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Editorial

What is next for Hematology, Hemotherapy and Cell Therapy? A message and an invitation from incoming editors

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Along with the start of 2026, we assume the great responsibility of continuing the work of editors who in the past brought our journal *Hematology, Hemotherapy and Cell Therapy* (HTCT) to the place it occupies today in the scientific publishing landscape. Throughout this time, the journal has achieved national and international recognition, illustrated, but not exclusively defined, by indexing and impact metrics. It is fair to say today that, nationally, HTCT has ceased to be a channel for publishing articles of local interest, becoming a privileged destination for disseminating national scientific publications. And internationally, the journal's visibility is confirmed by the year-on-year increase in the number of submissions, which reached 646 in 2025, compared to 412 in 2021. The quality of peer review has followed this trend, leading to an acceptance rate of 17% of submitted articles in the last year, further illustrating the challenge faced by our editorial board. Finally, in addition to this list of HTCT's qualities, we have what is perhaps our greatest asset: the exclusively scientific basis of its editorial policy, in an environment where other interests compete with science in the publishing industry. In this scenario of growth and consolidation, it is important that, as we assume this responsibility, we ask ourselves, and bring to the scientific community linked to HTCT the question: what new paths should be followed?

Having posed the question, we take the liberty of presenting some of the impressions that guide us in this endeavor. The first is to maintain and expand the relevance of HTCT in the global environment scientific publications in our field, which is broadly speaking, a scenario that has been undergoing important changes. According to a report prepared by Carlos Henrique Brito Cruz for the UN Multistakeholder Forum on Science, Technology and Innovation (2025), in the year 2024, 60% of scientific articles published worldwide had authors from low- and middle-income countries, a stark contrast to what occurred 30 years ago, when 87% of publications originated from wealthy countries.[1] This change is not only demographic, but also brings with it a broadening of scientific agendas, represented by the yet timid incorporation of themes such as the management of global health systems, innovation in resource-limited contexts, "One Health", and neglected diseases. Cross-cutting themes such as education in healthcare profession, disease burden in under-represented epidemiological scenarios, mental health and well-being consequences of disease, and solutions for incorporating new technologies such as AI are also gaining strength in this process, without prejudice to the fundamental themes of science in our area such as basic science, translational, epidemiology, and clinical intervention studies. In this scenario, we believe that increasing the visibility and relevance of HTCT involves attracting and incorporating high-quality publications that bring together these emerging areas with our core essential interest, Hematology, Hemotherapy, and Cell Therapy, which will always be protected and guide our editorial line. In fact, new scientific journals from highly relevant entities in our field have been recently vocalizing similar objectives.

At the same time, we understand that the link of HTCT to scientific entities of recognized seriousness and the possibility of subsidized open access is an element that should be further explored, as a guarantee of HTCT's scientific seriousness in attracting publications from the most affluent and traditional countries in science in our area, as well as in consolidating HTCT as the preferred publication vehicle for high-quality articles produced in Brazil and in the countries most directly linked to our community, whose quality of scientific

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production in hematology is growing more and more.[2] Here, it is also worth highlighting the recent changes in the Brazilian national policy for evaluating postgraduate programs, which modulates the emphasis on scientific journal impact metrics, beginning to value other elements that, in our view, are already being progressively more recognized in HTCT.

Finally, considering the increasingly important role of social networks as an environment for discussion and dissemination of scientific production, it is part of our objectives to seek strategies to further integrate HTCT into this environment.

For all these objectives, we need the collaboration of the entire community currently connected to HTCT. The generous contribution of those who are invited to act as reviewers. The tireless commitment of our editorial board, in the pursuit of both quality and speed in the evaluation of submissions, taking as a reference the deference of the position attributed to them by our entire community. And to everyone, in the invitation to consider HTCT more and more as a vehicle for publishing their best articles and reviews.

We conclude by inviting you to express your suggestions and criticisms through our contacts. And reiterating our most sincere commitment to contributing to this new phase of HTCT.

Data availability statement

N/A.

Conflicts of interest

The authors declare no conflicts of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Identification of an in-frame insertion in ACKR1 in five individuals from Agri community, India

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ABSTRACT

Introduction: Atypical chemokine receptor 1 (*ACKR1*) which carries the Duffy antigens, is not just a blood group antigen but serves many more functions. It is a receptor for various pro-inflammatory and inflammatory chemokines and for *Plasmodium vivax*. Genetic variations in *ACKR1* are the basis for Duffy blood group antigens.

Methods: Routine serological Fy^a/Fy^b typing and *ACKR1* genotyping by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism was employed in a population study that included 331 samples from the Agri community.

Results: Weak Fy^b expression was detected by serological findings in five unrelated samples, which prompted further investigation by molecular means. By Polymerase Chain Reaction an aberrant pattern was demonstrated on polyacrylamide gel electrophoresis, which led to the identification of an alteration by sequence analysis. This study describes a 3-bp insertion, present in the *FY*B* allele (c.144_146dupTGC), resulting in the insertion of the amino acid alanine (p.A49dup) within the full-length protein.

Conclusion: The 3-bp in-frame insertion (c.144_146dupTGC, p.A49dup) (rs765671589) in the *ACKR1* gene was identified in five individuals from the Agri community. Despite apparently carrying an *FY*B* allele, a very weak Fy^b antigen expression was found in association with this genotype. This insertion may also have implications for some physiological roles of *ACKR1* and be of interest in malaria research and population genetics.

Introduction

Atypical chemokine receptor 1 (*ACKR1*) [1] is a multi-pass trans-membrane protein found on the erythrocyte surface that is involved in the invasion of red blood cells (RBCs) by *Plasmodium vivax* (*P. vivax*) merozoites [2]. It is considered one

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of the most paradigmatic examples of positive selection in the human genome due to its strong geographic differentiation and relationship with resistance to vivax malaria [2]. It is a G protein-coupled receptor (a 35–50 kDa glycoprotein) found on the surface of RBCs and endothelial tissue [3]. ACKR1 is also expressed in erythroid progenitor cells of bone marrow, where *P. vivax* invasion may occur [4,5]. *ACKR1* expresses the antigens of the Duffy blood group system where the major alleles are *FY*A (FY*01)* and *FY*B (FY*02)*, which are co-dominantly expressed.

The Duffy blood group system is clinically significant in transfusion medicine because antibodies against its antigens may cause haemolytic transfusion reactions (HTR) and haemolytic disease of the newborn (HDN) [3,6]. *FY* (*FY*A and FY*B*) alleles differ by a single base change at nucleotide c.125 due to a *G > A* substitution (rs12075) [3]. The resultant antigens differ by a single amino acid at the 42nd position that encodes glycine in (Fy^a) and aspartic acid in (Fy^b) (D42G). This amino acid change does not affect chemokine binding affinity [7]. Anti-Fy^a and anti-Fy^b antisera allow detection of the four main phenotypes: Fy(*a + b+*), Fy(*a + b-*), Fy(a-*b+*), and Fy(a-b-) [6,8]. Although the Fy(a-b-) phenotype is the dominant phenotype in black human populations, particularly those of West African descent, it is rare in non-black people [8,9]. Most West Africans and two-thirds of Afro-Americans do not express ACKR1 on the surface of RBCs resulting in the Fy(a-b-) phenotype [3,10–12]. The absence of ACKR1 from the RBC surface is due to a homozygous substitution (c.–67T>C) (rs2814778) in the 5′ untranslated region of the *FY* gene, also known as the *GATA-1 box* (FY*02 N.01).

The Fy^x phenotype is caused due to the polymorphisms at nucleotide c.265 *C > T* (rs34599082) and at nucleotide c.298 *G > A* (rs13962) [12–14]. These nucleotide changes reduce Fy^b antigen expression to the extent that only a few anti-Fy^b reagents detect the antigen by haemagglutination. Weak Fy (*b +* ^{w}) [Fy^x] expression has also been linked to the loss of a nucleobase C in the sequence spanning the regulatory element *Sp1* site [15]. Other polymorphisms in the *ACKR1* gene that silence *FY* [or a Fy(a-b-)] phenotype include a 14-nucleotide deletion in Fy^a, which causes a frameshift and a premature stop codon [16], and three separate nucleotide changes, which are responsible for converting the Trp codon found at different positions to a stop codon in either *FY*A* or *FY*B* alleles [16,17].

The distribution of the ACKR1 allele *FY*02 N.01* has been used to assess the admixture and ethnicity of various populations, e.g., Saudi Arabs, Israeli Jews, Njazidia, and descendants of Africa [18–22]. Characterizing the pattern of genetic variations among numerous ethnic populations is vital for constructing human evolutionary records, investigating population records, and evaluating the right design and acumen of genetic disease affiliation studies [23,24].

Genetic variations among different communities in India are opaque. There exists vast human diversity in India, with more than four thousand anthropologically well-defined populations, each differing in language, culture, customs, and genetic makeup [25]. Amongst them, one of the populations is the Agri community. This community is seen mainly in Thane, Raigad and Palghar districts of Konkan division, Maharashtra, India. They are mainly engaged in farming, fishing, and salt making. Their population in India is estimated to be about 524,000, and they are reported to be found only in India [25]. They consider themselves just below Brahmins and Kshatriyas in the Indian caste hierarchical system. The Agris is subdivided into several divisions (*snull kul*), which regulate marriages. Exogamous marriages are allowed, but within the same *snull kul*, consanguineous marriages are not allowed [26]. Inter-community weddings are rare in Agris.

While studying the occurrence of *ACKR1* gene polymorphisms in the Agri community, a 3-bp insertion (c.144_146dupTGC) insertion (rs765671589) was identified. This in-frame insertion is predicted to insert Alanine at position 49 of the major isoform of the ACKR1 protein. ACKR1 expression on RBCs may be decreased or absent due to this nucleotide change in the *FY* gene. This example is remarkable with a 3-bp in-frame insertion (c.144_146dupTGC) to be documented in an Indian tribal population with serological data to support the effect of alteration on Duffy antigen stability. This study highlights the importance of establishing the incidence and nature of molecular events that could impact ACKR1 antigen expression in Agri.

Materials and methods

Study population

A total of 331 samples from the Agri community were collected during various camps organized by Indian Council for Medical Research –National Institute of ImmunoHematology (ICMR-NIIH) in Navi-Mumbai, Maharashtra to investigate the incidence of different red cell antigens. Unrelated males and females aged over 18 years participated in this study. Information on the patient's ethnic background, presence of other diseases such as diabetes, infectious disease (including malaria), and smoking were obtained by interviewing the individuals.

Ethical considerations

Detailed information was provided and explained about the research to be carried out on their blood sample to all the individuals who agreed to participate in the study. All studies were performed according to the recommendations put forth by the Institutional Ethics Committee for Research on Human Subjects, National Institute of Immunohaematology (ICMR), Mumbai.

Blood collection

Peripheral blood was collected (3 mL in EDTA and 4 mL in plain vacutainers) and stored at 4 °C till samples reached the Institute. The collected blood was used for serological and molecular typing of various blood group antigens including Duffy.

Serological testing

Duffy phenotyping was determined by haemagglutination assay using a monoclonal antibodies Anti-$Fy^a$and Anti-Fy^b (Gamma-clone); Cat.: 3013–2; Immucor, Inc. Norcross, GA,

USA) by the direct tube method and gel cards (Diamed SA, Morat, Switzerland) according to manufacturer's recommendations. Suitable controls were included during the serological phenotyping of RBCs.

DNA extraction

The phenol-chloroform method was employed to isolate genomic DNA from the peripheral blood [27]. DNA quantity and quality were assessed by Nanodrop-1000 (Thermo Fisher Scientific, Massachusetts, US), and the samples were diluted to attain a final concentration of 30 ng/μL.

Polymerase chain reaction-restriction fragment length polymorphism genotyping

ACKR1 polymorphisms which included promoter *GATA-1 box* c.−67T>C (*FY* Null* (*FY*02 N.01*), nucleotide changes c.125 *G > A* (*FY*01/FY*02*), c.265 *C > T*, and c.298 *G > A* (*FY*01 W.01 or FY*02 W.01*) were identified by polymerase chain reaction followed by restriction fragment length polymorphism (PCR −RFLP) as described by Castilho et al. [28] with minor modifications which included the choice of primer sequence (Table 1) and restriction enzymes (Table 2).

Polymerase chain reaction (PCR) was performed using 150 ng of DNA, 5 pmol of each primer, 2.5 nmol of each dNTP, 1.0 U Taq polymerase, and buffer (Genei Labs, Bangalore, India), a total volume of 25 μL. Two sets of primers were used for genotyping *ACKR1* variants. The first set of primer pair (ACKR1_FP and ACKR1_RP, Table 1) flanked c. −67 *T > C* GATA-1 of the erythrocyte-specific transcription factor eTFII. The second primer pair (FY_AB_FP and FY_AB_RP) spanned the *ACKR1* gene region containing the remaining three polymorphic sites.

PCR was performed in a thermal cycler (S-96 Gradient Thermal Cycler, Quanta Biotech, USA) with the cycling conditions as 95 °C for 5 min, followed by 35 cycles [95 °C for 45 s; annealing at X °C (Table 1) for 45 s; elongation at 72 °C for 45 s]; final extension of 5-min incubation at 72 °C; and 4 °C 5-min. The PCR products were loaded on 1 % agarose in Tris-acetate-EDTA (TAE) buffer and were electrophoresed for 35 mins at 80 V to check for amplification efficiency before treatment with restriction enzymes as per the manufacturer's instruction. The digested products were run on 12 % polyacrylamide gel. The restriction digestion patterns of the PCR products (in base pairs) with specific enzymes are enlisted in Table 2.

DNA sequencing

The polymorphic sequence within exon 2 was amplified by PCR using FY_AB_F and FY_AB_R primers (Table 1). The amplified PCR product was cleaned using ExoSAP-IT (USB Corporation, Cleveland, Ohio) and then sequenced using 3700XL automated DNA sequencer (Applied Biosystems, USA). Sequencing was performed using the fluorescent Big-Dye Terminator v.1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, US) as per the manufacturer's protocol. The National Center for Biotechnology Information reference sequence used for *ACKR1* was NM_002036.2. Mutation surveyor analysis tool was used to analyse the raw data obtained from DNA sequencer.

NEBcutter V2.0

The online tool NEBcutter V2.0 was used to assess if the insertion of 3-bp in the coding sequence (sequence flanked by FY_AB_FP and FY_AB_RP primers), generates aberrant

Table 1 – Primer sequences flanking *ACKR1* and *GATA-1* and three Single Nucleotide Polymorphisms.

Name	Sequence (5′ → 3′)	PCR Product Size (bp)	T_m (°C)	T_a (°C)
ACKR1_FP	CATGGCACCGTTTGGTTCAG	189	61.3	58.1
ACKR1_RP	CAAGGCCAGTGACCCCCATA			
FY_AB_FP	TCCCCCTCAACTGAGAACTC	392	58.2	55.3
FY_AB_RP	AAGGCTGAGCCATACCAGAC			

Table 2 – Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) genotyping of ACKR1 antigens using different restriction enzymes.

rs Number	Amino Acid Change	Restriction Enzyme	Genotype	Digestion	PCR Restriction Fragment Lengths (-bp)
rs2814778 (c.−67T>C)	-	*Sty I* 5′…C^CWWGG…3′ 3′…GGWWC^C…5′	T/T	Undigested	189
			T/C	Digested	189, 108,81
			C/C	Digested	108, 81
rs12075 (c.125G>A)	G42D	*Ban I* 5′…G^GYRCC…3′ 3′…CCRYG^G…5′	A/A	Undigested	392
			A/G	Digested	86, 94, 212, 306
			G/G	Digested	86, 92, 212
rs13962 (c.298G>A)	A100T	*Mwo I* 5′…GCNNNNN^NNGC…3′ 3′…CGNN^NNNNNCG…5′	A/A	Undigested	392
			A/G	Digested	51, 67, 274, 341
			G/G	Digested	51, 67, 274
rs34599082 (c.265C>T)	R89C	*Aci I* 5′… C^CGC…3′ 3′… GGC^G…5′	G/G	Undigested	392
			C/G	Digested	156, 236, 392
			C/C	Digested	156, 236

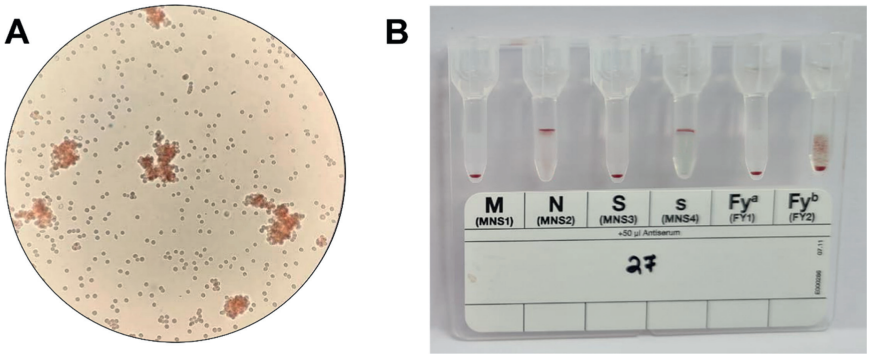

Fig. 1 – Serological typing of ACKR1 antigen. A. Standard serological typing of ACKR1 antigen using tube method. Weak to very weak agglutination reaction was observed under (X10) microscopic field. B. Validation of tube method of ACKR1 investigation using Diamed gel cards showing no agglutination reaction (Fy^a) and weak agglutination reaction (Fy^b).

band size on gel when digested by restriction enzyme *Ban I*.

In silico analysis

PROVEAN (Protein Variation Effect Analyzer) is an online software tool that predicts the impact of nucleotide substitution or indel on the protein's functionality. The nonsynonymous or indel variants that are expected to have functional significance can be found using PROVEAN. PROVEAN uses the score thresholds for prediction (deleterious or neutral). The default threshold value is set to −2.5. Variants with scores equal to or below −2.5 were considered "Deleterious," while the variants with a scores above −2.5 were considered "Neutral." For our analysis, the variant score was generated by comparing 215 sequences and constructing 30 clusters. (link: https://www.jcvi.org/research/provean) (supplementary data)

Results

Serology

Three hundred and thirty-one samples were tested using FY monoclonal antibodies by standard tube technique. Of these, 326 samples showed either Fy(*a* + *b*-), Fy(a-*b*+), or Fy(*a* + *b*+) phenotypic distribution. FY null [Fy(a-b-)] was not found in the tested population. Five samples showed weak to very weak (+) agglutination reactions with anti-Fy^b for Fy^b (Figure 1A); however, when tested on gel cards, it was found to be a mixed field (mf) reaction type (Figure 1B); which prompted us to investigate these samples further using molecular analysis.

Polymerase chain reaction-restriction fragment length polymorphism

All five analysed samples produced 189-bp products using ACKR1_FP and ACKR1_RP primers, which on digestion with *Sty I* produced a product of size viz. 189-bp, indicating the presence of wild-type T at c. −67 *T* > *C* (*GATA*-1) position. The five amplified samples using the primers FY_AB_F and FY_AB_R were genotyped as Fy(a-*b*+) using *Ban I* restriction enzyme. The aberrant band migration patterns observed post-digestion with *Aci I* and *Mwo I* enzymes were characterized by slight shifts in the electrophoretic mobility of the restriction fragments compared to control samples. Specifically, the 156-bp and 236-bp fragments from *Aci I* digestion and the 274-bp fragment from *Mwo I* digestion showed altered migration (Figure 2) distances due to the 3-bp in-frame insertion that modified the overall conformation of the DNA fragments, despite not creating or destroying restriction sites. These subtle but consistent mobility differences prompted further sequence analysis to identify the exact nature of the genetic variation.

DNA sequencing

The five samples with aberrant band patterns were taken up for Sanger sequencing to delineate the sequence changes. Mutation surveyor analysis tool was used for electrophoretogram analysis which showed the insertion of three bases TGC [c.144_146dupTGC; c.144_146dup] (Figure 3) in *ACKR1* CDS when compared to the reference sequence (NM_002036.2). This 3-bp in-frame insertion resulted in the insertion of an amino acid Alanine (A49dup) (Figure 3). The 3-bp insertion resulted in an increment of an amino acid to 337 as against wild type with 336 amino acids (Supplementary Figure 1). This variant was deposited in the dbSNP database under reference Single Nucleotide Polymorphism (SNP) cluster ID: **rs765671589**. However, neither publication report nor clinical significance is available pertaining to the **rs765671589** on the dbSNP website. The tested sample also did not show presence of any other known variant other than c.144_146dupTGC. The aberrant pattern observed on acrylamide gel might be due to the in-frame insertion of three bases. It is important to emphasize that the 3-bp insertion in the tested samples was in heterozygous state.

In silico analysis

In silico analyses using the online platform PROVEAN predicted the three-base insertion to be deleterious with a score value of −5.021 (cut-off = −2.5) (Supplementary Figure 2 and

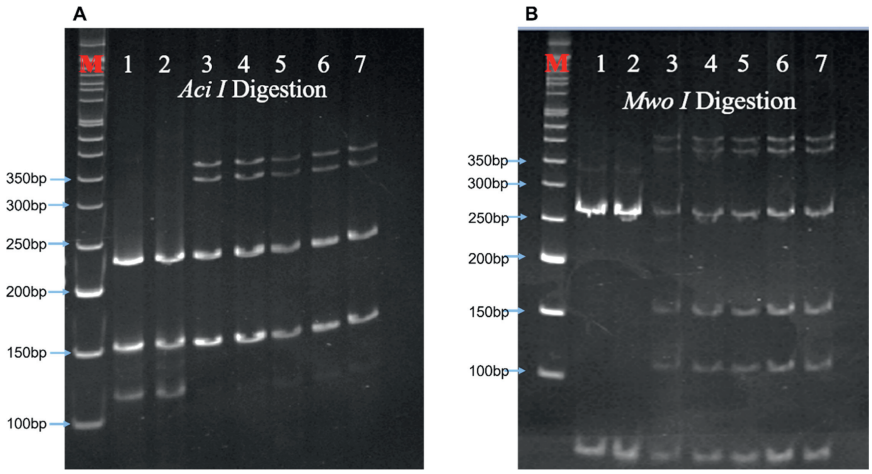

Fig. 2 – Polyacrylamide gel electrophoresis. Aberrant band pattern separation of restriction enzymes A. *Aci I* and B. *Mwo I* digested polymerase chain reaction (PCR) product on 12 % polyacrylamide gel. The gel picture showed the restriction digestion patterns of the PCR products from samples under investigation (samples 3, 4, 5, 6, and 7) and samples with known genotypes, which were used as the digestion and migration controls (samples 1 and 2). *Aci I* digestion produced two fragments viz. 156-bp and 236-bp, while *Mwo I* produced three fragments of sizes 51-bp, 67-bp and 274-bp. M: 50 bp DNA ladder (Cat. No.: DM012-R500, GeneDirex). The gels were stained with ethidium bromide (0.5μg/mL final concentration in 0.5x TBE buffer) for 15 mins with gentle shaking, followed by destaining for 20 mins with deionized water.

supplementary data). NEBcutter V2.0 analysis showed that the insertion of 3-bp did not alter the recognition and restriction sites of the enzyme.

Discussion

This paper describes a 3-bp in-frame insertion (c.144_146dupTGC, p.A49dup) identified in five individuals (probably unrelated) of Agri descent who were identified to be *FY*B* homozygous. The RBCs from these individuals showed weak to very weak (+) agglutination reactions with anti-Fy^b, indicating that the reduction of Fy^b antigen on their surface produced a visible, very weak haemagglutination reaction under the microscope. We hypothesize that the altered ACKR1 protein (p.A49dup) might: 1) not be well integrated on the RBC membrane, 2) be inefficiently transported to the membrane, or 3) be substantially degraded before being transported on RBCs membrane. The former events may affect the detection of the Fy^b antigen on RBCs by commercially available antisera.

The insertion of Ala at amino acid 49 position in the extracellular segment of Fy^b is predicted to be deleterious by the *in silico* platform PROVEAN. Alanine is an ambivalent and nonpolar amino acid with an optically active chiral C atom. It has a β-carbon (methyl group), which hinders conformation changes that the backbone can adopt. Being a hydrophobic amino acid, alanine contributes to closeness in protein folding by repelling water. The presence of extra alanine residues might mitigate the structural integrity.

The expression pattern observed in this study bears similarities to findings reported by Parasol et al. [12], who described a novel change in the *FY*B* allele leading to an altered erythrocyte phenotype, though in their case it was due to a *C > T* substitution at nucleotide 265. Similarly, Tournamille et al. [13] reported that an Arg89Cys substitution results in very low membrane expression of the Duffy antigen/receptor for chemokines in Fy^x individuals. Our findings represent a different molecular mechanism (insertion rather than substitution) leading to reduced Fy^b expression, adding to the spectrum of known Duffy-related variations with phenotypic consequences.

It is important to note that the insertion described here is in a heterozygous state, and the effect of this alteration needs to be addressed in individuals with homozygous insertion, providing a piece of concrete evidence on protein stability on RBCs. We made attempts to approach the individuals; however, due to unavoidable situations, we were not able to collect specimens from family relatives, keeping this avenue to be explored in the future.

Genotyping of blood group antigens, including Duffy, has been used for decades to assess population admixture and ethnic backgrounds. *ACKR1* polymorphisms are characterized in African populations, and the identity of the Fy^{bES} variation in non-African populations has been considered a probable situation for the admixture of African–American [12,28,29]. Consequently, our data suggest that c.144_146dupTGC, p.A49dup in *ACKR1* may be peculiar to the Agri community. The data does not confirm the racial identity specific to the community. Even though the observation provides insight that this alteration may be specific to this community, we need to establish/prove this hypothesis. The Agri community comprises people who prefer marriage within the community, and this endogamy might be the reason for the observed higher occurrence of this variation in this specific community.

The identification of a novel 3-bp insertion (c.144_146dupTGC; rs765671589) in the *ACKR1* gene within the

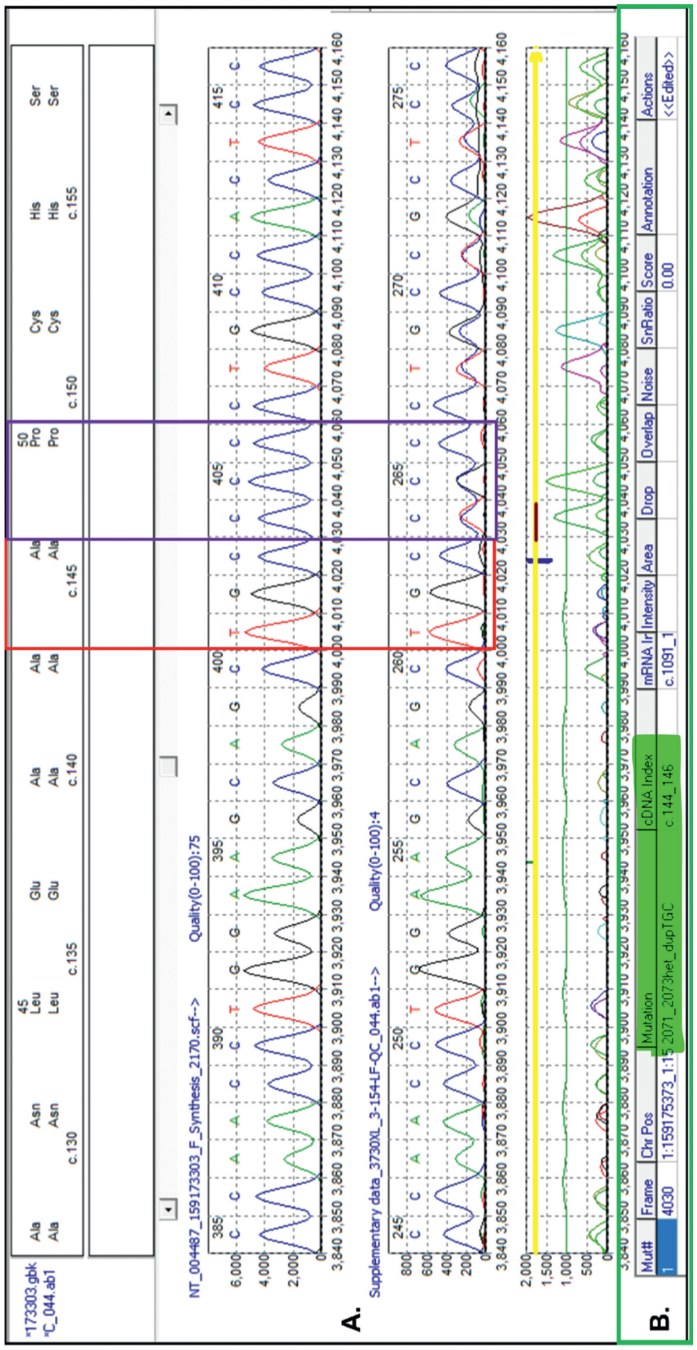

Fig. 3 – Electrophoretogram of *ACKR1* sequence. A. Comparison of electrophoretogram of polymerase chain reaction (PCR) product from a control sample (wild-type sequence) and sequence with 3-bp insertion (altered sequence). B. The result out-put generated by Mutation Surveyor shows insertion of three base pairs (TGC) in *ACKR1* sequence.

Agri community represents a significant molecular discovery, particularly given its absence in published literature despite being catalogued in the dbSNP. Our documentation of this insertion provides the first comprehensive characterization of rs765671589 in a specific population, contributing valuable data to the growing landscape of *ACKR1* polymorphisms. This finding has multifaceted implications: it enhances our understanding of genetic diversity in minority populations, potentially influences individual and population-level disease susceptibility profiles, and provides crucial insights into regional genetic adaptations. Furthermore, this discovery opens new avenues for investigating the functional consequences of *ACKR1* modifications and their potential impact on receptor expression, chemokine binding affinity, and disease associations in the Agri community. The presence of rs765671589 (c.144_146dupTGC, p.A49dup) might also have implications for managing transfusion therapy in this community.

The *FY*B* product is often difficult to detect using commercially available anti-Fy^b reagents used in routine RBC typing. Often Fy^x identification by anti-Fy^b goes undetected, and such samples are reported as negative. This is true even when the tests are repeated under the same conditions [30]. It is recommended to type RBCs using PCR/PCR-RFLP-based methods to distinguish Fy^x samples from that of Fy null [Fy(a-b-)] instead of employing several anti-Fy^b sera or a labour-intensive and cumbersome adsorption and elution technique. William et al. showed that ACKR1 antigens weaken on storage at 4 °C [31]. It is, thus, suggested to phenotype the samples for ACKR1 antigens immediately without delay.

Gene alternations conferring malaria resistance are often under balancing selection, maintaining deleterious alleles at high frequencies [31]. Erythrocyte polymorphisms, strongly shaped by malaria [32,33], affect structural proteins or metabolic enzymes, limiting parasite growth [34]. These polymorphisms are prevalent in malaria-endemic regions [35]. King et al. demonstrated the higher affinity of Fy^b for *P. vivax* -ACKR1 binding protein compared to Fy^a [36]. The p.A49dup insertion, predicted to be deleterious *in silico*, may protect against malaria by affecting Fy^b stability. Further research is needed to quantify its protective effect compared to Fy^a through binding and inhibitory assays, particularly for *P. vivax* infections.

Our study provides valuable insights into the c.144_146dupTGC (rs765671589) variant in the *ACKR1* gene, though there are several areas where future research could further enhance our understanding. While we successfully characterized the variant in heterozygous individuals, the identification and analysis of homozygous cases would provide additional insights into its full impact on ACKR1 protein expression and function. Although we made considerable efforts to collect family samples from the identified cases, unforeseen circumstances prevented this extension of our study, presenting an opportunity for future family or community-based investigations. The study opens several promising avenues for further research, including 1. Expanded molecular characterization using quantitative protein expression analysis to complement our current serological findings. 2. Additional functional studies to explore the variant's potential effects on chemokine binding and *P. vivax* interactions. 3. Broader geographical sampling within the Agri community to better understand the variant's distribution pattern. 4. Longitudinal follow-up studies to observe Fy^b expression patterns over time, if possible. 5. Extended family studies to better understand the inheritance patterns of this variant within the community. These research opportunities could provide valuable additional context to our findings and further illuminate the role of this variant in *ACKR1* function. Our current results lay a strong foundation for such future investigations, which could build upon the molecular and serological characterization we have established.

Conclusion

In summary, we report five individuals with the *FY*02* allele carrying c.144_146dupTGC, p.A49dup in exon 2 of the *ACKR1* gene, with significantly reduced Fy^b expression on the RBCs surface. This in-frame insertion, resulting in p.A49dup, has not been previously described. The in-frame change identified may not be directly related to the exposure of a human genome to *P. vivax*. However, it may have arisen due to *de novo* insertional alteration specific to the Agri community. However, this hypothesis needs further supporting data. Identifying rs765671589 genotypes in *FY* will provide clues for better genotype-phenotype correlation and may aid in explaining the molecular pathogenesis in diseases and in population genetics. These discoveries feature the significance of different investigations for better comprehension of the genetic basis of blood group antigens.

Author contributions

S.R. conducted the experiment. S.P. provided technical help. G.A. and G.K. supervised the experiments. S.R. conducted data analysis. G.A. conceptualized the project, was responsible for the overall supervision, and procured funding. S.R. wrote the manuscript. G.A. and G.K. approved the final manuscript.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Generative AI and figures, images and artwork

The authors did not employ generative AI or AI-assisted technologies to write the manuscript and take full responsibility for the content of the publication.

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Conflicts of interest

The authors report no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2025.106075.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Evaluation of the effects of fucoidan extracted from sargassum angustifolium on coagulation factors and biochemical parameters in male Wistar rats

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ABSTRACT

Introduction: This study investigates the effects of fucoidan extracted from Sargassum angustifolium on coagulation factors and biochemical parameters in male Wistar rats. Fucoidan, a sulfated polysaccharide from brown algae, is known for its anticoagulant, anticancer, and antioxidant properties

Methods: The study involved 25 rats, divided into control, sham, and three experimental groups, receiving varying doses of fucoidan (100, 150, and 200 mg/kg body weight) over 28 days. The research focused on Prothrombin Time, Thrombin Time, and Partial Thromboplastin Time, along with biochemical markers like glucose, total protein, iron-related parameters, and albumin

Results: This study found that fucoidan administration did not significantly affect the hemostasis tests, suggesting minimal impact on coagulation pathways in vivo. However, a dose-dependent reduction in glucose levels was observed, highlighting the potential of fucoidan as a hypoglycemic agent. Additionally, significant increases in transferrin, iron, and ferritin levels were noted, implying enhanced iron absorption and storage

Conclusion: The findings underscore the therapeutic potential of fucoidan, particularly in managing glucose metabolism and iron homeostasis, while its minimal anticoagulant effect suggests safe usage in clinical settings where anticoagulation is undesirable. Further research is recommended to explore the full clinical benefits of fucoidan.

Introduction

Seaweeds have long been a dietary staple in many East Asian cultures, revered not only for their culinary versatility but also for their rich nutritional profiles. These marine organisms are abundant in soluble dietary fibers, proteins, minerals, vitamins, antioxidants, phytochemicals, unsaturated fatty acids, and various bioactive compounds. Recent studies have further illuminated the potential health benefits of seaweeds, highlighting their roles in reducing inflammation, preventing blood clots, combating obesity, and lowering blood pressure [1,2]. Beyond these traditional applications, seaweeds are increasingly being explored for

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their therapeutic potential in addressing serious conditions such as cancer, allergies, diabetes, oxidative stress, and degenerative diseases [3–5].

Among the diverse types of seaweeds, brown algae (Phaeophyceae) are particularly noteworthy. This group includes approximately 2000 species across 265 genera, such as *Ascophyllum, Macrocystis, Laminaria, Eclonia, Sargassum*, and *Fucus* [6]. A key compound in brown algae is fucoidan, a sulfated polysaccharide found in their cell walls. Fucoidan has garnered significant attention due to its wide range of biological activities, including anticoagulant, antiviral, anticancer, antitumor, anti-inflammatory, and antioxidant effects [7].

The coagulation process, which can lead to conditions such as heart attacks and strokes, is a major health concern globally. The formation of blood clots involves a complex cascade of enzymatic events, beginning with the activation of proenzymes and culminating in the conversion of fibrinogen to fibrin by thrombin [8]. Heparin, a widely used anticoagulant, functions by inhibiting several key factors in this coagulation cascade via antithrombin III. Despite its effectiveness, heparin is associated with a range of side effects, including bleeding, heparin-induced thrombocytopenia (HIT), eosinophilia, skin reactions, and disturbances in liver function [9,10].

Fucoidans, complex sulfated polysaccharides primarily derived from brown seaweeds, have been studied for their anticoagulant properties. Initial investigations suggested potent anticoagulant activity similar to that of heparin. However, subsequent studies have revealed that the effects of fucoidan on coagulation may be more nuanced, influenced by factors such as molecular weight, sulfation pattern, and the source of extraction [11,12].

Among its most notable therapeutic effects are its anticancer and antioxidant activities. Fucoidan has demonstrated the ability to induce apoptosis, inhibit tumor growth, and prevent metastasis in various cancer models, making it a promising candidate for cancer therapy. Its antioxidant properties, on the other hand, are crucial in combating oxidative stress-related diseases by scavenging free radicals and enhancing the body's endogenous antioxidant defenses. The anticoagulant effect raises concerns about the safety of fucoidan, particularly when used in conditions where normal blood clotting is essential [13–16].

Of fucoidan anticoagulant activity is primarily attributed to its structural characteristics, particularly the degree of sulfation and the presence of specific functional groups such as fucose, uronic acid, and sulfate. The anticoagulant effect of fucoidan is known to occur through several mechanisms, including the potentiation of antithrombin III activity, inhibition of thrombin generation, and interference with the binding of coagulation factors to cell surfaces. The sulfated groups in fucoidan molecules mimic the negative charge of heparin, allowing them to interact with antithrombin III and enhance its inhibitory effect on thrombin and factor Xa, crucial components in the blood coagulation cascade [17,18].

However, despite the extensive in vitro evidence of fucoidan anticoagulant activity, clinical studies have provided a different perspective. In human trials, the anticipated anticoagulant effects of fucoidan have not been consistently observed, and in many cases, fucoidan has been found to have minimal or no significant impact on the coagulation process. These findings suggest that the anticoagulant properties of fucoidan, while evident in controlled laboratory settings, may not translate to a clinical context, where complex physiological factors come into play [19].

This study aims to evaluate the effects of fucoidan extracted from *Sargassum angustifolium* on specific coagulation factors—Prothrombin Time (PT), Thrombin Time (TT), and Partial Thromboplastin Time (PTT)—as well as on biochemical parameters including total protein, glucose, transferrin, iron, ferritin, and albumin in male Wistar rats. By investigating these parameters, this research seeks to elucidate the potential of fucoidan as a therapeutic agent with a dual impact on coagulation and metabolic pathways. The results will provide valuable insights into the safe and effective use of fucoidan in clinical settings, particularly in managing complex metabolic conditions such as diabetes and anemia, while also addressing concerns related to its anticoagulant effects.

Materials and methods

Animal preparation and grouping

This study was conducted using 25 male Wistar rats, which were transferred to the animal care facility at the Islamic Azad University, Kazerun campus. Upon arrival, the rats were allowed to acclimatize to the laboratory environment for one week under standard housing conditions. These conditions included a controlled light-dark cycle (12:12 h), a temperature of 22 ± 2 °C, and consistent ventilation. During the acclimation period, the rats were provided with free access to a standard laboratory diet and water ad libitum. All rats received equal portions of the same standard diet at fixed times each day to minimize variations in glucose levels due to dietary factors.

Following acclimatization, the rats were randomly assigned to one of five groups, with each group housed individually in labeled cages to ensure accurate identification and monitoring throughout the study:

Control Group: Consisting of five rats, this group received only the regular diet without any additional treatment. Sham Group: Also consisting of five rats, this group received the regular diet plus 1 cc of distilled water administered orally (gavaging) daily for 28 days. This group served to control for any potential effects of the gavaging procedure. Experimental Group 1: This group included five rats that were administered 100 mg/kg body weight (BW) of fucoidan orally (gavaging) daily for 28 days along with the regular diet. Experimental Group 2: Consisting of five rats; this group received 150 mg/kg BW of fucoidan orally (gavaging) daily for 28 days in addition to the regular diet. Experimental Group 3: This group included five rats that were administered 200 mg/kg BW of fucoidan orally (gavaging) daily for 28 days along with the regular diet [20,21].

Blood sample collection and coagulation factor analysis

Blood samples were collected from each rat on day 28 at the end of the study period. The blood was drawn via tail vein

puncture under light anesthesia to minimize stress. Each blood sample was immediately placed on ice and transported to the laboratory for coagulation factor analysis.

The coagulation parameters analyzed included Prothrombin Time (PT), Partial Thromboplastin Time (PTT), and Thrombin Time (TT). These parameters were measured using standard coagulation assays to assess the impact of fucoidan on the extrinsic, intrinsic, and common pathways of the coagulation cascade.

Fucoidan extraction

The fucoidan was extracted from the brown algae *Sargassum angustifolium*, which was collected from the shores of Bushehr, Iran. The algae were identified at the Persian Gulf Research Institute, washed with seawater, and cleaned of debris and other impurities. The samples were dried in the shade for four days and stored in zip-lock plastic bags.

For extraction, 20 *g* of algae were mixed with 400 mL of distilled water and heated at 45 °C for 45 min on a shaker. Sodium chloride (NaCl, 1 g) was added to adjust the pH to 7. The pH was then adjusted to 7.5 with 0.1 M NaOH, and the mixture was kept at 45 °C for 3 h. After an hour, 24 *g* of NaCl were added. The resulting compounds were precipitated by adding 100 mL of absolute ethanol and left at room temperature overnight. The compounds were washed twice daily for two days with 500 mL of distilled water for 30–60 min on a shaker at room temperature.

On the second day, the mixture was centrifuged, and the supernatant was removed. An additional 100 mL of absolute ethanol was added and kept overnight at room temperature. On the third day, the mixture was filtered, and the remaining filtrate was dissolved in 150 mL of distilled water and incubated for an hour at 40 °C. The pH was adjusted to 3 with HCl and filtered using a 0.2-micron filter. The solution was then lyophilized to obtain fucoidan powder.

Biochemical analysis

Biochemical analysis of fucoidan, including monosaccharide composition and structural characteristics, was performed using high-performance liquid chromatography (HPLC) and Fourier-transform infrared spectroscopy (FTIR). The hydrolyzed polysaccharide sample (90 min in 2 M trifluoroacetic acid at 120 °C) was injected into the HPLC system (VARIAN, Pro Star, USA) using a mobile phase of acetonitrile/deionized water (90:10) at a flow rate of 2 mL/min. FTIR analysis (PerkinElmer FT-IR, Spectrum RXI, USA) was performed on the ground sample in potassium bromide (KBr), with signals collected automatically using 60 scans in the range of 4000–400/cm with a resolution of 32/cm.

Preparation of fucoidan solutions

One gram of fucoidan powder was dissolved in 50 mL of distilled water and placed on a shaker at 25 °C for 12 h. The resulting solution (0.02 g/mL) was filtered through a 0.4-micron filter and used for coagulation assays.

Coagulation assays

Prothrombin Time (PT): 200 μL of PT solution (thermo scientific) was warmed to 37 °C in a water bath. An aliquot of 100 μL of fresh plasma was added to test tubes containing 200 μL of PT solution, 190 μL of PT solution plus 10 μL of normal saline, and 190 μL of PT solution plus 10 μL of fucoidan. The coagulation time was measured as the time taken for fibrin formation.

Partial Thromboplastin Time (PTT): 100 μL of PTT solution (thermo scientific) was warmed to 37 °C in a water bath. An aliquot of 100 μL of fresh plasma was added to test tubes containing 90 μL of PTT solution, 90 μL of PTT solution plus 10 μL of normal saline, and 90 μL of PTT solution plus 10 μL of fucoidan. After 2 min, 100 μL of 37 °C calcium chloride solution was added and the coagulation time was measured.

Thrombin Time (TT): Plasma was mixed with bovine thrombin reagent containing bovine albumin, calcium chloride, and buffer. The clotting time was measured optically at a wavelength of 405 nm.

Biochemical analysis of blood samples

Total Protein: Total protein levels were measured using a standard Biuret method. Plasma samples were mixed with Biuret reagent and incubated at 37 °C for 10 min. The optical absorbance was then measured at 540 nm using a microplate reader (BioTek, USA). The total protein concentration was calculated based on a standard curve prepared using bovine serum albumin (BSA).

Glucose: Glucose levels were determined using a glucose oxidase-peroxidase (GOD-POD) method. Plasma samples were incubated with glucose oxidase and peroxidase enzymes at 37 °C for 15 min, and the colorimetric reaction was measured at 505 nm. A glucose standard solution was used to generate a standard curve for glucose quantification.

Iron-Related parameters

Transferrin: Serum transferrin levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Azma plast), following the manufacturer's instructions. Optical absorbance was read at 450 nm, and transferrin concentration was calculated based on the standard provided with the kit.

Iron (Fe): Serum iron concentration was measured using a colorimetric iron assay kit (Delta.dp). The plasma samples were mixed with a chromogen that binds iron to form a colored complex, and optical absorbance was measured at 562 nm.

Ferritin: Ferritin levels were determined using an ELISA kit (Delta.DP), following the manufacturer's protocol. Optical absorbance was read at 450 nm, and ferritin concentration was calculated from a standard curve.

Albumin: Plasma albumin levels were measured using the bromocresol green (BCG) method. Plasma samples were mixed with BCG reagent, and optical absorbance was measured at 630 nm using a microplate reader. Albumin concentration was determined using a standard curve prepared with known albumin standards.

Transferrin Saturation: Transferrin saturation percentage was calculated using the following formula:

$$\text{Transferrin Saturation } (\%) = \left(\frac{\text{serum iron}}{\text{total iron binding capacity } (TIBC)}\right) * 100$$

$$TIBC = \text{Transferrin} \times 1.25$$

Serum iron and TIBC levels were measured as described previously, and transferrin saturation was determined accordingly.

Results

The effects of fucoidan extracted from Sargassum angustifolium on coagulation factors and biochemical parameters were evaluated in male Wistar rats.

Coagulation factors

Prothrombin time (PT)

The PT is a critical parameter used to evaluate the extrinsic pathway of coagulation. In this study, PT values were measured across the control group and the fucoidan-treated groups, which received varying doses of fucoidan. As illustrated in Figure 1, while there were slight variations in PT across the different groups, these differences were not statistically significant. The 200 mg/kg BW fucoidan group exhibited a marginally higher PT compared to both the control group and the lower-dose fucoidan-treated groups. However, the overall consistency in PT values suggests that fucoidan, at the doses administered, does not significantly alter the extrinsic coagulation pathway.

Thrombin time (TT)

TT is a measure of the time it takes for thrombin to convert fibrinogen into fibrin, an essential step in the final stages of the coagulation cascade. The TT results, as shown in Figure 1,

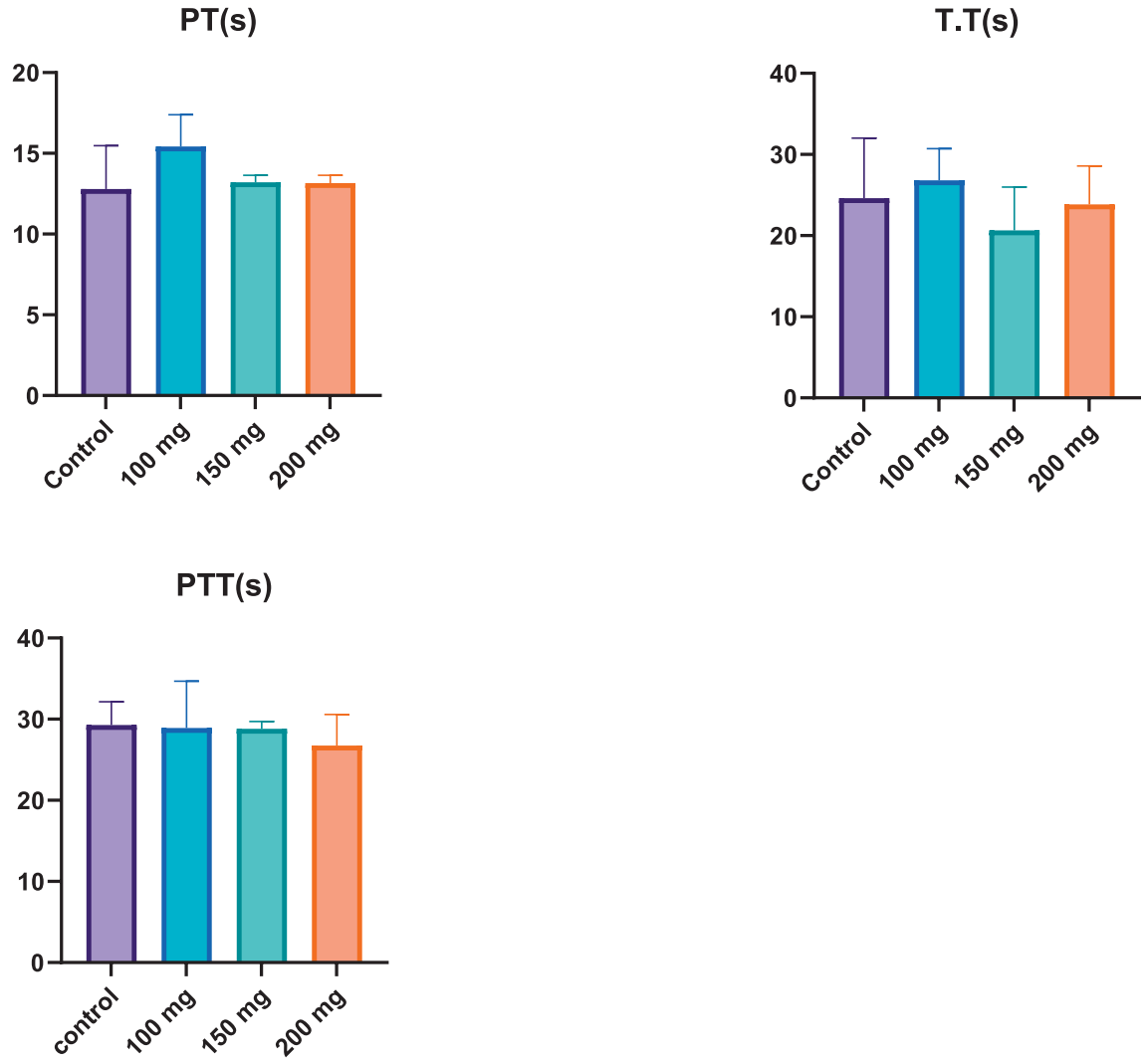

Figure 1 – Prothrombin Time (PT), Thrombin Time (TT), and Partial Thromboplastin Time (PTT) in male Wistar rats after 28 days of fucoidan administration. The results show no statistically significant changes in PT, TT, or PTT across different fucoidan dosages (100, 150, and 200 mg/kg BW), indicating that fucoidan does not significantly alter the coagulation pathways.

indicated minimal variations between the control group and the fucoidan-treated groups. All groups exhibited similar TT values, indicating that fucoidan administration did not substantially influence this coagulation parameter. This finding suggests that fucoidan does not significantly affect the thrombin-mediated conversion of fibrinogen to fibrin, an essential component of blood clot formation.

Partial thromboplastin time (PTT)

The PTT assesses the intrinsic and common pathways of coagulation. As displayed in Figure 1, the control group exhibited the highest PTT value, while the fucoidan-treated groups showed a slight, dose-dependent decrease in PTT. Among the fucoidan-treated groups, the 200 mg/kg group demonstrated the lowest PTT value, indicating a modest reduction in the time required for clot formation via the intrinsic pathway. Despite these observations, the differences between the groups were modest and may not be statistically significant. Therefore, while fucoidan administration appears to exert a mild influence on the intrinsic coagulation pathway, this effect is not pronounced.

Biochemical parameters

Total protein

Total protein levels are an essential indicator of overall health and nutritional status, reflecting the balance between protein synthesis and degradation. As shown in Figure 2, total protein levels were measured across the control group and the fucoidan-treated groups. The results indicated comparable protein levels for all groups, with no substantial differences observed between the control group and the groups treated with various fucoidan dosages. This finding suggests that fucoidan administration does not significantly impact overall protein metabolism or synthesis in the rats, maintaining a consistent total protein concentration across different treatment groups.

Glucose

Glucose levels were also assessed as a key biochemical parameter, given the known metabolic effects of fucoidan. As presented in Figure 3, a notable dose-dependent decrease in glucose levels was observed in the fucoidan-treated groups compared to the control group. Specifically, the 150 mg/kg and 200 mg/kg BW fucoidan-treated groups exhibited statistically significant reductions in glucose levels (p-value < 0.0001) compared to the control group. The 200 mg/kg BW group showed the most pronounced decrease in glucose concentration, highlighting the potential of fucoidan as a hypoglycemic agent. This dose-dependent hypoglycemic effect suggests that fucoidan may influence the glucose metabolism, potentially offering therapeutic benefits for conditions characterized by elevated blood glucose levels, such as diabetes (Figure 2).

Iron-Related parameters

Transferrin: The graph shows a dose-dependent increase in transferrin levels with increasing fucoidan dosage. The 200 mg/kg BW group exhibited significantly higher transferrin levels (p-value <0.001) compared to the control group. The 100 mg/kg BW group showed a slight decrease, while the 150 mg/kg and 200 mg/kg BW groups demonstrated progressive increases in transferrin concentration.

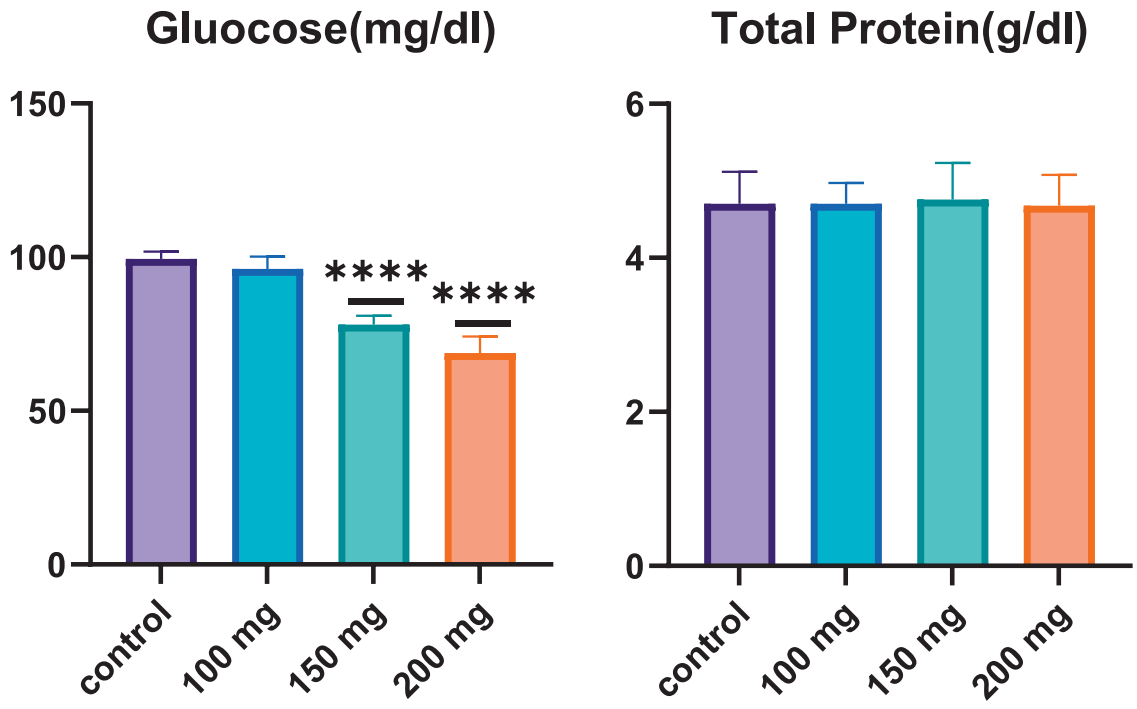

Figure 2 – Effect of Fucoidan on glucose and total protein levels in male Wistar rats after 28 days of fucoidan administration. A significant dose-dependent decrease in glucose levels is observed, particularly at 150 mg/kg and 200 mg/kg BW (p-value < 0.0001). Total protein levels remain consistent across all groups, indicating no significant impact on overall protein metabolism. ****: p-value < 0.0001.

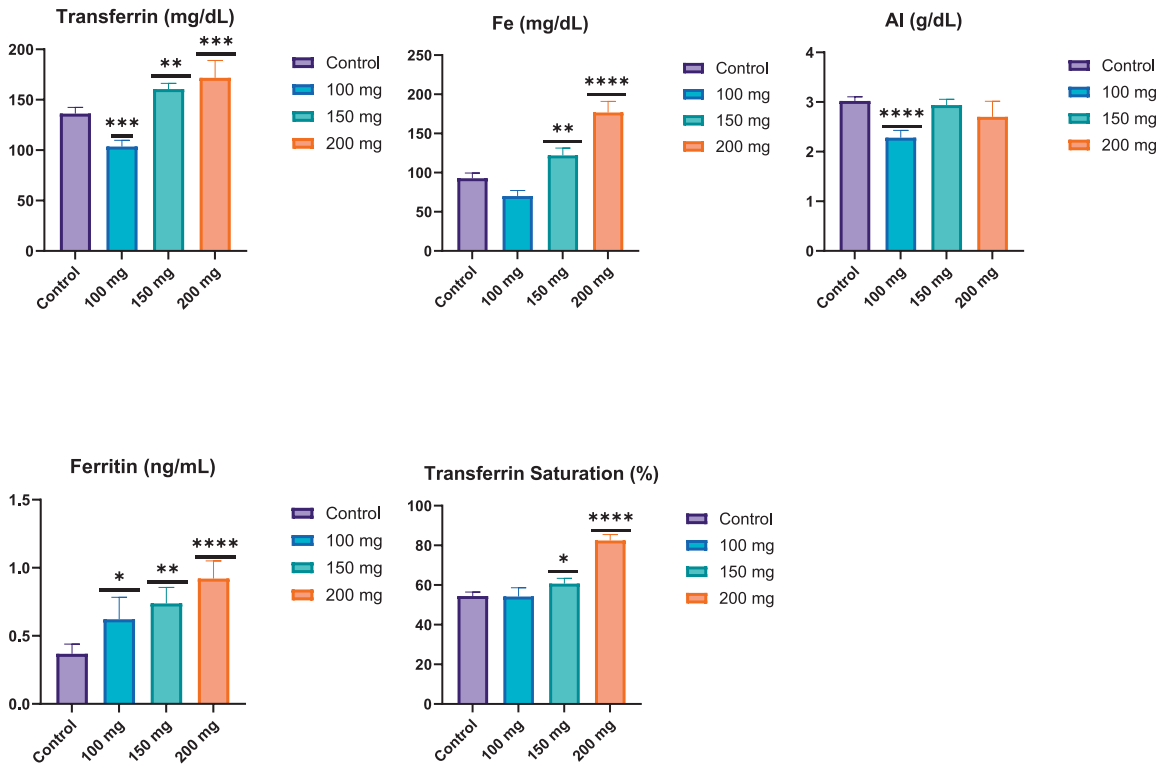

Figure 3 – Effect of fucoidan on iron-related parameters (Transferrin, iron (Fe), ferritin, Transferrin Saturation and albumin levels) in male Wistar rats after 28 days of fucoidan administration. Fucoidan treatment resulted in a dose-dependent increase in transferrin, iron, and ferritin levels, suggesting enhanced iron absorption and storage. Albumin levels decreased at the 100 mg/kg dose, with no significant changes at higher doses, indicating a complex effect of fucoidan on protein metabolism. ****: p-value <0.0001; ***: p-value <0.001; **: p-value <0.01; *: p-value <0.05.

Iron (Fe): The results for serum iron (Fe) levels showed a significant increase in response to fucoidan administration in a dose-dependent manner. Specifically, the group receiving 150 mg/kg BW of fucoidan exhibited a statistically significant increase in serum iron levels compared to the control group (p-value <0.01). Furthermore, the 200 mg/kg BW dose resulted in an even more pronounced elevation in serum iron levels, with a highly significant difference compared to the control group (p-value <0.0001). These findings suggest that fucoidan may enhance iron absorption and storage at higher doses (Figure 3).

Ferritin: Ferritin levels increased dose-dependently with fucoidan treatment. The 200 mg/kg BW group showed the most significant increase (p-value <0.0001), followed by the 150 mg/kg BW group (p-value <0.01), and the 100 mg group (p-value <0.05). This suggests that higher doses of fucoidan lead to greater ferritin production or storage (Figure 3).

Transferrin Saturation: A dose-dependent increase in transferrin saturation was observed across the experimental groups. The 150 mg/kg BW group showed a significant increase compared to the control group (p-value <0.05), while the 200 mg/kg group exhibited a highly significant rise (p-value <0.0001). No significant changes were noted in the 100 mg/kg BW group compared to the control. These findings suggest that higher doses of fucoidan may enhance iron transport and absorption (Figure 3).

Albumin: The graph shows changes in albumin levels across different fucoidan dosages. The 100 mg/kg BW fucoidan group demonstrated a significant decrease in albumin levels (p-value <0.0001) compared to the control. While the 150 mg/kg BW and 200 mg/kg BW groups also showed lower albumin values than the control, the differences were less pronounced and not marked as statistically significant in the graph. This suggests that fucoidan, particularly at lower doses, may influence albumin production or metabolism (Figure 3).

These results indicate that fucoidan administration has significant effects on iron metabolism and related parameters. The observed increases in transferrin, iron, and ferritin levels suggest that fucoidan may enhance iron absorption, transport, or storage in a dose-dependent manner.

Discussion

A salient finding of this study is the significant, dose-dependent reduction in blood glucose levels following fucoidan administration. This observation aligns with emerging literature underscoring the antidiabetic potential of fucoidan. For instance, Kim et al. reported that fucoidan improved insulin sensitivity and reduced hyperglycemia in diabetic mouse models [22]. The underlying mechanisms are multifactorial, involving the modulation of key enzymes in glucose metabolism, enhancement of insulin signaling pathways, and improvement of pancreatic β-cell function [23].

The results of this study also revealed significant changes in iron-related parameters following fucoidan administration. A dose-dependent increase was observed in transferrin levels, with the 200 mg/kg BW group showing significantly higher concentrations compared to the control. Similarly, all fucoidan-treated groups exhibited elevated iron levels, with the most pronounced increases in the 100 mg/kg BW and 200 mg/kg BW groups. Ferritin levels also increased dose-dependently, with all treated groups showing significant elevations compared to the control.

The concurrent increase in transferrin and iron levels indicates enhanced iron transport capacity, which could be beneficial in conditions such as iron deficiency anemia. The increase in transferrin and decrease in albumin are particularly interesting, as both are considered 'negative acute phase' proteins; typically, these proteins decrease during inflammation. The lack of similar significant changes in albumin and transferrin levels in rats that received fucoidan probably reflects changes in iron homeostasis induced by fucoidan, not due of inflammation.

The liver plays a central role in iron metabolism, and the results of this study indicate that fucoidan may influence hepatic function related to iron handling. Increased iron levels stimulate the production of transferrin, which is primarily synthesized in the liver. This increase in transferrin production may be a compensatory mechanism to manage the elevated iron levels observed. The liver also regulates ferritin production, and the increased ferritin levels observed in this study further support the notion of enhanced iron storage capacity in response to fucoidan treatment [24].

Additionally, the observed changes in iron-related parameters suggest that fucoidan may enhance iron absorption in the intestine. The interaction of fucoidan with the intestinal epithelium could potentially modify the expression or activity of iron transporters, such as divalent metal transporter 1 (DMT1) or ferroportin, leading to increased iron uptake. This enhanced absorption could explain the elevated plasma iron levels observed across all fucoidan-treated groups. The dose-dependent increase in ferritin further supports this hypothesis, as increased iron absorption would necessitate greater iron storage capacity [25,26].

The findings of this study suggest that fucoidan supplementation significantly influences iron metabolism in a dose-dependent manner. The marked increases in transferrin, serum iron, and ferritin levels, particularly in the 150 mg/kg and 200 mg/kg BW groups, indicate that fucoidan enhances iron absorption, transport, and storage. The significant rise in transferrin saturation further supports this notion, suggesting improved iron bioavailability. Interestingly, while the 100 mg/kg group did not exhibit substantial changes in transferrin saturation or serum iron levels, the 150 mg/kg and 200 mg/kg groups showed progressive increases, highlighting the importance of dosage of fucoidan on iron homeostasis. These results align with previous studies that have suggested the potential role of polysaccharides in enhancing iron metabolism, further reinforcing the therapeutic potential of fucoidan in addressing iron deficiency.

The lack of a significant increase in albumin, despite the elevation in other liver-produced proteins like transferrin, warrants further investigation. This discrepancy might be due to the differential effects of fucoidan on various liver functions or could indicate a complex interplay between iron metabolism and overall protein synthesis in the liver.

The anticoagulant properties of fucoidan have been widely studied, particularly in vitro, where it has shown significant potential to inhibit various steps of the coagulation cascade. These effects are largely attributed to the structural features of fucoidan, such as its sulfation pattern and molecular weight, which allow it to interact with key proteins like thrombin and antithrombin III, thereby modulating blood coagulation [27,28]. However, the results from this study, along with emerging in vivo data, suggest that the anticoagulant efficacy of fucoidan observed in vitro does not necessarily translate to a significant impact in vivo. However, it is important to note that not all species of brown algae possess anticoagulant properties. Some species, as mentioned in related literature, lack these effects, suggesting that the anticoagulant potential may vary significantly depending on the species and specific composition of fucoidan [29,30].

The findings of the present study demonstrated that fucoidan, at the administered doses, did not significantly alter prothrombin time (PT), thrombin time (TT), or partial thromboplastin time (PTT) in Wistar rats. This discrepancy between in vitro and in vivo effects might be due to several factors. In the complex biological environment of a living organism, fucoidan may undergo metabolic modifications that reduce its interaction with coagulation factors. Additionally, the bioavailability of fucoidan and its distribution within the body could limit its ability to reach effective concentrations at sites where coagulation occurs.

It is worth noting that our study utilized lower doses of fucoidan (100, 150, and 200 mg/kg BW) compared to some previous studies that have used very high doses (1000 and 1500 mg/kg BW). Our decision to reduce the dose was based on the principle of finding the minimal effective dose that could produce beneficial effects while minimizing potential side effects. The significant changes observed in glucose levels and iron-related parameters at these lower doses suggest that fucoidan can exert physiological effects even at more moderate concentrations.

The absence of a pronounced anticoagulant effect in vivo paves the way for the use of fucoidan in therapeutic contexts where anticoagulation is undesirable. For instance, the lack of significant interference of fucoidan with blood clotting makes it a promising candidate for cancer treatment, where anticoagulation might pose risks. Fucoidan has been recognized for its potential anti-cancer properties, including its ability to induce apoptosis, inhibit tumor growth, and reduce metastasis through various mechanisms. The minimal impact on coagulation parameters ensures that fucoidan could be safely incorporated into treatment regimens without exacerbating bleeding risks.

Furthermore, the anti-inflammatory and antioxidant properties of fucoidan add another layer of therapeutic potential, especially in the context of chronic diseases such as cancer. Fucoidan has been shown to modulate inflammatory pathways, reducing the production of pro-inflammatory cytokines and inhibiting the activation of key inflammatory cells. This, combined with its ability to scavenge free radicals and protect

cells from oxidative stress, highlights the role of fucoidan as a multi-functional therapeutic agent [29].

In summary, while fucoidan from *Sargassum angustifolium* at doses of 100, 150, and 200 mg/kg BW may not exhibit significant in vivo anticoagulant effects, its safety profile regarding coagulation, along with its beneficial properties, including hypoglycemic, anti-cancer, anti-inflammatory, and antioxidant activities, positions it as a valuable compound for the development of novel therapeutic strategies. Further research, particularly clinical studies, is warranted to explore the full spectrum of the benefits of fucoidan in human health.

Data availability statement

The data supporting the findings of this study, including raw datasets, figures, and supplementary materials, are available from the corresponding author, Ameneh Khoshvaghti, upon reasonable request. Due to confidentiality agreements, some data may not be publicly accessible, but inquiries regarding specific data points or methods can be directed to the corresponding author.

Conflicts of interest

The authors declare no conflicts of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Integrating comprehensive care in the management of sickle cell disease patients in Nigeria

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ABSTRACT

Introduction: Comprehensive sickle cell care is a holistic, multidisciplinary approach spanning from birth to adulthood. It includes newborn screening, routine investigations, medications, specific therapies and structured referrals. It is recognised since the 1972 US Sickle Cell Control Act and reinforced by the American Society of Haematology initiatives. This study evaluates the adoption of these strategies by physicians in Nigeria.
Aim: To examine the extent to which comprehensive care strategies are implemented in the management of sickle cell disease by adult and paediatric haematologists in Nigeria.
Methodology: This cross-sectional study was conducted from September to November 2022 across six tertiary hospitals. An adapted and pretested primary care assessment tool was used to collect data on physician demographics and strategic components of comprehensive care. Descriptive statistics and chi-square tests were used to analyse the data.
Results: A total of 157 doctors participated with most working in tertiary hospitals. Folic acid and proguanil hydrochloride were the most prescribed drugs; fewer than 50% used hydroxyurea. A complete blood count was the most requested investigation with 58% routinely scheduling investigations. Adult haematologists ordered more echocardiograms and paediatric haematologists requested more transcranial Dopplers. Adult haematologists referred more across specialities (p-value = 0.0001). All participants routinely counselled patients on clinic attendance, medication adherence and healthy lifestyle practices.

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Conclusion: Key components of comprehensive care are practised at varying levels by health professionals in Nigeria, mainly in urban/tertiary hospitals. To strengthen nationwide delivery of care, health policies should prioritise equitable workforce distribution and integration of additional services, like neonatal screening and emerging therapies.

Introduction

Sickle cell anaemia is a monogenic disorder caused by a point mutation in the β-globin gene (*Glu6Val*) leading to the production of abnormal haemoglobin S. Under hypoxic conditions, haemoglobin S polymerizes, deforming red blood cells and triggering chronic haemolysis, inflammation, endothelial dysfunction, and activation of leukocytes and platelets. These mechanisms drive a wide range of complications affecting nearly every organ. Clinical heterogeneity is further influenced by co-inheritance of α-thalassaemia, foetal haemoglobin levels, hydroxyurea use, transfusion history, environmental factors, and use of emerging therapies [1,2]. Nigeria bears the highest burden of sickle cell disease (SCD) globally, with an estimated 150,000 affected births annually and a general population prevalence of 2–3 % [3,4]. Comprehensive care, a multidisciplinary and preventive approach, has shown to improve survival and quality of life in SCD. Key elements include newborn screening, caregiver education, prophylactic antibiotics, vaccinations, malaria chemoprophylaxis, routine medications, clinical monitoring, and laboratory investigations. Effective pain management and timely treatment of vaso-occlusive crises are also essential [1,5,6].

Where available, other disease-modifying options such as the use of hydroxyurea, chronic transfusions, stem cell transplantation, and targeted molecular therapies are also used [5,7]. Hydroxyurea, increasingly recommended in comprehensive care, improves outcomes through multiple mechanisms, including increased foetal haemoglobin, reduced leukocyte and platelet activation and counts, improved red cell hydration, and reduced haemolysis [8].

Laboratory tests such as haemoglobin electrophoresis, complete blood counts (CBCs), liver and renal function tests, and reticulocyte counts support early diagnosis and monitoring.

Imaging tools, like transcranial Doppler and echocardiography, assist in stroke and pulmonary hypertension risk assessment, respectively. A multidisciplinary team including haematologists, paediatricians, nurses, psychologists, pain specialists, and social workers is essential for delivering coordinated care [5,6].

Despite the 2014 Nigerian national guidelines [9] addressing aspects of SCD care, including routine management and specialist referral [9], the absence of a structured care model and the lack of newborn screening severely limit the implementation of comprehensive care. Additionally, there is no unified national protocol, contributing to inconsistent practices among clinicians [10,11].

This study aims to assess the extent to which comprehensive care strategies are implemented in SCD management by adult and paediatric haematologists in Nigeria. It will evaluate the use of routine medications, diagnostic tools, and multidisciplinary services, with the goal of identifying gaps and informing future improvements in care delivery nationwide.

Methodology

This multicenter cross-sectional study was conducted from September to November 2022across six tertiary institutions representing Nigeria's six geopolitical zones. It assessed the extent to which adult and paediatric haematologists implemented comprehensive SCD care strategies in routine practice.

A modified and pre-validated version of the Primary Care Assessment Tool (PCAT) [12] was used. It was developed following focused group discussions by a selected group of locally based public health physicians and sickle cell care specialists. Using closed-ended questions, the tool collected data on the utilization of comprehensive services like routine clinical assessments, organ function assessments, use of routine and disease-modifying treatments, access to multidisciplinary care and physician demographics including years of practice, location, speciality, and cadre.

Content validity was established through expert review by three adult and three paediatric haematologists. The modified PCAT tool was deployed through Google Forms. An informed consent statement outlining the study title, purpose, benefits, risks and voluntary nature of participation was presented at the beginning. Completion of the form is possible within ten minutes, and consent was indicated by checking the "YES" option. In each participating centre, a designated focal person, a local SCD specialist, disseminated the tool and coordinated data collection. These focal persons, familiar within their communities, enhanced participation by sending reminders and making phone calls to limit attrition. They received no financial incentives.

Given an estimated number of 10 to 20 adult haematology department consultants and residents, and 8 to 15 paediatric haematology department consultants and residents per centre, the calculated estimated total population n was derived as shown below.

The average number of sickle cell care doctors per centre

$\frac{8+15}{2} = 11.5$ for paediatric haematology

$\frac{10+20}{2} = 15$ for adult haematology

The total number of specialists per centre: 11.5 + 15 = 26.5.

The total number of specialists across six centres: $26.5 \times 6 = 159$.

Estimated total population: $n = 159$

Table 1 – Recruitment targets.

Centre	Estimated number from paediatric haematology	Estimated number from adult haematology	Total number of the centre	Allocated sample size
A	8	10	18	13
B	10	15	25	18
C	12	20	32	23
D	15	18	33	24
E	11	14	25	18
F	12	14	26	17
Total			159	113

With a 95 % confidence level, a 5 % margin of error, and a p-value = 0.05, and using the Cochran formula with a finite population correction, the total estimated sample size was 113 participants.

To ensure an equitable centre-wide representative participation, the sample was proportionally distributed based on the number of eligible participants per centre. The recruitment targets illustrated in Table 1 present the estimated staff strength per centre and the corresponding sample size.

Sample size per centre = (Centre size divided by 159) x 113

Mixed sampling was used. Institutions with the largest staff strength per region were purposively selected. Subsequently, a census approach was used to recruit all eligible specialists within the centres. Sampling was proportionally stratified based on staff strength to ensure balanced representation across the regions. A total of 157 duly answered questionnaires were returned during the study thus the response rate was 93 % (Figure 1).

The University of Nigeria Teaching Hospital's Ethics Review Board granted ethical approval.

Inclusion and exclusion criteria

Adult haematology physicians and paediatric haematology physicians were included in this study. The following were the exclusion criteria: physicians in other clinical specialities who do not routinely manage patients with SCD; physicians who did not complete the online questionnaire; and physicians who did not give consent

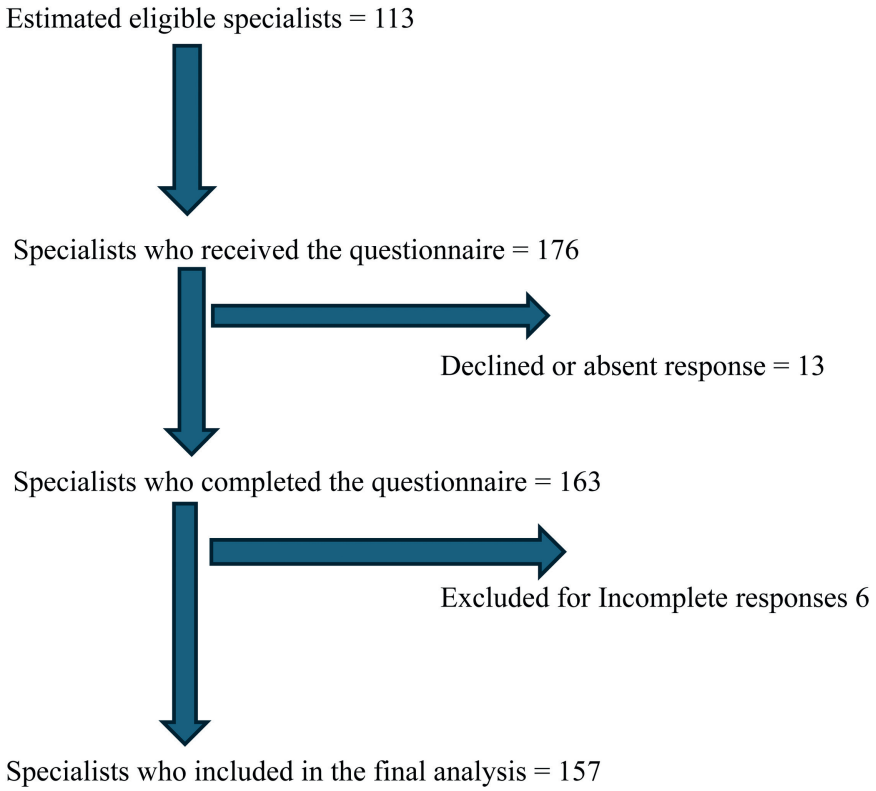

Figure. 1 – The flow of participant inclusion from eligibility to data analysis.

The response rate = The total number of completed questionnaires ÷ by the number who received the questionnaire × 100 = approximately 93 %.

Data analysis

Descriptive statistics were done using the STATA computer program (version 16.1). Sociodemographic and study-related characteristics, such as the use of routine medications, frequency and type of routine investigations, and physician referral habits and triggers for such referrals, were analysed and presented as frequencies, charts and in text. The chi-square test was used to assess the association between categorical variables.

Results

Recruitment summary

A total of 176 physicians received the questionnaire, 163 completed it, and 157 were included in the final data analysis after excluding six incomplete responses. Thirteen participants declined to participate. The participant flow chart is shown in Figure 1

Physician demographics

A total of 157 doctors participated in the study, comprising 54.1 % males and 45.9 % females. The mean age of respondents was 42 years, with an age range of 36–48 years. Among the participants, 85 were from the adult haematology departments and 72 from the paediatric haematology departments.

In the adult haematology group, consultants comprised the majority of participants (n = 60). Within the paediatric haematology group, 44.4 % were consultants, while the remaining 55.6 % were other cadres of doctors.

Geographically, 56 % of respondents were based in southern Nigeria, while 44 % were based in northern Nigeria. Within the southern region, South-Eastern Nigeria contributed the highest proportion of participants (28 %), while in the northern region, North-Western Nigeria had the largest representation (20.4 %).

Regarding the type of health facility, 79 % of participants were affiliated with tertiary hospitals, 3.8 % with private hospitals, and 0.6 % with primary healthcare centres. In terms of professional experience, approximately 50 % of respondents reported 11–15 years of clinical practice, while 25 % had 16 –20 years of experience.

Figure 2 shows the percentage distribution of physicians from adult haematology and paediatric haematology. Figure 3 is the distribution of respondents by facility type, years of practice and geographical region.

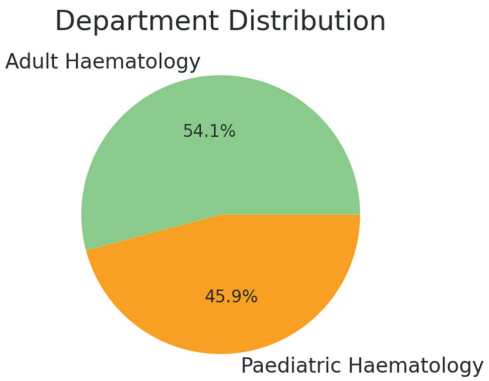

Figure. 2 – Physician demographics.

Use of both routine and disease-modifying medications

Prescription of routine medications

The pattern of routine drug prescriptions among adult and paediatric haematologists is illustrated in Table 2. Among adult haematologists, the most frequently prescribed medications, in descending order, were folic acid, proguanil hydrochloride, vitamin C, hydroxyurea, vitamin B complex, a fixed multivitamin preparation, Omega-3 fatty acids, penicillin, fixed multivitamin-amino acid supplements, and ferrous fumarate. In comparison, paediatric haematologists mainly prescribed folic acid, proguanil hydrochloride, penicillin, hydroxyurea, vitamin C, fixed multivitamin preparations, vitamin B complex, fixed multivitamin-amino acid supplements, ferrous fumarate, and omega-3 fatty acids, in that order.

Folic acid and proguanil hydrochloride were the two most prescribed medications across both groups. However, fewer than 50 % of physicians in either group reportedly routinely prescribing hydroxyurea. Notably, penicillin was prescribed significantly more often by paediatric haematologists compared to their adult counterparts.

Organ function assessments

As shown in Table 3, the CBC was the most frequently requested investigation by both groups. In contrast, fundoscopy and hepatitis screening were the least commonly requested. A Chi-squared analysis revealed that adult haematologists requested echocardiographies significantly more frequently than paediatric haematologists, whereas paediatric haematologists were significantly more likely to request transcranial Doppler (TCD) assessments.

Frequency of organ function assessments

Table 4 presents the intervals at which routine organ function investigations were requested. Only 20.4 % of physicians ordered tests monthly, 28.0 % did so quarterly, and 10.8 % did so yearly. A notable 35.5 % of respondents reported no consistent schedule for requesting routine investigations, while 6.3 % did not order routine investigations.

Access to multidisciplinary care

The referral patterns of participating physicians are summarized in Table 5. Clinical need was the predominant reason for referral among both groups. Across all other specialties, adult haematologists referred patients significantly more frequently than paediatric haematologists (Chi-squared test: p-value = 0.0001). The most frequently referred specialties by adult haematologists, in descending order, included obstetrics and gynaecology, ophthalmology, psychiatry, otorhinolaryngology, and dentistry. Interestingly, both adult and paediatric haematologists rarely referred patients to urologists or to fellow haematologists. Routine referrals were not

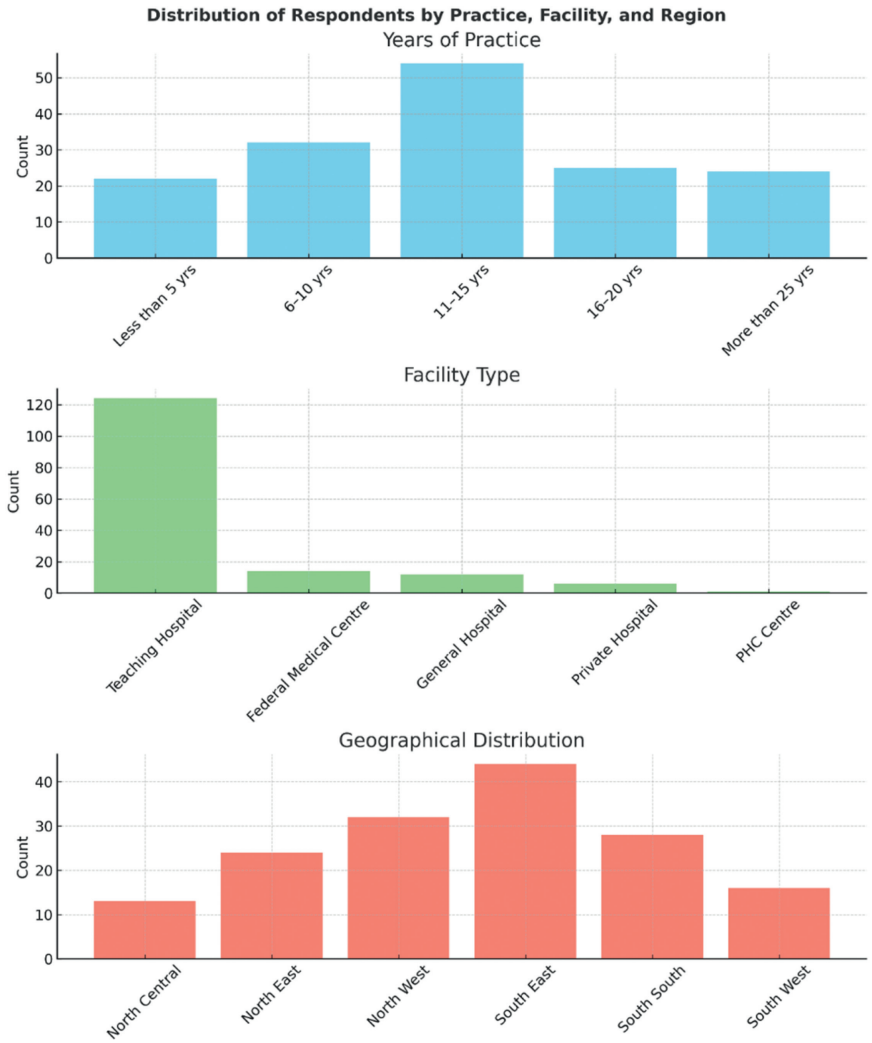

Figure. 3 – Physician demographics: Facility type, years of practice and geographical region.

Table 2 – Use of routine and disease-modifying drugs.

Routine Drugs	Adult Haematology n (%)	Paediatric Haematology n (%)
Folic acid	85.0 (100.0)	69.0 (95.8)
Proguanil hydrochloride	78.0 (91.8)	62.0 (86.1)
Vitamin C	47.0 (55.3)	26.0 (36.1)
Hydroxyurea	42.0 (49.4)	34.0 (47.2)
Vitamin B complex	33.0 (38.8)	13.0 (18.1)
Multivitamins	20.0 (23.5)	16.0 (22.2)
Omega 3 fatty acids	16.0 (18.8)	3.0 (4.2)
Penicillin	8.0 (9.4)	42.0 (58.3)
Fixed multivitamin-amino acids supplements	3.0 (3.5)	13.0 (18.1)
Ferrous fumarate	2.0 (2.4)	4.0 (5.6)

practiced by 62.5 % and 37.6 % of paediatric haematologists and adult haematologists, respectively.

Patient education and counselling

As shown in Figure 4, all participating physicians routinely counselled patients on regular clinic attendance, adherence to prescribed medications, proper nutrition, healthy habits, malaria prevention strategies and other lifestyle modifications.

Discussion

Comprehensive care remains a cornerstone of SCD management as endorsed by the World Health Organization, the American Society of Hematology, and the Nigerian government. It encompasses a wide range of clinical interventions, from scheduled services such as neonatal screening, routine disease monitoring to prophylactic and disease-modifying

Table 3 – Organ function assessments.

Routine Tests	Adult Haematology n (%)	Paediatric Haematology n (%)	P-value
Complete Blood Count	82.0 (96.5)	57.0 (79.2)	
Urinalysis	54.0 (63.5)	24.0 (33.3)	
Kidney Function Test	45.0 (52.9)	28.0 (38.9)	
Liver Function Test	32.0 (37.6)	17.0 (23.6)	
Transcranial Doppler Ultrasound	16.0 (18.8)	27.0 (37.5)	
Echocardiography	13.0 (15.3)	2.0 (2.8)	<0.0001
Chest X-ray	1.0 (1.2)	0.0 (0.0)	
Abdominal Ultrasound	0.0 (0.0)	0.0 (0.0)	
Packed cell Volume (PCV)	1.0 (1.2)	8.0 (11.1)	
Thick film for malaria Parasite	1.0 (1.2)	0.0 (0.0)	
Peripheral Blood Film	1.0 (1.2)	0.0 (0.0)	
Fundoscopy	0.0 (0.0)	1.0 (1.4)	
Hepatitis Screen	0.0 (0.0)	1.0 (1.4)	
Routine request not performed	2.0 (2.4)	7.0 (9.7)	

Table 5 – Access to multidisciplinary care.

Referred to Speciality	Haematology	Paediatric Haematology	p-value
Referrals not routine	32.0 (37.6)	45.0 (62.5)	
Obstetrician	47.0 (55.3)	12.0 (16.7)	
Ophthalmologists	33.0 (38.8)	12.0 (16.7)	
Psychiatrist	20.0 (23.5)	5.0 (6.9)	
ENT Surgeon	14.0 (16.5)	5.0 (6.9)	
Dentists	12.0 (14.1)	0.0 (0.0)	<0.0001
Nephrologist	3.0 (3.5)	0.0 (0.0)	
Orthopaedic surgeon	3.0 (3.5)	1.0 (1.4)	
Pulmonologist	1.0 (1.2)	0.0 (0.0)	
Neurologist	0.0 (0.0)	1.0 (1.4)	
Haematologist	0.0 (0.0)	3.0 (4.2)	
Paediatrician	0.0 (0.0)	1.0 (1.4)	
Urologist	1.0 (1.2)	0.0 (0.0)	

therapies, all aimed at reducing morbidity and improving quality of life [9,13,14].

This study revealed that 90.4 % of respondents were SCD specialists from either adult haematology or paediatric haematology, primarily working in tertiary centres. Over 85 % had more than six years of clinical experience, aligning with evidence that specialist-led care improves outcomes in SCD [15,16]. However, about 95 % were in urban centres, reflecting the ongoing maldistribution of healthcare professionals in sub-Saharan Africa, which continues to disadvantage rural populations and hinder equitable access to care [6,17].

Contrary to reports by Kanter et al. [6] that highlight more paediatric haematologists in SCD care, this study found a slight predominance of adult haematologists. This may reflect institutional variability or gaps in our national specialist registries and is thus not representative of the actual number in the country. More critically is the broader challenge of a limited workforce for SCD and other haematological disorders in Nigeria and Sub-Saharan Africa [3,8,18].

All participating specialists routinely prescribed core medications, demonstrating that they are integral to comprehensive care [3,8]. Folic acid, antimalarials (e.g., proguanil), and penicillin were most used, while hydroxyurea, despite its proven disease-modifying potential, was underutilized. This discrepancy likely stems from issues of cost, availability, limited treatment guidelines, safety concerns regarding use in pregnant and lactating mothers and as a possible cause of infertility [19,20].

The preference for penicillin among paediatricians aligns with evidence from the PROPS study, which demonstrated its efficacy in preventing life-threatening infections in children with SCD [21]. Notwithstanding, the 58 % uptake recorded by the paediatric haematologists, the rate of penicillin prophylaxis is deemed low, though this is in keeping with other studies [22]. Contributing factors include cost, limited availability of paediatric formulations, poor adherence due to SCD chronicity, sociocultural barriers, provider reluctance linked to the absence of national guidelines, the inclusion of vaccines for encapsulated organisms in routine immunization which has reduced the emphasis on prophylaxis and *Salmonella typhi* not *Streptococcus pneumonia* having been the most common isolate in a Nigerian study [23–25].

Folic acid supplementation remains a standard component of care despite ongoing debate about its clinical benefits. A Cochrane review suggests that aside from an increase in serum folate levels, it has limited impact on anaemia severity [26].

Routine folic acid use is usually justified by a theoretical need to prevent deficiency from increased folate turnover due to chronic haemolysis. Additionally, hypermetabolism in

Table 4 – Frequency of organ function assessments.

	Total (*n* = 157)	Adult Haematology (*n* = 85)	Paediatric Haematology (*n* = 72)	p-value
Not routinely requested	10 (6.3 %)	2 (2.4 %)	8 (11.1 %)	
Not consistent	54 (34.4 %)	25 (29.4 %)	29 (40.3 %)	
Monthly	32 (20.4 %)	25 (29.4 %)	7 (9.7 %)	
Quarterly	44 (28.0 %)	25 (29.4 %)	19 (26.3 %)	0.008
Yearly	17 (10.8 %)	8 (9.4 %)	9 (12.5 %)	

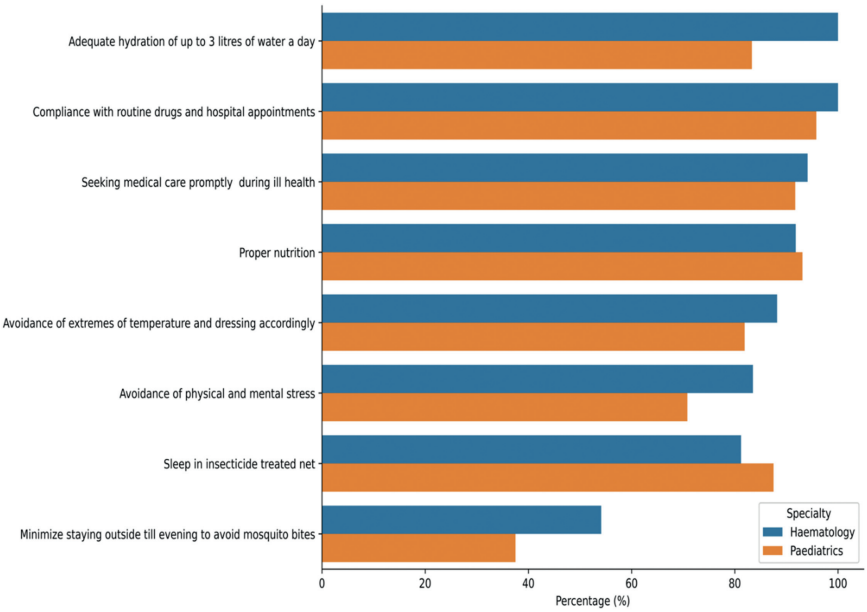

Figure. 4 – Patient education and counselling.

haemoglobin SS, elevated interleukin-6 levels (which may suppress appetite), and increased resting energy expenditure contribute to micro- and macro-nutrient deficiencies, underscoring the need for nutritional replacement in sickle cell anaemia management [26,27].

Vitamin B complex, other multivitamin preparations, and anti-inflammatory agents like omega-3 fatty acids are not as regularly prescribed because they are primarily used as supplements and, unlike the others, do not directly influence the pathophysiological mechanisms and complications of SCD.

Regarding the use of vitamin C, it is postulated to be a potent antioxidant that shields red cell membranes from damage by reducing the generation of reactive oxygen species [28]. In addition, vitamin C also promotes the absorption and metabolism of iron with improvements in specific red cell indices [29]. Iron supplementation is the least prescribed and is usually for coexisting iron deficiency anaemia.

Routine laboratory monitoring is a key pillar of comprehensive care. In this study, most specialists conducted regular tests, including CBCs, urinalysis, and renal function assessments.

Adult haematologists prioritized echocardiography to detect complications like pulmonary hypertension common in older SCD patients [1,3]. In contrast, paediatricians more frequently requested TCD ultrasound to screen for stroke risk, consistent with its established utility in children with SCD. This, in part, is due to established guidelines recommending annual screening for children with SCD, which the ASH guidelines, as well as the STOP and SPRING trials, have significantly influenced. TCD, as a standard of care in paediatric clinics, is supported by public health initiatives and advocacy primarily targeting children with sickle cell anaemia. In contrast, adult SCD care has limited TCD validation studies and lacks structured protocols. These factors collectively drive the increased TCD requests in Paediatric settings [30,31].

Referrals to other specialities were frequently prompted by specific clinical concerns such as stroke, priapism, leg ulcers, or mental health issues.

This multidisciplinary approach includes psychiatrists, surgeons, nephrologists, gastroenterologists, neurologists, social workers, nutritionists, pain specialists etc. It reflects best practice models and is central to holistic SCD care. Counselling also featured prominently, highlighting the psychosocial burdens of SCD and the importance of integrated mental health support. Each group consistently emphasized health-seeking and other well-being habits. Of key importance is malaria prevention because malaria infection is endemic in the population

While this study confirms a growing commitment to comprehensive, specialist-led SCD care in Nigeria, significant gaps remain. Chief among these is the limited rural coverage of specialist services, inconsistent access to hydroxyurea and diagnostics, and the absence of standardized care protocols. These deficiencies contribute to fragmented care delivery and suboptimal outcomes, particularly outside tertiary centres. Furthermore, even within tertiary facilities, the lack of standardized care protocols and harmonized clinical guidelines has led to inadequacies and considerable variability in the application of comprehensive care strategies by physicians. Addressing these gaps will require targeted national surveys and the constitution of expert panels to develop standardized and universally accepted comprehensive care protocols. Additionally, greater advocacy is needed to raise awareness of these deficiencies and to address specific practice gaps, such as the reduced use of TCD screening in adult haematology care. This should be supported by further validation studies focused on the utility of TCD in adult SCD populations.

This study did not capture essential aspects of comprehensive care, such as newborn screening, vaccinations, and the use of emerging therapies, including gene and targeted

therapies, in the management of SCD. This is due to the widespread unavailability of these services, which is a consequence of financial, infrastructure, and socioeconomic challenges, resulting in a weakened health service framework. The small sample size, purposive site selection a cross-sectional design and the answers of the surveys not validated against the medical records may create bias and limit the generalizability of study findings

Conclusion

This study underscores the importance of structured, comprehensive care in SCD management and affirms the critical role of haematology specialists in delivering this care. Although implementation is significant in urban tertiary centres, systemic barriers, including workforce shortages, urban concentration of expertise, and inconsistent access to medications, impede equitable care delivery nationwide. Addressing these challenges requires strategic policy actions, including decentralisation of services, and the expansion of care services and the SCD workforce, with improved funding for essential medicines and enhanced diagnostics.

Author contributions

Efobi CC participated in conceptualization, manuscript drafting, study design, data collection, interpretation, Statistical analysis, and manuscript reviews.

Nri-Ezedi CA participated in conceptualization, manuscript drafting, study design, data collection, interpretation, Statistical analysis, and manuscript reviews.

Chilaka UJ participated in conceptualization, manuscript drafting and study design, data collection, interpretation and manuscript reviews.

Okoye HC participated in conceptualization, manuscript drafting and study design, data collection, interpretation and manuscript reviews.

Anigbogu IO participated in conceptualization, manuscript drafting and study design, data collection, interpretation and manuscript reviews.

Okwummuo EP participated in conceptualization, manuscript drafting and study design, data collection, interpretation and manuscript reviews.

Ogundeji P S participated in conceptualization, manuscript drafting, study design, data collection, interpretation, and manuscript reviews.

All authors participated in the final approval of the manuscript.

Data availability

Data sets and other study documents are appropriately secured and will be available upon request.

Ethics statement

The University of Nigeria Teaching Hospital's Ethics Review Board granted ethical approval.

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Conflicts of interest

The authors declare there is no conflict of interest in undertaking this study.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Outcomes after bone marrow versus peripheral blood haploidentical hematopoietic cell transplantation using post-transplant cyclophosphamide-based graft-versus-host disease prophylaxis

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ABSTRACT

Background: This study aims to compare the outcomes of bone marrow (BM) to peripheral blood stem cells (PBSC) grafts in haploidentical hematopoietic cell transplantation using post-transplant cyclophosphamide-based graft-versus-host disease (GvHD) prophylaxis.
Methods: A single-center retrospective analysis of all adult patients who underwent haploidentical transplants with at least one year of follow-up was conducted. Bivariate analyses were performed using chi-square tests and *t*-tests. Data were analyzed using SPSS with statistical significance being defined at p-value <0.05.
Results: The study included 176 transplant recipients: 65 % received PBSC and 35 % received BM grafts. After a median follow-up of 21 months (range: 0–73 months), neither median overall survival nor disease-free survival had been reached. One-year overall survival (BM 75 % versus PBSC 74 %; p-value = 0.898) and one-year disease-free survival (63 % both groups; p-value = 0.994) were similar between groups. PBSC recipients exhibited earlier neutrophil engraftment (17 days versus 18 days; p-value = 0.022). The incidence of cytokine release syndrome was higher in PBSC (90 % versus 37 %) grafts (p-value <0.001). The incidences of Grade II-IV acute GvHD, relapse, non-relapse mortality, platelet engraftment, one-year chronic GvHD, and GvHD-free relapse-free survival were similar across both groups.
Conclusions: Haploidentical HSCT recipients observed similar outcomes regardless of graft source. Marginally faster neutrophil engraftment was observed in PBSC recipients. These

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findings suggest flexibility in using graft source for haploidentical transplants, though prospective studies are needed to confirm these results.

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) serves as a crucial therapeutic approach for a range of hematologic malignancies and non-malignant disorders [1]. However, its success relies upon the availability of suitable human leukocyte antigen (HLA)-matched donors, a challenge aggravated by donor scarcity [2]. Haploidentical stem cell transplant (haplo-HSCT) donation, when combined with post-transplant cyclophosphamide (PT-Cy), has revolutionized transplant accessibility for patients without fully matched donors [3]. This approach has confirmed the feasibility of using partially matched-related donors, significantly expanding available donor options. It demonstrates low rates of severe graft-versus-host disease (GvHD) which are comparable to those seen when bone marrow (BM) is used instead of peripheral blood stem cells (PBSC) in haplo-HSCT [4]. Additionally, high-dose PT-Cy selectively removes alloreactive T cells, effectively reducing the risk of GvHD while preserving the graft-versus-leukemia effect [5,6]. This method has particularly broadened transplant accessibility in regions with limited donor availability [7–9].

Published studies report that survival rates are comparable between the two graft sources, with PBSCs demonstrating lower relapse rates and faster engraftment [10,11]. However, PBSC grafts are associated with increased transplant-related mortality, primarily due to a higher incidence of GvHD [12]. In this study, the outcomes of haplo-HSCT using PT-Cy-based GvHD prophylaxis were analyzed, comparing the results based on the graft source. The aim was to clarify the relative efficacy and safety profiles of each graft source and to explore their clinical implications, thereby enhancing transplant practices and optimizing patient outcomes in haplo-HSCT.

Methods

Design, setting, and patients

A single-center retrospective study was conducted at the University of KS Medical Center, examining all adult haplo-HSCT recipients from August 2016 to July 2021. The study included 176 patients with at least one year of post-transplantation follow-up. The cohort consisted of adult patients who received their first allo-HSCT from donors mismatched at two or more HLA loci, independent of conditioning regimen and indication for allo-HSCT. Patients who received manipulated grafts (ex vivo or in vivo T-cell depletion, or ex vivo engineered T cells) were excluded as were those with matched unrelated, mismatched unrelated, matched related, or umbilical cord blood donors. All recipients of haplo-HSCT were administered standard GvHD prophylaxis consisting of PT-Cy, mycophenolate mofetil (MMF), and tacrolimus (continuing until Day +60 after the transplant). The myeloablative and reduced-intensity conditioning (RIC) regimens utilized are listed in Table 1. The institutional review board approved the study.

Data collection, outcomes, and key definitions

Data were collected by review of the electronic medical records. Demographic, clinical, and pathologic factors were ascertained at the time of HSCT. The primary objective of this study was to compare overall survival (OS) between the groups. The secondary objectives were to compare rates of acute and chronic GvHD, cytokine release syndrome (CRS), neutrophil and platelet engraftment, non-relapse mortality (NRM), relapse, and disease-free survival (DFS). Neutrophil recovery was defined as achieving an absolute neutrophil count (ANC) $>0.5 \times 10^9$/L for three consecutive days. Platelet recovery was defined as achieving a platelet count $>20 \times 10^9$/L without transfusion requirement for seven consecutive days. Disease relapse, progression, and death were treated as events. NRM was defined as time to death without relapse or progression. Relapse was defined as the molecular, cytogenetic, or hematologic recurrence of the primary disorder. DFS was defined as survival without relapse or progression. Acute GvHD (aGvHD) was staged and graded according to the Mount Sinai aGvHD International Consortium criteria [13]. CRS was graded based on American Society for Transplantation and Cellular Therapy Consensus Guidelines [14]. Chronic GvHD (cGvHD) was staged and graded according to the 2014 National Institutes of Health criteria. Causes of death were coded according to the Center for International Blood and Marrow Transplant Research recommendations; specifically, if a patient had an active or uncontrolled GvHD and concurrent infection at the time of death, then GvHD was coded as the primary cause of death and infection was coded as a contributing cause.

Statistical analysis

Descriptive statistics were used to compare baseline demographic characteristics. Categorical data were compared using

Table 1 – Transplant conditioning regimens.

Regimen	n
Myeloablative (n = 55)	
Flu/TBI	49
Bu/Flu/Cy	6
Reduced intensity (n = 121)	
Flu/Cy/TBI	105
Flu/Mel/TBI	9
Flu/Cy/TBI/ATG	6
Flu/Cy/TBI/ATG/Thio	1

Bu: Busulfan; Cy: Cyclophosphamide; Flu: Fludarabine; TBI: Total body irradiation; Mel: Melphalan; ATG: Anti-thymocyte Globulin; Thio: Thiotepa

the Chi-square test. Continuous data were compared using ANOVA or *t*-test. Univariate and multivariate Cox regression analyses were conducted to investigate factors associated with OS, DFS, NRM, and relapse including the graft source; the hazard ratio (HR) with 95 % confidence intervals (95 % CIs) were obtained. For regression models, PBSC recipients were compared with reference to BM graft recipients. Univariate regression analyses included correlation of key variables with the post-transplant outcomes, including age, sex, ethnicity, Karnofsky performance status (KPS), hematopoietic stem cell transplantation-specific comorbidity index (HSCT-CI), hematologic diagnosis, disease status (complete remission versus others), recipient cytomegalovirus (CMV) serostatus, donor CMV serostatus, donor age, donor sex, conditioning regimen, and GvHD prophylaxis. Significant factors identified in univariate analyses were entered into a multivariate analysis for the respective outcome. Data were analyzed using SPSS version 28 with statistical significance being defined as a p-value >0.05.

Results

Baseline and clinical characteristics

The study encompassed 176 haplo-HSCT involving 114 (65 %) PBSC and 62 (35 %) BM grafts. The median age of recipients was 54 years (range: 18–74); 119 (68 %) were male. The racial distribution was primarily Caucasian (123; 70 %), followed by Hispanic (23; 13 %), Afro-American (16; 9 %), and other ethnicities (14; 8 %). The most common hematologic diagnoses were acute myeloid leukemia (AML) in 74 patients (42 %), other myeloid disorders in 41 (23 %), acute lymphoid leukemia (ALL) in 17 (10 %), lymphoma in 33 (19 %), and other conditions in 11 (6 %). A pre-transplant HSCT-CI score of 3 or higher was recorded in 93 patients (53 %). KPS was ≥90 % in 59 (34 %) and 60–80 % in 117 (67 %) patients. RIC was administered to 121 recipients (69 %) and myeloablative conditioning (MAC) to 55 (31 %). CMV seropositivity was identified in 109 recipients (62 %) and 101 donors (57 %). At the time of transplantation, 111 recipients (63 %) were in complete remission. The median $CD34^+$ cell dose was 5.0×10^6 per kg for PBSC recipients and 2.8×10^6 per kg for BM recipients (p-value <0.001). These data are summarized in Table 2.

Primary and secondary outcomes

With a median follow-up of 21 months (range: 0–73 months), OS and DFS were not reached in either the BM or PBSC haplo-HSCT groups. The one-year OS rates were 75 % for BM and 74 % for PBSC (p-value = 0.898), and the one-year DFS rates were identical at 63 % for both groups (p-value = 0.994). PBSC recipients experienced earlier neutrophil engraftment at 17 days compared to 18 days for BM recipients (p-value = 0.022).

The incidences of Grade II-IV acute GvHD were nearly identical at 50 % for BM and 51 % for PBSC (p-value = 0.875). Relapse rates were 22 % for PBSC and 26 % for BM (p-value = 0.579), while NRM rates were 17 % for PBSC and 20 % for BM (p-value = 0.682). The median times to platelet engraftment were 28 days for PBSC and 31 days for BM (p-value = 0.092). Neutrophil recovery by Day +28 was 95 % for PBSC versus 90 % for BM (p-value = 0.349), and platelet recovery by Day +100 was 90 % for PBSC compared to 81 % for BM (p-value = 0.099). Rates of primary graft failure (PGF) and one-year chronic GvHD were also similar, with PGF at 2 % for PBSC versus 5 % for BM (p-value = 0.347) and chronic GvHD at 35 % for PBSC versus 38 % for BM (p-value = 0.410). The one-year GvHD-free relapse-free survival rate (GRFS) was 67 % for PBSC and 61 % for BM (p-value = 1.00). The overall incidence of CRS was 71 %, significantly higher in PBSC at 90 % compared to 37 % for the BM group (p-value <0.001). CRS grades were distributed as follows: Grade 1 occurred in 74 % of PBSC recipients versus 34 % for BM (p-value <0.001), Grade 2 in 14 % for PBSC and 0 % for BM (p-value <0.001), and Grade 3 and Grade 4 were both seen in 1–2 % for both groups. In the subgroup analysis stratified by conditioning intensity, neutrophil engraftment (17 days versus 18 days; p-value = 0.017) was faster with PBSC compared to BM grafts and no statistically significant association was noted in rates of acute or chronic GvHD, NRM, relapse, DFS or OS among the myeloablative transplant recipients (n = 55). Among the RIC transplant recipients, no statistically significant differences were noted in neutrophil and platelet engraftment, acute GvHD, NRM, Relapse, DFS, and OS between the BM and PBSC groups (Table 3).

Discussion

This retrospective single-center study analyzes the outcomes of haploidentical allo-HSCT using either PBSC or BM as the graft source, with PT-Cy for GvHD prophylaxis. The study findings show comparable one-year OS and DFS rates between the two graft sources. This aligns with several other studies that have also found no significant differences in OS, DFS, and NRM between different graft sources [7,15,16].

The choice of conditioning regimen is a critical factor in haplo-HSCT and may interact with graft source to influence outcomes. In the current cohort, RIC was more frequently used in the BM group (87 % versus 59 % in PBSC; p-value <0.001), reflecting its common application in older and comorbid patients. Subgroup analysis (Table 2) revealed faster neutrophil engraftment with PBSC in MAC recipients (17 versus 18 days; p-value = 0.017), but no significant differences in acute or chronic GvHD, relapse, NRM, DFS, or OS were observed between graft sources in either the MAC or RIC cohorts. Prior studies suggest RIC may mitigate GvHD risk using PBSC grafts by reducing inflammatory responses, though potentially at the cost of higher relapse rates in certain malignancies [15–17]. Conversely, MAC may increase GvHD risk with PBSC due to higher $CD34^+$ cell doses (median 5.0 versus 2.8×10^6/kg in the present study; p-value <0.001). The imbalance in conditioning regimens limits direct comparisons, and further studies are needed to elucidate how conditioning intensity modulates graft source effects.

A notable imbalance in disease status was observed, with 77 % of BM recipients in complete remission (CR) at transplant compared to 55 % of PBSC recipients (p-value = 0.005). This disparity could bias outcomes toward the BM group, as CR is a strong predictor of improved OS and DFS. To address this, multivariate Cox regression analyses adjusted for disease status, conditioning intensity, and other covariates were

Table 2 – Patient and transplant-related characteristics of haploidentical transplants.

	Total (n = 176)	PBSC (n = 114)	BM (n = 62)	P-value
Age - median years (range)	54 (18–74)	53 (19–73)	54 (18–74)	0.706
Sex - n (%)				
Male	119 (68)	81 (71)	38 (61)	0.238
Female	57 (32)	33 (29)	24 (39)	
Ethnicity - n (%)				
Caucasian	123 (70)	76(67)	47(76)	0.561
African American	23 (13)	17 (15)	6 (10)	
Hispanic	16 (9)	12 (10)	4 (6)	
Others	14 (8)	9 (8)	5 (8)	5
Karnofsky performance status - n (%)				
≥90 %	59 (33.5)	34 (30)	25 (40)	0.138
60–80 %	117 (66.5)	80 (70)	37 (60)	
Hematopoietic stem cell transplantation-specific comorbidity index - n (%)				
0–2	83 (47)	55 (48)	28 (45)	0.753
≥3	93 (53)	59 (52)	34 (55)	
Hematologic Diagnosis - n (%)				
Acute myeloid leukemia	74 (42)	43 (38)	31 (50)	0.027
Myeloid disorders[a]	41 (23)	33 (29)	8 (13)	
Acute lymphoblastic leukemia	17 (10)	10 (9)	7 (11)	
Lymphoma	33 (19)	24 (21)	9 (15)	
Others	11 (6)	4 (3)	7 (11)	
Disease Status - n (%)				
Complete remission	111 (63)	63 (55)	48 (77)	0.005
Others[b]	65 (37)	51 (45)	14 (23)	
Recipient cytomegalovirus serostatus - n (%)				
Negative	67 (38)	48 (42)	19 (31)	0.147
Positive	109 (62)	66 (58)	43 (69)	
Donor cytomegalovirus serostatus - n (%)				
Negative	75 (43)	46 (40)	29 (47)	0.429
Positive	101 (57)	68 (60)	33 (53)	
Donor age - median years (range)	32 (10–65)	34 (11–65)	31 (10–64)	
Donor sex - n (%)				
Male	111 (63)	73 (64)	38 (61)	0.746
Female	65 (37)	41 (36)	24 (39)	
Conditioning - n (%)				
Myeloablative	55 (31)	47 (41)	8 (13)	<0.001
Reduced intensity conditioning	121 (69)	67 (59)	54 (87)	
Graft cell dose (Median CD34 cells x10^6 per kg)	4.9 (4.3–5.0)	5.0 (5.0–5.2)	2.8 (2.3–3.2)	<0.001

[a] myeloid disorders include myelodysplastic syndromes, myeloproliferative neoplasms, and chronic myeloid leukemia.
[b] Includes partial response (n = 36; 8 %), stable disease (n = 89; 20 %), progressive disease (n = 31; 7 %), and not available (n = 14; 3 %).

performed, revealing no independent effect of graft source on OS (HR: 1.02; 95 % CI: 0.68–1.53; p-value = 0.898), DFS (HR: 1.00; 95 % CI: 0.67–1.49; p-value = 0.994), relapse (HR: 0.88; 95 % CI: 0.47–1.65; p-value = 0.579), or NRM (HR: 0.92; 95 % CI: 0.49 –1.74; p-value = 0.682). These findings suggest that while CR status and conditioning are critical prognostic factors, they do not significantly alter the comparative effectiveness of PBSC versus BM in the current cohort. Nonetheless, these factors should be considered when selecting graft sources to optimize patient outcomes.

While some studies have reported better OS with PBSC (due to higher doses of $CD34^+$ cells potentially leading to improved outcomes) [18], others have indicated poorer outcomes associated with higher NRM rates in PBSC recipients compared to those receiving BM grafts [8,18,19]. For example, Nagler et al. noted worse life expectancy, OS, and GRFS with PBSC [7,9,20]. The results of this study highlight the importance of considering multiple independent factors, including pre-transplant disease status, the Hematopoietic Cell Transplantation-Specific Comorbidity Index, and conditioning regimen, beyond the choice of graft source, which can significantly impact transplant outcomes [8,15,16,20].

Recipients of PBSC exhibited an earlier neutrophil engraftment by one day, indicating a potential advantage in the speed of hematopoietic recovery with PBSC grafts. However, this modest difference may not be clinically relevant. Several other studies also reported earlier neutrophil engraftment in patients who receive PBSC versus BM grafts [9,16,21]. Kato et al. demonstrated an association of total $CD34^+$ dose to engraftment regardless of graft source [21]. The results here differ from other studies where similar median times for neutrophil and platelet engraftment were noted [8,18,22].

In line with previous literature [8,11,22–25], similar incidences of Grade II-IV acute GvHD were observed between BM and PBSC recipients in this study. Consistent with previous studies, similar incidences of one-year chronic GvHD were also observed [8,16]. The incidence of GRFS, a composite outcome of GvHD and RFS, was comparable in both groups.

Table 3 – Outcomes after haploidentical transplants.

	Total (n = 176)	PBSC (n = 114)	BM (n = 62)	p-value
Follow-up - median months (range)	21 (0.3–73)	21 (0.3–69)	22 (0.3–73)	0.331
Neutrophil engraftment - median days (95 % CI)	17 (17–18)	17 (17–19)	18 (18–20)	0.022
Day +28 neutrophil recovery (>0.5 × $10^3/\mu$L) - n (%)	164 (93)	108 (95)	56 (90)	0.349
Platelet engraftment - median days (95 % CI)				
>20 × $10^3/\mu$L	27 (26–28)	27 (26–28)	28 (26–30)	0.195
>50 × $10^3/\mu$L	29 (27–31)	28 (27–31)	31 (29–36)	0.092
Day +100 platelet recovery (>20 × $10^3/\mu$L) - n (%)	153 (87)	103 (90)	50 (81)	0.099
Primary graft failure - n (%)	5 (3)	2 (2)	3 (5)	0.347
Day +100 acute GvHD - n (%)				
Grade 2–4	88 (50)	58 (51)	30 (48)	0.875
Grade 3–4	14 (8)	9 (8)	5 (8)	1.000
One-year chronic GvHD - n (%)				
All	62 (35)	43 (38)	19 (31)	0.410
Extensive	56 (32)	38 (33)	18 (29)	0.614
Relapse - n (%)	41 (23)	25 (22)	16 (26)	0.579
Non-relapse mortality - n (%)	40 (23)			0.998
One-year non-relapse mortality	31 (18)	19 (17)	12 (20)	0.682
Cytokine Release Syndrome - n (%)	125 (71)	102 (90)	23 (37)	<0.001
Cytokine Release Syndrome grade - n (%)				
Grade 0 (No CRS)	51 (29)	12 (10)	39 (63)	<0.001
Grade 1	105 (60)	84 (74)	21 (34)	
Grade 2	16 (9)	16 (14)	0 (0)	
Grade 3	2 (1)	1(1)	1 (1.5)	
Grade 4	2 (1)	1(1)	1 (1.5)	
Cause of death - n (%)				
Relapse/progression	22 (32)	13 (30)	9 (38)	0.599
GvHD	10 (15)	5 (11)	5 (21)	
Infections	23 (34)	17 (39)	6 (25)	
Organ failure	3 (4)	2 (5)	1 (4)	
Graft failure	3 (4)	1 (2)	2 (8)	
Others, non-transplant-related	5 (7)	4 (9)	1 (4)	
Not available	2 (3)	2 (5)	0 (0)	
Disease-free survival				
Median months (range)	NR	NR	NR	0.994
One-year disease-free survival - n (%)	111 (63)	72 (63)	39 (63)	1.000
Overall survival				
Median months (range)	NR	NR	NR	0.898
One-year overall survival - n (%)	131 (74)	85 (75)	46 (74)	1.000
One-year GvHD-free Relapse-free survival - n (%)	117 (66.5)	76 (67)	41 (61)	1.000

BM: Bone marrow; PBSC: Peripheral blood stem cells; NRM: Non-relapse mortality; GvHD: Graft-versus-host disease.

Several other studies reported higher incidences of acute and chronic GvHD in the PBSC compared to BM group [9,15,16,18,21,22]. Various other pre-transplant factors such as low body mass index (BMI) and older age, are independently associated with a higher risk of GvHD [26,27]. No difference in graft failure was found between both cohorts in the present study. In a large retrospective study, Olsson et al. reported lower rates of PGF in patients receiving PBSC compared to BM [28]. They attributed this improvement to the higher doses of graft cells typically administered in PBSC transplants. Another factor contributing to PGF reported in literature is donor-specific anti-HLA antibodies [29,30].

CRS after haplo-HSCT is often associated with early T-cell reconstitution and low incidence of post-transplant relapse [31]. However, research has noted an increased risk of infections following CRS, potentially due to its impact on neutrophil recovery [32]. In this study, the majority of CRS cases were of mild severity (Grades 1–2), aligning with findings from previous research [33]. Additionally, the higher incidence of CRS observed in the PBSC group is consistent with earlier studies [34]. In the present study population, infections and relapse/progression of the disease emerged as the most common causes of death, consistent with findings in previous literature where GvHD-related death also featured prominently [9,22,35].

This study has several limitations inherent to its retrospective design, including selection bias, confounding by indication, and limited statistical power. With a sample size of 176 patients, the analysis may be underpowered to detect small but clinically meaningful differences in outcomes such as GvHD, relapse, or GRFS, particularly in subgroups defined by conditioning intensity or disease status. The heterogeneous patient population, encompassing diverse hematologic malignancies, ethnicities, and conditioning regimens, further

complicates the generalizability of the findings. Additionally, the relatively short median follow-up of 21 months may not capture late events.

Conclusion

These findings suggest that haplo-HSCT with PT-Cy yields comparable rates of acute and chronic GvHD, relapse, NRM, DFS, and OS between BM and PBSC graft sources. A slightly faster neutrophil engraftment with PBSC offers a potential advantage, though its clinical significance is limited. Imbalances in disease status and conditioning intensity highlight the importance of patient-specific factors in graft selection. These results suggest flexibility in choosing either BM or PBSC, allowing clinicians to tailor decisions based on donor availability and patient characteristics, such as CR status and conditioning regimen. Prospective randomized trials are needed to confirm these findings and optimize transplant strategies.

Conflicts of interest

No relevant conflicts of interest. MH has consultancy roles in Incyte Corporation, ADC Therapeutics, Pharmacyclics, Omeros, Genmab, Morphosys, Kadmon, Kite, Novartis, Abbvie, Legend, Gamida Cell, SeaGen, Autolus, Byondis, and Forte Biosciences Inc., and is on the speaker's bureau for Sanofi Genzyme, AstraZeneca, BeiGene, and ADC Therapeutics. JPM has consulting and advisory roles in Bristol Myers Squibb, Kite, Novartis, AlloVir, Envision, Autolus, Nektar Therapeutics, CRISPR therapeutics, Caribou Bio, Sana Technologies, Legend Biotech, and Cargo Therapeutics. MUM has research funding from Iovance Biotherapeutics. The remaining authors declare no other commercial or financial relationships that could be construed as a potential conflict of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Mesenchymal stromal cells secretory pattern contributes to oncoinflammatory bone marrow microenvironment in polycythemia vera

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ABSTRACT

Introduction: Polycythemia vera is a myeloproliferative neoplasm marked by an increased proliferation of erythroid mature and precursors cells in bone marrow and peripheral blood. The pathophysiology is linked to the presence of the JAK2 driver mutation, epigenetic deregulation, and alterations in the bone marrow hematopoietic niche. Multipotent mesenchymal stromal cells (MSC) in the bone marrow, which are crucial for maintenance and development of hematopoietic stem cells, play a role in the communication between neoplastic cells and resident bone marrow cells by releasing various mediators that either suppress or promote tumor progression. These mediators include several essential immunomodulatory molecules, pro-angiogenic and growth factors. We hypothesized that MSC from polycythemia vera patients (Patient Group) would exhibit distinct properties compared to those from healthy donors (Control Group), thereby influencing the hematopoietic niche and contributing to disease pathogenesis.

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Methods: This study characterized MSC from patients, focusing on their secretory, proteomic, and phenotypic properties.
Results: MSC from the Patient and Control Groups had similar immunophenotypes and multipotentiality. However, MSC from the Patient Group exhibited reduced immunomodulatory properties, and released distinct soluble immune and angiogenic mediators when compared with the Control Group. Global proteomic analysis revealed that MSC from patients presented upregulated expressions of *FAM175B, VP526A, CTTN, MAP4, BAX*, and *TPD52L2* but a downregulated *TNC* expression. These results indicate that MSC contribute to the inflammation pattern in the hematopoietic niche. The secretory and proteomic profile of MSC from patients, indicate that these cells may influence immune cell function, induce neoangiogenesis, and alter cell-to-cell interactions within the bone marrow, thereby fostering a pro-tumor microenvironment and favoring disease pathogenesis.
Conclusion: These findings highlight the potential of targeting MSC-mediated pathways as a therapeutic strategy in polycythemia vera.

Introduction

Multipotent mesenchymal stromal cells (MSCs) are key elements that regulate hematopoietic stem cells (HSC) and resident BM cells in the hematopoietic niche [1,2]. They are known for their self-renewal ability and potential to differentiate into bone, cartilage, and fat cells [1,2]. The paracrine effect of MSCs is related to the production of stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), C-X-C motif chemokine ligand 12 (CXCL12), and interleukin (IL)-1, −6, −7, −8, −11 and −15 [3−7]. These mediators contribute to cell proliferation and differentiation, immunomodulation and angiogenesis [6,7].

MSCs play a role in the communication between neoplastic and resident BM cells by engaging multiple signaling pathways and release various mediators that can either suppress or promote tumor progression [8]. In myelodysplastic syndromes, MSCs favor the inflammatory milieu, immune system suppression, and disease progression [9]. Mice lymphoma-derived MSCs release CCL2, which increases the proliferation of tumor cells and attracts immunosuppressive cells to the lymphoid tissues [10].

On the other hand, MSC antitumor action is associated with angiogenesis inhibition through the activation of endothelial cell apoptosis in melanoma [11]. In a chronic myeloid leukemia model, MSCs attenuate K562 proliferation by inhibiting Wnt signaling [12]. In myeloproliferative neoplasms (MPN) the TGF-β1 produced by monocytes or macrophages convert MSCs into contractile α-SMA+ myofibroblasts that contribute to BM fibrosis [13,14]. In particular, BM alterations in polycythemia vera (PV) patients are marked by an inflammatory profile with elevated levels of inflammatory cytokines, chemokines and pro-angiogenic factors when compared with BM from apparently healthy donors (HD) [15]. These alterations potentially interfere with hematopoiesis, neoangiogenesis, cell-to-cell interactions, and cell-stroma interactions in the BM from MPN patients that contribute to a pro-tumor microenvironment and disease pathogenesis [15−18].

In this context, our hypothesis is that there is an imbalance between MSC and neoplastic cells in the bone marrow hematopoietic niche in PV. Thus, this study examined the immunophenotype, proteomic and secretory profile of bone marrow MSCs from PV patients to describe their potential contribution to the inflammatory niche in this MPN.

Material and methods

Subjects

Samples were collected from apparently healthy BM donors and MPN patients at the Ribeirão Preto Medical School Hospital, University of São Paulo (HC-FMRP/USP). The study included five HD volunteers and seven PV patients, with the latter diagnosed according to the 2016 WHO criteria [19]. All PV patients were JAK2V617F-positive, newly diagnosed, and therefore not under any treatment or using antiplatelet drugs at the time of bone marrow collection. Moreover, their medical records did not report any major comorbidity, such as diabetes mellitus or hypertension (Table 1).

The study was approved by the Ethics Committee for Human Research of the School of Pharmaceutical Sciences of Ribeirão Preto (protocol CAAE 55,545,716.6.0000.5403).

Table 1 – Clinical characteristics of patients with polycythemia vera (PV) and healthy donors (HD).

	PV (n = 7)	HD (n = 5)
Age - years (range)	61 (31−66)	29.6 (24−37)
Gender - n (male %)	4 (57.14)	3 (60.0)
Risk - n (%)		
High	5 (71.43)	
Low	2 (28.57)	
Vascular events - n (%)		
Yes	3 (42.86)	
No	4 (57.14)	
Bone marrow fibrosis rate - n (%)		
0	3 (42.86)	
1	4 (57.14)	

Bone marrow mesenchymal stromal cell isolation and culture

Bone marrow mononuclear cells (BMMC) were separated by gradient centrifugation using Ficoll® Paque Plus (Sigma-Aldrich), according to the manufacturer's instructions. BMMC were collected from the upper layer, washed twice with phosphate buffered saline (PBS), and centrifuged at 300 x *g* for 10 min at room temperature. The cells from the pellet were counted and then seeded at a density of 2–4 $\times$ 10^7 cells in T75 cm^2 flasks. MSCs adhered to the plastic surface and grew, while the other cell types were eliminated during medium changes and cell passages.

BM-MSCs were maintained in base medium alpha-mem (GIBCO™, Thermo Fisher Scientific) supplemented with 15 % fetal bovine serum (HyClone, Cytiva), 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (GIBCO™, Thermo Fisher Scientific), and cultured under 5 % CO_2 at 37 °C. For the assays, MSCs were used at passages 3 and 4.

Bone marrow mesenchymal stromal cell immunophenotyping

BM-MSC immunophenotyping was performed by flow cytometry (FACSCalibur™, BD Biosciences) using antibodies against: CD13 (clone WN15), CD29 (clone MAR4), CD31 (clone WM59), CD34 (Clone 8G12), CD44 (clone M1), CD45/14 (clone 2D1/MϕP9), CD49e (clone IIA1) CD54 (clone HA58), CD73 (clone AD2), CD90 (clone 5E10), CD105 (clone 266), CD166 (clone 3A6), and HLA-DR (clone G46-6) (BD Biosciences).

In vitro adipogenic differentiation assay

BM-MSCs (6×10^3) were cultured in 24-well plates under adipogenic conditions using StemPro™ Adipogenesis differentiation kit (GIBCO™, Thermo Fisher Scientific). Then, cells were washed twice with PBS and fixed in 4 % paraformaldehyde solution for 30 min. Cells were rinsed three times with distilled water, and once with 70 % (v/v) ethanol. Then, cells were stained with Sudan III (Merck, Sigma-Aldrich) for 30 min. Subsequently, cells were rinsed once in 70 % (v/v) ethanol, twice in distilled water, and then counterstained with Hematoxylin-Mayer solution (Merck, Sigma-Aldrich) for 3 min. After the Hematoxylin-Mayer staining, the cells were gently washed three times in distilled water and then kept in fresh water until they turned blue (from one to three minutes). Images were captured with an CX30 optical microscope (Olympus) at 10x magnification.

In vitro osteogenic differentiation assay

BM-MSCs (6×10^3) were cultured in 24-well plates for 14 days under osteogenic conditions with StemPro™ Osteogenesis Differentiation kit (GIBCO™, Thermo Fisher Scientific). Then, cells were washed twice with PBS and fixed in 4 % paraformaldehyde solution for 30 min. Cells were rinsed three times with distilled water and incubated in 5 % (w/v) silver nitrate solution (Merck, Sigma-Aldrich) for 60 min while exposed to a 100 W incandescent light. The silver nitrate solution was removed and cells were rinsed three times with distilled water. Cells were incubated with 5 % (w/v) sodium thiosulfate solution (Merck, Sigma-Aldrich) for 2 min and washed three times with distilled water. Then, cells were counterstained with hematoxylin-Mayer solution (Merck, Sigma-Aldrich) for 3 min and gently washed three times with water. Images were captured with an optical microscope CX30 (Olympus) at 10x magnification.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells, used in co-cultivation and in inhibition of T-lymphocyte proliferation assays, were isolated from peripheral blood from two HD, using gradient centrifugation with Ficoll® Paque Plus (Sigma-Aldrich).

Inhibition of T-lymphocyte proliferation

CD4 and CD8 T-lymphocytes, isolated from peripheral blood of two HD, were co-cultured with BM-MSC at different ratios to analyze inhibition of cell proliferation. Peripheral blood mononuclear cells (PBMC) were suspended in 4 mL of 0.1 % (w/v) bovine serum albumin (MERCK, Sigma-Aldrich) solution in PBS and stained with 5 μM of 5-(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE; MERCK, Sigma-Aldrich) for 10 min at 37 °C. Next, PBMC labeled with CFSE ($PBMC^{CFSE}$) were centrifuged at 400 x *g* for 10 min at 4 °C, and washed twice with 20 mL of complete Roswell Park Memorial Institute Medium 1640 followed by centrifugation at 400 x *g* for 10 min at 4 °C.

BM-MSCs were seeded in a 48-well culture plate at five concentrations: 1.25×10^5 cells/mL (ratio 1:2), 5×10^4 cells/mL (ratio 1:5), 2.5×10^4 cells/mL (ratio 1:10), 1.25×10^4 cells/mL (ratio 1:20) and 0.5×10^4 cells/mL (ratio 1:50). Cells were cultured in 10 % fetal bovine serum α-MEM media under 5 % CO_2 for 8 h at 37 °C. The supernatant was removed, and 1 mL of $PBMC^{CFSE}$ was seeded per well (2.5×10^5 cell/mL). To stimulate $PBMC^{CFSE}$ cell proliferation, 3.12 μL of CD3/CD28 magnetic beads (Dynabeads Human T-Activator CD3/CD28, Thermo Fisher Scientific) were added to the mixture at the 1:1 bead:T-cell ratio recommended by the manufacturer. The beads are necessary to activate CD4 and CD8 proliferation.

The plate was incubated under 5 % CO_2 for five days at 37 °C. The supernatant was removed and centrifuged at 400 x *g* for 10 min at 4 °C. The resulting supernatant was aliquoted and stored at −80 °C to further analyze soluble mediators. The cell pellet ($PBMC^{CFSE}$) was suspended in PBS, stained with anti-CD4 and anti-CD8 antibodies (BD Biosciences), and analyzed in a FACSCalibur™ flow cytometer (BD Biosciences). $PBMC^{CFSE}$ not stimulated with beads were used as the negative control. The percentage of CD4 and CD8 cell proliferation inhibition was calculated by the proliferation ratios of CD4: CD8 cells cultured alone or in combination with different MSC concentrations.

Bone marrow mesenchymal stromal cell doubling time

BM-MSCs at passages 3 and 4 were seeded in a 24-well plate, at a density of 3000 cells/cm^2 with one mL of complete base medium, and cultured at 37 °C for seven days under 5 % CO_2. Three wells were trypsinized with 0.25 % trypsin solution (GIBCO™, Thermo Fisher Scientific) every 24 h. The BM-MSC

concentration and viability were determined using 0.4 % trypan blue (GIBCO™, Thermo Fisher Scientific).

The cell doubling time was calculated using the following equation: DT = (*t* x log2) / (log(n/n_0)), where DT = doubling time, *t* = cultivation time (days), *n* = total cells at the end of seven days, and n_o = total cells in the beginning of the experiment (Day 0). The exponential cell growth curve was plotted using the GraphPad Prism 6.0 (Dotmatics) software.

Quantification of soluble immune and angiogenic mediators

Supernatants from co-cultures of BM-MSCs and PBMCs at 1:10 ratio were used to quantify epidermal growth factor (EGF), fibroblast growth factor 2 (FGF-2), Fms-related tyrosine kinase 3 ligand (Flt-3 L), interferon alpha (IFN-α), macrophage-derived chemokine (MDC), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor-alpha (TNF-α), and vascular endothelial growth factor (VEGF) using the multiplex human customized Magnetic Luminex® Assay (R&D Systems), according to the manufacturer's instructions. The samples were analyzed in a MX® Luminex (MERCK) and the concentration was determined using xPONENT (Luminex, MERCK, Sigma-Aldrich).

Proteomic analysis of bone marrow mesenchymal stromal cells from polycythemia vera patients and healthy donors

Protein was extracted from cultured MSCs at passage 3. Pelleted cells were washed twice with PBS to remove fetal bovine serum and then suspended in cell lysis buffer with four protease inhibitors. After centrifugation, the supernatant was used to quantify proteins by a fluorescence-based method using the Qubit Protein Assay kit and the Qubit fluorimeter (Thermo Fisher Scientific). Ten micrograms of protein lysates were applied in denaturant 10 % polyacrylamide gel (SDS-PAGE) and stained with Coomassie. The gel bands were cut, discolored, dehydrated, and dried. The dry fragments were reduced and alkylated, and the resulting liquid part was discarded. Then the fragments were digested, dehydrated, dried (twice), further incubated with a trypsin solution, washed, and digested. The liquid part was mixed with the extraction solution (water, 3 % TFA, 30 % acetonitrile) and incubated. The supernatants were transferred to new tubes and incubated twice with acetonitrile, followed by drying using a SpeedVac until the volume was reduced to 10–20 % of the original. The peptides were purified with StageTips-C18, dried and diluted in aqueous/dilution solution for analysis in a LC-MS/MS equipment (Liquid Chromatograph Ultimate 3000, Thermo Scientific). The parameters used were gradient phase A: 0.1 % formic acid, 5 % DMSO and phase B: 0.1 % formic acid, 5 % DMSO in acetonitrile, under the flow rate of 250 nL/min, with linear gradients of 5–40 % of phase B in 120 min. The analytical columns were 15 cm length with internal diameter of 75 μm. Mass spectrometry was performed in a hybrid LTQ Orbitrap XL ETD, Thermo Scientific Full Scan (MS1) with full scan window (*m/z*) of 300.0–2000.0, resolution of 60,000, lock mass of 401.922718 *m/z*.

Perseus software was used to analyze proteomic data according to the developer's instructions. The fluorescence intensity recorded in the mass spectrometer was normalized, values that were not detected in any sample of a group were eliminated, and groups of two samples at a time were compared using t-test [20].

Gene ontology biological process analysis

Gene Ontology analysis was performed using the Webgestalt online tool (https://www.webgestalt.org/) with the following settings: Over-representation Analysis (ORA) method, Gene Ontology Database non-redundant Database, with significance level cut-off false discovery rate (FDR) <0.05, and viewint the top ten categories. The 102 differentially expressed proteins (DEPs) were loaded in separate runs to compare DEPs of BM-MSCs from PV patients to BM-MSC from HD: upregulated DEPs (58 proteins) and downregulated DEPs (44 proteins) [21].

Statistical analysis

Proteomic data were analyzed and graphs plotted using GraphPad Prism version 6.01 and Excel for Microsoft 365. The Kruskal-Wallis test with Dunn's post-test was used for non-parametric analysis of variance to compare mean values across experimental conditions, and the Mann-Whitney test was used for two-group comparisons. Proteomics statistics were based on label-free quantification (LFQ) of protein abundance. Raw mass spectrometry data were analyzed using MaxQuant software, and proteins with FDR ≥1 % were filtered out. The resulting "proteinGroups.txt" table was imported into R (version 4.2) for differential protein expression analysis using the Differential Enrichment Analysis of Proteomics data package. Contaminant and reverse proteins were removed, and data were filtered for proteins with LFQ >0 in at least one group. The LFQ intensities were normalized and imputed with random Gaussian distribution draws around a minimal value (p-value <0.01). Differential enrichment analysis was performed using the Limma function in the Differential Enrichment Analysis of Proteomics data package, selecting proteins with p-adjust ≤0.05 and log2 fold change >1 [22,23].

Results

Bone marrow mesenchymal stromal cell characterization

The proliferative potential of BM-MSCs were examined from five PV patients and three HD. The BM-MSC doubling time was 46.2 and 55.5 h in HD and PV patients, respectively (Figure 1A) and the growth rate (μ) was 0.36 and 0.30/day in BM-MSC from HD and PV patients, respectively.

The immunophenotypic analysis revealed that BM-MSC from both PV patients and HD highly expressed the cell surface markers CD90, CD73, CD105, CD13, CD29, CD44, CD166 and CD49e, intermediately expressed CD54, and weakly expressed HLA-DR, CD31, CD34, CD14, and CD45 (Figure 1B).

There were no differences between the BM-MSC immunophenotypes from PV patients and HD (Figure 1B), and the BM-MSC from both groups were able to transdifferentiate into osteogenic and adipogenic cell lines (Figure 1C).

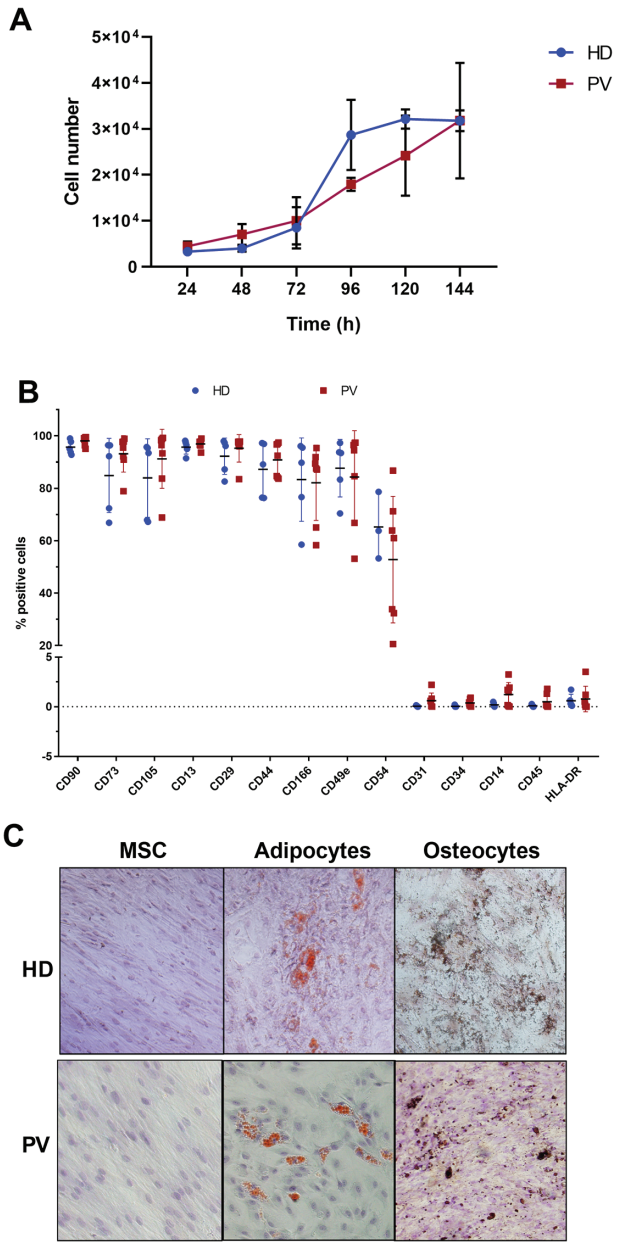

Figure 1 – Characterization of bone marrow mesenchymal stromal cells (BM-MSC) of polycythemia vera (PV) patients and healthy donors (HD). A. BM-MSC growth curve, where data were expressed as means (PV: *n* = 4; HD: *n* = 3). B. BM-MSC immunophenotype, expressed as percentage of positive cells relative to 10,000 events. The number of the samples varied according to the marker C. Representative images of multipotential analysis. Left: BM-MSC grown in base medium (α-MEM supplemented with 10 % fetal bovine serum) and stained with hematoxylin. Center: MSC after 12 days of growth in adipogenic medium. Lipid accumulation in adipocytes (orange) stained with Sudam III and counterstained with hematoxylin. Right: MSC after 15 days of growth in osteogenic medium. Calcium deposits in osteocytes (brown) stained with von Kossa stain and counterstained with hematoxylin.

Bone marrow mesenchymal stromal cells from polycythemia vera patients alter CD8⁺ T lymphocyte proliferation

BM-MSC were co-cultured with activated CD4⁺ and CD8⁺ *T* lymphocytes to evaluate their immunomodulatory effects on T cell proliferation (Figure 2). In both PV patients and HD groups, T cell proliferation (CD4⁺ and CD8⁺) decreased at high BM-MSC:lymphocyte ratios (1:2 and 1:5). Notably, BM-MSC from PV patients modulated CD8+ *T* cell proliferation in a concentration-dependent manner. At high BM-MSC:lymphocyte ratios (1:2 and 1:5), CD8⁺ *T* cell proliferation was reduced, while at low ratios (1:20 and 1:50), it was enhanced. At a BM-MSC:lymphocyte ratio of 1:10, CD8⁺ *T* cell proliferation was similar to that of CD8⁺ *T* cells cultured alone, indicating a balance and lack of immunomodulation. When comparing suppression effects between groups, BM-MSC from HD more effectively inhibited CD4⁺ *T* cell proliferation at a 1:2 BM-MSC: lymphocyte ratio. Conversely, BM-MSC from PV patients more effectively decreased CD8⁺ *T* cell proliferation at a lower BM-MSC:lymphocyte ratio (1:5) compared to BM-MSC from HD. At lower BM-MSC:lymphocyte ratios, there were no significant differences in the immunomodulatory effects on CD8⁺ and CD4⁺ *T* cells between HD and PV.

Bone marrow mesenchymal stromal cell secretory profile

Inflammatory and angiogenic factors were quantified in the supernatants from mono- and co-cultures of BM-MSC and PBMC to examine the BM-MSC secretory profile (Figure 3). Compared with BM-MSC from HD, those from PV patients released higher concentrations of EGF (p-value ≤0.05) and IL-6 (p-value ≤0.01). Both BM-MSC groups – from HD (p-value ≤0.001) and PV patients (p-value ≤0.05) – released higher concentrations of IL-6 and TNF-α when they were co-cultured with PBMC.

BM-MSC from PV patients co-cultured with PBMC released (i) had higher macrophage-derived cytokine (MDC; p-value ≤0.05) and vascular endothelial growth factor (VEGF; p-value ≤0.01) levels, compared with BM-MSC from PV patients alone; and (ii) lower FMS-like tyrosine kinase 3 ligand (Flt-3 L; p-value ≤0.01) levels than MSC from HD co-cultured with PBMC (Figure 3A).

The categorical analysis of the angiogenic mediators produced by BM-MSC from HD revealed that they were altered and PBMC-dependent. No relevant levels of angiogenic factors were detected in the supernatant from monocultures of BM-MSC from HD. In contrast, the supernatant from BM-MSC from HD co-cultured with PBMC had high concentrations of EGF, FGF-2, Flt-3 L, IL-6, IL-1β, and VEGF (Figure 3B). The supernatants from BM-MSC from PV patients alone or co-cultured with PBMC presented high concentrations of angiogenic and immune cell mediators.

Protein global expression in polycythemia vera mesenchymal stromal cells

Proteomic analysis was carried out to compare BM-MSC from PV patients and HD using Perseus (less stringent) and R (more

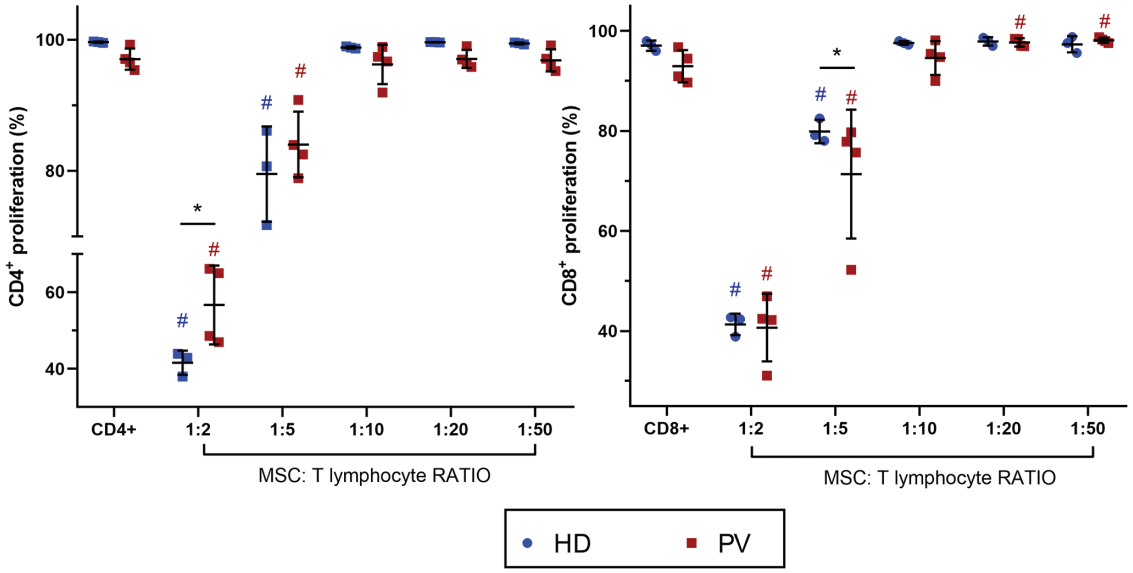

Figure 2 – Bone marrow mesenchymal stromal cells (BM-MSC) immunomodulation of CD4⁺ and CD8⁺ proliferation. Comparison between CD4⁺ and CD8⁺ proliferation cultured with and without BM-MSC from polycythemia vera patients (PV: *n* = 4) and healthy donors (HD: *n* = 3). T lymphocytes from a blood donor were used. P-value ≤0.05 indicated statistical significance (multiple linear regression analysis). All the experimental conditions were performed in triplicate. Dark blue # means significant differences between HD with CD4⁺ or CD8⁺ alone. Red # means significant differences between PV patients with CD4⁺ or CD8⁺ alone. Black * means significant differences between PV and HD.

stringent) software systems (Figure 4). Compared with BM-MSC from HD (Controls, HD), the number of differentially expressed proteins (p-value ≤0.05) in BM-MSC from PV patients was 102 (Figure 4B-C). Just seven proteins were differentially expressed in BM-MSC from PV patients, when the R package was used for the most stringent analysis. The differentially expressed proteins in PV patients evidenced by the Volcano plots were upregulated FAM175B, VP526A, CTTN, MAP4, BAX, TPD52L2 and downregulated TNC (Figure 4A).

Gene ontology biological process analysis

Figure 5A depicts the main gene ontology categories linked to the upregulated proteins in BM-MSC from PV patients. The three main biological processes detected in this group were biological regulation, metabolic processing, and response to stimulus. The enrichment analysis based on Biological Process and Gene Ontology Datasets of the 58 upregulated DEPs in BM-MSC from PV patients compared to BM-MSC from HD identified the top categories “neutrophil degranulation”, “neutrophil activation involved in immune response”, and “neutrophil activation” (Figure 5B). All the ten top categories were related to white blood cell activity and exocytosis. The 44 downregulated DEPs in BM-MSC from PV patients were subjected to the same analysis and the results for the biological processes were similar (Figure 5C). The enrichment analysis of the downregulated proteins in BM-MSC from PV patients identified the top category “protein targeting to endoplasmic reticulum”. All the top ten categories were involved in protein and mRNA metabolism (Figure 5D).

Discussion

BM-MSC from the BM microenvironment not only regulate HSCs but also various resident cells associated with tumorigenesis and malignant transformation within the hematopoietic niche. They contribute to immune evasion through paracrine activity, promoting the growth and survival of neoplastic cells and activating angiogenesis [24–26].

BM-MSC from PV patients and HD exhibited similar morphology, immunophenotype, and multipotentiality. However, PV BM-MSC proliferated more slowly, with longer doubling times compared to those from HD. This is consistent with findings in MPN and other hematological diseases [27–29].

BM-MSC suppress the proliferation of immune cells, including dendritic cells, natural killer cells, and T lymphocytes, mainly through cell-to-cell contact and secretion of mediators like PGE2 and TGF-β [30,31]. In this study, BM-MSC from HD inhibited both CD4⁺ and CD8⁺ T cell proliferation in a concentration-dependent manner, with higher BM-MSC concentrations reducing proliferation and inhibiting mononuclear cell functions. PV BM-MSC showed similar effects but paradoxically enhanced T cell proliferation at lower concentrations, suggesting that they provide additional stimulation for T cell proliferation.

T cells are crucial for antitumor immunity, and their negative regulation is linked to immune evasion mechanisms favoring tumorigenesis [30–33]. Data from this study show that BM-MSC exerted differential effects on immune cells depending on their concentration within the co-culture system. At high BM-MSC proportions, they inhibited

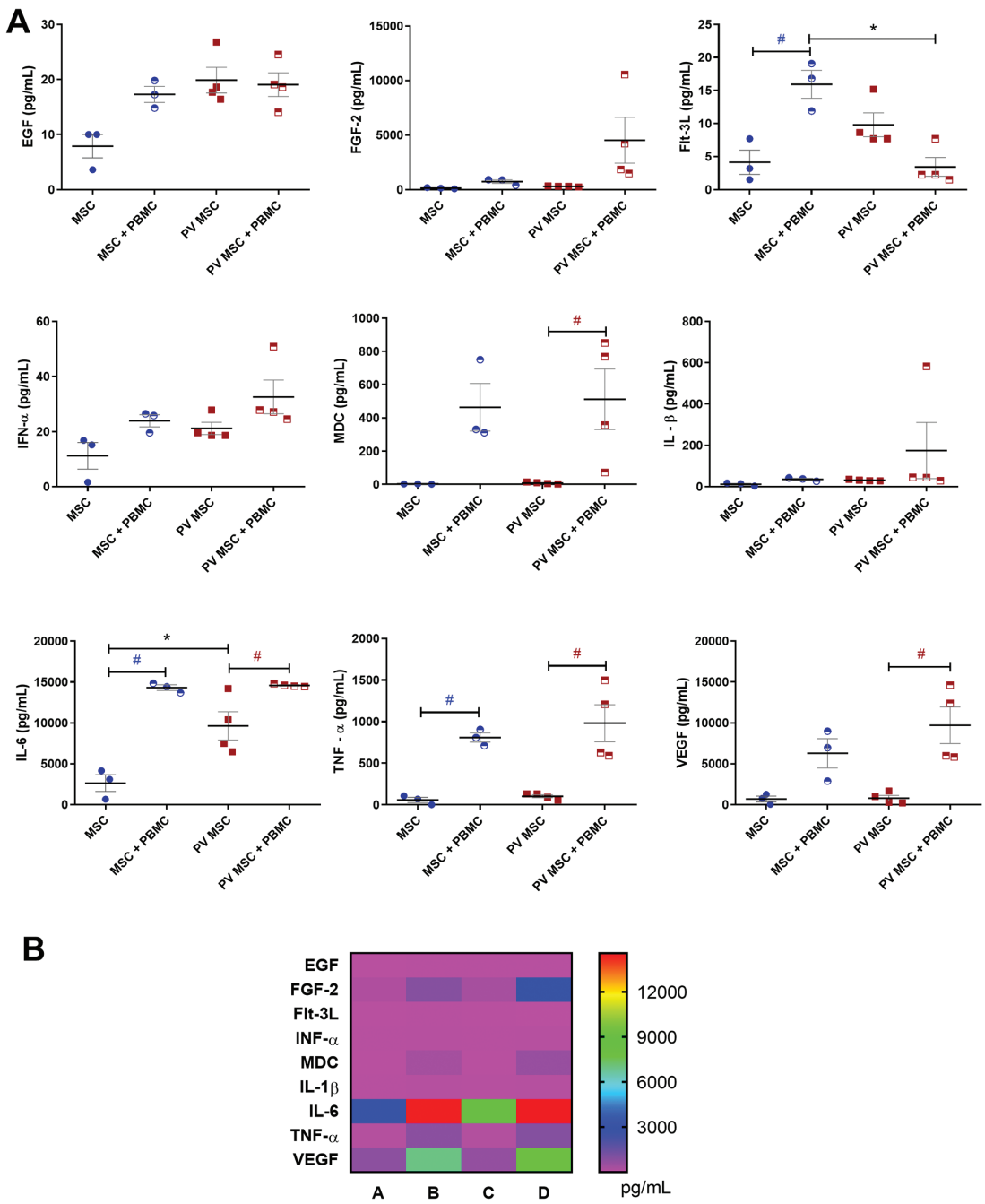

Figure 3 – Quantification of cytokines and angiogenic factors in bone marrow (BM) multipotent mesenchymal stromal cells (MSC) and PBMC (peripheral blood mononuclear cells) under mono- and co-culture conditions. BM-MSC from patients with polycythemia vera (PV: *n* = 4) and from healthy donors (HD: *n* = 3) were cultured for 24 h in the presence or absence of PBMC activated with CD3/CD28 magnetic beads. A: **Black * means significant differences between PV and HD MSC. Dark blue # means significant difference between MSC (HD) conditions. Red # means significant differences between PV MSC conditions. p-value <0.05 indicates statistical significance; * or # indicates p-value ≤0.05 (ANOVA followed by the Tukey test).** B: **Heatmap of cytokines and angiogenic factor concentrations (pg/mL) in (A) monoculture of BM-MSC from HD; (B) co-culture of BM-MSC from HD and PBMC; (C) monoculture of BM-MSC from PV patients; (D) co-culture of BM-MSC from PV patients and PBMC.**

immune cell functions, whereas low concentrations stimulated cell proliferation. This suggests that neoplastic cells may regulate MSC to release proliferative stimuli rather than suppress immune responses, thus aiding tumor progression.

The differential effects of PV BM-MSC on T cell proliferation highlight the role of the BM niche in immune modulation and tumor progression. Low BM-MSC ratios enhanced T cell proliferation, supporting a pro-tumor microenvironment and potentially contributing to MPN pathogenesis and

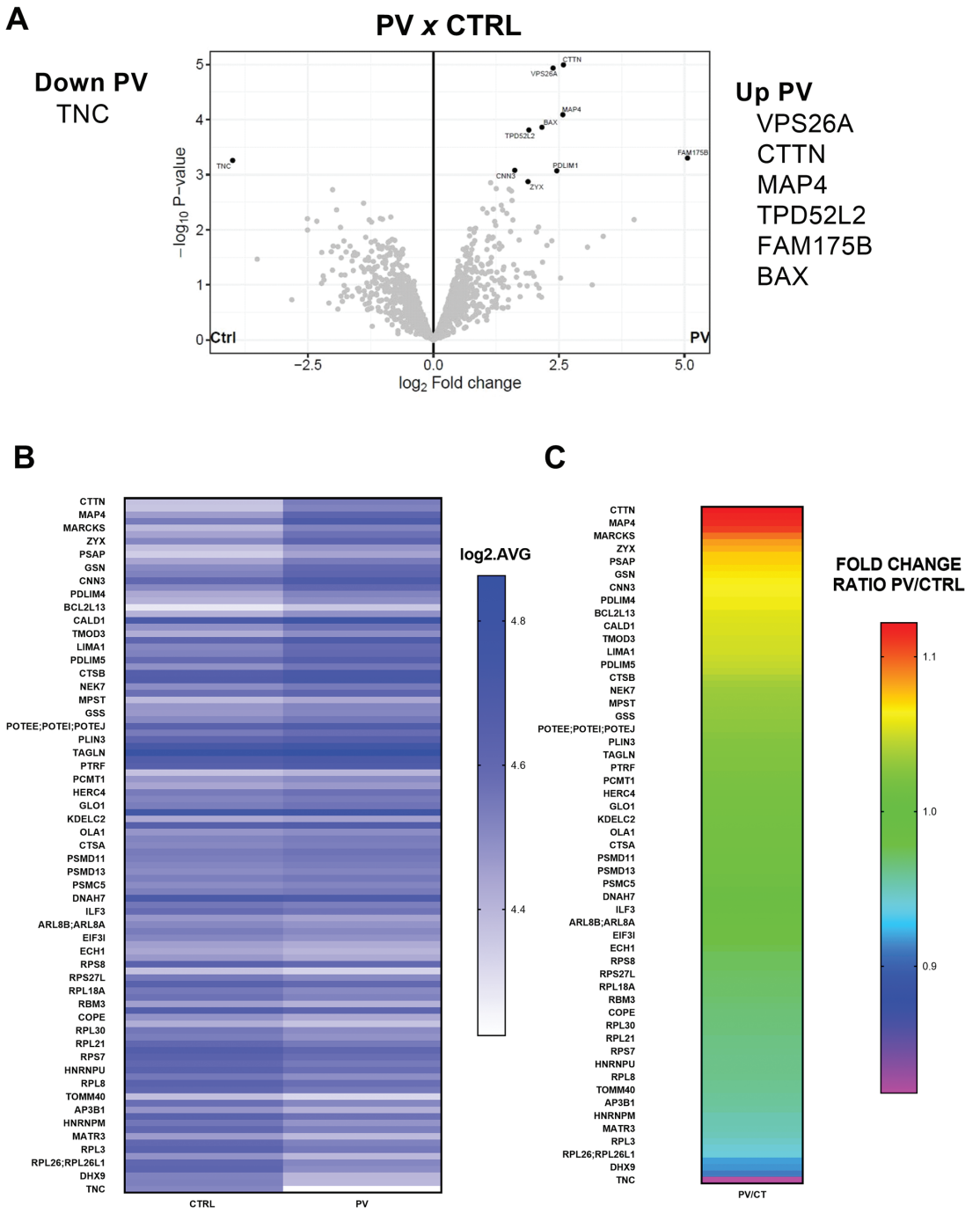

Figure 4 – Proteomics analysis of bone marrow mesenchymal stromal cells (BM-MSC) from patients with polycythemia vera (PV: *n* = 4) compared with BM-MSC from healthy donors (HD: *n* = 3). A: **Volcano Plots of proteins differentially expressed in MSC from PV patients compared to MSC from HD. R analysis (more stringent) was used to identify the differences. Six proteins were upregulated, and one was downregulated.** B: **Heatmap of Perseus analysis (less stringent). The number of differentially expressed proteins (p-value ≤0.05) in BM-MSC from PV patients was 102. Results expressed in log2 average.** C: **Heatmap of fold change ratio PV/HD from 102 differentially expressed proteins (Perseus analysis).**

oncoinflammation. Furthermore, cell-to-cell interactions in the BM reveal how MSC alter immune responses in PV and influence disease progression.

In PV patients, the BM microenvironment was marked by an inflammatory profile, with MSCs producing elevated levels of cytokines and chemokines, dysregulating hematopoiesis and favoring neoplastic cell proliferation [15]. The pro-inflammatory profile of MSCs (MSC1) has antitumor effects, while the immunosuppressive profile (MSC2) promotes tumor growth by inhibiting T cell activation [34,35]. The inflammatory microenvironment in PV patients likely biases MSC toward the immunosuppressive MSC2 profile, contributing to

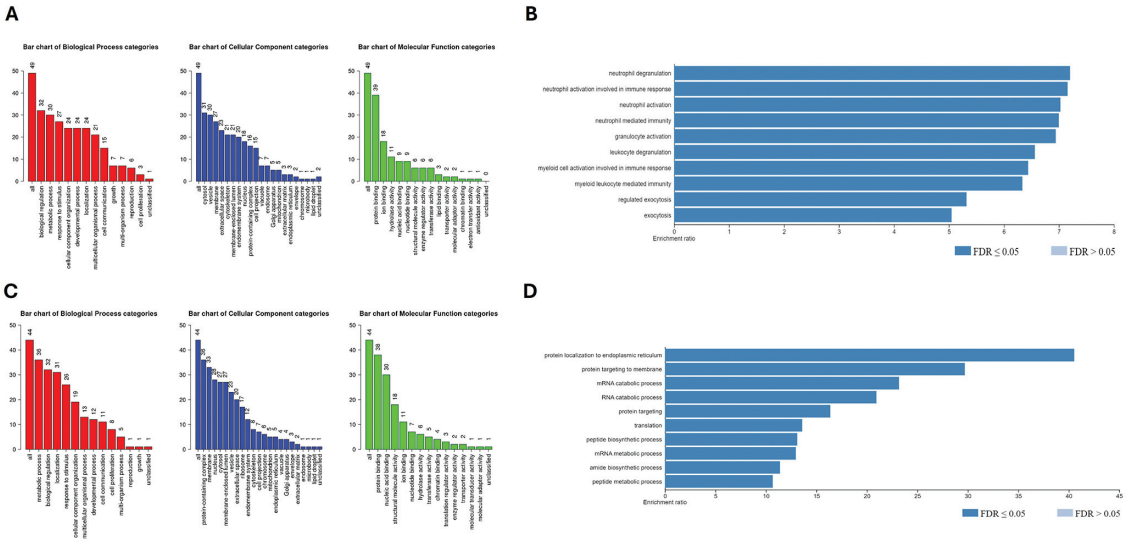

Figure 5 – Gene ontology (GO) enrichment analysis of differentially expressed proteins (DEPs) in bone marrow mesenchymal stromal cells (BM-MSC) from polycythemia vera (PV) patients. A: **GO categories enriched among upregulated proteins in PV-MSC, including biological processes, cellular components, and molecular functions.** B: **Functional enrichment of 58 upregulated DEPs, highlighting terms related to neutrophil-mediated activity.** C: **GO categories associated with 44 downregulated DEPs in PV-MSC, reflecting trends in reduced biological and molecular processes.** D: **Enrichment analysis of downregulated proteins showing pathways related to protein targeting the endoplasmic reticulum and mRNA/protein metabolism.FDR: False discovery rate.**

disease progression. Interestingly, angiogenic factors were undetectable in monocultures of BM-MSCs from HD, which may reflect their quiescent secretory profile under basal, non-inflammatory conditions. We hypothesize that these cells require interaction with immune cells or exposure to inflammatory signals, such as those present in PV, to activate their proangiogenic secretome. This reinforces the idea that the BM microenvironment plays a central role in modulating MSC behavior and should be taken into account when interpreting functional assays.

Proteomic analysis revealed that PV BM-MSC exhibited differential expression of proteins like CTTN, MAP4, TPD52L2, and BAX, associated with cell migration, intracellular transport, and apoptosis regulation. These proteins could play a role in MSC-mediated immune modulation and neoplastic cell evasion in PV.

The function of cortactin (CTTN), an actin-binding protein widely expressed in human cells, is associated with cell adhesion and migration. CTTN has recently been detected in different hematopoietic cells such as lymphocytes, dendritic cells, and macrophages. Its expression is upregulated in chronic lymphoid leukemia, acute lymphoid leukemia, and non-Hodgkin's lymphoma [36].

Upregulation of microtubule-associated protein 4 (MAP4) expression was also related to tumor invasion and migration, in addition to the worse prognosis of different solid tumors, such as lung adenocarcinoma [37] and breast cancer [38]. Its high expression correlates with tumor resistance to treatments with microtubule-targeting agents, such as vinca alkaloids, in lymphomas, breast cancer, and acute lymphoid leukemia [37,38].

TPD52L2 is the gene that encodes the protein known as tumor protein D54, which is a biomarker for breast tumors, different types of carcinomas, and lymphoid and acute myeloid leukemia [39]. This protein participates in the intracellular transport and membrane trafficking by intracellular nanoparticles [39,40] that mediate integrin recycling and control cell migration and invasion [40].

Considering the abovementioned information the CTTN, MAP4, and TPD52L2 are essential for cell mobility, intracellular transport, and cell-to-cell interactions. Further research is required to unravel the precise mechanisms and functional implications of these proteins in MSCs.

The pro-apoptotic protein BAX was also upregulated in MSCs from PV patients. BAX is a pro-apoptotic member of the Bcl-2 family of proteins that regulate the activation of the apoptosis intrinsic pathway [41]. Recently, Pang et al. reported that low expressions of the apoptotic effectors BAK and BAX in MSCs impair cell death and reduce the immunosuppressive action of MSCs in a murine model of allergic asthma. In this model, MSC death is crucial for them to exert their functions [42]. In this sense, we speculate that high levels of BAX protein favor MSCs to suppress the proliferation and response of various immune cell subsets in the BM microenvironment and thereby contribute to PV neoplastic cell evasion from immune response.

BAX, a pro-apoptotic protein, was upregulated, which may help MSCs suppress immune responses in the BM microenvironment and support the evasion of PV cells from immune detection.

Gene Ontology analysis revealed that upregulated proteins in PV were enriched in processes linked to disease activity,

particularly related to neutrophils, immunity, granulocytes, and exocytosis. Downregulated proteins were involved in transcription and protein translation, particularly ribosomal proteins and RNA splicing factors, which are often mutated in MPN [39].

Overall, these findings provide insights into the changes in the BM microenvironment in PV patients, improving our understanding of MPN pathophysiology and suggesting new therapeutic strategies targeting the inflammatory BM niche to restore immune balance and control disease progression. A potential limitation of this study is the age difference between PV patients and HD, as aging may modulate MSC functions. To address this issue, the data were reanalyzed including age as a covariate. This statistical reanalysis confirmed that age did not influence the outcomes of the study. These results support the robustness of our conclusions despite the age disparity between groups.

Conflicts of interest

The authors declare no conflicts of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Obstetrical use of intravenous immunoglobulin: A single-centre retrospective study

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ABSTRACT

Introduction: Intravenous immunoglobulin is widely used for various conditions but faces challenges such as limited supply, high cost, and substantial off-label use. Obstetrical intravenous immunoglobulin use remains underexplored, despite its relevance to maternal and neonatal care and resource management.

Methods: This single-center retrospective cohort study examined intravenous immunoglobulin administration in 136 pregnancies (122 patients) from 2007–2020, focusing on adherence to Health Canada licensed indications and Ontario Immunoglobulin Utilization Management Guidelines.

Results: Maternal thrombocytopenia (56.6 %) and treatment for fetal/neonatal alloimmune thrombocytopenia (16.2 %) were the most common indications, accounting for 16.9 % and 64.3 % of total intravenous immunoglobulin volume, respectively. Intravenous immunoglobulin use represented 1.6 % of the center's total consumption during the study period, with notable non-adherence to guidelines in 38.2 % (Health Canada) and 17.6 % (provincial guidelines) of pregnancies.

Conclusion: Findings highlight the need for optimized intravenous immunoglobulin use in obstetrics and future research to ensure safety, efficacy, and evidence-based guidance in clinical practice and policy.

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Introduction

Intravenous immunoglobulin (IVIG), derived from pooled human plasma, is used to treat immunodeficiencies, autoimmune diseases, and potentially other conditions [1]. However, IVIG is in limited supply, has a high cost, and considerable off-label use, which is often not supported by strong evidence of benefits [1,2]. These challenges are pronounced in obstetrics, where balancing maternal and fetal health complicates treatment decisions.

Although IVIG use in obstetrics is generally reserved for specific scenarios or refractory cases, it may be favored over other options anecdotally due to the lack of alternatives and perceived safety. The failure to include pregnant women in randomized controlled trials examining IVIG use in labelled indications [3–9], and the absence of high-quality studies for off-label obstetrical indications [10,11], leave significant gaps in evidence-based guidance. Existing literature largely overlooks IVIG applications in obstetrics [2,12–15], limiting understanding of its scope and potential misuse. This understanding is essential not only for optimizing patient care but also for ensuring the judicious use of a scarce resource.

This study assesses the obstetrical use of IVIG against established guidelines to inform policy, practice, and future research, which can help enhance obstetrical care and ensure stewardship of an expensive and limited resource. The objectives are two-fold: [1] to assess the frequency, dose, and indications of IVIG use in pregnancy, and [2] to assess concordance of IVIG use with the approved Canadian indications and the approved conditions of the Ontario Immunoglobulin Utilization Management Guidelines.

Methods

A retrospective cohort study was conducted using administrative data from The Ottawa Hospital Data Warehouse complemented by chart reviews from electronic health records to evaluate IVIG use in pregnancy. The study population comprised all pregnant women who received IVIG between 2007 and 2020 and delivered at the Ottawa Hospital. Data were collected on IVIG volumes, regimens, indications, and timing of administration during pregnancy. Health Canada licensed indications, which include primary immunodeficiencies, secondary immunodeficiencies, chronic lymphocytic leukemia, immune thrombocytopenia, chronic inflammatory demyelinating polyneuropathy, Guillain Barre Syndrome, and multifocal motor neuropathy were used to examine guideline adherence [16]. In addition, appropriateness was assessed using the Ontario Immunoglobulin Utilization Management Guidelines, which include both licensed and non-licensed indications for IVIG as approved for provincial use [17]. Non-licensed indications could include conditions such as fetal/neonatal alloimmune thrombocytopenia (F/NAIT), and hemolytic disease of the fetus and newborn (HDFN) [17]. Data were analyzed using descriptive statistics. The study was approved by Ottawa Health Science Network Research Ethics Board (CRRF 2826/Protocol 20210315-01H).

Results

Overall use and trends

From 2007 to 2020, a total of 122 pregnant patients representing 136 deliveries were treated with IVIG during their pregnancy at the Ottawa Hospital. Cumulatively, these patients used 41,107.50 grams of IVIG. The volume accounted for 1.6 % of the total IVIG consumption at the center over this period. While the total IVIG usage at the Ottawa Hospital increased during the period of the study, the relative proportion of IVIG used in pregnancy also increased, with greater obstetrical use seen in the latter period of the study (Figure 1A). Overall, the annual mean proportion of IVIG volume used in pregnancy relative to the total population was 1.53 % (Standard deviation [SD]: 1.02). The annual mean volume of IVIG used in pregnancy was 2936.25 grams (SD: 2129.82), while the annual mean volume for the total population at the center was 183,983.32 grams (SD: 31,527.03 grams). Specific years exhibiting peaks in IVIG use in pregnancy were predominantly driven by a higher number of F/NAIT cases, where pregnant patients received weekly doses of IVIG for the entire second and third trimesters (Figure 1B).

Indications for intravenous immunoglobulin use

The most prevalent indications for IVIG administration in pregnancy were related to hematologic conditions. Specifically, maternal thrombocytopenia was identified in 56.6 % (77/136) of deliveries, and antenatal therapy for F/NAIT was noted in 16.2 % (22/136) of deliveries. Other less common reasons, outlined in Table 1, included neurologic conditions (9.6 %), rheumatologic conditions (4.4 %), dermatologic conditions (2.9 %), obstetrical indications (2.2 %), immunodeficiencies (1.5 %), and renal conditions (0.7 %).

Intravenous immunoglobulin utilization

In terms of IVIG consumption, the antenatal treatment of F/NAIT accounted for the majority (64.3 %) of the total IVIG used in all pregnancies. This translated to 26,435 grams with a median of 1015 grams per pregnancy (Interquartile Range [IQR]: 542.5-1938.75 grams). Maternal thrombocytopenia followed, accounting for a total of 6,952.50 grams (16.9 %) used in all pregnancies and a median of 70 grams per pregnancy (IQR: 40-90 grams).

Guideline adherence

Regarding the congruency of IVIG use with labelled Health Canada indications, 38.2 % (52/136) of the pregnancies received IVIG for off-label indications. This use for indications not approved by Health Canada, which includes F/NAIT, represented a substantial portion of the total volume of IVIG use in pregnancies, amounting for 33,025 grams (80.3 % of the total volume used). Other off-label indications under Health Canada included Myasthenia Gravis, Multiple Sclerosis, repeated Implantation Failure, Antiphospholipid syndrome, Rheumatoid arthritis, Pemphigoid Gestationis, Anti-Ro antibodies, HDFN, Antibody-mediated rejection (renal

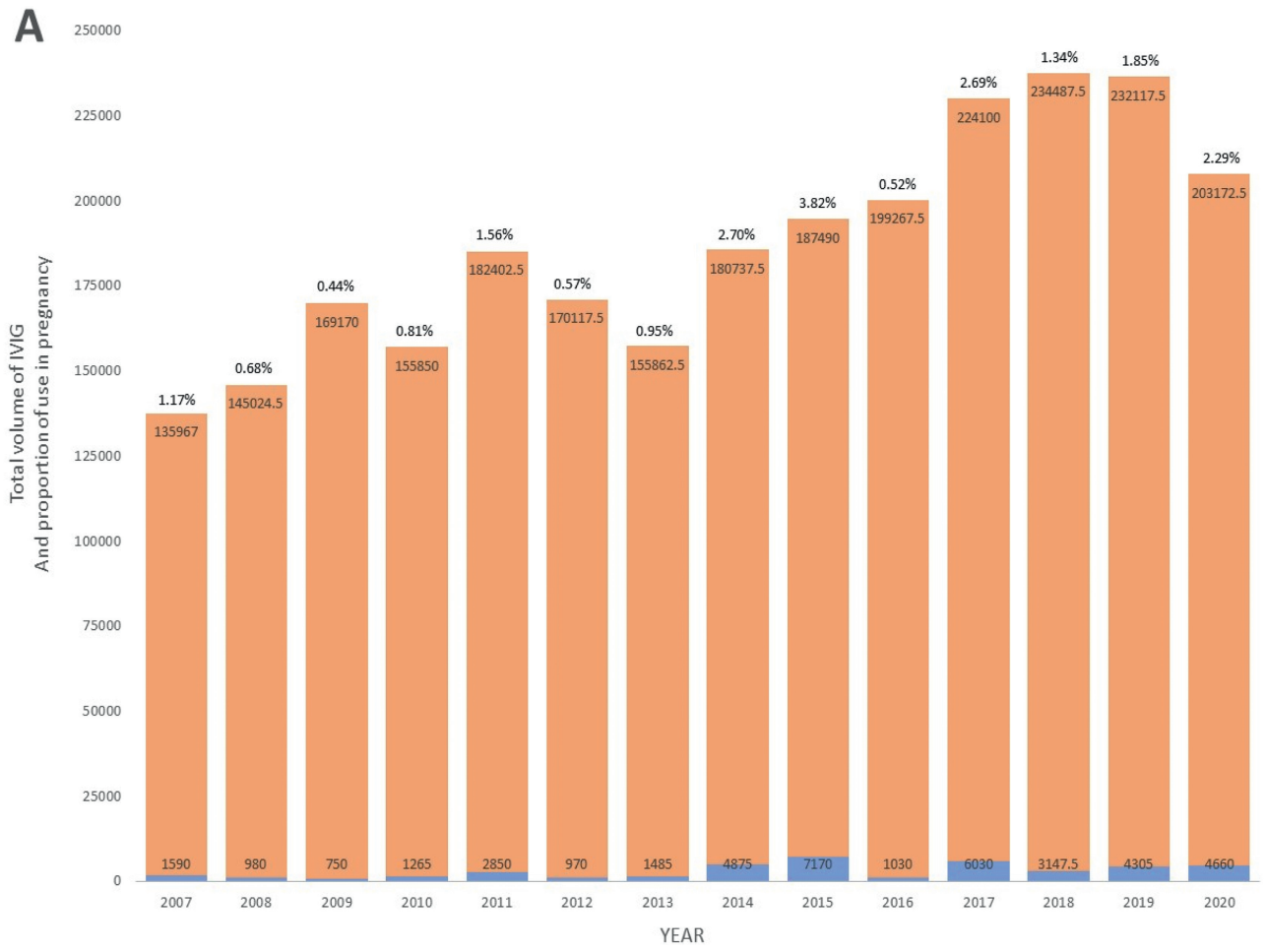

Figure 1 – Temporal trends in IVIG administration from 2007-2020. (A) Total volume of IVIG administered in pregnancy and for the total population at our center, including proportion (%) of IVIG volume used in pregnancy. (B) Box and whisker plots of the volume of IVIG use per pregnancy.

transplant), Chronic Villitis, Small fiber polyneuropathy, Idiopathic Angioedema, and Autoimmune Necrotizing Myositis.

In contrast, only 17.6 % (24/136) of pregnancies receiving IVIG, accounting for 5,475 grams (13 % of total volume used), did not adhere to the approved indications in the Ontario Immunoglobulin Utilization Management Guidelines. These conditions included Pemphigoid Gestationis, Idiopathic angioedema, Antiphospholipid antibodies, Anti-Ro antibodies, Autoimmune Myositis, Multiple Sclerosis, repeated implantation failure and Chronic Villitis.

Discussion

This study offers a comprehensive picture of the patterns and scope of obstetrical use of IVIG, an area less explored in existing literature [2,13,14,18]. It demonstrates that IVIG use for obstetrical patients at the Ottawa Hospital has increased over the study period but accounts for only a small fraction of the overall IVIG consumption. The primary indications for IVIG administration during pregnancy included hematologic conditions, notably maternal thrombocytopenia, and the treatment of F/NAIT. A considerable portion of IVIG use did not align with the approved Health Canada indications, and a smaller but still important proportion did not align with the Ontario Immunoglobulin Utilization Management Guidelines. This difference is due to F/NAIT being an off-label Health Canada indication but appropriate use in Ontario guidelines. Overall, the off-label use suggests a potential for optimizing its application in obstetrical care.

Maternal thrombocytopenia and prevention of F/NAIT accounted for a substantial portion of IVIG use in this study cohort and may be important clinical scenarios necessitating further research. Thrombocytopenia occurs in about 10 % of pregnancies but rarely requires treatment [19]. In our experience, IVIG may be preferentially administered at the clinician's discretion to avoid corticosteroid exposure with the aim of improving the platelet count over certain thresholds for labor and delivery particularly to allow for neuraxial anesthesia (generally a platelet count >70-80$\times 10^9$/L), despite the lack of evidence to suggest meaningful clinical benefits for the mother or newborn [20,21]. As for the prevention of F/

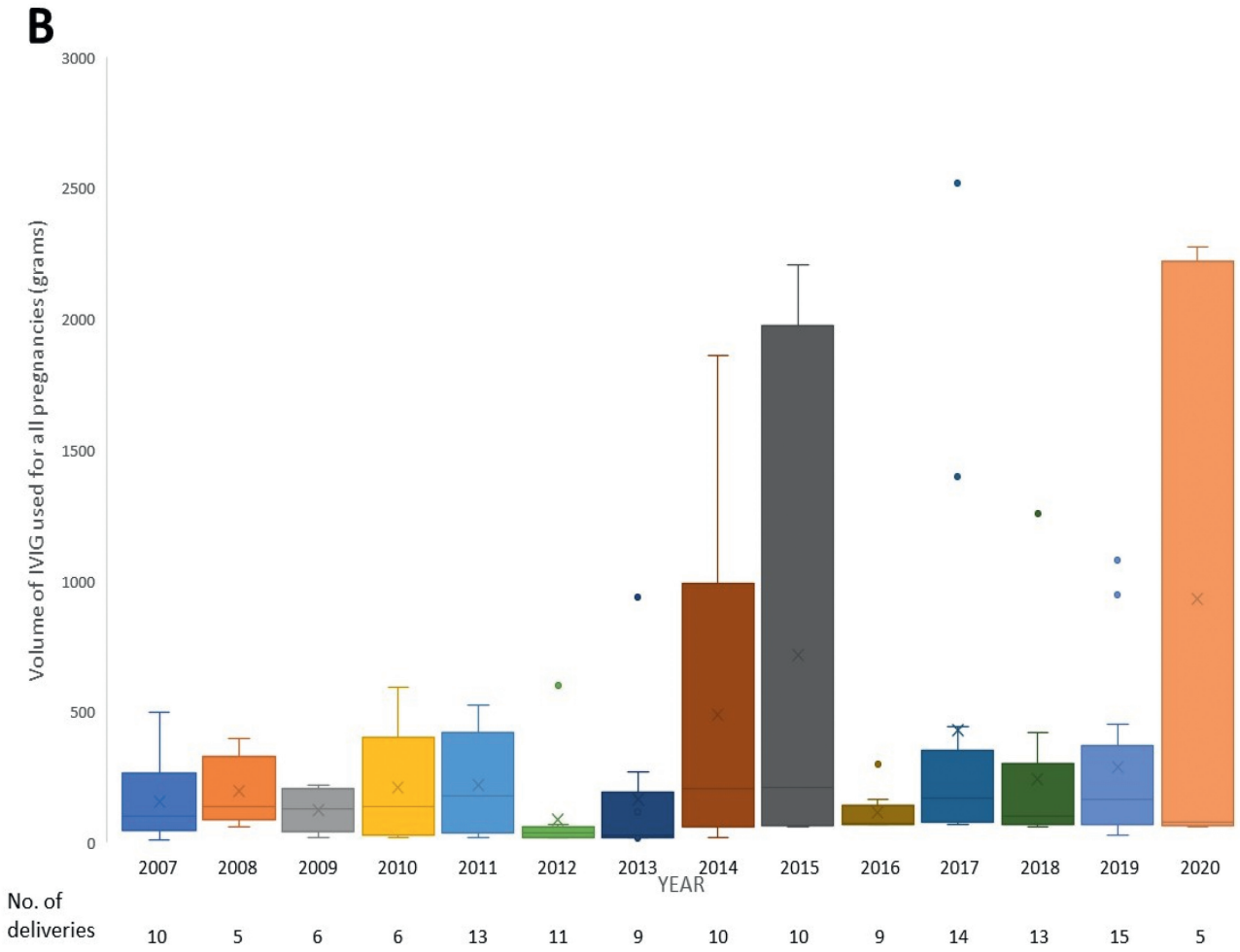

Figure 1 Continued.

NAIT, IVIG appears to be effective based on small observational studies [11] and has achieved consensus as the treatment of choice despite the lack of high-quality evidence [22]. Such practices raise questions about the broader clinical decision-making processes guiding IVIG use for maternal thrombocytopenia and F/NAIT, particularly in the context of balancing efficacy, safety, and resource allocation.

The findings of the current study complement other studies in non-obstetrical settings that have documented high rates of reliance on IVIG for various conditions without high-quality evidence [1,14,15,18,23,24]. The proportion of non-adherence to guidelines in this study, accounting for 38.2 % of deliveries in terms of Canadian indications and 17.6 % in terms of Ontario guidelines, underscores a potential area for improvement in clinical practice. While the upward trend of IVIG use and the divergence from licensed indications and/or guidelines could reflect a growing recognition of the obstetrical and non-obstetrical indications and evolving understanding of the therapeutic roles of IVIG [11,21,25], it also raises concerns about resource utilization and the need for ongoing surveillance to ensure that IVIG is used appropriately and sustainably [1,15,26]. IVIG stewardship programs that involve an intermediary healthcare professional to monitor, review and guide IVIG administration have shown great promise for optimizing adherence to guidelines, and reducing inappropriate administration of IVIG and associated costs without negatively impacting patient care [23,24]. Stewardship programs that relied primarily on order request forms and handouts of clinical practice guidance had little to no influence on IVIG use [1].

The strengths of this study include its comprehensive data collection spanning over a decade and its focus on a large, diverse population served by a major Canadian tertiary care and academic institution. From this dataset, it was possible to conduct a detailed analysis of IVIG usage patterns and guideline adherence. However, the retrospective nature of the study limits the possibility to fully assess the clinical contexts leading to off-label IVIG use. Additionally, the single center focus may restrict the generalizability of the findings. This study also does not capture different practice patterns across centers, including center-specific approval processes for IVIG.

In conclusion, the present study sheds light on important aspects of IVIG use in pregnancy, highlighting areas of both adherence and deviation from licensed indications and established guidelines. These findings underscore the need for stewardship programs to optimize IVIG use in pregnancy,

Table 1 – Indication, volume, and dosing of IVIG administration for all deliveries.

Diagnosis	No. of deliveries n (%)	Total volume of IVIG administered grams (%)	Median dose of IVIG in grams (IQR 25^{th}, 75^{th})	Frequency of IVIG administration
Hematologic	**107 (78.7)**	**35132.5 (85.5)**	**80 (60, 272.5)**	
Maternal isolated thrombocytopenia (Gestational thrombocytopenia, Immune thrombocytopenia	77 (56.6)	6952.5 (16.9)	70 (40, 90)	Single*
Antenatal therapy for Fetal/Neonatal Alloimmune Thrombocytopenia	22 (16.2)	26435 (64.3)	1015 (542.5, 1938.75)	Recurrent*
Antiphospholipid syndrome / Anti-phospholipid antibodies	5 (3.7)	730 (1.8)	120 (100, 220)	Recurrent
Maternal red cell antibodies / Prevention of Hemolytic Disease of the Fetus and Newborn	3 (2.2)	1015 (2.5)	300 (280, 377.5)	Recurrent
Neurologic	**13 (9.6)**	**2230 (5.4)**	**165 (115, 180)**	
Myasthenia Gravis	5 (3.7)	695 (1.7)	175 (70, 175)	Recurrent*
Guilain-Barre Syndrome	3 (2.2)	350 (0.9)	115 (110 – 125)	Single
Chronic inflammatory demyelinating polyneuropathy	2 (1.5)	465 (1.1)	N/A	Single
Multiple Sclerosis	2 (1.5)	440 (1.1)	N/A	Recurrent
Small fiber polyneuropathy	1 (0.7)	280 (0.7)	N/A	Recurrent
Rheumatologic	**6 (4.4)**	**1365 (3.3)**	**220 (165, 290)**	
Maternal Anti-Ro Antibodies	4 (2.9)	1025 (2.5)	280 (200, 336.25)	Recurrent*
Rheumatoid Arthritis	1 (0.7)	180 (0.4)	N/A	Recurrent
Autoimmune necrotizing myositis	1 (0.7)	160 (0.4)	N/A	Recurrent
Dermatologic	**4 (2.9)**	**740 (1.8)**	**190 (165, 210)**	
Idiopathic Angioedema Urticaria	1 (0.7)	180 (0.4)	N/A	Recurrent
Pemphigoid Gestationis	2 (1.5)	440 (1.1)	N/A	Recurrent
Undiagnosed recurrent cutaneous eruptions	1 (0.7)	120 (0.3)	N/A	Recurrent
Obstetrical	**3 (2.2)**	**905 (2.2)**	**300 (280, 322.5)**	
Chronic Villitis	2 (1.5)	645 (1.6)	N/A	Recurrent
Repeated Implantation Failure	1 (0.7)	260 (0.6)	N/A	Recurrent
Immunodeficiencies	**2 (1.5)**	**315 (0.8)**	N/A	
Selective IgA deficiency	1 (0.7)	35 (0.1)	N/A	Single
Secondary Immunodeficiency (Hypogammaglobulinemia)	1 (0.7)	280 (0.7)	N/A	Recurrent
Renal	**1 (0.7)**	**420 (1.0)**		
Acute antibody-mediated rejection in renal transplant	1 (0.7)	420 (1.0)	N/A	Recurrent

* Indicates the predominant frequency of IVIG administration for the listed indication.

ensuring that this valuable resource is used effectively and responsibly in clinical practice. Several questions remain, particularly regarding the mechanisms driving off-label IVIG use in pregnancy and its clinical outcomes. Future research should aim to fill these gaps by exploring the safety, efficacy, and cost-effectiveness of IVIG in obstetrical care, especially for conditions lacking alternative treatments. Prospective studies and randomized controlled trials involving pregnant women are essential to establish evidence-based guidelines for IVIG use in this population, ensuring both maternal and fetal well-being while maintaining resource stewardship.

Author contributions

RK, BN, IP, and AT performed the research. RK, DEC, DF, AK, JM, KW, and AT designed the research study and grant proposal. IP and MT contributed essential data. RK, BN and AT analyzed the data. RK wrote the paper. All authors revised the paper critically and approved the submitted and final versions.

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Ethics approval

The study was approved was approved by Ottawa Health Science Network Research Ethics Board (CRRF 2826/Protocol 20210315-01H).

Data availability statement

The data that support the findings of this study are available from the corresponding author.

Conflicts of interest

The authors do not have any conflict of interest to declare pertaining to this study.

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Original article

Assessment of the neutrophil-to-lymphocyte ratio as a prognostic marker in patients with newly diagnosed diffuse large B-cell lymphoma: A Colombian Cohort Study

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ABSTRACT

Introduction: Diffuse large B-cell lymphoma is a complex disease, and prognostic scores are inadequate for identifying high-risk patients. Recently, leukocyte indices, like the neutrophil-to-lymphocyte ratio, have become a marker of prognosis. The purpose of this study is to evaluate the performance of this marker as a risk predictor in adult patients with newly diagnosed diffuse large B-cell lymphoma in Colombia.

Materials and methods: A retrospective cohort study, calculated the neutrophil-to-lymphocyte ratio and its performance as a predictor for 2-year progression-free survival. Patients were divided into two groups; patients with high ratios in Group One and patients with low ratios in Group Two. Both groups were followed for at least 24 months from diagnosis.

Results: The cohort comprised 198 patients with a median age at diagnosis of 61 years. A neutrophil-to-lymphocyte ratio cutoff point of 6.2 was calculated. Patients with ratios higher than 6.2 (n = 45) were placed in Group One, and the patients with ratios below 6.2 (n = 153) in Group Two. The median follow-up time was 45 months. The 24-month progression-free survivals were 55.2 % (95 % confidence interval: 42.3–71.9 %) and 73.2 % (95 % confidence interval: 66.2–81.0 %) for high and low ratios, respectively (Hazard ratio 0.62; 95 % confidence interval: 0.44–0.89; p-value = 0.009). The 24-month overall survivals were 70.7 %; (95 % confidence interval: 58.5 - 85.5 %) and 80.4 %; (95 % confidence interval: 66.2 –87.3 %), respectively.

Conclusion: A neutrophil-to-lymphocyte ratio with a cutoff point at ≥6.2 could differentiate a diffuse large B-cell lymphoma population with an unfavorable prognosis for progression-free survival.

Introduction

The most prevalent non-Hodgkin's lymphoma (NHL) in Colombia is Diffuse Large B-Cell Lymphoma (DLBCL), which

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accounts for 40 % of all cases [1]. Immuno-chemotherapy with the R-CHOP (rituximab, cyclophosphamide, doxorubicin, prednisone, and vincristine) regimen is the standard first-line treatment with remission rates between 60 % and 70 % and overall survival (OS) rates of 85–88 % [2–4]. About 30 % of the patients experience relapse, which may be caused by the biology of the tumor [5]. Knowledge of the tumor microenvironment could give clues about the uncontrolled growth of abnormal clones, resistance and refractoriness to treatment with chemo-immunotherapy [6].

Various criteria are currently employed in clinical practice for prognostication. Based on this stratification, efforts have been made to establish intensified chemotherapy protocols for patients classified as having intermediate risk (IR) or high risk (HR), given their increased susceptibility to relapse. Despite these efforts, the OS of patients has not improved [6–8]. Intensive chemotherapy regimens for HR patients have not improved progression-free survival (PFS) rates assessed using the International Prognostic Index (IPI) [9].

Different prognostic factors besides the traditional ones could improve the classification of patients with DLBCL and optimize treatment. The neutrophil-to-lymphocyte ratio (NLR) is proposed as a prognostic marker. The growth of tumor cells is sustained through interaction with surrounding cells; the tumor clone produces cytokines such as CXCL-10, which increase and activate inflammatory cells like macrophages, neutrophils, and dendritic cells, resulting in tumor proliferation, cell migration, neutrophil infiltration, and lymphocyte inhibition [8,10,11]. Elevated neutrophil levels are associated with angiogenesis induction, tumor necrosis factor (TNF) secretion, nitric oxide production, neutrophil trap formation, apoptosis inhibition, and DNA damage [12–14]. Low lymphocyte counts impair host immunity, leading to decreased recognition and destruction of tumor cells [4,15,16]. NLR balances inflammation pathway activity and autoimmune function [17].

The inflammation and immune changes caused by the tumor are reflected in scores based on peripheral blood count ratios. Additional information about prognosis could be gained through the presence of systemic inflammatory responses that are caused by tumorigenesis [18]. A high NLR has been reported as a factor for poor prognosis in DLBCL with decreased OS and PFS. However, the majority of studies reported were carried out in Asian populations and a wide range of cutoff values were reported (between 2.32 and 5.54) [16,17,19–21]. In one study of Latin American patients, the NLR was shown to be an adverse prognostic factor with a cut-off value >4 [8], but another study of Peruvian patients identified a cutoff value >6 [22].

The Latin American population is heterogeneous, and the distribution of NHL subtypes varies significantly by geographic region. The DLBCL subtype of lymphomas is more common in Brazil, Guatemala, Peru and Colombia than in Argentina and Chile [1,23]. It is possible that DLBCL patients from tropical zones of Latin American, and Caribbean countries have outcomes that are not the same as those from other regions. This study aimed to evaluate the behavior of the NLR regarding PFS in patients with DLBCL in a Colombian cohort.

Materials and methods

Study type

This is a retrospective analytical cohort study. The purpose of the study was to analyze the NLR as an indicator of prognosis in newly diagnosed DLBCL patients and its effect on the PFS and OS.

Inclusion criteria

Patients with *de novo* diagnoses of DLBCL treated with curative-intent R-CHOP or similar regimens were included in this study if they met the following criteria: diagnosis between January 1, 2016, and December 31, 2021, at Colsanitas clinics in Colombia, and availability of a complete blood count prior to treatment initiation.

Exclusion criteria

Patients with previous diagnoses of rheumatologically diseases, cancer, or HIV were excluded as were all patients under treatment with chemotherapy or steroids before the first blood test.

Variables and data collection

The hematology department database was reviewed and patients who met the inclusion criteria were selected. The follow-up began at the time of the diagnosis and continued until the last available follow-up appointment in the electronic medical record system. All patients were monitored throughout this period

Electronic medical records of patients with DLBCL were reviewed collecting basic patient information, including age at diagnosis, sex, baseline lactic dehydrogenase (LDH) level, Eastern Cooperative Oncology Group performance status (ECOG), Ann Arbor stage, IPI, Revised International Prognostic Index (R-IPI), National Comprehensive Cancer Network-IPI (NCCN-IPI), relapse, and death. The Lugano response criteria were used to assess treatment response [24].

For the proposed analysis, the NLR for each patient was calculated at the time of diagnosis, prior to the initiation of any treatment, including chemotherapy or steroids. The Receiver Operating Characteristic (ROC) curve analysis was performed to determine the optimal NLR cut-off value for predicting the two-year PFS. Subsequently, patients were divided into two groups, patients with higher levels of NLR were included in Group One and those with lower levels in Group Two.

Outcomes

The main outcome was PFS, defined as the time in months elapsed from diagnosis until disease progression or death from any cause.

The secondary outcome was OS defined as the time elapsed from the date of diagnosis to death from any cause. Both PFS and OS were measured for the same period.

The institutional ethics committee approved this study.

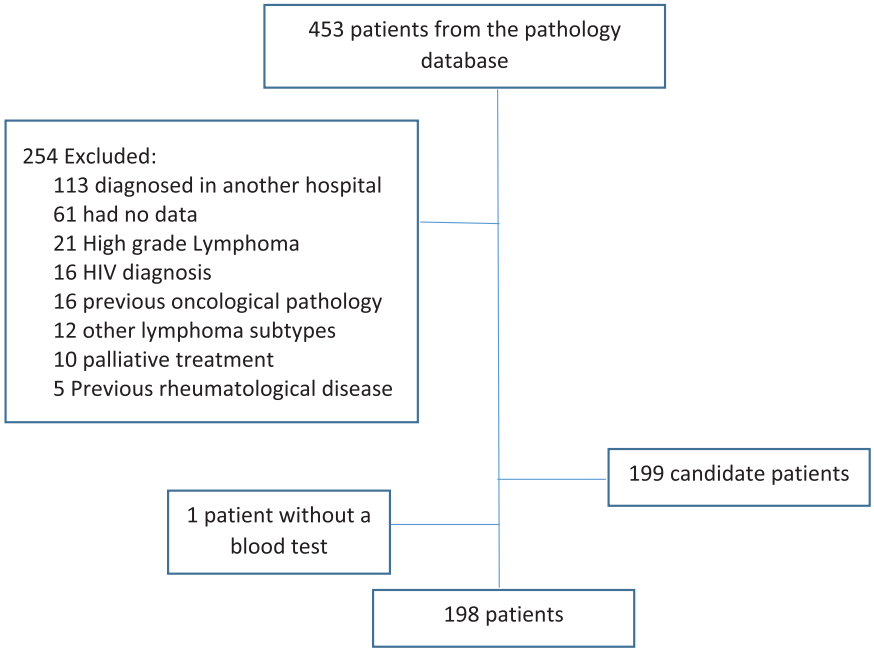

Figure 1 – CONSORT flowchart of patients included in the study.Most patients did not have their initial diagnosis at the Colsanitas clinic, so it was not possible to determine if they had received steroid therapy before the blood test.

Statistical analysis

The sample size was calculated with expected sensitivity and specificity of 51.2 % and 79.9 %, respectively using a confidence interval of 95 % and power of 80 %, resulting in a minimum sample size of 122 patients.

Once the data were collected, a mathematical formula was used dividing the absolute neutrophil count by the absolute lymphocyte count for each patient. The ROC curve was constructed using the NLR and the outcome of interest (two-year PFS) to calculate sensitivity, specificity, negative and positive predictive values, and the optimal cutoff point to discriminate the population. The sample was then divided accordingly for analysis. Because the primary outcome was PFS, a time-dependent event, the time-dependent ROC curve was also calculated which showed cumulative sensitivity and dynamic specificity for the performance evaluation of the NLR.

Categorical variables were compared using the chi-square test or Fisher's exact test, as appropriate, while continuous variables were compared using the Student's *t*-test or the Wilcoxon rank-sum test. The non-parametric Kaplan-Meier method was used to estimate the OS and DFS and the survival probabilities were compared using the log-rank test.

Proportional hazard regression (Cox) was used to evaluate factors that influenced OS and PFS. Bivariate Cox proportional hazards regression analysis was performed to screen for variables associated with prognosis; those yielding a p-value <0.2 were subsequently included in the multivariate model. A p-value of <0.05 was considered statistically significant. R version 4.3.2 was used for data analysis.

Table 1 – Main characteristics of the entire group of Diffuse Large B-Cell Lymphoma patients at diagnosis.

Variable	*n* = 185
Age – median (interquartile range)	61 (51–72)
Sex – n (%)	
Female	89 (48)
Male	86 (52)
Ann Arbor Staging – n (%)	
I	12 (6.5)
II	34 (18)
III	32 (17)
IV	107 (58)
Extranodal involvement – n (%)	
No	64 (35)
Yes	120 (65)
International Prognostic Index (IPI) – n (%)	
Low	50 (27)
Intermediate - low	66 (36)
Intermediate - high	50 (27)
High	18 (9.8)
Unknown	1
R-IPI – n (%)	
Very Good	5 (2.7)
Good	111 (61)
Poor	66 (36)
Unknown	3
NCCN-IPI – n (%)	
Low	20 (11)
Intermediate - low	85 (47)
Intermediate - high	61 (34)
High	15 (8.3)
Unknown	4

IPI: International Prognostic Index; R-IPI: Revised International Prognostic Index; NCCN-IPI: National Comprehensive Cancer Network-International Prognostic Index.

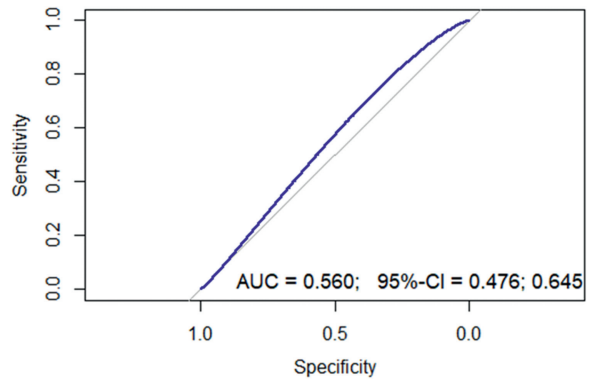

Figure 2 – Receiver operating characteristic curve of neutrophil-to-lymphocyte ratio for 24-months progression free-survival.AUC: Area under the curve; 95 % CI: 95 % confidence interval.

Results

Between 2016 and 2021, 453 patients who were diagnosed with DLBCL were identified; 254 patients were excluded from the study leaving 198 patients (Figure 1). The median follow-up was 45 months and main characteristics of the entire group are listed in Table 1.

Operational characteristics of the neutrophil-to-lymphocyte ratio

The median absolute neutrophil count at diagnosis was 5020 (interquartile range: 3648–6740) and the median absolute lymphocyte count was 1330 (interquartile range: 860–1855).

A ROC curve (Figure 2) showed an area under the curve (AUC) of 0.56 (95 % confidence interval [95 % CI]: 0.47–0.64) with a cutoff point of 3.3: the sensitivity was 66.6 %, specificity 47.8 %, positive predictive value was 48.3 %, and negative predictive value was 65.1 % (Table 2).

Because of the poor performance of the AUC with a cutoff of 3.3, a cutoff point in the 75th percentile was chosen to improve specificity. Any patient with an NLR >6.2 was considered to have a high NLR: the sensitivity was 35.3 %, specificity was 81.7 %, negative predictive value was 68.1 %, and positive predictive value was 53.8 %. Following the determination of the optimal cut-off value, the cohort was divided into two prognostic groups based on the NLR: Group 1, comprising 45 patients with a high NLR, and Group 2, comprising the remaining 139 patients (Table 3).

Treatment response

At the end of treatment, complete response was achieved in 104 patients (67.9 %) in Group 2 versus 23 patients (51.1 %) in Group 1. The rates for other responses were as follows: partial response (Group 2: 15.4 %; Group 1: 15.1 %), stable disease (Group 2: 4.6 %; Group 1: 7.6 %), and progression (Group 2: 7.5 %; Group 1: 16.9 %). The difference in overall response rates between the groups was statistically significant (p-value = 0.035)

Survival analysis

Progression-free survival

The 24-month PFS for Group 1 was 55.2 %; (95 % CI: 42.3 –71.9 %), and for Group 2, it was 73.2 % (95 % CI: 66.2–81 %). The hazard ratio (HR) for Group 2 was 0.62 (95 % CI: 0.44–0.89, p-value = 0.009 - Figure 3).

Overall survival

For both groups, the median OS was not reached. The 24-month OS was 70.7 % (95 % CI 58.5 –85.5 %) and 80.4 % (95 % CI: 66.2–87.3 %) for Groups 1 and 2, respectively. A HR of death of 0.71 (95 % CI: 0.45–1.1) was estimated for Group 2 (Figure 3).

Multivariate analysis

Initially, bivariate Cox proportional hazards regression analysis was performed to identify variables for inclusion; those with a p-value <0.2 were subsequently entered into the multivariate model.

NLR, age over 60 years, ECOG ≥2, involvement of more than two extranodal sites, bone marrow involvement, and bulky mass were chosen to construct the multivariate model.

Table 2 – Operative characteristics of Neutrophil-to-lymphocyte ratio (NLR) at different cutoff points.

NLR	Sensitivity (%)	Specificity (%)	LR +	LR -	PPV (%)	NPV (%)
3.3	70.5	47.8	1.35	0.61	44.4	73.3
3.5	64.7	52.1	1.35	0.67	44.4	71.4
4	54.4	59.1	1.33	0.77	44.1	68.6
4.5	48.5	66.1	1.43	0.77	45.8	68.4
5	47.1	70.4	1.59	0.75	48.5	69.2
5.5	45.6	73.9	1.74	0.73	50.8	69.7
6	39.7	78.2	1.82	0.77	51.9	68.7
6.2	**35.3**	**81.7**	**1.93**	**0.79**	**53.3**	**68.1**
6.5	25.0 %	82.6 %	1.43	0.91	45.9 %	65.0 %
7	22.1 %	86.0 %	1.58	0.90	48.3 %	65.1 %
7.5	20.6 %	88.6 %	1.82	0.89	51.8 %	65.3 %

LR+: Likelihood ratio positive; LR–: Likelihood ratio negative; PPV: Positive predictive value; NPV: Negative predicted value; NLR: Neutrophil-to-lymphocyte ratio.

Table 3 – Main characteristics of the patients according Neutrophil-to-lymphocyte ratio (NLR).

Variable	n	NLR low (<6.2) *n* = 139	NLR High (>6.2) *n* = 45	p-value[2]
Age - Median (interquartile range)	184	61 (52–72)	63 (44–73)	0.7
Sex – n (%)	184			0.9
Female		67 (48)	21 (47)	
Male		72 (52)	24 (53)	
Ann Arbor Staging – n (%)	184			>0.9
I		10 (7.2)	2 (4.4)	
II		26 (19)	8 (18)	
III		23 (17)	9 (20)	
IV		80 (58)	26 (58)	
Extranodal involvement – n (%)	183			0.5
No		50 (36)	14 (31)	
Si		88 (64)	31 (69)	
IPI – n (%)	184			0.018
Low		44 (32)	6 (13)	
Intermediate - low		51 (37)	15 (33)	
Intermediate - high		34 (24)	16 (36)	
High		10 (7.2)	8 (18)	
R-IPI – n (%)	181			0.050
Very good		5 (3.6)	0 (0)	
Good		89 (65)	22 (50)	
Poor		43 (31)	22 (50)	
NCCN-IPI – n (%)	181			0.006
Low		20 (15)	0 (0)	
Intermediate - low		66 (48)	19 (43)	
Intermediate - high		42 (31)	19 (43)	
High		9 (6.6)	6 (14)	

IPI: International Prognostic Index; R-IPI: Revised International Prognostic Index; NCCN-IPI: National Comprehensive Cancer Network-International Prognostic Index.

[2] Wilcoxon rank sum test; Fisher's exact test; Pearson's Chi-squared test.

The NLR continued to have a relationship with PFS after adjustment in the multivariate model (Table 4).

Discussion

In this study, the standard ROC curve of the NLR yielded an AUC of 0.56 with a cutoff point of 3.3; this was considered unsatisfactory. Consequently, the decision was made to select a cutoff point at the 75th percentile giving a higher specificity. The cutoff point for NLR was determined to be 6.2 with 35.3 % sensitivity and 81.7 % specificity.

There were statistically significant differences in 24-month PFS (p-value = 0.009). However, this study did not demonstrate significant differences in 24-month OS.

Other studies showed poor performance for the NLR in the ROC curve analysis. In 2021, Hasan published an analysis of 136 DLBCL patients who were divided into low and high NLR groups using a cutoff value of 2.8. The AUC for NLR was 0.512 (95 % CI: 0.41–0.61) with 57.4 % sensitivity and 55.1 % specificity (p-value = 0.81), but did not show differences in the five-year PFS (61.8 %; 95 % CI: 47.3–73.4 % versus 58.5 %; 95 % CI: 45.3–72.4 %; p-value = 0.41) or OS (63.5 %; 95 % CI: 50.4–76.9 % versus 56 %; 95 % CI: 43–69 %; p-value = 0.42) [25].

According to the ROC analysis of the Latin American Group of Lymphoproliferative Diseases (GELL), the optimal cutoff point is 4 with sensitivity and specificity of 50 % and 60 %, respectively and an AUC of 0.59. Significant differences were found between the groups for the 5-year OS (75 %; 95 % CI:

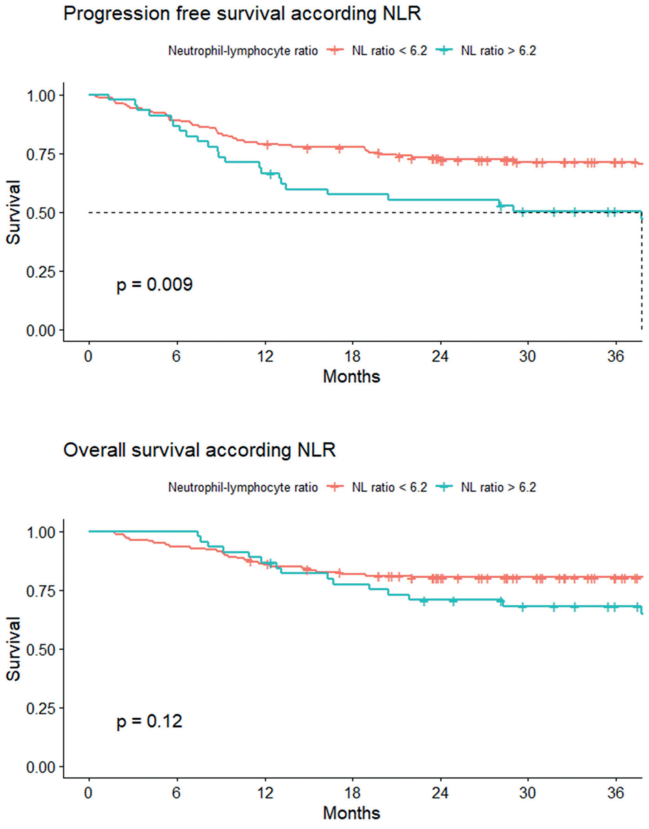

Figure 3 – Progression-free survival (PFS) and overall survival (OS) according to neutrophil-to-lymphocyte ratio.

Table 4 – Univariate and multivariate Analysis - Cox model for progression-free survival.

Analyzed variables	Univariate analysis		Multivariate analysis	
	HR (95 % CI)	p-value	HR (95 % CI)	p-value
NLR ≥6.2	1.58 (1.11–2.26)	0.009	1.65 (1.15–2.35)	0.006
Age >60 years	1.024 (1.008–1.042)	0.004	1.03 (1.01–1.04)	0.002
Non-germinal center	1.01 (0.57–1.77)	0.97		
ECOG ≥2	1.97 (0.84–4.59)	0.11		
Stage IV Ann Arbor	0.81 (0.32–2.08)	0.67		
Extranodal involvement	1.03 (0.62–1.72)	0.89		
≥2 Extranodal sites	1.65 (1.01–2.74)	0.049		
Bone marrow involvement	1.98(0.98–4.01)	0.05	2.26 (1.11- 4.63)	0.025
Bulky mass	1.45 (0.89–2.34)	0.12	1.87(1.13–3.08)	0.014

HR: Hazard ratio; 95 % CI: 95 % confidence interval; NLR: Neutrophil-to-lymphocyte ratio; ECOG: Eastern Cooperative Oncology Group performance status.

68 %–81 % versus 48 %; 95 % CI: 35 %–60 %, respectively; HR: 2.09; 95 % CI:1.43–3.30; p-value <0.001) [8].

According to meta-analyses data of nine studies with 1984 patients, NLR was a predicted factor for PFS (HR: 1.64; 95 % CI: 1.36–1.98; I^2 = 36.9 %). Two of these studies did not demonstrate significant differences [21,26]. The reported cutoff point in all studies was highly variable with values between 2.32 to 4.35 and the absolute value of the relationship was not associated with survival [16].

The meta-analyses data showed a NLR as a prognostic factor of OS (HR: 1.84; 95 % CI: 1.52–22; I^2 = 7.3 %) [27]. Two of these studies did not show differences in OS: Melchardt et al. and Ho et al. [28,29].

The limitations associated with this study include its retrospective design, being conducted in a single medical center, and obtaining a ROC curve with a low AUC. However, this is the only known study that evaluated an easily accessible biomarker for any hospital in a large sample of patients in the Colombian population. The collected patient data were sufficient to identify significant differences according to the previously calculated sample size, and the data quality was satisfactory. There were low losses during follow-up, and the methodology used, conducting a standard ROC curve analysis, was appropriate for a time-to-event outcome.

In conclusion, in this study, the NLR with a cutoff point of 6.2 was found to be a significant prognostic marker for 24-month PFS but not for OS. After multivariate analysis, the NLR remained a prognostic variable for PFS, suggesting that it could be used as a complementary tool of daily prognostic scores in clinical practice.

Conflicts of interest

There are no conflicts of interest to report.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Efficacy and safety analysis of the use of ibrutinib associated with rituximab for the first-line treatment of patients with chronic lymphocytic leukaemia

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ABSTRACT

Introduction: Chronic lymphocytic leukaemia, a common blood cancer in adults, particularly affects the elderly and is marked by the accumulation of B lymphocytes. While therapeutic options have expanded, the fludarabine, cyclophosphamide, and rituximab (FCR) regimen remains the standard first-line treatment for fit patients in the Brazilian public health system.

Aim: This systematic review aimed to assess the efficacy and safety of ibrutinib plus rituximab (IR) as a first-line therapy for chronic lymphocytic leukaemia.

Methods: Following PRISMA guidelines and registered in PROSPERO (CRD42023494868), searches were conducted in multiple databases in December 2023 to identify relevant randomized controlled trials comparing the IR and FCR regimens. Eligible studies reported at least one of the following outcomes: progression-free survival, overall survival, severe adverse events, or quality of life.

Results: Two double-blind randomized controlled trials (FLAIR and E1912) totalling 1300 patients met inclusion criteria. Meta-analysis showed that the IR regimen significantly improved progression-free survival compared to the FCR regimen (Hazard ratio: 0.41; 95% CI: 0.31–0.53) with moderate certainty of evidence. However, overall survival did not differ substantially (Hazard ratio: 0.71; 95% CI: 0.33–1.49), and the certainty of the evidence was very low. Quality of life data were unavailable. Due to variations in follow-up, results for severe adverse events were not pooled and the individual studies reported results with low certainty of evidence. The global risk of bias was rated as there was some concern due to the lack of concealed allocation in all outcomes.

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Conclusion: The IR regimen demonstrated superior progression-free survival and comparable safety to the FCR regimen suggesting it is an effective and safe option for first-line treatment of chronic lymphocytic leukaemia.

Introduction

Chronic lymphocytic leukaemia (CLL) is one of the most common blood cancers in adults. It is most frequently diagnosed among people aged 65–74 (median age: 69) and is characterized by the proliferation and accumulation of small immunocompetent B lymphocytes in the peripheral blood, bone marrow, lymph nodes, and spleen [1–7].

Recent estimates indicate the incidence of CLL at 4.5 per 100,000 per year (male 5.8; female 3.3) [8], reaching 30 per 100,000 per year at an age greater than 80 years [4]. The death rate is 0.8 per 100,000 per year, and the 5-year relative survival rate is 88.1 % [8]. In Brazil, 11,540 cases of all types of leukaemia, myeloid or lymphocytic, acute or chronic, are expected per year between 2023–2025, corresponding to an estimated risk of 5.33 per 100,000 per year [9].

The International Workshop on Chronic Lymphocytic Leukaemia (iwCLL) guidelines define recommendations on how to establish the diagnosis of CLL and detailed description of the assessment of the treatment response [10]. Initially, LLC frequently tends to be asymptomatic and an isolated peripheral blood lymphocytosis [7]. Otherwise, the most common clinical presentation is lymphadenopathy, spectral B symptoms (i.e., fever, night sweats, weight loss, fatigue) or cytopenias (i.e., anaemia, thrombocytopenia, neutropenia) due to marrow infiltration, although with lower frequency [11]. The prognosis for CLL is variable [2,5,6]; while some patients have rapidly progressive courses and die soon after diagnosis, other patients survive for a long time and die from causes not related to CLL [5].

Therapeutic options for the treatment of CLL have expanded over time. The best option should be based on disease stage, presence or absence of del (17p) or *TP53* mutations, immunoglobulin heavy-chain variable region (*IGHV*) mutation status, patient age, performance status and comorbid conditions, and the agent's toxicity profile. Fludarabine plus cyclophosphamide (chemotherapy) associated with rituximab (immunotherapy) regimens (FCR) remain first-line therapy due to their response rates and improved overall survival (OS) in specific subgroups of fit patients with previously untreated CLL. However, a continuous regimen with ibrutinib (targeted therapy) associated with rituximab (IR regimen) has also been considered an option in first-line treatment with improved efficacy and safety compared to the FCR regimen [12].

FCR is available in the Brazilian public health system (SUS) for the first-line treatment of CLL [13]. However, ibrutinib has recently been evaluated by the Commission for the Incorporation of Health Technologies into the SUS (Conitec) for the second-line treatment of CLL but was not recommended because of its incremental cost-effectiveness ratio [14]. However, Conitec has not yet evaluated IR as the first-line treatment of CLL. Thus, to identify the gaps that should be addressed, the present study aims to identify all peer-reviewed literature reporting the efficacy and safety of IR versus FCR in the first-line treatment of CLL patients.

Material and methods

Research strategy

The present systematic review was conducted according to the Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines. The systematic review protocol is registered in the International Prospective Register of Systematic Reviews (PROSPERO) database under the number CRD42023494868. Searches were conducted in December 2023 in Medline (PubMed), EMBASE, Cochrane Library and ClinicalTrials.gov databases using indexed descriptors and a combination of free terms (Supplementary material –Tables S1 and S2). The research question was: is ibrutinib associated with rituximab (IR) more effective and safer than chemoimmunotherapy (FCR) for first-line treatment of CLL in patients?

Two investigators independently selected the articles, first reading the title and abstract, and then studies that met the inclusion criteria were included for a full-text review. After open-blinding, any discrepancies identified were resolved by the investigators through discussion and consensus. Excluded studies are listed in Supplementary material. The Rayyan software was used to optimize the selection [15].

Inclusion and exclusion criteria

According to the PICO framework (Table S1), randomized controlled trials (RCTs) were eligible if they compared the IR with FCR regimens for the first-line treatment of naïve CLL patients. Eligible studies had to include at least one of the following outcomes: progression-free survival (PFS), OS, severe adverse events (SAE) or quality of life (QoL).

The search had no restrictions related to the year of publication, language of study, patient age, gender, ethnicity or presence of comorbidities. Results published as conference abstracts were excluded.

Data extraction

After reading the full text, the data from eligible studies related to the author, year of publication, study design, inclusion and exclusion criteria, study location, number of participants, interventions, age, sex, follow-up, and efficacy and

safety outcomes were extracted in a predefined Microsoft Office Excel spreadsheet.

Analysis plan

The data were analysed qualitatively and were reported in tables. The effect size was presented by the hazard ratio (HR) and 95 % confidence interval (95 % CI) for survival outcomes, by the relative risk (RR) and 95 % CI for the outcome of SAE, and by the mean difference and 95 % CI for quality of life, if data outcomes were available in included studies.

When possible, a meta-analysis was performed using the random effects model employing the Review Manager software (version 5.4). The heterogeneity of the studies was verified by visual inspection of forest plots and by the Chi square (p-value <0.05) and I^2 values.

For outcomes with sufficient data, subgroup analyses were performed based on mutations.

Risk of bias and certainty of evidence analysis

The risk of bias was evaluated using the Cochrane Risk of Bias (RoB 2.0) tool [16], and the certainty of the evidence was assessed with the Grading of Recommendations Assessment, Development and Evaluation (GRADE) tool [17]. Each assessment was conducted independently by two investigators, ensuring the highest level of objectivity and transparency. Any discrepancies were resolved through consensus.

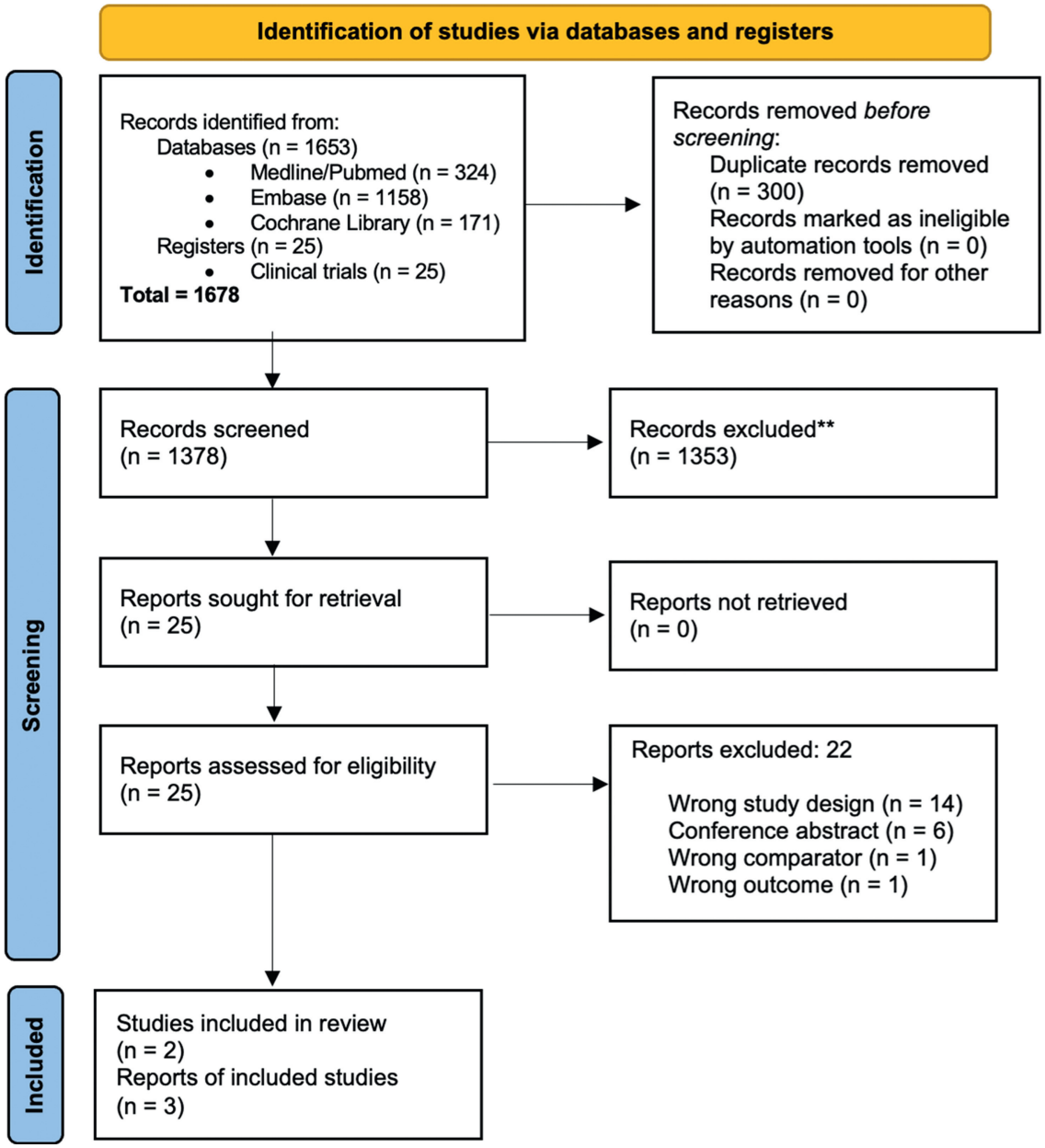

Figure 1 – PRISMA 2020 flow diagram of study selection.

Results

Of the 1678 records identified, 300 duplicates were removed, and the remaining 1378 records were screened to verify eligibility criteria. Of these, 1353 records were excluded because they did not meet the eligibility criteria, and the full texts of the remaining 25 records were read. Finally, three articles were included from two RCTs, the Flair [18] and E1912 [19,20] studies. The complete selection flowchart is presented in Figure 1.

The characterization of clinical trials included in the systematic review is presented in Table 1.

The population of the E1912 trial [19,20] consisted of 529 patients recruited in the USA diagnosed with CLL or SLL, previously untreated and in need of therapy according to the iwCLL criteria. The mean age was 56.7 ± 7.4 years, and the majority were male (67.3 %). According to the Rai classification, the disease stage was intermediate risk, I or II (*n* = 281; 53.1 %), and high risk, III or IV (*n* = 228; 43.1 %). The majority of patients were classified as unmutated (*n* = 281/395; 71.1 %) in terms of the *IGHV* mutation status. A significant portion of the overall population (*n* = 436; 82.4 %) underwent testing for *IGHV* mutation status. Among the 436 patients, *IGHV* status was determined in 395, ensuring the accuracy and reliability of the data.

The Flair study [18] included 771 naive CLL or SLL patients recruited in the United Kingdom and considered fit to receive the FCR regimen. Participants were aged between 18 and 75 years with a WHO performance status of 2 or less and disease status requiring treatment according to iwCLL criteria. The mean age was 62 (interquartile range: 56–67) years, and the majority were male (73 %). Regarding the disease stage, according to the Binet classification, the population was progressive A or B (*n* = 423; 55 %) and stage C (*n* = 348; 45 %). Regarding the *IGHV* mutation status, half of the patients were classified as unmutated (*n* = 388; 50 %).

The meta-analysis performed with a random model shows that the IR regimen is more effective than FCR for PFS (Tables 2 and 3) with the risk being reduced by 59 % with IR compared to FCR (HR: 0.41; 95 % CI: 0.31–0.53), with moderate certainty of evidence (Figure 2a). However, the mean OS for IR compared with FCR (Figure 2b) was (HR: 0.71; 95 % CI: 0.33–1.49) with very low certainty of evidence, showing no significant difference for this outcome (Tables 2 and 3).

Concerning the *IGHV* mutation, for unmutated CLL patients, the two studies show a benefit of IR compared with FCR in terms of PFS. While in the Flair trial, the difference was significant and precise for unmutated *IGHV*, the difference was not significant in the E1912 trial, even though the mean effect was in favour of IR (Table 2). Figures 2C and 2D present the meta-analysis for PFS in the subgroups without and with *IGVH* mutations, respectively. In the meta-analysis, the PFS was significantly better for IR than for FCR in individuals with unmutated *IGHV* (HR: 0.33; 95 % CI: 0.22–0.50). For those with mutated *IGHV*, the results showed no statistically significant differences (HR: 0.44; 95 % CI: 0.19–1.02).

There were no OS data available for a meta-analysis because only the E1912 trial presented data. This trial found that IR is more effective in reducing the risk of death in

Table 1 – Characterization of clinical trials included in the systematic review.

Study	Publication	Participants	Population	Interventions	Male n (%)	Age	Maximum Follow-up
E1912 (NCT02048813)	Shanafelt et al., 2019 [19] and Shanafelt et al., 2022 [20]	Total: 529; IR: 354; FCR: 175	Patients aged ≤70 years with previously untreated CLL or SLL and in need of therapy according to the iwCLL	Experimental: IR Comparator: FCR	Total: 356 (67.3); IR: 236 (66.7); FCR: 120 (68.6)	Mean total: 56.7 ± 7.4; IR: 56.7 ± 7.5; FCR: 56.7 ± 7.2	Median: 5.8 years (70 months)
Flair (ISRCTN01844152 and EudraCT, 2013 –001,944–76)	Hillmen et al., 2023 [18]	Total: 771; IR: 386; FCR: 385	Naive CLL or SLL patients considered fit to receive FCR, between 18 and 75 years of age with a WHO performance status of 2 or less and disease status requiring treatment according to iwCLL	Experimental: IR Comparator: FCR	Total: 565 (73); IR: 283 (73); FCR: 282 (73)	Median total: 62 years (IQR 56–67); IR: 63 (IQR 55-67); FCR: 62 (IQR 56–67)	Median: 4.4 years (53 months; IQR 41–61)

CLL: Chronic lymphocytic leukaemia; SLL: Small lymphocytic lymphoma; IR: Ibrutinib plus rituximab; FCR: Fludarabine, cyclophosphamide and rituximab; IQR: interquartile range; WHO: World Health Organisation; iwCLL: International Workshop on CLL criteria.

Table 2 – Results of global outcomes progression-free survival, overall survival andsevere adverse events.

Outcome	Study	Follow up time (years)	Intervention	Participants	Events	Measure of Effect	Measure of Effect by meta-analysis
Global PFS	NCT02048813-E-1219 [20]	5.8	IR	354	84	HR: 0.37; 95 % CI:	–
			FCR	175	74	0.27–0.51	
	NCT02048813-E-1219 [19]	3	IR	354	37	HR: 0.35; 95 % CI:	HR: 0.41; 95 %
			FCR	175	40	0.22–0.56	CI: 0.31–0.53
	FLAIR [18]	4.4	IR	386	59	HR: 0.44; 95 %	
			FCR	385	118	CI: 0.32–0.60	
PFS - unmutated IGHV	NCT02048813-E-1219 [20]	5.8	IR	210	56	HR: 0.27; 95 % CI:	HR: 0.33; 95 %
			FCR	71	42	0.18 - 0.41	CI: 0.22 - 0.50
	FLAIR [18]	4.4	IR	194	38	HR: 0.41; 95 %	
			FCR	194	77	CI: 0.28 - 0.61	
PFS - mutated IGHV	NCT02048813-E-1219 [20]	5.8	IR	70	44	HR: 0.27; 95 % CI:	HR: 0.44; 95 %
			FCR	44	15	0.11 - 0.62	CI: 0.19 - 1.02
	FLAIR [18]	4.4	IR	146	27	HR: 0.64; 95 %	
			FCR	148	19	CI: 0.35 - 1.16	
OS	NCT02048813-E-1219 [19]	3	IR	354	4	HR: 0.17; 95 % CI:	–
			FCR	175	10	0.05–0.54)	
	NCT02048813-E-1219 [20]	5.8	IR	354	21	HR: 0.47; 95 % CI:	HR: 0.71; 95 %
			FCR	175	18	0.25–0.89)	CI: 0.33–1.49)
	FLAIR [18]	4.4	IR	386	31	HR: 1.01; 95 %	
			FCR	385	29	CI: 0.61–1.68)	
SAE	NCT02048813-E-1219 [19]	3	IR	352	282	RR = 1.00; 95 % CI:	–
			FCR	158	126	0.91–1.10); p-value = 0.924	
	NCT02048813- E-1219 [20]	5.8	IR	352	257	RR = 0.88; 95 % CI:	–
			FCR	158	131	0.80–0.97); p-value = 0.015	
	FLAIR [18]	4.4	IR	384	205	RR = 0.99; 95 % CI:	–
			FCR	378	203	0.87–1.13); p-value = 0.93	

patients with unmutated *IGHV* (HR: 0.35; 95 % CI: 0.15–0.80) when compared with FCR. For patients with mutated *IGHV*, the result was not statistically different (HR: 0.72; 95 % CI: 0.15–3.47).

The RR for SAE was 0.88 (95 % CI: 0.80–0.97; p-value = 0.015) in the Flair study, and 0.99 (95 % CI: 0.87–1.13; p-value = 0.93) for the E1912 study; for this outcome, the certainty of evidence was classified as low. The incidence of Grade 3 or higher adverse events were different between the studies. However, both showed less SAE of neutropenia (14 %) and anaemia (3 %) for the IR group compared with the FCR group (54 % and 14 %, respectively). SAEs of interest with the use of ibrutinib, such as hypertension (11.4 % versus 1.9 %) and cardiac event (7.7 % versus 0 %), were reported more frequently in the IR compared to FCR arm of the E1912 study, respectively [18–20]. These results are presented in Table 3.

The global risk of bias was evaluated, as there was some concern regarding the lack of concealed allocation in outcomes of both RCTs. Additionally, for SAE, the analysis was not by intention-to-treat (Figure 3). The GRADE certainty of evidence was evaluated as moderate for PFS because of the risk of bias; very low for OS because of the risk of bias, inconsistency and imprecision; and low for SAE because of the risk of bias and imprecision (Figure 3).

Discussion

This systematic review showed a clinically relevant effect of IR on PFS compared to using FCR as first-line treatment in patients. Furthermore, the results indicate no difference in relation to the OS and SAE. The European Society for Medical Oncology created a scale to assess the magnitude of the clinical benefit of relevant outcomes for oncology, and when considering only the relative benefit observed as the HR, it is possible to affirm that a lower limit of the HR confidence interval (0.31) of IR reached the threshold of clinical benefit (≤0.70) for PFS when compared to the FCR regimen. Although the OS did not show a statistical difference between the interventions, it is possible to observe that the lower limit of the HR 95 % CI also reached the threshold of clinical benefit (≤0.70 for control >12 months) using the IR intervention, that is, a lower limit of 0.33 [21].

A study conducted to assess the preferences of CLL patients demonstrated that the most important outcome of treatment for them would be increased PFS. In addition, the study indicates a preference for using daily oral medications compared to intravenous medications. In view of this, one study emphasizes the importance of the systematic review findings concerning increased PFS and the use of orally administered ibrutinib [22].

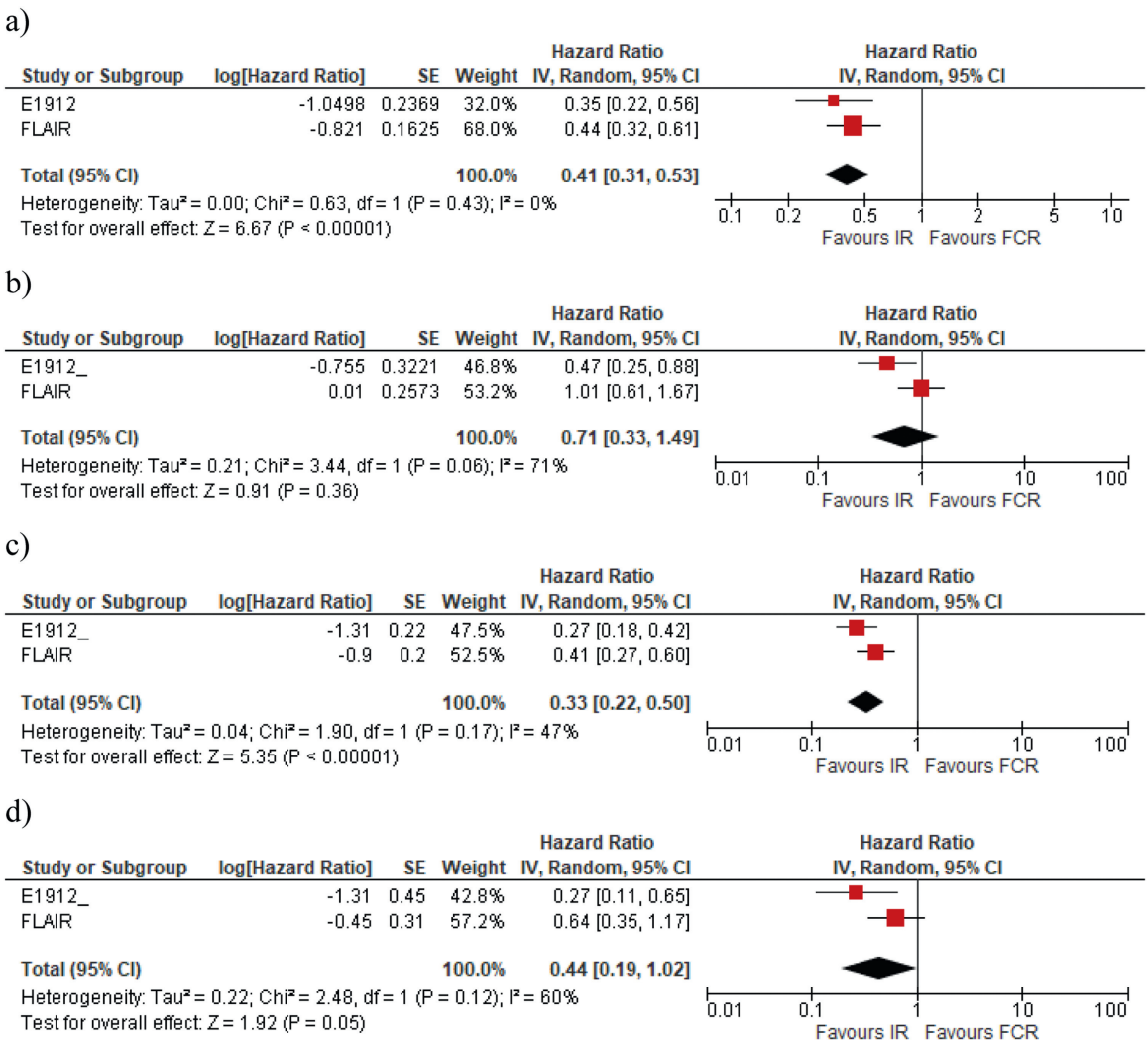

Figure 2 – Forest plot of hazard ratio for: a) global PFS; b) global OS; c) PFS in subgroups of with unmutated IGVH; d) PFS in subgroups with mutated IGVH.

Although ibrutinib and rituximab have been drugs with known efficacy and safety for the treatment of CLL for some years, the first clinical trial, ALLIANCE, that studied the combination of these two drugs in CLL was only published in 2018 [23]. In this study, the IR regimen compared to ibrutinib monotherapy and bendamustine plus rituximab (BR) showed no significant difference between IR and ibrutinib monotherapy regarding PFS in the treatment of older CLL patients [23]. Thus, this was the only study on the IR regimen used to treat CLL available for inclusion in the two systematic reviews with network meta-analysis published until 2021 [24,25]. As it employed the network meta-analysis methodology, the amount of data is quite relevant to the study results; it is desirable to have the most significant number of clinical trials of evaluated interventions.

Another analysis based on indirect comparisons included the data from the publication of the long-term results of the E1912 study in 2022 [20], and, for the first time, data from the comparison between IR and FCR in CLL treatment could be assessed [26]. This analysis demonstrated that there was no significant difference between IR (data from the ALLIANCE [23] and E1912 [20] studies) and ibrutinib plus obinutuzumab concerning PFS. Furthermore, these combinations were quite similar to ibrutinib monotherapy and venetoclax plus obinutuzumab. Together, these therapeutic options were better than FCR, BR, chlorambucil plus obinutuzumab and chlorambucil monotherapy, in decreasing order of PFS results [26]. However, due to the publication date, the analysis of the indirect comparison study did not include the FLAIR trial [18] (published in 2023), which would provide more data on the comparison between IR and FCR and potentially provide information with a higher level of certainty.

In this context, the clinical trial data from the comparison between IR and FCR regimens are highly relevant for healthcare decision-makers, highlighting the need to identify the best and most current evidence of efficacy and safety for this comparison. To our knowledge, the present study is the first systematic review to evaluate the efficacy and safety of IR for the first-line treatment of CLL patients compared to FCR. This systematic review demonstrated that IR is more effective than FCR in terms of PFS. However, the results, show no statistically significant difference between IR and FCR regarding

Table 3 – Certainty of evidence by Grading of Recommendations Assessment, Development and Evaluation (GRADE) Critical.

Certainty assessment							Events / No. of patients		Effect		Certainty	Importance
No. of studies	Study design	Risk of bias	Inconsistency	Indirectness	Imprecision	Other considerations	IR	FCR	Relative (95 % CI)	Absolute (95 % CI)		
Progression-free survival (follow-up: median 3 years)												
2 [18,20]	randomized trials	serious[a]	not serious	not serious	not serious	none	96/740 13.0 %	158/560 28.2 %	**HR 0.41** (0.31–0.53)	313 more per 1000 (from 229 more to 393 more)	⊕⊕⊕x̂ Moderate[a]	Critical
Overall survival (OS) (follow-up: range 4.4 years to 5.8 years)												
2 [18,20]	randomized trials	serious[a]	serious[b]	not serious	serious[c]	none	52/740 7.0 %	47/560 8.4 %	**HR 0.71** (0.33–1.49)	81 more per 1000 (from 51 fewer to 345 more)	⊕x̂x̂x̂ Very low[a,b,c]	Critical
Severe adverse events - Grades 3–4 (follow-up: range 4.4 years to 5,8 years)												
2 [18,20]	randomized trials	serious[d]	not serious	not serious	serious[e]	none	**E1912 - Shanafelt et al., 2022:** RR: 0.88; 95 % CI: 0.80–0.96 **FLAIR - Hillmen et al., 2023:** RR: 0.99; 95 % CI: 0.87–1.13	⊕⊕x̂x̂ Low[d,e]	Critical			

IR: ibrutinib and rituximab; FCR: fludarabine, cyclophosphamide and rituximab; CI: confidence interval; HR: hazard ratio.

Explanations:.

a. According to the assessment performed using the ROB-2 tool, the two RCTs had some concerns regarding the overall risk of bias, both for the progression-free survival and for overall survival outcomes. The limitations of the studies are related to the randomization process since neither study reported allocation concealment.

b. Considering a clinically important difference in the threshold of 0.85, the point estimates of the studies are located on opposite sides (Hillmen 2023 [18] - HR: 1.01 and Shanafelt 2022 [20] - HR: 0.47), indicating an inconsistency in the studies' results for the overall survival.

c. Considering a clinically important difference threshold of 0.85, the summary estimate of the meta-analysis of the overall survival outcome crossed this threshold and the null effect line, indicating an imprecision in the results.

d. The two RCTs were classified as having some concerns regarding the overall risk of bias according to the assessment performed using the ROB-2 tool in respect to severe adverse events. The limitation of the studies is related to the randomization process and the deviation from the intended interventions domains since neither study reported allocation concealment and did not perform intention-to-treat analysis.

e. Considering the clinically important difference threshold of 0.85, the 95 % confidence interval of Shanafelt crossed this threshold, and the 95 % confidence interval of the Hillmen study crossed the null effect line, indicating that the studies have imprecision regarding their results.

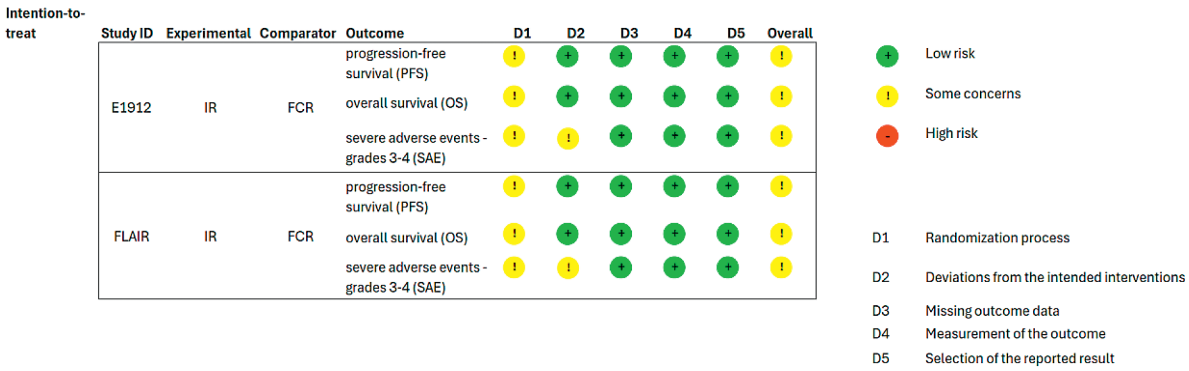

Figure 3 – Risk of bias summary.

OS. Unlike previous systematic reviews, an additional trial (FLAIR [18]) was included. In addition to providing more information about IR and increasing the statistical power of the clinical results, adding the FLAIR trial allowed the possibility to compare the IR with FCR regimens in another healthcare setting since the trial was conducted in the United Kingdom.

This study reduced the certainty of evidence in all outcomes evaluated mainly because of the potential risk of bias due to the randomization process since neither study reported allocation concealment. Besides that, this current study has limitations that should be highlighted. Some differences between the baseline characteristics of the populations of both clinical trials and their follow-ups may have contributed to inevitable heterogeneity in the results. First, the fact that E1912 [19,20] was conducted in the United States and FLAIR [18] in the United Kingdom requires us to consider potential differences in the healthcare systems and clinical protocols of each country. These differences in the context of the clinical trials may be related to the baseline characteristics and prognosis of CLL patients. For example, patients were staged using the Rai system in E1912 [19] and the Binet system in FLAIR [18], which are two staging systems that employ distinct stages and criteria definitions. Regarding *IGHV* status, 28.9 % of patients had a mutation in E1912 [19] while 38 % were mutated in FLAIR [18]. A difference in the ages of patients in clinical trials was also observed. In E1912 [19], the average age was 56.7 years, in both groups. In FLAIR [18], on the other hand, the median age was 62 years; 63 years in the subgroup with IR and 62 years with FCR. Concerning the proportion of patients in the IR arms of the two studies, E1912 [19] had 66.92 % (354/529) and FLAIR [18] 50.06 % (386/771). Furthermore, the difference in follow-up between clinical trials may also have contributed to some heterogeneity in the results. While patients were followed for 70 months (median) in E1912 [20], the follow-up was 53 months in FLAIR [18].

As mentioned in the results of this study, the follow-up and *IGHV* status were mainly related to the divergence on whether IR was favoured in comparison to FCR. The benefit of IR seems to be greater for the *IGHV* group, but this needs to be confirmed with more clinical data. Thus, the difficulty in weighing these differences between studies became another important limitation of the study. To minimize these differences, a meta-analysis of the results is presented, prioritizing data from the global population without stratification for OS by *IGHV* status. On the other hand, it was impossible to pool the safety data (SAE) in a meta-analysis due to the differences in follow-up times. In any case, we consider that this lack of a safety meta-analysis did not hinder the interpretation of the effects of IR.

Conclusion

Regarding PFS, IR was more effective than FCR in the first-line treatment of CLL. On the other hand, no additional OS or SAE benefits of IR were observed compared to FCR. Regarding safety, IR was shown to be at least as safe as FCR. Despite some concerns about heterogeneity observed between clinical trials and the certainty of evidence assessed, the results of this systematic review indicate that ibrutinib with rituximab should be considered an effective and safe regimen in the first-line treatment of CLL.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2025.106234.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Mechanistic insights into the antiproliferative effect of the redox-active iron chelator Dp44mT on multiple myeloma cell lines

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ABSTRACT

Background: Impaired iron metabolism has been linked to the pathogenesis of multiple myeloma. Redox active iron chelators have gained attention as potential anti-cancer agents as they target the high iron dependency of cancer cells. This study explored the potential mechanisms underlying the anti-multiple myeloma effect of the redox active iron chelator Dp44mT (Di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone).
Methods: The effect of Dp44mT was tested on both immunomodulatory drug-sensitive and drug-resistant multiple myeloma cell lines using the MTT assay. Proteomic and phosphoproteomics characterization were utilized to explore the mechanisms of Dp44mT action on multiple myeloma cells. In addition, a real-time polymerase chain reaction assay was performed to examine the expressions of major iron metabolism genes. Reactive oxygen species, lipid peroxidation, mitochondrial membrane potential, and intracellular iron compartmentalization were measured using flow-cytometry.
Results: The high potency of Dp44mT in killing multiple myeloma cell lines was confirmed. Treatment with Dp44mT showed evidence of deregulated cellular iron metabolism, reactive oxygen species homeostasis, and mitochondrial membrane potential in multiple myeloma cell lines. As possible mechanistic pathways of Dp44mT, there was overrepresentation of the AMPK pathway, cell cycle, endoplasmic stress, and down regulation of *ACSL4* (*acyl-CoA synthetase long chain family member 4*).

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Conclusion: This study suggests an in vitro, anti-multiple myeloma effect of Dp44mT that appears to be mediated by dysregulated iron metabolism, reactive oxygen species, and other biological pathways.

Introduction

Multiple myeloma (MM) is a plasma cell malignancy characterized by excessive production of circulating monoclonal immunoglobulin. Acquired resistance to the available treatment regimens poses the largest problem in disease management. Identifying the mechanisms and developing strategies to overcome resistance is key for improving disease outcomes.

Iron plays a crucial role in various cellular processes, including DNA synthesis, oxygen transport, and energy production under a tight regulatory system. The pathogenesis of MM has consistently been linked with deregulated iron metabolism [1]. High availability of intracellular iron can augment the growth of cancer cells and boost the dissemination cascade. However, under certain conditions, iron also promotes the production of toxic reactive oxygen species (ROS) that initiate ferroptosis (a form of cell death mediated by iron-induced lipid peroxidation) as a defense against cancer [2]. Thus, iron metabolism is tightly regulated to avoid the detrimental effects of iron induced toxicity while preserving its vital role in healthy cells.

Recently, a class of redox-active iron chelators that bind and eliminate excess iron from the cells, and yet have the potential to generate ROS to kill cancer cells has been investigated as possible therapeutic agents in hematological malignancies. Multiple redox-active iron chelators, like di-2-pyridyl thiosemicarbazone analogues, have been studied for their favorable applications in cancer treatment. Among them, Dp44mT (di-2-pyridylketone-4,4,-dimethyl-3-thiosemicarbazone), an iron chelating complex, has shown promise as an anti-tumor agent in both in-vitro and in-vivo models [3–5]. Previous studies have also established Dp44mT as a potential inducer of apoptosis as a single agent in many different cancers including, breast, osteosarcoma, neuroepithelioma, melanoma, glioblastoma and oral squamous cell carcinoma [4,6–9]. A preliminary study investigated the effects of Dp44mT in MM cell lines, finding that Dp44mT inhibited the growth and viability of MM cells, induced cell cycle arrest, and promoted apoptosis [3]. Although Dp44mT has shown promising results in preclinical models, further research including human clinical trial studies are required to establish its suitability in the clinical setting.

Despite its potential as an anti-cancer agent, the precise molecular targets and pathways through which Dp44mT exerts its effects are underexplored. This study undertakes a comprehensive evaluation of the anti-cancer effects of potent iron chelators in MM. A multi-modal experimental approach was employed including high-resolution mass spectrometry for proteomics and phosphoproteomics profiling, cell viability assays to assess cytotoxicity and proliferation, and a range of flow cytometry approaches to monitor ROS, lipid peroxidation, mitochondrial membrane potential ($\Delta\Psi m$), and intracellular iron compartmentalization. Taken together, the potent anti-cancer effect of Dp44mT in MM and the intracellular mechanism of its anti-MM effect were established by the utilization of advanced mass spectrometry-based technologies.

Methods

Study design

The experimental plan of this study involved a comprehensive investigation of a wide range of anti-cancer compounds in eight different MM cell lines. MM cells showed high sensitivity to iron chelating drugs independent of immunomodulatory drug (IMiD) sensitivity as IMiD resistant cells were also susceptible to treatment. To understand the detailed mechanism involved behind anti-MM activity of redox-active iron chelating drugs, two cell lines one based on IMiD sensitivity (*MM.1S*) and another on resistance (*RPMI-8226*) were selected for further study. Dp44mT was most effective in inducing MM cell death and was therefore chosen for further studies. To identify mechanisms, a highly advanced approach was used to establish the total proteomics and phosphoproteomics signatures influenced by Dp44mT treatment.

Cell culture

The human MM cell lines *JJN3*, *RPMI-8226, MM.1S* and *KMS11* were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10 % AB serum (Gem cell human) and 1 % penicillin–90 streptomycin (P/S) solution (Gibco) at 37 °C in a humidified incubator (SANYO, Japan) containing 5 % CO_2.

RNA extraction, cDNA synthesis and real-time polymerase chain reaction

Total RNA was extracted using Qiagen total RNA extraction kit following the manufacturer's recommended protocol. cDNA was synthesized by utilizing iscript cDNA synthesis kit (BioRad). Real-time polymerase chain reaction (RTqPCR) assay was performed using Fast SYBR Green Master mix (Applied Biosystems) in 20 μL total volume. The cDNA was diluted 10 fold to carry out RTqPCR amplification. The final concentration of each primer in the reaction mix was 0.5 μM. Reactions were run in a RTqPCR System (LightCycler® 480 Instrument II Roche). The cycling conditions were set at 95 °C for 25 s, 35 cycles at 95 °C for 1 s, 60 °C for

20 s, 95 °C for 15 s, 60 °C for 1 minute, and a gradient from 60 °C to 95 °C with a continuous detection at 0.015 °C/s increases for 15 min.

Cell proliferation assay

The cell proliferation assay was carried out using the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent. The drugs Lenalidomide (Cat#SML2283), Salinomycin, Di-2-pyridylketone (DpC, Cat#SML0483) and Di-2-Pyridylketone 4,4-Dimethyl-3-Thiosemicarbazone, (Dp44mT, Cat#SML0186), were obtained from sigma Aldrich Burlington, MA, United States in lyophilized form and were reconstituted at the desired concentrations as instructed by manufacturer: small single use aliquots were stored at −80 °C until further use. The different MM cells were independently seeded into 96-well plates at a density of 3×10^4 cells per well. After seeding, cells were treated with various drugs according to the planned experimental workflow and cultured for up to 72 h. After 72 h, 15 μL of the MTT reagent were added per well followed by incubation for 4 h at 37 °C in a humidified incubator. One hundred microliters of stop solution were added to each well to dissolve the formazan crystals. The microplate was then incubated at 37 °C to dissolve the formazan crystals in DMSO properly. The 96-well plate reader (Flax station) measured the color intensity at 590 nm. Mean optical density of three replicate readings were plotted by normalizing each condition against an untreated control.

Flow cytometric analysis

To measure mitochondrial superoxide, cells were stained with MitoSox Red dye (Molecular Probes: Cat# M36008) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS (Gibco: Cat#10,010–23) and resuspended in media without Phenol Red. For cytoplasmic superoxide staining, cells were stained with CellRox Deep Red dye (Molecular Probes: cat#C10422) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS, resuspended in media without Phenol Red. For intracellular ROS staining, cells were stained with CM-H2DCFDA dye (Thermo Fisher: cat#C6827) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS, and resuspended in media without Phenol Red. For lipid peroxide staining, cells were stained with Liperfluo dye (Dojindo: cat#50–190–3721) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS, and resuspended in media without Phenol Red. For mitochondrial labile iron staining, cells were stained with MitoFerro Green dye (Dijindo: Cat#M489) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS. For endoplasmic reticulum labile iron staining, cells were stained with FerroFar Red dye (Millipore: cat#SCT037) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS. For Golgi labile iron staining, cells were stained with BioTracker Red dye (Millipore: Cat#SCT030) in culture media without Phenol Red at a final concentration of 5 μM for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS. For cytoplasmic calcium staining, cells were stained with X-rhod1 AM dye (Molecular Probes: cat#14,210) in Ca^{+2}/Mg^{+2} free PBS at a final concentration of 1 μM and 1 mM Probenecid (Molecular Probes, Cat#P36400) for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS, resuspended in PBS, and incubated further at 37 °C for 30 min to allow de-esterification of intracellular ester. For Mitochondrial calcium staining, cells were stained with Fluo4 AM dye (Molecular Probes: cat#14,201) in Ca^{+2}/Mg^{+2} free PBS at a final concentration of 1 μM and 1 mM Probenecid (Molecular Probes, Cat#P36400) for 30 min at 37 °C. Cells were washed two times in Ca^{+2}/Mg^{+2} free PBS, resuspended in PBS and incubated further at 37 °C for 30 min to allow de-esterification of intracellular ester. Once again cells were washed before acquiring. For apoptosis detection. equle concentration of Annexin V/7-AAD dyes was added in 100 μL binding buffer. The cells were incubated for 15 min at room temperature in the dark followed by addition of 400 μL binding buffer. The data were analyzed by flow cytometry within 1 hour. Mitochondrila membrane potential ($\Delta\Psi$m) was studied using MitoView™ dye in phenol red free media. The cells were incubated for 30 min at 37 °C followed by washing with Ca^{2+}/Mg^{2+} free PBS. The cells were again resuspended in phenol red free media. The acquiring was done with a Fortessa flow cytometer (BD Bioscience) and data was analyzed using FACSDiva software (BD Bioscience). Mean fluorescence intensity was normalized with untreated parent cells.

Proteomics and phosphoproteomic analysis

The experimental protocol followed was essentially as previously described and is outlined in supplementary Figure 1 [10]. Briefly, the samples were subjected to protein extraction followed by trypsin digestion. Tryptic peptides were labelled with TMT 6-plex reagents, and the pooled peptides were fractionated into 12 fractions using basic pH reversed-phase fractionation. Five percent of each fraction was used for global proteomics analysis while the remainder was subjected to phosphopeptide enrichment using Fe(III)-NTA cartridges on an Agilent Bravo automated liquid handling platform. Fractions for global proteomics analysis and phosphoproteomic analysis were analyzed on an Orbitrap Exploris 480 mass spectrometer and UltiMate 3000 RSLC nano system (Thermo Scientific, San Jose, CA, USA). Fractions were resuspended in 0.1 % formic acid and injected on to a trap column (Optimize Technologies) using solvent A (0.1 % formic acid). Peptides were then separated on an analytical column (PepSep 40 cm × 100 μm, C_{18} 1.5 μm) for a total run time of 180 min using a gradient of solvent B (Acetonitrile, 0.1 % formic acid) from 5–35 % at 0.35 μL/min flow rate for 160 min. This is followed by increasing the solvent B to 90 % maintained for 9 min to wash the column. Mass spectrometry analysis was performed in data dependent mode in which a survey mass spectrometry scan was performed in an Orbitrap mass analyzer operated at 120,000 resolution. Precursor ions were collected with a normalized AGC target

of 200 % or a maximum injection time of 50 ms and analyzed across a mass range of 350–1500 m/z. Precursor ions from charge states 2–6 were sequentially isolated based on abundance using quadrupole mass filter with an isolation width of 0.7 m/z. Isolated precursor ions were fragmented using HCD mode with a normalized collision energy of 34 %. Fragmented ions were collected for a normalized AGC target of 200 % or a maximum injection time of 100 ms and recorded in an Orbitrap mass analyzer at 30,000 resolution. Dynamic exclusion setting was enabled with an exclusion duration of 35 s and exclusion mass width of 10 ppm.

For the analysis of phosphopeptide fractions, a gradient of 5–35 % solvent B was used for separating the phosphopeptides for 135 min followed by a high organic wash of 90 % solvent B for 5 min. An overall run time of 150 min was used for the analysis. Mass spectrometry analysis was performed as described above except an isolation width of 1.2 m/z was used for isolating the precursor ions for MS/MS analysis.

Mass spectrometry raw data from proteomic and phosphoproteomic analyses were analyzed using the Sequest HT search engine in Proteome Discoverer software (version 2.5). MS/MS spectra was searched against the human Uniprot protein database with full tryptic cleavage specificity, two missed cleavages, precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.05 Da. Oxidation (M), acetylation (protein N-terminus) as dynamic modification and carbamidomethylation (C), TMT modification (K, peptide N-terminus) were specified. Additionally, phosphorylation (S,T,Y) was specified as a dynamic modification for the phosphoproteomics data. Percolator was used for the false discovery rate calculation which was maintained at 1 % for protein and peptide levels. Reporter ion quantitation was performed by integrating the signal with 20 ppm tolerance. PSMs with an average report ion S/N threshold of 10 were filtered out for quantitation. Finally, normalized abundances were calculated by using total peptide amount option which were used for further data analysis.

In summary, protein samples were digested, TMT-labeled, and fractionated for total proteomics and phosphoproteomic analyses. Enrichment and analysis of phosphopeptides were done using the Agilent system and Orbitrap Exploris 480, respectively. The identified phosphopeptides with normalized abundance values reflected the relative expression across samples.

Data analysis

The data were analyzed in Prism 8 (GraphPad Software, San Diego, CA, USA). Ingenuity pathway analysis (IPA) was performed on differentially expressed proteins comparing untreated versus treated groups. The networks, functional analyses and graphical summary were generated using QIAGEN IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). The default parameters were used for data interpretation. The phosphoproteomics data was analyzed using a "Phosphomatics" webtool available online (https://phosphomatics.com). Kinase-Substrate Enrichment Analysis (KSEA) was performed as described by Casado et.al [11].

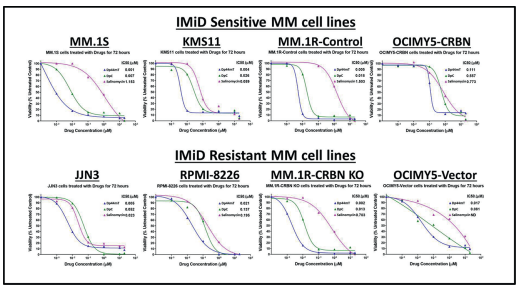

Figure 1 – Iron chelators are potent killers of multiple myeloid cell lines. Multiple myeloid (MM) cell-lines (IMiD sensitive and IMiD resistant) were treated with Drugs (Dp44mT, DpC and Salinomycin) for 72 h. MTT assay was performed at the end of 72 h incubation and half maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism software.

Results

Iron chelators are most potent killers of multiple myeloma cell lines

The antiproliferative activity of various drugs was investigated in MM cells. Of the tested compounds, the most potent drugs found to kill MM cell lines, came under one common umbrella of iron chelators (data not shown). Therefore, three different iron chelators were tested, namely: Dp44mT, DpC, and Salinomycin. All of these drugs were effective in killing MM cells in exceptionally low concentrations. Interestingly, the effect of iron chelators was stronger than the clinically approved immunomodulatory drugs, and their effect was independent of *CRBN* expression (experiments with cereblon-knockout cells; data not shown). Of the iron chelators, Dp44mT exhibited the most potent ability to kill MM cell lines with highest potency (half maximal inhibitory concentration [IC_{50}] = 0.001 to −0.1 μM - Figure 1).

In comparison, treatment with IMiDs did not induce 100 % killing in MM cell lines. Only IMiD sensitive cell lines - *MM.1S, KMS11, MM.1R, OCIMY5-CRBN*-overexpressed showed sensitivity to lenalidomide with saturation at certain concentrations, and once the drug reached its maximum antiproliferation effect, there was no further killing even by increasing the lenalidomide concentration to as high as 100 μM. As anticipated, IMiD resistant cell lines *JJN3, RPMI-8226, MM.1R CRBN-KO* showed no response to IMiD treatment (Data not shown).

These data suggest a potent anti-MM effect of Dp44mT and similar redox-active iron chelators that can be effective even in IMiD -resistant clones. (Figure 1).

Dp44mT-induced proteomic changes in multiple myeloma cell lines

To firmly establish the mechanism of the anti-MM effect of Dp44mT, global proteomics- and phosphoproteomics-based evaluations of molecular pathways were adopted. Two MM cell lines, *MM.1S* (IMiD sensitive), and *RPMI-8226* (IMiD

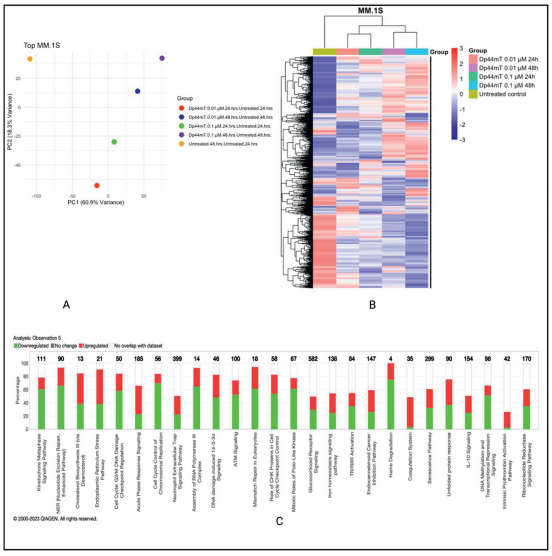

Figure 2 – Dp44mT-induced proteomic changes in multiple myeloid (MM) cell lines: A. Principal component analysis. Unit variance scaling was employed to rows and the principal components were calculated using the singular value decomposition imputation approach. The X and Y axes correspond to principal component (PC)-1 and PC-2, respectively thus explaining the total variance (*n* = 5) data points. B. Heatmap of differentially regulated proteins after Dp44mT treatment (Only those genes with absolute fold change |log2 (FC)| >1.5 were included to reduce the size of the data). C. Canonical ingenuity pathway analysis showing differentially regulated pathways in MM cell lines. Green represents downregulation, red represents upregulation, and white represents no overlap with the ingenuity pathway knowledge-based database. *MM.1S* (top) and *RPMI-8226* (bottom).

resistant) were selected and treated with Dp44mT for two different time periods (24 and 48-hours). Two concentrations were selected from cell viability data obtained from studies with Dp44mT treatment for 72 h; the concentration at which ~90 % cells died at 72 h i.e., 0.1 nM and the concentration at which ~50 % cells died after 72 h of Dp44mT treatment i.e., 0.01 nM. The cells were treated with or without Dp44mT and pellets were collected first at 24 h and then after 48 h for downstream experiments.

The global proteomic analysis identified 8066 proteins in the *MM.1S* cell line and 7972 proteins in the *RPMI-8226* cell line. Principal component analysis for both cell lines revealed a substantial variability after Dp44mT treatment. The normalized abundance ratios were used to calculate the fold change between the groups with the respective untreated group. The most prominent proteomics expression level changes were observed at the 48-hour time point at both studied concentrations i.e., 0.1 μM and 0.01 μM.

Ingenuity pathway analysis highlighted differentially expressed pathways specific to the *MM.1S* cell line such as cell cycle, control of chromosomal replication, ATM signaling, and unfolded protein response, amongst others. On the other hand, the *RPMI-8226* cell line showed differential expression involving pathways such as ferroptosis signaling, glutathione biosynthesis, and death receptor signaling. Given that both cell lines included in this study for proteomics and phosphoproteomics analyses differ a lot in their underlying biology, the mechanism of action of Dp44mT also showed some differences between them (Figure 2). The graphical summary of biological processes and their activation (Orange) or inhibition (Blue) state in Dp44mt treated versus untreated cell lines are shown in Supplementary Figure 2 A & B. The similar/crosstalk biological processes represented in both cell lines were related to iron homeostasis and endoplasmic reticulum stress. The data of this study show evidence of the ability of Dp44mT to modulate iron metabolism by iron starvation, decrease antioxidant defenses via NRF2-mediated oxidative stress response, and increase endoplasmic reticulum stress in MM cell lines.

However, considering the lack of an obvious common biological pathway to explain the anti-MM effect of Dp44mT between the two cell lines, further studies with phosphoproteomics were undertaken.

Global phosphoproteomics after Dp44mT treatment establishes a link with iron metabolism

Iron plays an important role in reprogramming phosphorylation signaling [12]. Phosphorylation is a post-translational modification that has a crucial role in regulating protein function and cellular signaling.

To investigate Dp44mT-induced phosphoproteomic alterations in IMiD resistant and IMiD sensitive cell lines, phosphopeptides identified from the *MM.1S* and *RPMI-8226* cell lines were measured using TMT-based multiplexed quantitation. Approximately ~27,000 phosphopeptides were identified in both experiments. The phosphoproteins and phosphosites with fold changes |(FC)| >1.5 were identified as differentially expressed phosphosites. After normalization and filtering out missing data, ~17,000 phosphorylation sites were found to be present in each group with approximately ~5000 unique proteins (Supplementary Figure 3 A-F).

The top active kinases after 48 h of Dp44mT treatment of the *MM.1S* cell line include CDK1 AURKB1, AURKB, CDK2, and TTK. CSNK2A2, ATM, AKT1, PRKD1 and SGK1 were more active in the untreated groups. Most of the top kinases identified in the Dp44mT treatment group are involved in controlling cellular processes necessary for cell growth, survival, and DNA damage response.

On the contrary, the top downregulated kinase CSNK2A2 is the catalytic subunit of the protein kinase CK2 (formerly known as casein kinase 2). CK2 has been demonstrated to interact with and modify the activity of iron-related proteins, such as the iron regulatory protein 1 (IRP1) and the iron-sulfur cluster assembly enzyme ISCU [13]. Abnormal expression of *CSNK2A2* has been implicated in various diseases, including cancer [14]. Moreover, the downregulation of kinases like ATM and AKT1 might make cells more vulnerable to damage, leading to cell death.

The *RPMI-8226* cell line showed CDK2, PRKCD, AKT1, MAPK8, PRKD1, CDK4 as most represented kinases in the Dp44mT treatment group and SRPK1, HIPK2, CDK9, PRKG1, MAPK3, in the untreated group. These protein kinases contribute in various signaling pathways, and regulatory

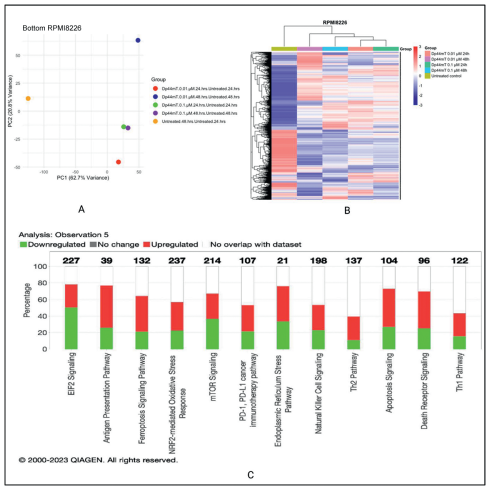

Figure 2 Continued.

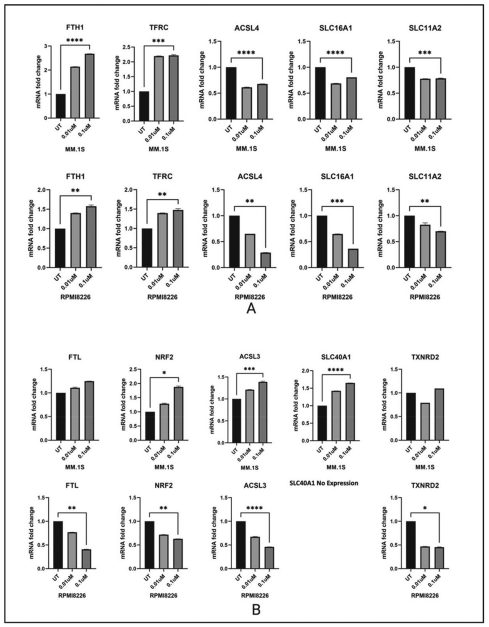

Figure 3 – Global Phosphoproteomics changes after Dp44mT treatment: A & D: Kinase-substrate enrichment analysis (as described by Casado et al. and Wiredja et al.) the plot shows the Z-score for enrichment of different kinases in present data. Kinases with Z-scores greater than 0 are more active in the control group (Untreated group). B & E: Upstream motif analysis to identify kinases responsible for phosphorylation. C & F: Enriched pathways of most represented phosphorites. *MM.1S* (Top) and *RPMI-8226* (Bottom).

mechanisms. Their functions are diverse and can include roles in cell cycle regulation, transcriptional control, signal transduction, apoptosis, and cellular growth and survival.

Of interest, the enrichment analysis showed over representation of spliceosomes, autophagy, AMPK pathway, cell cycle, and protein processing in endoplasmic reticulum of both cell lines. All the above pathways are critical aspects of cellular physiology. AMPK regulates cellular energy homeostasis. It functions as a sensor of AMP/ATP ratios, activating energy-generating processes and inhibiting energy-consuming pathways to restore energy balance. This is in line with a previous study that concluded that activation of the AMPK pathway after Dp44mT treatment is a cellular mechanism to rescue the loss of iron and to deal with oxidative stress generated by Dp44mT [15]. Additionally, dysregulation of fundamental cellular processes like cell cycle and protein processing in endoplasmic reticulum after Dp44mT treatment points towards the potential of novel mechanisms for antitumor activity of Dp44mT (Figure 3A-F).

Dp44mT-induced changes in levels of major iron regulating genes

Since Dp44mT is known to target the iron metabolism, and both AMPK and endoplasmic reticulum stress are implicated in ferroptosis [16], Dp44mT-induced changes in the expression of major iron metabolism genes were determined. The mRNA of ten genes involved in major iron metabolism-related biological processes were quantified. Change in iron storage (*FTH1* and *FTL*), iron transport (*SLC11A2*, *SLC40A1* and *SLC16A1*), iron uptake (*TFRC*), oxidative stress defense (*NRF2*), redox regulation (*TXNRD2*), and ferroptosis (*ACSL3* and *ACSL4*) were measured.

The *MM.1S* (IMiD sensitive) and *RPMI-8226* (IMiD resistant) cell lines showed distinct gene expression profiles of iron metabolism genes after 24 and 48 h of Dp44mT treatment. The common differential expression pattern observed between both cell lines was seen in genes like *FTH1*, *TFRC*, *ACSL4*, *SLC11A2*, *SLC16A1*. *NRF2*, *SLC40A1* (*RPMI-8226* lack *SLC40A1* gene expression), *ACSL3*, *FTL* showed opposite trends in expression after Dp44mT treatment. The observed cell death after Dp44mT treatment could primarily be due to iron mis-localization and deprivation in some cellular compartments. Both iron depletion and iron excess can trigger apoptotic cell death through disrupted redox balance, mitochondrial dysfunction, and activation of apoptotic pathways. The ability of Dp44mT to chelate iron can lead to cellular iron maldistribution ultimately leading to cell death.

It is possible that a constitutive lack of expression of the iron export protein SLC40A1 (ferroportin) in the *RPMI-8226* cell line could lead to compensatory mechanisms to deal with iron dysregulation making Dp44mT less effective compared to its effect on other MM cell lines. The IC_{50} of the *RPMI-8226* cell line was 0.02 compared to 0.001 for the *MM.1S* cell line. A decrease in ferroportin expression has been reported to promote myeloma growth in previous research articles [17,18].

Of interest, there was substantial downregulation of the ferroptosis mediating gene *ACSL4* in both cell lines after Dp44mT treatment. *ACSL4* is a highly expressed gene in MM and its downregulation in response to Dp44mT might suggest a dual function. A recent study by Zhang et al. has also experimentally proven that *ACSL4* is abnormally expressed in MM patients and directly promotes MM cell proliferation. Further, knockdown of *ACSL4* suppressed MM cell viability despite reducing susceptibility to RSL3-induced ferroptosis [19]. Thus, suppression of *ACSL4* may be a key mechanism through which Dp44mT mediates its anti-myeloma effect. Collectively, data suggest that the disordered iron metabolism in

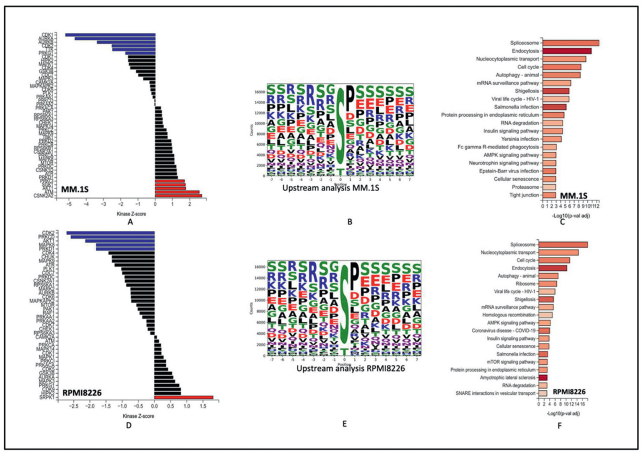

Figure 4 – Changes in major iron metabolism related genes 48 h after Dp44mT treatment in *MM.1S* and *RPMI-8226* cell lines. A: The genes with similar expression patterns in both studied cell lines are shown at the top and B: Genes having differential expression patterns in response to Dp44mT treatment are shown at the bottom.

MM cell lines can be a therapeutic target in the development of effective therapies against MM progression (Figure 4).

Dp44mT-induced multiple myeloma cell apoptosis

A flow cytometric approach was utilized to explore the underling mechanism of the Dp44mT-induced anti-proliferative effect to find the apoptotic profiles of MM cells. Annexin 5/7AAD was used to quantify apoptotic cells. The data showed that Dp44mT (0.1 and 0.01 μM) significantly increased the number of apoptotic cells in both tested cell lines following a distinct dose-dependent manner. Moreover, a decrease in mitochondrial calcium and an increase in cytoplasmic calcium were also observed in both cell lines at both time points and concentrations. The increased influx of cytosolic calcium and decrease in mitochondrial calcium is likely due to activation of apoptosis under Dp44mT-induced stress (Figure 5A–C).

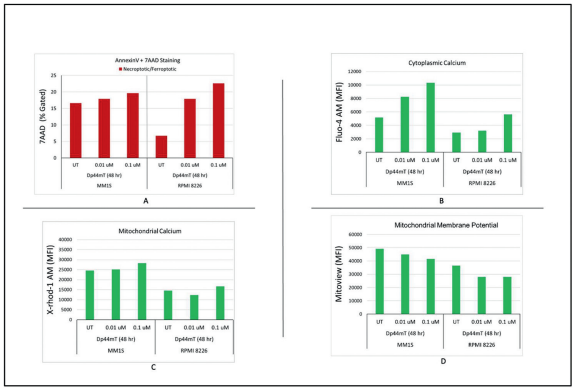

Figure 5 – Dp44mT-induced multiple myeloid (MM) cell apoptosis: Flow cytometric analyses to detect the apoptotic profiles of MM cells. A: Annexin 5/7AAD was used to quantify the apoptotic cells. B & C: The increased influx of cytosolic calcium and decrease in mitochondrial calcium is likely due to activation of apoptosis under Dp44mT-induced stress D: Time- and Dose-dependent decreases in mitochondrial membrane potential ($\Delta\Psi$m) after Dp44mT treatment.

Time- and dose-dependent mitochondrial membrane potential ($\Delta\Psi$m) after Dp44mT treatment

To study the effect of Dp44mT treatment on overall mitochondrial health, the $\Delta\Psi$m was measured using Mitoview dye. A decrease in $\Delta\Psi$m was observed in both cell lines. A decrease in $\Delta\Psi$m after Dp44mT treatment indicates the activation of early events in the intrinsic pathway of apoptosis. These results indicate that Dp44mT impairs mitochondrial function, thereby weakening the capacity to maintain the proton gradient across the inner mitochondrial membrane, a process that ultimately leads to cell death (Figure 5D).

Dp44mT-induced imbalance in reactive oxygen species homeostasis

Dp44mT lies among thiosemicarbazones that are actively involved in redox cycling of their bound iron and the generation of ROS. Previous studies have proved that Dp44mT can induce ROS accumulation, leading to increased cytotoxicity [15,20]. To test this, intracellular H_2O_2 was measured by flow cytometry using the H2DCF-DA dye, which becomes fluorescent when oxidized by O_2^-, H_2O_2, or HO^-. Interestingly, time- and dose-dependent decreases in intracellular ROS levels were observed after Dp44mT treatment in the IMiD sensitive cell line (*MM.1S*). However, the IMiD resistant cell line (*RPMI-8226*) did not show any change in intracellular ROS levels after Dp44mT treatment. On the other hand, time- and dose-dependent increases in mitochondrial and cytoplasmic superoxide were observed. The combination of increased superoxide and decreased H_2O_2 suggests a shift in the balance of ROS metabolism that is cellular compartment-specific (Figure 6A-C).

Lipid peroxidation levels after DP44mT treatment in MM cells were measured to get deeper insights into the mechanisms underlying oxidative damage and the disease processes. There were increases in lipid peroxidation levels with

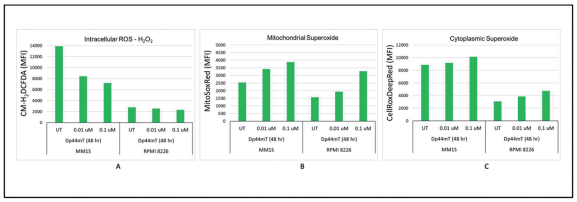

Figure 6 – Dp44mT-induced imbalanced reactive oxygen species (ROS) homeostasis: A: Time- and dose-dependent decreases in intracellular ROS levels after Dp44mT treatment of the IMiD sensitive cell line (*MM.1S*). However, the IMiD resistant cell line (*RPMI-8226*) did not show any change in intracellular ROS levels after Dp44mT treatment. B & C: Time- and dose-dependent increases in mitochondrial and cytoplasmic superoxide in both cell lines.

the IMiD sensitive cell line (*MM.1S*) at both time points and concentrations. However, the results for the IMiD resistant cell line (*RPMI-8226*) cells were inconsistent and did not show any specific pattern (Supplementary Figure 4A). This observed pattern suggests a complex oxidative stress response where increased superoxide levels are leading to lipid peroxidation in the IMiD sensitive cell line. Increased lipid peroxidation despite a reduction in *ACSL4* expression indicates alternate pathways for this effect as shown before [21]. Thus, Dp44mT might mediate MM cell death both through ferroptosis and via an independent effect mediated by *ACSL4* suppression.

Furthermore, labile iron compartmentalization was quantified by measuring the labile iron levels within the Golgi complex and the endoplasmic reticulum. Some dysregulation in labile iron levels was observed, but the results did not show any specific pattern (Supplementary Figure 4B-C).

Discussion

Iron chelators have obtained considerable attention as potential anti-cancer agents in few decades due to their ability to target iron-dependent processes that are essential for cancer cell growth. Cancer cells have a higher requirement for iron than healthy cells because they proliferate rapidly. In the present study, the potential of redox-active iron chelator Dp44mT in killing MM cell lines was confirmed. The anti-myeloma potential of Dp44mT was found to be independent of *CRBN* expression or IMiD sensitivity. The drug was found to be effective in nanomolar concentrations. Specific proteins/phosphosites that are altered in response to DP44mT treatment were identified by comparing the total proteome and phosphoproteome of DP44mT-treated cells with untreated control cells. The disruption of cellular iron metabolism, ROS homeostasis, and $\Delta\Psi m$ was also substantially characterized as part of the mechanism of action for the Dp44mT-induced anti-myeloma effect. This information may help to elucidate the molecular mechanisms underlying the effects of the compound and potentially identify key signaling pathways affected by DP44mT.

Phosphoproteomics data showed significant enrichment of the AMPK pathway, cell cycle, protein processing in the endoplasmic reticulum in both cell lines after D44mT treatment. All the above pathways are critical aspects of cellular physiology including ferroptosis [22,23]. These pathways are interconnected, and their dysregulation can have significant implications for cellular function and disease processes. For example, AMPK can influence the cell cycle by regulating key cell cycle proteins and checkpoint control and can also regulate ferroptosis [22,24]. Dysregulation of protein processing in the endoplasmic reticulum can also impact cellular proteostasis and contribute to cell death including ferroptosis [25,26]. Understanding the molecular mechanisms underlying these pathways and their interplay is crucial for developing targeted interventions and therapies to treat various diseases, including cancer, metabolic disorders, and protein misfolding diseases.

An imbalance of the ROS metabolism, decrease in $\Delta\Psi m$, and disruption in calcium efflux were also observed. These results demonstrate that Dp44mT disturbs intracellular redox balance thereby activating apoptosis. Malignant transformation of MM has already been linked with overproduction of intracellular ROS because of enhanced metabolism in tumor cells [27]. However, cells adapt to deal with oxidative stress by activating alternate mechanisms. Hence, further induction of oxidative stress can be an effective strategy to manage MM. A previous study proved that MM cell lines with decreased antioxidant defense are more vulnerable to IMiD-induced oxidative stress therefore highlighting the need of further exploring redox balance in MM cells [28]. Many studies have confirmed that Dp44mT, different from other iron chelators like deferoxamine, can generate ROS by a process called redox cycling: it therefore falls under the category of a redox-active compound [29]. Taken together Dp44mT, exhibits a dual role in killing MM cells by starving MM cells from iron and by the overproduction of ROS. An increase in lipid peroxidation was also observed despite a reduction in *ACSL4* expression, thus indicating alternate *ACSL4*-independent pathways for lipid peroxidation as shown previously. Thus, Dp44mT could mediate MM cell death both through ferroptosis and via an independent effect mediated by *ACSL4* suppression.

DP44mT has been the subject of numerous studies investigating its potential as an anti-cancer drug. A study by Lovejoy et al. in 2002 described the initial characterization of DP44mT and its analogs as novel iron chelators with potent antiproliferative activity against cancer cells. In the study, the researchers investigated the anti-cancer properties of DP44mT and its analogs as novel iron chelators. They aimed to explore the potential of these compounds to disrupt iron metabolism and selectively inhibit cancer cell growth [30]. Since then, there have been numerous research articles published related to the potential of Dp44mT as a potent anti-cancer drug. Another study by Whitnall et al. showed that DP44mT and its analogs overcome resistance to standard chemotherapeutic agents in cancer cells. The findings suggest that DP44mT and its derivatives have broad-spectrum antitumor activity and the potential to enhance the effectiveness of conventional chemotherapeutic drugs [31]. To study the underlying molecular mechanism behind Dp44mT, Dixon et al. investigated the molecular mechanisms underlying the anti-cancer effects of DP44mT in prostate cancer cells. The researchers demonstrated that DP44mT targets multiple signaling pathways, including AKT, TGF-β, and ERK, and engages the metastasis suppressor *NDRG1*, leading to inhibition of cell growth and metastasis [32].

These references provide a glimpse into the research on DP44mT as an anti-cancer drug. However, it is important to note that the scientific literature that has investigated the properties, mechanisms of action, and therapeutic potential of DP44mT in different cancer models and contexts has reported divergent findings. Therefore, it must be understood that Dp44mT can influence multiple pathways or biological processes which are highly dependent on the underlying biology of the study subject. However, the findings of this study provide novel insights, establishing a potential link between previously disparate findings via the dysregulation of iron metabolism, lipid peroxidation, and the inhibition of *ACSL4* expression. Iron must be tightly regulated for any cell to function properly and any disruption of iron machinery, whether it is upregulation or downregulation, can be detrimental. Therefore, targeting iron in MM is an excellent approach to develop anti-myeloma therapies.

Conclusion

In-vitro anti-MM effect of Dp44mT appears to be mediated by dysregulated iron metabolism, ROS, and other biological pathways. The present study highlights the antitumor role of Dp44mT and its likely therapeutic potential that could be tested in future clinical trial studies.

Conflicts of interest

All authors declare no conflict of Interest.

Acknowledgments

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Availability of data and materials

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [33] partner repository with the dataset identifier PXD059741

Reviewer access details

Username: reviewer_pxd059741@ebi.ac.uk
Password: WBSzxHspWtwY

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.htct.2025.106233.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Beyond the crisis: Tracking chronic neuropathic pain in sickle cell disease using *Douleur Neuropathique* 4 and PainDETECT questionnaires

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ABSTRACT

Background: Neuropathic pain represents a complex and often underdetected component of the pain spectrum in Sickle Cell Disease, particularly among individuals with chronic or treatment-resistant symptoms. Despite its clinical relevance, neuropathic pain is not routinely screened for in hematology practice, where pain is frequently attributed solely to vaso-occlusive mechanisms.

Method: A cross-sectional study was conducted with 214 individuals diagnosed with Sickle Cell Disease at a hematology referral center in northeastern Brazil. Two validated instruments, *Douleur Neuropathique* 4 and PainDETECT were utilized to screen for neuropathic pain. Clinical and demographic data were collected, and the correlation between the instruments was assessed using Pearson's coefficient.

Results: The *Douleur Neuropathique* 4 tool identified neuropathic pain in 29 % of participants. PainDETECT indicated 8.4 %, which increased to 22 % when including uncertain-range scores. The correlation between the two tools was strong ($r = 0.87$). Neuropathic pain was more prevalent among older individuals, those who reported recurrent painful episodes in the past year (p-value <0.001), and those with recent opioid use (p-value = 0.042). Sensory descriptors such as tingling, numbness, and electric shock sensations were commonly reported.

Conclusion: The combined use of *Douleur Neuropathique* 4 and PainDETECT, both of which are quick and simple to administer, proved to be a complementary strategy for identifying neuropathic pain, with each instrument capturing distinct features. Incorporating this approach into hematology care may facilitate the detection of pain profiles beyond vaso-occlusion and support more individualized treatment decisions.

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Introduction

Pain is one of the most common manifestations of Sickle Cell Disease (SCD) and often serves as the primary reason for hospitalizations within this population. The traditional explanation links these painful crises to vaso-occlusive episodes, primarily involving inflammatory and nociceptive mechanisms. However, recent studies underscore the complexity of this phenomenon, suggesting that some patients may experience pain with neuropathic characteristics [1,2].

Simply put, nociceptive pain is understood to arise from the activation of peripheral receptors by damaging stimuli, as seen in cases of tissue hypoxia. In contrast, neuropathic pain (NP) is associated with lesions or dysfunctions of the somatosensory nervous system, exhibiting symptoms such as paresthesia, burning pain, or the sensation of electric shock. These represent two distinct entities, both in terms of pathophysiology and therapeutic approach, although this distinction is still inadequately incorporated into the clinical practice of many professionals [3].

In many patients with SCD, pain is not limited to acute vaso-occlusive episodes. It may also include symptoms such as allodynia, persistent limb discomfort, tingling, burning, or pain triggered by light touch, complaints that are typically associated with a neuropathic component. These cases challenge the traditional perspective on sickle cell pain and suggest that neuroimmune mechanisms may also play a role, rather than pain being solely attributed to vascular occlusion. Evidence has demonstrated that the activation of glial cells, especially microglia and astrocytes, can lead to the release of pro-inflammatory cytokines such as Tumor necrosis factor-alpha, interleukin (IL)-1β, and IL-6. These mediators enhance neuronal excitability and promote abnormal pain signaling, contributing to central sensitization and the persistence of pain even in the absence of acute tissue injury [3,4].

Although neuropathic mechanisms have been described in SCD, this type of pain often remains unrecognized in clinical practice. It is estimated that between 25 % and 40 % of individuals with SCD suffer from NP, many of them without proper identification or treatment [4].

Tools like the *Douleur Neuropathique* 4 (DN4) and PainDETECT questionnaires are commonly used to assess NP across various populations. Both are simple and quick to administer, but they differ in structure and focus. The DN4 combines symptom descriptors with physical examination findings, while the PainDETECT is entirely self-administered and emphasizes sensory descriptors and pain patterns [5,6]. Few studies have compared these instruments in patients with SCD, making it challenging to standardize diagnoses and identify more effective therapeutic approaches.

Although pain is a primary concern for patients with SCD in clinical contexts, the potential for neuropathic mechanisms is often overlooked in many settings, particularly outside pain specialties. This oversight is not necessarily due to a lack of knowledge but may reflect the prioritization of other clinical manifestations that are also prevalent in the hematology practice. Increased awareness of the different pain mechanisms in this patient group could directly impact therapeutic management and improve quality of life. In light of this, this study aimed to evaluate the prevalence of NP in SCD patients by utilizing the DN4 and PainDETECT questionnaires. Additionally, the study sought to identify clinical characteristics related to the presence of NP and to compare the diagnostic findings provided by each instrument.

Material and methods

Study design and setting

This is a cross-sectional study conducted with patients diagnosed with SCD who are regularly followed at the Hematology and Hemotherapy Center of Maranhão (HEMOMAR), a state reference institution for hemoglobinopathies located in São Luís, Maranhão, Brazil. Data were collected between December 2022 and December 2024.

Sample and inclusion and exclusion criteria

The sample was composed for convenience of patients who attended routine appointments during the study period. Individuals aged 14 years and older, of both genders, with a confirmed diagnosis of SCD by hemoglobin electrophoresis (via high-performance liquid chromatography) were included. Participants needed to be in adequate clinical, cognitive, and emotional condition to answer the interview and must have formally consented to participate. For participants under 18, an assent form was obtained in addition to informed consent from their legal guardians. Individuals experiencing a pain crisis at the time of the interview, pregnant women, patients with severe psychiatric disorders, or those with hearing or speech deficits were excluded.

Data collection procedures

After the signing of the consent and assent forms, participants completed a structured questionnaire that collected sociodemographic data, including age, gender, skin color, education, city of origin, occupation, and family income, as well as clinical data such as age at diagnosis, hemoglobin genotype, number of hospitalizations, transfusions in the past 12 months, occurrence of pain crises in the past 12 months, and use of opioids. Routine laboratory data were obtained from medical records, which included hemoglobin levels, leukocyte count, platelet count, and hemoglobin electrophoresis.

Instruments used

In this study, two validated instruments were used to screen for NP: the DN4 and the PainDETECT Questionnaires. The DN4 consists of ten items: seven related to self-reported sensory symptoms and three derived from physical examination. These items assess sensations such as burning, painful cold, electric shocks, tingling, pins-and-needles, numbness, and itching, as well as the presence of mild touch hypoesthesia, pinprick hypoesthesia, and allodynia. Each item is scored using a binary system (0 or 1), resulting in a total score that

ranges from 0 to 10. A score of 4 or higher is considered indicative of probable NP. The Portuguese version of the DN4 has been validated, and its brevity and diagnostic accuracy make it suitable for use in outpatient hematology settings [5,7,8].

The PainDETECT Questionnaire is a self-administered tool originally developed for patients with chronic low back pain but has also been validated for other NP conditions. It consists of four main sections. The first section contains three items that assess current, average, and maximum pain intensity over the past four weeks, using a numerical scale from 0 to 10. The second section presents four temporal patterns of pain, while the third examines the presence and direction of pain irradiation illustrated on a body diagram. The fourth section includes seven sensory descriptors (burning, tingling, allodynia, pain attacks, temperature-evoked pain, numbness, and pressure pain), evaluated on a Likert scale from 0 (none) to 5 (very strong). The final score ranges from 0 to 38: scores of 19 or higher indicate probable NP; scores of 12 or lower suggest that this mechanism is unlikely; and scores between 13 and 18 represent a zone of diagnostic uncertainty. The questionnaire also includes a body diagram, where patients indicate the location and radiation of pain, and a panel of line graphs representing different temporal pain patterns (e.g., persistent, fluctuating, intermittent) to help the patient select the pattern that best characterizes their experience. Although these visual components are not included in the scoring algorithm, they provide useful clinical information. The Portuguese version of the PainDETECT Questionnaire was adapted and validated, demonstrating good diagnostic accuracy. Similar to the DN4, its ease of use and applicability in outpatient non-acute contexts make it a valuable instrument for routine pain screening in patients with SCD [6,9].

All assessments were conducted in an outpatient setting, and only patients in stable clinical conditions were included. Individuals experiencing vaso-occlusive crises at the time of evaluation were excluded, as acute pain episodes can distort symptom reporting and introduce bias in responses to both the DN4 and PainDETECT questionnaires. This approach ensured that participants' answers reflected their typical pain experience rather than transient exacerbations, and minimized the risk of cognitive or emotional interference associated with acute stress.

Statistical analysis

Categorical variables were analyzed using Fisher's exact test based on their suitability. For continuous variables, the Mann-Whitney test was applied. Binary logistic regression was performed to estimate adjusted odds ratios (OR) with 95 % confidence intervals (95 % CI). The significance level was set at 5 % (p-value <0.05). The analyses were carried out using appropriate statistical software.

Ethical aspects

The Research Ethics Committee at the University Hospital of the Federal University of Maranhão (HUUFMA) approved the study under opinion CAAE 51,633,821.1.0000.5086, in accordance with the ethical guidelines outlined in Resolution 466/2012 by the National Health Council.

Results

This study evaluated 214 patients with SCD who were regularly monitored at the Hematology and Hemotherapy Center of Maranhão and completed questionnaires to identify NP.

Among the participants, there was a predominance of females: 125 (58 %) compared to 89 males (42 %). The average age was 26.2 years, with 28 % aged between 14 and 18 years old. Regarding self-identified race, 24 % identified themselves as Black, while 76 % identified themselves as non-Black. Approximately 14 % had an income of less than one minimum wage; 78 % earned between one and two salaries; and 8 % earned between three and five. About 49 % reported receiving disability benefits. In terms of education, 24 % had low education levels (illiterate to incomplete primary education), 61 % had intermediate education (completed primary to secondary education), and 15 % had completed or incomplete higher education. Although the study took place in the capital, approximately 69 % of the participants lived in the interior of the state of Maranhão.

The NP questionnaires revealed the following findings: DN4 was positive for 62 patients (29 %), with an average score of 5.64. PainDETECT was positive for 18 patients (8.4 %), with an average score of 21.8 points. When considering individuals with intermediate PainDETECT scores (between 13 and 18 points, the diagnostic uncertainty zone), a total of 48 patients (22 %) exhibited possible NP, with an average score of 18 points.

For analytical purposes, participants were classified according to the DN4 questionnaire (score ≥4), which was used as the primary reference instrument for the group comparisons presented in Tables 1 and 2. The PainDETECT questionnaire was applied as a complementary and comparative tool to analyze its correlation and agreement with the primary reference instrument.

Regarding genotype, 76 % were hemoglobin (Hb) SS, 14 % were Hb SC, and 10 % were Hb Sβ^0. Neonatal diagnosis through the heel prick test had identified 40 % of cases; 15 % were diagnosed by age five, 23 % between the ages of 6 and 15, and 22 % only after age 15.

Approximately 77 % of patients reported experiencing two or more episodes of pain in the past 12 months. This proportion was higher among those with NP (68 % compared to 30 %), indicating statistical significance (p-value <0.001). Opioid use was more common among individuals with NP (55 %; p-value = 0.042). The frequency of hospitalizations and transfusions in the last year was also elevated in this group, although it did not reach statistical significance.

Laboratory data, including hemoglobin concentration, total leukocyte and platelet counts, and percentage of Hb S, showed no differences between the groups, as shown in Table 3.

A total of 128 patients (60 %) reported using hydroxyurea. This variable showed no statistically significant difference when comparing the groups with and without NP (p-value = 0.4).

Table 1 – Demographic data of the patients included in the study.

Variable	n	% of total (%)	No neuropathic pain* n = 152 (%)	With neuropathic pain* n = 62 (%)	OR	95 % CI	p-value
Age group							
Between 14 and 18 years old	60	28	50 (33)	10 (16)	—	—	—
Between 19 and 34 years old	99	46	69 (45)	30 (48)	1.61	0.66–4.11	0.3
Over 34 years old	55	26	33 (22)	22 (35)	2.91	1.15–7.74	0.027
Gender							
Female	125	58	87 (57)	38 (61)	—	—	—
Male	89	42	65 (43)	24 (39)	1.12	0.57–2.19	0.7
Race/skin color							
Non-black	162	76	121 (80)	41 (66)	—	—	
Black	52	24	31 (20)	21 (34)	1.85	0.92–3.71	0.083
Education							
Low	52	24	38 (25)	14 (23)	—	—	—
Average	131	61	93 (61)	38 (61)	1.36	0.61–3.18	0.5
High	31	15	21 (14)	10 (16)	2.27	0.74–7.08	0.2
Residence							
São Luís (Capital)	67	31	42 (28)	25 (40)	—	—	—
Other	147	69	110 (72)	37 (60)	0.63	0.32–1.23	0.2
Occupation							
Household/unemployed	62	29	44 (29)	18 (30)	—	—	—
Paid work	38	18	24 (16)	14 (22)	1.06	0.39–2.86	>0.9
Disability benefit	105	49	76 (50)	29 (47)	0.68	0.31–1.48	0.3
Retired	9	4	8 (5)	1 (1)	0.15	0.01–1.07	0.10
Family income							
<1 minimum wage	29	14	17 (11)	12 (20)	-	-	
1–2 minimum wages	166	78	120 (79)	46 (74)	0.56	0.23–1.39	0.2
3–5 minimum wages	19	8	15 (9.9)	4 (6)	0.23	0.05–0.99	0.058

* according to DN4; OR: odds ratio adjusted by binomial logistic regression; 95 % CI: 95 % confidence interval; p-value <0.05 = statistically significant.

Among patients with NP indicated by DN4, the most common symptoms were tingling (90 %), a pinprick sensation (87 %), numbness (80 %), painful cold (74 %), electric shocks (70 %), and burning (56 %). Other symptoms reported included itching (39 %), pain caused by brushing (32 %), hypoesthesia to pricking (19 %), and hypoesthesia to touch (14 %) (Figure 1).

Table 2 – Clinical data on sickle cell disease of the patients included in the study.

Variable	n	% of total	No neuropathic pain* n = 152 (%)	With neuropathic pain* n = 62 (%)	OR	95 % CI	p-value
Age at diagnosis							
<1 year old	85	40	65 (43)	20 (32)	—	—	—
1 - 5 years old	33	15	26 (17)	7 (11)	0.72	0.24–1.96	0.5
6 - 15 years old	49	23	35 (23)	14 (23)	0.98	0.40–2.35	>0.9
>15 years old	47	22	26 (17)	21 (34)	1.79	0.70–4.58	0.2
Subtype							
Hb SC	30	14	17 (11)	13 (21)	1.00	1.00–1.00	>0.9
Hb Sβ^0	22	10	15 (10)	7 (11)	0.55	0.15–1.89	0.3
Hb SS	162	76	120 (79)	42 (68)	0.51	0.22–1.19	0.11
Pain crises in 12 months							
None	50	23	45 (30)	5 (8)	—	—	—
<3 episodes	76	35	61 (40)	15 (24)	2.27	0.79–7.61	0.15
≥3 episodes	88	42	46 (30)	42 (68)	8.12	3.07–25.9	<0.001
Opioid use in 12 months							
Yes	99	46	65 (43)	34 (55)	—	—	—
No	115	54	85 (56)	30 (48)	0.51	0.27–0.97	0.042
Hospitalizations in 12 months							
<3	189	88	137 (90)	52 (84)	—	—	—
≥3	25	12	15 (10)	10 (16)	1.65	0.66–4.03	0.3
Transfusions in 12 months							
<10 units	56	26	36 (24)	20 (32)	—	—	—
≥10 units	13	6	6 (4)	7 (11)	2.47	0.67–9.49	0.2
None	145	68	110 (72)	35 (56)	0.55	0.27–1.14	0.10

* according to DN4; OR: odds ratio adjusted by binomial logistic regression; 95 % CI: 95 % confidence interval; p-value <0.05 = statistically significant.

Table 3 – Laboratory data on the patients included in the study.

Variable	Value	No neuropathic pain* n = 152	With neuropathic pain* n = 62	p-value
Hemoglobin (g/dL)	9.1	9.00 (7.80–10.25)	9.20 (8.00–10.20)	0.5
Leukocytes (/mm^3)	8072.50	7980 (5780–10,980)	8165 (5800–10,750)	0.2
Platelets (x 10^3/mm^3)	327	344 (257–462)	310 (212–419)	0.3
Hemoglobin S (%)	78	79 (70–86)	77 (53–86)	0.2

* Values are presented as Median (Q1; Q3).

The PainDETECT questionnaire also enabled the capture of various aspects of the pain reported by patients. Among those diagnosed with DN, this instrument indicated that the average pain at the time of the interview was 3.2 (on a scale of 0 to 10). When asked about the pain experienced over the last four weeks, the average score was 6.4, with the most intense peak reported being on average 8.9.

Another aspect of PainDETECT involved depicting the pain pattern using a graphic image that best represents it. It was observed that 40 % of those with ne NP described frequent pain crises occurring at intervals, while 33 % reported pain crises without complaints during those times. Additionally, 27 % noted constant pain accompanied by crises, while none described constant pain with slight variations. Furthermore, a significant 94 % of patients with NP characterized their pain as radiating. Among the most notable sensations were moderate numbness in 61 %, moderate tingling in 55 %, and temperature (cold or heat) causing occasional pain in 50 %. Shock waves were also reported by 44 % of patients, adding to the variety of pain experiences (Figure 2).

The scores obtained from the DN4 and PainDETECT instruments were compared using Pearson's coefficient ($r = 0.870655$). This indicates a strong positive correlation, confirming the high agreement in identifying and quantifying NP in this population. This finding provides reassurance regarding the reliability of these instruments, despite the differences in prevalence observed when each tool is applied independently (Figure 3).

Discussion

NP remains underrecognized in SCD, either due to limited clinician awareness or the considerable variability in symptom presentation. This lack of recognition often creates the perception that these patients are particularly challenging to manage [1,10]. This investigation aimed to explore this dimension by applying two validated screening tools, DN4 and PainDETECT, to assess how each performs in identifying neuropathic features and where their findings converge or diverge.

Interest in diagnosing NP in individuals with SCD has increased in recent years, with studies indicating its presence in up to 40 % of patients, a rate that exceeds that observed in other neuropathic conditions, such as painful diabetic neuropathy [11,12]. In the current study, the prevalence of NP was 29 % according to the DN4 assessment and 8.4 % with PainDETECT; including patients with scores in the uncertain range raised this figure to 22 %. These differences reflect findings from previous studies and illustrate how diagnostic

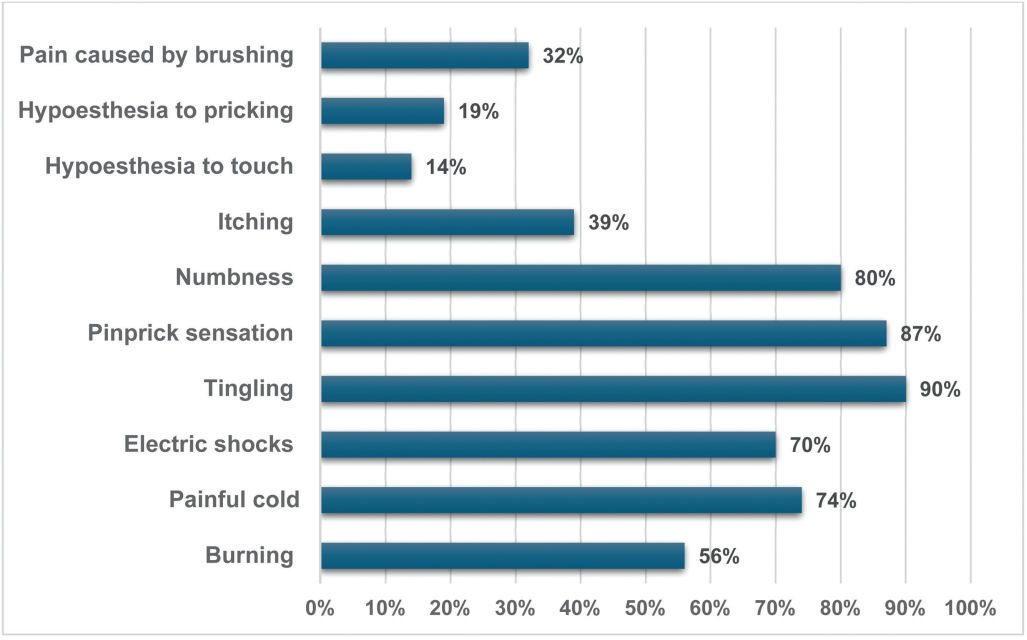

Fig. 1 – The most common complaints identified using the DN4 questionnaire in patients with neuropathic pain related to sickle cell disease (n = 62).

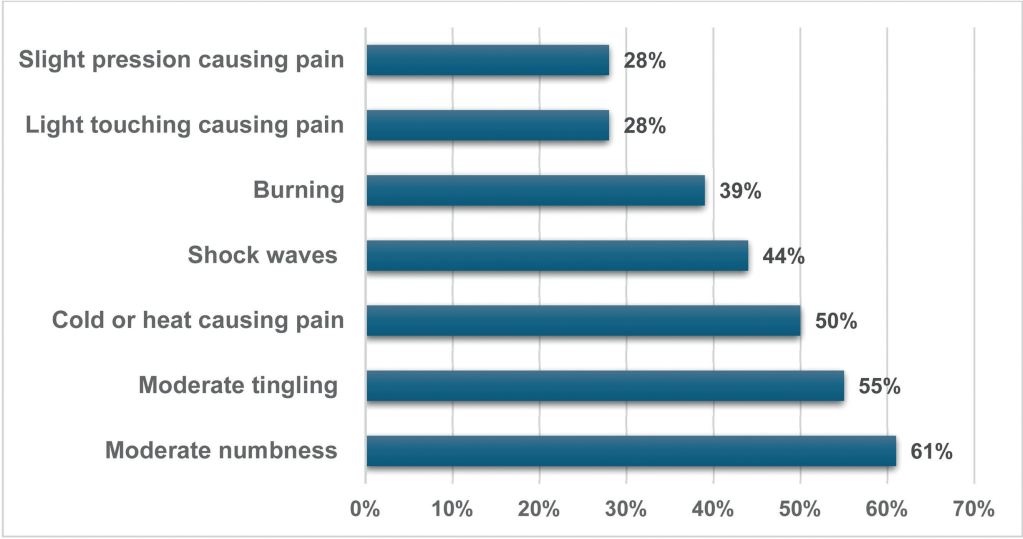

Fig. 2 – The most common complaints identified using the PainDETECT questionnaire in patients with neuropathic pain related to sickle cell disease (n = 18).

criteria and instrument characteristics can influence detection rates [1]. Notably, the prevalence of NP observed in the present sample regardless of the instrument used was comparable to or higher than general population estimates that typically range from 6–10 % [13]. This finding reinforces the need to improve both recognition and management of NP in individuals with SCD.

A study conducted in Bahia, Brazil, using the DN4 questionnaire, found that NP was present in 69 of 100 individuals with SCD. However, many of those patients also reported experiencing depression and anxiety, which may have influenced their reporting of symptoms [14].

In another study, patients with SCD from Brazil and France were assessed using PainDETECT via telephone. In Brazil, 55 % showed a likely neuropathic component, 23 % were negative, and 22 % were uncertain. Similarly, in France, results indicated 51 % positive, 29 % negative, and 20 % unclear, with all participants reporting constant pain. Opioid use was reported by 62 % of Brazilian patients and 32 % of French patients. These findings highlight the challenge of identifying pain subtypes and the implications of inadequate treatment [10].

Other instruments for identifying NP can also be used in this context. For example, the Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale (LANSS tool) identified NP in 25 % of 56 SCD patients [15], emphasizing the diversity of pain mechanisms in SCD and the need for more precise diagnostic approaches [16].

The present study identified a strong correlation between DN4 and PainDETECT (r = 0.87), suggesting that both instruments capture overlapping features of NP despite differing prevalence estimates. The DN4 emphasizes classical neuropathic descriptors, whereas PainDETECT also incorporates pain intensity and temporal patterns, potentially reflecting

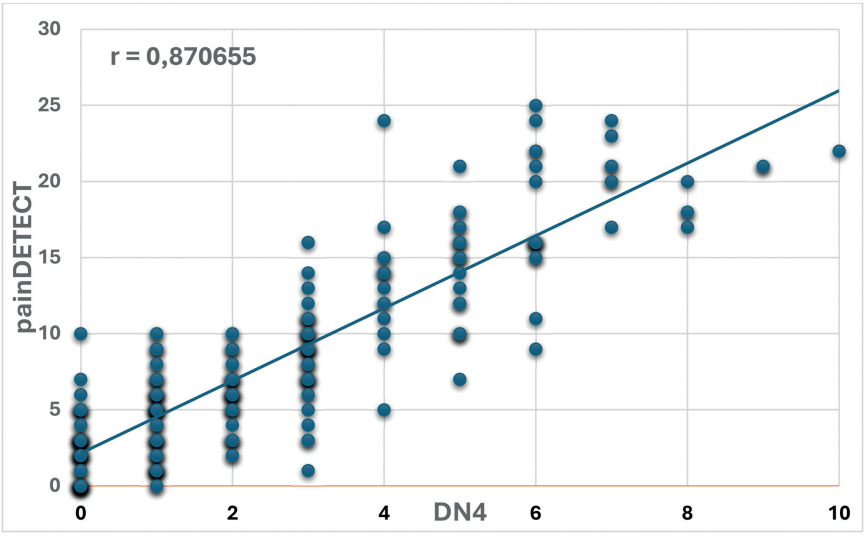

Fig. 3 – Correlation between DN4 and PainDETECT questionnaires for detecting neuropathic pain in sickle cell disease.

central sensitization. Although previous studies have utilized different instruments in individuals with SCD, few have examined their concurrent use through direct statistical comparison [17,18]. These findings contribute novel evidence by highlighting the complementary strengths of DN4 and PainDETECT in capturing NP characteristics in a stable outpatient population with SCD.

The DN4 assessment also includes a brief physical examination, which may enhance its diagnostic utility in clinical settings. PainDETECT features a gray area (13–18 points) that does not confirm a diagnosis but can still raise clinical suspicion. The absence of a disease-specific questionnaire for NP in SCD present an opportunity to utilize general screening tools. However, instruments like DN4 and PainDETECT serve as more than just general screening tools; they are easy to administer, require no more than five minutes, and can provide valuable insights to guide clinical management, including in hematology outpatient settings. Their integration by hematologists into chronic pain evaluations may significantly enhance the care of patients with complex or refractory pain presentations. Their potential as practical diagnostic tools offers hope for the future management of NP in SCD.

In this study, older patients were more likely to experience NP. This aligns with the broader literature on pain, which indicates that aging is associated with an increased risk of NP. Neuroplasticity may alter pain perception over time, and repeated vaso-occlusive episodes can lead to cumulative nerve damage, chronic nociceptor activation, and increased sensitivity [2,18].

While some previous studies suggest a higher prevalence of NP in women, the findings of this study did not indicate a sex difference, which aligns with other reports [15,18]. This highlights the need for ongoing research to identify clinical predictors of NP in SCD.

Among patients identified with NP in this sample, 94 % reported radiating pain. Symptoms such as tingling (90 %), pins-and-needles (87 %), and numbness (80 %) were common, as revealed by the DN4 tool. These findings support the hypothesis that NP in SCD may result from chronic ischemia or nerve injury, as previously reported in the literature [19]. Similar symptoms were also the most frequently observed in a study of 54 younger patients [20]. These data may enhance clinical interviews when specific tools are unavailable.

Another significant finding was the strong association between NP and frequent pain crises (p-value <0.001). Patients with NP experienced more episodes in the past year, suggesting a cycle in which repeated pain could lead to central sensitization, chronicity, and poorer function [21].

This study also found that 55 % of patients with NP had used opioids in the previous year, a significantly higher rate compared to those without NP (p-value = 0.0042). While opioids are often used to manage acute vaso-occlusive pain, they are less effective for NP and may even contribute to hyperalgesia. In these cases, alternative medications such as gabapentin (Neurontin)oids, tricyclic antidepressants, and serotonin-norepinephrine reuptake inhibitors might be more appropriate [22,23]. However, none of the patients identified with NP in this study were receiving any specific adjuvant treatment, including gabapentin (Neurontin)oids, at the time of data collection. This indicates that NP had not been previously diagnosed in these individuals and was only identified through the application of DN4 and PainDETECT during the study. This finding underscores the gap in clinical recognition and the potential of systematic screening tools to enhance more appropriate and individualized pain management strategies in patients with SCD [22,23].

This study has limitations. As a cross-sectional analysis, it cannot establish causality between NP and other variables. Additionally, because the tools used vary in sensitivity, some variation in diagnostic rates is expected. Nonetheless, the strong correlation between DN4 and PainDETECT supports the reliability of our findings. Further multicenter studies and the development of specific screening tools for SCD may enhance the detection and management of NP across diverse care settings.

Conclusion

This study shows that NP is an important yet often overlooked aspect of the pain experience in individuals with SCD. Using NP and PainDETECT allowed us to identify various pain patterns and demonstrated agreement between the instruments. These simple and efficient tools could be valuable additions to the routine evaluation of patients, particularly those whose pain does not respond as expected to standard treatments. Incorporating this type of screening into hematology practice may help refine therapeutic choices and improve clinical outcomes. This highlights the need to adopt diagnostic approaches that better reflect the complexity of pain in SCD.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the author(s) used ChatGPT 4.0 and Grammarly to enhance language and readability. After utilizing these tools, the author(s) reviewed and edited the content as needed and take full responsibility for the publication's content.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

None.

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Original article

Plasma levels of soluble podoplanin are higher in acute promyelocytic leukemia compared to other forms of acute myeloid leukemia

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ABSTRACT

Background: Acute promyelocytic leukemia (APL) is a subtype of acute myeloid leukemia (AML) marked by a high incidence of coagulopathy. Podoplanin, a glycoprotein involved in platelet activation through interaction with CLEC-2, has recently been identified on leukemic promyelocytes and suggested as a potential contributor to APL coagulopathy. Identification of novel biomarkers and therapeutic targets for APL coagulopathy can potentially improve the outcomes of this condition

Aim: To explore whether levels of soluble podoplanin in plasma are different in APL, and to evaluate its association with laboratory and clinical outcomes in these patients

Methods: Samples were obtained from consecutive patients with APL at the time of diagnosis in an academic hospital. Biobank samples from 35 patients with non-APL AML matched for age and sex were used as comparators. Circulating podoplanin levels were measured in plasma using a commercial ELISA kit. The study was approved by the institutional ethics committee and all participants provided written informed consent

Results: APL patients showed significantly higher plasma soluble podoplanin concentrations compared to non-APL AML. Using the median soluble podoplanin value as a cutoff, a higher proportion of APL patients presented elevated levels. Soluble podoplanin levels correlated with CD40L in APL cases, but not in non-APL AML patients, suggesting a possible interaction with thrombo-inflammatory activation pathways

Conclusion: These findings represent a proof-of-concept that measuring soluble podoplanin in plasma samples can contribute to the diagnosis of APL, while also providing novel data on the association of podoplanin with the pathogenesis of APL coagulopathy.

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Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) characterized by the accumulation of promyelocytes in the bone marrow and a high risk of severe hemorrhagic complications. Despite significant therapeutic advancements with all-trans retinoic acid (ATRA) and arsenic trioxide (Arsenic Trioxide) [1–3], early mortality remains a major concern, potentially reaching 5–20 %, depending on clinical and geographical factors. The primary cause of early death is coagulopathy, which often leads to fatal intracranial hemorrhages [4]. Therefore, early diagnosis and prompt initiation of treatment are crucial for reducing mortality and improving patient outcomes. In addition, the identification of novel biomarkers and therapeutic targets of APL coagulopathy could bring benefits to these patients.

Podoplanin is a small transmembrane glycoprotein expressed in various cell types, including lymphatic endothelial cells, kidney podocytes, and certain tumor cells [5]. Functionally, podoplanin interacts with the C-type lectin-like receptor 2 (CLEC-2) on platelets, triggering their activation and contributing to thrombotic processes [6]. This mechanism is particularly relevant in cancer-associated thrombosis and inflammatory diseases, where podoplanin overexpression has been linked to prothrombotic states [5–7].

In the context of APL, podoplanin expression was first identified in leukemic promyelocytes in 2018, suggesting its potential role as a distinguishing marker for this leukemia subtype [8]. More recently, its expression was validated in a prospective cohort using flow cytometry, reinforcing its diagnostic and pathophysiological relevance in APL [9]. However, the extent to which circulating podoplanin correlates with disease characteristics and coagulation abnormalities remains unexplored.

Soluble forms of membrane proteins can serve as biomarkers, reflecting their biological activity and disease status. For instance, soluble fms-like tyrosine kinase-1 (sFLT1) is well established as a biomarker in preeclampsia and soluble urokinase-type plasminogen activator receptor (suPAR) as a biomarker in different types of cancers [10–13]. Similarly, soluble podoplanin (sPDPN) has been identified as a biomarker in malignancies and inflammatory diseases such as cancer and COVID-19, highlighting its potential and prognostic significance [14–18].

Based on this evidence, this study investigated whether quantification of sPDPN in plasma could be associated with the diagnosis of APL, as well as its relationship with biomarkers involved in APL coagulopathy.

Methods

Study population

This study analyzed patients diagnosed with acute leukemia between 2014 and 2022 at an academic tertiary hospital. Inclusion criteria were: age ≥18 years and a confirmed diagnosis of APL or AML. Exclusion criteria included: presence of severe infection with hemodynamic instability requiring vasoactive agents at the time of sample collection, and a diagnosis of other forms of acute leukemia. Patients with non-APL AML were used as a comparator group. These patients were selected from the population of consecutive new AML cases during the same study period, matched for age, sex and year of enrollment, before any of the laboratory analyses. The diagnostic workup comprised morphological evaluation, immunophenotyping, cytogenetic analysis, and molecular testing. APL cases were confirmed through molecular detection of the *PML-RARA* fusion gene. Patients with a strong clinical and morphological suspicion of APL, based on peripheral blood smear findings, were promptly initiated on ATRA therapy in accordance with the institutional protocol. This study was approved by the institutional ethics committee (CAAE: 39948520.8.1001.5404) and performed in accordance with the Declaration of Helsinki.

Sample collection and processing

Whole blood samples were collected in EDTA tubes. Plasma was obtained by centrifugation at 2500 g for 15 min at 22 °C within two hours of collection. All samples were aliquoted and stored at −80 °C until analysis.

Clinical and laboratory data

Clinical and laboratory data were retrospectively retrieved from hospital electronic medical records. Laboratory variables included initial bone marrow blast percentage, hemoglobin concentration, white blood cell (WBC) count, platelet count, prothrombin time (TP), activated partial thromboplastin time (aPTT), and fibrinogen concentration, based on the first set of results available upon hospital admission. Clinical outcomes comprised early mortality (defined as death within 30 days of diagnosis) [19] and central nervous system (CNS) and retinal bleeding.

Measurement of soluble podoplanin levels

Plasma levels of podoplanin were measured in duplicate in EDTA plasma using a commercial enzyme-linked immunosorbent assay kit (ELISA) (Sigma Aldrich, Cat. RAB 1632–1KT - Lot: 0325/2117), according to the manufacturer's instructions.

Measurement of P-selectin and CD40L

Plasma concentrations of P-selectin and CD40L were determined using a custom-designed multiplex assay panel (Invitrogen – Thermo Fisher Scientific – Cat. PPX-07-MX323GU – Lot: 283,814–000), following the manufacturer's protocol. All measurements were performed in duplicate, and analyte concentrations were calculated based on standard curves generated using known concentrations of recombinant proteins.

Statistical analysis

Quantitative variables were described using both median with interquartile range and mean with standard deviation (SD). Patients with APL were matched 1:1 to non-APL AML patients, as detailed above. Matching was done prior to any

laboratory analysis. Comparative analyses between groups were performed using parametric and nonparametric statistical methods. The Student's *t*-test was applied for normally distributed data, while the Mann-Whitney *U* test was used for data not meeting parametric assumptions. For the analysis of the association between podoplanin expression with clinical outcomes, APL patients were arbitrarily categorized as podoplanin positive or negative, based on the 50th percentile cut-off. Correlations were evaluated by Spearman's test. A p-value <0.05 was considered statistically significant. Statistical analyses were conducted using IBM SPSS Statistics version 26 and GraphPad Prism version 8.0.

Results

In total, 35 patients with APL were included in this study, as well as 35 patients with non-APL AML, matched by age, sex and year of diagnosis, used as a comparator group. The clinical and laboratory characteristics of these groups are presented in Table 1. As expected, APL patients presented more frequently with alterations in hemostasis biomarkers, and a lower rate of early mortality. Plasma concentrations of sPDPN ranged from 0.3–113.6 ng/mL. Patients with APL exhibited significantly higher levels of sPDPN (median: 16.4 ng/mL) compared to patients with other AML subtypes (median: 2.2 ng/mL; p-value = 0.003) (Figure 1). To investigate the potential relationship between sPDPN and platelet activation, plasma levels of P-selectin and CD40L, two established markers of platelet degranulation, were measured. Both P-selectin (7194 pg/mL versus 11,220 pg/mL; p-value = 0.003) and CD40L levels (100.4 pg/mL versus 196.6 pg/mL; p-value = 0.002) were significantly lower in APL patients compared to those with other AML subtypes (Figure 2A,B). Interestingly, a significant positive correlation was observed between podoplanin and CD40L levels in APL patients (R_s = 0.60; p-value = 0.0004), but not for other AML subtypes (Figure 2C & D). Podoplanin levels were not associated with predefined clinical outcomes (Table 2).

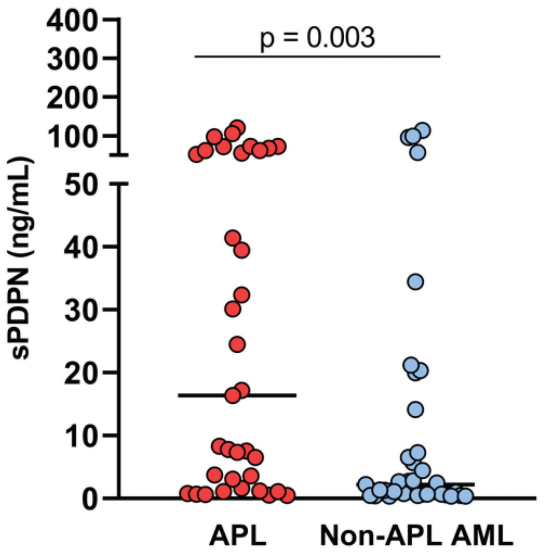

Figure 1 – Plasma levels of podoplanin of acute promyelocytic leukemia (APL) and non-APL acute myeloid leukemia (AML) patients (p-value: Mann-Whitney test).

Discussion

APL coagulopathy still represents a major challenge to the early management of APL, and the availability of biomarkers capable of differentiating APL from other forms of AML, and of providing information about APL coagulopathy could potentially facilitate the management of these patients [20].

Table 1 – Laboratory and clinical characteristics of study participants.

	APL (n = 35)	Non-APL AML (n = 35)	p-value‡
Age*	37.3 (21–61)	41.4 (18–59)	0.31
Male:Female	17:18	17:18	0.99
Risk group - (low/int/high)	3/18/14	11/11/13	0.04
Peripheral blast count, 10^9/L*	2.23 (0.01–129.6)	7.75 (0.1–130.8)	0.25
Bone marrow blast count - %*	88.0 (46.0–98.2)	80.5 (26.5–95.6)	0.002
Leukocytes - $10^3/\mu$L*	3.1 (0.32–137.9)	14.4 (0.91–150.3)	0.02
Hemoglobin - g/dL**	8.4 ± 2.39	8.1 ± 2.10	0.64
Platelets - 10^9/L*	22.0 (4.0–89.0)	27.0 (5.0–230.0)	0.02
PT (INR)**	1.29 ± 0.19	1.19 ± 0.13	0.01
aPTT*	0.90 (0.75–1.24)	0.89 (0.67–1.38)	0.48
Fibrinogen - mg/dL*	121.7 (26.0–378.5)	384.5 (58.9–705.8)	<0.0001
Clinical outcomes			
CNS/retinal bleeding - n (%)	4 (11.4)	2 (5.7)	0.39
30-day survival - (no/yes)	5/30	12/23	0.05

* Median (interquartile range).
** Mean ± SD.
‡ Mann-Whitney test, *t*-test or chi-square test.
APL: acute promyelocytic leukemia; AML: acute myeloid leukemia; CNS: central nervous system; PT: prothrombin time; aPTT: activated partial thromboplastin time; INR: International Normalized Ratio

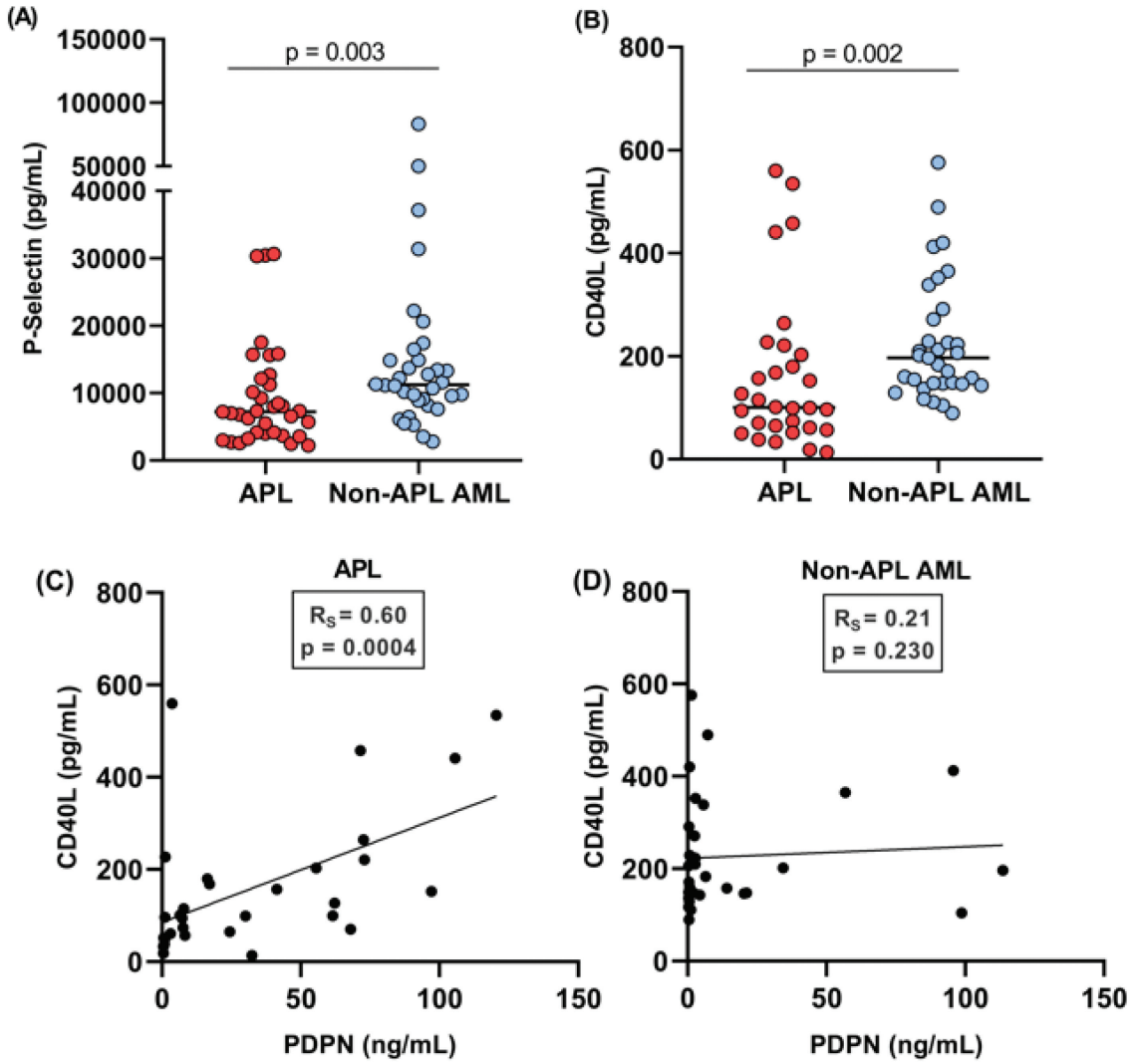

Figure 2 – (A) Plasma levels of P-selectin were significantly lower in acute promyelocytic leukemia (APL) patients compared to non-APL acute myeloid leukemia (AML) patients (Mann-Whitney test; p-value = 0.003). (B) Plasma levels of CD40L were significantly lower in APL patients compared to non-APL AML patients (Mann-Whitney test; p-value = 0.002). (C) A significant positive correlation between podoplanin and CD40L was observed in APL patients (Spearman's correlation; p-value = 0.0004). (D) No significant correlation between podoplanin and CD40L was found in non-APL AML patients.

Table 2 – Association of podoplanin expression with clinical outcomes in acute promyelocytic leukemia.

Clinical outcome	Podoplanin expression category*		p-value**
	Podoplanin +	Podoplanin -	
Early mortality (30-day)			0.46
Yes	4	1	
No	19	11	
CNS/ retinal bleeding			0.67
Yes	3	1	
No	20	11	

CNS: Central nervous system.

* For the purpose of association analyses, acute promyelocytic leukemia patients were arbitrarily categorized as podoplanin positive or negative, based on the 50th percentile cutoff.

** Statistical comparison was performed using the chi-square test.

The main contribution of the present study was the demonstration of sPDPN as a potential biomarker. Its expression is markedly higher in APL compared to other forms of AML, and it correlates with an important thrombo-inflammatory mediator. This suggests that podoplanin expression may be a unique biological feature contributing to the well-known predisposition of these patients to hemorrhagic and thrombotic complications.

The first demonstration that podoplanin expression is a hallmark of APL was published in 2018 and included data obtained by flow cytometry and RNA sequencing [8]. That study also presented evidence linking podoplanin expression to platelet activation in vitro. While the diagnosis of APL using flow cytometry and molecular techniques is well established, reliance on methods that are not usually available outside specialized cancer centers may delay diagnosis and jeopardize

patient outcomes [20]. Immune-based methods such as ELISA are widely available and have the advantage of being potentially performed with diagnostic kits in almost every setting. Based on the demonstration that circulating levels of podoplanin is capable of identifying discrete subgroups of patients with other conditions [14–18], and that soluble levels of membrane proteins can serve as useful disease biomarkers [10–13], we hypothesized that sPDPN levels might contribute to the differential diagnosis of APL from other forms of AML. In fact, this study demonstrates a significant difference in sPDPN levels between these two patient subgroups, although a subgroup of patients with non-APL AML also presents higher sPDPN values.

In addition, a positive correlation between sPDPN and CD40L levels was found in APL patients, suggesting a biological interaction between these two markers. CD40L is a transmembrane protein expressed on activated platelets that has been implicated in platelet-leukocyte crosstalk and inflammatory signaling [21]. CD40L is also expressed by other hematopoietic cells such as lymphocytes, neutrophils, dendritic cells, monocytes and macrophages, in response to cytokines [22], which are key elements of thrombo-inflammatory pathways [23]. So, the correlation described in the current study could represent either evidence of the participation of podoplanin in platelet activation, in alignment with Lavallé et al. [8], who demonstrated that podoplanin-expressing APL blasts can activate platelets, or a yet unknown association between podoplanin and CD40L in another hematopoietic compartment. In the present dataset, sPDPN levels were not associated with clinical outcomes, although this study was not powered for this type of analysis.

The results also demonstrate that a significant proportion of AML patients present detectable sPDPN levels, while some APL patients present low sPDPN levels. This is in contrast with data from flow cytometry and reverse transcription polymerase chain reaction (RT-PCR) results from our lab that show less overlap between APL and other forms of AML (data not shown). Additional studies are warranted to explore and explain this lower accuracy of sPDPN ELISA for the segregation of APL from other forms of AML compared to flow cytometry.

This study has limitations that should be acknowledged. Although APL is a relatively rare condition and the present sample size was sufficient to demonstrate relevant biological differences in sPDPN levels, this study was not powered to analyze clinical outcomes. Another limitation lies in the absence of additional hemostatic biomarkers, which might have enhanced the interpretation of coagulation-related mechanisms. However, these markers are not routinely assessed in the standard clinical workflow of the hospital, and citrate plasma samples were no longer available. Future studies involving larger cohorts and broader biomarker panels are important to further investigate the potential diagnostic and clinical relevance of sPDPN in APL.

In conclusion, the results of this study represent proof-of-concept that the immunodetection (in this case, using ELISA) of circulating levels of sPDPN can serve as a complementary tool in the differential diagnosis of APL. The data also adds support to the concept that podoplanin expression is a distinct hallmark of APL and contributes to the pathogenesis of the unique thrombo-inflammatory complications seen in these patients.

Data availability

All reported data are available for sharing upon a reasonable request to the corresponding author.

Ethics statement

The study was performed in accordance with the Declaration of Helsinki and approved by the local institutional research board (protocol CAAE: 39948520.8.1001.5404).

Author contributions statement

CRPM: performed all assays; revised medical records; contributed to data analysis and drafted the manuscript; revised and approved the manuscript. CMAS: revised medical records; contributed to data analysis; revised and approved the manuscript. ITBJ: contributed to data analysis; revised and approved the manuscript. BKLD: contribute to study design; revised and approved the manuscript. PMC: provided laboratory support and infra-structure; revised and approved the manuscript. STOS: provided laboratory support and infra-structure; revised and approved the manuscript. EVDP: designed the study, oversaw and provided resources and infra-structure for ELISA analysis, contributed to data analysis; drafted the manuscript; revised and approved the manuscript.

Conflicts 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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Original article

In patients with suspected thrombotic thrombocytopenic purpura, what is the optimal time to therapeutic plasma exchange?

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ABSTRACT

Background: In patients with suspected immune thrombotic thrombocytopenic purpura, guidelines suggest that therapeutic plasma exchange should be initiated within eight hours. However, this time threshold may be difficult to attain. This study sought to identify the optimal time to plasma exchange to maximize outcomes.

Study Design and Methods: Patients with international classification of disease codes for thrombotic microangiopathy were identified in a retrospective cross-sectional analysis of public use data files from the Recipient Epidemiology and Donor Evaluation Study-III (REDS-III). The assumption of linearity between time to therapeutic plasma exchange and the composite outcome of bleeding, thrombosis, and mortality were evaluated. Subsequently, the optimal time for plasma exchange was identified using a nonparametric approach with bootstrapping.

Results: For 149 patients with a suspected diagnosis of thrombotic thrombocytopenic purpura, the association between time to plasma exchange and the primary outcome was non-linear. With regard to the primary composite outcome, this time had a low predictive capacity (area under the curve: 0.62). The optimal time that maximized outcomes was 13.5 h.

Conclusion: Although this study found that time to therapeutic plasma exchange did not independently predict outcome, future studies might evaluate how this time interacts with other variables to predict clinical outcomes.

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Introduction

In patients with suspected thrombotic thrombocytopenic purpura (TTP), therapeutic plasma exchange (TPE) and immunosuppression should be initiated promptly [1]. Although prompt TPE initiation has been associated with improved outcomes of thrombosis, major bleeding, and mortality, the optimal time to TPE that also minimizes poor outcomes is unknown [2–4]. Based on guidelines from the British Society of Haematology, the recommended time window between referral for a suspected diagnosis and TPE initiation is 4–8 h [1]. However, this specific time window may lack robust supporting evidence. Also, due to barriers in diagnosis and treatment initiation, this time window is often not attained [3,5].

Prior evidence suggests that in patients with suspected TTP, TPE initiation within 8 h is achieved in only 27–41 % of patients [3,5]. In fact, time to TPE has been found to be delayed as long as >72 h [4,6]. In studies evaluating treatment delays >24 to 48 h, delays longer than 8 h may account for differences in thrombosis, major bleeding, and mortality outcomes [2,4]. Therefore, there is a need to identify the optimal time window within which TPE initiation is both practicable and associated with improved outcomes. Identifying an optimal TPE initiation window may both simplify clinical practice and guide timely diagnostic strategies.

To characterize the association between time to TPE and outcomes and identify the optimal time threshold to TPE initiation, this study analyzed public use data from the Recipient Epidemiology and Donor Evaluation Study-III (REDS-III) [7,8].

Methods

Study design and data source

This study is a retrospective cross-sectional analysis of public use data files from the REDS-III study [9]. REDS-III was a prospective longitudinal four-year (2013–2016) National Heart, Lung and Blood Institute-sponsored observational multicenter electronic health record study involving four United States blood centers and 12 hospitals [7,8]. Participating sites contributed data on blood donors/donations (6.5 million blood components) and transfusion recipients (120,290 patients in over 234,277 encounters) [7,9,10]. Included as a comparison group were all non-transfused patients in inpatient encounters (1,285,359). REDS-III data include demographics, laboratory results, medication administration, and blood product transfusions [11]. Because REDS-III public use data files contain de-identified data, the study was reviewed by the Institutional Review Board and determined to be exempt.

Participant selection

All children and adults were included in this study if they had a suspected TTP diagnosis. To identify patients with suspected TTP, a validated strategy was used that has been identified to have a positive predictive value of 65 % [12]. Thus, participants with suspected TTP were identified using common procedural terminology (CPT), and ICD-9 and 10 (International Classification of Diseases, ninth and tenth edition) codes for TPE (CPT code 36514, or ICD-9 code 99.71, or ICD-10 codes 6A550Z3 or 6A551Z3) and thrombotic microangiopathy (TMA: ICD-9 446.6 or ICD-10 M31.1; see codes in the Appendix) [2,12,13]. Although REDS-III provides data at the encounter level, this analysis was done at a patient level. Patient level data were obtained by merging recipient data files by encounter and subject ID [11]. Additionally, because each patient may have multiple healthcare encounters, this analysis included only data from the first suspected TTP inpatient encounter (unique hospitalization event).

Primary outcome and variable definitions

The primary outcome was defined as a composite of arterial and venous thrombosis, major bleeding and all-cause mortality (see ICD code definitions in the Appendix) [2,4]. Time to TPE was defined as the time from the recorded hospital admission to the first plasma issue time reported in hours [2,5]. Time to platelet recovery was defined as days from first plasma issue to platelet count >150 $\times$ 10^9/L [14]. Refractory TTP was defined as no platelet count doubling and lactate dehydrogenase concentration greater than the upper limit of the normal range after four days of treatment [14]. TTP exacerbation was defined as a subsequent TTP encounter occurring <30 days after discharge [15]. TTP relapse was defined as a subsequent TTP encounter occurring ≥30 days after discharge [15]. Plasma issued from the blood bank was identified using validated Information Standard for Blood and Transplant (ISBT) codes [8,10,11].

Statistical analysis

To determine the optimal time to TPE, this study sought to identify the time threshold that maximized Youden's statistic (sensitivity + specificity − 1) − the cutoff-point that provides the best balance between true positive and true negative rates [16]. To this end, the assumption of linearity between time to TPE and log odds of our primary outcome was evaluated.

To determine the optimal time threshold, a nonparametric approach was used with the R (version 4.4.0) "cutpointr" package [17]. The "cutpointr" package allows direct estimation of cutoff values in binary classification problems. To this end, various metrics were calculated for a range of potential time cutoff values [17]. Thus, each possible time threshold was evaluated by 1) calculating its sensitivity and specificity, 2) computing Youden's index, and 3) selecting the time threshold that maximizes Youden's index.

To optimize the robustness of the identified time threshold, bootstrapping (1000 resamples) was performed. By repeatedly resampling and replacing the dataset and recalculating the optimal threshold, bootstrapping enhances the reliability of the identified time threshold across multiple samples.

Sensitivity analysis

Clustering on time to TPE

In the course of the analysis, a non-linear association was identified between time to TPE and the odds of the primary

outcome. Therefore, clusters, based on time to TPE, were investigated to identify subpopulations within the cohort. The aligned box criterion was used to select the optimal number of clusters. This criterion helps identify the optimal number of clusters (k) that are not only compact and distinct but also aligned with the underlying data [18]. This analysis was performed using k-means clustering within PROC HPCLUS in SAS version 9.4 (SAS Institute Inc., Cary, NC). These clusters were used in the sensitivity analysis below.

Comparison of multiple models and datasets

To increase the specificity of the TTP cohort and validate the consistency of the findings, a series of sensitivity analyses was performed. First, time to TPE (≥5 days) was used as an exposure variable to identify outliers. Second, outliers based on the cluster analysis were excluded; and third, cases that received only one TPE procedure (ICD 10 codes distinguish only single versus multiple procedures) were excluded [19,20]. Different parametric and nonparametric tests were performed for each sub-analysis. Each test was done with and without bootstrapping (see Supplementary Methods).

Results

Baseline characteristics and outcomes

From 2012 to 2016, the number of patients meeting inclusion criteria for a suspected TTP diagnosis was 149. Among these, the mean age in years was 50. Most patients were female (70.5 %), White (64.4 %), and non-Hispanic (88.6 %). Average admission lab values (± standard deviation [SD]) were as follows: 1) hemoglobin (9.7 ± 2.3 g/dL); 2) platelet count ($69.8 \pm 85.9 \times 10^9$); 3) lactate dehydrogenase (1056.8 ± 898.1 U/L); and 4) creatinine (2.1 ± 1.8 mg/dL). The average time to TPE was 71.3 ± 136.4 h. Detailed baseline characteristics and demographic information are shown in Table 1.

Association between time to therapeutic plasma exchange and the primary outcome

Outcomes data are shown in Table 2.

The association between time to TPE and the log odds of the primary outcome was non-linear (see Figure 1). In the nonparametric model, the optimal time threshold for TPE initiation was 13.5 h (also see bootstrapping analyses in Supplementary Results); and the area under the curve (AUC) was 0.62.

Sensitivity analysis

The cluster analysis based on time to TPE identified six subpopulations. These subpopulations had distinct baseline characteristics (age and sex), admission labs (platelet count, hemoglobin, creatinine and lactate dehydrogenase levels) and prognosis. In summary, some subpopulations had worse outcomes despite shorter time to TPE initiation (see detailed clustering analysis data in Supplementary Results).

The sensitivity analysis identified a similar non-linear association between time to TPE and the primary composite outcome. Additionally, it identified similar AUCs (range: 0.59

Table 1 – Baseline characteristics of patients with suspected thrombotic thrombocytopenic purpura.

	Suspected TTP (*n* = 149)
Demographic	
Age	
Mean (SD)	50.2 (18.2)
Median	50.0
Range	(7.0–87.0)
Gender – n (%)	
Male	44 (29.5)
Female	105 (70.5)
Race – n (%)	
White	96 (64.4)
Black or African American	33 (22.1)
Asian	3 (2.0)
Other	7 (4.7)
Not Reported	10 (6.7)
Ethnicity – n (%)	
Hispanic	7 (4.7)
Non-Hispanic	132 (88.6)
Not Specified	10 (6.7)
Comorbidity – n (%)	
Diabetes	34 (22.8)
Heart Failure	18 (12.1)
Renal Disease	99 (66.4)
Hepatic Disease	17 (11.4)
Stroke	19 (12.8)
Transient Ischemic Attack	2 (1.3)
Venous Thrombosis	26 (17.4)
Pulmonary Embolism	8 (5.4)
Immune Thrombocytopenia	11 (7.4)
Evans Syndrome	1 (0.7)
SLE	6 (4.0)
APS	12 (8.1)
Admission Labs	
Hemoglobin (g/dL)	
n	149
Mean (SD)	9.7 (2.3)
Median	9.4
Range	(4.7–16.4)
Platelet count (x 10^9)	
n	148
Mean (SD)	69.8 (85.9)
Median	36.5
Range	(4.0–464.0)
Lactate Dehydrogenase (U/L)	
n	148
Mean (SD)	1056.8 (898.1)
Median	729.0
Range	(126.0–5002.0)
Creatinine (mg/dL)	
n	149
Mean (SD)	2.1 (1.8)
Median	1.3
Range	(0.5–14.2)
Troponin (ng/mL)	
n	53
Mean (SD)	2.6 (13.1)
Median	0.1
Range	(0.0–96.0)
Time to TPE (h)	
Mean (SD)	71.3 (136.4)
Median	23.5
Range	(0.6–1179.6)

TTP: thrombotic thrombocytopenic purpura; APS: Antiphospholipid syndrome; SD: Standard deviation; SLE: Systemic Lupus Erythematosus; TTP: Thrombotic thrombocytopenic purpura.

Table 2 – Outcomes of 149 patients with suspected thrombotic thrombocytopenic purpura.

	Suspected TTP
Composite outcome n (%)	90 (60.4)
Time to platelet recovery (days)	
Mean (SD)	7.6 (8.0)
Median	4.6
Range	(0.3–39.5)
Refractory TTP n (%) *	25 (28.1)
TTP relapse n (%)	8 (5.4)
TTP exacerbation n (%)	19 (12.8)

SD: Standard deviation; TPE: Therapeutic plasma exchange; TTP: Thrombotic thrombocytopenic purpura.

* Included data from 89 patients.

−0.66) and time thresholds for TPE initiation (range: 12.98 −15.93 h; see Table 3).

Discussion

In this retrospective analysis of patients with suspected TTP, the optimal TPE initiation time threshold was identified as 13.5 h. Additionally, the association between time to TPE initiation and the primary composite outcome (bleeding, thrombosis, and mortality) was found to be non-linear. With regard to the composite outcome, time to TPE initiation had an AUC of 0.62 (poor predictive capacity). Taken together, these data suggest that, although there is a clearly identified threshold, time to TPE initiation may be only one of several factors impacting outcomes.

An important and novel finding of this analysis was the optimal TPE initiation time threshold of 13.5 h. With the sensitivity analyses taking into account outliers and number of TPE procedures, the optimal time threshold may fall between 12.98–15.93 h (see Table 3). To our knowledge, the optimal time to TPE has not been specifically investigated in previous studies. However, compared to the evaluation of time to TPE initiation as a continuous variable in this study, previous studies have evaluated differences in outcomes based on prespecified time thresholds (that is time to TPE initiation as a categorical variable). In a study of 61 patients with confirmed TTP, outcomes did not differ significantly when the selected categorical time threshold was 8 h [3]. Specifically, when compared to >8 h, TPE initiation within 8 h did not significantly improve rates of myocardial infarction (31 % versus 24 %) neurological events (28 % versus 33 %), venous thromboembolism (6 % versus 10 %) and mortality (7 % versus 4 %). In a previous large database study of 793 cases of suspected TTP, when categorical time thresholds of <1 day, 1 day, 2 days, and >2 days were evaluated, a higher odds of mortality, major bleeding, and thrombosis was associated with time to TPE initiation >2 days (OR: 1.68; 95 % confidence interval: 1.11–2.54; p-value = 0.0150) [2]. Although these studies differ from the current study in their use of time to TPE as a categorical variable, they appear to suggest that the optimal time to TPE that maximizes outcomes is greater than 8 h but <48 h [2,3]. While imperfect (a definitive TTP diagnosis was not established), the methodology used in this study may offer practical insights for improving patient outcomes through timely intervention.

Notwithstanding the above, the ability to compare outcomes across studies is limited by the absence of a definition for standardized time to TPE. In this study, time to TPE was defined as hours from hospital admission to first plasma issue. In other studies, time to TPE has been variably defined as follows: 1) days from hospital admission to first TPE procedure [2,4,13]; 2) hours from laboratory blood sample receipt to blood bank plasma release [5]; and 3) hours from suspected diagnosis to first TPE procedure [3]. Each of these definitions, which serve as an imperfect surrogate for time from suspected diagnosis to TPE initiation, has its own limitations [1]. For example, time to plasma issue could represent, not plasma for TPE but rather, plasma for infusion as a

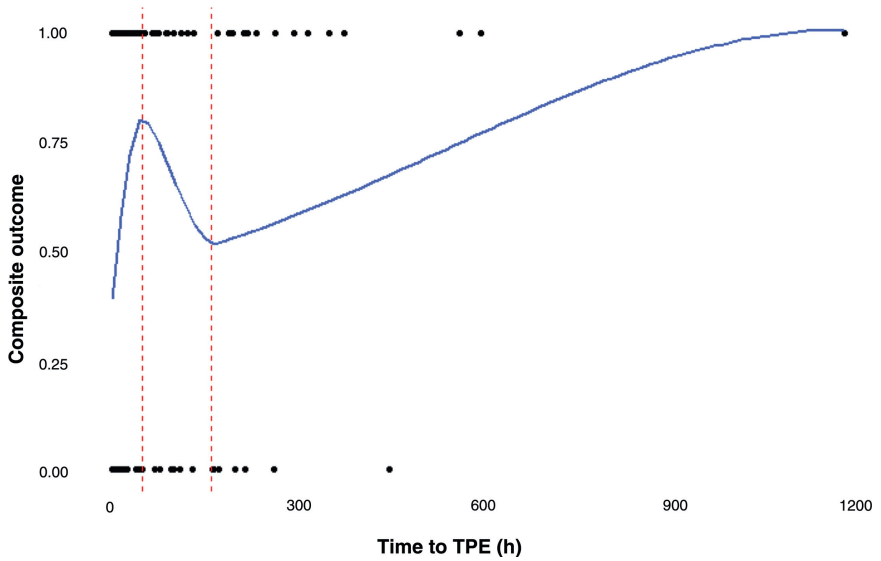

Figure 1 – The association between time to therapeutic plasma exchange and log odds of composite outcome is non-linear. TPE: Therapeutic plasma exchange; TTP: Thrombotic thrombocytopenic purpura.

Table 3 – Sensitivity analysis to identify the optimal time threshold for therapeutic plasma exchange initiation.

Cohort (n)	Model	AUC	Threshold identified (h)
Total cohort (149)	Parametric	0.65	15.93
Remove those with only one TPE (65)		0.59	13.55
Remove time to TPE >5 days (125)		0.65	13.5
Remove clusters with outliers (139)		0.66	12.98
Remove clusters with 100–200 h (137)		0.66	13.69
Total cohort (149)	Nonparametric (GAM)	0.65	15.93
Removing 100–200 h cluster (137)	Nonparametric with bootstrapping*	0.64	13.5

AUC: Area under the curve; GAM: Generalized Additive Modeling; TPE: Therapeutic plasma exchange;.

* : "cutpointr" package in R.

temporizing strategy prior to initiating TPE. Additionally, it may not account for time from blood bank issue to the actual TPE procedure start time. Therefore, in evaluating the impact of time to TPE on TTP outcomes, differences in time definition may confound the results. Furthermore, when the total time from symptoms onset to initial healthcare-seeking behavior and final suspicion is considered, healthcare-associated measures of time to TPE may represent only a small fraction (see Figure 2) [3,6]. In a previous study of 38 patients, the median time from symptoms onset to first hospital visit was 6.5 days (range: 2–18 days) [6]. Thus, measures of time to TPE used here are imprecise: They do not account for delays in healthcare seeking and time from initial healthcare presentation to final acute care referral.

An interesting finding from this analysis is that, with regard to the primary composite outcome, time to TPE had low predictive capacity (AUC: 0.62). While this AUC falls within the range of an "acceptable" predictor, it is lower than what is seen in other commonly used prediction models such as the PLASMIC score (AUC: 0.91–0.96) [21,22]. However, it is important to note that, similar to the PLASMIC score, most prediction models include not one but a combination of several predictive factors [23,24]. Indeed, in patients with TTP, factors known to impact outcomes include age [24–26], renal failure [24,25], lactate dehydrogenase levels [24,26], platelet count [24], stupor or coma [24,26], and platelet transfusions [25]. This study did not assess the interaction of these variables with time. Therefore, future studies could evaluate how time to TPE interacts with other factors to predict outcomes.

This study also found the association between time to TPE and outcomes to be non-linear. This non-linear association may suggest diversity in TTP clinical presentations. Of 66 patients with TTP on the Oklahoma TTP Registry, clinical diversity was illustrated in ten patients as follows: 1) prolonged prodrome of mild symptoms, 2) sudden onset of critical illness with multi-organ dysfunction, 3) stroke without hematologic manifestations, and 4) association with other life-threatening diseases (such as infections and systemic lupus erythematosus) [27]. TTP may also present with asymptomatic thrombocytopenia or small bowel ischemia [28,29]. The diversity of presentation may suggest that, compared to patients with mild symptoms at diagnosis, patients who are acutely ill at presentation may require more urgent treatment initiation. Nevertheless, how distinct clinical presentations impact TTP outcomes remains poorly understood. Understanding the relationship between clinical presentations, patterns and outcomes may guide optimal management strategies for specific subpopulations. Nevertheless, it is important to note that the cohort, which is focused on patients with suspected TTP, may also include patients with other TMAs [30]. Therefore, studies to evaluate the non-linear relationship of time to TPE with outcomes are needed in a cohort of patients with confirmed TTP.

The primary strength of this study is the use of a robust and rigorous methodology enhanced by combining a nonparametric approach with bootstrapping and clustering-based analysis. Additional strengths include the use of public use data files from REDS-III – a multicenter database optimized to provide accurate data regarding plasma issue. Nevertheless, REDS-III

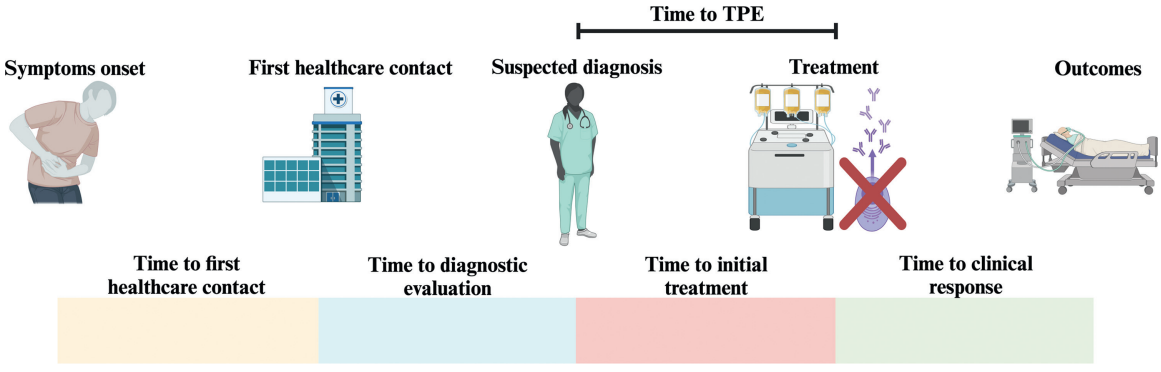

Figure 2 – The patient journey since symptoms onset and potential factors contributing to time to diagnosis. TPE: Therapeutic plasma exchange; TTP: Thrombotic thrombocytopenic purpura.

data is dated. Therefore, it may not account for current trends in TTP diagnosis and management. With the 2019 Food and Drug Administration (FDA) approval and evolving use of caplacizumab, the effect of time to TPE initiation on outcomes may have changed. Additionally, although this study uses a validated strategy to identify patients with suspected TTP, the database lacks key diagnostic variables to identify patients with true TTP including ADAMTS13 activity and variables to calculate the PLASMIC score (mean corpuscular volume) or French score (anti-neutrophil antibodies) [22,26,31]. Without these data, these findings can only be applied to a broad unselected population of patients with TMAs in whom TPE has been initiated. It cannot be generalized to patients with confirmed TTP [12]. Also, to avoid reverse causation bias from outcomes that may occur prior to TTP diagnosis, future studies could evaluate the association between time to TPE and TTP-specific outcomes such as platelet recovery, refractoriness, exacerbation, and relapse. Notwithstanding these limitations, this study is an important milestone in understanding the association between time to TPE initiation and outcomes.

Conclusion

In patients with suspected TTP, the threshold for time to TPE initiation may be greater than recommended by the guidelines. Nevertheless, when the non-linear association between time to TPE and outcomes and its low predictive capacity is considered, other factors besides time to TPE alone may be important in predicting outcomes. Future studies may help evaluate how time to TPE interacts with other factors to predict clinical outcomes.

Conflicts of interest

Oluwatoyosi A.Onwuemene has received honoraria from and has served on an Advisory Board for Sanofi. This relationship is unrelated to the content presented in this manuscript.

Supplementary materials

All reported data are available for sharing upon reasonable request to the corresponding author. Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.htct.2025.106223.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Changes in the microbiota following allogeneic hematopoietic stem cell transplantation: A potential bioguide for clinical outcome?

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ABSTRACT

Introduction: This study aims to support our hypothesis regarding compositional changes in the intestinal microbiota by characterizing these changes through pre- and post-transplantation analyses. Additionally, it seeks to determine whether monitoring the intestinal flora could provide predictive or therapeutic insights into graft versus host disease.
Methods: This study included adult patients who underwent allogeneic hematopoietic stem cell transplantation. Microbiota assessments were performed through stool analyses. Stool samples were collected twice: once before transplantation and once after engraftment. Following nucleic acid isolation, the samples were processed using New Generation Sequencing. Microbiota-associated pathways were examined using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Statistical analyses were performed using R statistical software. In addition to microbiota analysis, resistance genes common in Gram-negative bacteria in the region (such as *OXA-48-like, KPC-like, NDM-like*, and *CTX-M-like*) were identified via classical polymerase chain reaction in stool samples collected after transplantation. The pathways were analyzed using the KEGG database.
Results: Fifteen transplant recipients participated in the study. The Proteobacteria phylum increased in patients who tested positive for the *CTXM-1 group* and *OXA-48-like* resistance genes. *Blautia caecimuris* and *Enterococcus* exhibited significant changes following transplantation, while *Tyzzerella spp.* and *Dialister spp.* showed significant alterations after the onset of graft-versus-host disease. A marked change in *Eubacterium spp.* was also noted in patients with disease relapse. Two key metabolic pathways—acridone alkaloid

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biosynthesis and the D-arginine and D-ornithine metabolism—were associated with clinical outcomes.

Conclusions: This study demonstrates that allogeneic hematopoietic stem cell transplants lead to significant alterations in intestinal microbiota composition, including increased pathogenic bacteria associated with graft-versus-host disease exacerbation. These findings suggest that microbiota monitoring may be a promising strategy for the prevention and treatment of graft-versus-host disease. Moreover, modulation of specific microbial metabolic pathways may influence disease clinical outcomes. As the first study of its kind conducted within the Turkish population, this research contributes novel insights to the existing literature and highlights the potential of microbiota-based approaches in post-transplant patient management.

Introduction

Stem cells are undifferentiated cells that can self-renew and differentiate into specialized cell lineages, possessing the unique capability of unlimited cell division. Hematopoietic stem cells (HSCs) are responsible for regenerating blood and immune cells; their therapeutic potential is widely utilized in the treatment of hematological malignancies through stem cell transplantation. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) aims to re-establish hematopoiesis from a donor-derived source and reconstruct a healthy donor immune system capable of eliminating residual tumor cells [1]. However, donor-derived immune cells—primarily alloreactive T cells—may also react against non-tumor tissues. Organs such as the skin, intestines, and liver are particularly susceptible to this response, leading to tissue damage and constituting a significant cause of morbidity and mortality following allo-HSCT. If the reaction occurs within the first 80–100 days after transplantation, it is called acute graft-versus-host disease (aGvHD) [2,3]. In allo-HSCT patients, conditioning regimens are used to ablate hematopoiesis, immunosuppressive therapies are administered to prevent the development of aGvHD alongside prophylactic antibiotic treatments aimed at reducing or preventing the risk of infections in this immunocompromised setting. The incidence and severity of aGvHD are associated with various risk factors [4].

Chemotherapy, given as a conditioning regimen, destroys epithelial cells and their integrity, resulting in bacterial translocation. The damaged epithelium secretes uric acid, adenosine triphosphate (ATP), and various proinflammatory cytokines. Pathogen recognition receptors such as 'Toll-like receptors' (TLR), 'NOD-like receptors' (NLR), and P2XR are activated by the pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP). Antigen-presenting cells are activated. All of these reactions contribute to the development of aGvHD [5].

There is a suspicion of compositional changes in the intestinal microbiota after transplantation and also that the change in intestinal microbiota may have a role in aGvHD. Thousands of microbial species essential to human life have colonized the human body [6] with over 1000 different types of bacteria having been identified in stool samples. A high degree of variation is observed between individuals at the species level. The gut microbiome plays essential roles in human physiology, including food digestion, maintenance of the intestinal barrier, prevention of pathogen colonization, regulation of the gut–brain axis, and immune system development. The intestinal microbiota is influenced by external factors such as diet, lifestyle, environment, medications, and stress, which can significantly reduce bacterial diversity [7–9]. Through the production of short-chain fatty acids (SCFAs), the microbiota contributes to ATP synthesis, the production of vitamins B and K, the modulation of immune cells such as macrophages, and the regulation of immune responses [10]. Metabolic pathways are closely linked to cancer and other diseases, particularly after stem cell transplantation. The biosynthesis of acridone alkaloids is notable for its wide-ranging bioactivities, including cytotoxic, antibacterial, antiviral, anti-tumor, and enzyme inhibitory effects [11]. T cell activity highly depends on metabolic function, which is crucial for anti-tumor responses, however, the tumor microenvironment can impair T cell metabolism and function [12]. Arginine is a precursor of various compounds, such as nitric oxide, polyamines, creatinine, and urea. Ornithine, an intermediate in the urea cycle, contributes to immune regulation and is not incorporated into natural proteins [13]. Some of these metabolites also contribute to initiating and regulating immune responses.

This study aimed to document the microbiota before and after transplantation in allo-HSCT patients diagnosed with leukemia and lymphoma. Additionally, it seeks to determine whether monitoring the intestinal flora could provide predictive or therapeutic insights into GvHD.

Method

Fifteen patients and fifteen sibling donors in the transplant preparation process were included in this study. Stool samples from patients were taken before the initiation of the preparation regimen and after engraftment. Stool samples from donors were taken before the mobilization regimen and before transplantation. All stool samples were stored under appropriate conditions (−80 °C) until being processed. The

microorganism DNA isolation process was performed using the Spin Column Nucleic Acid Isolation Kit (ZymoBIOMICS DNA Kits, USA) in groups of ten in accordance with the manufacturer's recommendations. All samples were subjected to microbial nucleic acid sequencing using Oxford Nanopore Technology [14]. In addition, the *extended-spectrum β-lactamases* (ESBLs; *CTXM-1* group) genes which are the most common of the *Enterobacteriaceae* family in Turkey and resistance genes that make up the enzyme carbapenemase (*OXA-48-like, NDM* and *KPC*) and the presence of the *vanA* gene responsible for vancomycin resistance in enterococci were investigated in the stool samples of patients after transplantation [15,16]. The associated pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

The volunteers (patients and healthy individuals) included in the study were informed and consent forms were signed after ethics committee approval. Study approval was obtained from the Istanbul University, Istanbul Faculty of Medicine Clinical Research Ethics Committee (Date: 19.07.2019, No: 146,386) and supported by Istanbul University, Scientific Research Projects Coordination Unit.

Nucleic acid isolation

In the first stage, nucleic acid was isolated from samples using a stool nucleic acid isolation kit (ZymoBIOMICS DNA Kits, USA). For DNA isolation, various combinations of previously defined methods were tested. These included physical (sonication or bead disruption), chemical (SDS or CTAB), and biochemical (proteinase K, lysozyme) cell lysis techniques with the most effective method being subsequently determined. Following this, silica columns were used to separate DNA from protein molecules in the lysed cells, and RNA contamination was eliminated through the application of RNase. At the final stage of isolation, the DNA attached to the silica columns was dissolved in water without the DNase/Pyrogen and the nucleic acid concentration was determined using a spectrophotometer. DNA samples were selected with a minimum concentration of 10 ng/μL (preferably 50–300 ng/μL) to meet the following purity criteria: an OD_{260}/OD_{280} ratio of 1.8–2.0 and an OD_{260}/OD_{230} ratio of 2.0–2.2.

Microbiome sequencing

A two-primer polymerase chain reaction (PCR) with rapid adapter binding chemistry was simplified following the instructions of the manufacturer with small changes, and the modified 5-end 16S rRNA gene amplicons were produced for adapter attachment after PCR. The 16S rRNA gene-specific forward primer (27F) and reverse primer (1492R) were used for amplification of the V1-V9 region of the 16S rRNA gene. PCR amplification of 16S rRNA genes was performed using ZymoTaq™ Hot Start PreMix (USA) in a total volume of 25 μL containing inner primer pairs (50 nM each) and outer primer mixture with barcode (3 %) from PCR Barcoding. The amplified DNA was purified using the AMPure® XP (Beckman Coulter), and was measured using a NanoDrop® 1000 (Thermo Fischer Scientific, Waltham, MA, USA) and (Qubit, Thermo Fisher, Waltham, MA, USA). A total of 100 ng of DNA was incubated for 5 min at room temperature with a 1 μL Rapid Adapter. The prepared DNA was loaded into the R9.4 flow cell (FLO-MIN106; Oxford Nanopore Technologies) and was sequenced. The MINNOW software version 1.11.5 (Oxford Nanopore Technologies) was used for data collection.

Bioinformatics and statistical analysis

Sequencing was performed on a MinION device using Oxford Nanopore Technologies (ONT) FLO-MIN106D and 16S raw readings were obtained as fast5 files. The initial bioinformatics analyses were performed using ONT guppy version 5.0.11. Consensus arrays were created using bbtools 38.91, magicblast 1.6.0, and samtools 1.13. The NCBI blastn (version 2.0.12) was applied in preparation of the operational taxonomic unit (OTU) tables in accordance with the NCBI general 16S bacterial taxonomy reference dated 10/8/2021. The generated OTU tables were used to calculate alpha diversity using R Statistical Computing Language (version 4.04) (readr, phyloseq, microbiome, vegan, descr and ggplot2 packages). Statistical analyses also used R Statistical Computer Language version 4.0.4 and Rstudio IDE 1.4 (tidyverse, readr, xlsx and ggplot2 packages). Shapiro-Wilks normality testing showed non-normal distribution and therefore the Bonferroni correction method was used after performing the Wilcoxon Rank Sum or Mann-Whitney U tests thereby identifying the bacteria which showed statistical differences. In addition, the pathways associated with the KEGG database were examined.

Results

All patients received transplants from fully HLA-matched (10/10) sibling donors.

The mean age of patients was 46.66 ± 14.30 years (20–69 years), and 44.40 ± 11.56 years (24–61 years) for donors. The gender distribution was 9/6 (M/F) in patients and 11/4 (M/F) in donors.

The most common indication for transplantation was acute myeloid leukemia, followed by myelodysplastic syndrome, non-Hodgkin lymphoma, primary myelofibrosis, and Hodgkin lymphoma.

Three patients received a myeloablative conditioning regimen, three received reduced-intensity conditioning, and the remaining underwent non-myeloablative conditioning. All patients received antimicrobial prophylaxis. In all cases, peripheral blood stem cells were used as the stem cell source.

The 100-day post-transplant survival rate was 80 % with aGvHD developing in 20 % of the patients. Complete chimerism was documented in 13 % of the patients who achieved engraftment.

The transplant-related protocols are given in Table 1.

The *KPC* and *NDM* genes were not found in the post-transplant stool samples of the patients using PCR. *CTXM-1 group* and *vanA* genes were found in 13 % of the stool samples of patients and *CTXM-1 + OXA-48-like* genes were detected in 13 %. 16S targeted sequencing phylum distributions of the intestinal microbiota of 15 patients with hematological malignancy before and after transplantation were calculated as percentage values. A total of 27 phyla were found. Only the Bacteroidetes phylum showed a statistically significant (p-

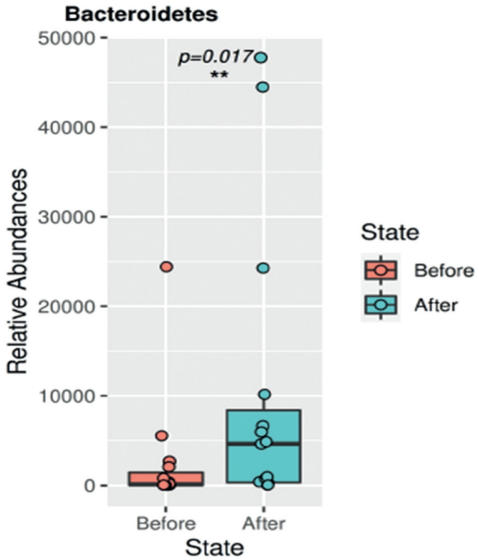
Table 1 – Transplantation protocols.

aGvHD Prophylaxis	CSA/MTX
	TAC alone or in combination with MMF
	CSA (or TAC) alone or in combination with MMF
	PTCy
Conditioning Regimens	Busulfan and cyclophosphamide
	TBI and cyclophosphamide
	Fludarabine, melphalan and ATG
	Treosulfan and fludarabine
Antimicrobial Prophylaxis	
Antibacterial prophylaxis	Fluoroquinolones
Herpes simplex virus prophylaxis	Acyclovir
Pneumocystis jiroveci	Trimethoprim-sulfamethoxazole
Fungal infection	fluconazole (Diflucan) for yeast

aGvHD: acute graft versus host disease; CsA/MTX: cyclosporin-A and methotrexate (Methotrexate); TAC: tacrolimus; MMF: mycophenolate mofetil (CellCept); PTCy: posttransplant cyclophosphamide; TBI: Total body irradiation; ATG: rabbit anti-thymoglobulin (ATG). Three out of 15 patients developed aGvHD (skin; skin and liver; intestine).

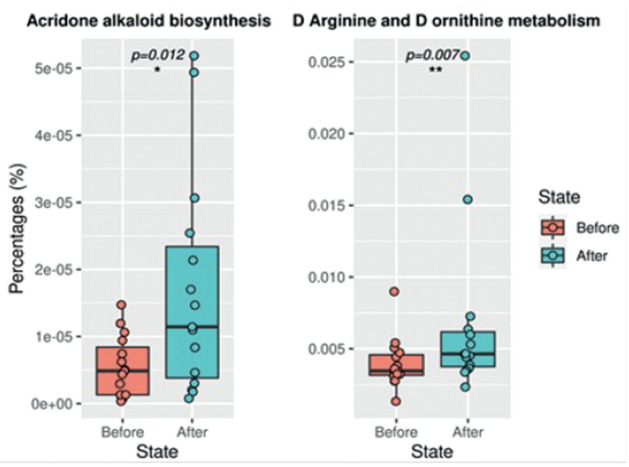
value = 0.017) increase in individuals after transplantation (Figure 1).

A pathway analysis was performed after a 16S microbiome study of 30 stool samples from 15 patients before and after transplantation. Of 272 pathways, the obtained data showed



Bacteroidetes		
	Before Transplant	After Transplant
Min	9	23
Max	24407	47775
Median	136	4643
Mean	2430.33	10090

Figure. 1 – The change of the Bacteroidetes phylum before and after transplantation. The asterisk in the figure indicates that this phylum is a crucial feature, statistically different between the groups.



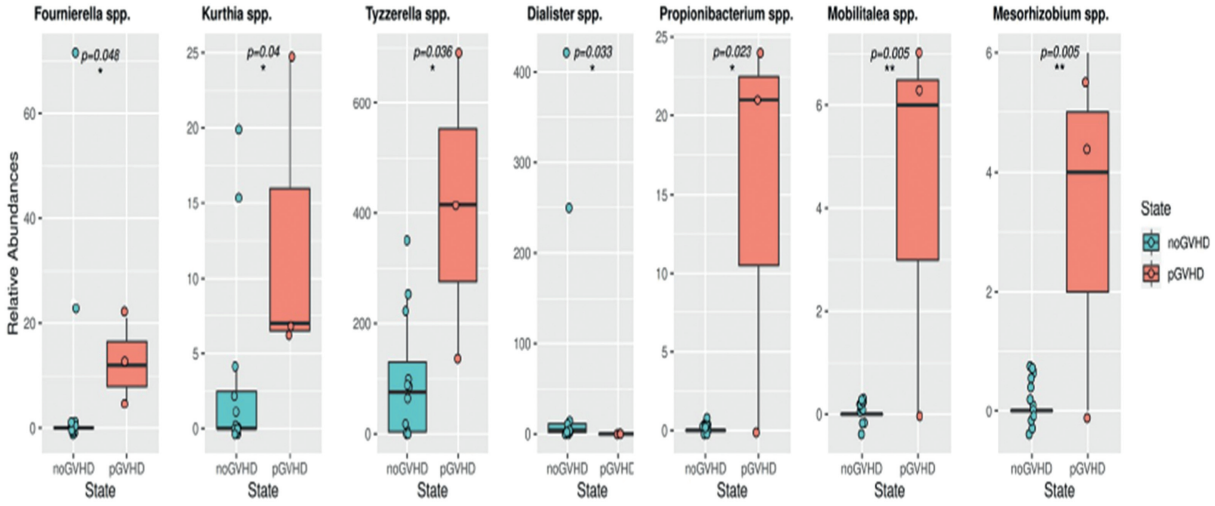
	Acridone alkaloid biosynthesis		D-arginine and D-ornithine metabolism	
	Before Transplant	After Transplant	Before Transplant	After Transplant
Min	$3.33x10^{-7}$	$7.63x10^{-7}$	$1.33x10^{-3}$	$2.34x10^{-3}$
Max	$1.47x10^{-5}$	$5.19x10^{-5}$	$8.99x10^{-3}$	$2.54x10^{-2}$
Median	$4.88x10^{-6}$	$1.44x10^{-5}$	$3.46x10^{-3}$	$4.64x10^{-3}$
Mean	$5.46x10^{-6}$	$1.69x10^{-5}$	$9.95x10^{-3}$	$6.71x10^{-7}$

Figure. 2 – According to the KEGG database results, differences in Acridone alkaloid biosynthesis and D-arginine and D-ornithine metabolism. The asterisk in the figure indicates that this phylum is a crucial feature, which is statistically different between the groups.

statistically significant differences in two between the groups. These two pathways were found to be acridone alkaloid biosynthesis and D-arginine and D-ornithine metabolism (p-value = 0.012 and p-value = 0.007, respectively). A general increase was observed in acridone alkaloid biosynthesis (Figure 2) in the pathway analysis.

A total of 36 OTU differences were found in deceased and surviving patients within the first 100 days after transplantation. In the species-based analysis, significant decreases were detected in *Enterocloster bolteae* (p-value = 0.018), *Streptococcus salivarius* (p-value = 0.018), *Blautia caecimuris* (p-value = 0.018), and *Erysipelatoclostridium* spp. (p-value = 0.018). However, significant increases were detected in Faecalibacterium *spp*. (p-value = 0.02), *Enterococcus* spp. (p-value = 0.018), *Desemzia spp*. (p-value = 0.014), *Oceanobacillus spp*. (p-value = 0.007), *Brochothrix* spp. (p-value = 0.003) and *Anoxybacillus spp*. (p-value = 0.002).

Using the Mann-Whitney U test, seven OTUs showed differences in the microbiota between patients who developed and did not develop GvHD after transplantation. The bacterial species that were found to have statistically significant increases in patients who developed GvHD were *Fournierella spp*. (p-value = 0.048), *Kurthia spp*. (p-value = 0.04), *Tyzzerella spp*. (p-value = 0.036), *Dialister spp*. (p-value = 0.033),



	Fournierella spp.		Kurthia spp.		Tyzzerella spp.		Dialist□r spp.		Propionibacterium spp.		Mobilitalea spp.		Mesorhizobium spp.	
	Non	GvHD	Non	GvHD	Non	GvHD	Non	GvHD	Non	GvHD	Non	GvHD	Non	GvHD
Min	0	4	0	6	0	138	0	0	0	0	0	0	0	0
Max	71	21	20	25	351	691	421	0	1	24	0	7	0	6
Median	0	12	0	7	76.5	415	4	0	0	21	0	6	0	4
Mean	7.92	12.33	3.5	12.6	99.33	414.67	59.5	0	0.08	15	0	4.33	0	3.33

Figure. 3 – Species of bacteria in patients who developed and did not develop GvHD after transplantation. According to the Mann-Whitney U test the post-transplant minimum, maximum, median, and averages of the relative quantity values of the species *Fournierella* spp. (p-value = 0.048), *Kurthia* spp. (p-value = 0.04), *Tyzzerella* spp. (p-value = 0.036), *Dialister* spp. (p-value = 0.033), *Propionibacterium* spp. (p-value = 0.023), *Mobilitalea* spp. (p-value = 0.005) and *Mesorhizobium* spp. (p-value = 0.005) are given. The * and ** symbols in the figures show the statistically significant levels (p-value <0.05 and p-value <0.01, respectively).

Propionibacterium spp. (p-value = 0.023), *Mobilitalea spp*. (p-value = 0.005) and *Mesorhizobium spp*. (p-value = 0.005) (Figure 3).

Of the 26 phyla detected in 15 patients with hematological malignancies, the Actinobacteria phylum showed a statistically significant difference (p-value <0.018) in individuals who developed relapse after transplantation. This phylum was found in smaller numbers in this group. Analysis of the microbiota at the species level revealed statistically significant differences between patients who developed relapse and those who did not (Figure 4). These differences were observed for *Eubacterium* spp. (p-value = 0.031), *Schaalia* spp. (p-value = 0.025), *Intinibacter* spp. (p-value = 0.021), *Saccharococcus* spp. (p-value = 0.02), *Polycladomyces* spp. (p-value = 0.02), and *Desulfurobacterium* spp. (p-value = 0.02).

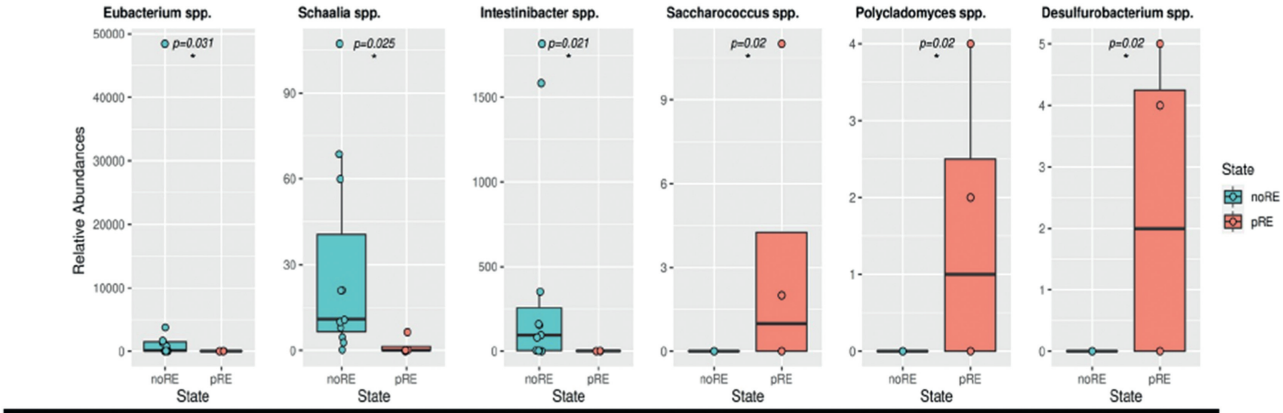
The bacterial distribution at the phylum level was studied by grouping patients according to the presence or absence of the resistance gene. Of the 26 phyla detected, the Proteobacteria phylum showed an increase in patients with the resistance gene, though this change was not statistically significant.

Discussion

The greater understanding of the intestinal microbiota developed over the last 20 years has caused significant changes in how various diseases are followed up and treated. The role of microbiota in hematological malignancies has also been revealed [17]. The first hint that the intestinal microbiota affects GvHD dates back to the early 1970s [18,19]. It is impractical to follow the changes in all bacteria in the microbiota with the classically applied culture methods in today's technology as not all bacteria can be produced in vitro. Therefore, genomic studies are needed for the identification of bacteria. In microbiota studies, it is crucial to detect by molecular methods not only the bacteria found in normal flora but also bacteria resistance genes; other viral, fungal, and parasite species are also important. All microorganisms and their metabolites are in a state of balance with the host, however under some conditions, a disturbance leads to dysbiosis.

Due to budget constraints, this study conducted investigations only in terms of bacteria, and not of other microorganisms. Some bacteria in the intestinal microbiota acquire resistance genes against glycopeptide group antibiotics such as cephalosporins, carbapenems, or vancomycin. These bacteria can cause life-threatening, invasive infections in transplant recipients and other immunocompromised patient groups via endogenous spread from the gastrointestinal system.

During allo-HSCT, factors such as the conditioning regimen, nutrition, infections, and antibiotics disturb the balance of the intestinal flora [20,21]. Thus the diversity of microorganisms may decrease with changes that occur in the



	Eubacterium spp.		Schaalia spp.		Intestinibacter spp.		Saccharococcus spp.		Polycladomyces spp.		Desulfurobacterium spp.	
	Non	Res	Non	Res	Non	Res	Non	Res	Non	Res	Non	Res
Min	6	6	0	0	0	0	0	0	0	0	0	0
Max	48408	33	107	6	1815	2	0	11	0	4	0	5
Median	109	16	11	0	95	1	0	1	0	1	0	2
Mean	5125.73	17.75	28.64	1.5	386.45	1	0	3.25	0	1.5	0	2.25

Figure. 4 – Distribution of bacteria in patients who developed relapse and those who did not. The relative abundances of the species that showed statistically significant differences are provided above. Statistically significant differences were observed for *Eubacterium* spp. (p-value = 0.031), *Schaalia* spp. (p-value = 0.025), *Intestinibacter* spp. (p-value = 0.021), *Saccharococcus* spp. (p-value = 0.02), *Polycladomyces* spp. (p-value = 0.02), and *Desulfurobacterium* spp. (p-value = 0.02). The asterisk (*) in the figure indicates a statistical difference between the groups.

gastrointestinal tract of patients [22]. Poor intestinal diversity may affect engraftment, suggesting that gut microbiota may be an essential factor in the success or failure of allo-HSCT [23]. The loss of intestinal diversity is usually associated with the loss of *Clostridia* species known to produce SCFA by taking advantage of the fibers in food [24]. One of the most well-known SCFAs, butyrate, is recognized as the energy source of intestinal epithelial cells. In an experimental study using a mouse model, the abundance of *Lactobacillus* species in the intestinal flora decreased following hematopoietic stem cell transplantation (HSCT) and was associated with the development of GvHD. Researchers suggested that the microbiota can control the severity of intestinal inflammation by protecting against GvHD damage mediated by *Lactobacillus* species [25]. A recent article indicates that reduced amounts of butyrate are found in intestinal epithelial cells after allo-HSCT in mice, which may increase intestinal damage due to the development of GvHD [26]. In this study, considering the complications after the transplant, differences were identified in the Firmicutes phylum, which also includes *Lactobacillus*. In addition, the Bacteroidetes phylum showed a significant increase after transplantation compared to the levels before transplantation.

Microbiota analysis of stool samples obtained from patients after engraftment and before transplantation revealed a significant loss of diversity. Systemic inflammation of the gastrointestinal tract plays a vital role in the onset and exacerbation of GvHD. Therefore, the progression of GvHD with gastrointestinal involvement was more severe than with the other types of organ involvement [27].

The current research project showed an increase, albeit statistically insignificant, in *Enterococcus* and *Clostridium* species in one patient who died of intestinal GvHD. Also, qualitative changes in the microbiota are known as a result of allo-HSCT, especially the loss of microbiota diversity, which is characterized by the depletion of short-chain fatty acid-producing anaerobes [28]. Therefore, different protective and pathogenic components of the microbiota affect GvHD and survival following HSCT. Studies showed that loss of microbiota diversity was affected by both treatments and the development of GvHD [16]. In addition, an extensive prospective study targeting anaerobic bacteria in HSCT patients showed a regression in GvHD damage, indicating that some specific species in the microbiota may have beneficial effects [22,29]. Given these data, determining risk groups is essential for investigating the relationship between microbiota, stem cell transplantation, and GvHD development. Developing new follow-up and treatment algorithms is essential for graft survival.

In the present study, the incidence of aGvHD was 16 %. Dysbiosis associated with GvHD is characterized by the loss of overall diversity in the microbiota [30]. Essentially, a decrease was found in *Clostridia* species. Still, the change was not statistically significant in the stool samples of patients before and after transplant, and after engraftment. An increased presence, although statistically insignificant, of *Enterococcus* species bacteria was detected in patients who developed GvHD, including those in the current study. This study showed that the predominance of the *Enterococcus* population in the microbiota was significantly associated with severe aGvHD thereby indicating a causal role of *Enterococcus* in the pathogenesis of GvHD [31,32]. In a study conducted on a larger cohort of adults, the relative abundance of the Lachnospiraceae species and the decrease in *Blautia* species have

been associated with decreased mortality rates from GvHD [33]. In the present case, a decrease was detected in Lachnospiraceae and *Blautia* species in the post-transplant samples of the recipients. However, no relationship was found with GvHD and mortality. In addition, the examination of microbiota differences between patients who developed GvHD and those who did not showed that a total of seven OTU showed differences between the individuals. In patients who developed GvHD, the OTU counts of bacterial some species (*Fournierella* spp., *Kurthia* spp., *Tyzzerella* spp., *Dialister* spp., *Propionibacterium* spp., *Mobilitalea* spp., and *Mesorhizobium* spp.) increased.

A total of 36 OTU differences were found in patients who died compared to those who survived within the first 100 days after transplantation. In the review conducted on the species, significant decreases were detected in *Enterocloster bolteae, Streptococcus salivarius, Blautia caecimuris, Erysipelatoclostridium* spp, while significant increases were detected in *Faecalicoccus* spp., *Enterococcus* spp., *Desemzia* spp., *Oceanobacillus* spp., *Brochothrix* spp. and *Anoxybacillus* spp. Of the 26 identified phyla found in patients who developed relapse within the first 100 days after transplantation, Actinobacteria was found to have a statistically significant lower number in relapsed individuals.

Conditioning chemotherapy administered before HSCT leads to prolonged neutropenia and damage to mucosal surfaces, facilitating the passage of microorganisms through these barriers and predisposing to bloodstream infection with the most common causes in HSCT recipients being enterococci and viridans group streptococci [34–36]. The results of one study showed that broad-spectrum beta-lactamase (GSBL) genes were positively correlated with *Klebsiella* species in samples taken from intensive care unit patients of eight hospitals in Turkey [37]. *KPC* and *NDM* gene positivity were not found in tests performed on the stool samples taken after transplantations however 13 % of *CTXM-1 group* and *vanA* gene positivity, and 13 % *CTXM-1 group + OXA-48-like* gene positivity were detected. In the current study, *Klebsiella* species were higher in number in patients who were detected to have resistance genes. In Turkey, *CTXM-1 group* and *OXA-48-like* resistance genes are widely observed, especially in *Klebsiella pneumoniae* strains [16]. Resistance to vancomycin develops due to the presence of *vanA* gene in bacteria of the *Enterococcus* species, which also leads to treatment failures. This study found the *vanA* gene in 13 % of patients. In patients, increases were detected in *Enterococcus* and *Klebsiella* species who died of septicaemia; however, these findings were not statistically significant.

Citrobacter murliniae, K. pneumoniae, and *Enterobacter cloacae*, which are known as hospital pathogens, are very important for the risk of infection in HSCT. Other members of the *Enterococcus, Citrobacter*, and Enterobacteriaceae families, such as *Enterobacter* and *Klebsiella*, are the most opportunistic members of the human intestinal microbiota. In nosocomial infections, *Citrobacter, Enterococcus, Klebsiella,* and *Enterobacter* are well-known possible sources and have been reported as the reason for the increase in morbidity and mortality rates [38]. Our results showed that colonization of the intestine with resistant strains was observed in some patients however, no nosocomial infections were detected.

Many metabolic activities have a crucial role in the state of health and disease. Various bioactivities of acridone alkaloids have been studied for the last 22 years [39]. Acridone, which is commonly found in healthy individuals, is also known to have cytotoxic and anticancer activity in addition to its antiparasitic and antimicrobial properties [40]. Other ongoing studies in mice with lymphoma suggest that acridone alkaloids are effective anti-cancer, and anti-proliferative agents [41]. Glutamate-consuming bacteria are predicted to utilize the acridone alkaloids pathway.

Another crucial metabolic pathway is the amino acid D-arginine, which is active in the body only in the 'L' isomer. L-arginine can be generated from the breakdown of proteins with ornithine being the central intermediate product in the arginine degradative pathway. Arginine and ornithine metabolisms are crucial in bacterial homeostasis [42]. D-Amino acids are found at high levels in humans and play a role in some biological functions. D-Amino acids may be present in some bacteria or may have adverse effects because they can be formed spontaneously in some reactions. According to a study conducted on mice, it was noted that the potential of the mitochondrial membrane is reduced after mitochondrial accumulation [43]. Again, in the same article, it was shown that D-ornithine caused no membrane potential changes. In addition, many studies have highlighted the potent anti-cancer activity of the acridone nucleus. Pathway analysis was performed after a 16S microbiome study of a total of 30 stool samples from 15 patients before and after transplantation in the present project. As a result of the obtained data, two of a total of 272 pathways showed statistically significant differences between the two groups. It was observed that these two pathways were acridone alkaloid biosynthesis and metabolism of D-Arginine and D-ornithine. The conducted pathway analysis showed that a general increase was observed in acridone alkaloid biosynthesis. L-arginine is a versatile amino acid that can be utilized as both carbon and nitrogen sources for bacteria and arginine can be de-novo synthesized by bacteria from several compounds, such as glutamate and glutamine [44]. L-arginine can also be metabolized by various enzymes such as Nitric Oxide Synthase or Arginase [45]. In this research, acridone alkaloid biosynthesis and D-arginine and D-ornithine metabolism pathways significantly increased after treatment. Considering all this information, this significant increase in both pathways may reflect the increase of a specific group of bacteria, such as glutamate-utilizing bacteria, and the increased expression of related pathways. This may suggest that these pathways have been functionally affected by the treatment and by some bacterial metabolites in the microbiota. It may be more relevant to evaluate all the data together.

The diversity of the microbiota community present in the environment before the HSCT procedure, the relative increases of saccharolytic commensals such as *Blautia* and *Fusobacterium nucleatum* includes risk factors for localized mucosal damage. Such microorganisms may have a greater chance of becoming a "pathobiome", such as a 'pathogenic community adapted to becoming healthy' during pre-HSCT hospitalization and the HSCT procedure, which cannot support immunological recovery in patients [46,47].

Conclusion

In the future, supportive probiotics or prebiotics may be developed to increase the diversity of the commensal flora or control the gastrointestinal metabolome. Thus, these sensitive probiotics can be used as a biological key in patients with suspected bacteremia. Microbiota, which is regulated by non-pathogenic microorganisms, can even be used in patients who have undergone HSCT and have become immunocompromised. Dysbiosis after allo-HSCT can be treated with the enrichment of microorganisms required to prevent bacteremia and sepsis. In addition, a better understanding of the human ecosystem may allow the microbiota composition of patients to be used as a biomarker of disease. To exemplify, the microbial signature of patients may serve as a risk estimator for steroid-resistant GvHD, allowing them to start secondary treatments earlier. In general, microbiota-based therapeutics show great promise for preventing and treating GvHD and infections in patients after HSCT. It is essential to conduct further research aimed at developing targeted and individualized dysbiosis prevention and treatment regimens applicable to these patients.

This work was aimed to determine the changes in intestinal microbiota due to transplantation, and in treatment relapse, and the development of GvHD in patients with blood diseases.

The results of this study conducted in Turkey are correlated with those of similar studies in European countries and the United States. There is hope for therapeutic treatments with fecal transplantation, prebiotic support, and gut microbiota regulation for the treatment and prognosis of disease. Intestinal flora monitoring may provide guiding data on GvHD protection and/or treatment. Conducting the study with broader cohorts will contribute to the literature.

Funding information

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Data availability

The data used to support this study's findings are included within the article.

Author's contributions

The authors indicated in parentheses made substantial contributions to the following research tasks: initial conception (E.E.G.K, F.S.O), design (E.E.G.K, F.S.O, S.K.B, Z.A, O.O), provision of resources (Z.G), collection of data (E.E.G.K), analysis and interpretation of data (U.S.), and writing and revision of papers (all researchers).

Conflicts of interest

The authors declare no conflict of interest.

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Review article

Hereditary thrombocytopenias: the challenge of increasing frequency and differential diagnosis

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ABSTRACT

Hereditary thrombocytopenias are often difficult to diagnose. Since most of them are rare diseases, their characteristics are less known by physicians who deal with adults. While pediatricians are accustomed to considering genetic diseases in the differential diagnosis of diseases affecting their young patients, clinicians who treat adults often overlook this possibility. Making a definitive diagnosis usually requires complex laboratory techniques. In addition, because this is a very dynamic field half of the patients have forms that have not yet been identified with new disease definitions being made every month with next-generation sequencing (NGS). These patients are often mistakenly diagnosed with immune thrombocytopenia, and, accordingly, they are at risk of receiving unnecessary immunosuppressive therapy. Misdiagnosis is widespread in patients whose low platelet count is discovered in adulthood because, in these cases, the hereditary origin of thrombocytopenia can be overlooked. The age of manifestation and the duration/chronicity of symptoms are crucial clinical features for identifying hereditary thrombocytopenia disorders. It is important to establish the correct diagnosis because it has recently been shown that some hereditary forms of thrombocytopenia predispose to other diseases such as leukemia, renal failure, and bone marrow failure; therefore, affected individuals should be kept under close surveillance and, when necessary, treated for concomitant diseases. This review aims to determine when to suspect and how to diagnose and manage inherited thrombocytopenias. It also intends to detail the less common kinds of isolated thrombocytopenias highlighting that not all isolated thrombocytopenias that emerge in adults are immune thrombocytopenia.

Introduction

An acquired condition rather than an inherited genetic mutation is more likely to be the underlying cause of newly diagnosed thrombocytopenia in a person of any age. Autoimmune disorders, elevated platelet consumption, splenomegaly, bone marrow suppression (infectious or drug-mediated), and bone marrow failure are among the different reasons for acquired thrombocytopenia [1]. The most frequent causes of acquired thrombocytopenia will differ according to the patient's age at onset, reflecting the prevalent illnesses in each age group. Acute viral infections and immune thrombocytopenia (ITP) are more prevalent in children. Because chronic diseases and cancer are more common and diverse in adults, the differential diagnosis is rather extensive [1].

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Hereditary thrombocytopenias (HTs) are a rare, heterogeneous group of disorders that result in early-onset thrombocytopenia with marked variability in bleeding. The age at manifestation and the duration/chronicity of symptoms are crucial clinical features for identifying HT disorders. The prenatal period or the time the child starts to crawl and walk are often when acute thrombocytopenia or profound platelet dysfunction is identified. Throughout life, milder abnormalities are observed at periods of hemostatic stress (beginning of menses, surgery/trauma, delivery). However, a significant proportion of individuals with HT may not have clinically significant bleeding and are diagnosed with mild to moderate thrombocytopenia ($>20\times10^9$/L) during routine blood tests [1]. Because alterations in platelet shape can identify HTs, all patients with recently diagnosed thrombocytopenia should have their peripheral blood smear carefully examined. The diagnostic options are reduced when big platelets or microthrombocytes are identified. Furthermore, due to the fixed particle size, automatic cell counts may underreport very big and very tiny platelets.

In 1948, a patient with Bernard-Soulier syndrome (BSS), a congenital bleeding condition, was described, marking the beginning of the history of HTs [2]. Nearly 70 years later, 33 distinct types of HT caused by genetic abnormalities affecting at least 32 genes were defined as a result of developments in clinical and scientific research [2]. The advent of whole-exome sequencing (WES) and whole-genome sequencing (WGS) significantly advanced our understanding of HTs. This is a very dynamic field with new disease definitions being made every month with next-generation sequencing (NGS). Numerous national and international consortiums devoted to identifying the genes causing HT in patients who did not receive a molecular diagnosis after examining one or more candidate genes have embraced them. These high-throughput sequencing techniques have identified genes that cause illness, such as *PRKACG*, *GFI1b*, *STIM1*, *FYB*, *SLFN14*, *ETV6*, *DIAPH1*, and *SRC* [3–11]. New genes will continue to be identified in the future. This review aims to determine when to suspect and how to diagnose inherited thrombocytopenias. It also intends to detail the less common kinds of isolated thrombocytopenias highlighting the fact that not all isolated thrombocytopenias that emerge in adults are ITP.

When to suspect and how to diagnose hereditary thrombocytopenias

Inappropriate therapies and incorrect prognostic criteria might arise from misdiagnosing hereditary illnesses as acquired conditions. Furthermore, misdiagnosis hinders genetic counseling, which might impact subsequent generations. The differential diagnosis between ITP and HT can be challenging due to the absence of specific tests, particularly in patients with mild bleeding symptoms. Recently, the feasibility of using parameters of the complete blood count (CBC) to support this differential diagnosis was illustrated by a series of studies, which demonstrated and validated that the mean platelet volume (MPV) can help the segregation of patients with ITP and HT [12,13]. In recent years, new parameters have been incorporated into the CBC, including the immature platelet fraction (IPF), which represents a population of newly formed platelets containing a greater amount of residual RNA [14]. Initially, the IPF was measured by flow cytometry, and described as reticulated platelets [15]. According to Ferreira et al., IPF helps distinguish hereditary macrothrombocytopenia from ITP; it is elevated in the former when compared to both ITP and other thrombocytopenias [16]. It is still unclear what processes underlie the rise in IPF in hereditary macrothrombocytopenia. One consistent feature linked to the biological causes of thrombocytopenia in these patients may be the higher RNA content of these platelets [16]. Two characteristics of the HTs that are more commonly found in adulthood are that they do not cause spontaneous bleeding and are not syndromic at birth [17]. The abnormalities linked to thrombocytopenia are apparent from the first few months of life in the majority of syndromic types, which makes accurate diagnosis easier [18–20]. However, in some syndromic types, thrombocytopenia-related problems show up later in life, making it easy to overlook their genetic roots [21,22].

The best diagnostic approach to HTs is still debated. Mutation screening is always necessary for a conclusive diagnosis. Instead of sequencing one or a small number of genes selected based on patient features, a single-step diagnostic process that can assess all potential mutations causing HTs is now feasible. Massive sequencing, however, often identifies some genetic alterations, and it is frequently difficult to discern between pathogenetic and non-pathogenetic variations. With the use of these techniques, new HTs continue to be identified. Because NGS-targeted systems enable the simultaneous investigation of a predetermined set of genes, molecular diagnosis of hereditary illnesses may be completed more quickly and at a lower cost. Ninety percent of patients who had not previously undergone molecular-level investigation were able to have the disease-causing gene detected thanks to a targeted sequencing platform that covered 63 genes associated with thrombotic and bleeding disorders. The platform demonstrated 100 % sensitivity in identifying causal variants previously found by Sanger sequencing [2,23]. An appropriate clinical approach combined with NGS techniques may provide the best chance for diagnostic success. Since NGS is widely used because of its high throughput and high efficiency, additional gene variants linked to HT have been found. It will grow to be a significant addition to first-line diagnostic techniques like the patient's history, physical examination, and morphological evaluations. Laboratory and clinical features suggestive of HT are shown in Table 1.

Classification of hereditary thrombocytopenias: clinical, biological, and cytogenetic features

From minor disorders that may go undiagnosed even in adulthood to severe bleeding diatheses that are identified in the first few weeks of birth, HT has a wide variety of clinical manifestations. To prevent needless and sometimes hazardous therapies, it is crucial to differentiate between HT and acquired thrombocytopenia, particularly ITP, for the category of disorders. An effective diagnosis of HT cannot ignore the meticulous collection of personal and family history, careful physical examination, and analysis of peripheral blood smears. The HT categories that work best for diagnostic reasons are those based on the existence of other abnormalities

Table 1 – Laboratory and clinical features suggestive of hereditary thrombocytopenia.

Clinical features suggestive of hereditary thrombocytopenia
Patients with hereditary thrombocytopenia vary widely in the presence and intensity of their bleeding propensity
Lifelong bleeding/bruising/petechiae symptoms
Presence of thrombocytopenia that is not related to the patient's concomitant disease or medications
Congenital defects with hereditary thrombocytopenias include skeletal deformity, cognitive impairment, central nervous or cardiovascular system malformations, and immunodeficiency
Hereditary thrombocytopenia increases the risk of developing additional diseases, including renal failure or leukemia
Having a family history of bleeding or increased thrombocytopenia
Absence of normal platelet values in a complete blood count
No response or minor response to treatment modalities such as steroids, ıntravenous ımmunoglobulin, anti-D, splenectomy
Response to platelet transfusions shows good growth/normal survival
Laboratory features suggestive of hereditary thrombocytopenia
In addition to normal platelet size, small, large, and giant platelets may be seen in peripheral smear
Pale platelets
Döhle bodies, which are protein clumps in neutrophils,
Red cell stomatocytosis
Anemia, high hemoglobin level or high leukocyte count

in addition to thrombocytopenia and platelet size, which vary significantly across the different types. Even if NGS is becoming more widely available, a logical and economical method of diagnosing HTs is still needed. Molecular analysis is necessary to confirm the diagnosis and offer patients individualized treatment, counseling, and follow-up. At the very least, a definite diagnosis must always be sought for persons in whom a predisposing form is suspected. The large number of sporadic instances caused by de novo mutations in some conditions and the inadequate penetrance of the same mutations, frequently making it difficult to discern the inheritance pattern, limits the usefulness of the inheritance-based categorization. In this article, HTs are classified into three groups based on more recent perspectives and research on the condition as depicted in Figure 1: forms that only have the platelet defect; disorders in which the platelet phenotype is

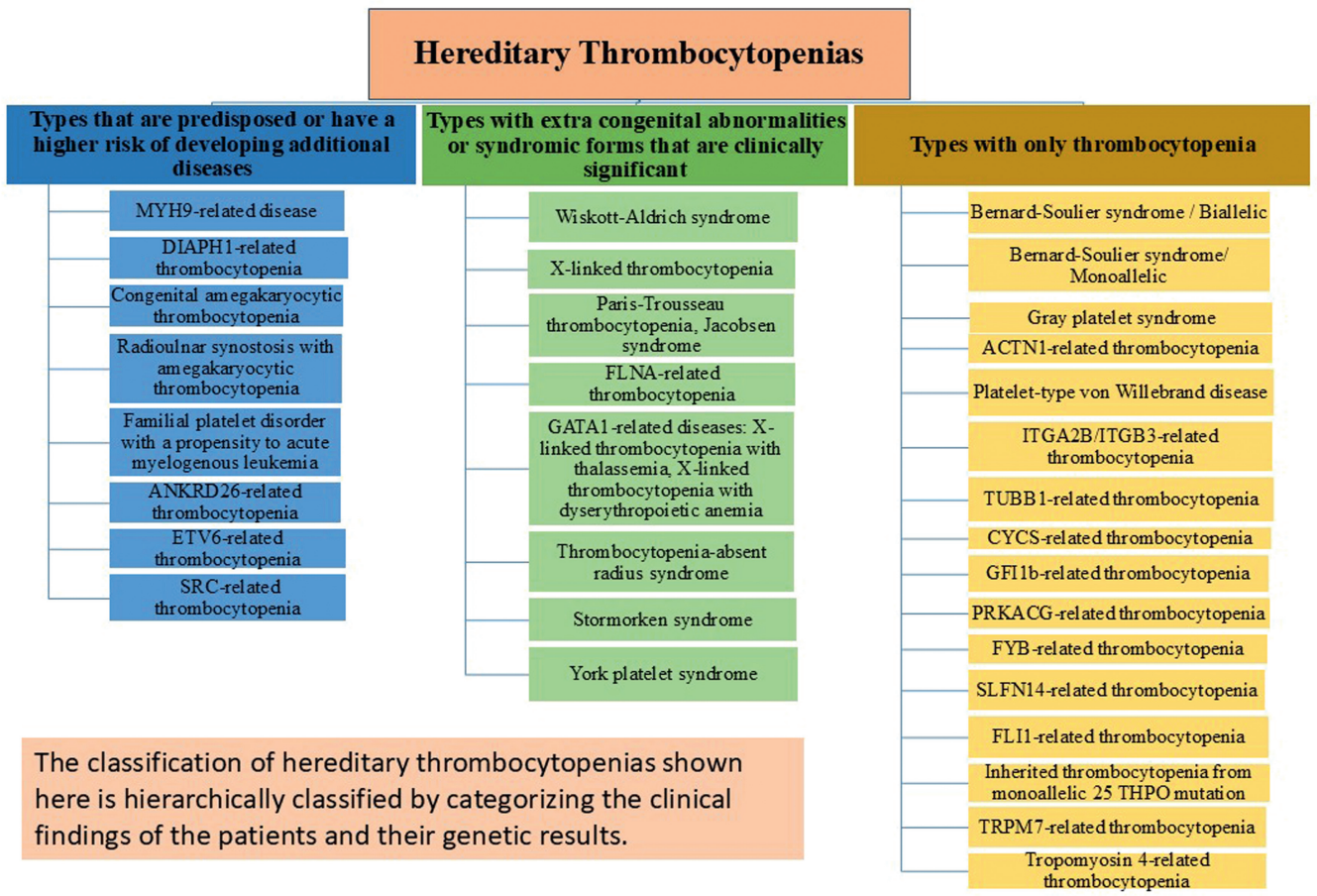

Figure 1 – Classification of Hereditary Thrombocytopenias based on more recent perspectives and research.

associated with other congenital defects (syndromic forms); and forms that have an elevated risk of developing other diseases in life [2,3,11,20,24–46]. As a result, this categorization has both diagnostic and prognostic value.

Types that are predisposed or have a higher risk of developing additional diseases

The distribution of hereditary thrombocytopenia types associated with an elevated risk of developing secondary comorbidities or related clinical disorders is delineated in Table 2. A non-syndromic type of macrothrombocytopenia with a tendency to bleed generally correlated with the severity of platelet shortage is the initial manifestation of *myosin*-9-related disease (*MYH9*-RD) [21]. This diagnosis is supported by the finding of Döhle-like structures in the neutrophil cytoplasm [47]. Monoallelic mutations in *DIAPH1*, a gene implicated in proplatelet expansion and cytoskeleton assembly, cause abnormally big platelets in *DIAPH1*-related thrombocytopenia [10]. Congenital megakaryocytic thrombocytopenia (CAMT) typically manifests as isolated, severe, and symptomatic thrombocytopenia from birth. If a hematopoietic stem cell transplant (HSCT) is unsuccessful, this condition almost always progresses into bone marrow aplasia during childhood and results in death before adulthood [44]. In addition to additional skeletal abnormalities and/or sensorineural deafness, radio-ulnar synostosis with amegakaryocytic thrombocytopenia disease is typified by congenital bilateral or unilateral proximal synostosis of the radius and ulna, which results in restricted forearm supination [45]. *ANKRD26*-related thrombocytopenia (*ANKRD26*-RT), *ETV6*-related thrombocytopenia (*ETV6*-RT), and familial platelet disorder predisposing to acute myeloid leukemia (FPD/AML) are three HT linked to specific predispositions to hematological malignancies [13,48]. Platelets in *SRC*-related thrombocytopenia are dysmorphic, exhibit a wide range of sizes, and have few granules [11].

Types with extra congenital abnormalities or syndromic forms that are clinically significant

Clinically significant hereditary thrombocytopenia types with extra congenital abnormalities or syndromic forms are listed in Table 3. Almost primarily affecting men, Wiskott-Aldrich syndrome (WAS) is characterized by moderate to severe thrombocytopenia, immunosuppression, and eczema. Microthrombocytes, with a steady platelet count of $5.0–50.0\times10^9$/L and a markedly decreased mean platelet volume, are a defining feature of various associated illnesses [49]. In Jacobsen syndrome (and Paris-Trousseau thrombocytopenia, a major and consistent feature of Jacobsen syndrome), probably, the psychomotor retardation and facial and cardiac defects characteristic of Jacobsen syndrome are caused by the loss of a contiguous gene [50,51]. Periventricular nodular heterotopia (PNH) and the otopalatodigital syndrome spectrum diseases, which include congenital deformities, mental retardation, and skeletal abnormalities, are the two primary phenotypes associated with *FLNA* mutations [52]. From birth, patients with *GATA*-related diseases are anemic and have symptoms of severe thrombocytopenia. The bone marrow includes

Table 2 – Hereditary thrombocytopenia types that are predisposed or have a higher risk of developing additional diseases.

Disease name	Genetic transmission type/Gene	The size of platelets	Clinical features
MYH9-related disease [24]	AD/*MYH9* (22q12)	Giant, large	Nephropathy, cataracts, and/or sensorineural deafness could develop. Elevated liver enzymes are detected in half of the patients
DIAPH1-related thrombocytopenia [10]	AD/*DIAPH1* (5q31.3)	Large	Infantile sensorineural deafness risk. Leukopenia may be moderate and temporary
Congenital amegakaryocytic thrombocytopenia [44]	AR/MPL (1p34.2)	Normal, somewhat diminished	Diminished or nonexistent megakaryocytes. All patients develop significant bone marrow aplasia in infancy
Radioulnar synostosis with amegakaryocytic thrombocytopenia [20,45]	AD/AR/ *HOXA11* (7p15) or *MECOM* (3q26.2)	Normal, somewhat elevated	Megakaryocytes in the bone marrow are diminished or nonexistent. Potential development of bone marrow aplasia. Due to *MECOM* mutations, the hematological phenotype is more severe in the AR variant
Familial platelet disorder with a propensity to acute myelogenous leukemia [46]	AD/*RUNX1* (21q22)	Normal, somewhat elevated	More than 40 % of patients develop myelodysplastic syndromes or acute myelogenous leukemia. T-cell acute lymphoblastic leukemia risk is elevated. Reduced platelet activity
ANKRD26-related thrombocytopenia [26]	AD/*ANKRD26* (10p12)	Normal, somewhat elevated	Approximately 8 % of patients develop myeloid cancers. Hemoglobin and/or leukocyte counts are elevated in certain cases. Decreased granules in certain patients
ETV6-related thrombocytopenia [9]	AD/*ETV6* (12p13)	Normal, somewhat elevated	Acute lymphoblastic leukemia and other hematological malignancies affect about 25 % of patients
SRC-related thrombocytopenia [11]	AD/*SRC* (20q11.23)	Large	Severe osteoporosis, premature edentulism, juvenile myelofibrosis and splenomegaly, and congenital facial dysmorphism. Agranular or hypogranular platelets. lots of vacuoles

AD: Autosomal dominant; AR: Autosomal recessive.

Table 3 – Hereditary thrombocytopenia types with extra congenital abnormalities or syndromic forms that are clinically significant.

Disease name	Genetic transmission type/Gene	The size of platelets	Clinical features
Wiskott-Aldrich syndrome [37]	XL/WAS (Xp11)	Normal, somewhat diminished	Severe immunodeficiency that results in premature mortality. Eczema. An elevated risk of cancer and autoimmune diseases
X-linked thrombocytopenia [38]	XL/WAS (Xp11)	Normal, somewhat diminished	Mild lack of immunity. mild, temporary eczema. Elevated risk of autoimmunity and cancer. There are descriptions of non-syndromic patients who solely have thrombocytopenia
Paris-Trousseau thrombocytopenia, Jacobsen syndrome [39]	AD/Deletions in 11q23	Normal, somewhat elevated	Anomalies of the cardiovascular system, central nervous system, gastrointestinal tract, kidney, and/or urinary tract; growth retardation; cognitive impairment; facial and skull dysmorphisms; and other abnormalities. Reduced platelet activity. decreased dense granules and giant a-granules. Over time, thrombocytopenia may go away.
FLNA-related thrombocytopenia [40]	XL/*FLNA* (Xq28)	Large	Heterotopia of periventricular nodules. There are reports of non-syndromic patients who solely have thrombocytopenia.
GATA1-related diseases: X-linked thrombocytopenia with thalassemia, X-linked thrombocytopenia with dyserythropoietic anemia [41]	XL/*GATA1* (Xp11)	Normal, somewhat elevated	Hemolytic anemia with splenomegaly, dyserythropoietic anemia, and laboratory abnormalities similar to β-thalassemia. Congenital erythropoietic porphyria.
Thrombocytopenia-absent radius syndrome [42]	AR/*RBM8A* (1q21)	Normal, somewhat diminished	Further anomalies in the upper and lower limbs, as well as bilateral radial aplasia. Potential heart, renal, and/or central nervous system abnormalities. An increase in thrombocytopenia may be linked to a potential cow's milk intolerance. Megakaryocytes in bone marrow are decreased. Over time, the platelet count may increase.
Stormorken syndrome [6]	AD/*STIM1* (11p15)	Not available	Tubular aggregation myopathy, congenital miosis, headache, mild anemia, facial dysmorphisms, anatomical or functional asplenia, ichthyosis, physical growth abnormalities, and cognitive impairment
York platelet syndrome [43]	AD/*STIM1* (11p15)	Normal	Myopathy, anomalies in the ultrastructure of platelets, including enormous electron-dense and target-like entities, increased vacuoles, and a moderate decrease in granules

AD: Autosomal dominant; AR: Autosomal recessive; XL: X-linked.

many tiny, dysplastic megakaryocytes in addition to dyserythropoiesis and dysplastic platelet alterations [53]. Bilateral radial aplasia is the defining feature of thrombocytopenia with missing radius syndrome, however, most patients may also have other anatomical abnormalities [54]. The endoplasmic reticulum protein that *STIM1* encodes controls the entry of Ca^{2+} into cells via the calcium release-activated channels of the plasma membrane in a variety of cell types. In Stormorken syndrome and York platelet syndrome, platelets exhibit many in vitro abnormalities related to aggregation, activation, and granule secretion [6,20].

Forms with only thrombocytopenia

Bernard-Soulier syndrome, the most prevalent isolated form of thrombocytopenia, is characterized by recurrent and spontaneous hemorrhages and is frequently fatal since it is linked to severe platelet function abnormalities and thrombocytopenia [28].

Patients with HT vary widely in the presence and intensity of their bleeding tendency. While some patients may only experience hemorrhages during hemostatic challenges, others may present with no bleeding at all. A small percentage of individuals experience spontaneous bleeding at first, and some may even experience potentially fatal bleeding episodes. Petechiae, ecchymoses, epistaxis, menorrhagia, and gastrointestinal hemorrhages are all examples of mucocutaneous bleeding. In those HTs that are not linked to severe platelet dysfunction, the degree of bleeding tendency correlates with the platelet count [24]. On the other hand, in forms linked to altered platelet function, the tendency to hemorrhage is typically more severe than anticipated based on platelet count [28]. For the vast majority of HTs, the severity of thrombocytopenia does not change throughout the patients' lives. A classification of hereditary thrombocytopenias presenting with isolated thrombocytopenia is provided in Table 4.

Treatment and follow-up of individuals with hereditary Thrombocytopenias

The treatment and follow-up of these patients is a very important issue in itself and should be discussed in detail in

Table 4 – Types of hereditary thrombocytopenia presenting with only thrombocytopenia.

Disease name	Genetic transmission type/Gene	The size of platelets	Clinical features
Bernard-Soulier syndrome/Biallelic [28]	AR/*GP1BA* (17p13) *GPIBB* (22q11)	Giant, large	Platelet function impairment
Bernard-Soulier syndrome/ Monoallelic [25]	AD/*GP9* (3q21)	Large	–
Gray platelet syndrome [3]	AR/*NBEAL2* (3p21)	Large	Impaired function of platelets. Greatly decreased granule content. Over time, the platelet count declines. development of splenomegaly and increasing bone marrow fibrosis. Elevated amounts of vitamin B12 in the serum.
ACTN1-related thrombocytopenia [29]	AD/*ACTN1* (14q24)	Large	–
Platelet-type von Willebrand disease [30]	AD/*GP1BA* (17p13)	Normal, somewhat elevated	Most patients have a normal platelet count, but in stressful situations (pregnancy, surgery, and infection), it might drop significantly.
ITGA2B/ITGB3-related thrombocytopenia [31]	AD/*TGA2B* (17q21) 1,11/ *ITGB3* (17q21)	Large	Impaired function of platelets
TUBB1-related thrombocytopenia [32]	AD/*TUBB1* (20q13)	Large	–
CYCS-related thrombocytopenia [33]	AD/*CYCS* (7p15)	Normal, somewhat diminished	–
GFI1b-related thrombocytopenia [5]	AD/*GFI1B* (9q34)	Large	Impaired function of platelets. Granules are reduced. Anisocytosis of red cells
PRKACG-related thrombocytopenia [4]	AR/*PRKACG* (9q21)	Large	Impaired function of platelets
FYB-related thrombocytopenia [7]	AR/*FYB* (5p13.1)	Normal, somewhat diminished	–
SLFN14-related thrombocytopenia [8]	AD/*SLFN14* (17q12)	Normal, large	Impaired function of platelets
FLI1-related thrombocytopenia [34]	AR/*FLI1* (11p24.3)	Large	Impaired function of platelets. Enormous granules.
Inherited thrombocytopenia from monoallelic 25 *THPO* mutation [35]	AD/*THPO* (3q27.1)	Normal, somewhat elevated	–
TRPM7-related thrombocytopenia [36]	AD/*TRPM7* (15q21.2)	Large	Increasing number of microtubules, anarchic microtubule architecture, and aberrant granule dispersion.
Tropomyosin 4-related thrombocytopenia [27]	AD/*TPM4* (19p13.1)	Large	–

AD: Autosomal dominant; AR: Autosomal recessive

another article. Avoiding the use of medications that might reveal the platelet abnormality is a crucial first step [55]. Thus, it is important to carefully weigh the advantages and disadvantages of various therapies. Preventing the need for medical procedures that increase the risk of bleeding is another crucial step. New therapy avenues in this area were made possible by the recent discovery that eltrombopag, an oral nonpeptide agonist of the thrombopoietin receptor, might raise the platelet count in a small case series of individuals with *MYH9*-RD [56]. Patients with HTs who are predisposed or have a higher risk of developing additional diseases need close follow-up, whereas individuals with HTs who are not at risk of developing new disorders need medical care for hemostatic problems or bleeding episodes [57].

Conclusions and future perspectives for the hereditary thrombocytopenias

Once thought to be rare, HT syndromes are now more widely recognized as a group of clinical abnormalities ranging from mild disorders discovered incidentally in adults to severe diseases in neonates. In recent years, our knowledge of HTs has shown that these syndromes will not only present with thrombocytopenia and bleeding disorders, but also with complex diseases and even malignancies, bone marrow failure, and renal, hearing, and vision disorders. A multidisciplinary approach to patients with HT is needed, taking into account additional disorders that have already developed or are at risk of developing soon. The classification of HTs should be evaluated together with the clinical findings of the patient and categorized together with the genetic results. Studying the clinical features of patients and identifying new genes and their association will greatly contribute to the understanding of the pathophysiology of HT syndromes and the development of new treatments.

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Review article

Lymphoma-associated hemophagocytic lymphohistiocytosis

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ABSTRACT

Hemophagocytic lymphohistiocytosis is a severe, rare condition characterized by excessive immune activation, leading to significant morbidity and mortality. Lymphoma is the most common trigger for malignancy-related hemophagocytic lymphohistiocytosis in adults, with large B-cell non-Hodgkin, T- and NK-cell lymphomas being the most diagnosed. Hodgkin lymphoma is less frequently observed. Lymphoma-associated hemophagocytic lymphohistiocytosis poses diagnostic and therapeutic challenges due to its complex pathogenesis and heterogeneous presentation. Treatment aims to control the overactive immune system, identify and treat modifying factors, optimize clinical support, and treat the underlying lymphoma. Early etoposide (Etoposide) combined with dexamethasone for immunomodulation results in rapid control of hyperinflammation and clinical improvement. It has increasingly been adopted as a standard initial approach followed by lymphoma-specific treatment. However, the outcomes for patients with lymphoma-associated hemophagocytic lymphohistiocytosis remain poor, especially for patients with T- and NK-cell lymphomas. In relapsed or refractory cases, emerging therapies have been explored, with ruxolitinib showing the most promising results. This paper reviews current understanding of the epidemiology, pathogenesis, clinical features, diagnosis, and treatment of lymphoma-associated hemophagocytic lymphohistiocytosis in adults and proposes an appropriate treatment protocol based on the most recent data from the literature.

Introduction

Hemophagocytic lymphohistiocytosis (HLH) is a spectrum of conditions characterized by intense, pathological immune activation with clinical manifestations such as extreme

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inflammation, hemophagocytosis, and end-organ damage. HLH is rare and is most frequently diagnosed in children, but 30–40 % of cases are observed in adults [1]. HLH is classified as an H-group histiocytic disorder and is historically divided into primary or secondary HLH.

Primary HLH is predominantly observed in children whose genetic defects lead to inflammasome impairment or disturbance, particularly of cytotoxic T-cells and natural killer (NK) cells. Secondary HLH is mainly observed in adults, with immune activation being caused by external triggers, including persistent infection, autoimmune conditions, and malignancy [2,3]. However, recent findings suggest that 40 % of adult patients have genetic abnormalities that mainly affect the perforin cytosolic pathway. Based on its pathophysiology, HLH is currently defined as a clinical spectrum of conditions with a predisposition to hyperinflammation with predisposing factors that include genetic defects, immune impairment, and acute triggers. [4,5]

Malignancy is the leading cause of HLH in adults, diagnosed in up to 50 % of cases. Hematological neoplasms and lymphomas are the most common triggers of malignancy-associated HLH (M-HLH) in adults [1,6]. This paper reviews the pathogenesis and diagnosis of lymphoma-associated HLH (L-HLH) in adults and proposes an appropriate treatment protocol based on the most recent data from the literature.

Epidemiology

HLH is a rare and often underdiagnosed disease. Therefore, the incidence and prevalence in adults remain unclear [7,8]. Available studies based on small cohorts estimate an annual incidence lower than 1 case per 100,000 people per year [1,9]. The median age is approximately 50 years, with a clear predominance of females (7:1).

In assessing the epidemiology of M-HLH, a Swedish population-based study analyzed all patients with neoplastic and histiocytic disorders registered in the national database and reported an incidence of 0.21 cases per 100,000 inhabitants, with a male predominance. Lymphoma was the most common malignancy diagnosed in M-HLH [10].

In hematologic malignancies, approximately 1 % develop HLH, detected either at the time of initial cancer diagnosis or during treatment [9]. Lymphomas are the most common triggers, accounting for 45–50 % of patients. Of these, T- and NK-cell lymphomas/leukemias are diagnosed in approximately 35 % of cases; large B-cell non-Hodgkin lymphomas (NHL) in 32 %; and Hodgkin lymphoma in 6 % [1]. Uncommon subtypes of B- (intravascular B-cell lymphoma) and T-cell (nasal NK/T-cell, angioimmunoblastic T-cell, gamma/delta T-cell, and subcutaneous panniculitis-like T-cell lymphoma) NHL must also be considered because they account for 20 % of adults with L-HLH [1,11].

A key epidemiological finding is the geographic variability in the incidence of different subtypes of lymphomas associated with HLH. T-cell and NK-cell lymphomas prevail in Eastern countries, such as China and Japan. In contrast, Western countries tend towards an equal distribution of B- and T-cell lymphomas [1]. The reasons for this geographic variability are unclear, but they may be related to Epstein-Barr virus infection, which is more prevalent in eastern countries, and genetic differences between these populations [12,13]. A recent study evaluated 173 patients with intravascular B-cell lymphoma, of whom 50 were from Western countries and 123 from Eastern countries. None of the patients from Western countries presented with L-HLH in this series. Conversely, 45 patients from eastern countries were diagnosed with L-HLH at some point. These findings suggest a genetic predisposition to L-HLH [14].

HLH can also be triggered by lymphoma treatment (known as treatment-related HLH), including chemotherapy, hematopoietic stem cell transplantation (HSCT), and, more recently, checkpoint inhibitors and targeted cell therapy, such as chimeric antigen receptor (CAR) T-cell and bispecific antibody therapy. The incidence is highly variable, reaching 30 % in some series. Moreover, the diagnosis is challenging due to potential confounding factors, such as lymphoma activity and secondary infections [15,16].

Lymphoma-associated hemophagocytic lymphohistiocytosis pathogenesis

Under normal physiology, the immune response is an orchestrated process involving interactions between immune cells and proteins, such as granulocytes, lymphocytes, macrophages, immunoglobulins, cytokines, and complement molecules. Each condition that increases the inflammatory response is counteracted by a process that avoids excessive and dangerous immune stimulation, which could lead to tissue destruction. In HLH, this process is disrupted, particularly the autoregulatory mechanisms, resulting in excessive and persistent inflammation and organ damage [3,17].

Primary (or familial) HLH is considered a model for understanding the pathophysiology of this condition. In these patients, recessive mutations in genes involved in T- and NK-cell cytotoxicity cause defective perforin and granzyme secretion, impairing the ability to clear the antigenic stimulus and downregulate the inflammatory response [4,15,18]. In turn, the pathophysiology of secondary (or acquired) HLH is not entirely understood and is likely to be multifactorial. One of the main mechanisms proposed so far is the constant presence of an antigenic stimulus, resulting in $CD8^+$ T- and NK-cell hyperactivation and, consequently, excessive secretion of proinflammatory cytokines and increased macrophage activation. Furthermore, hereditary genetic alterations associated with immune response defects previously considered only in primary HLH have also been identified in secondary HLH and may contribute to its pathogenesis [4,8,18–21].

In M-HLH, particularly L-HLH, the immune dysfunction is also intrinsic to neoplasia or triggered by the different treatment modalities, leading to immune activation and loss of immune inhibitory function [19].

Finally, increased predisposition to bacterial and viral infection at diagnosis and during lymphoma treatment is a significant risk factor for L-HLH. Among the primary pathogens, Epstein-Barr virus stands out because chronic infection with this virus is directly related to the development of some types of lymphomas, such as Burkitt and T-cell lymphomas,

and plays a key role in regulating the immune response to neoplasias [13,22].

Clinical presentation and diagnosis

L-HLH has a challenging diagnosis; it may be detected at diagnosis, or during relapse or treatment of lymphoma. L-HLH is often triggered by an uncommon lymphoma histological subtype with an atypical clinical presentation [11]. Moreover, some signs and symptoms of L-HLH are not specific to this disease and may be explained by lymphoma or because of treatment.

Clinically, L-HLH presents as an acute or subacute febrile condition associated with multiple organ dysfunction. Fever is present in >90 % of patients, usually associated with cytopenia in at least two lineages [6,23]. Other commonly observed findings in L-HLH include markedly elevated lactate dehydrogenase (LDH), ferritin, and soluble CD25 (sCD25), which are almost universally present [23]. Organic involvement may manifest as hepatocellular injury, which may or may not be associated with impaired liver function, hepatosplenomegaly, neurological changes, skin lesions, bleeding, or acute respiratory distress syndrome [1,7,11,24,25]. Moreover, most patients with L-HLH are diagnosed in advanced Ann-Arbor stages and frequently have lymphoma infiltration in the bone marrow and other extranodal sites, like the liver and skin [23].

Different criteria grouped into scoring protocols have been proposed for a more accurate diagnosis of HLH. These are also used for the diagnosis of L-HLH. The oldest and most widely used are the revised HLH-2004 diagnostic criteria from the Histiocyte Society, which are based on eight clinical, laboratory, and cytopathological criteria (Table 1). Diagnostic confirmation requires meeting at least five of the eight criteria [26].

These scoring protocols were initially developed for children with familial HLH. While they capture the most frequently observed alterations in adult HLH, their sensitivity and specificity have not been prospectively validated in adults. Additionally, HLH-2004 uses nonspecific parameters that may overlap with other inflammatory conditions or manifestations of lymphoma and overlooks alterations frequently observed in HLH, such as increased transaminase, lactic dehydrogenase, D-dimer, and C-reactive protein levels and neurological manifestations. Therefore, while HLH-2004 should be applied in clinical practice, the findings should be interpreted with caution, particularly where there is high suspicion of HLH without fully meeting the criteria [11,20,27].

Alternative guidelines have been proposed to improve HLH diagnosis in adults. Based on data from a retrospective cohort, the HScore was developed to define and predict the likelihood of adult HLH. After weighing the significant clinical and laboratory parameters, the authors identified an optimal cutoff of 169 points with 93 % sensitivity and 86 % specificity (Table 2). Notably, 44 % of the study cohort were patients with cancer [28]. In a retrospective analysis, the accuracy of the HScore was directly compared with that of HLH-2004, with the former outperforming the latter, reaching 90 % sensitivity and 79 % specificity for adults at the initial presentation. However, the values were similar when clinical status was worse. Moreover, the optimal cutoff can be affected by the trigger and pattern of the individual inflammatory response. These factors may impact the sensitivity and specificity of the HScore in different cohorts [29,30].

In a retrospective database analysis that included only patients with complete documentation of cancer, HLH-2004 criteria were compared with extended diagnostic criteria comprising 18 variables. The authors of this study, conducted at the MD Anderson Cancer Center, University of TX, reported that among patients with suspected HLH, only 21 % met the standard HLH-2004 diagnostic criteria, whereas 57 % met the extended diagnostic criteria. No significant difference in outcome (overall survival [OS]) was found between the 13 patients who met the HLH-2004 criteria and the 20 patients who did not meet the HLH-2004 criteria but met the extended 18-point HLH criteria, suggesting that these patients are likely to have had a more aggressive systemic process. Case in point, the OS was significantly improved among the 26 patients with hemophagocytosis or lymphohistiocytosis

Table 1 – HLH-2004 diagnostic guidelines for hemophagocytic lymphohistiocytosis.

At least five of the following criteria:
Fever
Splenomegaly
Cytopenias (affecting at least two lineages):
Hemoglobin <9 g/dL
Platelets <100.000/mm^3
Neutrophils <1.000/mm^3
Hypertriglyceridemia and/or hypofibrinogenemia
Fasting triglycerides ≥265 mg/dL
fibrinogen <150 mg/dL
Hemophagocytosis in bone marrow, spleen, liver or lymph nodes
Ferritin >500 mg/dL
Reduced or absent NK-cell function
Increased serum levels of CD25 (soluble IL-2 receptor) ≥2400 U/mL

Table 2 – HScore.

Parameter	Points for scoring
1. Fever (°C)	0 (<38.4), 33 (38.4 −39.4) or 49 (>39.4)
2. Cytopenia	0 (1 lineage), 24 (2), 34 (3)
3. Organomegaly	0 (no), 23 (hepatomegaly or splenomegaly) or 38 (hepatomegaly and splenomegaly)
4. Ferritin (ng/mL)	0 (<2000), 35 (2000−6000) or 50 (>6000)
5. Fibrinogen (mg/dL)	0 (>250) or 30 (≤250)
6. Triglycerides (mg/dL)	0 (<150), 44 (150−400), 64 (>400)
7. Aspartate aminotransferase	0 (<30) or 19 (≥30)
8. Hemophagocytosis on biopsy	0 (no) or 35 (yes)
9. Known underlying immunosuppression	0 (no) or 18 (yes)

on pathological examination but failed to meet either HLH-2004 or expanded HLH criteria [31].

More recently, to improve and simplify the specific diagnosis of M-HLH, the roles of sCD25 and ferritin as potential diagnostic biomarkers were studied in a multicenter retrospective cohort of 225 patients [32]. Patients with and without HLH were included, all of whom met the HLH-2004 criteria. Among different HLH diagnostic parameters, the optimized HLH inflammatory (OHI) index, a composite score defined by the simultaneous elevation of sCD25 (>3900 U/mL) and ferritin (>1000 ng/mL), provides an accurate diagnosis, yielding a prognostic tool with 84 % sensitivity and 81 % specificity. OHI highly predicted mortality across hematologic malignancies, but this combined index still requires validation in larger cohorts. The kinetics of sCD25 have also been explored as a predictor of survival. Verkamp et al. demonstrated that the failure to improve sCD25 from baseline strongly predicted survival in children and young adults treated with etoposide (Etoposide)-based therapy. Additionally, the combination of sCD25 with other biomarkers such as platelet count, absolute lymphocyte count, and blood urea nitrogen also predicted mortality, suggesting a potential role in the early identification of high-risk patients [33].

Despite these tools, diagnosis of L-HLH remains challenging because symptoms are usually nonspecific, and lymphoma can be difficult to detect on physical examination. For an early diagnosis, physicians must identify hyperinflammation based on clinical and laboratory findings, including rapid clinical deterioration, persistent fever, a high ferritin level, and cytopenia. Atypical clinical presentations and less frequent histological subtypes should also be considered (Table 3). Ancillary methods, such as positron emission tomography-computed tomography-guided tissue biopsy, bone marrow biopsy, and flow cytometry, may facilitate the diagnosis. Another relevant issue is the limited access to specific diagnostic tests required by the HLH-2004 criteria, such as sCD25 levels and NK cell activity, particularly in resource-limited settings. Without a gold standard scoring protocol for diagnosing L-HLH, new and accurate biomarkers must be urgently developed to provide rapid confirmation of diagnosis and timely initiation of treatment towards improving outcomes for patients with L-HLH.

Table 3 – The most common lymphoma subtypes associated with lymphoma-associated hemophagocytic lymphohistiocytosis.

T-cell and NK-cell lymphomas	B-cell lymphomas
NK/T-cell lymphoma, aggressive NK cell leukemia	DLBCL
Peripheral T-cell lymphoma	Intravascular B-cell lymphoma
Anaplastic large cell lymphoma;	Indolent lymphomas (FL, MZL)
Angioimmunoblastic T-cell lymphoma	Burkitt lymphoma
Panniculitis-like T-cell NHL	Hodgkin lymphoma
Gamma-delta T-cell lymphoma	

NK: Natural killer; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; MZL: marginal zone lymphoma.

Treatment

L-HLH is a life-threatening condition with challenging treatment. Most patients undergoing treatment are aged and very ill, with chemotherapy and immunosuppressive therapy increasing the risk of complications. The condition frequently requires rapid intervention, so measures to improve health status before initiating treatment cannot be implemented. L-HLH treatment aims to control the overactive immune system, identify and treat modifying factors, optimize clinical support, and treat lymphoma [5,34,35] (Figure 1). However, due to the lack of prospective, randomized, or controlled clinical trials, there is no consensus on whether an HLH, malignancy-directed, or combined approach should be adopted first. Most available data derive from very small retrospective series and case reports, with potential selection bias. The strength of the recommendations is usually based on expert opinions [11,20,36].

In general, the prognosis of patients with L-HLH is poor. Different series in the literature show heterogeneous data, with mean OS ranging from one to 12 months. The main causes of death are lymphoma and associated infections [9,22,37–42] (Table 4). The main contributing factors to the unfavorable outcomes of L-HLH are late diagnosis and, consequently, a delay in providing adequate therapy, in addition to the heterogeneity of approaches resulting from the lack of randomized studies [22,38,39,42–44].

Moreover, the subtype of lymphoma seems to be a relevant prognostic factor. Even in the context of L-HLH, B-cell lymphomas are usually associated with a better prognosis than NK/T-Cell lymphomas. In a multicenter retrospective study conducted in Japan with 132 cases of M-HLH, 108 patients had L-HLH, with 48.2 % and 12.2 % five-year OS for B-cell lymphoma and NK/T-cell lymphoma, respectively [44]. In another study, Wang et al. assessed the role of chemotherapy with a dose-adjusted etoposide (Etoposide) phosphate, prednisone, vincristine sulfate (Oncovin), cyclophosphamide, and doxorubicin hydrochloride (hydroxydaunorubicin) (DA-EPOCH) regimen in 55 patients with B-cell non-Hodgkin lymphoma (B-NHL), most of which were diffuse large B-cell lymphoma, and different subtypes of T-cell-NHL. The patients with B-NHL were more tolerant to treatment and received more treatment cycles, with five-year OS reaching 73 %. Conversely, patients with T-cell-NHL were less tolerant to treatment and responded less well to treatment, with only 3 % of patients surviving after 12 months [45]. Whether this difference derives from the increased clinical aggressiveness of NK/T-cell lymphoma or differences in the therapeutic approach to these subtypes, particularly the availability of anti-CD20 monoclonal antibodies for B-NHL, remains unclear.

Historically, the treatment of choice for HLH is based on the HLH-94 protocol, initially designed for children, but which has since proved feasible in adults, albeit more toxic and with lower response rates [37]. The main concept of this protocol is the weekly administration of etoposide (Etoposide) combined

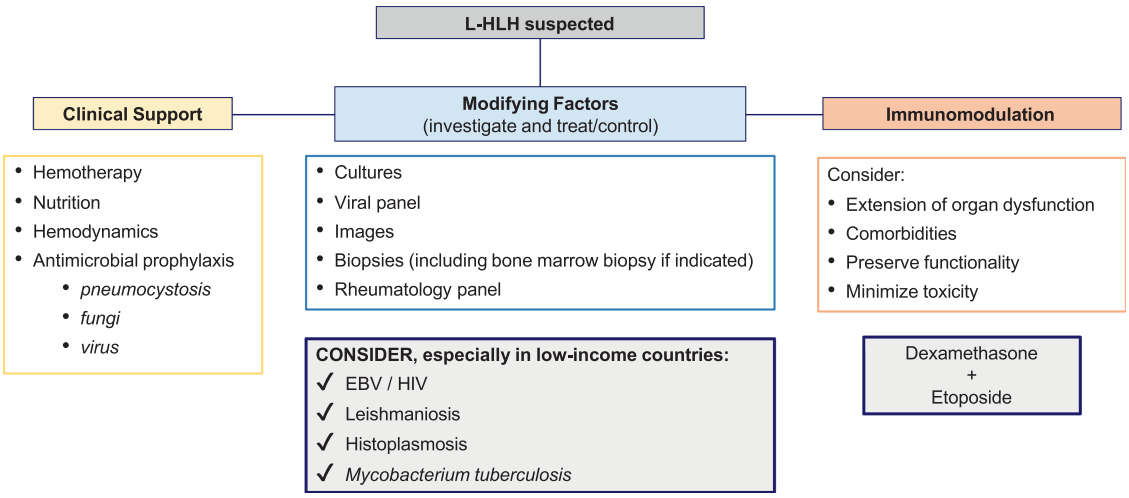

Figure 1 – General Principles of Lymphoma-associated hemophagocytic lymphohistiocytosis (L-HLH) Treatment. The mainstay of L-HLH treatment is to control the overactive immune system, identify and treat modifying factors, optimize clinical support, and treat lymphoma. EBV: Epstein–Barr virus.

Table 4 – Retrospective studies including lymphoma-associated hemophagocytic lymphohistiocytosis patients and overall survival outcomes.

Study	Population	Outcome
Han AR, et al. 2007	Retrospective, single-center, Korea *n* = 52 HLH 29 L-HLH 83 % T-cell lymphomas	Median OS = 36 days
Li F, et el. 2014	Retrospective, single-center, China *n* = 69 HLH 16 L-HLH, 15 T/NK-cell lymphomas	Median OS = 37 days
Yu JT, et el. 2013	Retrospective, single-center, China *n* = 30 L-HLH 69 % T-cell lymphomas	Median OS B-cell = 11 months Median OS T-cell = 3 months T-cell usually refractory to first line chemotherapy
Wei L, et al. 2020	Retrospective, single-center, China *n* = 43 All ENKTL	OS 34.4 % at 6 months All received etoposide (Etoposide) and/or dexamethasone-based treatment
Jin Z, et al. 2020	*n* = 8 All Hodgkin lymphomas	12-month OS = 56.3 %
Li B, et al. 2020	Retrospective, two centers in China *n* = 31 All B-cell NHL	Median OS = 1.5 months

OS: overall survival; ENKTL: extranodal natural-killer T-cell lymphoma; NHL: non-Hodgkin lymphoma.

with dexamethasone, for its high potential for immunosuppression and, particularly, macrophage activity suppression, and the subsequent addition of cyclosporine (Cyclosporine capsules). A 6.2-year follow-up of the original study showed 54 % OS, which was higher for children undergoing consolidative allogeneic bone marrow transplantation (BMT) [46].

An updated HLH-2004 protocol has been proposed to improve response rates to HLH-94. The updated version primarily differed in bringing forward cyclosporine (Cyclosporine capsules) administration to the first few weeks of treatment. However, there was no additional clinical benefit. In fact, the strategy proved to be more toxic than the original protocol [47].

Although the HLH-94 protocol has not been validated for adult patients with L-HLH, some modified versions have been used. These modified versions consider the crucial role of etoposide (Etoposide) in depleting T-cell lymphocytes and suppressing immune hyperactivation, in addition to its anti-lymphoma effect [17,19,24]. Early etoposide (Etoposide) use combined with dexamethasone for immunomodulation in L-HLH leads to rapid control of hyperinflammation, an improved clinical condition, and reduced risk of permanent organ damage [15,48]. A Chinese retrospective study evaluated 66 patients with L-HLH divided into two groups. The first group included patients who had been treated with etoposide (Etoposide)-based protocols, whereas the second group included patients who had not been treated with the drug. The results showed a significant difference in response rate (73.1 % versus 42.9 %; p-value = 0.033) and median OS (25.8 months versus 7.8 months; p-value = 0.048) [49]. Bigenwald et al. analyzed a cohort of 71 patients with L-HLH and observed that treatment with etoposide (Etoposide) was independently associated with improved prognosis [50].

The aggressive presentation of L-HLH is often associated with rapid clinical deterioration and significant laboratory changes. These changes delay lymphoma-specific treatment. Considering such factors, the MD Anderson Cancer Center published guidelines suggesting a two-stage approach, which

has been widely adopted. The initial phase aims to control hyperinflammation and T-cell proliferation based on weekly etoposide (Etoposide) use in reduced doses (50–100 mg/m^2) combined with corticosteroids. In the absence of an initial response, treatment is intensified with the liposomal doxorubicin, etoposide (Etoposide), and methylprednisolone (DEP) protocol. The second phase aims to treat the lymphoma as soon as clinical improvement and organ dysfunction reach permissive levels, notably lower ferritin, transaminases, and fibrinogen levels. Regimens with etoposide (Etoposide), such as EPOCH or DA-EPOCH, are recommended, along with rituximab in cases of B-NHL [15,19] (Figure 2). However, the use of high-dose regimens proved more toxic, with no additional benefit in NK/T-cell lymphomas [45].

Despite being included in the original HLH-94 protocol, the role of HSCT in adult HLH remains controversial and is reserved for cases of disease refractory to first-line therapy. In L-HLH, some studies suggest that autologous HSCT is beneficial as a first-line consolidation therapy, but data are scarce, thus preventing the generalization of this approach. Consolidation should follow the usual indications in lymphoma treatment and be discussed individually in severe cases [45,51]. Allogeneic HSCT should be considered for first-line candidates with NK/T-NHL, given the poor prognosis of this cohort, and in cases refractory to induction treatment, particularly in reduced-intensity conditioning (RIC) [12,15,16].

Considering that exacerbated cytokine production in HLH plays a key role in the Janus Kinase-Signal Transducer and Activator of Transcription (JAK-STAT) pathway [52], ruxolitinib, a JAK 1 and 2 inhibitor, has been tested as a potential targeted treatment, with promising results. Single-arm studies and some case series demonstrate that this drug is effective in secondary HLH with different associated triggers in first-line settings and refractory cases [53–55]. The most representative cohort is found in a Chinese study on 70 patients with L-HLH, 36 of whom were treated with the ruxolitinib, liposomal doxorubicin, etoposide (Etoposide), and dexamethasone (R-DED) protocol and 34 with etoposide (Etoposide) combined with dexamethasone, followed by lymphoma-specific treatment in both arms. Patients in the R-DED group had \ higher overall response rate (83.3 % versus 54.8 %; p-value = 0.011) and median OS (5 months versus 1.5 months; p-value = 0.003). Moreover, this cohort was mainly composed of patients with T-cell lymphoma (78.6 %), generally associated with a more reserved prognosis when associated with HLH [56].

The effect of anti-cytokine therapies on L-HLH is still unclear. The interleukin-1 receptor agonist anakinra has been the most frequently used in HLH associated with rheumatologic diseases (often termed macrophage activation syndrome). However, its efficacy in the context of malignancy is questionable [57,58]. Interleukin-6 blockade with tocilizumab has been extrapolated from its use in cytokine release syndrome and coronavirus disease 2019, but data on HLH are limited, with a recent series showing increased mortality from infections when tocilizumab was used in the context of M-HLH [59].

HLH triggered by lymphoma treatment should be subjected to differential diagnosis for L-HLH. This condition has been more frequently observed with the development and increasingly widespread use of new therapies, such as checkpoint inhibitors, CAR T-cells, and bispecific antibodies. Mild-to-moderate cytokine release syndrome is expected because of these therapies, with good response to tocilizumab and

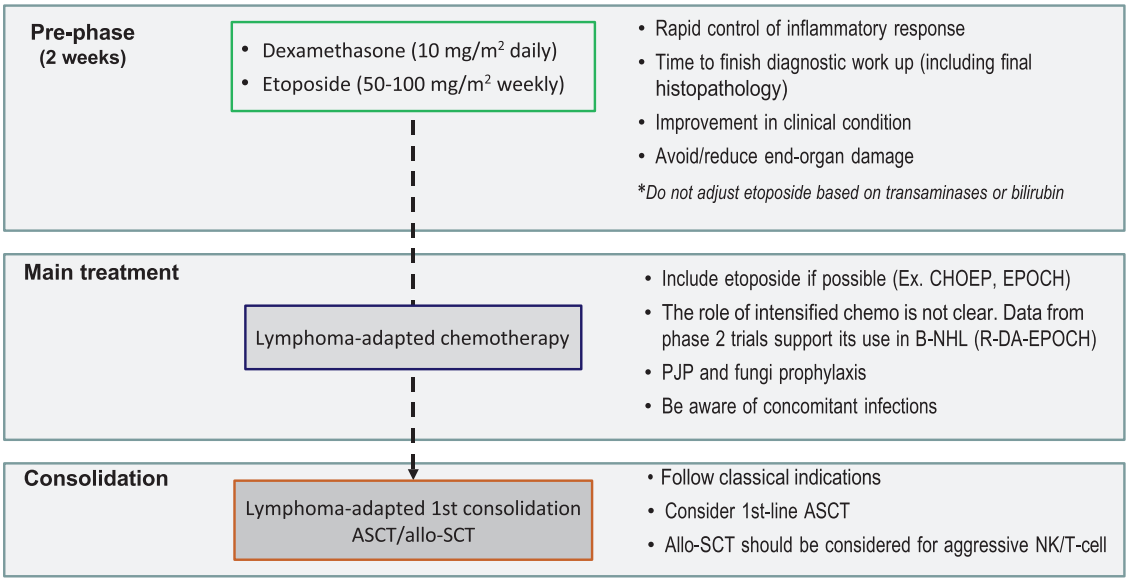

Figure 2 – A suggested treatment approach for lymphoma-associated hemophagocytic lymphohistiocytosis. The two-stage approach aims to initially control hyperinflammation and T-cell proliferation based on weekly etoposide (Etoposide) and dexamethasone, followed by lymphoma treatment. CHOEP: Cyclophosphamide, Hydroxydaunorubicin, Oncovin, etoposide (Etoposide), Prednisone; EPOCH: etoposide (Etoposide), Prednisone, Oncovin (Vincristine), Cyclophosphamide, and Hydroxydaunorubicin (Doxorubicin); B-NHL: B-cell non-Hodgkin lymphoma; R-DA-EPOCH: Rituximab (Dose-Adjusted), etoposide (Etoposide), Prednisone, Oncovin (Vincristine), Cyclophosphamide, Hydroxydaunorubicin (Doxorubicin); ASCT: autologous stem-cell transplantation; PJP: *Pneumocystis jirovecii* pneumonia; Allo-SCT: allogenic stem-cell transplantation; NK: Natural killer cells.

anakinra [60]. More severe cases that progress with HLH characteristics are less frequent and generally managed using anti-interleukin drugs, however there is the lack of robust data in the literature [61]. For other cases, first-line anti-interleukin therapy in L-HLH is not routinely recommended until more robust evidence demonstrates efficacy and safety.

Despite available therapies, most patients with L-HLH, particularly those with NK/T-cell lymphoma, fail to respond to first-line therapy or relapse after a brief response. Relapsed or refractory disease is associated with high mortality, usually due to the progression of HLH/lymphoma or secondary infections. No standard treatment is available, and, as a rule, the approach should be individualized, focusing on controlling the neoplasia and other concomitant predisposing conditions.

High-dose chemotherapy was evaluated using the DEP protocol in 63 patients, 29 of whom had L-HLH. The overall response rate was 76.2%, reaching 75.7% in L-HLH. The reported median OS was 28 weeks [62]. Other therapeutic options studied so far include ruxolitinib, alemtuzumab, and emapalumab, an anti-interferon gamma, albeit with little data on L-HLH [16,52,63]. A recent study investigated the use of emapalumab in patients with hematologic M-HLH, the majority of whom had L-HLH and had undergone extensive prior treatment. Emapalumab did not yield promising results, showing limited clinical efficacy in this population, although a few patients demonstrated improvements in HLH-related biomarkers [64].

Regardless of the therapeutic approach, treatments for refractory L-HLH are more toxic and less likely to lead to long-lasting remission. Therefore, allogeneic BMT should be considered for candidates with an available donor, preferably using reduced intensity conditioning (RIC). Studies have shown that this approach reaches 50–75 % OS and that the best results are found in combination with alemtuzumab prior to RIC [65,66].

High clinical suspicion, prompt immunosuppressive therapy, and adequate clinical support remain the pillars of successful L-HLH treatment. An improved understanding of the pathophysiology of this condition, elucidation of molecular pathways, and genetic alterations that may contribute to the exacerbated inflammatory response have spurred the development of individualized protocols and the exploration of new agents as adjuvants. Future collaborative studies are crucial for assessing the best therapeutic strategies, particularly in patients with HLH associated with NK/T-cell lymphoma, which has a poor prognosis.

Conflicts of interest

none

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Review article

Cold-stored platelets: A systematic review of recovery in healthy adults and chest drain output in cardiothoracic surgery patients

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Meta-analysis

ABSTRACT

Cold-stored platelets were abandoned in the 1960s after demonstration of an increased clearance in vivo due to an irreversible activated phenotype. Difficulties in storage, logistics, and the increased requirement of therapeutic platelet transfusions for haemostasis have sparked renewed interest in cold-stored platelets. This systematic review compared two primary outcomes: in vivo recovery for autologous cold-stored platelets *versus* room-temperature platelets in healthy volunteers, and chest drain output at 24 h for allogeneic cold-stored platelets *versus* room-temperature platelets after complex cardiothoracic surgery. A total of 4215 articles were found in the ProQuest, PubMed, Scopus, Embase, and Cochrane electronic databases. Seven eligible papers were included in this meta-analysis. Cold-stored platelets showed a decreased in vivo recovery two hours after retransfusion following storage for two to seven days compared to a room-temperature platelet control group (mean difference: −25.85 %; 95 % confidence interval: −41.98 to −9.71 %; p-value = 0.002). Further, cold-stored platelets showed a decreased chest cavity output when transfused within 24 h after complex cardiothoracic surgery (mean difference: 249.68 mL; 95 % confidence interval: 85.68 to 413.67 mL; p-value = 0.003). While cold-stored platelets are not a substitute for room-temperature platelets in a prophylactic scenario, their ability to significantly reduce chest cavity output suggests they may be optimal for the management of bleeding in surgical patients, especially in the context of logistical difficulties.

Introduction

A brief history of the platelet

Platelet concentrates serve an important function in transfusion medicine today, but as late as 1910, the role of platelets in haemorrhages was only just being described [1]. Duke

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observed platelets modulating haemorrhage in transfusions, and went on to suggest some haemorrhagic diseases were likely caused by extremely low platelet counts. However, it was not until the 1950s that the transfusion of platelets was systemically approached [2]. Like other therapeutic products, platelets were stored cold, and they were often used for haematological disorders, so they required a long lifespan in vivo [3]. Transfusion of these platelets demonstrated a nearly 50 % decrease in haemorrhage-related leukaemia fatalities, which sparked much research interest [4]. During the 1950s, research began to demonstrate that overall survival of cold-stored platelets (CSPs) was poorer than room-temperature platelets (RTPs), and the switch to plastic containers in the 1960s allowed better storage conditions for studies to demonstrate this phenomenon [2,5,6]. Given the desired outcome for most platelet transfusions was a sustained increment of patient platelet count, in the 1960s it was strongly suggested that the switch from CSPs to RPTs be made, and the entire industry quickly followed this recommendation [6].

Platelet function and the platelet storage lesion

Platelets are small cytoplasmic fragments from bone marrow megakaryocytes that play several roles in haemostasis, thrombosis, and immune modulation [7]. In circulation, they are in a quiescent, thin disc shape. As thermosensors, they are primed at peripheral sites with lower temperatures, a process that induces the release of intracellular granules and biochemicals for subsequent activation [5,7–9]. In particular, α-granules containing P-selectin (CD62P), Platelet Factor 4 (PF4), von Willebrand factor (vWF), and CD63 are released and either bind to the surface of platelets to modulate their function or signal the immune system [10–13]. They also undergo conformational changes in surface receptors, such as the fibrinogen receptor GPIIb/IIIa involved in aggregation, and the vWF receptor GPIb/IX/V involved in adhesion [14,15]. Activated platelets will remodel their actin filaments to form filopodia, which increase the surface area and allow better aggregation, in conjunction with degranulation [16–18].

The platelet storage lesion (PSL) is defined as any deterioration in platelet quality or viability that occurs during preparation and *ex vivo* storage [19]. PSLs manifest distinct effects that are dependent on the stage of component preparation. These effects are triggered by a range of factors including temperature fluctuations (*e.g.*, during whole blood transport), artificial surface contact, pathogen reduction technology, centrifugation forces, the storage media composition, and agitation (rocking) [19–22]. PSLs include cellular activation, which leads to degranulation and biochemical release; cellular fragmentation, which results in decreased platelet count and reduced in vivo survival; and loss of functional receptors, which diminishes overall in vivo function [19–20,23]. An additional problem of RTPs is bacterial contamination, which necessitates shorter storage times [20,21].

Cold-Stored platelet troubles

Evidence in the 1960s led scientists to recommend room-temperature storage over cold-storage, due to the unique characteristics of platelets when exposed to colder temperatures. Platelets are thermosensors, and thus undergo various changes in metabolism, structure, and expression when they reach particular temperature thresholds [8,9]. Below 20 °C, platelets activate, which involves degranulation, increased surface expression of GPIIb/IIIa and P-selectin, and serotonin release [10,24,25]. Exposure to increased P-selectin levels, along with its associated receptor GPIb/IX/V, is a major component of the activated phenotype seen in CSPs [12]. Actin rearrangement leads to a spherical shape with pseudopods forming to increase surface area for aggregation [5,26,27]. The vWF receptors, GPIb, IX and GPV, are irreversibly clustered, with this condition being recognised by hepatic macrophage complement type 3 (CR3) receptors, leading to sequestration and phagocytosis in vivo [8,28,29]. Further, β-GlcNAc moieties on the cell surface are exposed through desialylation, leading to recognition and phagocytosis in the liver [30,31]. It has also been demonstrated that increased Ca^{2+} in storage, which may be an element of the storage medium, is correlated with increased aggregation in vivo [32]. These factors play a role in the observed increase in clearance and reduced survival of CSPs in vivo; studies which aim to demonstrate efficient function need to address these in vivo survival times and recovery levels to demonstrate efficacy of the product.

Resurgence of cold storage interest

Despite many challenges, there has been an increase in both research and clinical interest in CSPs within the past two decades, due to both the changes in prophylactic platelet indications and clinical trial data. CSPs have several advantages compared to RTPs, and these may outweigh the drawbacks in conditions such as active bleeding, remote laboratories, or war zones [33,34]. CSPs demonstrate superior haemostatic function in vitro and in vivo, and refrigeration can reduce vasoactive substance release, leading to decreased febrile non-haemolytic transfusion reactions, and increased clearance, which can lower the risk of thrombosis [35–37]. Studies have demonstrated that CSPs maintain viability for up to 14 days in storage, which could markedly reduce wastage [38,39]. There is decreased metabolism and mitochondrial dysfunction, with less reactive oxygen species (ROSs) causing cellular damage [40–42]. CSPs have even been shown to better reverse anti-platelet drug-related bleeding [43]. One of the biggest advantages is the markedly decreased risk of bacterial sepsis, with refrigeration halting much bacterial overgrowth [44–46].

Use of CSPs would mitigate logistical challenges associated with transport of platelets, especially to remote areas, and in war zones [33]. Use of a dual inventory would allow platelets to be prescribed based on clinical appropriateness, and despite this being recommended in the 1970s when RTPs were universally instated, modern practices with prophylactic RTPs better support the use of a dual inventory [39,47]. A dual inventory study demonstrated the effectiveness of this design during the COVID-19 pandemic [39].

Research aims

With the resurgence of interest in CSPs, there is limited evidence on the in vivo recovery of CSPs in the context of

modern platelet preparation and in actively bleeding patients. Cardiothoracic surgery is common in the Western world, and platelet products better suited to these patients could lighten the burden on transfusion services and post-surgical interventions [38]. This study aims to compare current data using the PICO framework [48] by addressing the question: does transfusion of CSPs (intervention) in healthy volunteers (population) show variation in vivo recovery (outcome) when compared with RTPs (comparison)? A secondary question aims to address: does transfusion of CSPs (intervention) in cardiothoracic surgery patients (population) show a variation in chest cavity output (outcome) when compared with transfusion of RTPs (comparison)?

Methods

Search strategy

This systematic review and meta-analysis employed the Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA) protocol for study screening and eligibility assessment [49]. In addition, the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist was applied to all full papers and conference abstracts to assess the quality of included studies [50]. To identify appropriate studies, searches were made of the PubMed, Cochrane, Scopus, Embase, and ProQuest electronic databases from inception date until July 2024. Searches used a combination of the following keywords: “cold-storage platelet”, “cold-stored platelet”, “thrombocytopenia”, “chilled platelet”, “room-temperature platelet”, “cardiac”, “malignancy”, “trauma”, and “cancer”. The ProQuest search employed additional restrictions: filtered by peer-reviewed, full-text only, and terms in abstract. Manually searching scientific databases did not turn up additional studies.

Eligibility criteria

All articles were saved in Endnote automatically removing duplicates. The title and abstract of papers were screened and assessed for eligibility based on the research aim. The following criteria were assessed:

Types of studies
Observational studies, including both prospective and retrospective studies, were eligible for inclusion. Papers published in any time frame were eligible, and all journal articles were fully accessible and published in English. Papers must have had a minimum of two participants per study arm (*i.e.* CSP in vivo recovery, RTP in vivo recovery, CSP chest drain output, RTP chest drain output), and they must have measured both CSPs and RTPs for each cohort. Review articles and individual case studies were not included, but conference abstracts were acceptable.

Types of participants
For the primary research aim, studies analysing healthy individuals were considered eligible. Subjects must have been over 18 years old, not on any anti-platelet drugs, and have a normal platelet count. They must have consented to autologous collection of apheresis platelets stored in plasma, subsequent product treatment, and then their return for in vivo testing.

For the secondary research aim, studies analysing patients undergoing semi-urgent complex cardiothoracic surgery who had no history of congenital coagulopathies or haemostatic disorders, and who had not taken anti-platelet drugs within 48 h of the surgery, were considered. The patients must have received at least one unit of apheresis platelets stored in platelet additive solution (PAS) or PAS-C within 24 h after surgery without requiring reoperation. All patients must have consented during admission screening.

Types of outcomes measured
For the primary research aim, the papers must have included in vivo recovery for both CSPs and RTPs, with recovery presented as percentage of subject’s fresh autologous platelets two hours after reinfusion. Data must be presented as mean $\pm$ standard deviation (SD), or with standard error of mean (SEM) or 95 % confidence intervals (95 % CIs), from which SD was calculated. For the secondary research aim, the papers must have included the type of surgery, the chest drain output in mL 24 h post-surgery, and the corresponding data for both RTPs and CSPs. Studies were excluded if they did not measure both RTPs and CSPs.

Data extraction

The following relevant data were extracted from eligible studies: primary author, year of publication, study design, study period (if indicated), country of origin, number of participants, participant population, cold-storage time, platelet type, percent in vivo recovery of RTPs, percent in vivo recovery of CSPs, and chest drain output 24 h post-surgery in mL for patients that received RTPs and those that received CSPs.

Statistical analysis

Cochrane RevMan software was used to perform the meta-analyses [51]. A two-arm study of in vivo recovery of CSPs and RTPs was performed, as well as a two-arm study of chest drain output at 24 h after CSP or RTP treatment in cardiothoracic surgery patients. Each analysis used the inverse variance method measuring mean difference and employed the continuous effects analysis model presented as Forest plots. The software calculated statistical significance as p-value and 95 % CIs, with p-value <0.05 indicating statistical significance; and heterogeneity of studies as I^2 with a corresponding p-value. The software was also used to calculate SDs for any studies that provided 95 % CIs or SEMs instead of SD. Risk of bias for included papers was assessed using Funnel plots generated by the software.

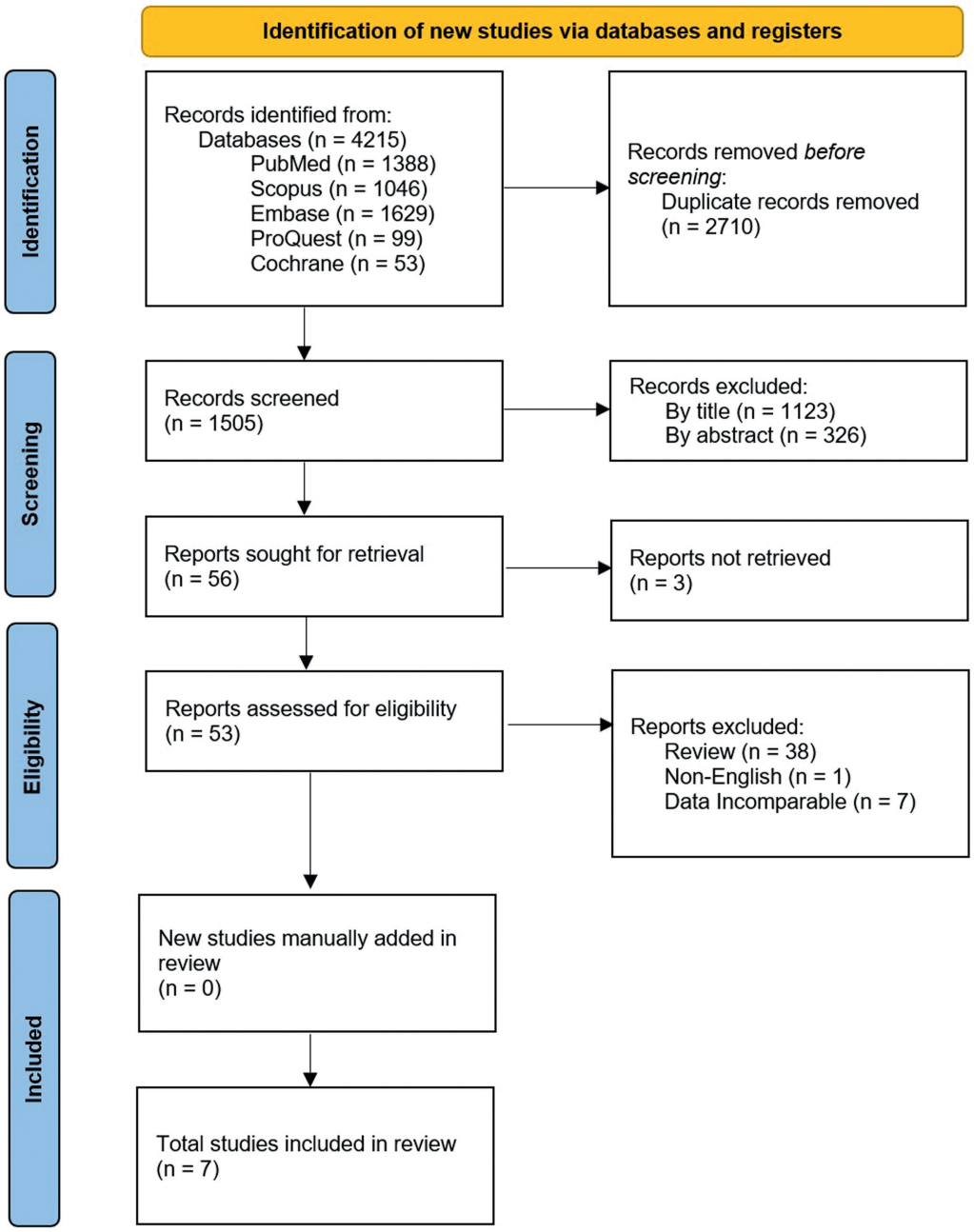

Figure. 1 – PRISMA flowchart for the identification and inclusion of relevant studies for the meta-analysis of in vivo recovery of transfused autologous platelets stored either at room temperature or refrigerated for two to seven days, and chest drain output 24 h after complex cardiothoracic surgery with transfusion of allogenic platelets stored either at room temperature or refrigerated [49].

Results

Study selection

The search strategy found 4215 articles published in electronic databases (PubMed, Scopus, Embase ProQuest, and Cochrane). EndNote was employed to remove 2710 duplicates before screening. Articles were screened first by title, removing a further 1123, as they were not relevant to the topic. Next, abstracts were screened based on eligibility and 326 were excluded after failing to meet the study criteria. Fifty-six full-length articles or conference abstracts were thoroughly assessed for eligibility. Three were removed after not being retrievable, 38 as they were review articles that did not present data, one for not being published in English, and then seven for having data that did not meet all the criteria for this meta-analysis. Therefore, seven articles were identified as eligible. A breakdown of the articles excluded in each stage of screening can be found in Figure. 1. The reference lists of the

Table 1 – Characteristics of eligible studies investigating the in vivo recovery of transfused autologous platelets.

Study	Study Design	Study Period	Country	Number of Patients	Patient Population	Parameter Measured	Cold-Storage Time	Platelet Type
Apelseth et al. 2017 [52]	Prospective	Finished 2017	USA	35	Complex cardiothoracic surgery patients	RTP and CSP post-op chest drain	<7 days	Apheresis in PAS
Bailey et al. 2022 [53]	Prospective	Not indicated (but funding published in 2021)	USA	6	Healthy volunteers	RTP and CSP platelet in vivo recovery	5 days	Apheresis in plasma, apheresis in PAS-C
Stolla et al. 2020 [54]	Prospective	2016 – 2018	USA	5	Healthy volunteers	RTP and CSP platelet in vivo recovery	5 days	Apheresis in plasma
Strandenes et al. 2016 [55]	Prospective	Finished 2016	USA	26	Major cardiothoracic surgery patients	RTP and CSP post-op chest drain	<7 days	Apheresis in PAS
Strandeneset al. 2020 [38]	Prospective	2015 – 2018	Norway	50	Elective and semi-urgent cardiac surgery patients	RTP and CSP post-op chest drain	<7 days	Apheresis in PAS-C
Vostal et al. 2018 [56]	Prospective	Not indicated	USA	16	Healthy volunteers	RTP and CSP platelet in vivo recovery	7 days	Apheresis in plasma
Wandall et al. 2008 [31]	Prospective	Not indicated	USA	4	Healthy volunteers	RTP and CSP platelet in vivo recovery	2 days	Apheresis

RTP: Room-temperature platelets; CSP: Cold-stored platelets; PAS-C: platelet additive solution C (Intersol).
Platelets were stored either at room temperature or refrigerated for two to seven days and chest drain output was assessed 24 h after complex cardiothoracic surgery with transfusion of allogenic platelets stored either at room temperature or refrigerated.

selected papers, as well as excluded review papers, were examined but no additional articles were identified. All seven identified papers were included in this meta-analysis.

Study characteristics

The seven studies included in this review and meta-analysis measured the in vivo recovery of transfused autologous CSPs or RTPs two hours post-transfusion [31,53,54,56], or the chest drain output 24 h post-surgery for complex cardiothoracic surgery patients who received CSPs or RTPs [38,52,55]. All seven studies were prospective and conducted in the USA or Norway (Table 1). Four of the studies comprised healthy volunteers [31,53,54,56]; the other three consisted of cardiothoracic surgery patients [38,52,55].

The length of time platelets were stored cold before transfusion varied between the studies: two studies transfused after five days [53,54], one study transfused after two days [31], one study transfused after seven days [56], and all three chest drain output studies transfused within seven days but without stating a specific time [38,52,55]. All seven studies used platelets collected by apheresis, with in vivo recovery studies storing platelets in plasma [31,53,54,56] and chest drain output studies storing platelets in PAS or PAS-C [38,52,55].

All four in vivo recovery studies gave recovery as a mean percentage of the study arm ± SD (Table 2). For the three chest drain output studies, one reported mean output ± SD [55], one reported mean output ± SEM [52], and the other reported mean output with 95 % CI [38]. The SDs for papers that did not include them were calculated by RevMan software using the mean, SEM or 95 % CI, and population size. For Apelseth et al. the SD for CSPs was calculated to be 252 mL and for RTPs it was 511 mL [52]. For Strandenes et al. the SD for CSPs was calculated to be 327 mL and for RTPs it was 546 mL [38].

Quality assessment of included studies

The quality, assessed for the seven studies using the STROBE checklist, is shown in Tables 3A & 3B for full-length articles and conference abstracts, respectively. All criteria except two were met by every study. In full-length articles, only one paper clearly stated the eligibility criteria of the included patients, setting of the study, and collection methods [38]. Also, only two full-length articles described the participant characteristics and possible confounders in the results section [31,38]. Both conference abstracts met all quality criteria and were considered high quality. The paper by Bailey et al. was a letter to the editor providing further data on a previous study and had no subsections, but the title was clear and the study design was indicated so it was considered eligible for the first criterion [53]. Stolla et al. reported potential biases in their results and not methods, so it was not considered ineligible for reporting of bias [54]. The paper by Wandall et al. did not have sample sizes large enough for statistical analysis (two participants per study arm), so it did not report statistical methods [31]. However, the study met the inclusion criterion of a minimum of two participants per study arm and thus it was included.

Table 2 – Results of extracted data from eligible studies included in the meta-analyses.

Study	Patients Receiving RTP	Patients Receiving CSP	RTP in vivo Recovery%	CSP in vivo Recovery%	RTP Drain	CSP Drain
Apelseth et al. 2017 [52]	22	17	-	-	820 mL (109)*	546 mL (61)*
Bailey et al. 2022 [53]	21	5	92 ± 12	46 ± 7	-	-
Stolla et al. 2020 [54]	5	5	70 ± 7	46 ± 3	-	-
Strandenes et al. 2016 [55]	12	14	-	-	1055 mL ± 677 mL	775 mL ± 534 mL
Strandenes et al. 2020 [38]	25	25	-	-	865 mL (640–1091 mL)†	649 mL (514–784 mL)†
Vostal et al. 2018 [56]	12	4	55.7 ± 13.9	23.1 ± 8.8	-	-
Wandall et al. 2008 [31]	2	2	47 ± 13	53 ± 5	-	-

RTP: Room-temperature platelets; CSP: Cold-stored platelets; SEM: standard error of the mean; 95 % CI: 95 % confidence interval.
Results are given as mean ± SD unless otherwise indicated. The SEM and 95 % CI results are represented as they were found in the studies; however, calculated SDs for these papers are presented in the results section and in the meta-analyses.

* Mean (SEM).
† Mean (95 % CI).

In vivo recovery meta-analysis

A meta-analysis was performed and a Forest plot generated for the in vivo recovery of autologous CSPs and RTPs in healthy volunteers two hours after retransfusion (Figure. 2A). Across the four studies which reported in vivo recovery, three favoured RTPs for increased in vivo recovery whereas one favoured CSPs [31]. The mean difference between CSPs as a study group and RTPs as a control group was −25.85 % (95 % CI: −41.98 to −9.71 %), showing overall favour for RTPs. This finding was deemed statistically significant with a p-value of 0.002. The data between the studies demonstrated high heterogeneity (I^2 = 91 %; p-value <0.00001). Risk of bias was assessed using a Funnel plot (Figure. 3A). Only four studies were included, so estimation of intervention effect is hard to determine from plot symmetry. The paper by Wandall et al. demonstrated the highest SEM and the greatest variation in mean difference between studies [31].

Chest drain output meta-analysis

A meta-analysis was performed and a Forest plot generated for the chest drain output 24 h post-surgery after treatment with CSPs or RTPs (Figure. 2B). Across the three studies which reported chest drain output, all favoured CSPs for decreased output. The mean difference between CSPs as a study group and RTPs as a control group was 249.68 mL (95 % CI: 85.68 to 413.67 mL), overall, in favour of CSPs. This finding was deemed statistically significant with a p-value of 0.003. The data between studies demonstrated no heterogeneity (I^2 = 0 %; p-value = 0.94), which likely indicates the studies are homogenous, and any differences in values are probably due to random sampling errors. Risk of bias was assessed using a Funnel plot (Figure. 3B). Only three studies were included, so estimation of intervention effect is hard to determine from plot symmetry. The paper by Strandenes et al. cannot be seen on the graph as the software was unable to place it in any viewable area, a fact that could not be mitigated with RevMan software [55].

Discussion

Reduced recovery of cold-stored platelet transfusion in vivo

This systematic review with meta-analysis shows that current studies are still in agreement with the long-understood decrease in vivo recovery of autologous platelets when stored cold before retransfusion. The overall recovery for CSPs was statistically significantly lower than RTPs, with a mean difference of −25.85 % (95 % CI: −41.98 to −9.71 %; p-value = 0.002). There was high heterogeneity of the data (I^2 = 91 %; p-value <0.00001), which indicates that 91 % of the variation in results cannot be attributed to chance alone but is the result of other factors such as bias [57]. This heterogeneity may be high due to the small number of included studies, the small number of participants in each study arm, and reported patient-to-patient variations in many of the included studies. Since 2003, the Food and Drug Administration of the United States (FDA) have required platelet in vivo recovery to be ≥66 % [58], and CSPs did not meet this criterion in any study (53 ± 5 % [31], 46 ± 7 % [53], 46 ± 3 % [54], and 23 ± 9 % [56]).

Though beyond the scope of this review, all included papers also tested in vitro parameters to measure the activated phenotype observed in cold storage. This consists of assessing surface receptors such as CD62P (P-selectin) and phospholipid phosphatidylserine (PS), which are stored intracellularly and released during activation or in response to temperature drops; along with metabolic markers such as glucose and lactate, which are consumed to supply the energy needed for shape changes but are often considerably decreased at room temperature [11,12,31,38,40,53,54]. While there are increased activation marker levels in CSPs, including P-selectin and annexin V binding (an indirect test of PS expression), metabolic markers and pH are decreased, which may allow for better storage times and product quality.

One of the included studies showed slightly increased recovery for CSPs, likely due to the very short cold storage time (48 h) compared to the five- or seven-days others were stored [31]. While only demonstrated in one study, a shorter cold storage time displaying greater in vivo recovery may be a

Table 3A – Evaluation of the methodology of included full-length studies.

	Title and Abstract	Introduction	Methods				Results	Discussion
	Clear title and abstract with study design indicated	Explain the scientific background and rationale	Study methods presented clearly	Eligibility criteria, setting, dates, and data collection described	Statistical methods described	Describes and addresses potential bias	Describes characteristics of study participants and potential confounders	Summarise key results and discusses limitations
Bailey et al. 2022 [53]	Y[a]	Y	Y	N	Y	Y	N	Y
Stolla et al. 2020 [54]	Y	Y	Y	N	Y	Y[b]	N	Y
Strandenes et al. 2020 [38]	Y	Y	Y	Y	Y	Y	Y	Y
Vostal et al. 2018 [56]	Y	Y	Y	N	Y	N	N	Y
Wandall et al. 2008 [31]	Y	Y	Y	N	N[c]	Y	Y	Y

Y: Criteria fulfilled; N: Criteria not fulfilled.

[a] Small study in letter to the editor format, no subsections like abstract.
[b] Addressed, but in the results section, not in the methods.
[c] Sample size sufficient for proof of hypothesis but not large enough for statistical analysis.

Table 3B – Evaluation of the methodology of included conference abstracts.

	Clear title with study design indicated	Study methods presented clearly	Eligibility criteria and setting briefly mentioned	Primary outcome of report clearly defined	Statistical methods described	Number of participants in study reported	Measures of variability or uncertainty reported	General
	interpretation of study given							
Apelseth et al. 2017 [52]	Y	Y	Y	Y	Y	Y	Y	Y
Strandenes et al. 2016 [55]	Y	Y	Y	Y	Y	Y	Y	Y

Y: Criteria fulfilled; N: Criteria not fulfilled.
According to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist for full-length articles [50].

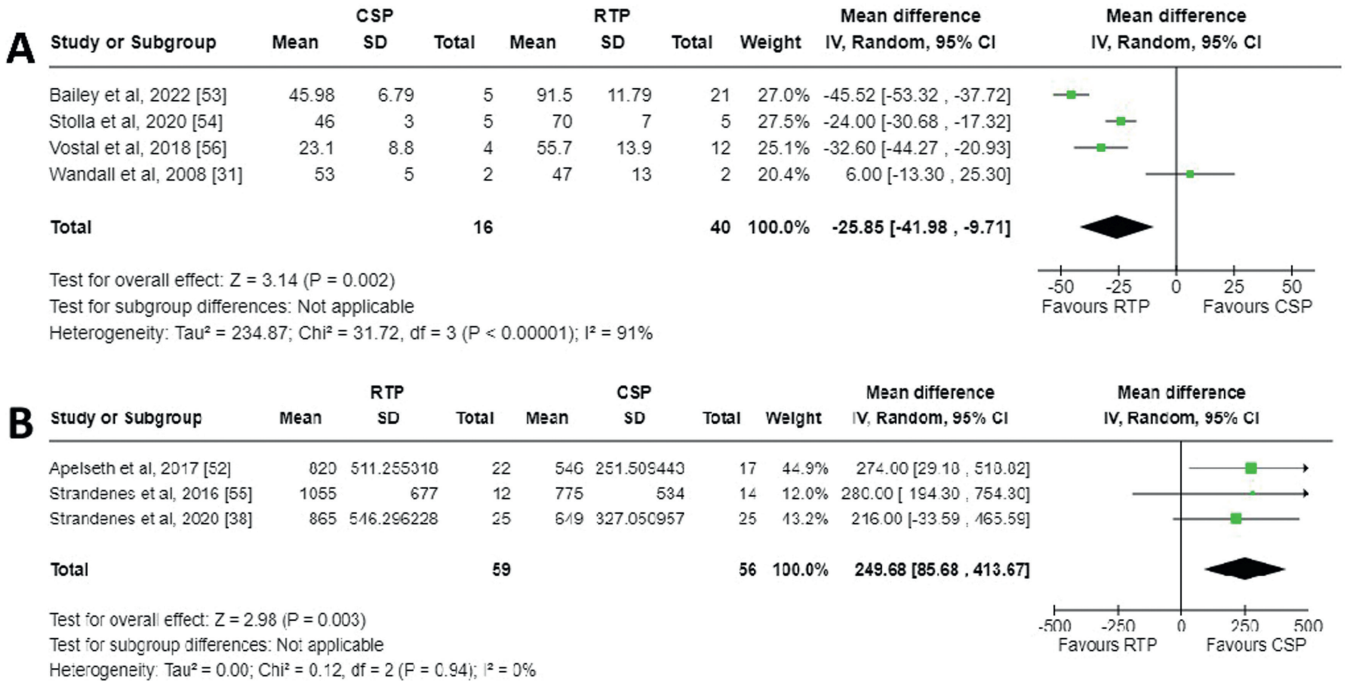

Figure. 2 – Forest plots for meta-analyses generated using RevMan [51]. A) **Forest plot for in vivo recovery of transfused autologous platelets stored either at room temperature or refrigerated for two to seven days.** B) **Forest plot for chest drain output 24 h post complex cardiothoracic surgery with transfusion of allogenic platelets stored either at room temperature or refrigerated.**

further avenue of research. Irreversible cold storage lesions usually occur after 18 h of storage at cold temperatures and so they can be ameliorated by warming within this period [6,59]. Combining cold storage with room temperature storage in a temperature cycling pattern therefore may yield better recovery and metabolic results. Vostal et al. studied the effects of temperature cycling, but only the data on CSPs was used for this meta-analysis [56]. They also reported that the study arms with CSPs and RTPs were not performed on the same participants, were performed at different times due to funding issues, used two different manufacturers for the collection of apheresis platelets, and thus demonstrated up to 10 % variation in results between study arms, which undoubtedly contributed to the heterogeneity seen in this meta-analysis.

Two of the studies found unexpected results when adapting their models from previous animal studies, which showed better outcomes than were reported in humans [31,56]. Differences in both metabolic markers and in vivo parameters demonstrated that structures and functions exist in human platelets which contributed to clearance of platelets; they were not seen in animal studies. This suggests that animal platelets are not a good substitute for human platelet testing, a major reason animal studies were excluded from the scope of this analysis.

Reduced chest cavity output in cold-stored platelet transfusion

This systematic review with meta-analysis shows that transfusion of CSPs results in a lower chest cavity output within 24 h after complex cardiothoracic surgery compared with RTPs. The chest drain output in CSP transfusion was statistically significantly lower, with an overall mean difference of 249.68 mL (95 % CI: 85.68 to 413.67 mL; p-value = 0.003). There was no heterogeneity in the data (I^2 = 0 %; p-value = 0.94), indicating that all the variation in the results is due to chance and not bias [57]. Despite overall findings showing significance when transfusing CSPs, each individual study did not report significance between participant groups. Both individual studies reporting no significant difference between groups and this meta-analysis reporting significant decrease in chest cavity output for CSPs indicate that CSPs are a suitable substitute for RTPs in the setting of acute bleeding in complex cardiothoracic surgeries. This is the logical conclusion for the transfusion of a haemostatically superior product in the context of haemostasis.

All platelet products employed PAS or PAS-C as the storage media, which has a reported lower incidence of transfusion reactions, and is routinely used for RTP storage. PAS-C has been demonstrated to show better in vivo recovery and survival, potentially influencing the decreased output compared to platelets in plasma used for the in vivo recovery meta-analysis [60]. All three included studies were conducted by mostly the same research team, which may have been part of the reason that no heterogeneity was seen in the data. There are ongoing trials in Australia and the USA using CSPs in surgical and bleeding patients, but data had not been published at the time of this study. The data from these clinical trials will be very useful in further demonstrating the effects of CSPs in halting bleeding.

A major problem with the design of these studies is the non-specific nature of chest drain output as a marker of haemostasis in surgery patients. In 2020, Strandenes et al. reported five different types of cardiac surgery with

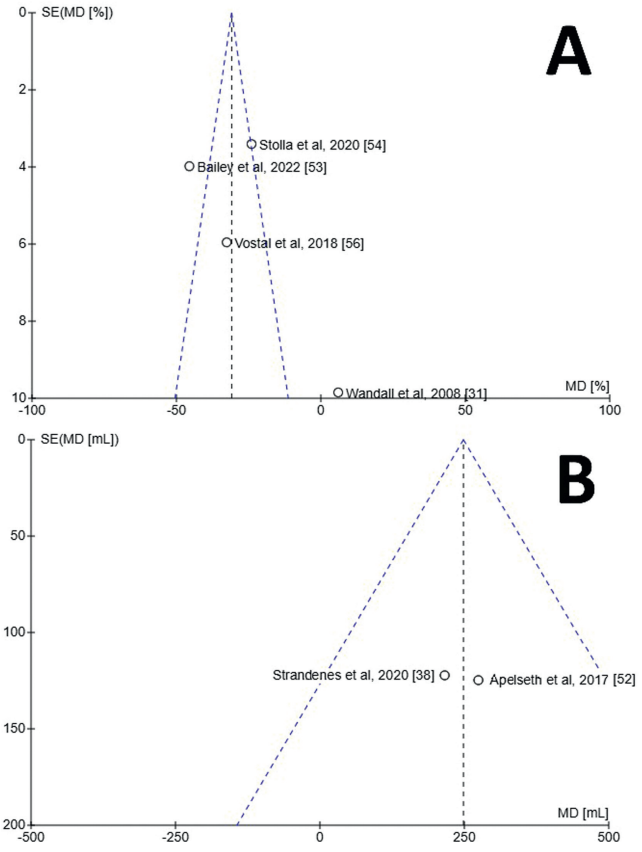

Figure. 3 – Funnel plots for estimation of bias generated using RevMan [51]. Programme did not generate 95 % confidence interval lines, likely due to small number of samples. A) Funnel plot for in vivo characteristics of transfused autologous platelets stored either at room temperature or refrigerated for two to seven days. B) Funnel plot for chest drain output 24 h after complex cardiothoracic surgery with transfusion of allogenic platelets stored either at room temperature or refrigerated. Programme did not display paper by Strandenes et al. [55].

approximately the same number in each study arm [38]. Factors such as history of sternotomies, length of surgical procedure, patient age, logistic EuroSCORE, and patient blood volume all impact the required number of platelets required for restoration of haemostasis, as well as expected blood loss. These factors are very difficult to control for, and chest cavity output may not represent effectivity of platelets. Additionally, platelets were rarely given in isolation, but along with other transfusion products, and these products undoubtedly impacted blood loss. While this review points to a significant lowering of chest drain output, the most important finding is that CSPs currently demonstrate acceptable testing parameters for use during complex cardiothoracic surgery. Given the logistical difficulties of RTP storage and transport, they may be a viable option for some hospitals and centres.

Limitations of review

The major limitation of this review was the small number of papers included, and the small number in each study arm in the papers. Only 56 healthy patients were tested across four studies for in vivo recovery, split between CSPs (n = 16) and RTPs (n = 40), and only 115 cardiothoracic surgery patients were tested across three studies for chest cavity output, split between CSPs (n = 56) and RTPs (n = 59). High heterogeneity for in vivo recovery was likely due to the size of these studies. Failure to test for both CSPs and RTPs, or differences in testing parameters, accounted for the exclusion of otherwise acceptable studies. Another important marker of in vivo platelet function is survival, which could not be tested as insufficient papers reported comparable results, with some reporting survival in days and others as a percentage of fresh count. Three of the in vivo recovery papers reported CSPs as a control for modified platelets stored cold, and therefore did not address problems with cold storage but with the modification of the platelets [31,53,56].

Further research

CSPs in plasma have shown in vivo recovery levels lower than FDA requirements, but this meta-analysis did not report on the addition of other storage media or modifications to platelets, which may increase circulation time [58]. Research has demonstrated the various mechanisms by which CSPs are cleared rapidly from circulation, and continued research into stopping irreversible cold storage changes, such as galactosylation of clustered GPIb/IX receptors, is one avenue just starting to be reported in human trials; further research comparing these methods to a control group may lead to better preparation methods to promote cold storage [31]. Modification of platelets will be dependent on patient treatment. Increased recovery and survival are a requirement of prophylactic platelet treatment, but may also increase the effects of therapeutic platelets by virtue of increased time in circulation to participate in haemostasis. However, therapeutic platelets benefit from priming as they are effective immediately upon transfusion.

Research into storage conditions, such as temperature cycling (which may mitigate irreversible changes to platelet structure), is required to better understand the effects on storage time, in vivo recovery and survival, and product viability. Results from this research could elucidate new ways of storing platelets, allowing dual inventories or switching to an entirely new storage method. Two clinical trials are looking at how extended storage of CSPs for cardiac surgery may impact bleeding [61,62]. Purpose-built refrigerators can automate the storage process, reducing waste and increasing product effectiveness. Another clinical trial is determining efficacy of autologous CSPs that are reinfused after platelet count is normalised, in a similar fashion to acute normovolaemic haemodilution methods [63]. CSPs are only just being reintroduced for the treatment of bleeding, but their potential is still being understood even today, and future research needs to address the different ways platelets can be stored and modified to allow longer shelf life, better therapeutic function and life, and reduced risk to the patient.

Conclusion

This systematic review and meta-analyses demonstrates that CSPs will have a statistically significantly reduced in vivo

recovery when stored cold for two to seven days and tested two hours after retransfusion. This is due to an activated phenotype which occurs when they are stored below 20 °C for over 18 h, and recognised by hepatic macrophages which quickly clear platelets from circulation. Therefore, CSPs are not an effective prophylactic replacement for RTPs. Because of this activated phenotype, CSPs demonstrated a statistically significantly lower chest drain output when transfused within 24 h after complex cardiothoracic surgery. Ongoing clinical trials will hopefully provide further data to demonstrate increased haemostatic effectiveness of CSPs for therapeutic transfusion.

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Data availability statement

The data used to support this study's findings are included within the article.

Conflicts of interest

Authors declare no conflict of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Case Report

First report of perioperative iptacopan interruption in paroxysmal nocturnal hemoglobinuria without breakthrough hemolysis

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Introduction

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematopoietic stem cell disorder characterized by complement-mediated intravascular hemolysis, thrombosis, and marrow failure. Iptacopan, an oral selective factor B inhibitor, has emerged as a novel therapeutic agent that effectively controls hemolysis and reduces transfusion burden by targeting the alternative complement pathway [1]. Its oral administration provides convenience but raises concerns over adherence and peri-interruption risk, especially in the surgical setting where oral intake may be restricted. Perioperative management of iptacopan remains undocumented, and no case reports have described the safety of temporary drug interruption in surgical settings.

We herein report, to our knowledge, the first case of successful laparoscopic cholecystectomy following brief preoperative interruption of iptacopan without breakthrough hemolysis (BTH), highlighting a practical approach to perioperative management of this novel oral complement inhibitor.

Case report

A 57-year-old woman with a history of depression and ovarian cysts presented in September 2022 with fatigue, anemia, and progressive thrombocytopenia. Initial laboratory testing showed hemoglobin 5.6 g/dL with macrocytosis (MCV: 115.9 fL), platelet count 32 $\times$ $10^9/\mu$L, and white blood cell count 4.7 $\times$ 10^9/L without differential abnormality. Lactate dehydrogenase (LDH) was slightly elevated (241 IU/L) and haptoglobin was at an

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undetectable level (<2 mg/dL). Flow cytometry identified 5.9 % of CD55/CD59-deficient erythrocytes, which later increased to 31.8 %. Bone marrow examination showed mild dyserythropoiesis and slightly reduced megakaryocytes, no blast excess, and a normal karyotype. A diagnosis of PNH with possible coexisting myelodysplastic syndrome was made.

Immunosuppressive therapy with cyclosporine was ineffective. In January 2024, ravulizumab was initiated after a meningococcus vaccination (Menactra®), reducing the red blood cell (RBC) transfusions from 4 to 2 units per month but without transfusion independence. In June 2024, danicopan (150 mg three times daily, later increased to 200 mg) was added due to persistent anemia but this remained insufficient. During this period, the patient developed cholecystitis and experienced BTH.

In December 2024, therapy was switched from ravulizumab and danicopan to iptacopan (200 mg twice daily) following pneumococcal (Pneumovax®23) and haemophilus influenzae type b (ActHIB®) vaccinations, with the first dose given six weeks after the last ravulizumab infusion. Hemoglobin stabilized, and transfusions were no longer required.

Elective laparoscopic cholecystectomy was scheduled for March 2025. On admission, the hemoglobin level was 8.1 g/dL, which would not typically require transfusion. However, considering the upcoming surgery, two units of RBC were administered. Considering the pharmacokinetics of iptacopan (T_max ≈ 2 h, half-life 18–25 h) [2], a one-day interruption (two missed doses) was planned. The final preoperative dose was taken at 7:00 pm on the day before surgery; both doses on the surgery day were withheld. Eculizumab was kept on standby in case of prolonged oral intake restriction or BTH. Oral iptacopan was resumed at 7:00 am on postoperative Day 1. Blood tests at 5:00 am that day showed stable hemoglobin and a mild LDH increase (256 IU/L), which normalized by postoperative Day 3. No hemoglobinuria, vital sign changes, or thrombotic events occurred. The patient recovered uneventfully and has remained on iptacopan, with sustained transfusion independence and improvement in fatigue. The perioperative laboratory data, with stable hemoglobin, LDH and indirect-bilirubin levels, are summarized in Table 1. The clinical course from diagnosis to the present is illustrated in Figure 1.

Table 1 – Perioperative laboratory data.

	Day −3	Day 0	POD 1	POD 3	POD 14
Hb (g/dL)	8.1*	9.0	9.8	9.4	9.5
LDH (IU/L)	229	220	256	197	205
ID-Bil (mg/dL)	0.64	0.77	0.90	0.69	0.68
CRP (mg/dL)	0.18	0.12	2.31	4.08	1.59

POD: Post-operative day; Hb: hemoglobin; LDH: lactate dehydrogenase; ID-Bil: indirect bilirubin; CRP: C-reactive protein.

* Preoperative transfusion of 2 units of red blood cells.

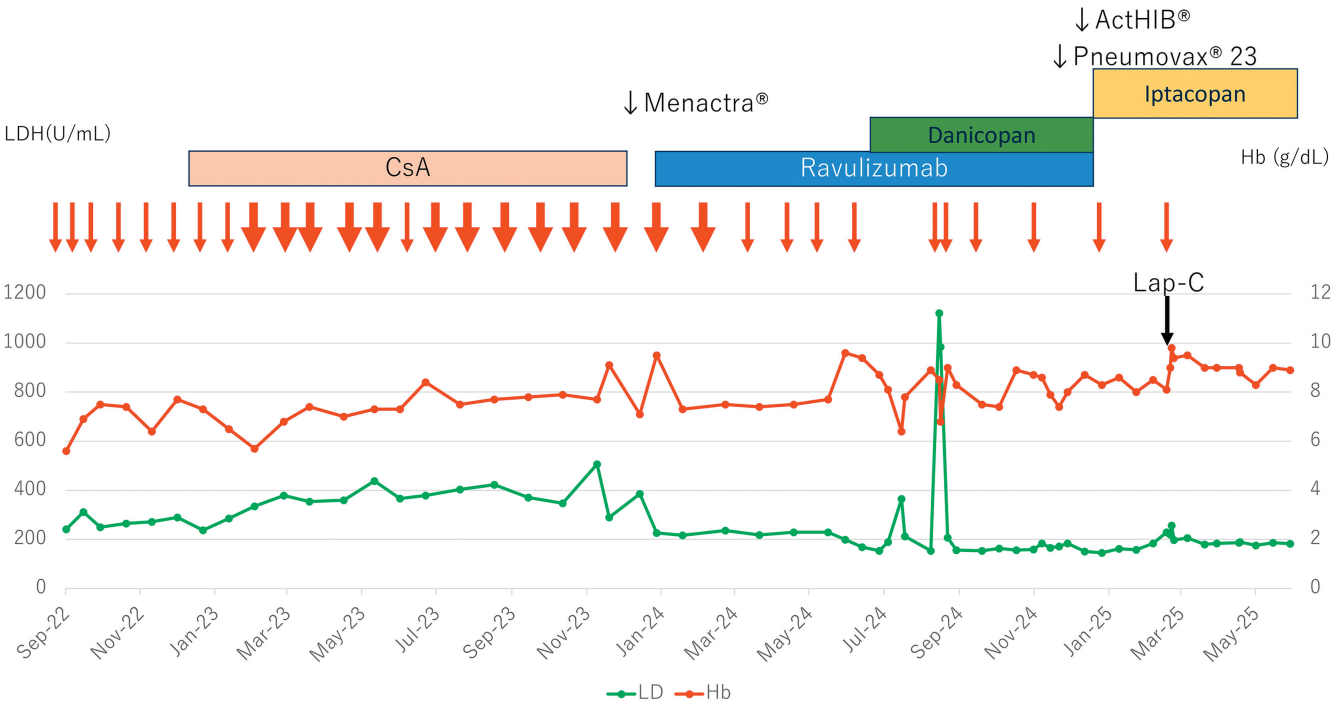

Figure 1 – Clinical course of the patient.

LDH: lactate dehydrogenase; CsA: Cyclosporine A; Hb: hemoglobin; Lap-C: laparoscopic cholecystectomy thin arrow: 2 units red blood cell transfusion; thick arrow: 4 units red blood cell transfusion.

Discussion

Although the anti-C5 antibodies eculizumab and the long-lasting ravulizumab have been established as the standard of care for PNH patients, effectively reducing adverse events and mortality, unmet needs persist in cases of treatment failure [3,4]. Several novel drugs such as pegcetacoplan [5], danicopan [6], crovalimab [7], and iptacopan [1] have recently emerged, significantly changing the management of patients with PNH. Among these, Iptacopan is a first-in-class, oral, selective factor B inhibitor that targets the alternative complement pathway and has demonstrated substantial efficacy in controlling hemolysis and reducing transfusion burden in PNH patients [1]. Its oral administration offers advantages in convenience over intravenous or subcutaneous administrated drugs, but also raises concerns of adherence and rapid loss of complement control upon discontinuation due to its shorter half-life. Perioperative management of iptacopan remains unaddressed in the literature, and to our knowledge, this is the first reported case of successful elective surgery without BTH after short-term (two doses) iptacopan interruption in the perioperative period.

Perioperative management is a critical consideration in PNH because surgery is considered a complement amplifying condition that can trigger hemolysis and thrombosis in PNH patients. There are several case series that major surgeries were performed without complications under the treatment of eculizumab [8–10]. Today, several guidelines recommend giving a dose of eculizumab one day before surgery [11]. Kimura et al. [12] reported that following the eculizumab recommendation, ravulizumab administration one day before laparoscopic cholecystectomy led to an uneventful perioperative period in a PNH patient. In contrast, due to their recent approval and limited clinical experience, data regarding perioperative management with novel agents remain scarce. For pegcetacoplan, a subcutaneous anti-C3 antibody administered twice weekly, there is one perioperative case report available. Vara et al. [13] described successful perioperative management in a PNH patient undergoing multiple surgeries, achieved through dose adjustments of pegcetacoplan and careful monitoring of hemolytic markers. Further reports on the perioperative management of these agents are awaited; the accumulation of such data is expected to contribute to the development of clinical evidence and new guidelines. Therefore, our case supports the notion that a one-day iptacopan holiday, in the setting of scheduled surgery, may not compromise complement control in well-stabilized patients. In this patient, the decision to interrupt iptacopan for one day was based on its pharmacokinetic profile (T_max $\approx$ 2 h, half-life 18 −25 h), the planned minimally invasive procedure, and the ability to resume oral intake promptly. Importantly, backup intravenous complement inhibition with eculizumab was arranged in case of prolonged oral intake restriction or evidence of BTH. Close perioperative laboratory monitoring allowed for timely detection of potential hemolysis, with only a mild transient LDH elevation that resolved spontaneously.

The main limitation of this observation is that it represents a single patient undergoing a laparoscopic procedure with an uncomplicated postoperative course. The safety of perioperative iptacopan interruption in higher-risk scenarios, such as major surgery, delayed resumption of oral intake, or active hemolysis, remains unknown. Even so, we present this case with the view that the accumulation of such individual reports will provide valuable evidence to permit the development of future clinical guidelines. Also, it highlights the importance of individualized planning, backup complement inhibition, and close monitoring. Given the expanding therapeutic landscape in PNH, with the introduction of multiple novel complement inhibitors, it is essential to develop perioperative management strategies tailored to each drug's pharmacologic characteristics.

Conclusion

This case demonstrates that a planned, short-term interruption (one day) of iptacopan during the perioperative period can be tolerated without breakthrough hemolysis in a stable PNH patient undergoing elective laparoscopic surgery. Careful preoperative planning, availability of backup intravenous complement inhibition, and close perioperative monitoring are essential. Further clinical data are needed to confirm the safety and define best practices for perioperative management of iptacopan and other newly approved complement inhibitors.

Informed consent

Informed consent was obtained from the patient for publication of this case report.

Author contributions

T.K. drafted the initial manuscript. J.O. provided critical revisions and edited the manuscript. H.T. supervised the project and finalized the manuscript. All authors were involved in the diagnosis, treatment, and follow-up of the patient, critically revised the manuscript, read, and approved the current version of the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT (OpenAI) in order to assist with English language editing during the manuscript preparation. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication

Conflicts of interest

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Letter to the Editor

National multiple myeloma cohort: Gaps and opportunities for research in Brazil

Dear Editors,

Multiple myeloma (MM) is a relatively uncommon hematologic malignancy. It accounts for approximately 1 % of all cancers and is the second most frequent hematologic malignancy [1]. However, national data on the epidemiological profile, access to innovative therapies, and clinical outcomes in Brazil remain scarce [2].

The study "Overall survival in multiple myeloma in Brazil: A cohort of 16 years", published in Hematology, Transfusion and Cell Therapy, provides a comprehensive overview of survival among MM patients in the country. This study evaluated 25,370 patients treated within the Brazilian Unified Health System (SUS) between 2000 and 2015, constituting one of the largest national MM cohorts ever published. It allows for important reflections on gaps and opportunities in research and health policy [3].

The epidemiological data of the study align with the national literature. Key characteristics included: a median age of patients of 62 years, a predominance of males, regional differences (highest concentration in the southeastern region), and overall survival (OS) of 37 months. These findings are consistent with other Brazilian analyses [4]. but contrast with countries such as the USA and France, where the median age at diagnosis is higher (66–74 years) and survival is longer [5,6]. This underscores differences in demographics, epidemiology, and access to therapy.

The study also evaluated the impact of different therapeutic regimens. Although bortezomib (Bortezomib) was only formally incorporated into the SUS in 2020, patients treated with this agent had an OS of 67 months, while those on thalidomide-based regimens reached 54 months. Notably, patients undergoing hematopoietic stem cell transplantation (HSCT) exhibited an even higher survival, with a median of 87 months [3]. These results reinforce the clinical benefits of innovative therapies and transplantation, highlighting the importance of early access to effective treatments.

However, despite the robustness of the administrative database used by the authors (DATASUS), some gaps remain. There are no data on treatment adherence, toxicity, cytogenetic stratification, or responses to specific lines of therapy. Additionally, the absence of information on time to diagnosis, functional status, and associated comorbidities limits individualized interpretation and prevents understanding the determinants of survival. Furthermore, the methodology adopted —classifying patients according to therapy exposure at any point rather than only the first line regimen—reflects real-world complexity, although it may introduce selection bias.

The difference in survival of individuals undergoing HSCT underscores its relevance and efficacy as a standard of care. However, it is noteworthy that HSCT was performed in only 26.9 % of the population, despite its established efficacy as a standard therapeutic cornerstone for eligible patients [3]. In the Brazilian context, this low rate reflects structural barriers, including an insufficient number of specialized centers, regional disparities in healthcare access, long waiting times, and socioeconomic limitations. Thus, concerns remain regarding the underutilization of transplantation in Brazil [7]. Additionally, the late incorporation of new drugs, such as bortezomib (Bortezomib), demonstrates the need for more agile health technology assessment processes without compromising safety and efficacy [8]. Consequently, it is plausible that many eligible patients never undergo HSCT, limiting the potential gains shown by the national cohort.

Importantly, this study offers key lessons for Brazil. In summary, three points should be considered: there is room for more detailed investigations that can translate evidence into concrete improvements in clinical practice; it is essential to discuss strategies that expand equitable access to HSCT, such as increasing the network of specialized centers, coupled with rapid incorporation policies and adequate funding. Robust national data can support health policy, guide therapeutic protocols, and reduce reliance on evidence from other countries, which often have distinct population profiles.

In conclusion, the 16-year national cohort analysis highlights the impact of innovative drugs, the importance of long-term studies for MM in Brazil, exposes failures in access to innovative therapies and HSCT, and underscores the urgency

of public policies capable of democratizing this access. Encouraging the collection of population-based data, expanding access to medications, and increasing the transplantation network are essential steps to improve care for patients with MM in Brazil.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

The authors declare no competing financial interests.

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Letter to the Editor

Platelet transfusion in end-of-life adult care

Dear Editor,

In a tertiary care hospital, where the complexity of patients treated has increased over the years, the decision to transfuse each individual that presents with anemia or thrombocytopenia must be guided not only by clinical and laboratory characteristics, but also by the availability of blood products, ensuring judicious and ethical use of this scarce resource. In our hospital, despite a continuous effort to increase platelet collections, transfusion demands continue to rise, requiring careful and continuous reassessment of transfusion practices.

Current protocols recommend platelet transfusion in case of active hemorrhage or, prophylactally, for high-risk bleeding procedures in patients with severe thrombocytopenia or under antiplatelet therapy, and for spontaneous bleeding prevention in severe thrombocytopenia cases [1–3]. In asymptomatic patients with platelet counts $<10 \times 10^3/\mu L$ or $<20 \times 10^3/\mu L$ if other bleeding risk factors exist, platelet transfusion is intended, in particular, to reduce the likelihood of intracerebral bleeding [1–4].

However, emerging evidence suggests that transfusion policies should not rely solely on platelet counts, but instead be adapted to each patient's bleeding risk (also associated with personal history of bleeding, renal failure, hypoalbuminemia, fever, or recent stem cell transplantation [5]) and care objectives, particularly in palliative and end-of-life settings [6,7].

In this population, prophylactic transfusions raise significant clinical and ethical concerns: based on the stage of the disease and estimated life expectancy, the level of care should focus on optimizing quality of life. In these cases, efforts should be made to discontinue all treatments that do not directly contribute to the patient's comfort and that do not aim for symptom control or achieving realistic goals [8,9].

In hemato-oncologic terminal patients, disease-specific treatments are often continued, resulting in frequent hospital visits and admissions, intensive care interventions, and high intrahospital mortality rates [10,11]. Unlike erythrocyte transfusions, that may provide symptomatic relief, prophylactic platelet transfusion has not consistently been associated with a reduction in bleeding complications or an improved survival in terminal patients [5,12,13].

It is known that terminal patients often face repeated hospital visits for transfusions, resulting in discomfort and stress for them and their caregivers. Nonetheless, each transfusion carries approximately a 1% risk of severe adverse reactions, and its hemostatic effect is short-lived [14–18]. As such, providing platelet transfusions in end-of-life care involves complex ethical principles:

- **Non-maleficence:** Restrictive prophylactic transfusion policies may prevent harm by avoiding frequent hospital trips, invasive testing, and treatment-related adverse effects that offer limited benefit in late-stage disease. Unnecessary transfusions may delay timely transition to palliative care, potentially diminishing quality of life.
- **Beneficence:** Avoiding futile treatments helps spare patients from side effects and unnecessary interventions, promoting well-being.
- **Justice:** Platelet components are scarce; clinical judgment should ensure equitable distribution while addressing individual needs.
- **Autonomy:** Patients must receive complete information about transfusion risks and benefits, allowing their preferences and advance directives to guide treatment decisions.

Based on a literature review and multidisciplinary discussion at our hospital, we proposed the following recommendations for platelet transfusion in adult patients in end-of-life care:

Recommendation 1: Clinical records must identify their disease stage and care objectives (curative intent, symptom control, or comfort measures only). Before deciding to transfuse platelet components, information concerning patients' indication for advanced life support and/or prognosis-modifying therapy is essential.

Recommendation 2: The decision to transfuse platelet components should focus on patients' comfort, burden of associated symptoms, and life goals.

Recommendation 3: Prophylactic platelet transfusions should be avoided:

- Decision should not depend on the patient's platelet count.
- In this context, peripheral blood smear platelet counts should not be performed.

Recommendation 4: In patients that present with bleeding symptoms or need to undergo high bleeding risk procedures:

- Clinical justification for invasive procedures with associated bleeding risk must be weighed against their potential futility, inherent risks, and associated discomfort for the patient [13]. All measures that represent therapeutic obstinacy should be avoided.
- Patient and platelet components should not be phenotyped/genotyped for platelet and HLA antigens/genetics.
- Platelet function tests should not be performed.
- Transfusion of platelet pools should be preferred, avoiding the use of single platelet concentrates.

Recommendation 5:

- Each patient's autonomy must be respected, ensuring the right to information and active participation in decisions regarding platelet transfusions, through truly informed consent.
- Considering each patient's autonomy, advance directives, when available, must be taken into consideration in situations where the patient is unable to express his wishes.

In conclusion, we believe that the management of platelet transfusion in end-of-life care requires a nuanced, ethically grounded approach that balances clinical benefit with the patient's comfort, dignity, and autonomy [19]. These recommendations aim to guide clinicians in judiciously using platelet components, avoiding non-symptom-relieving interventions, and promoting individualized, compassionate care for terminally ill adult patients.

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Informed consent

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Author contributions

DC reviewed the literature and drafted the manuscript; FT, MB, JA, JAP, EG, JB and JT contributed to the data discussion; FA conceptualized the study, evaluated the data, and revised the manuscript.

Data availability

The authors declare that data supporting the findings of this study are available within the article.

This study was approved by São João Local Health Unit Health Ethics Committee.

Conflicts of interest

None to declare.

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Letter to the Editor

Drug development for sickle cell disease: repeated setbacks, yet there remains an optimistic outlook for future breakthroughs

To the editor:

Sickle cell disease (SCD) is a chronic and debilitating genetic blood disorder that affects approximately 6.5 to 9 million individuals worldwide. It is estimated that more than 90% of them are located in Africa and India [1,2].

The basic event of the pathophysiology of the disease, namely the polymerization of deoxy HbS, has been recognized for approximately the last fifty years [3]. Since then, we have seen a continuous search for drugs that can interfere with and reduce hemoglobin S polymerization as a potential therapy for SCD. Approximately a hundred possible anti-sickling agents have been described. Several of these, with some effects *in vitro*, were tested in several clinical trials without success [3–15]. More recently, newer molecular biology methods and animal models of SCD have helped characterize the complex chain of events, beyond Hb polymerization, responsible for most of the acute and chronic organ lesions of SCD. Thus, several other essential steps responsible for the vaso-occlusion and organ damage have been discovered, such as endothelial cell lesions, the critical role of hemolysis in inflammation and organ damage, increased adhesion of red and white blood cells and platelets to the endothelium, sterile inflammation, reduced nitric oxide (NO) levels, activation of thrombosis and lesions secondary to oxidation [2]. These new data resulted in a new wave of possible new treatment targets, which started the search for new of families of molecules.

However, until now, the only molecule that has been able to reduce the polymerization of deoxy HbS in humans with confirmed clinical effects is hydroxyurea (HU). The predominant effect of HU is to increase the production of HbF, a hemoglobin well known as a molecule that can reduce the desoxy HbS polymerization. Moreover, HU presents other actions, such as, for example, reducing white and red blood cell adhesion to the endothelium, acting as a NO donor, and reducing inflammation secondary to hemolysis. HU was approved by the FDA for treatment of adults with SCD in 1998 and for children in 2017. It is effective and safe for all ages and in all regions of the planet, and there is robust evidence that treatment with HU reduces the number of vaso-occlusive crises and acute chest syndrome, increases patient survival, and decreases chronic organ damage. Although there is a percentage of patients that do not respond to the treatment and there are several collateral effects that should be monitored, there are no other drugs that have shown clinical effects comparable to those of HU. In fact, HU should be considered, today, the standard drug for SCD and universal access to this drug should be a priority, particularly for patients in countries with low and medium incomes (LMIC), such as regions of Sub-Saharan Africa and India [2,15].

In addition, several basic procedures should be recognized as essential to increase the survival of SCD patients, irrespective of any specific drugs for blocking polymerization. Solid and well known data have shown that early universal diagnosis of the disease (as achieved by newborn screening), well organized infrastructure and health care for children with SCD, procedures to prevent complications such as pneumococcus, Haemophiles Influenzae and meningococcal vaccines, prophylactic penicillin, transcranial doppler (TCD), adequate and well controlled transfusion programs, adequate patient registries, well-trained health providers, among others, can significantly increase the survival of patients, as has been seen in some high income countries such the US, UK and France. Unfortunately, in most low-middle income countries (LMICs), even basic procedures are not available, and the implementation of achievable goals aimed at reducing the mortality and morbidity associated with this long-neglected disease is urgent [2,16].

Despite all of the benefits derived from the treatment with HU and other well-consolidated measures, it is recognized that there are several unmet needs in the treatment of SCD and new treatments that target the underlying course of SCD, as well as its acute and chronic complications are necessary. Regrettably, with exception of HU, in the past few decades

there has been an unfortunate failure to provide new disease-modifying treatments, due to a lack of benefit or potential harm, as demonstrated by clinical trials [2,4,17–20]. Remarkably in the last eight years, three new drugs were approved by the US control agency, the FDA, including L-glutamine, crizanlizumab and voxelotor. The very disturbing fact is that two of them (crizanlizumab and voxelotor) were removed from the market due to lack of benefit or emerging safety concerns and re-evaluation of their risk-benefit profiles [17,18,20–22].

In September 2024, Pfizer voluntarily withdrew voxelotor, an anti-sickling agent for SCD, from the global market based on emerging clinical data indicating that the drug's risks outweigh its benefits in SCD management.3 Studies have indicated an elevated risk of vaso-occlusive crises and fatalities among individuals receiving voxelotor treatment. In a multicenter trial (NCT04218084) involving 236 children with sickle cell disease (SCD) who were at high risk of stroke, there were eight deaths in the voxelotor group, compared to two in the placebo group, prompting an early termination of the trial [23].

Another trial (NCT05561140) focused on adolescents and adults with SCD and leg ulcers, and reported eight deaths among 88 patients treated with voxelotor during the open-label phase [24]. These outcomes led Pfizer to discontinue the distribution, recall existing stock, and cease all ongoing clinical trials involving voxelotor.

Given the probable efficacy, benefits and favorable safety profile from the pivotal phase 2 SUSTAIN study (NCT01895361), along with pharmacokinetic and pharmacodynamic evaluations of the dosing regimen, crizanlizumab, an anti-adhesive monoclonal antibody, was seen as a potential disease-modifying therapy for reducing vaso-occlusive crises (VOCs) in SCD [25]. It received conditional approval from the regulatory agencies in the USA, Europe and Brazil. Unfortunately, the primary results from the Phase 3 STAND trial (NCT03814746) indicated no significant difference between the crizanlizumab groups (5.0 mg/kg and 7.5 mg/kg) and the placebo group in the annualized rate of vaso-occlusive crises (VOC) that necessitated a healthcare visit or home management for patients with sickle cell disease (SCD) aged 12 years and older. The safety profile of crizanlizumab was consistent with previous studies, and no new safety issues were identified with either dose. Although the drug has been withdrawn from the market, it remains available through compassionate use and other programs in several regions.

Despite these adverse results, the game seems not to be over for crizanlizumab. New data from clinical trials involving different regimens and age groups of SCD patients continue to emerge about crizanlizumab. This data reinforces the novel drug's safety and tolerability globally. The subsequent phase 2 open-label SOLACE-adults study analyzed long-term (i.e., ≥3 years) follow-up on the effects of crizanlizumab (5.0 mg/kg and 7.5 mg/kg) in adult patients with SCD (N=57) [26]. Similarly, the Phase 2 SOLACE-kids study examined children with SCD aged 12 to under 18 years (N=53) [18]. Both studies showed reductions from baseline in VOC-related healthcare visits, hospitalizations, emergency room visits, and a consistent safety profile for crizanlizumab. Crizanlizumab was made available through a managed access program (MAP, NCT03720626). This analysis assessed the effects of 12 months of crizanlizumab treatment on vaso-occlusive crises (VOCs) and opioid use for managing VOC-related pain in patients with sickle cell disease (SCD) participating in the MAP. Crizanlizumab showed promise in reducing VOC incidents, regardless of SCD genotype or previous hydroxyurea treatment, and in decreasing opioid consumption. The safety profile of crizanlizumab aligned to prior findings [27].

To test the effectiveness of crizanlizumab outside a pandemic context, Novartis is conducting the CSEG101A2303 study (SPARKLE, NCT06439082). This phase III, multi-center, randomized, placebo-controlled, double-blind trial is designed to evaluate the efficacy and safety of crizanlizumab (5 mg/kg) compared to a placebo, with or without hydroxyurea/hydroxycarbamide treatment, in patients with sickle cell disease (SCD) aged 12 and older who experience frequent vaso-occlusive crises (4-12 events in the 12 months leading up to the screening visit). Participants will be randomly assigned in a 2:1 ratio to receive either crizanlizumab 5 mg/kg or a placebo. Central randomization will be stratified based on concurrent use of HU/HC (yes/no) and region (South America, North America, and sub-Saharan Africa) at baseline [28].

On August 15, 2025, Pfizer Inc. announced results from the Phase 3 THRIVE-131 (NCT04935879) trial evaluating inclacumab, an investigational P-selectin inhibitor and has decided to terminate the study [29]. The study enrolled 241 participants with SCD, 16 years of age and older. Inclacumab was generally well-tolerated, but the study did not meet its primary endpoint of a significant reduction in the frequency of VOCs in participants administered inclacumab compared to those given a placebo every 12 weeks over 48 weeks [29].

It is very difficult to describe all the causes of this repetitive failure to find new drugs with significant clinical effects. Moreover, the approval and subsequent removal from the market of Voxelotor and Crizanlizumab sparked an intense debate among researchers and physicians working with SCD [19, 20–22]. Despite all these problems, important lessons emerge from this disappointing scenario of failure in developing new drugs for treating SCD and are described below [19,21,22]:

- The pathophysiology of SCD turned out to be very complex with multiple pathways.

- Geographic variability remains a significant challenge while evaluating real-world data on novel SCD therapies. SCD outcomes vary widely across different countries and continents. Recruitment across diverse geographical areas should consider patients' demographics, including various regions (e.g., Africa, Brazil and India) and ethnicities. Although the disease had similar manifestations across ethnicities, variations in the use of health-care facilities, socio-cultural factors, and management of pain across different regions could have marked differences in its results [15,21].

- The complex interplay between environmental and genetic factors, trial settings, healthcare usage and practices, and how they influence treatment efficacy.

- It's time to reach a consensus on the definitions of VOC in patients who receive these therapies. While clinical trials of therapies for patients with SCD often rely on the reduction or elimination of VOC pain events as a clinical

endpoint, definitions of what constitutes a VOC pain event differ between trials and have no impact on efficacy assessments between clinical trials [30,31].

- A possible premature approval of drugs by authorities, their promotion by healthcare professionals and patient associations, and the pursuit by pharmaceutical companies to access the market rapidly should be rethought and better discussed [15,19,21,22].

What suggestions can be considered to counteract this unsatisfactory situation, effectively reduce health disparities, and promote the global advancement of efficient and accessible therapies? [4,19]. As already mentioned, there are well-known and recognized measures in the comprehensive management and patient-focused care of SCD that are essential. Among these, it is essential to emphasize universal newborn screening, providing access to specialized healthcare for all patients with SCD, regardless of their location, making HU accessible and affordable, and improving the availability and safety of blood transfusions. In addition, there are several other actions that should be considered [2,15,19,21,22]:

1. Improving clinical trial designs: focus on meaningful clinical outcomes, incorporate diverse patient populations, always include patient participation, and consider variations in SCD outcomes between different countries and continents [30,31].
2. Real-world evidence should be considered as the primary reference for translating clinical trial results to achieve and reliability across various patient groups.
3. Innovative therapeutic approaches: explore personalized therapies based on combined treatments that target various pathophysiological pathways in SCD, such as combination therapies, which are currently underexplored.
4. Collaboration and communication involve fostering dialogue among experts, patients, funding bodies, universities, pharmaceutical companies, regulatory authorities, and other stakeholders.
5. Increasing research funding requires promoting a more effective blend of academic and industry-driven studies, engaging patients from the outset, and conducting more robust clinical trials with suitable patient populations and endpoints. [32,33]

As of now, hydroxyurea remains the only approved disease-modifying therapy for sickle cell disease (SCD). Given the significance of SCD as a global public health issue, it is imperative to develop new therapeutic options. Encouragingly, emerging drugs - such as pyruvate kinase activators and those that either inhibit the polymerization of sickle hemoglobin or enhance the production of hemoglobin F - are advancing to late-stage clinical trials [21,22]. Substantial progress is being achieved in so-called curative therapies, including bone marrow transplantation and gene therapy. The possibility of doing HSCT with haploidentical donors seems to be a major improvement [34]. Two gene therapy procedures are now approved by the FDA (gene editing and gene addition), but these procedures are extremely expensive, making access impossible for most patients with SCD [35].

It is crucial to emphasize the need for transparent and careful communication when approving or withdrawing therapies for SCD [36]. When new medications are approved, it is essential to proceed cautiously, clarifying known toxicities, uncertainties, and the provisional nature of the approvals, avoiding exaggerated promises. There must be long-term post-approval, real-world outcomes, supported by multiple data registries, to ensure that subtle problems and other benefits can be identified [22]. Obviously, it is essential to ensure informed consent from all the patients. In the case of medication withdrawals, it is very important to provide the data that justifies the decision promptly [36].

Conflicts of interest

The authors declare no conflicts of interest.

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