = 10$). Statistical analysis: A sample *t* test with a theorical mean of 1 was performed in **(A)**, ^{a}p-value < 0.05; ^{c}p-value < 0.001 and ^{d}p-value < 0.0001. Sidak multiple comparison test was performed to compare GC metabolite content at 24 or 48 h of storage with that after 6 h of storage in **(B)**, $^{**}p$-value < 0.01; $^{***}p$-value < 0.001; $^{****}p$-value < 0.0001.

prednisone GC neutrophils exhibited a significant increase in non-opsonized zymosan phagocytosis. Whether this increased phagocytic capacity compensates for the lower ANC to preserve the overall antimicrobial efficacy in these GCs remains unknown. In contrast, G-CSF GC neutrophils migrated less efficiently than unstimulated donor neutrophils. This may be, in part, due to their incomplete differentiation as chemotaxis is one of the last functions to develop in mature neutrophils (32). Whether reduced chemotaxis is compensated *in vivo* by the higher ANC of these GCs and/or their increase in IL-8 production remains unknown and underscores a gap in our knowledge about functional compensation in neutrophils.

Comparison of GC neutrophil functional data between studies is challenging due to different study designs. Nevertheless, a comparison with the few reports resembling our study revealed that neutrophil function varies between GCs prepared by different blood centers The significant decrease in fMLF-induced chemotaxis in G-CSF GCs was also observed by Leavey (26) in GC neutrophils prepared from donors administered a higher dose of G-CSF for 5 consecutive days. In contrast, other studies (22, 33) reported increased basal chemotactic activity in neutrophils mobilized by a combination of G-CSF and dexamethasone (DEX) (22, 33). As for ROS production, Mochizuki (19) also demonstrated that ROS production was preserved in neutrophils of G-CSF and G-CSF/DEX GCs. In contrast to our findings, Joos found an increase in *E.coli* and fMLF-induced ROS in G-CSF GC neutrophils (34) underscoring the unmet need in GTX to certify GCs for transfusion with a neutrophil functional, quality control test. The only consistent finding irrespective of GC preparation was an increase in cytokine levels during storage including IL-8, IL-6, IL-1β and TNF-α (18, 19, 21, 27). Activated neutrophils may be

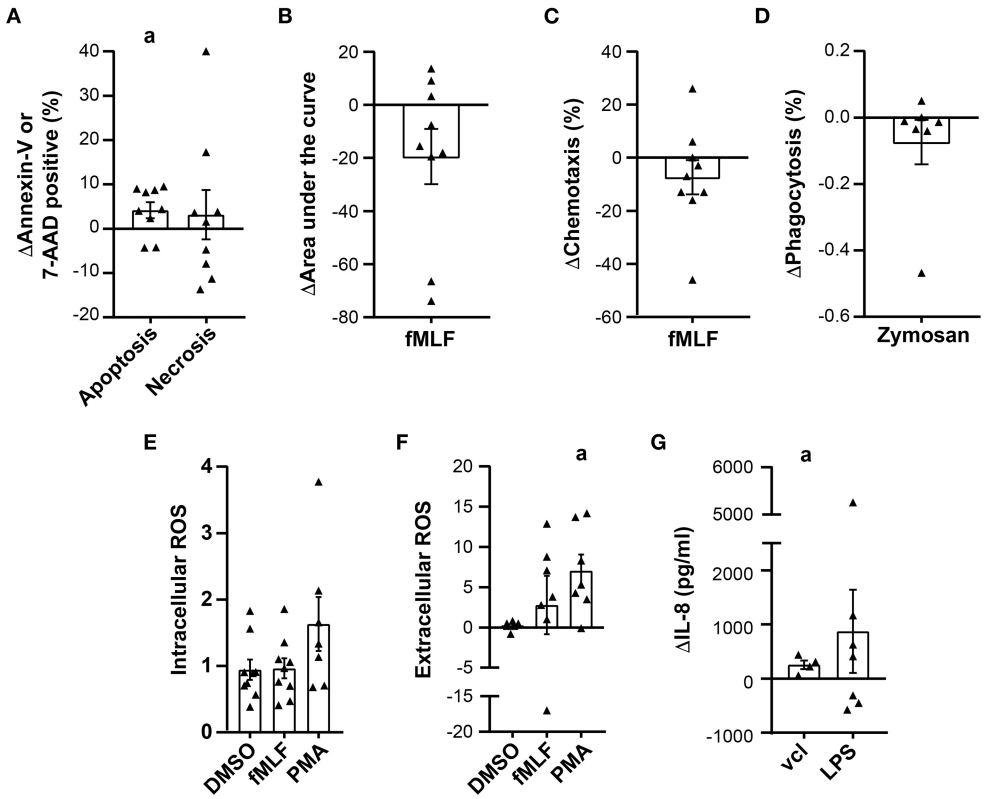

FIGURE 6 | Variation in prednisone GC neutrophil viability and function after 24 h of storage. Viability and functional assay data are presented as the difference between the values obtained on the day of leukapheresis (D1) and 24 h post-leukapheresis (D2), (D2-D1), in all graphs except in **(E)**. **(A)** Difference in the proportion of apoptotic and necrotic neutrophils determined by Annexin-V and 7-AAD staining, respesctively ($n = 9$). **(B)** Difference in the fMLF-induced increase in intracellular calcium expressed as the area under the curve ($n = 9$). **(C)** Difference in the maximal proportion of neutrophils that migrated across a chemotaxis chamber toward fMLF ($n = 9$). **(D)** Effect of storage on the proportion of neutrophils that phagocytosed non-opsonized pHRodo Red Zymosan conjugated particles ($n = 7$). **(E)** Neutrophil fMLF and PMA-induced intracellular ROS production expressed as a ratio of the MFI (D1/D2); $n = 9$ and $n = 7$, respectively. **(F)** Change in fMLF and PMA-induced superoxide anion production expressed as the difference in O^{2-} produced on D1 and D2 ($n = 7$). **(G)** Difference of IL-8 production expressed in pg/ml ($n = 8$). Statistical analysis: One sample t test with a hypothetical mean of 0 [in **(A–D,F,G)**] or 1 (in **(E)** was performed to determine the significance of the difference or ratio between values obtained on D1 and D2, ^{a}p-value < 0.05.

a source of these cytokines as we observed for prednisone GC neutrophils.

Another source of variability between GCs stems from the inherent functional variability between healthy donors (35, 36). Neither prednisone nor G-CSF diminished GC neutrophil, interdonor functional variability. The only exception was prednisone's ability to increase the phagocytic capacity of GC neutrophils of all donors to the same level. While the molecular mechanism(s) involved in this phenomenon remain unknown, we postulate that prednisone increases the expression of pattern recognition receptors. How this increase in phagocytosis modifies the antimicrobial competency of prednisone GC neutrophils remains unknown.

Neutrophils are fragile cells *ex-vivo*. Even though glucocorticoids and G-CSF increase neutrophil viability (37, 38), GC neutrophil viability was not increased in stimulated donors on the day of collection or during storage. Neutrophil viability decreased significantly during storage most likely due to the rapid decrease in glucose and pH. The high leukocyte concentration in GCs and metabolic activity of red blood cells are likely causes of the pH decrease. Enriching the GC medium is thus crucial to preserve neutrophil viability and function (39). Several reports already demonstrated the possibility to extend neutrophil viability *ex vivo* with hypothermic solutions or the addition of anti-apoptotic agents and/or G-CSF (27, 39, 40).

In conclusion, the variability in neutrophil function between GCs underscores the unmet need of establishing a functional test to certify GCs for their antimicrobial properties before GTX and for GC donor selection. In addition, optimization of GC storage conditions will extend neutrophil viability *in vitro*, improve GC efficacy and expand its use in transfusion medicine.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The study was performed according to the Declaration of Helsinki for studies with human subjects and approved by the CHU de Québec-Université Laval research Ethics Committee (# 2019-4493) and by the Héma-Québec research Ethics Committee (# 2018-012). Written informed consent was obtained from all the participants.

AUTHOR CONTRIBUTIONS

AM: significant contribution to performing the research, data compilation and analysis, participated in writing the manuscript, and prepared the figures. M-ÈA, GP, MV, LB, M-PC, JV, PL, and M-ML: all these co-authors contributed to performing the research and data compilation and part of the data analysis. NR: contributed to the data analysis and manuscript review. DB: contributed to the conceptualization, data analysis and manuscript review as well as funding acquisition. MG: contributed to the supervision of the personnel, conceptualization and methodology of the study, data analysis, manuscript review, and funding acquisition. MF: funding acquisition, resources and project administration, major contribution to the conceptualization and methodology of the study, the manuscript writing as well as review, editing, visualization, and supervised the personnel. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.839475/full#supplementary-material

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New Insights Into Pathophysiology of β-Thalassemia

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β-thalassemia is a disease caused by genetic mutations including a nucleotide change, small insertions or deletions in the β-globin gene, or in rare cases, gross deletions into the β-globin gene. These mutations affect globin-chain subunits within the hemoglobin tetramer what induces an imbalance in the α/β-globin chain ratio, with an excess of free α-globin chains that triggers the most important pathogenic events of the disease: ineffective erythropoiesis, chronic anemia/chronic hypoxia, compensatory hemopoietic expansion and iron overload. Based on advances in our knowledge of the pathophysiology of β-thalassemia, in recent years, emerging therapies and clinical trials are being conducted and are classified into three major categories based on the different approach features of the underlying pathophysiology: correction of the α/β-globin disregulation; improving iron overload and reverse ineffective erythropoiesis. However, pathways such as the dysregulation of transcriptional factors, activation of the inflammasome, or approach to mechanisms of bone mineral loss, remain unexplored for future therapeutic targets. In this review, we update the main pathophysiological pathways involved in β-thalassemia, focusing on the development of new therapies directed at new therapeutic targets.

Keywords: anemia, thalassemia, β-globin, GATA1, inflammasome

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INTRODUCTION

Thalassemias is an inherited hemoglobin disorder characterized by reduced or absent globin chain synthesis, resulting in variable clinical phenotypes from severe chronic anemia requiring lifelong transfusion and iron chelating therapy to asymptomatic individuals (1).

Traditionally, β-thalassemias have been more common in countries in the Mediterranean area, North and Central Africa, Southeast Asia, and the Middle East. However, as a result of migrations of populations, β-thalassemias are now encountered in other regions, such as Northern Europe and North America (2).

β -thalassemia has a broad clinical spectrum, and traditionally has been classified in the clinic in thalassemia major (TM), thalassemia intermedia (TI), and thalassemia minor (**Figure 1**). The TM grouped patients with more severe anemia from an early age who require periodic blood transfusions associated with iron chelation for life, while thalassemia minor, the less severe manifestation, is characterized by people with mild asymptomatic anemia and a heterozygous condition (trait) for thalassemia. TI constituted a group with a variable clinical spectrum, from mild to moderate to moderately severe anemia, who do not require blood transfusions on a regular basis, sometimes only occasionally, but who do develop various complications of thalassemia

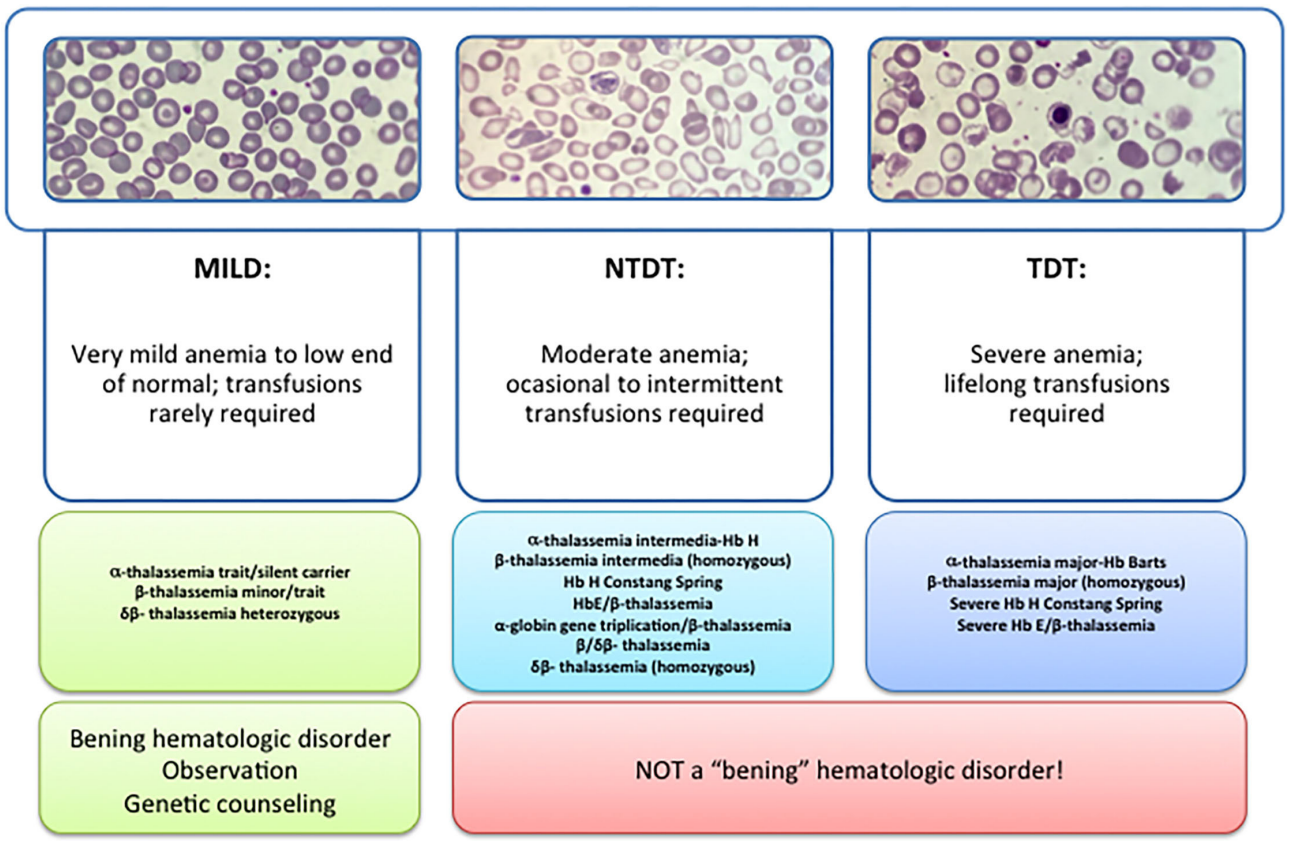

FIGURE 1 | Types of thalassemia. Genotype–Phenotype Association. α and β-thalassemias are genetically heterogeneous diseases. The clinical management with RBC transfusions is an essential factor in classifying them as either transfusion-dependent thalassemia (TDT) or non–transfusion-dependent thalassemia (NTDT). Patients with TDT need life-long regular transfusions for survival in early childhood while patients with NTDT do not need life-long regular transfusions for survival and normally have later in childhood or even in adulthood with mild/moderate anemia that requires only occasional or short-course regular transfusions under concrete clinical circumstances during times of erythroid stress (infection, pregnancy, surgery, or aplastic crisis); however, usually present the typical complications of TDT such as extramedullary hematopoiesis, iron overload, leg ulcers, and osteoporosis. Patients with TDT include those with β-thalassemia major or severe forms of β-thalassemia intermedia, HbE/β-thalassemia, or α-thalassemia/HbH disease. NTDT mainly encompasses three clinically distinct forms: β-thalassemia intermedia (β-TI), hemoglobin E/β- thalassemia (mild and moderate forms), and α-thalassemia intermedia (hemoglobin H disease).

such as extramedullary hematopoiesis, pulmonary hypertension, iron overload, leg ulcers, skeletal deformities, and growth retardation (3, 4).

Recently, this classification has changed due to better understanding of the pathophysiology of the disease and findings focused on the clinical management and complications of IT that show that these patients may present with the same serious complications as transfused patients later in life. In 2012, the International Thalassemia Federation adopted the new terminology for clinical classification of transfusion-dependent thalassemia (TDT) and non–transfusion-dependent thalassemia (NTDT) that groups in three different types in the clinic: α-thalassemia intermedia (hemoglobin H disease) and β-thalassemia intermedia (β-TI), hemoglobin E/β- thalassemia (mild and moderate forms). Differentiating a new patient with thalassemia as TDT or NTDT is essential and requires an accurate clinician's evaluation using various indicators such as hematological parameters, particularly baseline Hb levels, and follow-up for a minimum of 3 to 6 months to determine clinical severity is recommended before making a diagnosis of TDT or NTDT (5, 6).

The three important pathophysiologic factors in β-thalassemias are: chronic anemia/hypoxia, ineffective erythropoiesis, and iron overload. The harshness of the disease depends mainly on molecular deficiencies. Chain imbalance causes excess unstable α chains to provoke within erythroid progenitors, leading to cell membrane decline and cell lysis. This triggers an alteration in the mycomedial environment of the bone marrow due to an imbalance of cytokines that causes the erythroid progenitors to proliferate but with inadequate maturation, which is called ineffective erythropoiesis. This cytokine imbalance together with bone marrow hyperplasia causes extramedullary erythropoiesis and subsequently the associate bone deformations. Because of anemia/chronic hypoxia, infective erythropoiesis retroelements, is maintained and perpetuates over time (3, 6).

INEFFECTIVE ERYTHROPOIESIS

Erythropoiesis is a finely regulated process in which every stage is highly regulated by different signal transduction pathways and proteins. Erythropoiesis process in humans is divided into two parts: the early stage of erythropoiesis and the late stage. The first stage is EPO-dependent whereas the second stage is iron-dependent. Erythropoietin (EPO-dependent stage) is the main regulator of early-stage erythropoiesis whereas erythrocyte differentiation and maturation are negatively regulated by the transforming growth factor (TGF-family), the late-stage erythropoiesis or iron-dependent stage of erythropoiesis (**Figure 2**).

EPO-Dependent Erythropoiesis

In this stage, EPO is fundamental for the proliferation of erythroid progenitors. The recognition of EPO by EPO-R on the surface of these precursors induces JAK2 activation activating STAT5 phosphorylation, with associated induction of erythroid antiapoptotic genes and increase of erythroid progenitors proliferation and survival (7). The production of EPO and the expansion of erythropoiesis is regulated according to demand (hypoxia, hemorrhage, hemolysis). On the contrary, if the production of erythrocytes is adequate, down-regulation by a mechanism of apoptosis is performed. The predisposition of EPO-dependent erythroid progenitors to apoptosis is associated with different levels of FAS protein (CD95) expression and the FAS ligand (FASL), which belong to the family of TNF receptors, the binding of which activates the caspase cascade and consequently, the immature cell apoptosis limiting erythropoiesis expansion. On the other hand, if the need for erythrocytes increases, EPO production increases, and apoptosis is reduced because EPO stimulates the production of heat shock protein 70 (HSP70) that protects GATA1 from cleavage by FAS/FASL and the activation of the caspase cascade (8).

Fe-Dependent Erythropoiesis

In the large stage, the presence of iron is essential for the synthesis of hemoglobin, and the integrity of the erythroferrone (ERFE)-hepcidin-ferroportin axis is essential for iron homeostasis (9, 10). ERFE is a potent negative regulator of hepcidin. When it is chronically elevated, such as in situations of ineffective erythropoiesis, low plasma iron availability occurs. Transferrin (and its cellular receptor) is also involved in this stage as well as growth differentiation factors like GDF11, a member of the TGF-β superfamily, which negatively regulate erythrocyte maturation and differentiation (11).

TGF-β receptor ligands are a group of cytokines that include TGF, activins, bone morphogenetic proteins (BMPs), and GDF-11 and play an important role in the regulation of erythropoiesis within the hematopoietic stem cell niche. Various activins, and in particular GDF-11, exert inhibitory activity at the late stage of erythropoiesis.

In the TGF signaling pathway, ligand binding to the type II receptor leads to the recruitment and phosphorylation of the type I receptor and phosphorylation of regulatory SMADs (R-SMADs), SMAD2 and 3, to form the R-SMAD/SMAD4 complex, which modulates the expression of target genes inducing an inhibitory activity on erythroid differentiation by inducing apoptosis in erythroblasts.

During normal erythroid maturation, reduced GDF11 expression with consequent TGF- β signaling suppression, and EPO stimulation occur in parallel, and both are essential for the differentiation of hematopoietic erythroid progenitor cells (12).

In β-thalassemia, ineffective erythropoiesis is triggered by two main pathogenic mechanisms (7). On the one hand, α-globin chains aggregates sequester cytosolic heat shock protein 70 (HSP70). This inhibits its nuclear translocation and protects the erythroid transcription factor GATA-factor 1 (GATA1) from cleavage. On the other hand, these toxic aggregates of α-globin chains stimulate the formation of radical oxygen species (ROS) (whose formation is also produced by other mechanisms such as iron overload), which activate GDF11, which in turn activates the inhibitory pathway of SMAD2/3, and as a consequence, erythroid differentiation is inhibited (13).

IRON OVERLOAD

Iron overload is one of the main pathogenic events in β-thalassemia. Apart from the transfusion-dependent iron overload in patients with TDT, there is a mechanism by which an inappropriate increase in intestinal iron absorption occurs, both in patients with TDT and in NTDT.

This mechanism is triggered by ineffective erythropoiesis. The accumulation of erythroid precursors during ineffective erythropoiesis increases the production of erythropherrone, potent negative regulator of hepcidin secreted by bone marrow erythroblasts (10), which negatively regulates the expression of hepcidin. The decrease in hepcidin, the main negative ferroportin modulator (an iron transporter protein in the basolateral membrane of the enterocyte), increases iron absorption (hepcidin inhibits iron absorption and recycling ferroportin) and release of iron from the reticuloendothelial in situations of iron overload and iron sequestration in erythropoiesis. It has been described that iron availability during stress erythropoiesis is produced by increase of ERFE (13).

In NTDT, growth differentiation factor 15 (GDF-15) can induce hepcidin downregulation. This is a member of the transforming growth factor- (TGF-β) family which use to be upregulated during ineffective erythropoiesis, causing the down regulation of hepcidin (14).

BONE DISEASE IN β-THALASSEMIA

Recently, several advances in the understanding of the pathophysiology of bone disease in β-thalassemia have been done. Classically, it was directly attributed to ineffective erythropoiesis and secondary bone expansion, but recently it has been shown that there is an imbalance of cytokines that can directly alter bone metabolism, although the mechanisms involved are not yet well-established.

Both patients with TDT and NTDT show marked decreases in bone mineral density (BMD), despite optimization of

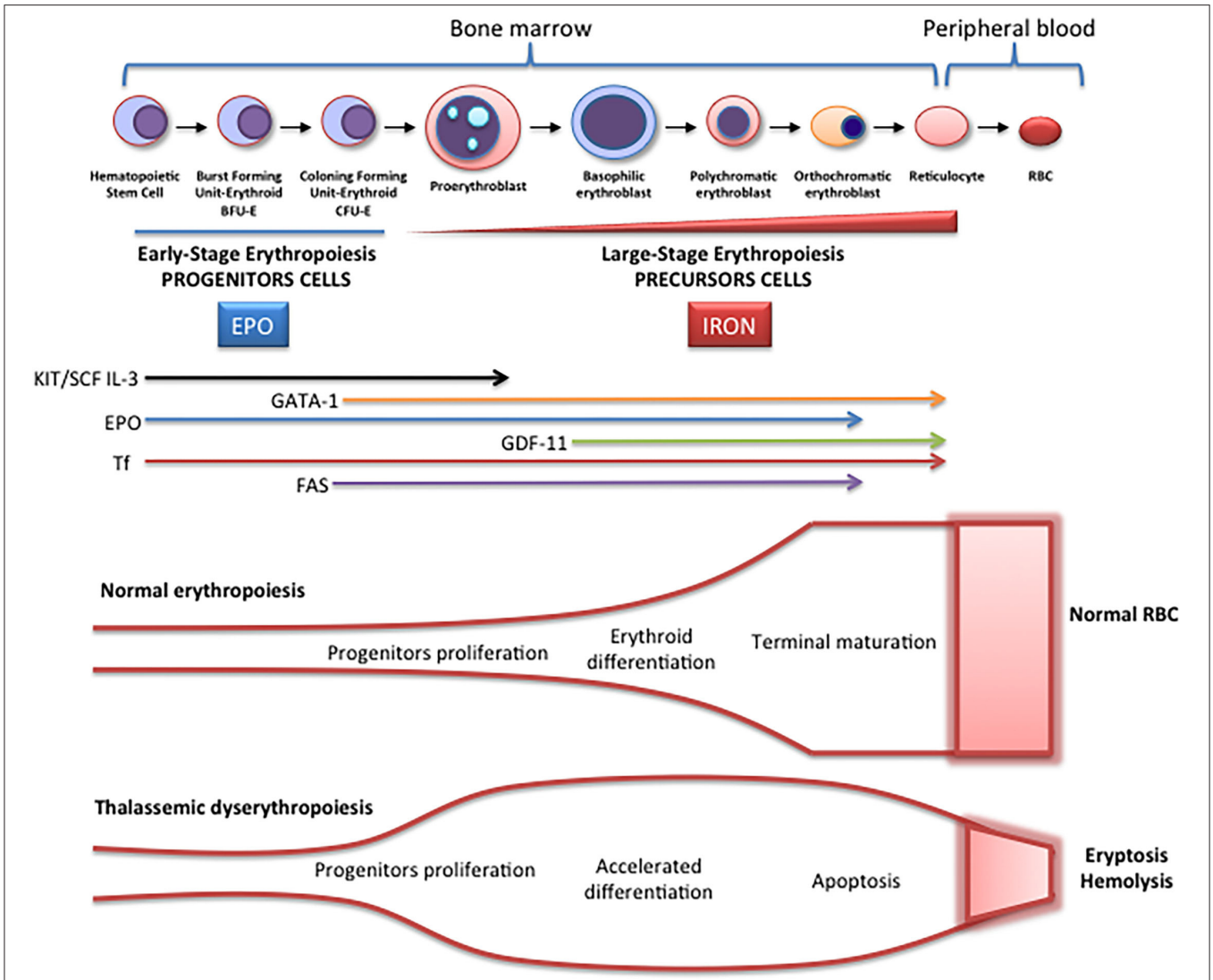

FIGURE 2 | Schematic representation of erythropoiesis. During erythroid development, several stages occur in which a complex network of molecules are expressed (EPO, iron, transcription factors) are involved. In early-stage erythropoiesis, EPO is the main regulator after BFU-E formation. In this stage, GATA-1 promotes erythropoiesis and increases the EPO receptor expression. In large-stage erythropoiesis molecules such as transferrin and growth/differentiating factor 11 (GDF11) are involved. Erythroid expansion is negatively regulated by association of FAS to FAS ligand, which has as a consequence the apoptosis on immature erythroid cells, and GDF11 and other members of the TGF-β family, which negatively regulate erythrocyte differentiation and maturation from the early to the late stages.

transfusions, and low BMD continues to be a frequent complication in these patients.

The mechanisms that have been postulated to explain the loss of bone mineral density in patients with β-thalassemia include explicit effects of abnormal erythroid proliferation with bone expansion, increased circulating erythropoietin (EPO), iron bone deposit with iron toxicity, and oxidative stress with endocrine secondary disorders (hypogonadism, deficit GH-IGF-1, vitamin D deficiency) that which in turn affect the bone mineral loss and secondary osteoporosis (15).

In recent years, there is growing evidence of the relationship between erythropoiesis, bone mineral metabolism and iron homeostasis. Recently, a mechanism responsible for the activation of osteoclasts in thalassemic patients has been described that could be associated to cytokine dysregulation and, in particular, to the modification of the RANK/RANKL/OPG axis, which is essential for the regulation of osteoclastogenesis (16, 17). The OPG/RANK/RANKL system is essential for the regulation of osteoclastogenesis. Osteoprotegerin (OPG) or osteoclastogenesis inhibition factor (OCIF or TNFRSF11B), is a member of the superfamily of tumor necrosis factor (TNFR) receptors that is expressed and secreted in numerous tissues (lung, heart, kidneys, liver, intestine, stomach, brain, thyroid gland and spinal cord) as well as in bone in which its main function is to inhibit the maturation and activation of osteoclasts.

Recently, it has been shown ERFE binds and sequesters some members of the bone morphogenetic protein (BMP) family, primarily BMP2, BMP6, and the BMP2/6 what suppresses hepcidin by inhibiting hepatic BMP/SMAD signaling. Therefore, iron availability by stimulated erythropoiesis can be regulated by ERFE (9). Bone formation by osteoblasts during skeletal development, modeling, and ongoing remodeling can be stimulated by BMPs. Therefore, ERFE seems to be key in the recently described erythropoiesis-iron-bone circuit, by modifying the availability of BMP. Therefore, ERFE appears to be an important link between abnormal erythropoiesis, iron metabolism alteration, and loss of BMD in β-thalassemia (16, 17).

The mechanism of action of ERFE, through the sequestration of BMP, could be that the loss of ERFE, by improving the availability of BMP, stimulates the formation of osteoblastic bone (18). However, high ERFE levels are osteoprotective and prevent bone loss in β-thalassemia when erythropoiesis is extended. Therefore, there is a paradoxical effect that has not yet been fully explained. Although in TDT patients, in whom ERFE is inhibited post-transfusion, and is lower than in NTDT patients, could explain the more severe bone alterations in these patients despite transfusions and ERFE has a protective function decreasing bone loss phenotype in b-thalassemia.

In addition, other data indicate that the loss of BMP signaling (high ERFE), increases bone mass through direct inhibition of osteoclasts and activation of the Wnt pathway, predicting that the loss of ERFE would lead to a decrease in bone mass (16, 17) by increased expression of RANKL and sclerostin.

In summary, to date the only known function of ERFE was hepcidin regulation expression through BMP sequestration, contributing to iron overload (9). However, ERFE seems to have bone metabolisms implications and a new role in bone protection has been described (16). In conditions of elevated ERFE, such as β-thalassemia and others ineffective erythropoiesis situations, BMP2 and BMP6 proteins are sequestered, decreasing signaling through the BMP/Smad and ERK pathways. This would result in decreased SOST and RANKL expression (Rankl/OPG) with decrease osteoclastogenesis and bone resorption. On the other hand, when level ERFE is low, increased BMP2, and BMP6 proteins, lead to stimulate osteoclastogenesis (RANKL/Opg), bone resorption and increased sclerostin osteocytes synthesis (expression SOST gene), with a consequent decrease in bone formation by inhibition of osteoblastic function.

GATA1 LEVELS REGULATION IN β-THALASSEMIA

As mentioned above, erythropoiesis starts from hematopoietic stem cells (HPSCs), in a finely regulated process, that involves various factors, and it is controlled at different molecular levels by growth factors and hormones such as erythropoietin, that activate different signaling pathways that end up activating erythroid transcription factors. The essential transcription factors for erythropoiesis are GATA-1, SCL, TAL1, LMO2, LDB1, KLF-1, and GFI-1B, although many more participate. These factors are organized in a complex called CEN ("Core ErythroidNetwork") (19), whose operation is finely regulated by SCF and EPO between others, to ensure adequate erythrocyte development. GATA1 is considered the "master regulator" of this process, GATA1 is a DNA-binding zinc finger transcription factor that plays an essential role in the normal development of hematopoietic lines. The protein contains 413 amino acids, with an N-terminal region where its transcriptional activity resides and a C-terminal region that mediates the binding of GATA1 to DNA and other proteins. In 1995, two isoforms of GATA1 resulting from alternative splicing were identified. GATA1 encodes a 47 kDa protein and GATA1s, a shorter 40 kDa protein that lacks the transactivation domain at the N-terminus. Both proteins are capable of binding to DNA and could form dimers or heterodimers, although the shorter GATA1s isoform is less active than the long (20). In 1999 cleavage of GATA1 by Caspase 3 was reported (21). More recently, it has been linked the stabilization of GATA1 to inhibition of Caspase-1 Activity (22).

β-thalassemia shares several common elements with other forms of anemia including a deficiency of GATA1 the 'master regulator' in erythropoiesis (23), GATA1 levels are finely regulated during erythropoiesis to develop sufficient functional erythrocytes (24). This transcription factor is necessary for normal early erythroid progenitors' differentiation [i.e., colony-forming unit-erythroid (CFU-E) and burst-forming unit erythroid (BFU-E) cells]. In β-thalassemia, α-hemoglobin chains accumulate in the cytosol due to the non-functionality of β-hemoglobin chains and sequester heat shock protein 70 (HSP70). Subsequently, HSP70 cannot be translocated into the nucleus thereby impairing GATA1 stabilization through caspase 3 cleavage, resulting in altered GATA1 levels with disturbed erythropoiesis and accumulation of unfunctional erythroid progenitors (23).

Importantly, GATA1 is highly expressed during these early stages of differentiation but GATA1 protein expression is shown to decline toward terminal erythroid differentiation (25). Recent data from Tyrkalska et al. demonstrated the role of the inflammasome, a complex of innate immune system which receptors and sensors playing important roles in infection and inflammation, in GATA1 regulation, and subsequent erythroid differentiation. Pharmacological inhibition of the inflammasome was shown to stimulate GATA1 expression and promote erythroid differentiation (22).

On the other hand, Fetal hemoglobin (HbF) increase has revealed as a promising results to treat β-hemoglobinopathies (26) with a recent implication of MiR-486-3p and miR-15a in Fetal hemoglobin induction (27). Several groups reported that increase of miR-210 levels is high in erythroid precursors from β-thalassemia patients what have an impact on fetal hemoglobin (HbF) levels (28–31).

Finally, other genetic studies indicate a relationship between variants in the gene BCL11A and HbF levels (29). There they described that reduced BCL11A expression induce HbF. The data published by Gasparello et al. are consistent with a globin gene regulation by BCL11A and therefore postulated BCL11A as a therapeutic candidate to reactivate HbF in disorders associated to beta-hemoglobin (29). Aligned with this, Bauer et

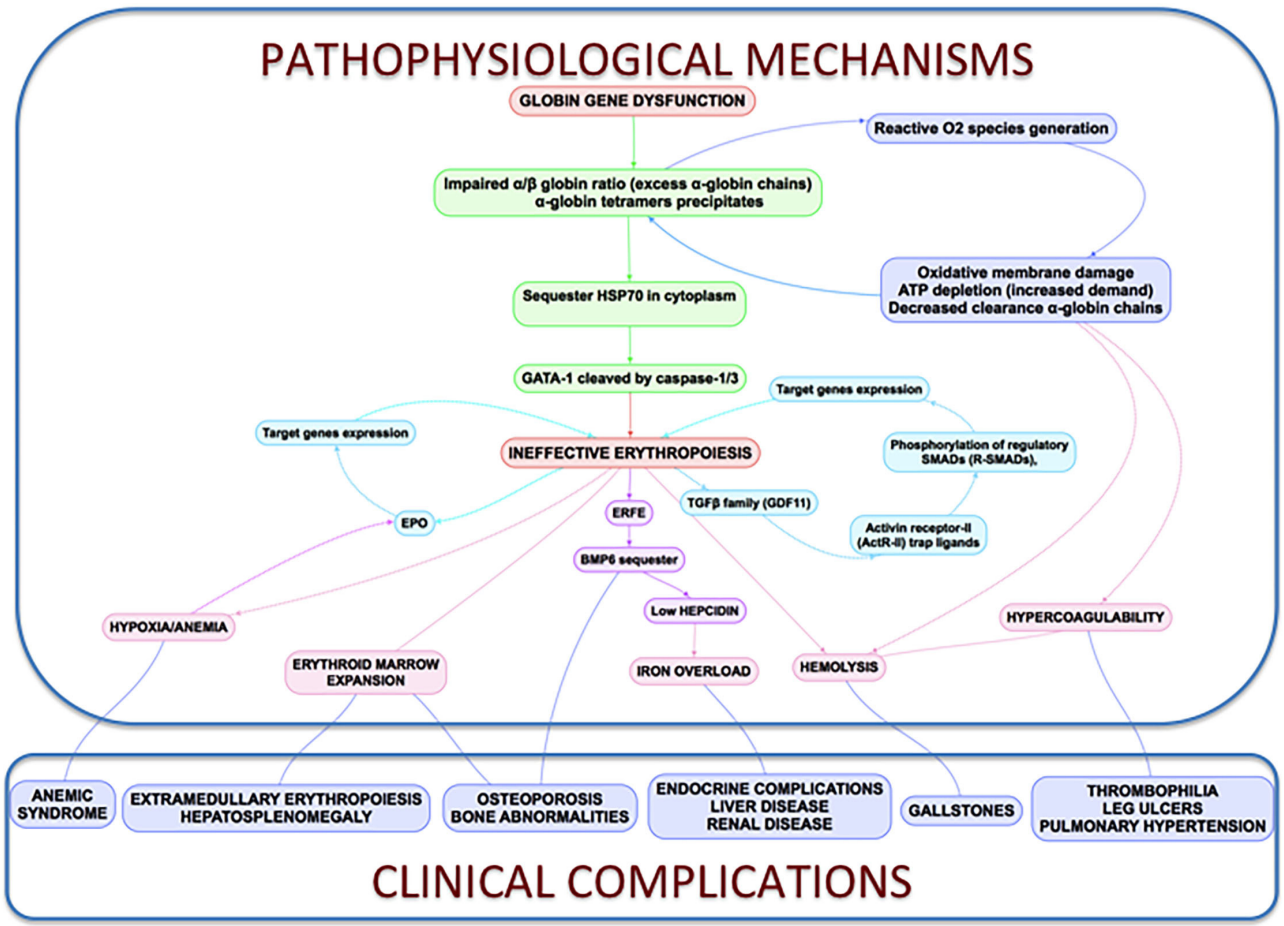

FIGURE 3 | Clinical complications and pathophysiological mechanisms of β-Thalassemia. In thalassemia, the imbalance α/β-globin synthesis is the fundamental initial pathogenic event. Excess α-globin chains precipitate in the cytoplasm, sequester HSP70 and GATA1 is cleaved by Caspase 3/1 which result in dysfunctional erythropoiesis and imposes metabolic stress on the erythrocytes, specifically in the form of excess generation of reactive oxygen species and increased demand on adenosine triphosphate (ATP)-dependent proteolytic mechanisms to clear excess globin chains. These pathophysiological changes lead to the characteristics of this disease: ineffective erythropoiesis, peripheral hemolysis, and subsequent anemia. Clinical implications of the α- and β-globin imbalance include lack of sufficient RBCs and Hb for effective oxygen transport, and ineffective erythropoiesis and hemolysis, which can lead to splenomegaly, bone marrow expansion (extramedullary hematopoiesis), concomitant bone deformities, and iron overload.

al. published that GWAS-marked BCL11A enhancer represents another potential optional treatment in this disease (32).

All the processes described above are reflected and connected in **Figure 3**.

DISCUSSION: PERSPECTIVES AND FUTURE NEW POTENTIAL β-THALASSEMIA TREATMENTS

Despite the high social and economic impact of β-thalassemia, there is no curative treatment available, except for bone marrow transplant for the few pediatric patients who have an identical HLA donor, and who assume the high morbidity and mortality of transplant that is sometimes not acceptable for non-malignant disease. Currently no approved treatments to handle anemia in NTDT and, even though it has recently been approved, the first drug that improves anemia in these patients (luspatercept), has limited efficacy. Therefore, there are no effective treatments to improve anemia or to reduce red blood cell transfusions and chronic complications. However, in recent years there is a growing interest in studying these diseases with an increasing number of clinical trials directed against various therapeutic targets (gene therapy, erythroid maturation agents, pyruvate kinase activators, JAK kinase 2 inhibitors, targeting iron dysregulation). All of them are being developed and in the future may change the quality of life of these patients and we aim to be part of this scenario. All of them summarized in a succinct way by Musallam et al. (33).

Although these targets may be effective, efficacy is partial, not all patients respond, and therefore further research and treatments are required considering its multifactorial pathophysiology. Perhaps the correct approach in the future is combined treatment and, in this scenario, it will be essential to

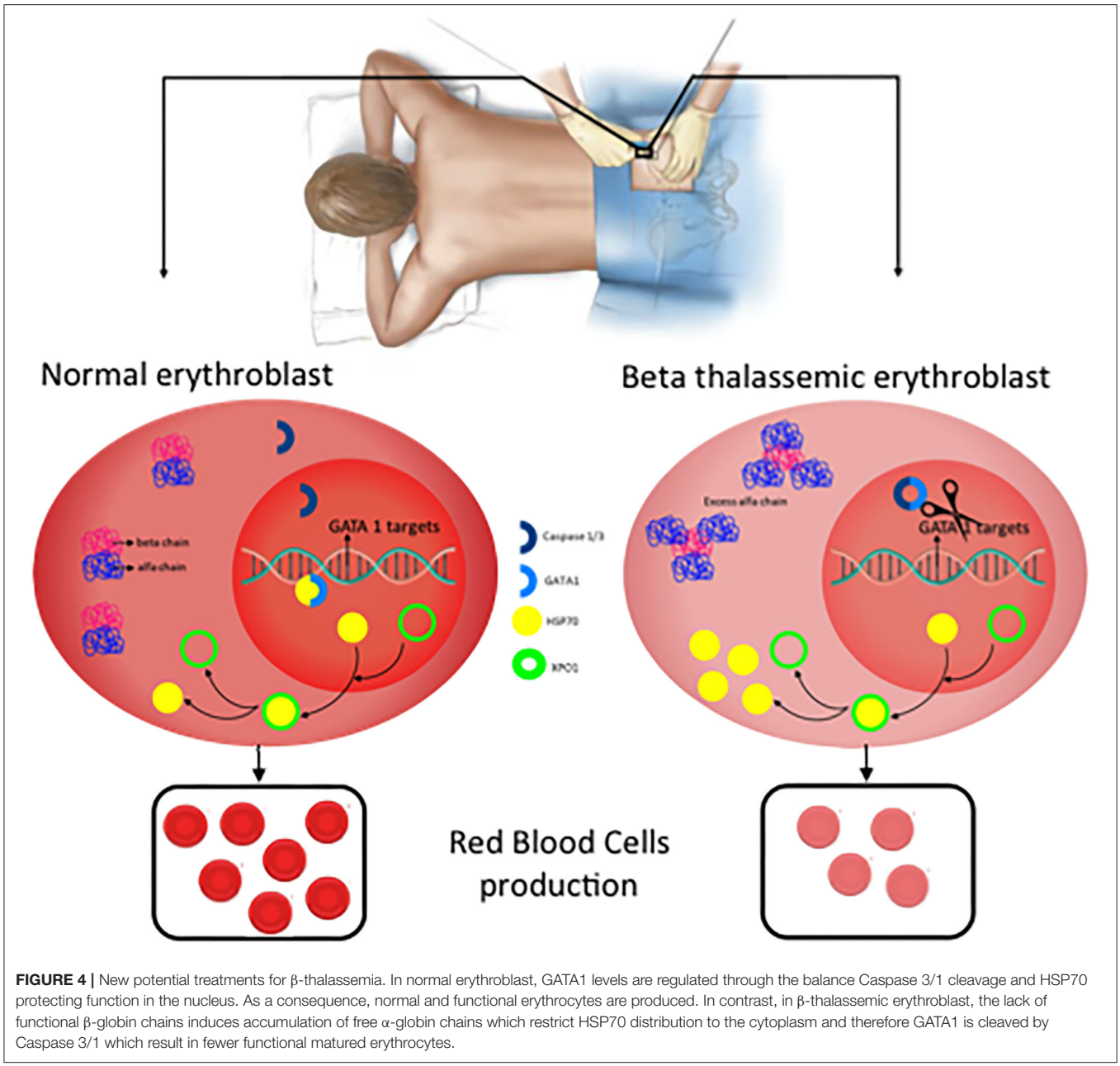

FIGURE 4 | New potential treatments for β-thalassemia. In normal erythroblast, GATA1 levels are regulated through the balance Caspase 3/1 cleavage and HSP70 protecting function in the nucleus. As a consequence, normal and functional erythrocytes are produced. In contrast, in β-thalassemic erythroblast, the lack of functional β-globin chains induces accumulation of free α-globin chains which restrict HSP70 distribution to the cytoplasm and therefore GATA1 is cleaved by Caspase 3/1 which result in fewer functional matured erythrocytes.

explore new therapeutic targets. In recent years, inflammasomes have emerged as a new potential therapeutic target for these types of diseases as well as the HSP70 nuclei regulation.

Inflammasome as a Potential Target Treatment in β-Thalassemia

Inflammasomes are multiprotein complexes firstly described a decade ago by Tschopp and colleagues. Inflammasomes are multiprotein complexes usually composed of sensor proteins, mainly from the NLR family, adaptor proteins such as ASC, and an effector cysteine-protease enzyme, usually caspase-1 (34). Gasdermin D (GSDMD) a pore-forming protein is cleavage and activated by caspase-1 (35), what induce cytokine release and pyroptosis (36). Several inflammasome receptors have been described with different roles including inflammatory diseases, sepsis protection or host defense (37–39).

Inflammasomes are broadly expressed in hematopoietic and non-hematopoietic cells and can induce different responses including production of IL-18, IL-1β, eicosanoids, and pyroptosis. Since this first description, research within the inflammasome field has been one of the most studied fields in immunology leading to huge advances.

In the innate immune context, the activation of inflammasomes is essential in the clearance of pathogens or damaged cells. On the other hand, uncontrolled inflammasome activation induces metabolic and autoimmune disorders,

indicating the importance of these complexes. The recent role of the inflammasome in erythropoiesis brings the focus to these complexes to treat anemia and neutrophilia (22).

The effector protein of the inflammasomes are caspases and previous data indicated a role of Caspase 3 in GATA1 stabilization (21). More recently, inflammasome has been postulated as a potential treatment of Diamond-Blackfan anemia, this type of anemia curses by a deficiency in GATA1 levels due to a ribosomopathy that produces an inefficient translation of GATA1 (22, 40). Tyrskalska et al. (22)demonstrated an increase of GATA1 levels in human and zebrafish larvae with caspase-1 inhibitor. Therefore, potentially inhibition of Caspase-1 or Caspase-3 could be considered as a potential treatment to stabilize GATA1 levels to increase red blood cells formation. In this scenario, the identification of the inflammasome type that mediates this process will be essential to translate these results with higher specificity to the clinic (**Figure 4**).

Inhibition of HSP70 Export From the Nucleus

As described above, GATA1 levels are essential in erythroid differentiation, and in this plays an important role the Heat Shock Protein 70 (HSP70) a chaperone, which is translocated to the nucleus to protect GATA1 transcription factor of caspase-3 cleavage (41). In β-thalassemia, the accumulation of free α-globin chains sequestered HSP70 in the cytosol which avoids the protective role of GATA1 into the nucleus. A recent publication demonstrated that HSP70 localization is regulated by the exportin-1 (XPO1) and inhibition of XPO1 increase HSP70 levels in normal erythroid progenitors what increase, which have as a consequence an increase in GATA1 levels (42). This introducesXPO1 inhibitors as a new therapeutic option to treat β-thalassemia (42, 43).

For many years, management of β-Thalassemia patients has been limited to blood transfusion and iron chelation. However, β-Thalassemia is now the focus of a flourishing research field that has already offered new treatments with the potential to modify the natural history of the disease and the quality of life of the patients. In this review we have summarized the present knowledge of the pathophysiology of the disease and proposed future possible new, and more directed approaches, to its treatment (**Figure 4**).

CONCLUSIONS

β-thalassemia (transfusion and non-transfusion dependent), is an inherited hemoglobinopathy caused by a quantitative defect in the synthesis of β-globin chains of hemoglobin, leading to the accumulation of free α-globin chains aggregates that cause ineffective erythropoiesis. The only curative treatment for these patients is hematopoietic stem cell transplantation, but this option only is feasible in a few patients with HLA-matched sibling donors. In most patients, the development of chelation and support treatments has improved survival, however, chronic complications have increased (iron overload, osteoporosis, extramedullary hematopoiesis, etc.) that limit their quality of life.

Despite increasing knowledge of the pathophysiology of β-thalassemia, only luspatercept has been recently approved in TDT patients, reducing transfusion needs but of limited effectiveness. NTDT patients, which do not require regular transfusions, lack an approved treatment and have the same (if not more) complications as TDT patients. Therefore, new treatments are needed that, alone or in combination with existing ones, can improve the expectations of these patients.

In human erythroblast, terminal erythroid maturation is altered due to HSP70 sequestration in the cytoplasm by free α-globin chains preventing its accumulation in the nucleus to protect GATA1 transcription factor from Caspase-3 cleavage, resulting in maturation arrest and apoptosis. ERFE and BMP play an important role in bone disease but are not well-established yet.

The knowledge of these critical new pathophysiological approaches can help develop new therapeutic options such as XPO1 inhibitors or inflammasome inhibitors that could rescue GATA-1 expression, improved erythroid terminal differentiation, and represent a new therapeutic option to ameliorate ineffective erythropoiesis, iron overload, and decreased mineral bone mass of β-thalassemia patients.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

AUTHOR CONTRIBUTIONS

MS-V: writing—original draft preparation. AP-O, ES, JM, and MB: writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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A Novel Phenotype of the Factor 5 Gene Mutation (Homozygote Met1736Val and Heterozygote Asp68His) Is Associated With Moderate Factor V Deficiency

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Chang Y-S, Lan Y-C, Chen Y-J, Huang J-S, Yang C-N, Huang C-YF and Yeh K-Y (2022) A Novel Phenotype of the Factor 5 Gene Mutation (Homozygote Met1736Val and Heterozygote Asp68His) Is Associated With Moderate Factor V Deficiency. Front. Med. 9:870269. doi: 10.3389/fmed.2022.870269

Background: Factor V (FV) deficiency is a rare disease, with a low incidence rate in Asia. Therefore, the *F5* mutation in the Taiwanese population is poorly understood.

Methods: A Chinese family with FV deficiency was included, and the patient and his family members underwent mutation analysis. Then, patients from Keelung City (Taiwan) were screened for *F5* polymorphism; the Chang Gung Human Database was used to determine single-nucleotide variants in the non-FV-deficient patient population.

Results: Eight mutation sites on the *F5* gene locus, including exon 16 homozygote Met1736Val and seven heterozygous mutations, including Asp68His, were found. Moreover, Met1736Val was found to be the dominant mutation in people living in the Taiwan community, and this result was compared with the records of the Chang Gung Human Database. The above-mentioned polymorphisms may result in a variable incidence of FV deficiency in Keelung City, thereby facilitating carrier diagnosis and prenatal diagnosis in most FV-deficient families.

Conclusion: The homozygote Met1736Val and the co-inheritance of the Asp68His *F5* gene are unique and worthy of screening in FV-deficient patients.

Keywords: factor V deficiency, factor V gene, polymorphism, coagulopathy, Asia

BACKGROUND

Factor V (FV) deficiency, or parahemophilia, first described by Owren in 1947 (1), is an autosomal recessive bleeding disorder characterized by low FV coagulant activity and antigen levels. Factor V deficiency is a rare bleeding disorder, with an estimated prevalence of one per one million (2, 3). The phenotypic expression of FV deficiency is variable, with mutations showing different severities of bleeding symptoms. Both homozygous and heterozygous mutations may affect the severity of bleeding symptoms (4). Thus, identifying the gene polymorphism of *F5* will provide additional insights into the mechanisms underlying this variable clinical expression.

Human coagulation FV (proaccelerin or labile factor) plays an important role in mediating coagulation (5). Factor V is a single-chain, high-molecular-weight glycoprotein (330 kDa) primarily synthesized by hepatocytes and megakaryocytes. Plasma FV is mainly represented by precursor molecules, and 20% of it is stored in platelet alpha-granules (6). The domain architecture of *F5* is ordered as A1–A2–B–A3–C1–C2 (7). The human *F5* gene has been mapped to chromosome 1q23 and spans >80 kb (8), and the entire gene contains 25 exons and 24 introns. The messenger RNA encodes a leader peptide of 28 amino acids and a mature protein comprising 2,196 amino acids (9). The heavy chain (A1–A2) is encoded by exons 1–12, whereas the light chain (A3–C1–C2) is encoded by exons 14–25. The other part—the entire B domain—is encoded by exon 13. After proteolytic cleavage by thrombin or activated factor X (FXa), the B domain is removed, and FV is converted to its active form (FVa) (10, 11). One heavy chain and one light chain, linked together in the presence of Ca^{2+} ions, constitute the fully activated FV (12). Factor V, converted to its active form, binds to FXa in the prothrombinase complex that converts prothrombin to thrombin in the presence of calcium and a phospholipid membrane. Activated protein C (APC) inactivates FVa by cleaving the active factor in three additional arginine residues and requires FV in APC-mediated inactivation of factor VIIIa (13, 14). Thus, FV plays an important role in both pro-coagulant and protein C anticoagulant pathways (15). The dual role of FV is reflected in disorders associated with molecular defects in FV, which can be either hemorrhagic or thrombotic.

In 2018, the updated Human Genome Mutations Database (http://www.hgmd.org) displayed that the most homozygous or heterozygous mutations occur in the heavy (A1–A2) and light (A3–C1–C2) chains (∼80% mutation annotated in these two chains). Another mutation profile of FV deficiency was reported by Paraboschi et al. in 2020 (16). In Taiwan, studies about FV deficiency are limited and focus on the A1 domain mutations, such as Asp68His and His147Arg (17). In this study, we report the cases of two patients with FV deficiency wherein we sequenced their *F5* genes. To facilitate a better understanding of FV deficiency in Taiwan, we traced their family history and analyzed the non-FV-deficient population.

METHODS

Measurement of Prothrombin Time, Activated Partial Thromboplastin Time, Prothrombin Time, and FV Coagulant

Tests of prothrombin time (PT) and activated partial thromboplastin time (APTT) were performed in our laboratories using the Clauss method (18). The normal ranges for coagulation tests were determined according to the threshold used by the manufacturers of the reagents.

The FV activity in plasma was examined *via* a functional assay based on PT using human recombinant tissue factor (Instrumentation Laboratory Company, Bedford, MA). Plasma FV antigen was measured *via* a sandwich enzyme immunoassay (EIA; Hyphen Biomed, Andresy, France) using a sheep anti-human FV polyclonal antibody (6). The FV activity was expressed as a percentage of normal pooled plasma.

Abbreviations: APC, activated protein C; APTT, activated partial thromboplastin time; F, factor; FV, Factor V; FX, factor X; PCR, polymerase chain reaction; PT, prothrombin time; SNV, single-nucleotide variant.

Factor FV Exon Analysis

Before the gene analysis, we provided adequate information to the patient and his family regarding the reasons for the analysis. The study was reviewed and approved by our institutional review board (Nos. 200903793B0, 201102005A3D001, 201102005A3C102, and 201102005A3C103). After explaining the study details, all participants provided informed consent. We conducted a mutation analysis of the patient and his family members. To better understand the polymorphism of the *F5* gene, including its promoter, a total of 111 non-FV-deficient people were enrolled at the Keelung Chang Gung Memorial Hospital. The Ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood samples were collected from the patient and his family members. Genomic DNA was isolated from whole blood using the QIAamp DNA Blood Mini kit (Qiagen, Hiden, Germany). All exons and exon–intron boundaries of the FV gene were amplified *via* the polymerase chain reaction (PCR) using a 50-μl reaction volume containing 0.5–1.0 μg DNA, 20 pmol/L of each primer, 0.2 mmol/L of each dNTP, 2.0 mmol/L $MgCl_2$, and 1.5 U Taq DNA polymerase in an appropriate buffer. The polymerase chain reaction was performed in 30 cycles of 30 s at 94°C, 40 s at 50–60°C, and 40 s at 72°C, followed by an elongated extension of 7 min at 72°C. The PCR products were analyzed *via* direct DNA sequencing using the ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). **Table 1** shows the gene analysis results of the family. We also performed an *F5* polymorphism genotype analysis, including its promoter, and the results for the non-FV-deficient population are shown in **Table 2** (25 exons of *F5*)/**Supplementary Table 1** (*F5* promoter).

Database Analysis of FV Polymorphism

We used the Chang Gung Human Database to determine single-nucleotide variants (SNVs) on exons 3, 8, 13, 16, and 25 in a non-FV-deficient patient population (including 391 individuals). Whole blood samples were collected from 391 patients from 2008 to 2018. All detected variants were filtered against a panel of 300 germline DNA samples (Chang Gung Human Database, an unpublished, institutional whole-genome database of normal controls). **Table 3** lists non-synonymous and synonymous mutations. We used the database described in the previous studies (16, 19).

Factor V Protein Conformational Changes at a Specific Site of Met1736

We searched the RCSB Protein Data Bank (RCSB PDB: Homepage). We then used the BIOVIA Discovery Studio Visualizer version (BIOVIA, San Diego, CA, USA) to illustrate the mutation site and protein folding process.

TABLE 1 | The pattern of mutation and clinical symptoms in FV deficiency family.

	Genotype	Patient 1	Patient 2	Father	Mother
Exon 3 Asp68His	Heterozygote **g**ac → **c**ac	Heterozygote	Heterozygote	Heterozygote	
Exon 8 Met385Thr	Heterozygote a**t**g → a**c**g	Heterozygote	Heterozygote		Heterozygote
Exon13 Asn789Thr	Heterozygote a**a**c → a**c**c	Heterozygote	Heterozygote		Heterozygote
Exon13 Lys830Arg	Heterozygote a**a**a → a**g**a	Heterozygote	Heterozygote	Heterozygote	
Exon13 His837Arg	Heterozygote c**a**t → c**g**t	Heterozygote	Heterozygote	Heterozygote	
Exon13 Lys897Glu	Heterozygote **a**ag → **g**ag	Heterozygote	Heterozygote	Heterozygote	
Exon16 Met1736Val	Homozygote **a**tg → **g**tg	Homozygote	Homozygote	Heterozygote	Heterozygote
Exon25 Asp2194Gly	Heterozygote g**a**t → g**g**t	heterozygote	Heterozygote		
Clinical presentation					
Age		19	20	51	44
Clinical symptoms		Easy bruising	1. Easy bruising 2. Postpartum hemorrhage	No bleeding episode	No bleeding episode
Factor V activity, % (50–150%)		3.2%	2%	58%	54%
PT: sec (range: 8–12)		22.2	13.7	10.2	10.4
APTT: sec (range: 25.0–31.3)		53.2	33.8	30.5	31.0

Patient 1 and 2 both had the same clinical symptoms and the Factor V activity.
PT, Prothrombin time; APTT, Activated partial thromboplastin time.

RESULTS

Clinical Course of FV-Deficiency Cases

The first patient was a 19-year-old man admitted to Chang Gung Memorial Hospital (Keelung branch) for an inguinal hernia operation. He had no history of spontaneous bleeding. Routine clotting tests confirmed a moderate FV deficiency. He received fresh frozen plasma before surgery, and no bleeding complications occurred.

Before we commenced our study, we suggested that his family members be screened for coagulopathy and informed them about the impact of genetic mutation and the hereditary risk. His parents did not suffer from bleeding problems and denied consanguinity. The PT, APTT, and FV activity are shown in **Table 1**. **Figure 1A** shows the pedigree chart. The patient's 20-year-old sister mentioned that she was easily bruised but did not pay much attention to this problem. Compared with other coagulation factor deficiencies, bleeding due to FV deficiency is typically harmless. However, she had experienced a massive postpartum hemorrhage in 2017 at a gynecology clinic. The bleeding could not be stopped by emergency surgical intervention after she was transferred to our hospital. She received a fresh frozen plasma transfusion after the operation, which reduced the bleeding. After this episode, we informed them about the importance of prophylactic blood transfusion before undergoing any future surgical interventions.

Detection of FV Deficiency Within a Family and Phenotype Identification

Both patients had a prolonged APTT and PT; the thrombin time was within the normal range. The plasma coagulation factor levels were normal, except for FV. The FV activity of patient 1 was only 3.2% (normal range, 50–150%), which indicated that this patient had moderate FV deficiency, and that of his sister was 2% (normal range, 50–150%). Although patient 1 denied any prior spontaneous bleeding episode, patient 2 suffered from easy ecchymosis. Their parents did not suffer from bleeding problems and denied consanguinity. **Table 1** summarizes mutation patterns and clinical symptoms in the family.

TABLE 2 | Factor V polymorphism distribution for Keelung non-FV deficiency population, including 25 exons.

Exon	Mutation	Heterozygote number	Homozygote number
exon 3	Asp68His (D68H)	1/111 (0.9%)	0
exon 8	Met385Thr (M385T)	2/111 (1.8%)	0
exon 13	Asn789Thr (N789T)	0	0
	Lys830Arg (K830R)	27/111 (24.3%)	4/111 (3.6%)
	His837Arg (H837R)	27/111 (24.3%)	4/111 (3.6%)
	Lys897Glu (K897E)	27/111 (24.3%)	4/111 (3.6%)
exon 16	Met1736Val (M1736V)	47/111 (42.3%)	8/111 (7.2%)
exon 25	Asp2194Gly (D2194G)	4/111 (3.6%)	0

Gene Mutation Analysis of This Family

Only a limited number of mutations have been documented because of the large size and complexity of the coagulation *F5* gene (1q23) and its low prevalence. Almost all mutations identified to date have been unique and specific to the family. All 25 exons were assessed separately for each patient. We detected eight mutation sites in this study. Here, we report the combination of mutation sites in one patient and predict FV deficiency in his sibling carrying the same mutations.

First, we recorded eight mutation sites in the patient as follows: exon 3 (Asp68His), exon 8 (Met385Thr), exon 13 (Asn789Thr), exon 13 (Lys830Arg), exon 13 (His837Arg), exon 13 (Lys897Glu), exon 16 (Met1736Val), and exon 25

TABLE 3 | The nonsynonymus mutations in non-FV deficiency patient population.

Exon	refGene	AAChange	Frequency SNP	
exon25	T6673C	X2225Q	0.00128	Stoploss
exon25	A6665G	D2222G	0.02046	Nonsynonymoussnv
exon24	A6452G	K2151R	0.00128	Nonsynonymoussnv
exon23	C6298T	R2100C	0.00128	Nonsynonymoussnv
exon23	G6277A	G2093R	0.00128	Nonsynonymoussnv
exon20	G5828C	G1943A	0.00128	Nonsynonymoussnv
exon17	G5558T	G1853V	0.01918	Nonsynonymoussnv
exon17	A5552C	E1851A	0.00128	Nonsynonymoussnv
exon17	A5431T	M1811L	0.00128	Nonsynonymoussnv
exon16	G5378T	R1793I	0.00128	Nonsynonymoussnv
exon16	A5290G	M1764V	0.23402	Nonsynonymoussnv
exon16	G5275C	D1759H	0.00128	Nonsynonymoussnv
exon13	C4210T	P1404S	0.12276	Nonsynonymoussnv
exon13	C4189T	L1397F	0.67008	Nonsynonymoussnv
exon13	T4000C	F1334L	0.00128	Nonsynonymoussnv
exon13	A3980G	H1327R	0.02174	Nonsynonymoussnv
exon13	C3853A	L1285I	0.09335	Nonsynonymoussnv
exon13	C3446T	S1149F	0.00384	Nonsynonymoussnv
exon13	A2998G	K1000E	0.00128	Nonsynonymoussnv
exon13	T2911C	W971R	0.00128	Nonsynonymoussnv
exon13	A2773G	K925E	0.21228	Nonsynonymoussnv
exon13	A2594G	H865R	0.21228	Nonsynonymoussnv
exon13	A2573G	K858R	0.21228	Nonsynonymoussnv
exon13	A2450C	N817T	0.02174	Nonsynonymoussnv
exon13	G2219A	R740Q	0.00128	Nonsynonymoussnv
exon13	A2032G	K678E	0.00384	Nonsynonymoussnv
exon10	A1601G	Q534R	1	Nonsynonymoussnv
exon10	G1538A	R513K	0.66624	Nonsynonymoussnv
exon11	T1699C	C567R	0.00128	Nonsynonymoussnv
exon8	T1238C	M413T	0.02558	Nonsynonymoussnv
exon7	C1106T	A369V	0.00512	Nonsynonymoussnv
exon7	A1000G	R334G	0.00256	Nonsynonymoussnv
exon6	C801A	F267L	0.00128	Nonsynonymoussnv
exon4	T398C	F133S	0.00128	Nonsynonymoussnv

Among these nonsynoymous mutations, 8 of them marked in red have high (exon16: M1764V exon13: P1404S, L1397F, K925E, H865R, K858R; exon10:Q534R, R513K) in whole-exome sequencing of OSCC tumor-normal (fresh-frozen tumors and matched whole blood) database in Taiwan.

(Asp2194Gly) (**Table 1**). Only exon 16 expressed the homozygous genotype. Half of the mutations are reportedly heterozygous mutations on the B domain (exon 13). Frequent B domain mutations have been reported before, but their functions remain unclear (20, 21). Other gene mutations in exons 3, 8, and 25 were heterozygous. The pattern and location of each gene mutation are depicted in **Figures 1A,B**, respectively.

We then analyzed the *F5* gene in other family members and demonstrated its mutation pattern throughout the entire family (**Table 1**). Interestingly, the patient's sister had the same phenotype of the *F5* gene, i.e., exon 16 (Met1736Val), which was homozygous. Based on the FV level, the patient's sister also had moderate FV deficiency. A co-inheritance of Met1736Val FV gene mutation and seven polymorphisms were identified within this family. This study is the first to report many mutations detected in exon 13.

Polymorphism Analysis of the Non-FV-Deficient Population

In the polymorphism screening, 111 non-FV-deficient participants were included. We focused on the eight mutation sites detected in our patients. The frequency of *F5* polymorphism is shown in **Table 2**. The frequency of heterozygote mutations ranged from 0.9 to 42.3%. The presence of a homozygous mutation was less frequent and only ranged from 0 to 7.2%. Among the eight mutation sites, the mutation of Met1736Val in exon 16 is more common than other mutation sites among people from Keelung City (up to 42.3%). Other hot mutation sites located on exon 13 include Lys830Arg, His837Arg, and Lys897Glu (24.3%). Less frequent mutation sites are Asp68His in exon 3 (0.9%), Met385Thr in exon 8 (1.8%), and Asp2194Gly in exon 25 (3.6%). Thus, we need more information on the prevalence of FV deficiency and polymorphism based on data available from other databases. To better understand the role of promoters, we performed an *F5* promoter gene analysis, the results of which have been provided in **Supplementary Table 1**. Seven promoter mutations were identified. The promoter bases that changed at −1,559 (–g), −1,506 (t → c), −1,487 (t → c), −790 (–c), and −95 (t → c) were considered to be polymorphic, given their high frequency in the Keelung non-FV-deficiency population. The frequencies of the other two mutations, −319 (a → c) and −281 (g → a), were 1 and 66.7%, respectively.

The SNVs of FV in the healthy Taiwanese population have not been analyzed previously. According to the eight mutation sites in the five exons, the aim was to determine the SNVs of the non-FV-deficient population to further elucidate the existence of mutations we found in the normal population. The results are shown in **Table 3**. By analyzing the sequence at the *F5* locus, we found 55 SNVs. The frequency of the SNVs can be found ranging from 1 to 66%. None of the SNVs were observed in the patient. Of the SNVs, 66% were non-synonymous amino acid substitutions. Some non-synonymous SNVs (Arg513Lys/A2, Pro1404Ser/B, Leu1397Phe/B, Lys925Glu/B, His865Arg/B, Lys858Arg/B, and Met1764Val/A3) were highly conserved in the Taiwanese population.

Location and Influence of Met1736 on the FV Protein

The structural analysis of FVa in Protein Data Bank code, 7KXY, as shown in **Figure 2A**, suggests that the solvent-front Met1736 (on A3 domain) plays a dual role in (i) anchoring its nearby Thr1984 and Arg1985 (on C1 domain) and (ii) positioning the adjacent Asn1733 into the core of the A3 domain to secure a hydrogen bond network linking Asn1733, Arg1621, and Tyr1554. In a molecular modeling-based prediction by Schreuder et al., FVa's A3 and C1 domains are associated with FXa and prothrombin (22). In **Figure 2B**, we also highlight A3's

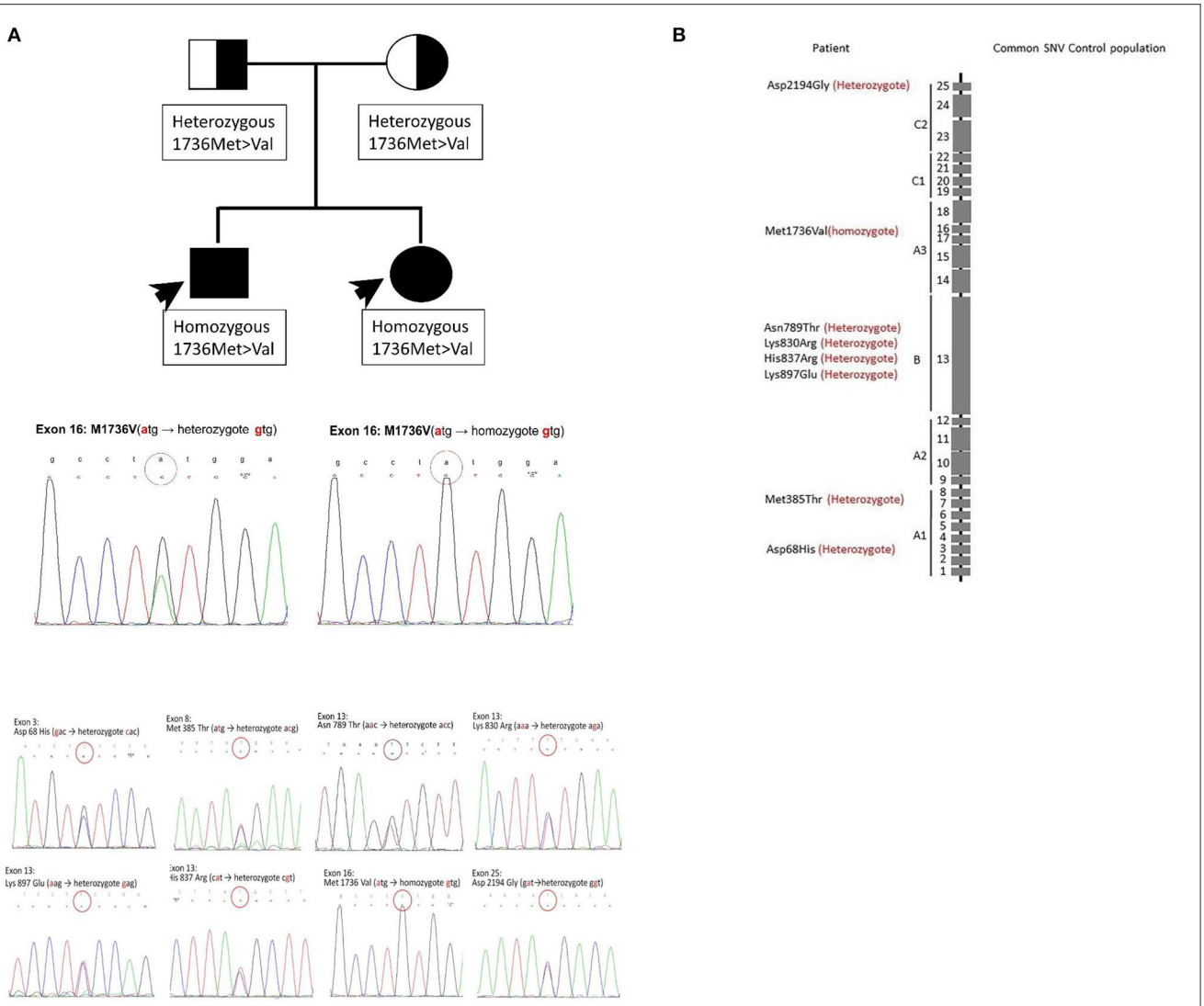

FIGURE 1 | Mutation results of the patient and his family. In this family, two members had a coagulopathy disorder. **(A)** The pedigree chart. The FV deficiency was only noted in their children. The results of the *F5* gene analysis for their children are the same and shown at the bottom. Crucial mutations in this family. Homozygous Met1736Val and other seven heterozygous mutations coexisted. **(B)** Those mutation sites are demonstrated. The graph shows the absolute location of the mutation sites on the FV gene. The location of eight mutation sites on the chromosome. The mutation sites are demonstrated sequentially.

residues interacting with FXa and prothrombin (encompassed by yellow and orange surface) and C1's residues interacting with prothrombin (encompassed by green surface and covering Thr1984). Notably, that part of the A3–FXa interface is made of the Asn1547–Tyr1555 segment (highlighted in orange surface), where most residues face outward to reach FXa. Rather than facing out, the aromatic ring moiety of Tyr1554 is embedded in the A3 domain core, and the hydroxy group forms a hydrogen bond with Arg1621, structurally stabilizing the Asn1547–Tyr1555 segment. In addition, T1984 is located on the FVa C3 domain and in the FVa–prothrombin interface. The interdomain interactions among Thr1984, Thr1985, and Met1736 shall help orient Thr1984.

To understand the structural impact due to the Met1726Val mutation, Gaussian accelerated molecular dynamics (GaMD)-enhanced sampling simulations were applied to the FVa structure carrying Val1736. The aforementioned wild-type FVa structure was used to create the Met1726Val variant by manually modifying Met1726 into a valine residue. With a previously used protocol (23), we collected a 100-ns long trajectory, as summarized in **Figure 2C**. Then Val1726 is detached from Thr1984 and Arg1985, resulting in a rearrangement of Thr1984 and Asn1986. The Arg1621–Tyr1554 linkage is lost and the Asn1547–Asn1555 segment moves to a new position. To summarize, the mutated Val1726 reorients the structural features essential for FVa–FXa–prothrombin complex formation.

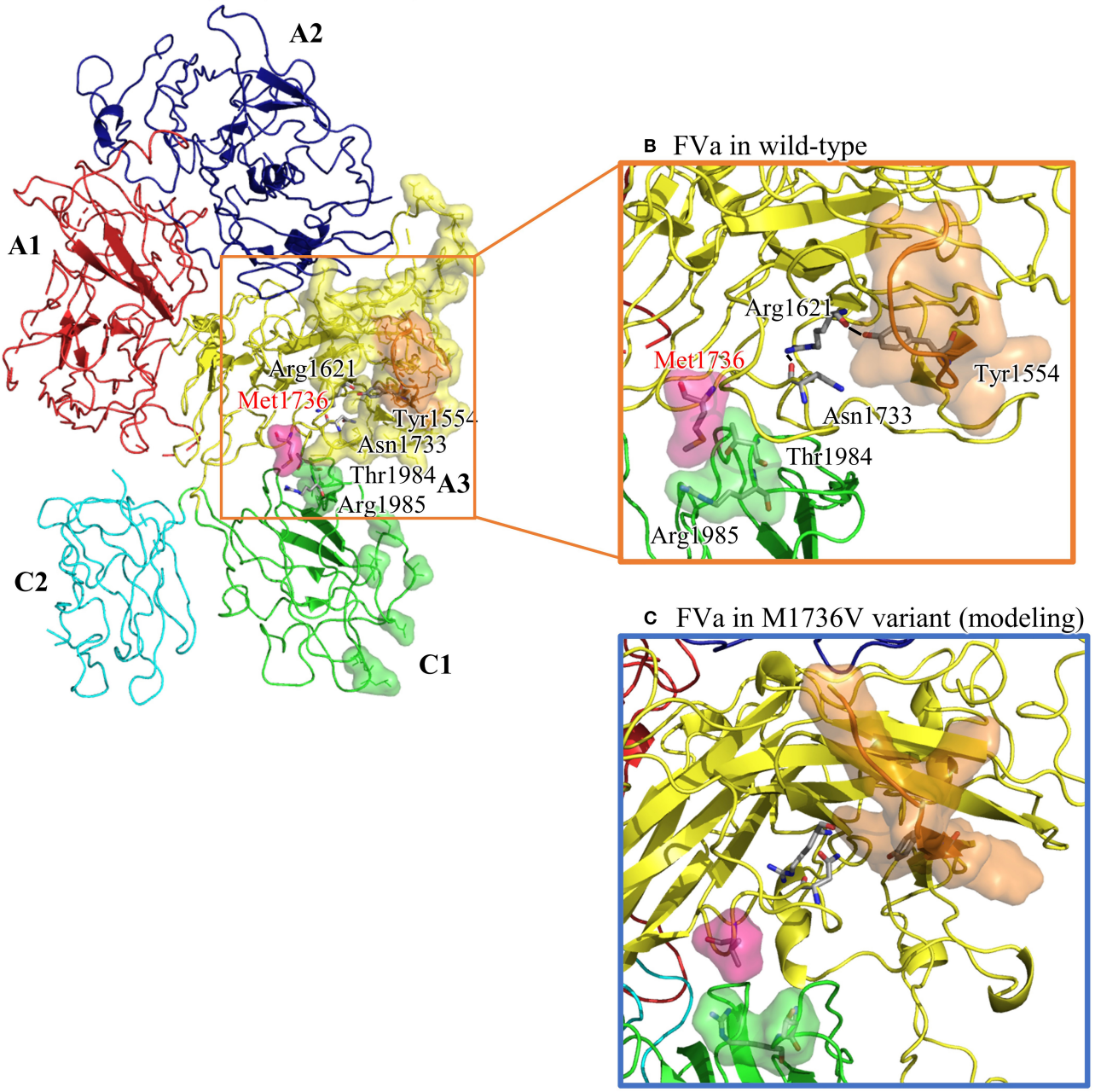

FIGURE 2 | The structural analysis of FVa in wild type (in PDB: 7KXY) and in Met1736Val variant obtained by molecular modeling using GaMD. **(A)** The overall FVa structure is composed of A1 (in red), A2 (in navy blue), A3 (in yellow), C1 (in green), and C2 (in turquoise) domains. A3 residues interacting with FXa and prothrombin are encompassed by yellow surface, whereas C1 residues interacting with prothrombin are encompassed by green surface. The Asn1547–Asn1555 (N1547–Y1555) segment in A3 domain is highlighted in orange surface. **(B)** A close-up view of the wild type showing Met136 embedded by Thr1984 and Arg1985, and a well-maintained hydrogen bond network linking Asn1733, Arg1621, and Tyr1554. **(C)** A close-up view of the Met1736Val variant. Val1736 averts from Thr1984 and Arg1985, and Tyr1554 is no longer restrained by Arg1621, which possibly causes reorientation of the Asn1547– Tyr1555 segment.

DISCUSSION

A Taiwanese family with FV deficiency was included, and a novel homozygous mutation of Met1736Val in exon16 was found. Although symptom severity does not correlate with the FV level (24, 25), massive postpartum hemorrhage unexpectedly occurred in our female patient. Prophylactic plasma transfusion should be prepared before invasive procedures (26).

Due to the coagulation *F5* gene (1q23) and its low prevalence, only a limited number of mutations have been documented.

Almost all mutations identified to date are unique and specific to each family. More than 200 genetic defects spanning the entire *F5* gene have been described. Most *F5* mutations are unique, occurring in only one patient or a few patients and family members (27). Our mutation analysis revealed multiple conserved mutation sites in the family included in our study. This study is the first to report the clinical impact of mutations in exon 16 (Met1736Val) and many mutations found in exon 13.

Initially, eight mutation sites in the patient were detected: exon 3 (Asp68His), exon 8 (Met385Thr), exon 13 (Asn789Thr), exon 13 (Lys830Arg), exon 13 (His837Arg), exon 13 (Lys897Glu), exon 16 (Met1736Val), and exon 25 (Asp2194Gly) (**Table 1**). Only exon 16 expressed the homozygote genotype. Other gene mutations were heterozygous. **Figure 1B** shows the pattern of each gene mutation sequentially. Meanwhile, the International Society on Thrombosis and Haemostasis website was searched for the genotypes of our patient with FV mutation through exon 3 (Asp68His), as this was the only well-reported mutation site (17, 28, 29) and other mutation sites were only newly discovered. The previous studies have helped us better understand FV deficiencies by documenting associated mechanisms (17, 30). For example, exon 3 (Asp68His) had been discovered, but the other seven sites have not been reported previously. Also, exon 25 (Asp2194 Gly) in recombinant FV molecules can lower the FV level (30–32). Our initial results suggest that exon 3 (Asp68His) or exon 25 (Asp2194 Gly) contribute to FV deficiency in this patient.

By comparing our patient with FV deficiencies and his family, only exon 16 (Met1736Val) was homozygous. His sister exhibited the same gene expression. Therefore, exon 16 (Met1736Val) mutation needs further evaluation to understand its contribution to FV deficiency. The previous studies regarding the genotypes of patients with FV deficiency were reviewed (16). It was reported that moderately decreased secretion rates were detected for the p.Met385Thr and p.His1299Arg FV proteins because of the quantitative defect of the p.Asp2194Gly substitution caused by the four missense variants constituting the haplotype (i.e., p.Met385Thr, p.His1299Arg, p.Met1736Val, and p.Asp2194Gly) (30). However, to our knowledge, no studies have reported about the FV deficiency related to the isolated homozygous Met1736Val; therefore, further investigation is needed.

In 2008, Liu et al. *first* identified the Asp68His mutation in Taiwan in two unrelated FV-deficient patients with compound heterozygous patterns (17). Furthermore, Cao et al. reported a homozygous pattern in a Chinese patient (28) and Huang et al. discussed a patient with a heterozygous mutation (29). Using *in vitro* recombinant mutant FV protein expression, Liu et al. demonstrated that Asp68His causes impaired secretion and ineffective FV protein translocation (17). Previously, the Asp68His mutation was reported only in a Chinese patient with FV deficiency. It is worth noting that including the two cases reported in this study (one patient from Chang Gung Memorial Hospital, Linko branch; data not shown here), four patients with heterozygous Asp68His mutation have been reported in different families. Further studies are needed to assess how the Asp68His missense mutation influences FV deficiency in Taiwanese individuals.

There remain four genotype patterns in this family, which could not be found in the genotypes of patients with FV deficiency. However, one kind of genotype, including three missense mutations (Met385Thr, Met1736Val, and Asp2194Gly), is associated with a low FV activity level (30, 31, 33, 34). Based on the results identified in the R2 haplotype allele, a low synthesis rate and impaired secretion were observed when these mutations appeared (30). Meanwhile, the Asp2194Gly mutation appears to be the major determinant of gene expression. The FV activity levels of our patients were similar to those of patients with the Asp2194Gly mutation, indicating that the phenotype influences FV activity. Other gene mutation types could be considered polymorphisms (30).

Still, many missense mutations correspond with FV deficiency. Patients with a homozygous mutation pattern have marked FV deficiency. However, the same presentation or gene mutation was not seen in our study, indicating the need to build a normal population genotype of FV in the Taiwanese population to understand gene function better. After analyzing the SNV of the normal FV population, our results indicated the uniqueness of the *F5* mutation sites that were obtained. The patient's parents also showed important heterozygous mutations in exon 3 and exon 8 but normal blood results. The difference found between the patient and his parents was that the patient's mutation was entirely inherited from his parents' mutations, indicating that the combination of mutations triggered coagulation failure. In the Keelung non-FV-deficient population, the polymorphism of Met1736Val was dominant (42.3%) but was not found in other databases. Based on a previous study, the Met1736Val mutation is associated with APC resistance, which is associated with the risk of a thromboembolic event (35). Thus, for a population with the *F5* Met1736Val mutation, such as the Keelung population, a critical issue is the prevalence rate of the risk of thromboembolism.

The prevalence of FV disease is known to be widely distinct among different populations. Also, *F5* SNVs in the Taiwanese population have not been analyzed previously. By analyzing the sequence at the *F5* locus, we found 55 SNVs from the Whole–Genome Research Core Laboratory of Human Diseases, Chang Gung Memorial Hospital, as shown in **Table 3**. Some non-synonymous mutations are highly conserved in the Taiwanese population, such as Arg513Lys/A2, Pro1404Ser/B, Leu1397Phe/B, Lys925Glu/B, His865Arg/B, Lys858Arg/B, and Met1764Val/A3 (R513K/A2, P1404S/B, L1397F/B, K925E/B, H865R/B, K858R/B, and M1764V/A3). Some SNVs have been recorded in the SNV database, such as rs118203905 (R334G, Arg334Gly/A1) and rs6025 (Q534R, Gln534Arg/A2), as genetic variants of the *F5* gene in Hong Kong (36). Through these two datasets, the distribution of *F5* genetic mutations is different and leads to the variable incidence of FV deficiency or FV Leiden disease.

The pathogenesis of factor V deficiency resulting from the homozygous Met1736Val mutation remains unclear. Based on the structure of the active FV obtained from the Protein Data Bank, the Met1736Val mutation site is not located in the interaction site. Moreover, the Met1736Val mutation does not influence protein synthesis (30). Further functional experiments

to understand the impact of homozygous Met1736Val mutation on *F5* gene are needed. Some hypotheses are proposed to explain this result. Based on the role of FV in thrombosis, not only the homozygous Met1736Val mutation but also the seven coexisting heterozygous mutations could be caused by an abnormal interaction with FXa. First, mutated FVa cannot bind to FXa, or the weak interaction of FVa and FXa complex cannot further attach to prothrombin. However, there are no reports of identical residues for FXa binding. The current evidence shows that residues 493–506 and 311–325 in the A2 domain of FVa are important for FXa binding (37). The carboxyl-terminal Asp683–Arg709 domain of the heavy chain is essential (38) for the interaction of FVa with factor Xa and prothrombin. Therefore, in the future, functional studies can be conducted to determine whether Met1736 is crucial for either FXa or FXa and prothrombin interactions.

Finally, two cases of FV deficiency were reported, and the *F5* gene was analyzed. Multiple sites of mutations were demonstrated in the *F5* gene in one family. The mutation Asp68His was first identified as a compound heterozygous mutation in FV-deficient patients, and the homozygous pattern was previously reported in a Chinese patient (15). The patient's father carried one copy of the mutation of Asp68His, but his FV activity level was 58% (normal range: 50–150%). Other mutations contributed by the patient's mother might have led to the coagulation defect. In our study, the effect of homozygous Met1736Val mutation was associated with FV deficiency in the bleeding event rather than in the thromboembolic event mentioned before. The incidence of homozygous Met1736Val mutation is also higher in Keelung city compared with other Taiwan areas. Thus, a retrospective study is warranted to understand the influence of this mutation on the risk of thromboembolism or bleeding.

CONCLUSIONS

New *F5* gene mutations, including homozygous Met1736Val and seven heterozygous mutations, were discovered in this study and are associated with moderate FV deficiency. The Val1736 mutation changes factor 5 protein structure and leads to coagulation disorders. Screening of the homozygote Met1736Val and the co-inheritance of the Asp68His *F5* gene is advised for patients with FV deficiency.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the zenodo repository, accession number: 10.5281/zenodo.6477462.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Chang Gung Memorial Hospital IRB (Nos. 200903793B0, 201102005A3D001, 201102005A3C102, and 201102005A3C103). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-SC analyzed the data, generated the figures/tables, and wrote the manuscript. Y-CL provided the FV-deficient cases. C-NY and Y-JC performed the protein modeling and discussed the impact of gene mutation. J-SH and K-YY helped with the study design. C-YH helped develop possible pathogenesis and edited the manuscript. K-YY helped with the study design and article writing and was a significant contributor to this study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.870269/full#supplementary-material

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Past, present and future in low-risk myelodysplastic syndrome

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Myelodysplastic syndromes (MDS) is a heterogeneous group of disorders characterized by increased risk of acute myeloid leukemia transformation and cytopenia. The prognosis of MDS patients can be evaluated with various scoring systems, the most commonly used are IPSS (International Prognostic Scoring System), revised-IPSS, and WPSS (WHO classification-based prognostic scoring system). MDS treatment is decided according to the risk classification. The goal of treatment in low-risk MDS is to improve cytopenia, reduce transfusion needs, improve quality of life, prolong overall survival, and maybe reduce the risk of progression to leukemia. In the near future, combining both genomics-based, *ex vivo* functional based and molecular stratification analysis will lead the way to a personalized and targeted approach.

KEYWORDS

myelodysplastic syndrome, low risk, treatment, anemia, thrombocytopenia

Introduction

Myelodysplastic Syndrome (MDS) can be defined as a common name given to a heterogeneous group of diseases in which ineffective hematopoiesis and cytopenia are predominate. There are specific characteristic changes in blood and bone marrow, mainly dysplasia (dyserythropoiesis, dysgranulopoiesis and monocytosis, dysmegakaryopoiesis) in all three series of bone marrow.

Although it can be observed in almost any age group, MDS, which is known to affect mostly the elderly, poses an important problem for hematologists especially in terms of diagnosis and ability to determine and administer an appropriate treatment in limited time (1). The median age at diagnosis is 71, and the annual incidence of disease is 0.1/100,000 for the population under the age of 40, while this rate is 30.2/100,000 for the 70–79 age group and 59.8/100,000 for those over 80 years old, according to the data of the United States (US) National Cancer Institute (NCI) (2, 3).

Cytopenia, bone marrow dysplasia, and some characteristic chromosomal anomalies define this disease according to the World Health Organization (WHO); however, understanding the pathogenesis, classification, and prognosis of the disease has actually become much easier as a result of the integration of next-generation DNA sequencing (NGS) technique into daily practice and its integration with morphology, cytogenetic and molecular genetic techniques (2).

The highly heterogeneous nature of MDS complicates the treatment of the disease and requires individualization as well. The only "curative" approach among many treatment options actually comes with allogeneic hematopoietic stem cell transplantation (AHSCT), but this form of treatment can unfortunately be applied to a limited number of "fit" patients. For the majority of patients, the preferred treatment methods are generally "non-intensive" options due to age, comorbidities, etc. of these patients, and the ideal choice is to use risk-based approaches/treatments. Almost all of a wide range of treatment options ranging from growth factor to lenalidomide and hypomethylating drugs are the preferred approaches to correct cytopenia, improve quality of life, and if possible, prevent disease progression, rather than radical treatment of the disease.

In this review, the past, present and possible future view of low-risk MDS management will be discussed including general definition, classification, clinical presentation, risk stratification, prognostic evaluation.

Definition, classification, pathogenesis, clinical presentation, risk stratification and prognostic assessment of myelodysplastic syndrome

Definition

Myelodysplastic syndrome is a wide spectrum of heterogeneous diseases in which different sizes of cytopenia and morphological dysplasia that have the risk of developing into acute myeloblastic leukemia (AML) (2). It is clear that the most important determinant for MDS is dysplasia, which can be detected in early and mature cells of the bone marrow, rather than the presence of cytopenia.

Dyserythropoiesis is identified by various anomalies in the nucleus and cytoplasm of erythroid cells. In the nucleus, budding, bridging, karyorexia, presence of multiple nuclei and megaloblastoid changes are present; cytoplasmic ones are classified as ring sideroblast, vacuolization and Periodic acid–Schiff (PAS) positivity (2). Dysgranulopoiesis, on the other hand, is characterized by very small or abnormally large myeloid lineage cells, nuclear hyposegmentation (pseudo-Pelger-Huet), nuclear hypersegmentation, reduction or absence in granulation, the presence of Pseudo-Chédiak-Higashi granules, Döhle bodies, Auer rods, and Barr bodies (2). Dysmegakaryopesis is classified as the presence of micromegakaryocytes, nuclear hypolobation, and multiple nuclei (2).

Classification

It was first named as preleukemia in 1953, and as a result of various definitions such as chronic erythremic myelosis, hypoplastic acute myeloid leukemia, and dysmyelopoietic syndrome, its first morphological classification (FAB, French–American–British) was made in 1982 using the name MDS (2, 4). WHO updated this classification in 2001 and 2008, and finally, in 2016, made a morphological and cytogenetic-based classification and defined 6 disease types in general, including subgroups (5). It should be emphasized, however, that the issue of exactly where MDS and AML diverge continues to be debated. The only difference between the two diseases is not only the number of blasts, but also the clinical progression rate, as well as the biological and morphological characteristics of the diseases. Especially, the US National Comprehensive Cancer Network (NCCN) panel team and also WHO state that a disease with a blast rate of between 20 and 29% and a stable clinical course for at least 2 months can be defined as "high-risk MDS" (3). However, in this situation, it would be more accurate to define patients with FLT3 or NPM1 mutations as AML rather than MDS (2, 6).

Pathogenesis

Myelodysplastic syndrome is known to be a hematopoietic stem cell disease that develops as a result of the interaction of bone marrow microenvironment and immune system with various genetic and epigenetic factors for many years (2). In MDS, it was reported that structural genetic defects, mostly caused by various "unbalanced changes" due to chromosomal losses and excesses, such as deletion (del) 5q, monosomy 7, trisomy 8, and del 20q and were identified in almost more than 50% of cases (7). With the adaptation of the NGS technique to clinical practice, recurrent mutations of more than 50 genes involved in DNA methylation, chromatin modification, RNA *"splicing"*, *"cohesion"* formation, transcription control, and DNA repair and signaling processes have been detected (2, 8). It has been suggested that there are various immunological imbalances, especially in the T lymphocyte series; for example, cytotoxic T cells increase in low-risk MDS and regulatory T cells dominate the presentation together with the immune "escape" mechanism in the high-risk subgroup (2, 9). Its shown that low risk MDS patients also have chronic inflammation (10).

Another MDS developmental process for which the underlying mechanism is largely unknown is "therapy related" disease, which is cytotoxic drug or radiotherapy related. The remarkable feature here is that the disease develops at a higher rate in individuals carrying a CHIP (clonal hematopoiesis of indeterminate prognosis) clone (11).

Clinical presentation

Long-standing macrocytic anemia, mild thrombocytopenia, and neutropenia may be identified during the development of MDS before the disease clinically develops. In fact, the diagnosis can be made by identifying cytopenia in routine blood tests by chance even when the patient is not more symptomatic and at relatively earlier times.

Complaints such as weakness, fatigue, decrease in effort capacity, dizziness, and cognitive dysfunction, which are triggered by tissue hypoxia related to anemia and caused by the fact that almost the whole organism, mainly the musculoskeletal system, cardiovascular system and central nervous system are affected, are remarkable. Although mild at first, skin/mucosal bleeding caused by overt/deep thrombocytopenia is not surprising in cases in whom the diagnosis is delayed. Thrombocytopenia can mistakenly be diagnosed as immune thrombocytopenia if dysplastic changes are ignored and not recognized. Neutropenia accompanied by functional disorders may be the cause of life-threatening bacterial and fungal infections. There is a substantial number of MDS cases diagnosed with fever, cough, dysuria, and even septic shock.

Risk stratification and prognostic assessment

In the historical process, many approaches have been developed that try to determine the prognostic classification through defining a MDS patient and consider various criteria such as clinical features, bone marrow blast rate, cytopenia, age, lactate dehydrogenase level, and cytogenetic features (2). The first of these was the International Prognostic Scoring System for MDS-IPSS, which divided patients into four subgroups in 1997 according to their cytopenia, bone marrow blast rate and cytogenetic features, and their median survival of 0.4–5.7 years (12). In the last 15 years, the need for a new classification has arisen as a result of the addition of ferritin, beta 2 microglobulin, bone marrow fibrosis, comorbidity, and performance status of patients, morphological re-classification of MDS, and finally the new cytogenetic features that can be detected with the developing technology (2). With the evaluation of 7,012 patients belonging to the database of the MDS study group (International Working Group for Prognosis in MDS-IWG-PM), which was formed with international multicentre participation, a more detailed scoring system - revised (R) IPSS- was developed which defines 5 prognostic categories with five different cytogenetic features, and classifies the depth of cytopenia and bone marrow blast ratio in more detail (13) (**Table 1**). Moreover, a WHO classification-based prognostic scoring system (WPSS) was developed with the participation of two centers from Italy and Germany in 2007, and five different subgroups were defined with a median survival of 12–103 months (14) (**Table 2**). Although anemia is a poor prognostic subgroup in this classification, the depth of anemia, in other words Hb level, was also included in the prognostic classification in a recent analysis as its depth and its clinical reflection were not sufficiently correlated (15). IWG-PM reported in its analysis comparing WPSS with R-IPSS that WPSS was also a very effective scoring method in the prognostic classification of untreated MDS cases and AML transformation (16).

Although there are many different approaches today, it is clear that the R-IPSS risk classification system is generally used in making treatment decisions by considering the age and performance status of a patient (2, 3).

The presence of various somatic mutations which are not in these systems, but have been identified with NGS in recent years, such as TP53, ASXL1, EZH2, ETV6, and RUNX1 that lead to poor clinical course, and SF3B1 that leads to a good clinical outcome, are tried to be integrated into scoring systems by some centers (17–19).

Treatment in low-risk myelodysplastic syndrome

International prognostic scoring systems that divide MDS into two large groups as low or high risk have actually revealed two disease subtypes with completely different treatment goals (20). While treatment policies in low-risk patients focus mostly on the correction of cytopenia which is reducing blood product support, especially erythrocyte suspension support, improving the quality of life, and maintaining it, if possible; those in the high-risk subgroup are correction of cytopenia, as well as delaying leukemic progression, and if possible, ensuring the survival of patients (**Table 3**) (21).

There is no conflict regarding the inclusion to low-risk MDS of patients who belong to "low" and "intermediate-1" risk groups according to the IPSS, and "very low" and "low" risk groups according to the R-IPSS. However, there is a conflict as to whether patients in "intermediate" risk group in R-IPSS have low or high risk. While some researchers place those in the "intermediate" risk group directly into the high-risk MDS group, others consider those in the "intermediate" risk group above 3.5 points only as high-risk MDS (20, 21). Nevertheless, it is obvious that the first things to consider when making a decision are the worsening of disease or side effects might be caused with the treatment.

Watch and wait/observation

In fact, there is no need to move beyond "supportive" therapy due to the presence of mild and asymptomatic

TABLE 1 R-IPSS scoring system (13).

Prognostic score value	0	0.5	1	1.5	2	3	4
Prognostic category							
Cytogenetics	Very good		Good		Intermediate	Poor	Very poor
BM blasts, %	≤2		>2 to <5		5–10	>10	
Hgb, g/dl	≥10		8 to <10	<8			
Platelets, $\times 10^9$/L	≥100	50 to <100	<50				
ANC, $\times 10^9$/L	≥0.8	<0.8					

ANC, absolute neutrophil count; BM, bone marrow; Hb, hemoglobin.
Cytogenetics: Very good: −Y, del(11q); Good: Normal, del(5q), del(12p), del(20q), del(5q) + 1 additional abnormality; Intermediate: del(7q), + 8, + 19, i(17q), other abnormalities not in other groups; Poor: −7, inv(3)/t(3q), −7/del(7q) + 1 additional abnormality, complex (three abnormalities); Very Poor: Complex (>3 abnormalities).

TABLE 2 WHO classification-based prognostic scoring system (WPSS) scoring system (14).

Score				
Parameter	**0**	**1**	**2**	**3**
WHO category	RA, RARS, 5q-	RCMD, RCMD-RS	RAEB-1	RAEB-2
Karyotype	Good[a]	Intermediate[b]	Poor[c]	–
Transfusion	Yes	Regular	–	–
Score		**Risk subgroup**	**Survival, Italian cohort (m)[d]**	**Survival, German cohort (m)[d]**
0		Very low	103	141
1		Low	72	66
2		Intermediate	40	48
3–4		High	21	26
5–6		Very high	12	9

[a]Good: normal, −Y, del(5q), del(20q).
[b]Intermediate: other abnormalities not seen in "good" or "poor".
[c]Poor: complex (≥3 abnormalities) or chromosome 7 anormalies.
[d]Median survival.

cytopenia in a substantial number of low-risk MDS patients. This approach can be considered applicable to all low- or high-risk MDS patients who do not have a long-life expectancy due to age and comorbidities. Patients who are fit, asymptomatic, without blast increase, and who do not have a high-risk cytogenetic/molecular profile do not require any special treatment other than regular controls (21, 22). Results from a recent real-world cohort study (n = 125) indicate that over a third of patients with low-MDS have been managed using watchful waiting only, with no systemic treatment or transfusions received (23). However, it is recommended that asymptomatic low-risk MDS patients should be followed up more closely if they have various mutations that are not currently included in the risk classification and show a genotypically high-risk prognostic profile, such as ASXL1 mutation (21). The most important markers in these patients are rapid worsening cytopenia, an increase in the number of blasts in the peripheral blood or bone marrow, and the change of findings in cytogenetic/molecular studies (21).

Treatment options in symptomatic patients

Treatment of anemia

Current treatment approach in low-risk MDS patients focuses on combating cytopenia, especially anemia, and the poor results of transfusion load.

Treatment with erythropoiesis stimulating agents

Symptoms reflecting anemia-related tissue hypoxia such as weakness, fatigue, and decreased exercise capacity are the most frequently reported complaints in MDS patients. These patients also need regular erythrocyte suspension (ES) support. Regular and frequent ES support, on the other hand, means a very problematic complication such as transfusional hemosiderosis, as well as a financial load and the need for social support.

The use of "erythropoiesis-stimulating agents" such as recombinant erythropoietin (EPO) or darbepoetin (DAR) as

TABLE 3 Treatment goals in low- and high-risk myelodysplastic syndromes (MDS) patients (20).

Order of priority	MDS risk classification	
	Low-risk	High-risk
1	Management of cytopenia Fewer transfusions Less iron load	Delaying disease progression Prolonging survival Recovery
2	Sustainability of the administered treatment Improving and maintaining quality of life	Reducing the load of disease Management of cytopenia Fewer transfusions
3	Delaying disease progression Prolonging survival	Sustainability of the administered treatment
4	Recovery	Improving and maintaining quality of life

TABLE 4 New treatment strategies in low grade myelodysplastic syndromes (MDS).

Drug	Mechanism of action
Luspatercept and sotatercept	Activin receptor fusion proteins that act as ligand traps to neutralize negative regulators of late-stage erythropoiesis
Roxadustat	Inhibitor of the "hypoxia-inducible factor" "prolyl hydroxylase"
Imetelstat	Telomerase inhibitor in cells with short telomere length and hyper telomerase activity
Ivosidenib and Enasidenib	Specific inhibitors of IDH1 or IDH2 genes

a single drug has been the mainstay of treatment for many years in low-risk patients who are at the forefront of anemia and need frequent transfusion support. On the other hand, phase three randomized studies on both drugs were conducted more recently and the use of EPO-alpha was eventually approved (20, 21). In a multicenter, randomized phase 3 study from European countries, 147 low-risk MDS patients with hemoglobin (Hb) level of ≤10 g/dL, EPO level of ≤500 mU/mL, and low transfusion load were randomized to receive DAR-alpha or placebo at the rate of 2/1 (24). Following placebo or subcutaneous administration of 500 μg DAR-alpha every 3 weeks for 24 weeks, the frequency of transfusion was significantly higher in the placebo arm (59.2 vs 36.1% and $p = 0.008$), while the recovery of anemia was significantly higher in the DAR arm (14.7 vs 0% and $p = 0.016$). In another multicenter, double-blind, placebo-controlled phase 3 study from European countries, 130 low-risk (IPSS low and medium-1) MDS patients with similar characteristics to the other study were randomized to EPO-alpha or placebo arms at the rate of 2/1 (25). After subcutaneous administration of 450 IU/kg/w EPO-alpha or placebo for 24 weeks, the recovery of anemia was significantly higher in the EPO arm (31.8 vs. 4.4% and $p = 0.001$).

Some features in low-risk MDS patients have become important in predicting the response to "erythropoiesis stimulating agents." These features are low endogenous EPO level (<500, <100 IU/L) and having a total ES transfusion load of less than 4 units in 2 months (26, 27).

In European Union countries, endogenous EPO level should be <200 IU/L for reimbursement approval of EPO-alpha, whereas in our country this value is determined as <500 IU/L. In many low-risk MDS patients, the effect of "erythropoiesis stimulating agents" becomes apparent in about 3 weeks, while it is also stated that this effect can be sustained for a median of 15–18 months (21).

In patients whose expected response cannot be obtained with "erythropoiesis stimulating agents" and especially in the subgroup with ring sideroblasts, recovery can be achieved in approximately 20% of patients with subcutaneous application of granulocyte colony stimulating factors (G-CSF) of 1–2 μg/kg/w (28).

Lenalidomide

In del(5q) positive low-risk MDS patients with low transfusion load and symptomatic anemia, the first-line treatment of anemia is again "erythropoiesis stimulating agents" (26). However, it is noteworthy that most of these patients have high endogenous EPO levels, usually associated with low and short-term response rates and correlated with high clonality rate in myeloid precursor cells (23). Lenalidomide seems to be a good choice with a treatment success of up to 70% in the treatment of anemia, especially for patients with high EPO levels and a constant need for ES transfusion (29, 30). The most common side effects of the drug are diarrhea, rash, nausea, constipation, fever, itching, shortness of breath, recurrent arterial thrombotic events, and hematological side effects such as neutropenia and/or thrombocytopenia, and it is obvious that these complications should be considered very important considering the average age and fitness of MDS patients (20).

In an international multicenter, randomized, phase 3 study published in 2016, 239 low-risk and del(5q) negative patients were randomized 2/1 to lenalidomide or placebo arm (31). ES transfusion independence was clearly superior in the lenalidomide arm, while no significant deterioration in quality of life was detected in the drug arm. In another recent randomized study, 131 patients resistant to "erythropoiesis stimulating agents", who were del(5q) negative low-risk and ES transfusion-dependent, were randomized to either lenalidomide alone or lenalidomide + EPO arms (32). The use of lenalidomide in combination with "erythropoiesis stimulating agents" provided a significant superiority in anemia response compared to the arm in which it was given alone (39.4 vs 23.1% and $p = 0.044$). In a recent study, the combination of lenalidomide and erythropoiesis stimulating agents yields 38.9%

of major erythroid responses who relapsed or unresponsive to erythropoiesis stimulating agents (33).

On the other hand, patients with TP53 mutations, which constitute approximately 20% of all patients, did not have the expected benefit from lenalidomide with high leukemic transformation rates (21). In the LEMON5 study, the overall response rates and survival in patients with TP53 mutation who were followed up with lenalidomide monotherapy were significantly lower than those in patients without TP53 mutation (34). Moreover, TET2 and RUNX1 mutations are associated with poor outcome and lenalidomide unresponsiveness (21). Even if there is no disease progression or leukemic transformation in patients who do not respond adequately to lenalidomide in the presence of TP53 or other mutations, it is recommended to administer hypomethylating (HMI) agents or HSCT in the absence of an ongoing clinical trial (21).

Supportive therapy with iron chelation

Many MDS patients live dependent on regular ES transfusions as part of supportive therapy. In these patients, "non-transferrin bound iron" and labile plasma iron fraction increase, and ultimately and stored especially in the heart, liver and endocrine glands. In a phase 3 study comparing placebo and deferasirox, there was a significant risk reduction of 36.4% in event-free survival (event: worsening cardiac function, hospitalization with heart failure, liver dysfunction, liver cirrhosis, and AML transformation) in the iron chelation group (35).

Many international guidelines recommend iron chelation based on the ferritin level; the opinion is that this amount should be at least 1,000 ng/mL (21). It is known that the most commonly used chelator is deferasirox, and the patient compliance problem is also improved with the film-coated tablets released in recent years. Iron chelation is an application that must be included in the algorithm before HSCT, and it positively affects the transplant outcome.

Immunosuppressive drugs

Immune dysregulation is better understood in the etiopathogenesis of MDS and is considered to be responsible for ineffective hematopoiesis. In this context, it was reported that very satisfactory response rates (16–67%), including all three series, were obtained with antithymocyte globulin (ATG) and/or cyclosporine treatment (31). Some features become important in predicting patients who will benefit from immunosuppressive therapy. Presence of dysplasia which is the subtype previously classified as refractory anemia, absence of ring sideroblast subtype, especially hypoplastic/hypocellular bone marrow, having HLA DR15 typing, young age (<60 years), female gender, presence of trisomy 8, and relatively short duration of transfusion need are among these characteristics (21). Interestingly, in a retrospective study involving a large number of patients, in which the presence of SF3B1, a somatic mutation known to be associated with good clinical outcome in MDS, adversely affected the overall response, it was reported that a total response rate of up to 45% was obtained with the use of horse-derived ATG (32). A meta-analysis was unable to associate specific biomarkers predictive of response given the overall lack of prospective, randomized controlled studies for the use of immunosuppressive treatment in low risk MDS (33).

Hypomethylating agents

Hypomethylating agents (HMIs), used at standard or reduced doses, are included in the treatment of low-risk MDS. Although it is generally approved by the US Food and Drug Administration (FDA), it has a more limited use in European countries. The reason may be disappointing results obtained in the studies. In a phase 2 study of the Nordic group, which included patients with low-risk MDS who were resistant to the EPO + G-CSF combination or were not suitable for transfusion, a response of only 20% was achieved with azacitidine (AZA) at a dose of 75 mg/m^2/day administered for 5 days every 28 days. Moreover, this response was both short-term and more toxic than expected (36). Similarly, in prospective studies of the French group, in which they randomized 98 low-risk patients with a median age of 72 years to AZA vs AZA + EPO treatment arms, transfusion independence at the end of six courses was only achieved in 16.3% of patients (14.3% in the AZA-EPO arm) (37). In a study which 113 low-risk MDS patients were included, a total response rate of 70% was achieved with 3 days of 20 mg/m^2/day decitabine (DEC) every 28 days, while transfusion independence was achieved in 32% of patients (38). Although the oral formulation of AZA, which has been developed in recent years, promises ease of administration and longer lasting efficacy at lower doses, the international multicenter phase 3 study which compared CC-486 (oral AZA) with placebo and included low-risk MDS patients with ES transfusion dependent and thrombocytopenia was terminated earlier than expected due to toxicity (21, 39).

In 2020, the FDA and Canadian authorities approved DEC/cedazuridine (ASTX727 or DEC-C, oral decitabine) for the treatment of all subtypes of adult MDS and CMML in any stage of disease based on the 60% of overall response rate (40). In the Ascertain trial, the low grade MDS patients had 57% overall response rate, 48% became ES independent and 67% platelet transfusion independent (41).

New drugs developed for the treatment of anemia

"Luspatercept" and "sotatercept", also named as "erythropoiesis maturation agents", are specific activin receptor fusion proteins that act as ligand traps to neutralize negative regulators of late-stage erythropoiesis (2, 20, 21). In the PACE-MDS study which included 57 MDS patients with low transfusion load and in IPSS low and intermediate-1 risk groups, hematological recovery and transfusion independence

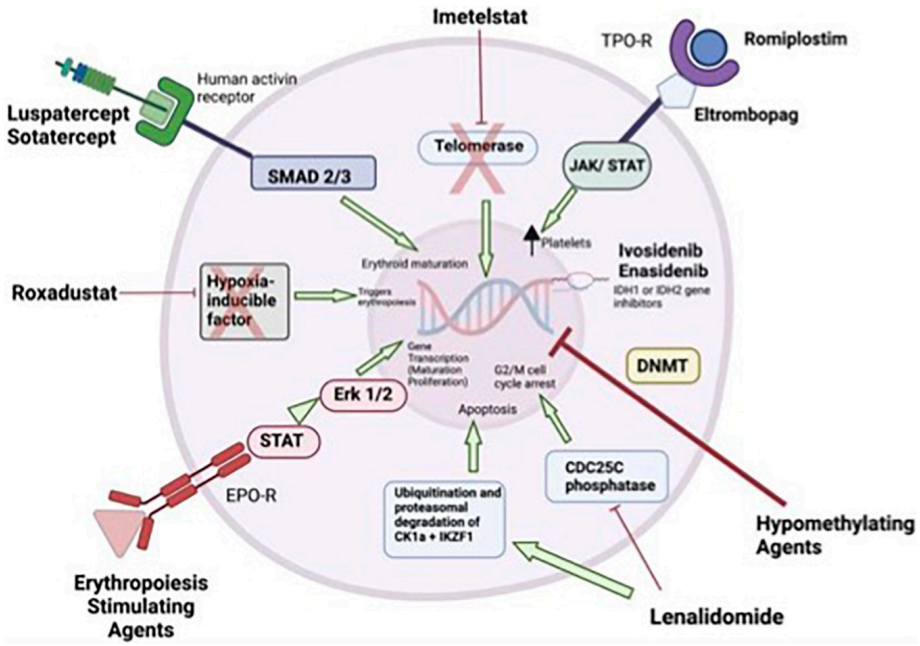

FIGURE 1
Mechanism of actions for treatments in low-risk myelodysplastic syndromes (MDS). Erythropoiesis stimulating agents stimulate gene transcription of maturation and proliferation of erythrocytes through JAK/STAT and Erk1/2. Lenalidomide inhibits the CDC25C phosphatase and by decrease in CK1α levels. Inhibition of the CDC25C phosphatase leads to a stoppage of proliferation by induction of an arrest in the cell cycle at the transition between G2 and M phase. Hypomethylating agents induce DNA hypomethylation. Ivosidenib, and enasidenib inhibits IDH1 or 1DH2 genes. Thrombopoietic receptor agonists activate signaling leads to increased platelet production. Imetelstat is a drug that help maintaining normal hematopoiesis by acting as a telomerase inhibitor in cells with short telomere length and hyper telomerase activity. "Luspatercept" and "sotatercept" specific activin receptor fusion proteins that act as ligand traps to neutralize negative regulators of late-stage erythropoiesis. Roxadustat is a small molecule that can be used orally and is an inhibitor of the "hypoxia-inducible factor" (20, 21, 61–64). EPO-R, erythropoietin receptor; JAK, janus kinase; STAT, signal transducer and activator of transcription; TPO-R, thrombopoietin receptor.

were reported as 63 and 38%, respectively, in the group using relatively higher dose (0.75–1.75 mg/kg) luspatercept (42). In an international multicenter, double-blind, placebo-controlled, randomized, phase 3 study (MEDALIST) published in January 2020, 229 MDS patients with ring sideroblasts who were in very low, low, and intermediate risk groups according to R-IPSS were randomized 2/1 to luspatercept and placebo arms (43). Transfusion independence (38 vs 13%) and hematological recovery (58 vs 17%) were significantly superior to placebo in the luspatercept arm, while AML transformation in both arms was not different. In April 2020, the FDA approved luspatercept for use in MDS patients with ring sideroblasts who are with very low, low, and intermediate risk and who require two units or more of ES for 8 weeks and have not benefited from "erythropoiesis stimulating agents".

Roxadustat, on the other hand, is a small molecule that can be used orally and is an inhibitor of the "hypoxia-inducible factor" "prolyl hydroxylase" (20). There are ongoing studies on the use of this drug, which stimulates endogenous EPO production, eliminates the negative effects of inflammation in endogenous EPO production, triggers erythropoiesis and Hb production, and regulates iron regulation through hepcidin metabolism, in anemia in MDS and chronic kidney disease (21). The data for the low burden transfusion dependent low risk MDS are promising which 38% of the patient achieved transfusion independency over 8 weeks in weeks 1–28 of treatment while 42% achieved this during 52 weeks of treatment (44). The results of a recent phase 3, randomized, double-blind, placebo-controlled study showed that transfusion independence was achieved in nine patients (37.5%) at 28 and 52 weeks; seven of the patients patients received 2.5 mg/kg dose (NCT03263091) (45).

Imetelstat is a drug that help maintaining normal hematopoiesis by acting as a telomerase inhibitor in cells with short telomere length and hyper telomerase activity (21). Short telomeres and high telomerase activity are associated with shorter overall survival in MDS (2, 20). In a study published at the annual meeting of the American Society of Hematology in 2017, transfusion independence was found in 34% and erythroid-hematological improvement in 63% of patients with imetelstat in MDS patients with low and intermediate-1 risk who were resistant/unresponsive to "erythropoiesis stimulating agents" (46). When administered intravenously every 4 weeks, transfusion independence was reported in 42%

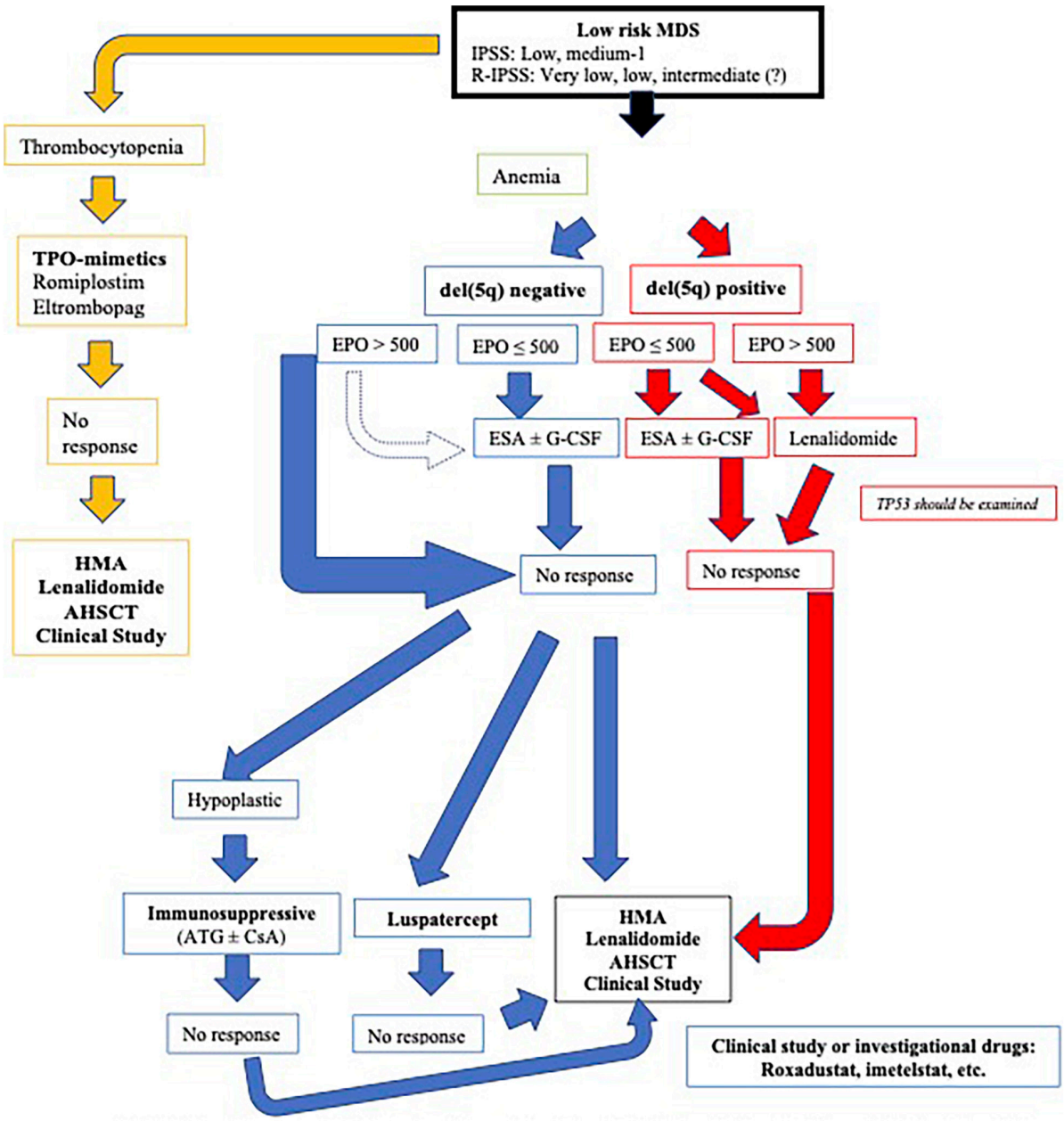

FIGURE 2
Current treatment approaches in low-risk myelodysplastic syndromes (MDS) (19, 20). MDS, myclodysplastic syndrome; IPSS, International Prognostic Scoring System; R-IPSS, Revised International Prognostic Scoring System; TPO, thrombopoietin; EPO, erythropoietin; ESA, erythropoiesis stimulating agents; G-CSF, granulocyte colony stimulating factor; HMA, hypomethylating agents; AHSCT, allogeneic hematopoietic stem cell transplantation; ATG, antithymocyte globulin; CsA, cyclosporine.

of patients and response duration may be over 1 year in 30% of patients up to 2.8 years (47). Furthermore, specific inhibitors of IDH1 or IDH2 genes, ivosidenib and enasidenib showed promising responses (50%) in patients that carry the somatic mutations (48, 49). KER-050, a modified activin receptor type IIA inhibitor, is designed to target transforming growth factor-β ligands, including activin A. In an open label phase II study in very low to intermediate risk MDS patients, overall erythroid response rate was 60% ($n = 6/10$). 33% ($n = 1/3$) non-transfused participants had a hemoglobin increase of ≥1.5 g/dL sustained ≥8 weeks. Increases in platelets were also observed (50). The new treatment approaches are given in **Table 4**.

Treatment of thrombocytopenia

In low-risk MDS with thrombocytopenia, platelet suspension transfusion and thrombopoietin (TPO) receptor

agonists, in addition to HMIs, constitute a remarkable treatment option (21). In a randomized study in which "romiplostim" was compared with placebo in low-risk MDS patients, it was found that the platelet counts increased, bleeding episodes and the need for thrombocyte suspensions decreased significantly in patients who received romiplostim compared to the placebo group (51). In the 5-year long-term analysis of the same study, transformation to AML and death rates were not different in the drug group than in the placebo group (52). In a prospective, multicenter EUROPE phase II trial, mutated SRSF2 occurred more often in responders of romiplostim compared with non-responders (41 vs 16%, $p = 0.018$) (53).

In a phase 2 study in which 90 low-risk thrombocytopenic MDS patients were randomized to "eltrombopag" and placebo at a rate of 2/1, decrease in thrombocyte response and bleeding episodes were found to be significantly superior in the eltrombopag arm (47 vs. 3%, $p = 0.0017$ and 14 vs 42%, $p = 0.0025$, respectively) (54). AML transformation was also reported to be same between the two groups. A similar response rate of 44% was observed in a second phase 2 dose escalation study. The predictors of response were the presence of a PNH clone, marrow hypocellularity, thrombocytopenia, and baseline elevated plasma TPO levels (55). The combination of eltrombopag and lenalidomide in low and intermediate risk MDS demonstrated good efficacy with ORR of 40.9%, response durability and an acceptable safety profile (56). The mechanisms of potential treatment alternatives for low grade MDS are summarized in **Figure 1**.

Allogeneic hematopoietic stem cell transplantation

It is clear that performing AHSCT earlier in the course of the disease will provide a more favorable outcome in the long term. On the other hand, it is necessary to avoid a treatment process that carries a substantial risk of death in low-risk patients who have a high chance of obtaining a response with standard first-line "soft" treatment options (57). General opinion is that IPSS low and moderate-1 risk subgroup patients do not have any indications for AHSCT at the time of diagnosis, except IPSS intermediate-1 risk subgroup with cytopenia and/or poor cytogenetic karyotype (58). In a multicenter biologic assignment trial, reduced intensity AHSCT showed advantage to hypomethylating therapy or best supportive care on 3-year OS (47.9 vs 26.6%, $P = 0.0001$) and 3-year leukemia-free survival (35.8 vs 20.6%, $P = 0.003$) in subjects 50–75 years of age with intermediate-2 or high risk MDS (59). In the retrospective analysis of the European Society for Blood and Marrow Transplantation (EBMT), which included 246 MDS patients with low and moderate-1 risk according to IPSS, a 3-year overall survival rate of 58% was achieved with AHSCT and the non-relapse mortality rate was reported to be 30% (60). Therefore, in patients with unresponsive disease to first-line therapies and having poor prognostic characteristics such as life-threatening infection, grade 2 or greater bone marrow fibrosis, severe thrombocytopenia, severe neutropenia, severe anemia, ES transfusion dependency, high-risk molecular anomalies, and treatment-related MDS, AHSCT should be considered an option as an individualized treatment approach (21, 57).

Conclusion and summary of recommendations for low-risk myelodysplastic syndrome treatment

In conclusion, for the majority of MDS patients, the therapeutic approach is based on IPSS (or IPSS-R/WPSS) stratification, with some non-curative options, except AHSCT. However, it should be known that the heterogeneity and complexity of low risk MDS requires a personalized management that, unfortunately, does not yet exist (20).

It should be kept in mind that there are a substantial number of asymptomatic patients who can only be kept under close follow-up with the option of "watchful waiting". It is known that "erythropoiesis stimulating agents" in low-risk MDS where anemia is at the forefront and lenalidomide in those with del (5q) positive are beneficial. The combination of lenalidomide + G-CSF may be a good alternative for patients who are del (5q) negative and are resistant to "erythropoiesis stimulating agents". Immunosuppressive treatment options should not be disregarded, especially in subtypes with low ring sideroblasts, high endogenous EPO levels and additionally HLA DR15 positivity. Although thrombocytopenia and neutropenia are encountered less frequently, they are two characteristics that are more difficult to treat (21). HMIs can be used at adjusted doses and with profit/loss calculations in low-risk MDS patients with both anemia and thrombocytopenia/neutropenia, those who do not respond to the first-line treatments that are specified, and those who have unfavorable somatic mutations. Although mortality rates are high, in patients who are resistant to first-line therapy, are transfusion-dependent, and have additionally high-risk molecular anomalies, the option of AHSCT should be considered without delay, by explaining the possible complications to the patient and family at the appropriate time and in the appropriate order. Recommendations are summarized in **Figure 2** (20, 21).

It seems impossible to consider a single gold standard treatment option that will be successful in a large proportion of patients because of underlying stem cell disease and the combination of many different mechanisms in its etiopathogenesis (2). However, with the analysis of the

results of ongoing *ex vivo* functional studies and genomic-based studies, it may be possible in the non-distant future to create more specific treatment options that will work in low risk MDS.

Author contributions

ST conceived and designed the review, collected the data, wrote the manuscript, and approved the submitted version.

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Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The place of allogeneic stem cell transplantation in aggressive B-cell non-Hodgkin lymphoma in the era of CAR-T-cell therapy

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Chimeric antigen receptor T (CAR-T) cells are a treatment option for patients with relapse/refractory (R/R) non-Hodgkin lymphoma (NHL), acute lymphoid leukemia and multiple myeloma. To date, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), follicular lymphoma (FL), and chronic lymphocytic leukemia (CLL) have been successfully treated with CAR-T cells directed against the CD19 antigen. However, when R/R disease persists after several treatment lines, patients with these diseases are often referred to transplantation centres to receive allogeneic stem cell transplantation (ALLO-SCT). ALLO-SCT and CAR-T cells share mechanism of actions, inducing immune effects of *T*-cells (and other cells after transplantation) against lymphoma cells, but they differ in several other characteristics. These differences justify unique positioning of each therapy within treatment algorithms. In this paper, we analyzed the results obtained after ALLO-SCT and CAR-T-cell therapy in patients with aggressive lymphomas (large B-cell lymphoma and MCL) to identify the ideal scenarios in which these 2 immunological therapies should be employed.

KEYWORDS

allogeneic stem cell transplantation, CAR-T cells therapy, non-Hodgkin lymphoma, refractory, toxicity

Introduction and background

Although progress has been made in recent years in the treatment and diagnosis of aggressive B-cell lymphomas, particularly diffuse large B-cell lymphoma (DLBCL), with the introduction of monoclonal anti-CD20 antibodies (i.e., rituximab) and consolidation therapy with high-dose chemotherapy in mantle cell lymphoma (MCL), many patients relapse or are considered refractory. The current research era has witnessed an increasing understanding of the molecular abnormalities present in lymphoma cells, and based

on this knowledge, more precise therapies have been developed. While these newly identified molecules have mostly been used in treatments for more advanced disease, the efficacy and safety of such treatments suggest that additional agents will be able to be employed in earlier phases of disease.

As such, researchers have long been aiming to develop strategies to manipulate the immune system to fight lymphoma cells, and the first example was allogeneic stem cell transplantation, in which was developed based on studies of leukemia. Several phenomena support the existence of the so-called "graft vs. tumor/lymphoma effect," such as the lower risk of relapse with ALLO-SCT vs. autologous transplantation, the lower risk of relapse in the presence of graft vs. host disease (GVHD), the higher risk of relapse in cases of mixed chimerism, the increase in efficacy when immunosuppression is withdrawn, and the utility of donor lymphocyte infusion (DLI). However, it is well known that the time of ALLO-SCT can induce severe side effects, leading to a concerning non-relapse mortality (NRM) incidence, which limits its use. From an immunology point of view, donor *T*-cells recognize recipient antigens on lymphoma cells *via* the conventional immunological synapse: HLA-Ag-TCR.

Recently, a new class of immune-active molecules has been developed in clinical trials. Bispecific antibody engagers (BiTEs) enable direct crosstalk of *T*-cells with lymphoma cells independent of the HLA system. Indeed, these antibodies link CD3 molecules on *T*-cells with antigens expressed on lymphoma cells. The first BiTE, used in R/R ALL, was blinatumomab, which is composed of two single-chain variable antibody fragments connected by a flexible linker and is able to link CD3 on *T*-cells with CD19 on leukaemic cells. This linkage enables *T*-cells to kill B cells by granzyme- and FAS/FAS-ligand-mediated mechanisms. Many other BiTEs have been developed, such as glofitamab, mosunetuzumab, odronextamab, epcoritamab, and plamotamab, which are characterized by improved pharmacokinetic proprieties, and additional BiTEs are being developed.

Genetically modified immune cells, both autologous and allogeneic, directed toward specific lymphoma and leukemia antigens are a promising new therapy. The most advanced product is genetically modified autologous T cells, which express a chimeric antigen receptor (CAR) recognizing the CD19 molecule on B cells, both tumoral and normal, and killing them without the need for HLA-mediated antigen presentation. Three CAR-T cells directed against CD19 have been approved in Europe: axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel), and lisocabtagene maraleucel (liso-cel).

These CAR-T cells are now used as therapies for R/R DLBCL, primary mediastinal lymphoma, MCL, CLL, and follicular lymphoma. Given its efficacy and safety, CAR-T-cell therapy is replacing ALLO-SCT in clinical practice. However, it should be noted that data from prospective studies are not fully mature.

In this paper, we will briefly present results obtained in several studies of ALLO-SCT and CAR-T cells in lymphoma patients and then present possible scenarios in R/R lymphomas considering the interplay between relevant immunotherapies.

Post-ALLO-SCT results in LBCL

Clinical results obtained in DLBCL after ALLO-SCT are reported in Tables 1, 2, and from these data, some general conclusions can be made.

The survival results vary substantially, ranging from 12 to 80% for progression-free survival (PFS) and 28 to 80% for overall survival (OS). Toxicity is also variable, as the NRM incidence ranges from 9 to 55%, the acute GVHD incidence ranges from 17 to 64%, and the chronic GVHD (overall) incidence ranges from 14 to 75%. This heterogeneity has several explanations. For example, some studies (1–3, 5, 10–12, 14) included subtypes other than DLBCL. In addition, most of the studies were retrospective or registry-based, which can lead to selection bias, and there were differences in transplant characteristics between the studies, such as donor characteristics and the intensity of conditioning regimens.

It is well known that immunotherapy is more active in indolent/follicular lymphoma (17) than in more aggressive subtypes such as DLBCL, MCL and T-cell lymphomas. Indeed, this was evident in the EBMT study (1) and in an Italian study (2), in which patients with indolent lymphoma achieved longer survival.

Some studies included patients with chemorefractory disease at the time of ALLO-SCT. This is important because the disease status before ALLO-SCT is consistently reported to be one of the most reproducible prognostic factors for survival (Table 3). Three studies are interesting in this regard because they analyzed only patients with chemorefractory disease (5, 10, 12). Two were retrospective analyses. The first was from a single centre and included 46 lymphoma patients with chemorefractory disease who received ALLO-SCT from 1988 to 2007. Only 16 DLBCL cases were included. As reported in Table 1, PFS and OS were better in patients with stable disease than in patients with progressive disease at the time of ALLO-SCT. The latter group of patients were more likely to relapse and/or die, with a substantial difference in survival (5). The second study, which included 226 DLBCL (and 207 grade III follicular lymphoma) cases, was a registry-based analysis. Most of the donors received a transplant from a MUD and had MRD, but 11% of patients received transplantation from a mismatched unrelated donor (mMUD), and 58% of patients received a myeloablative conditioning regimen. The outcomes are reported in Table 1. It is interesting to note that in a multivariate analysis, the NRM incidence was lower for grade III FL; in addition, with RIC, the PFS and OS were higher and the relapse rate was lower in grade III FL. More intensive conditioning regimens were associated with a reduced

TABLE 1 Clinical results after allo-SCT in B-cell NHL.

Authors	*N*	*N**	Median Age y	Median CT lines	Previous HDC	Disease status	Donor	CTX	OS	PFS	Relapse	NRM
Robinson et al. (1)	188	62	46	3 (1–5)	29%	CTS 73%	MRD 91% MUD 9%	RIC 90%	46%@2 y	12%@2 y	47%@2 y	36%@2 y
Corradini et al. (2)	170	61	51	3 (1–6)	49%	CTS 77%	MRD 100%	RIC 100%	69%@3 y	46%@3 y	31%@3 y	15%@3 y
Rezvani et al. (3)	68	16	54	6 (1–19)	44%	CTS 63%	MRD 55% MUD 45%	RIC 100%	45%@3 y	35%@3 y	41%@3 y	25%@3 y
Thomson et al. (4)	48	48	46	5 (2–7)	71%	CTS 83%	MRD 81% MUD 19%	RIC 100%	47%@4 y	48%@4 y	33%@4 y	32%@4 y
Hamadani et al. (5)	46	18	46	3 (3–8)	0	CTS 0%	MRD 88% MUD 15%	RIC 93%	38%@5 y	38%@5 y	50% PD 25% SD	43%@100 d PD 9%@100 d SD
Sirvent et al. (6)	68	68	48	2 (1–5)	79%	CTS 83%	MRD 82% MUD 18%	RIC 100%	49%@2 y	44%@2 y	41%@2 y	23%@2 y
Van Kampen et al. (7)	101	101	46	3 (2–6)	100%	CTS 74%	MRD 72% MUD 28%	RIC 64% MAC 36%	52%@3 y	43%@3 y	30%@3 y	28%@3 y
Rigacci et al. (8)	165	165	46	/	/	CTS 55%	MRD 65% MUD 35%	RIC 70%	39%@5 y	31%@5 y	67%@5 y	28%@1 y
Bacher et al. (9)	396	396	48–54	/	18–51%	RIC CTS 35% MAC CTS 40% NMA CTS 35%	/	RIC 36% MAC 41% NMA 22%	RIC 27%@3 y MAC 21% NMA 29%	RIC 23%@3 y MAC 19% NMA 27%	RIC 26%@3 y MAC 26% NMA 28%	RIC 42%@3 y MAC 55% NMA 34%
Hamadani et al. (10)[$]	533	533	46–53	3–4	15–38%	CTS 0%	MRD 48% MUD 24% mMUD 11%	RIC 42%	28%@3 y	23%@3 y	35%@3 y	42%@1 y
Bouabdallah et al. (11)[§][II]	31	14	57	3 (2–4)	96%	CTS 100%	MRD 66% MUD 34%	RIC 100%	80%@2 y	80%@2 y	7%@2 y	13%@2 y
Glass et al. (12)[§^]	84	61	48	4 (3–6)	52–55%	CTS 45%	MRD 27% MUD 40% mMUD 31%	MAC 100%	52%@1 y	45%@1 y	29%@1 y	10%@1 y
Fenske et al. (13)	503	503	52	4 (1–7)	100%	CTS 74%	MRD 50% MUD 23% mMUD 26%	RIC 75% MAC 25%	37%@3 y	31%@3 y	38%@3 y	30%@1 y

(Continued)

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TABLE 1 (Continued)

Authors	*N*	*N**	Median Age y	Median CT lines	Previous HDC	Disease status	Donor	CTX	OS	PFS	Relapse	NRM
Dodero et al. (14)§	121	35	52	/	61%	CTS 97%	MRD 55% MUD 28% mMUD 17%	RIC 100%	52%@3 y	40%@3 y	27%@3 y	21%@3 y
Kawashima et al. (15)	60	60	55	4 (2–9)	32%	CTS 64%	MRD 25% MUD 22% mD 28% CB 20%	RIC 93%	42%@2 y	59%@2 y	60% DEL 20% noDEL	22% DEL 9% noDEL
Dreger et al. (16)°	1,438	1,438	55–58	/	42–62%	CTS 75–82%	MRD 36% MUD 53% HAPLO 10%	RIC 100%	MRD 50%@3 y MUD 43– 46%@3 y HAPLO 46%@3 y	MRD 37%@3 y MUD 36%@3 y HAPLO 38%@3 y	MRD 47%@3 y MUD 38–34%@3 y HAPLO 41%@3 y	MRD 17%@3 y MUD 26–30%@3 y HAPLO 22%@3 y

HDC, high-dose chemotherapy; CT, chemotherapy; N*, N of patients with DLBCL; CTX, conditioning regimen; CTS, chemosensitive; mD, mismatched donor; MRD, matched related donor; MUD, matched unrelated donor.
In Hamadani et al. (5), patients were analyzed based on disease status at ALLO-SCT: progressive disease (PD) vs. stable disease (SD).
In Hamadani et al. (10), patients with DLBCL and grade III follicular lymphoma had a better outcome. In this study, a myeloablative conditioning regimen was used in a high percentage of patients. Though this reduced the relapse rate, the PS was worse than that in the RIC group because of the higher NRM incidence (53% in the MAC cohort).
In Kawashima et al., the results were analyzed based on the double expressor subtype.
In Thomsom et al. (4) all patients received in vivo T-cell depletion using CAMPATH.
In Rezvani et al. (3) patients with transformed NHL were included.
In Bacher et al. (9) the authors analyzed the impact of the intensity of CTX on clinical outcomes. The NRM incidence was higher after MAC, while the relapse rate was higher with less intensive CTX regimens, explaining the similar PFS and OS.
In van Kampen et al. (7) the NRM incidence was higher using MAC.
§Prospective trials.
IIIn this trial, CTX included Zevalin.
^In this trial, the NRM incidence was reported for patients receiving MRD or MUD transplants with ATG.
°In this study, the MUD group was separated based on whether GVHD prophylaxis was applied with or without in vivo T-cell depletion.
$In this study, 226 patients had DLBCL, and 207 had G3 follicular lymphoma.

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TABLE 2 Acute and chronic GVHD rates.

Authors	*N*	*N**	Grade 2–4 aGVHD	cGVHD
Robinson et al. (1)	188	62	25%	NR
Corradini et al. (2)	170	61	35%	49%
Rezvani et al. (3)	68	16	63%	47%
Thomson et al. (4)	48	48	17%	22%
Hamadani et al. (5)	46	18	43%	75%
Sirvent et al. (6)	68	68	39%	41%
Van Kampen et al. (7)	101	101	33%	42%
Rigacci et al. (8)	165	165	27%	NA
Bacher et al. (9)	396	396	RIC 43%	RIC 37%
			MAC 43%	MAC 41%
			NMA 44%	NMA 37%
Hamadani et al. (10)	226	226	30%	35%
Bouabdallah et al. (11)§	31	16	27%	14%
Glass et al. (12)§	84	61	42%	41%
Fenske et al. (13)	503	503	36%	47%
Dodero et al. (14)§	121	35	22%	44%
Kawashima et al. (15)	60	60	64%	40%
Dreger et al. (16)	1,438	1,438	MRD 32%	MRD 48%
			MUD 32–42%	MUD 27–57%
			HAPLO 34%	HAPLO 18%

NR, not reported.
§Prospective trials.

risk of relapse (10). In a phase 2 randomized study, Glass et al. included patients with aggressive lymphoma (DLBCL, 61 out of 84 patients) who relapsed after high-dose chemotherapy (53%) and were refractory to the first CT line (57%) or who relapsed within <12 months (16%). All patients received a MAC regimen. Only 45% showed chemosensitive disease at the time of ALLO-SCT. In this unfavorable group of patients, the 1-y PFS and OS were 52 and 45%, respectively, with a better outcome when the patient had achieved MRD negativity or a MUD and a conditioning regimen containing ATG were used (12). Notably, the 1-y NRM incidence was only 10%. Interestingly, the inclusion criteria of this prospective study are the same as those used in some phase 2 studies of patients treated with CAR-T cell (18, 19) and thus allow an indirect comparison of CAR-T-cell therapy and ALLO-SCT. The CIBMTR reported the outcome of DLBCL patients relapsing after HDC, and overall, the 3-y PFS and OS were 31 and 37%, respectively. In that paper, the authors identified some prognostic factors (time to relapse after HDC, disease status at the time of ALLO-SCT, and PS) and developed a scoring model to predict survival (13). Of note, the model can predict the outcome of patients treated with CAR-T cells (20).

The conditioning regimens were mostly reduced intensity or non-myeloablative in nature, but in some studies (7, 9, 13, 15), some patients were treated with intensive conditioning regimens, suggesting that the intensity of the conditioning regimen might be a relevant factor. For example, should a MAC regimen be used in patients with advanced disease to encourage the graft vs. lymphoma effect? In the prospective German study DSHNHL R3, all patients received a MAC regimen consisting of fludarabine, busulfan (3–4 days) and cyclophosphamide. More than half of the patients had refractory disease at the time of ALLO-SCT, and the median age was 48 years. Though the age limit for inclusion was 65 years, the oldest included patient was 57 years. The 1-y NRM incidence was 10%, and interestingly, the 1-y PFS was 45%, and the 1-y OS was 52% (12). In a retrospective study from CIBMTR comparing MAC, RIC, and non-myeloablative conditioning (NMAC) regimens in DLBCL, and as reported in other studies (7, 13, 15), the reduced risk of relapse/progression with MAC was offset by a higher risk of early and late NRM, and consequently, the survival was not different (9). In a recent analysis, including 1,823 NHL patients (every histology), Ghosh et al. compared the outcomes based on the intensity of the RIC regimen. Again, the most intensive regimen, namely, fludarabine plus melphalan 140 mg/m2, showed a less favorable profile in terms of NRM, without any improvement in relapse risk (21). This study suggests that more intensive conditioning regimens do not lead to superior results in lymphoma patients. However, we think that younger patients (maybe <45 years), patients who have not been treated with HDC or autologous stem cell therapy, patients without comorbidities, and patients who are not in complete remission could benefit from a MAC regimen.

Another variable that could affect the outcomes is the donor type. Although an HLA-identical sibling (HLAid sib) or matched unrelated donor (MUD) was usually used in the past, with the advent of the PTCY platform, haploidentical donors can be used. This advance has changed the treatment landscape, allowing more patients to be transplanted. The impact of donor type (HLAid sib vs. MUD) was analyzed in most of the studies reported in Table 1. In some of the studies, donor type did not impact survival or NRM incidence (3, 4, 6, 7, 12, 14), while in others, an MUD transplant was associated with a higher incidence of NRM (8–10, 13), lower PFS (8) or higher GVHD incidence (1). Recently, a joint retrospective analysis from CIBMTR and EBMT of patients with DLBCL receiving transplantation from an HLAid sib, an MUD with or without T-cell depletion (TCD), or a haploidentical donor. This study confirmed that the survival rate after transplantation from a haploidentical donor and administration of cyclophosphamide posttransplantation was similar to that observed after transplantation from an HLAid sib or MUD. It was confirmed that the graft-relapse free survival (GRFS) was better with haploidentical donors due to the low incidence of chronic GVHD. Furthermore, in this study, the NRM incidence of patients receiving an MUD transplantation without TCD was significantly higher (16).

TABLE 3 Risk factors (RF) for the major outcomes identified in selected studies.

Authors	*N*	*N**	Disease status	Donor	RF OS	RF PFS	RF Relapse	RF NRM
Robinson et al. (1)	188	62	CTS 73%	MRD 91% MUD 9%	Chemosensitive	Chemosensitive	Chemosensitive	Age > 50 y
Corradini et al. (2)	170	61	CTS 77%	MRD 100%	Chemosensitive Disease histotype Previous HDC Severe aGVHD	Chemosensitive Disease histotype Severe aGVHD	Chemosensitive Disease histotype	
Van Kampen et al. (7)	101	101	CTS 74%	MRD 72% MUD 28%	TtR after HDC > 12 M High LDH	TtR after HDC > 12 M High LDH BM	Disease status	Age > 45 y BM TtR after HDC > 12 M
Rigacci et al. (8)	165	165	CTS 55%	MRD 65% MUD 35%	CR > PR > other	CR > PR > other MRD		
Bacher et al. (9)	396	396	RIC CTS 35% MAC CTS 40% NMA CTS 35%	/	KPS Disease status Year of ALLO-SCT	KPS Disease status Year of ALLO-SCT	Disease status RIC/NMA	KPS Disease status Year of ALLO-SCT Donor type MAC better
Hamadani et al. (5)	533	226	CTS 0%	MRD 48% MUD 24% mMUD 11%	DLBCL	DLBCL	RIC (vs. MAC) DLBCL Previous HDC	DLBCL Donor type
Glass et al. (12)	84	61	CTS 45%	MRD 27% MUD 40% mMUD 31%	/	aGVHD 0–1 mMUD Refractory No ATG	/	/
Fenske et al. (13)	503	503	CTS 74%	MRD 50% MUD 23% mMUD 26%	PS <80% Chemoresistant TtR After HDC < 12 M MAC	PS <80% Chemoresistant TtR After HDC < 12 M MAC	PS <80% Chemoresistant TtR After HDC < 12 M	Chemoresistant Donor type
Dreger et al. (16)	1,438	1,438	CTS 75–82%	MRD 36% MUD 53% HAPLO 10%	CR > PR > SD/PD Age HCT-CI	CR > PR > SD/PD	MUD better	MRD better Age HCT-CI

TtR, time to relapse.

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A relevant clinical aspect of ALLO-SCT is the age of patients with NHL (DLBCL 30%); ALLO-SCT is a valid therapeutic option in elderly patients. In a CIBMTR study, Shah et al. showed that the 4-year relapse rate, PFS and OS were similar in a cohort of older patients (median age 68 years) compared to a cohort of younger patients (median age 60 years). Only the 4-year NRM was slightly higher in the older patient cohort (22).

Furthermore, immunological activity against lymphoma cells can be reflected in the relapse rate. Indeed, B and *T*-cells in lymphoma tissue express antigens recognized by donor T cells. Table 1 shows the relapse rate observed in several clinical studies. Because most of the studies were retrospective and thus may have selection bias that may have influenced the results, the relapse rate ranges from 7 to 60%. It is not surprising that the disease status at the time of ALLO-SCT is the main factor related to this phenomenon. This pattern was also observed in the study by Hamadani et al. (5), in which only patients with SD or PD were included, and in the study by Bouabdallah et al. which included only CR patients (11). In these 2 studies, the relapse rates were 25% when in SD, 50% in PD, and 7% in CR, respectively. Another factor could be the intensity of conditioning regimen. As described above, high-intensity conditioning regimens can have more activity against lymphoma, but their toxic side effects can be prohibitive. In conclusion, the disease status before ALLO-SCT is the most important factor related to relapse.

Finally, after allo-SCT, only one study reports that DE DLBCL receiving allo-SCT showed inferior PFS linked to higher relapse incidence compared to no-DE lymphomas (15).

Results after CAR-T-Cell therapy in LBCL

CAR-T-cell therapy has changed the treatment landscape for many patients with relapsed or refractory aggressive B-cell lymphomas. Since 2017, when the three commercial CAR-T-cell products were approved, many clinical trials and data have been reported.

The pivotal phase 2 prospective studies ZUMA-1 (18), JULIET (19) and TRANSCEND (23) for axicabtagene ciloleucel (axi-cel), tisagenlecleucel (tisa-cel) and lisocabtagene maraleucel (liso-cel), respectively, enrolled heavily pretreated patients who relapsed after or were refractory to at least two prior lines of standard therapy and autologous stem cell transplantation. In these studies, the histology-related inclusion criteria were different; in general, the studies included high-grade B-cell lymphoma (HGBCL) with or without translocations of MYC and BCL2 and/or BCL6 (double/triple-hit lymphoma) and transformed follicular lymphoma (tFL). Both ZUMA-1 and TRANSCEND also included patients with R/R primary mediastinal B-cell lymphoma (PMBCL). Only TRASCEND included patients with transformed diffuse large B-cell lymphoma arising from indolent histologies other than FL or FL3B (2, 3) and those with secondary CNS involvement or who had received prior ALLO-SCT. Table 4 shows the main results from the prospective and retrospective studies. The follow-up was shortest in the TRANSCEND trial, with data from a median follow-up of 18.8 months (vs. 27.1 months in ZUMA-1 and 32.6 months in JULIET). Even though there were differences in the inclusion criteria across the 3 studies, the ORR (ranging from 52 to 74%) and CR rate (ranging from 40 to 54%) were comparable across age and tumor histology subgroups. Interestingly, almost 40% of the refractory patients in these studies were disease free 3–4 years after infusion.

Clinical trials have stringent eligibility criteria, and the outcomes observed in these trials may not be observed in real-life clinical practice. Several retrospective studies of the use of commercial CAR-T-cell products have been published (Table 4). Some general conclusions can be made from real life studies. Overall, in the real world, the groups of patients treated with CAR-T cells are less refined than those in prospective studies, but the clinical results are similar in terms of ORR (ranging from 59 to 84%), CR rate (ranging from 32 to 65%) and survival. In the real life studies compared with the clinical trials, the vast majority of patients had DLBCL, the median age was higher, there was a greater number of patients with ECOG PS $>$ 2, and just over half of the patients treated with axi-cel received bridging therapy (BT), which was not allowed in ZUMA-1. The role of bridging therapy is unclear. CAR-T-cell therapy is typically used for patients who are resistant to chemotherapy, and thus, BT should improve disease control prior to CAR-T-cell infusion. On the other hand, it is possible, that patients treated with BT may have inferior outcomes because they have more aggressive and rapidly progressive disease or because the BT itself confers additional treatment toxicity or immunosuppression (34). Conventional BT have not been as effective as expected, and in a recent retrospective trial analyzing the impact of different BT before axi-cel infusion, it was clear that patients who received systemic BT showed more advanced and aggressive disease, and their survival was lower than that of patients who did not receive BT. Furthermore, the studies show that patients who received radiotherapy had longer PFS than patients who received systemic therapy as BT (35). Recently, the Spanish groups GELTAMO and GETH published a comparison of real-world CAR-T-cell therapy with standard of care (SOC) treatment for refractory large B-cell lymphoma, and their results confirmed the higher efficacy of CAR-T-cell therapy than SOC, showing longer PFS and OS in the CAR-T-cell therapy group independent of other prognostic factors. In the CAR-T cell therapy cohort, CAR-T-cell therapy type (axi-cel better), unfavorable R-IPI at LD, no previous ASCT, and higher Haematopoietic Cell Transplantation-specific Comorbidity Index (HCT-CI) before lymphodepletion (LD)

TABLE 4 Main clinical results from prospective and retrospective studies of aggressive lymphomas.

Authors	*N*	Median age	Phase	Drug	*N* CT lines	ORR	FU (months)	PFS (months)	OS (median)
Neelapu et al. (18)	101	58	P 1–2	Axi-cel	3	74% (CR 54%)	27.1	5.9	NR
Schuster et al. (19)	115	56	P 2	Tisa-cel	3	52% (CR 40%)	32.6	NR	NR
Jacobson et al. (24)	122	62	R	Axi-cel	2–3	70% (CR 50%)	10.4	4.5	NR
Abramson et al. (23)	269	63	P 1	Liso-cel	3	73% (CR 53%)	12–17.5	6.8	NR
Nastoupil et al. (25)	298	60	R	Axi-cel	3	82% (CR 63%)	12.9	8.3	NR
Pasquini et al. (26)	410	65	R	Tisa-cel	4	62% (CR 49%)	11.9		
Lamure et al. (27)	60	64	R	Axi-cel Tisa-cel	2–3	63% (CR 40%)	6.9	3.1	12.3
Iacoboni et al. (28)	72	60	R	Tisa-cel	3	BOR 60% (CR 32%)	14.1	3	10.7
Sehgal et al. (29)	61	74	P 2	Liso-cel	n.a.	80% (CR 54%)	12.3	9.03	
Bastos-Oreiro et al. (30)	204	axi-cel 54 tisa-cel 56	R	Axi-cel Tisa-cel	2	60%	10 in axi-cel; 14 in tisa-cel	8.5 axi-cel; 4.6 tisa-cel	NR axi-cel; 11.7 tisa-cel
Betghe et al. (31)			R	Axi-cel Tisa-cel					
Kwon et al. (32)	307		R	Axi-cel Tisa-cel	2	57% (38%)	9.2	4.8	11.7
Bachy et al. (33)	518	63	R	Axi-cel Tisa-cel	2				

MA, metanalysis; NR, not reached; NA, not applicable; ORR, overall response rate; P, prospective; R, retrospective.

adversely influenced PFS while CAR-T-cell therapy type (axi-cel better), unfavorable R-IPI at LD, ECOG-PS 2–4 before apheresis, primary refractory disease, and higher HCT-CI before LD impacted OS in the multivariate analysis (30). The efficacy of tisa-cel and axi-cel was assessed in 3 recent retrospective studies. In the first single-centre study, axi-cel was clearly more toxic than tisa-cel or a homemade 41-BB CAR-T-cell product, but the ORR was influenced by absolute lymphocyte count (ALC) before leukapheresis, with axi-cel being more active when ALC was high (36). In a second study, 307 patients were analyzed (152 who received axi-cel vs. 155 who received tisa-cel) in a multicentre setting. The patient characteristics were well balanced, and while the ORR, duration of response (DOR), PFS and OS were not significantly different between the treatments, the incidence of ICANS was higher while the CRS rate was similar after axi-cel (32). Different findings were reported in the second study (33), in which a large number of patients included in the DESCART national registry were analyzed (209 who received tisa-cell vs. 209 who received axi-cel) using propensity score matching to reduce differences in variables associated with outcomes. The overall CRS incidence was higher after axi-cel than after tisa-cell (86.1 vs. 75.6%), but the severe CRS incidence was similar (9.1 vs. 5.3%); in addition, the overall and severe ICANS incidences were higher after axi-cel than after tisa-cel (48.8 vs. 22 and 13.9 vs. 2.9%, respectively), as was the rate of cytopenia. The ORR and CR rate were significantly higher for the axi-cel group than for the tisa-cel group (80.4 vs. 66 and 60.3 vs. 42.1%, respectively), and the 1-year PFS and OS were longer for the axi-cel group than for the tisa-cel group (46.6 vs. 33.2 and 63.5 vs. 48.8%, respectively). Survival was confirmed to be better in the axi-cel subgroups with either age ≥70 years or bulky disease (33). These data confirmed the initial results from the matching adjusted indirect comparison (MAIC) trial (37).

TABLE 5 Clinical results from the metanalysis (38).

		Axi-cel	Tisa-cel	Liso-cel
All patients	Severe CRS	13%	9%	2%
	Severe ICANS	31%	8%	10%
DLBCL	ORR	70%	75%	72%
	CR rate	52%	40%	52%
Primary mediastinal	ORR	62%	/	/
	CR rate	58%	/	/
High grade BCL	ORR	88%	/	76%
	CR rate	/	/	61%

BCL, B-cell lymphoma; DLBCL, diffuse large B-cell lymphoma; ORR, overall response rate.

Meng et al. published a meta-analysis of the safety and efficacy of CAR-T cells. Overall, the authors did not find remarkable differences in the terms of ORR, CR rate, or survival, as reported in Table 5 (38).

CAR-T-cell infusion is associated with specific side effects that result from on-target off-tumor activity. The most frequent

toxicities are cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANS), and prolonged cytopenias, and less frequently, B-cell aplasia and hypogammaglobulinemia, infections, tumor lysis syndrome and infusion-related immune reactions are described. Table 6 shows the toxicities after CAR-T-cell treatment. It is difficult to compare toxicities across the studies because different scales were used, and there were more toxicities in the first years after commercial CAR-T cell product administration. Notably, newer and more refined grading criteria for CAR-T-cell therapy-associated neurological events (NEs) are now available, such as the ASCT consensus criteria for ICANS, which is currently widely used for reporting NEs in real-world studies (39, 40). In the prospective studies, the cumulative incidences of CRS and ICANS seem to be higher than those in real-life studies, probably because of the early use of tocilizumab and steroids and greater confidence of the clinicians. In general, axi-cel was associated with a significantly higher risk of CRS and severe neurotoxicity due to the rapid and massive T-cell expansion linked to the costimulatory moiety CD28, which is not seen with the 41-BB costimulatory moiety used for tis-cel and liso-cel. Furthermore, the frequency and severity of CRS and NEs were higher in patients with high tumor volume, and patients were more likely to experience a severe (grade > 3) NE after receiving > 5 prior lines of therapy (41–43). In a real-life German study, the NRM incidence at 2 years was significantly higher after axi-cel than after tisa-cel therapy (10.4 vs. 3.5%, respectively) (31).

Table 7 shows the factors predictive of response and survival in different studies, both retrospective and phase 1–2. There are many differences, and there is no overlap of predictive factors, but in general, age; factors related to the disease, such as high disease burden and primary refractory disease; and higher IPI were associated with a worse outcome. Some studies found a correlation between CAR-T-cell expansion *in vivo* with the duration of response, with the strongest correlation demonstrated for acute lymphoblastic leukemia. Overall, the biological characteristics of lymphoma cells did not impact the response, i.e., DH/TH subgroups showed the same sensitivity to CAR-T-cell therapy. However, the ORR (34%) and survival (1-y survival rate 44%) in p53-mutated lymphomas were significantly lower than those in unmutated lymphoma (46). In that study, primary refractory disease and SD or PD at infusion were also predictive factors of lower ORR.

In conclusion, CAR-T cells are extraordinarily active against advanced, aggressive lymphomas, even if disease control cannot be achieved before infusion.

Results after ALLO-SCT in MCL

Mantle cell lymphoma is still considered an incurable disease, even though the survival of patients has improved in recent years. In facts, the survival of transplant-eligible patients

TABLE 6 CRS and ICANS incidences in clinical studies.

	CRS						ICANS						Cytopenia		
	Axi-cel		Tisa-cel		Liso-cel		Axi-cel		Tisa-cel		Liso-cel		Axi-cel	Tisa-cel	Liso-cel
	Any	Severe	Any	Severe	Any	Severe	Any	Severe	Any	Severe	Any	Severe			
Neelapu et al. (18)	93%	13%					64%	28%					84%		
Schuster et al. (19)			58%	22%			70%	35%	21%	12%				44%	
Jacobson et al. (24)	93%	16%											/		
Abramson et al. (23)					42%	2%					30%	10%			63%
Nastoupil et al. (25)	91%	7%					68%	37%					/		
Pasquini et al. (26)			45%	4.5%					18%	5%				18%	
Iacoboni et al. (27)			71% 5%						15%	1%				/	
Bastos-Oreiro et al. (28)	90%	8%	65%	4%			42%	16%	16%	5%			/	/	
Bethge et al. (31)	81%	10%	65%	13%			76%	28%	40%	12%				17%	
Kwon et al. (32)	82%	8%	73%	6%			42%	16%	18%	5%			10%	15%	
Bachy et al. (33)	86%	5.3%	75%	9.1%			48.8%	13.9%	22%	2.9%			64.6%	39.2%	

Severe toxicities were defined as those ≥ grade 3.

TABLE 7 Pre-CART and post-CART factors predicting response and survival after CAR-T-cell therapy identified in clinical trials in aggressive lymphomas.

Authors	Overall response rate Pre-CART	Post-CAR-*T*-cell therapy
Neelapu et al. (18)	None	CAR-T-cell expansion
Schuster et al. (19)	None	None
Schuster et al. (44)	LDH levels, G3–4 thrombocytopenia	None
Jacobson et al. (24)	High tumor burden, ferritin, LDH levels	-higher peak CAR-T-cell:TB ratio -CAR-T-cell expansion -Better if high CCR7–CD45RA–T cells
Abramson et al. (23)	None	None
Nastoupil et al. (25)	Age > 60 y, LDH levels before LD	None
Pasquini et al. (26)	None	None
Vercellino et al. (45)	None	None
Locke et al. (41)	High tumor burden, IL6 levels, CRP, Day 0 IL-15, interferon-g in coculture	CAR-T-cell expansion
Lamure et al. (27)	Advanced age, refractoriness to previous treatment, multiple previous lines of treatment	None
Iacoboni et al. (28)	Sex (females did better), higher IPI	None
Shouval et al. (46)	P53 mutation, refractoriness and SD/PD at infusion	None
Bastos-Oreiro et al. (30)	None	
Betghe et al. (31)	IPI > 2, high LDH levels	

	Survival Pre-CART	Post-CART
Neelapu et al. (18)	None	None
Schuster et al. (19)	None	None
Schuster et al. (44)	None	None
Jacobson et al. (24)	Day 0 CRP > 30 mg/L	None
Abramson et al. (23)	None	None
Nastoupil et al. (25)	PFS: Bilirubin levels, LDH levels, ECOG, sex OS: Bilirubin levels, LDH levels, ECOG, sex, disease status, BT	None
Pasquini et al. (26)	None	None
Vercellino et al. (45)	CRP, END > 2, MTV > 41%	None
Locke et al. (41)	High tumor burden, LDH levels, IL6, effector: target ratio, number of infused CD8 T cells, CCR7-CD45RA-T cells	-higher peak CAR T cell:TB ratio
Lamure et al. (27)	Female gender, aaIPI at the time of infusion	None
Iacoboni et al. (28)	Primary refractory disease [HR: 2.24 (95%CI1.20–4.18), $p = 0.01$] and high LDH	None
Shouval et al. (46)	P53 mutation, refractoriness and SD/PD at infusion, LDH levels	None
Bastos-Oreiro et al. (30)	Previous HDCT, primary refractoriness, ECOG PS pre-apheresis, type of CAR-T cell, BT, HCT-CI, R-IPI	None
Betghe et al. (31)	Nonresponse to bridging therapy, elevated LDH, poor PS	None
Kwon et al. (32)	PFS: LDH levels before apheresis, ECOG PS ≥2 before LD therapy OS: high LDH at apheresis, ECOG PS ≥2 at apheresis, progressive disease at apheresis, ECOG PS ≥2 before LD	None

ORR, overall response rate; R, retrospective; P, prospective; LD, lymphodepletion; END, extranodal disease; CRP, C-reactive protein; HDCT, high-dose chemotherapy; BT, bridging therapy, R-IPI, Revised International Prognostic Index; PS, performance status.

with advanced MCL is almost 8 years (47). Treating R/R MCL is a clinical challenge, and Bruton kinase (BTK) inhibitors or anti-Bcl2 agents (venetoclax) can achieve a high objective response rate, though the survival is short and unsatisfactory. Clinical (progression of disease within <24 months after first-line treatment), histological (high Ki67, blastoid morphology), and molecular (p53 mutation) factors can identify subgroups of patients likely to have unfavorable outcomes (48).

TABLE 8 Clinical results of ALLO-SCT in R/R MCL.

	N	CTX	Disease status	2–5 y OS	PFS	Relapse rate	NRM
Robinson et al. (1)	22	RIC	CTS 73%	13%	/	100%	82%
Maris et al. (59)	33	NMAC	CTS 54%	64%	60%	16%	24%
Armand et al. (60)	15	RIC	/	42%	22%	33%	37%
Tam et al. (61)	35	RIC	CTS 83%	53%	46%	/	9%
Cook et al. (49)	70	MAC	CTS 83%	37%	14%	65%	18%
Hamadani et al. (53)§	202	MAC 74	CTS 0%	25%	20%	33%	47%
		RIC 128		30%	25%	32%	43%
Le Gouill et al. (54)	70	RIC	CTS				
Fenske et al. (62)	Early AUTO	RIC	CTS 100%	61%	52%	32%	3%
	Early ALLO			62%	55%	15%	25%
	Late AUTO			44%	29%	51%	9%
	Late ALLO			31%	24%	38%	17%
Kruger et al. (63)	39		CTS 92%	73%	67%	15%	24%
Mussetti et al. (56)	29	RIC	CTS 90%	54%	41%	28%	29%
Vaughn et al. (50)	70	NMAC	CTS 64%	55%	46%	26%	28%
Tessoulin et al. (51)	106	RIC	CTS 80%	62%	43%	30%	28%
Robinson et al. (52)	324	RIC	CTS 65%	40%	31%	40%	24%
Dreger et al. (57)	22	RIC 82%					5%
Arcari et al. (58)	55	RIC	CTS 93%	56%	53%	16%	23%

MAC, myeloablative conditioning regimen; RIC, reduced intensity conditioning regimen; NMAC, nonmyeloablative conditioning regimen. CTS, chemosensitive.
§In this study, all patients were chemorefractory at the time of ALLO-SCT.

R/R MCL can be treated with a BTK inhibitor +/− venetoclax or other conventional combinations (i.e., bendamustine + cytarabine + cyclophosphamide, BAC), and responding patients can be considered for immunotherapy. Before the advent of CAR-T-cell therapy, ALLO-SCT was frequently used as immunotherapy. Table 8 summarizes the clinical results obtained in the last 20 years from studies using ALLO-SCT in RR MCL. The majority of patients relapsed or progressed after receiving AUTO-SCT. In most of the studies reported in Table 8, most patients had experienced a CR or PR just before ALLO-SCT (range 54 to 100%). As expected, the disease status at the time of ALLO-SCT is an important prognostic factor for survival (49–52), and survival is significantly better in patients with a favorable disease status. However, the OS rate varies widely, ranging from 13 to 73%, indicating a strong selection bias. Of particular interest is the CIBMTR study, which included only patients with chemorefractory disease at ALLO-SCT (53), because these patients are similar to the patients that were included in the CAR-T-cell trials. RIC or MAC were used as conditioning regimens, and HLAid siblings or unrelated donors were used. The 3-y OS, PFS, relapse rate, and NRM incidence in the MAC and RIC groups were 25 vs. 30%, 20 vs. 25%, 33 vs. 32%, and 47 vs. 43%, respectively. In multivariate analysis, the use of bone marrow as a graft source and *ex vivo* T-cell depletion were associated with higher NRM incidence and inferior survival. Furthermore, the intensity of the conditioning regimen was not associated with outcome. The EBMT recently reported the outcome of 324 MCL patients treated with ALLO-SCT between 2000 and 2008. The results are interesting because after a median follow-up of 70 months of a heavily pretreated population (46% of patients received previous AUTO-SCT, 60% of patients received more than 3 CT lines), one-third of patients were progression free. Again, survival was better with chemosensitive disease (54). The toxicity of ALLO-SCT remains important and limits its applicability. Several factors can increase the risk of death due to toxicity. The first is the period in which ALLO-SCT is performed because it is clear that the mortality rate has improved in recent years (55). Indeed, the year of inclusion in the more recently published series (from 2015) ranges from 1999 to 2013 (50, 51, 54, 56). In the last 2 series, the years of inclusion were 2013–2016 and 1999–2013 (57, 58). In Table 9 (10), we present factors predicting NRM incidence from these last studies. Not surprisingly, only severe aGVHD was predictive of a high mortality rate [in 2 studies, (51, 58)], while only one study found age <60 years and heavy pretreatment to be predictive of severe toxicity (58).

Of note, MIPI, histological and/or molecular variables did not predict the clinical outcome. However, these informations were frequently unknown in registry studies.

TABLE 9 Factors predictive of higher NRM incidence in MCL.

	N	CTX	Risk factors	NRM
Mussetti et al. (56)	29	RIC	NR	29%
Vaughn et al. (50)	70	NMAC	No factors	28%
Tessoulin et al. (51)	106	RIC	G3–4 aGVHD	28%
Robinson et al. (52)	324	RIC	No factors	24%
Dreger et al. (58)	22	RIC 82%	NR	5%
Arcari et al. (57)	59	RIC	G3–4 aGVHD, > 2 CT lines, age > 60 y	23%

Results after CAR-T cell therapy in MCL

CD19 CAR-T cells have emerged as a highly active treatment modality for R/R MCL. The experience with CAR-T cells in MCL is more limited than that in large B-cell lymphomas.

Table 10 shows the results of two studies: the ZUMA-2 study, a phase 2 prospective trial that led to FDA approval (July 2020) of brexucabtagene autoleucel (brexu-cel), and a real-life retrospective study.

In the ZUMA-2 (16) trial, in terms of baseline characteristics, 31% of patients had blastoid or pleomorphic histology, 81% of patients received ≥3 prior lines of treatment, 6 of 36 patients had TP53 mutation (of those with available data), and all patients had prior BTKi treatment (acalabrutinib and/or ibrutinib). Among the 68 treated patients, the 1-y PFS was 61%, with a median PFS not reached at the time of study publication. Subgroup analysis demonstrated a similar ORR and 6-month PFS among high-risk subgroups, including patients with TP53 mutation, patients with blastoid morphology, and patients with high-risk MIPI, compared with patients without these high-risk features (64). Recently, a 3-year follow-up analysis of this study was published. The ORR and CR rate were 91 and 68%, respectively. The duration of response (DOR) for responding patients was 28.2 months, and the median PFS was 25.8 months. Although not significant, a trend toward a lower ORR was observed in the high-risk subgroups (p53 mutation, POD24, and blastoid histology subgroups), even if these data are still unstable due to low number of patients analyzed.

Pretreatment with a BTK inhibitor did not impact the ORR, while exposure to bendamustine seemed to have a negative impact on the DOR (65).

Iacoboni et al. recently published the first real-world study from Europe of brexu-cel in high-risk MCL (high MIPI score, poor Eastern Cooperative Oncology Group performance status and receipt previous allogeneic HCT). The ORR was 91% (CR rate 79%), and the 1-y PFS and OS were 50.8 and 61.4%, respectively. However, in this cohort of patients, the mortality rate related to CAR-T-cell therapy was 15% (28).

The safety profile is quite different for different cellular products (Table 11). The use of liso-cel appears to be associated with a lower incidence of CRS and neurotoxicity than the use of brexu-cel. Grade 5 toxicities occurred in two patients (3%).

In light of the results described above, the ideal R/R MCL patients for CAR-T-cell treatment are those with progressive disease following BTKi therapy, but these patients be well enough physiologically to tolerate expected complications, including CRS, and those patients with significant frailty or with severe end organ damage, such as severe systolic heart failure, should generally not be candidates for this type of therapy.

Possible scenarios for integrating ALLO-SCT and CAR-T-Cell therapy

Although it is now widely acknowledged that CAR-T-cell therapy is useful in DLBCL and MCL and thus that ALLO-SCT does not have a place in the treatment scheme, we do not think that these 2 immunological approaches to cure advanced lymphoma are mutually exclusive (66). In the recent European Bone Marrow Transplantation Society (EBMT) guidelines, the role of ALLO-SCT was modified: it is now considered only an option, while CAR-T-cell therapy is the standard of care (67).

Three clinical scenarios can be proposed: first, patients can be treated with ALLO-SCT before CAR-T-cell therapy; second, CAR-T-cell therapy can be applied first, and ALLO-SCT can be applied if there is progression/relapse; and third, CAR-T-cell therapy can be used as induction therapy in a tandem CAR-T-cell therapy/ALLO-SCT sequence, as frequently done for acute lymphoblastic leukemia.

The first scenario has already been proved lack utility, and in different countries, CAR-T cells are already approved by regulatory agencies as the first-line immunotherapy in R/R LBCL and MCL with specific indications. Dreger et al. compared the results obtained in an intention-to-treat analysis of patients with R/R LBCL at their centre. In the first period (2004–2020), ALLO-SCT was considered the preferred option ($n = 60$), while in the second period (2018–2020), CAR-T-cell therapy was considered the standard of care ($n = 41$). The researchers did not observe differences in terms of 1-y OS (68 vs. 54%), 1-PFS (39 vs. 33%) or relapse incidence (59 vs. 44%), but there was a significant difference in NRM incidence in favor of CAR-T-cell therapy (3 vs. 21%) (68). More recently, in a registry study, the Center for International Blood and Marrow Transplant Research (CIBMTR) analyzed results obtained from patients with DLBCL who relapsed after autologous transplantation and were treated with ALLO-SCT or CAR-T-cell therapy (axi-cel). In the CAR-T cell therapy cohort, at 1 year, the relapse rate was 39.5%, the NRM incidence was 4.8%, the OS was 73.4%, and the PFS was 55.7%. In the ALLO-SCT group, the results were similar, except

TABLE 10 Clinical results after CAR-T-cell therapy in MCL.

Authors	*N* PZ	Median age	Phase	CAR-*T*-cell therapy	N CT lines	TP53 mutated	ORR	PFS	OS
Wang et al. (64)	68	65	P 2	Brexu-cel	>3	6/36	85% (CR 59%)	61%	83%
Iacoboni et al. (28)	39	67	R	Brexu-cel	2	12%	91% (CR 79%)	77%	83%

NR, not reached.

TABLE 11 Main toxicities after CAR-T-cell therapy in MCL.

	CRS				ICANS				Cytopenia	
	Brexu-cel		Liso-cel		Brexu-cel		Liso-cel		Brexu-cel	Liso-cel
	Any	Sever	Any	Severe	Any	Severe	Any	Severe		
Wang et al. (65)	91%	15%			63%	31%			26%	
Iacoboni et al. (28)	91%	16%			91%	3%			50% at 1 month	

Severe toxicities were defined as those ≥ grade 3.

for NRM incidence (26.2, 20, 65.6, and 53.8%, respectively). The clinical characteristics were different mainly in terms of disease status at the time of cellular therapy, as only 26% of patients in the CAR-T-cell therapy group had a disease status of CR or PR, compared to 80% in the ALLO-SCT group. Furthermore, in that study, the CIBMTR score applied to 2 cohorts clearly separated three groups of patients with different survival (20). However, in patients in CR/PR after bridging therapy, considering that the relapse rate is low after allo-SCT, in presence of p53 mutation, this kind of therapy should be considered.

For R/R MCL, similar to LBCL, the scenario has also already been shown to be ineffective. In the recent American Society of Transplantation and Cellular Therapy, CIBMTR, and EBMT clinical practice recommendations for cellular therapies in MCL, CAR-T-cell therapy is recommended as the standard of care for patients with R/R MCL (69).

In the second scenario, CAR-T-cell therapy is used as induction therapy to perform ALLO-SCT. However, it is getting easier to predict the outcome of a single patient after CAR-T-cell therapy based on several predictive factors before and after infusion. In our opinion, one of the most interesting predictive factors is lack of complete remission at disease response evaluation 1–3 months after infusion, as reported by Nastoupil et al. (25). This factor can be combined with other factors, such as expansion of CAR-T cells early after infusion. For these high-risk patients, ALLO-SCT could be used after reinduction therapy to obtain CR or to reduce the lymphoma burden as much as possible. However, specific studies should be conducted in this field, using strong predictive factors of post-CAR-T cells outcome.

For both lymphoma subtypes, the 3rd scenario, in which CAR-T cell therapy is the first-line choice in refractory patients and ALLO-SCT is reserved for relapsed patients, provided that a clinical response is obtained, is probably more realistic, as reported in a recent survey by ASTCT (70). In this survey, the majority of physician considers allo-SCT in patients failing CAR-T and responding to salvage treatment. Shadman et al. first reported the outcomes of 13 patients who relapsed after CAR-T-cell therapy and underwent ALLO-SCT. Although the NRM incidence was relatively high (33% at 100 days), in part due to the use of a myeloablative conditioning regimen (39% of patients), the 1-year OS was encouraging (59%) (71). Chow et al. analyzed the outcomes of 61 patients who relapsed and progressed early (in the first 30 days, $n = 26$) or late ($n = 35$) after CAR-T-cell infusion. One-quarter of the patients did not receive any treatment at the time of progression for several reasons. Overall, only 6 patients underwent ALLO-SCT. The median OS of the entire population was only 5.3 months (72). More recently, Zurko et al. reported on 88 patients treated with ALLO-SCT after failing CAR-T-cell therapy. The median time between CAR-T-cell therapy and ALLO-SCT was 255 days (range 63–753). The majority of patients were chemosensitive at the time of ALLO-SCT (76%). After a median of 1 treatment line, reduced intensity conditioning regimens were used in 77% of patients, and there was similar use of various donor types throughout the cohort (MSD 26%, haploidentical 30%, matched unrelated donor 39%). At 100 days after ALLO-SCT, the cumulative incidence rates of grade II-IV and III-IV aGVHD were 34 and 10%, respectively. At 1 year, the cumulative incidence of moderate/severe cGVHD was 7.8 and 3.8%, respectively. The 1-year NRM was 22%, and the 1-year OS and PFS were 59 and 55%, respectively. In the multivariate analysis, the number of lines of therapy between CAR-T-cell therapy and ALLO-SCT and disease status at the time of ALLO-SCT were the most important prognostic factors for survival (73).

Di Blasi et al. (74) recently reported on 238 patients relapsed/refractory after CAR-T (both axi-cel and tisa-cel) in France. Relapse/progression was classified as very early (before d +30 days after CAR-T), early (between d +31 and d+90), and late (> d +90). Information on therapies received was available in 64% of patients, and mostly received lenalidomide (38%), target therapy (21%) and immune-chemotherapy (20%). The overall response rate was 14% (CR rate 65) and the median survival range from 3.7 and 8.5 months. In the multivariate analysis, predictive factors for PFS were LDH and ferritin levels at infusion, and for OS LDH, CRP, and very early relapse. To note, no association with outcome was observed for treatment type. This study confirms that R/R NHL after CARE-T is an unmet clinical need.

Furthermore, the toxicity observed after these two kinds of immunotherapy is deeply different, because allo-SCT is still complicated by a significant NRM due to infections and GVH, while the safety profile of CAR-T is acceptable. Of course, this should be take in account planning to treat the patients with adoptive immunotherapy.

In conclusion, even if ALLO-SCT for patients who relapse/progress after CAR-T seems reasonable, this population is very difficult to treat. Furthermore, ALLO-SCT can be complicated by the aggressiveness of disease, poor patient performance status and/or cytopenias, which can preclude the administration of induction therapy.

Author contributions

LC and RB wrote the manuscript. ST, GS, AI, AS, VT, LN, AM, and CP revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Influence of Voxelotor–hemoglobin complexes in the estimation of hemoglobin S levels by the current standard of care laboratory evaluation techniques

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Background: Sickle cell disease is an inherited disorder characterized by the presence of sickle hemoglobin (HbS). The process of Hb molecule polymerization is a pivotal step in the sickling process. Voxelotor, a recently approved novel therapeutic agent, is known to interfere with polymerization. We aim to study the impact of Voxelotor on Hb variants analysis using high performance liquid chromatography (HPLC).

Material and methods: We are reporting the impact of Voxelotor on Hb variants analysis using HPLC after an informed consent and medical research committee approval. Data was collected from eight patients who are enrolled in the GBT440-034OL study using electronic medical records, to evaluate the Hb levels, hemolytic markers and the clinical response.

Results: Our patients were well-balanced for gender, with a mean age of 31.1 years (19–50). Six patients showed a significant improvement in the Hb level, with reduced reticulocytes, bilirubin, LDH and an improved clinical outcome. Interestingly, these patients showed the appearance of a split band of Hb S and D on HPLC impacting significantly on HbS level. Two patients did not show any improvement on laboratory parameters, and no changes on their HPLC analysis.

Conclusions: We report here eight patients on Voxelotor therapy, six of which showed improved hemolytic markers and anemia and demonstrated the appearance of HbD peak on the HPLC chromatogram. Therefore, the absence of HbD on HPLC or other laboratory methods for estimating HbS in patients on Voxelotor therapy, gives the clinician a possible hint regarding the patient's compliance with the drug.

KEYWORDS

sickle cell disease, Voxelotor, high performance liquid chromatography, exchange transfusions, hemoglobin D Punjab

Introduction

Sickle cell disease (SCD) is an inherited disorder characterized by the presence of hemoglobin (Hb) S. It manifests as a homozygous state (HbS/S) or double heterozygous state, when HbS is co-inherited with other Hb variants such as β-thalassemia, Hemoglobin C (HbC), Hemoglobin D-Punjab (HbD Punjab), Hemoglobin E (HbE), and others (1). SCD results from a point mutation involving the β-globin chain of hemoglobin, where glutamic acid is replaced with valine (βGlu→ Val). At low oxygen tension, hemoglobin molecule undergoes a process of polymerization, which is thought to play a critical role in the process of sickling.

Hydroxyurea (HU) was the first approved disease-modifying intervention in adults and children with SCD; however, the introduction of L-glutamine, Crizanlizumab and Voxelotor provides an additional approved treatment options for the management of SCD complications (2).

The US FDA has approved Voxelotor for treating adults and children (≥4 years of age) with SCD based on the results from the HOPE and HOPE-KIDs trials, where it demonstrated an improved Hb level, and reduced hemolysis, with an improved rate of vaso-oclusive crises (VOCs) (3, 4). Post-licensure of Voxelotor, real-world studies in the US (5) and the EU (6) concur with the HOPE studies' result. Voxelotor brings its effects by reversibly binding to, and stabilizing HbS in an oxygenated state to ultimately inhibit the polymerization of HbS and increase oxygen affinity in RBCs, thereby preventing red blood cells from sickling. Assessment and monitoring of the level of HbF and Hb S are desirable and necessary in many situations including monitoring the response to hydroxyurea (measurement of Hb F level) and also in assessing the effectiveness of red cell exchange (measurement of Hb S) procedures. Although the addition of Voxelotor therapy to the drugs already available for SCD patients is highly desirable, physicians and scientists should be aware of its interference with laboratory tests that are routinely performed to measure HbS levels, and other Hb Variants.

Voxelotor seems to be a potent inhibitor of HbS polymerization *in vitro* as well as *in vivo* animal models in SCD individuals, but *in vitro* spiking experiments showed that Voxelotor could modify multiple variants and sub-variants of hemoglobins, including HbA, HbS, HbC, HbD-Punjab, HbE, HbA2, and HbF (7). Mass Spectrophotometric studies using matrix-assisted laser desorption/ionization (MALDI) confirmed that Voxelotor can bind with the N-terminus of hemoglobin alpha chain forming hemoglobin S-Voxelotor complexes (8). Consequently, these HbS-Voxelotor complexes could lead to inconsistencies in HbS quantification by the currently used standard of care laboratory evaluations techniques, including high-performance liquid chromatography (HPLC), capillary zone electrophoresis (CZE) and isoelectric focusing techniques (IEF) (7, 8). Thus, the question arises as to how do we monitor HbS in patients on Voxelotor therapy that may require blood or exchange transfusion, where the actual percentage of HbS plays a significant role in their management. Here we are reporting the impact of Voxelotor on various Hb Variants, and we also hypothesize that the appearance of abnormal Hb variant on HPLC may reflect patient compliance with the drug.

Materials and methods

We report our experience managing eight SCD patients receiving Voxelotor. Results were obtained from the electronic medical records using the data from the pre-and post-Voxelotor therapy periods, including Hb levels, biochemical markers of hemolysis, and results of Hb variant analysis using HPLC as our laboratory performs HbS quantitation by HPLC using the BioRad Variant II β-thalassemia short program (Bio-Rad Laboratories, Hercules, CA, USA). We also analyzed the impact of the various genotypes that are available in our cohort and the impact of Voxelotor therapy on the analysis. The patients are enrolled on the ongoing open label GBT440-034 OL study which was approved by the medical ethics committee (SQU-EC/043/18).

Results

We analyzed data from eight patients who were enrolled in this study. Our eight cases, showed a 1:1 male: female distribution, with a mean age of 31.1 years (19–50). Table 1 displays the SCD-significant laboratory parameters including hemoglobin (Hb), retics, lactate dehydrogenase (LDH), bilirubin, HbS and HbD. Across the eight subjects, there is a significant increase in Hb levels ($P = 0.02$, Wilcoxon signed ranks test) and a significant decrease in retics ($P = 0.03$), LDH ($P = 0.01$), bilirubin ($P = 0.02$) and HbS ($P = 0.0001$). Interestingly HbD variant was increased from zero to a mean of 23.7% post-Voxelotor dose due to forming HbS-Voxelotor complexes that run in the HbD window in HPLC. Cases 7 and 8 showed no change in Hb, or other laboratory markers, and no appearance of HbD variant on their HPLC. The results also show a significant reduction ($P < 0.05$) of the hemolytic parameters including reticulocytes, LDH, and bilirubin. Expectedly, this corresponded to a significant reduction in recurrent VOC episodes reported by these patients.

Table 2 shows the different changes in Hb variants HbS, HbF, HbA2, and HbD before and after Voxelotor therapy across the three genotypes commonly seen in our population namely, homozygous HbS/S, and double heterozygous HbS/β0 and HbS/D-Punjab. The patient with HbS/S displayed a decrease in HbS post-treatment, roughly compensated in HPLC by an increase in HbD. Unfortunately, Voxelotor therapy on this patient was interrupted for 2 weeks, and a blood exchange was administered, which affected the results of the HPLC by dramatically decreasing HbS, disappearance of HbD and spike in HbA. Further, patient with HbS/β0 genotype had the highest increase in HbD. Upon reviewing the patient's HPLC chromatograms, we observed a split peak spanning the HbD (retention time 4.10 min) and HbS (retention time 4.37) windows, as displayed in Figure 1A. The rise in the HbD variant is compensated by a reduced HbS, with limited impact on HbF and HbA2, as indicated in Tables 1, 2. We also noted that two of our patients (Cases 7 and 8) did not show any change in

TABLE 1 Demography and laboratory parameters in SCD cohort on Voxelotor.

Voxelotor cohort	Demography		Hb g/dl		Retics %		LDH μ/L		Bilirubin mg/dl		HbS %		HbD %	
	Sex	Age	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Case 1	M	35	7.8	9.6	13.7	3.5	1,137	303	87	20	92.8	73.5	0	22
Case 2	M	40	8.8	13.3	4.1	2.7	1,659	232	70	13	87.1	56.4	0	36.1
Case 3	F	25	7.1	10.8	15.7	8	492	282	59	18	85.5	42.4	0	52.1
Case 4	M	19	10.4	16.1	5	1.3	1,059	185	53	40	87.5	56.8	0	36.1
Case 5	F	26	7.7	13.2	6.3	2.4	419	343	18	9	91.2	56.2	0	28.5
Case 6	M	33	7.8	11.6	12	6.2	662	392	106	61	86.1	46.8	0	15.1
Case 7	F	50	7.1	6.7	9.4	7.6	539	497	33	21	89.6	47.8	0	0
Case 8	F	21	8.5	7.9	8	9.3	391	408	64	54	93.9	92.4	0	0
Mean		31.1	8.2	11.2	9.3	5.1	795	330	61.3	29.5	89.2	59	0	23.7
± SD		9.7	1.0	2.9	3.9	2.8	420	94	26.2	18.3	2.9	15.3	0	17.1
Min		19	7.1	6.7	4.1	1.3	391	185	18	9	85.5	42.4	0	0
Max		50	10.4	16.1	15.7	9.3	1,659	497	106	61	93.9	92.4	0	52.1
Median		29.5	7.8	11.2	8.7	4.9	600	323	61.5	20.5	88.6	56.3	0	25.3
25th		22	7.3	8.3	5.3	2.5	437	245	38	14.3	86.4	47.1	0	3.8
75th		38.75	8.7	13.3	13.3	7.9	1,117	404	82.8	50.5	92.4	69.3	0	36.1
P-value			0.02#	0.03#	0.01#	0.02#	0.0001#	0.002#						

#P < 0.05 vs. pre-dose is considered significant as determined by Wilcoxon signed ranks test. n = 8.

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TABLE 2 The Impact of Voxelotor on various Hb variants in different genotypes.

Hb variant	Pre Voxelotor				Post Voxelotor				Voxelotor interrupted			
	HbS %	HbF %	HbA2 %	HbD %	HbS %	HbF %	HbA2 %	HbD %	HbS%	HbF%	HbA2%	HbD%	HbA%
HbS/S	92.8	3.3	3.8	0	73.5	1.1	3.5	22	25.8	1.0	3.2	0	68.8
Hb S/β^0	87.5	6.3	5.5	0	56.8	3	4.1	53					
HbS/D Punjab*	43.7	5.4	3.4	47.5	20	3.0	3.0	67.7					

*The patient with genotype HbS/D-Punjab is based on a live case, but is not part of the Voxelotor study; therefore, the post-Voxelotor levels of Hb variants are a rough estimates only.

their hematological parameters and, as seen on in Figure 1B, and displayed no D-peak on the HPLC test.

Discussion

We present results seen from the data on eight patients with SCD, who are exposed to Voxelotor therapy and they unequivocally show a significant improvement in Hb. This was also correlated with a significant improvement of the hemolytic markers including a significant reduction of reticulocytes, bilirubin and LDH. In HbS/S patients with sickle cell anemia (SCA), there are two peaks, one representing HbS and the other Hb-Voxelotor fraction, the latter having similar mobility as native HbD in HPLC assays. This implies that HPLC could indeed be additionally used to monitor patients' compliance and adherence to the drug. Similarly, we observed an apparent disappearance of the HbD peak upon a 2-week interruption of the medications, due to an acute sickle cell crisis, as illustrated by one of the patients in Table 2, indicating the reversible nature of binding between Voxelotor and hemoglobin. In addition, this patient required a blood transfusion/exchange, as characterized by the discrepancy in the levels of HbS seen in Table 2. Our data also suggested that the impact of Voxelotor on both genotypes HbS/S and HbS/β was roughly identical; however, patient with HbS/β0 genotype had the highest increase in HbD, possibly indicating an enhanced drug effect and binding of Voxelotor to hemoglobin. We also generally observed the higher the rise in Hb, the more pronounced level of HbD on HPLC. Similarly, although we did not have any HbS/D-Punjab patient taking Voxelotor in this group, the HPLC profiles of these patients will be of interest and may cause further confusion when calculating the actual amount of HbS with HbS-Voxelotor complexes in these samples as indicated in Table 2. Therefore, we wish to highlight and emphasize to the laboratory scientists, transfusion medicine specialists, and clinicians providing care for patients with SCD that they need to understand the precise underlying mechanism of Voxelotor therapy and consider its effects on hemoglobin quantitation by existing techniques of hemoglobin estimation.

For this purpose, the laboratory scientist and technicians could easily be trained to recognize the distinct appearance of Voxelotor-modified HbS peaks that run as HbD, as shown in Figure 1, to avoid this pitfall. Further, there is a necessity for appropriate communication between the laboratory staff and the treating clinician to crucially avoid the misinterpretation of HPLC results. Lastly, the decision to report "total" HbS or HbS without HbS-Voxelotor complexes could lead to a confusion in the number of units of RBCs a patient receives in an exchange transfusion setting and, subsequently, estimate its efficiency. Godbey et al. (9), have also reported on this dilemma in their patients coming to the apheresis clinic for the RBC exchange program, who were also on Voxelotor therapy. Thus, additional studies into the structure-function relationship of the hemoglobin-Voxelotor complexes are necessary to understand how quantitative HbS results should be reported in the presence of this agent. We also observed that two of our patients (Cases 7 and 8) did not show any change in their hematological parameters and, showed no appreciable clinical responses, as seen on in Figure 1B, and displayed no D-peak on the HPLC test, possibly indicating that these patients may not

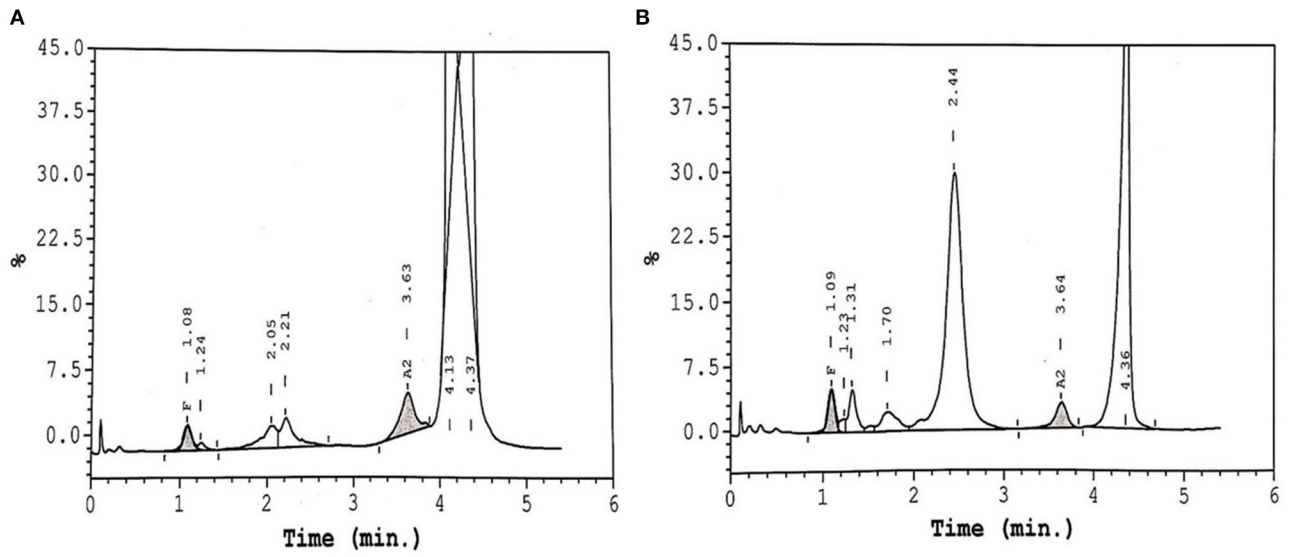

FIGURE 1
HPLC chromatogram of two patients on Voxelotor therapy. **(A)** Shows the presence of HbS and Hb-Voxelotor fraction in the HbD window. **(B)** Shows a SCD patient on Voxelotor therapy showing only HbS without any HbD peak.

be compliant with the drug, although other mechanism for lack of response is also possible. On direct interrogation of these two patients, they also admitted to non-compliance with the drug due to various reasons including drug related side effects.

In conclusion, we reported here eight patients on Voxelotor therapy, six of whom showed improved hemolytic markers and anemia and demonstrated the appearance of HbD peak on the HPLC chromatogram. Therefore, the absence of HbD on HPLC or other laboratory methods for estimating HbS in patients on Voxelotor therapy, gives the clinician a possible hint regarding the patient's compliance with the drug.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Medical Research Ethical Committee, College of Medicine & Health Sciences. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SA and AP were fully involved in the conception and design of the study, acquisition, analysis, interpretation of data, and drafting of the article and critical appraisal before submission. All authors have made substantial contributions and have seen and approved the final version of the manuscript.

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Conflict of interest

SA declares that he is the principal investigator in the GBT440-034 clinical trial.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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