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ABHH
Rua Diogo de Faria, 775/conjunto 133
04037-002
Vila Clementino - São Paulo/SP - Brazil
(11) 2369-7767 / (11) 2338-6764 (WhatsApp)
E-mail: abhh@abhh.org.br
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Original article

Annualized bleeding rate in hemophilia A patients in Brazil: a systematic review

Alessandra NL Prezotti [a,*], *Débora MC Rocha* [a], *Endi L. Galvão* [b], *Thaís Gimenez* [c], *Leo Sekine* [d], *Rodrigo A. Ribeiro* [e], *Elisa Sobreira* [f]

[a] *Centro de Hematologia e Hemoterapia do Espírito Santo, Vitória, ES, Brazil*
[b] *Departamento de Fisioterapia, Universidade Federal dos Vales do Jequitinhonha e Mucuri, MG, Brazil*
[c] *Departamento de Ortodontia e Odontopediatria da Faculdade de Odontologia da Universidade de São Paulo, SP, Brazil*
[d] *Serviço de Hemoterapia do Hospital de Clínicas de Porto Alegre/Faculdade de Medicina da Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[e] *HEMAP Consulting, Porto Alegre, RS, Brazil*
[f] *BioMarin Farmacêutica do Brasil, São Paulo, Brazil*

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ABSTRACT

Background: Hemophilia A is an X-linked chronic bleeding disorder due to deficiency of the coagulation factor VIII. According to the residual level of FVIII activity, patients can present with severe (FVIII levels <1 %), moderate (1–5 %) or mild (6–40 %) phenotypes. While long-term prophylaxis is the current standard of care and has been shown to be effective in minimizing bleeding episodes, episodes of hemarthrosis, that could lead to arthropathy and disability, are still reported. This systematic review aimed to evaluate available data concerning current treatment outcomes in severe hemophilia A patients without inhibitors in Brazil, focusing on the frequency of bleeding episodes and adherence to therapy of patients under prophylactic treatment.

Method: A literature search strategy was used in the MEDLINE (via PubMed), Embase, LILACS and SciElo databases from 2014 onwards, since it was the moment that prophylaxis effectively became available in the Brazilian National Health Service, even though prophylactic treatment had been officially incorporated in 2011 focused on concerning bleeding episodes and adherence rate of this population.

Results: Searches yielded 536 articles. After removal of duplicates, 417 articles were screened for eligibility. Eventually, 104 articles were selected for full-text assessment. Finally, only five publications met eligibility criteria and were selected for the descriptive review.

Conclusion: Available information on efficacy of severe hemophilia A management in Brazil currently relies on scarce and possibly biased information. It should be strongly empha-

* Corresponding author. Centro de Hematologia e Hemoterapia do Espírito Santo, Campos, 1468, Maruipe, Vitória, Espírito Santo, Brazil.
E-mail address: aprezoti@yahoo.com (A.N. Prezotti).

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sized that Brazil is in great need of a structured and coordinated effort to improve collection, analysis, and reporting of data on hemophilia A patients.

Introduction

Hemophilia A is an X-linked chronic bleeding disorder due to deficiency of the coagulation factor VIII (FVIII).[1] Although considered a rare disease, it is possible that numbers have been grossly underestimated,[2] with previously reported hemophilia A incidence rates at 1 case in 5000 male births,[3] and an observed prevalence rate of 10.5 patients per 100,000 males.[4] The estimated worldwide prevalence of patients with hemophilia (both hemophilia A and B) reaches a total of 1,125,000 individuals, while an estimated 418,000 individuals will present severe manifestations of the disease.[5]

Small amounts of residual FVIII activity exert a large clinical impact in hemostasis. Patients with severe deficiency (FVIII levels <1 %) usually fare worse than moderately (1–5 %) or mildly (6–40 %) affected patients.[1] Indeed, the cornerstone of treatment is replacement therapy, increasing FVIII levels with intravenous injections, either episodically to treat acute bleeding or prophylactically to prevent them.[5] Long-term prophylaxis is currently standard of care and has been shown to be very effective in minimizing bleeding episodes, especially hemarthrosis, that could lead to arthropathy and disability.[2] However, due to terminal half-life of traditional FVIII replacement, frequent injections are needed, making it rather burdensome and expensive for patients and the healthcare system, while also compromising treatment access and adherence.[5]

While much effort has been made during the last few years aiming at developing new alternatives for hemophilia A patients such as extended half-life clotting factor concentrates, bispecific monoclonal antibodies (e.g. emicizumab) and gene therapy, patients in Latin America still seem to struggle to attain adequate access to comprehensive multidisciplinary treatment. In Brazil, patients with hemophilia, and several other types of coagulopathies, are managed at blood centers, governmental dedicated healthcare facilities that hold and distribute all clotting factor concentrates. Despite this centralized care, access to contemporary therapeutic options and pipeline drugs and therapies is limited due to cost-effectiveness concerns. Furthermore, clinical data on severe hemophilia A patients have not been adequately summarized, especially after implementation of the 2014 national policy for primary prophylaxis.

Objective

The present systematic review aimed to evaluate available data concerning current severe hemophilia A treatment outcomes in Brazil, focusing on the frequency of bleeding episodes and adherence to therapy of patients under conventional treatment.

Methods

The main objective of the present study was to systematically review relevant data on severe hemophilia A management outcomes in Brazil, especially concerning bleeding episodes (annualized bleeding rate [ABR]) and adherence rate of this population.

Information sources and search strategy

A literature search strategy was performed in the MEDLINE (via PubMed), Embase, LILACS and SciElo databases. No language restrictions were used but the time of publication was restricted to 2014 onwards, since it was the time that prophylaxis effectively became available in the Brazilian National Health Service, even though prophylactic treatment had been officially incorporated in 2011.

The search strategy for each database is shown in Table 1. All searches were restricted to between 2014 and 2022. Overall, the search terms were as follows: population was defined as Brazilian hemophilia A patients; intervention included any type of prophylaxis (whether primary, secondary, or tertiary); the outcomes were ABR and adherence to treatment; and type of study comprised both observational studies and clinical trials.

Duplicates were excluded before proceeding to study selection. All titles and abstracts retrieved were screened independently by two researchers. Full-text articles also had their eligibility evaluated by two independent researchers. The last date of the search was May 18th, 2022. The review protocol was registered in the OSF registries database (https://osf.io/am4pg). This study followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement for conducting studies and reporting results.

Eligibility criteria

Observational studies and clinical trials that fulfilled the following criteria were selected: 1) they were concerned with hemophilia A patients with a congenital bleeding disorder resulting from FVIII deficiency; 2) Brazilian patients with severe hemophilia A without inhibitors, receiving some type of prophylactic FVIII; and 3) Prophylaxis could be conceptually primary, secondary, or tertiary. No comparators were required and the main outcome to be evaluated was the reported ABR. Proceedings from major international meetings in the field and letters to the editor were also included. In vitro or animal model studies, review articles, guidelines,

Table 1 – Search strategy employed for each database.

Database	Search strategy
PubMed/MEDLINE	((((((((((("Factor VIII deficiencies") OR ("Factor VIII deficiency")) OR ("FVIII deficiencies")) OR ("FVIII deficiency")) OR ("Hemophilia A")) OR ("Haemophilia A")) OR (a, hemophilia[MeSH Terms])) OR (hemophilia)) OR (hemophilia[Title/Abstract])) OR (haemophilia[Title/Abstract])) AND ("bleedings"[All Fields] OR "hemorrhage"[MeSH Terms] OR "hemorrhage"[All Fields] OR "bleed"[All Fields] OR "bleeding"[All Fields] OR "bleeds"[All Fields] OR "prophylaxis"[All Fields] OR "prophylaxes"[All Fields] OR "prophylaxis"[All Fields])) AND ((brasil* or Brazil* or Brazil[ad]))
EMBASE	('bleedings' OR 'hemorrhage'/exp OR 'hemorrhage' OR 'bleed' OR 'bleeding'/exp OR 'bleeding' OR 'bleeds' OR 'prophylaxis'/exp OR 'prophylaxis' OR 'prophylaxes' OR 'prophylaxis') AND ('brasil' OR 'brasileiro' OR 'Brazil'/exp OR 'Brazil' OR 'Brazilian'/exp OR 'Brazilian') AND ('factor viii deficiencies' OR 'factor viii deficiency'/exp OR 'factor viii deficiency' OR 'FVIII deficiencies' OR 'FVIII deficiency' OR 'hemophilia a'/exp OR 'hemophilia a' OR 'haemophilia a'/exp OR 'haemophilia a' OR 'a, hemophilia' OR 'hemophilia'/exp OR hemophilia OR 'haemophilia'/exp OR haemophilia)
Lilacs	'factor viii deficiencies' OR 'factor viii deficiency' OR 'FVIII deficiencies' OR 'FVIII deficiency' OR 'hemophilia a' OR 'hemophilia a' OR 'haemophilia a' OR 'haemophilia a' OR 'a, hemophilia' OR 'hemophilia'/exp OR hemophilia OR 'haemophilia' OR haemophilia [words] and Brazil OR Brazil [words]
Scielo	factor viii deficiencies OR factor viii deficiency OR FVIII deficiencies OR FVIII deficiency OR hemophilia a OR hemophilia a OR haemophilia a OR haemophilia a OR a, hemophilia OR hemophilia/exp OR hemophilia OR haemophilia OR haemophilia

qualitative studies, expert opinion articles and case reports were excluded.

Study selection and data extraction

Two reviewers independently participated in the screening and full-text evaluations. A third reviewer participated in the case of any discordance.

Data were tabulated in Excel spreadsheets (Microsoft Corp, Washington, USA) by the two independent reviewers. A data extraction form included the following information:

Study characteristics: author and year of publication, country, and follow-up period;
Sample characteristics: n, mean age, gender, and treatment status (Y/N); outcomes evaluated;

Main findings: descriptive and quantitative results, effect size, and *p*-value whenever available.

Quality assessment and risk of bias

The risk of bias was assessed using the Risk of Bias in Non-randomized Studies of interventions (ROBINS-I)[6]. The authors answered signaling questions for each domain (confounding, selection, classification of interventions, deviation from intended interventions, missing data, measurement of outcome, and selection of the reported results). They then estimated the overall risk of the bias according to the results for each domain as low, moderate, serious, or critical. The risk of bias analysis considered studies with a before-after design, without a comparative group.

Strategy for data synthesis

Descriptive synthesis, and when considered feasible, a meta-analysis with the ABR and adherence rate values were planned.

Results

The PRISMA flowchart illustrating the study selection process is shown in Figure 1. The searches yielded 536 records (including duplicate entries). After removal of duplicates, 417 references were screened for eligibility. Eventually, 104 records were selected for full-text assessment. Only five publications[4,7-10] met eligibility criteria and were selected for descriptive review. Meta-analysis of data retrieved could not be performed due to the heterogeneity of the studies.

Data pertaining adherence to prophylactic treatment could not be retrieved according to established selection criteria.

Study by *Kenet et al.*[4]

This was a multinational, prospective, non-interventional study that aimed at collecting standardized real-world data on bleeding episodes, hemophilia medication use, and health-related quality of life (QoL) from a global, heterogeneous population of participants with severe hemophilia A on currently available FVIII prophylaxis. Participating sites were located in Australia, Belgium, Brazil, France, Germany, Israel, Italy, South Africa, South Korea, Spain, Taiwan, the UK, and the US. This study was also a run-in for the sponsor's Phase 3 gene therapy studies (Clinicaltrials.gov NCT03370913/EudraCT 2017−003215−19, NCT03392974/EudraCT 2017−003573−34).

Enrolled patients were males, 18 years of age or older, with severe hemophilia A (FVIII activity ≤1 IU/dL), continuously treated with prophylactic exogenous FVIII for six months or more and no history of detectable FVIII inhibitors. Patients were excluded if they were HIV-positive, had significant liver dysfunction, chronic or active hepatitis B, or active hepatitis C. High-quality historical documentation concerning bleeding and exogenous FVIII usage over the previous six months was required.

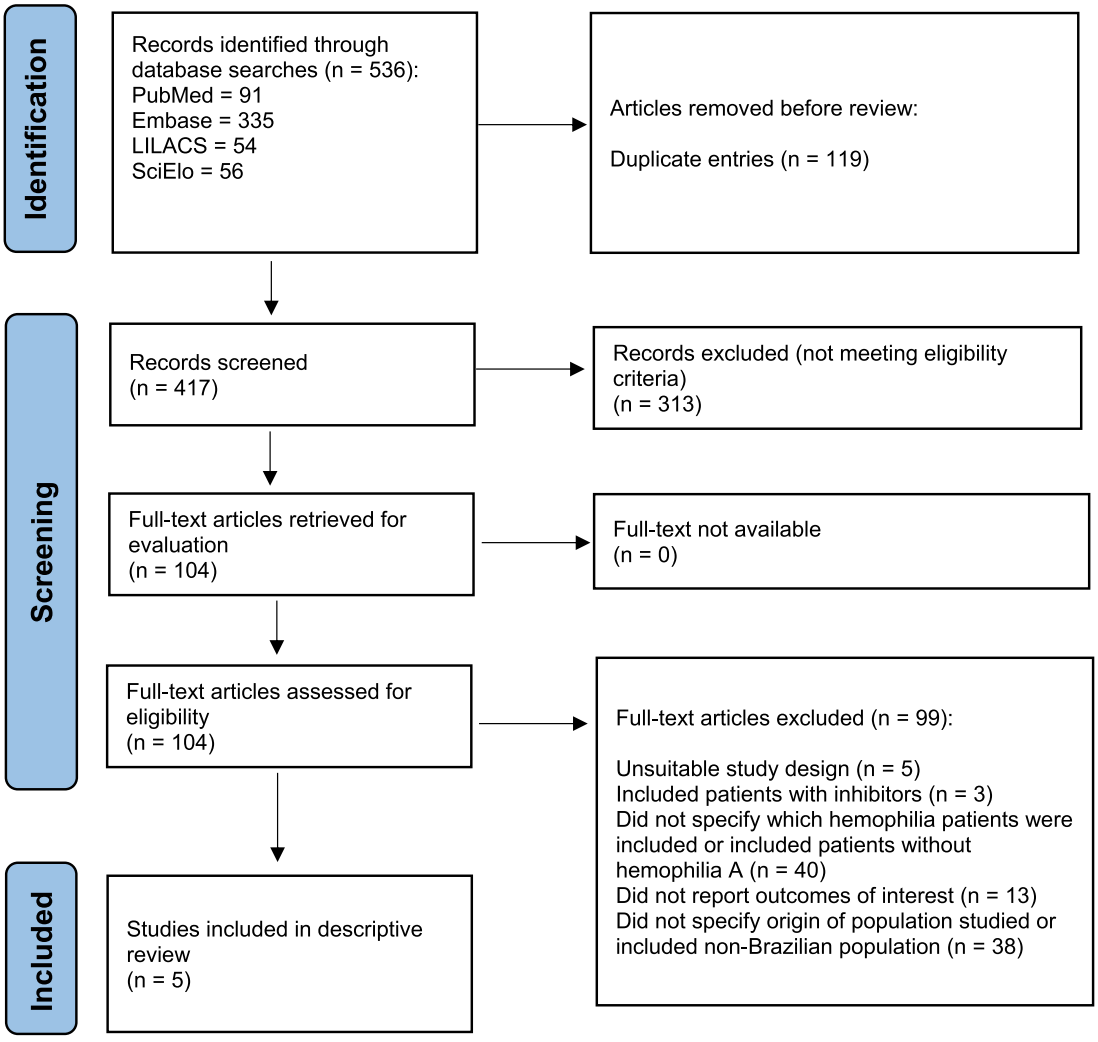

Figure 1 – Included studies - flow diagram.

Study procedures included a review of bleeding episodes (including start date/time, type [e.g., joint or muscle], location, and whether there was preceding trauma or ensuing treatment), FVIII replacement (start date/time, product name, dose, indication [e.g. usual prophylaxis, one-time prophylaxis, or treatment for bleeding]) at least at a monthly basis (weekly evaluations were recommended whenever possible), as well as the monitoring of concomitant medications, adverse events (AEs), serious AEs (SAEs), and interim medical history at each visit or with telephone calls on at least a monthly basis. Except for screening/baseline and end-of-study visits, all other study visits occurred according to participants' local standard of care. No clinical intervention or study drug was provided.

The primary clinical endpoint was ABR requiring exogenous FVIII replacement treatment. Secondary endpoints included annualized utilization (IU/kg/year) and infusion rate (count/year) of exogenous FVIII replacement therapy. Also, patient-reported outcomes such as the hemophilia-specific health related quality of life questionnaire for adults (Hemo-QoL-A), EQ-5D-5 L, Hemophilia Activities List (HAL), and Work Productivity and Activity Impairment plus Classroom Impairment Questions: Hemophilia Specific (WPAI+CIQ:HS) were evaluated. Safety assessments consisted of monitoring AEs (coded using the Medical Dictionary for Regulatory Activities v20.1) and measuring vital signs and hematology, clinical chemistry, and urinalysis variables.

A total of 370 patients were screened for eligibility and eventually 294 patients were enrolled. From those enrolled, 225 (76.5 %) completed at least six months of follow up and were included in the six-month analysis population. Results are presented by region, and as the only study site from South America was Brazil, whole data originated from the Hemocentro, a reference tertiary healthcare provider established in the city of Campinas and coordinated by the State University of Campinas. Patient demographics and baseline characteristics for the Brazilian subgroup are found in Table 2. The Brazilian patients had the lowest median age at enrolment (27 years old) while East Asia participants had the highest median age (40 years old). Also, lowest rates of problem joints (defined as joint with chronic pain, chronic synovitis, hemophilic arthropathy, limited motion or recurrent bleeding)

Table 2 – Patient demographics and baseline characteristics of the Brazilian hemophilia patients.[4]

Parameter	*n* = 54
Age at enrolment (years) - median (min-max)	27.0 (18.0–47.0)
Male sex - n (%)	54 (100.0)
Race - n (%)	
Black or Afro-American	10 (18.5)
White	44 (81.5)
Weight (kg) - mean (SD)	78.9 (20.4)
History of hepatitis B[a] - n (%)	1 (1.9)
History of hepatitis C[a] - n (%)	12 (22.2)
History of HIV - n (%)	0
Participants with problem joints[b] - n (%)	5 (9.3)
Number of problem joints[b] - n (%)	
0	49 (90.7)
1	5 (9.3)
2	0
3	0
>3	0

[a] Includes cleared or cured infections.
[b] Problem joints were identified by investigators at baseline and were defined as joints with any of the following symptoms: chronic joint pain, chronic synovitis, hemophilic arthropathy, limited motion, or recurrent bleedingHIV: human immunodeficiency virus; SD: standard deviation.

were found in Brazilians (9.3 %) while East Asia had the highest rates (56.3 %).

For the six-month analysis, the median follow-up time was 225.0 days (range: 169–469 days). Follow-up time specifically for Brazilian population was not reported. The ABR concerning treated bleeds, for Brazilian patients (*n* = 41) was reported for pre-baseline (mean: 2.44; standard deviation [SD]: 3.83; median: 0.00; range: 0.0–14.0), on-study (mean: 2.41; SD: 4.61; median: 0.00; range: 0.0–23.8), and total study duration (mean: 2.42; SD: 4.05; median: 0.80; range: 0.0–19.3) intervals. As shown, pre-baseline rate was consistent with on-study ABR.

Although no formal comparison was performed by the authors (it is mentioned that the study was underpowered to assess differences between the variables collected), mean and median treated ABR values reported for Brazilian patients seemed lower than the whole population (pre-baseline: mean: 5.03; SD: 9.35; median: 2.00; range: 0.0–86.0]; on-study: mean: 4.33; SD: 6.39; median: 1.85; range: 0.0–37.8; total study duration: mean: 4.64; SD: 7.00; median: 2.27; range: 0.0–57.8). Data for all bleeding events and stratified by treated bleed categories (whether spontaneous, traumatic, joint bleeds and problem joint bleeds) was not reported by region.

The pattern of patient's individual FVIII consumption was also reported for Brazil (Table 3). Brazilian patients showed low rates of FVIII infusion when compared to the whole population. Variations for this outcome between the different regions studied were not as significant as for ABR. Brazilian patients relied mostly on standard half-life recombinant FVIII, while most patients in Africa received plasma-derived products.

Concerning the frequency of FVIII infusions, Brazil had the highest mean rate: pre-baseline: *n* = 163 (per year: 60.0); on-study: *n* = 172 (per year: 63.1); total study duration: *n* = 168 (per year: 60.2) of the regions which, considering FVIII utilization rates were low, implies that probably lower doses were used for each infusion when compared to other countries.

Data on adverse events were not reported separately by region, and overall adverse events were seen in 43.5 % of patients, although only 4.8 % were considered serious events (according to the Common Terminology Criteria for Adverse Events - CTCAE). No adverse event led to discontinuation of treatment.

Patient reported QoL outcomes (total and stratified by region) concerning the Hemo-QoL-A tool are depicted in Figure 2 (higher scores representing better health-related QoL). For Brazil, the highest domain scores were observed for emotional impact (86.7 points) and role functioning (89.1 points), while the lowest scores were observed for physical functioning (63.3 points) and treatment concern (46.7 points). Noticeably, the treatment concern domain (that assesses confidence of patients in respect to safety and accessibility to treatment, e.g. "I worry about the availability of hemophilia products") for Brazilian patients was the lowest score among all the regions evaluated. Also, total score for Brazil fared unfavorably when compared to other countries with the lowest score observed (67.7 points). Results for the additional QoL scales applied were not reported separately for Brazil or other regions.

Upon discussion of the results, the authors argue that it is somewhat contradictory that countries and regions with such a low rate of FVIII utilization, such as Brazil and Africa, eventually presented with ABRs comparable to other regions, and especially such a low prevalence of problem joints (the lowest

Table 3 – FVIII replacement therapy profile in Brazil.[4]

Variable	FVIII Replacement Product (IU/kg/year)	Pre-baseline mean (SD)	On-study mean (SD)	Total duration mean (SD)
Pre-baseline and on-study annualized FVIII utilization rates of the 6-month analysis	Overall (*n* = 41)	3325 (1526)	3457 (1612)	3396 (1546)
	Standard half-life only (*n* = 35)	3265 (1225)	3391 (1434)	3335 (1307)
	Extended half-life only (*n* = 3)	5925 (2299)	5795 (2234)	5851 (2262)
	Plasma-derived only (*n* = 0)	NA	NA	NA
	Combination of products (*n* = 3)	1421 (370)	1888 (269)	1663 (78.7)
Pre-baseline and on-study annualized FVIII infusion rates of the 6-month analysis	Overall (*n* = 41)	163 (60.0)	172 (63.1)	168 (60.2)
	Standard half-life FVIII only (*n* = 35)	170 (60.5)	177 (61.8)	174 (60.3)
	Extended half-life FVIII only (*n* = 3)	102 (19.1)	100 (19.4)	101 (19.3)
	Plasma-derived FVIII only (*n* = 0)	NA	NA	NA
	Combination of FVIII products (*n* = 3)	140 (42.1)	185 (77.1)	163 (54.3)

NA: Not applicable; FVIII: factor VIII.

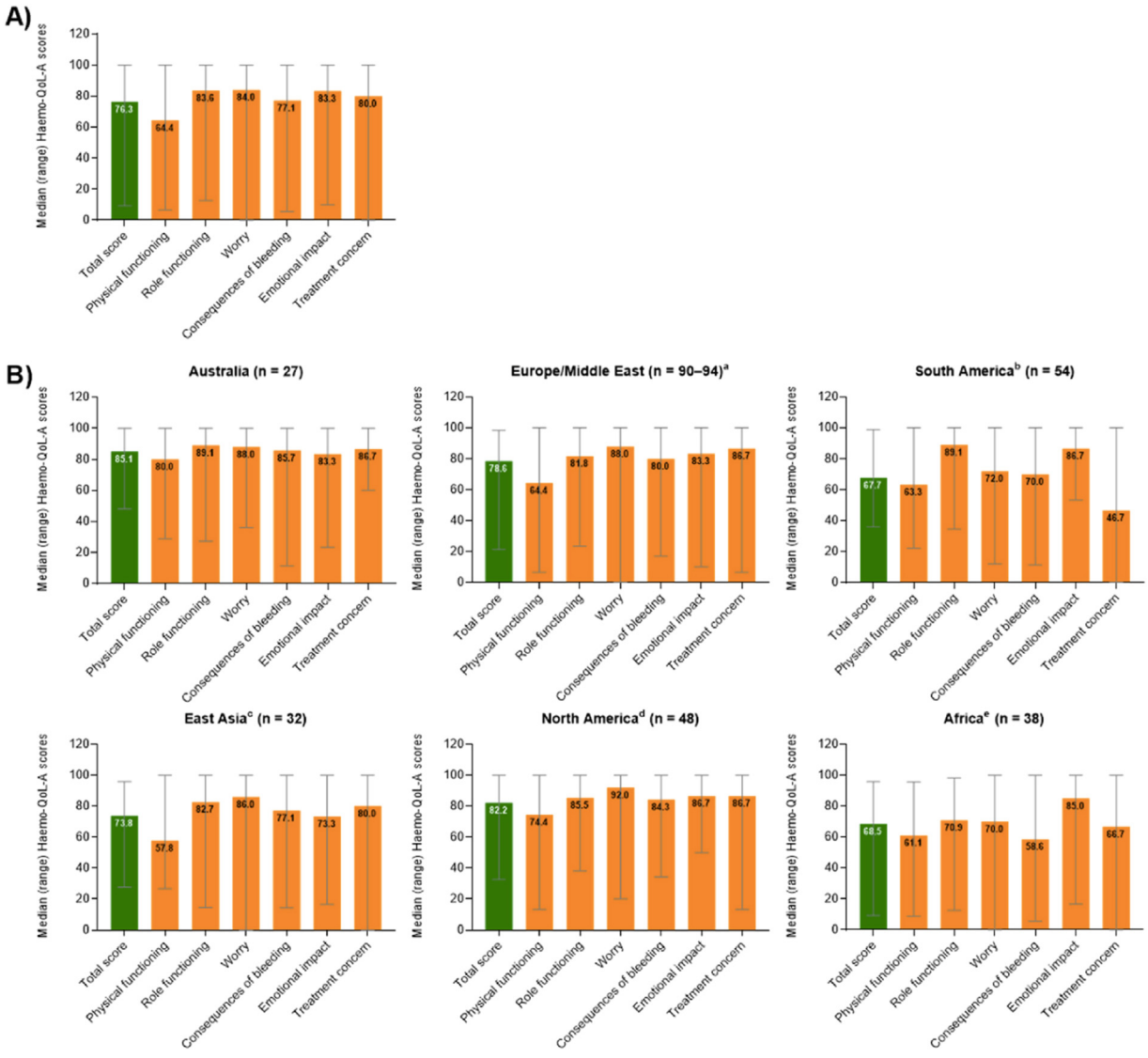

Figure 2 – Median (range) overall transformed Hemo-QoL-A total and domain scores at baseline (A) for all participants globally (*n* = 298) and (B) for participants by region.[4]

rates among the countries studied). Possibility underreporting should be considered. Another relevant drawback is the fact that this study enrolled patients that were motivated to take part in a gene therapy study that would follow this first 6-month observational follow up. As so, patients would probably be more prone to have a good adherence to treatment and to be dissatisfied with current therapeutic options in use. Site selection also could have influenced results as only facilities capable of providing structures demanded by gene therapy studies were selected.

Study by *Borges et al.*[7]

This research, published only as an abstract, evaluated the impact of a pharmacokinetic-guided prophylaxis strategy for hemophilia A patients using the myPKFiT™ tool developed for alfa-octocog™ recombinant FVIII (Advate, Takeda). Effects in replacement costs and bleeding episodes were assessed. Men with hemophilia A due to a severe or moderate deficiency but without detectable inhibitors on current use of alfa-octocog were evaluated for enrollment at two Brazilian hemophilia treatment centers (in the states of Paraná and Minas Gerais).

The inclusion criteria were that patients should present ≥50 exposure days, age ranging from 1 to 65 years, weigh from 12 to 120 kg, have a bleeding-free period of at least 2 wk, with the last registered surgical procedure being ≥6 months before enrollment. The detection of inhibitors (>0.6 BU/mL at two time points) during follow up resulted in patient exclusion from the study.

All information pertaining anthropometric and hemophilia-related data were obtained using a standardized form and pharmacokinetics analysis by the myPKFiT™ software using a one-step test. This analysis guided dose adjustments based on bleeding phenotype, arthropathy, and physical exercise. The replacement regimen and FVIII utilization was evaluated before and after guided adjustments. Under 15-year-old

patients were followed up for six months, while older patients were monitored for 12 months. ABR was calculated based on reported bleeding episodes.

A total of 37 patients were included. For the younger subgroup (*n* = 20), 75 % had severe hemophilia A and 65 % had no hemophiliac arthropathy (half of these were on primary prophylaxis). For those in the older subgroup (*n* = 17), 7 % were severe cases, one patient was treated exclusively on-demand before adjustment, none were on primary prophylaxis, and 12 % had no hemophiliac arthropathy. Three patients were excluded from the analyses: one due to development of inhibitors during the follow up, one transferred to on-demand only treatment, and one received prescriptions of plasma-derived FVIII after adjustments.

The median ABR for younger patients in this cohort was 3.0 (interquartile range: 0.5–10.0) before dose adjustment and 1.0 (interquartile range: 0.0–2.0) during the follow up. In the younger population, FVIII replacement costs increased after pharmacokinetics-guided adjustments (*p*-value <0.0001) mainly due to increased costs of prophylaxis (*p*-value <0.0001), while episodic therapy costs were reduced (*p*-value <0.05). For older patients, the ABR did not change significantly comparing before and after the intervention (values for rates were not reported). Although total treatment costs did not differ comparing before and after treatment adjustments, episodic therapy costs were reduced (*p*-value = 0.039).

Study by *Cerqueira et al.* – ahead study[8]

This study reports data from the International Anti-Hemophilic factor (recombinant) Hemophilia A outcome Database (AHEAD), a prospective, non-interventional, multicenter study (NCT02078427) designed to assess long-term effectiveness and safety of Anti-Hemophilic factor (recombinant) (rAHF) in patients with hemophilia A in the real-world clinical practice. Patients with moderate or severe hemophilia A (FVIII ≤5 %) were enrolled. Primary endpoint was joint health outcomes evaluated using the Gilbert score (pain: 0–3; bleeding: 0–3; physical exam: 0–12) or Hemophilia Joint Health Score (HJHS) according to hemophilia treatment center preferences. Secondary endpoints included ABR, annualized joint bleeding rates, and safety endpoints. This publication was presented as an abstract in the International Society on Thrombosis and Haemostasis (ISTH) Meeting and reports demographic and clinical characteristics at screening from the safety analysis set for patients in the AHEAD Brazil subset at the 6th interim analysis (cutoff date July 2019).

The Brazilian subset included 203 male patients with a median age of 13.0 years (range: 0–43 years). One hundred and ninety received prophylaxis (median age: 14.0; range: 0–43 years), two received on-demand treatment (median age: 12.0; range: 0–24 years), and 11 patients with inhibitors received immune tolerance induction (ITI; median age: 12.0; range: 3–34 years). In the 12 months prior to screening, bleeding events had occurred in 130 (68.4 %) patients on prophylaxis, one (50.0 %) on-demand patient, and four (36.4 %) patients receiving ITI. Computed median ABR for the 190 prophylaxis patients was 2.0 (range: 0.0–30.0), for the on-demand patients it was 5.0 (range: 0.0–10.0), and for the ITI patients it was 0.0 (range: 0.0–26.0). Results for other variables in the study can be found in Table 4.

Table 4 – Outcomes in the Brazilian Anti-hemophilic factor Hemophilia A outcome database (AHEAD) subset of patients.[8]

Outcome	Prophylaxis	On demand	ITI
Mean Gilbert score (n)	35	–	1
Median (range)	1.0 (0.0–5.0)	–	1.0 (1.0–1.0)
HJHS: Global Gait Score (n)	86	0	8
Median (range)	1.0 (0.0–4.0)	–	1.0 (0.0–4.0)
AJBR (n)	190	2	11
Median (range)	1.0 (0.0–30.0)	4.5 (0.0–9.0)	0.0 (0.0–19.0)

ITI: immune tolerance induction.; HJHS: Hemophilia Joint Health Score; AJBR: annualized joint bleeding rate.

Study by *Ozelo et al.* – BRAVE[9]

This observational retrospective study aimed at collecting real-world evidence of Brazilian hemophilia A patients and was presented as an abstract on the 13th Annual Congress of European Association for Hemophilia and Allied Disorders. Three Brazilian Hemophilia treatment centers participated in data collection that was performed from January 2014 to December 2017. Outcomes of a total of 30 inhibitor patients (*I*+) and 60 non-inhibitor patients (I-) were reported.

Median age at enrolment was 18 (*I*+) and 26 (I-) years. Prophylaxis was used for 83.3 % of the *I*+ patients (with immune tolerance of 93.3 %) and 95 % of the I- patients. At least one bleeding episode was observed in 97.8 % of all patients. For the I- Group, the ABR was 2.98 (range: 2.15–3.8) with 10.17 % having an ABR of ≤3, while for the *I*+ Group, the ABR was 4.84 (range: 3.93–5.74) with only 3.33 % of patients having an ABR of ≤3. Additionally, FVIII prophylaxis and on-demand ABR were respectively 4.04 (range: 3.51–4.56) and 1.92 (range: 0.35–3.48), for the I- Group, and 6.72 (range: 5.7–7.74) and 3.93 (range: 1.44–4.46) for the *I*+ Group. Statistically significant differences in estimates were not reported. Authors state that results demonstrate significant healthcare resource utilization indicating that an improvement in Brazilian hemophilia A management strategies is needed.

Study by *Rodrigues et al.*[10]

This abstract, presented in the 2016 World Congress of the World Federation of Hemophilia, reports a retrospective study evaluating the efficacy and FVIII concentrate consumption for daily tertiary prophylaxis in a group of severe hemophilia A adolescents (FVIII <1 % IU/dL) managed at the State University of Campinas referral center.

Enrolled patients should have been guaranteed a daily prophylaxis regimen as a modification from a previous replacement protocol. The ABR and monthly FVIII consumption rate from the period under daily prophylaxis was compared to the 12-month period previous to enrollment.

Six of 33 (18 %) adolescent patients received daily prophylaxis and were eligible for analysis. The median age was 14 years (range: 12–18). Previous regimen of enrolled patients was 15–23 IU/kg FVIII every other day (four patients) or 20 IU/kg twice or three times per week (two patients). During daily prophylaxis,

patients received 500–1000 IU/day FVIII. Mean dose was 12.14 IU/kg (range: 7.8–16.9). At publication, patients had a median period under treatment of 16.33 months (range: 4–28) and all were still being treated in a daily prophylaxis regimen.

Observed ABR was 10.0 (range: 4.0–26.0) in the non-daily period and 1.7 (range: 0–8.5) with the daily prophylaxis regimen (p-value = 0.015). For annualized joint bleeds, rates of 4.98 (range: 2.04–24) and 0.42 (range: 0–6) were registered for non-daily and daily prophylaxis, respectively (p-value = 0.04). No significant difference was observed in monthly FVIII concentrate consumption between regimens (non-daily: 11,698 IU/month; range: 6500–20,416 IU/month; daily: 11,673 IU/month; range: 2833–23,979 IU/month; p-value = 0.94).

Summary of findings concerning ABR for Brazilian patients are shown in Table 5.

Quality assessment

A moderate risk of confounding was observed in three studies[8-10] due to a lack of clear information about inclusion and exclusion criteria of the study participants; thus, it was not clear if confounding was successfully controlled at baseline. In addition, it was not clear if analyses were performed with appropriate statistical methods. All studies recruited consecutive patients that met screening criteria and were judged as low risk of bias in the selection of participants. As prophylaxis was the only evaluated intervention, misclassification of interventions was unlikely and did not apply to these studies. All studies were judged as low risk in respect to deviations from intended intervention domain as no co-interventions were addressed by the participants and no deviations from intended intervention were reported. The results of the studies were not biased by missing data as there was no incomplete data collection and no participant was excluded from the analyses. Finally, there was no selective reporting related to ABR outcome. A summary of quality assessment is shown in Table 6.

Discussion

Treatment of severe hemophilia A has witnessed important steps towards a less immunogenic and more efficacious therapy over the last years. But, as a rare disorder, information on hemophilia A is usually scarce, especially real-world evidence. Brazilian data are no exception, and as a result, a very limited number of studies was retrieved for this systematic review regarding ABR, and no study correlating ABR with adherence to therapy was found. Also, it is noteworthy that data come mainly from the southern region of Brazil, limiting the scope of patients and probably favoring patients with improved access to healthcare facilities.

Apart from the scarce number of reports, quality of evidence was also considered moderately prone to bias in the majority of studies found. Although ROBINS-I is the tool indicated for risk assessment of non-randomized clinical trials, the use of this tool with the objective of evaluating 'before and after' interventions has not been validated yet. Thus, it is recommended that the qualitative assessment of each domain should be prioritized over the general results.

ABR for Brazilian non-inhibitor patients under conventional prophylactic treatment showed great variance with median values ranging from 0.8 to 10, in different population settings (Table 5). These estimates are grossly comparable to those observed in other regions as reported by Kenet et al.[4] However, results from Kenet et al.[4] may have been influenced by selection bias, with a possible underestimation of bleeding episodes due to a better treatment-compliant population.

However, it is known that, although ABR has been used by many contemporary studies as a default principal efficacy outcome, it suffers from great variability between hemophilia treatment centers.[11] Estimation of bleeding rates poses a complex challenge and depends on a myriad of patient-related and extrinsic factors, such as the individual clotting factor level, pharmacokinetic profile and pain perception, the subject's age, health status, activity level, dosing regimen, bleeding event definition, follow-up time, and number of patients analyzed. ABR estimation is prone to subjective assessment, as patients and physicians are required to define each bleed.[11]

Indeed, additional data reported by the studies retrieved deserve a special mention. First, Kenet et al.[4] showed that access to treatment is a major concern for Brazilian hemophilia A patients, which may reflect previous difficulties in receiving timely and adequate infusions of FVIII. Also noteworthy, patients in Brazil, differently from other countries studied by Kenet et al.[4], mainly have access to standard half-

Table 5 – Summary of ABR reported in eligible publications.

Study (Year)	n	Age (years) n	Baseline* ABR median (range)	Post-Intervention ABR median (range)	Setting
Kenet et al.[4]	41	27	0.8 (0–19.3)	NA	Adult-only patients. Considers six months of retrospective data added to at least six months of prospective follow up
Borges et al.[7]	37	≤15 = 20† >15 = 17†	3.0 (0.5–10.0)	1.0 (0–2.0)	ABR reported only for the younger cohort. Improvement with myPKFiT™ tool statistical significance not reported
Cerqueira et al.[8]	190	14	2.0 (0–30.0)	NA	Results for prophylaxis cohort
Ozelo et al.[9]	60	26	4.04 (3.51–4.56)	NA	Results for non-inhibitor prophylaxis group
Rodrigues et al.[10]	6	14	10.0 (4.0–26.0)	1.7 (0–8.5)	Adolescent patients only. Conventional versus daily replacement (p-value = 0.015)

ABR: annualized bleeding rate; NA: not applicable.

* Rates depicted here are those registered before intervention for patients on prophylaxis treatment. †Number in each category.

life products (>85 % of patients in the cohort) and demonstrate a lower comparative FVIII utilization rate; this could be evidence of inadequate adherence. Furthermore, studies by Borges et al.[7] and Rodrigues et al.[10] demonstrated that maintaining more stable and continuous levels of FVIII activity effectively reduce the ABR, at least for one subgroup of patients. Such a premise has been for a long time the main core of many initiatives in the development of therapeutic options for hemophilia A, aside from the efforts on reducing immunogenicity of replacement factors.[12] However, efficacy of such replacement regimens demanding frequent factor infusions pose a significant burden upon patients, compromising long-term effectiveness, treatment adhesion and QoL. Also, financial costs increase as more infusions are required to maintain a lower ABR. As a recent alternative addressing such obstacles, gene therapy has emerged as a promising pathway of treatment in the near future.[13,14]

Conclusion

Available information on efficacy of severe hemophilia A management in Brazil currently relies on scarce and possibly biased information. It should be strongly emphasized that Brazil is in great need of a structured and coordinated effort towards better collection, analysis and reporting of data of severe hemophilia A patients. Overcoming the scarcity of information about this specific topic is key to maintain improvement in policies directed toward Brazilian hemophilia A patients.

Despite of this, one could infer that the great variance in ABR in different studies, potential selection bias of patients (with better access to healthcare facilities and more compliant to treatment) and the lower comparative FVIII utilization rate suggest that Brazilian non-inhibitor patients still need better treatment.

Conflicts of interest

None.

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Table 6 – Risk of bias summary for non-randomized clinical trials for prophylaxis in severe hemophilia A patients according to the ROBINS-I tool.

Author	Bias due to confounding	Bias in selection of patients into the study	Bias in classification of intervention	Bias due to deviations from intended interventions	Bias due to missing data	Bias in measurement of outcomes	Bias in selection of the reported result	Overall risk
Kenet et al.[4]	Low	Low	NA	Low	Low	Low	Low	Low
Borges et al.[7]	Low	Low	NA	Low	Low	Low	Low	Low
Cerqueira et al.[8]	Moderate	Low	NA	Low	Low	Low	Low	Moderate
Ozelo et al.[9]	Moderate	Low	NA	Low	Low	Low	Low	Moderate
Rodrigues et al.[10]	Moderate	Low	NA	Low	Low	Low	Low	Moderate

*A study was assigned low risk if the study was judged to be at low risk for all domains.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Outcomes and vaccination patterns against COVID-19 in a cohort of sickle cell disease patients in the state of Rio de Janeiro

Claudia de Alvarenga Maximo [a,*], *Jorge Francisco da Cunha Pinto* [b], *Fabiana Canedo Pinto* [a], *Patrícia Brasil* [c]

[a] *Instituto Estadual de Hematologia Arthur de Siqueira Cavalcanti – HEMORIO, Rio de Janeiro, State of Rio de Janeiro, Brazil*

[b] *Universidade Federal do Estado do Rio de Janeiro – UNIRIO, Rio de Janeiro, State of Rio de Janeiro, Brazil*

[c] *Instituto de Pesquisa Clínica Evandro Chagas – FIOCRUZ, Rio de Janeiro, State of Rio de Janeiro, Brazil*

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ABSTRACT

Background: Patients with sickle cell disease were presumed to be at high risk for severe COVID-19 outcomes due to their compromised immunity and chronic comorbidities. This study aimed to evaluate vaccination patterns, healthcare utilization, and clinical outcomes in a cohort of sickle cell disease patients during the COVID-19 pandemic in Rio de Janeiro.

Methods: A total of 289 over 18-year-old patients from the Epidemiology and Donor Evaluation Study (REDS-III) Brazil sickle cell disease cohort were followed between January 2021 and August 2023. Sociodemographic data, emergency department visits, hospitalizations, mortality rates, and COVID-19 vaccination status were collected. SARS-CoV-2 infection was confirmed by reverse transcription polymerase chain reaction testing for symptomatic or hospitalized patients.

Results: Of the participants, 89.2% completed the primary vaccination schedule, 62.2% received the first booster, 30% the second booster, and 4.1% completed all five doses. Emergency visits increased slightly during the pandemic but were primarily due to vaso-occlusive crises. Of the 119 patients tested for SARS-CoV-2, six were positive, presenting mild symptoms with no COVID-19-related deaths. Vaccination rates in the cohort were similar to those in the general population, with Oxford/AstraZeneca and Pfizer being the most used vaccines.

Discussion: The findings suggest that COVID-19 infection was not a significant trigger for vaso-occlusive crises or severe disease outcomes. High vaccination adherence likely played a key role in preventing severe COVID-19, alongside other factors such as social isolation and herd immunity. However, the overlap between symptoms of vaso-occlusive crises and COVID-19 may have caused diagnostic challenges. Importantly, the low morbidity and mortality observed emphasize the protective effect of vaccines, despite the presence of thromboplastic activity and pro-inflammatory states inherent to sickle cell disease.

* Corresponding author.
E-mail address: claudia.maximo@hemorio.rj.gov.br (C.d.A. Maximo).

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Addressing vaccine hesitancy remains crucial, particularly as booster doses show declining adherence.

Conclusion: COVID-19 had a limited clinical impact on this cohort, with no significant role in triggering vaso-occlusive crises or severe outcomes. High vaccination rates and potential environmental or biological factors may have contributed to this protective effect.

Introduction

COVID-19 emerged in China at the end of 2019, and due to the high transmissibility of the SARS-CoV-2 virus through respiratory droplets, it was responsible for nearly 800 million cases worldwide by February 2024.[1] In Brazil, there were approximately 38.5 million cases with over 709,000 deaths in the same period.[2] Initially, the only strategy for governments to mitigate transmission was social isolation, however by the end of the first pandemic year, the first vaccines were made available by the pharmaceutical industry, significantly reducing morbidity and mortality. Vaccination began in January 2021, with the first doses of COVID-19-Coronavac-Sinovac/Butantan (Coronavac) being administered, prioritizing the most vulnerable groups, such as the elderly and those with chronic diseases, including sickle cell disease (SCD). This hereditary hemoglobinopathy, characterized by multiple comorbidities, posed a high presumed risk of complications if patients contracted SARS-CoV-2. The polymerization of red blood cells containing hemoglobin (Hb) S leads to the main pathophysiological mechanisms of the disease, namely vaso-occlusion and hemolysis. These events cause vascular and endothelial dysfunction through inflammatory and prothrombotic mechanisms resulting in acute events and chronic organ damage.[3] Pain crises due to vaso-occlusion are the hallmark of SCD with the main pathophysiological mechanisms being: 1) polymerization of deoxyhemoglobin S, 2) Sickling of red blood cells, 3) microvascular occlusion by sickled cells, 4) tissue ischemia, 5) tissue damage due to hypoxia and 6) stimulation of peripheral nerve endings that lead to pain perception.[4]

The lungs are frequently affected, with acute chest syndrome (ACS) being a severe complication and the leading cause of death. Patients have compromised immunity due to hyposplenism, making them susceptible to infectious diseases, sepsis, chronic kidney disease, and pulmonary hypertension, justifying their inclusion in the priority group.[4]

COVID-19 primarily affects the respiratory system with the release of inflammatory cytokines, presenting symptoms ranging from mild to severe, potentially leading to circulatory and thromboembolic complications, and death from pulmonary involvement and multiple organ failure. By January 2021, Brazil began offering vaccines, and by 2023, monovalent vaccines such as Coronavac, COVID-19-RNAm Pfizer-Comirnaty (Pfizer), Covishield-Oxford/Fiocruz (AstraZeneca), Janssen (Janssen), and the COVID-19-RNAm Pfizer (Comirnaty) bivalent vaccine (Bivalent), introduced after the emergence of the Omicron variant, were available. The primary vaccination schedule consists of two doses, followed by a first booster, a second booster, and a third booster including the bivalent vaccine.[5]

The objective of this study was to investigate the vaccination pattern and its association with clinic outcomes and healthcare utilization of a cohort of SCD patients at the blood center of Rio de Janeiro in the pandemic period. Given that patients with SCD are at high risk for COVID-19 complications, it was hypothesized that this population may have experienced worse clinical outcomes and increased utilization of healthcare services due to COVID-19 infection.

Methods

Study population and selection criteria

All over 18-year-old patients, who were participating in the multicenter cohort study, Epidemiology and Donor Evaluation Study (REDS-III) Brazil Sickle Cell Disease, of the blood center of Rio de Janeiro were selected. REDS-III is a longitudinal cohort project involving six SCD treatment centers across Brazil in collaboration with researchers from the Blood Systems Research Institute in California (USA) since 2013.[6] Out of the total patients of Hemorio, 721 were selected by REDS-III based using sample size calculations. Of these, 337 met the eligibility criteria for this study. A consent form was signed by 289 patients.

Variables of interest

Sociodemographic data, including SCD genotype, age, race and educational attainment, were collected from medical records as part of the REDS database. Additionally, information on numbers of emergency visits and hospitalizations was gathered for three time periods to compare the pre-pandemic period (2018–2019) to the pandemic (2020–April 2022) and to the post-pandemic period, after the end of the international public health emergency (May 2022–2023). The reasons for emergency visits in the periods were described.

Only patients presenting with respiratory symptoms and those hospitalized for any reason were tested for SARS-CoV-2 using reverse transcription polymerase chain reaction (RT-PCR) as part of standard care.

Mortality rates were calculated based on the number of deaths within the cohort during the study period, and deaths were analyzed in terms of their dates and causes.

The COVID-19 vaccination status of participants was obtained from the National Immunization Program (NIP) website from January 2021 to August 2023. For this study, the

vaccines offered in sequence were categorized as the first dose, second dose, first booster, second booster, and bivalent. Participants who received all five doses available from NIP were considered to have completed the vaccination schedule. The sociodemographic profiles of the vaccinated and unvaccinated populations were assessed. A comparison of the vaccination rate of the cohort participants with the population of the municipality of Rio de Janeiro was made.

This study was approved by the Hemorio Ethics Committee under register number 6089,121 and conducted according to the revised 2008 Helsinki Declaration.

Statistical analysis

Sociodemographic and clinical variables are described using descriptive statistics, including means, standard deviations, and absolute and relative frequencies.

For each of the two periods, the incidence rate of emergency room visits was calculated as the number of visits per person-months at risk. The incidence rates between periods were compared using Poisson regression.[7] In the Poisson model, the dependent variable was the number of emergency visits, and the independent variable was a binary variable indicating whether the visit was during the pre-pandemic or pandemic period. The incidence rate ratio and its 95% confidence interval were estimated using the GLM package in R 4.3.1 8.[8] A ratio greater than one with a confidence interval that does not overlap with one would represent a significantly greater risk of emergency visits during the pandemic, whereas a confidence interval overlapping one would indicate no significant association between the pandemic and emergency visits.

Results

Of the 289 participants, 116 (40%) were male, and 173 (60%) were female, with a mean age of 34.3 ± 13.2 years (range: 18–72 years). About one-half (*n* = 147; 51%) were identified as mixed race, 114 (39%) as Black, 25 (9%) as White, and three (1%) as Asian. Regarding genotype, 227 (78.5%) were Hb SS, 42 (14.5%) Hb SC, 12 (4.1%) Hb SB^0, seven (2.4%) Hb SB^+, and one Hb SD. Educational attainment varied with 87 (30.1%) individuals having basic education, 50 (17.3%) with completed basic education, 103 (35.6%) with secondary education, 19 (6.6%) had completed technical courses, 27 (9.3%) had higher education, one participant with a master's degree, and two were illiterate.

Most participants (89.2%) completed the primary COVID-19 vaccination schedule with two doses. Of these, 62.2% received the first booster dose (third dose), while 30% completed up to the second booster dose (fourth dose). Only 4.1% received all five recommended doses according to the Brazilian vaccination schedule, and 11.7% of participants received the bivalent vaccine between the fourth and fifth doses. Five males and four females with varied educational backgrounds did not get vaccinated. The distribution of vaccine doses by sex, education level, and age group is shown in Table 1. There was no significant association between education level, age group, and sex regarding vaccination, except for the second booster dose, where more women were vaccinated. The most used vaccine from the first to the fourth dose was the Oxford/AstraZeneca, followed by Coronavac. The distribution of vaccine types by dose is shown in Figure 1.

In the pre-pandemic period, 77 patients made a total of 1020 emergency room visits. During the pandemic, 88 patients made 1416 visits, while in the post-pandemic period, 92 patients accounted for 1286 visits (Table 2). Most individuals seen in the pre-pandemic and pandemic periods were the same, with SCD-related pain crises and the need for blood transfusions being the leading reasons for emergency department visits.

Before the pandemic, the incidence rate of emergency visits was 0.23 visits per person-month at risk. During the pandemic, the rate was 0.31 visits/person-month. The incidence

Table 1 – Distribution of vaccine doses by sex, educational attainment, and age group.

Variable	1st Dose n (%)	2nd Dose n (%)	1st Booster n (%)	2nd Booster n (%)	Bivalent n (%)	None n (%)	All n (%)
Sex							
Male	111 (39.6)	99 (35.3)	67 (23.9)	29 (10.4)	5 (1.8)	5 (2.9)	5 (4.3)
Female	168 (60.0)	161 (57.5)	115 (41.1)	58 (20.7)	7 (2.5)	4 (3.4)	7 (4.0)
Education							
Illiterate	2 (100)	2 (100)	1 (50)	–	–	–	–
Primary school	83 (95.4)	76 (87.3)	53 (60.9)	26 (29.9)	3 (3.4)	5 (5.7)	3 (3.4)
Secondary school	48 (97.9)	42 (85.7)	29 (59.1)	9 (18.3)	3 (6.1)	1 (2.0)	2 (4.0)
High school	101 (98.0)	98 (95.1)	68 (66.0)	39 (37.8)	3 (2.9)	2 (1.9)	5 (4.8)
Adult education	1 (100)	1 (100)	1 (100)	–	–	–	–
Technical school	17 (89.4)	13 (68.4)	10 (52.6)	2 (10.5)	–	1 (5.2)	–
Higher education	26 (96.2)	26 (96.2)	17 (62.9)	8 (29.6)	3 (11.1)	–	2 (7.4)
Postgraduation	1 (100)	1 (100)	1 (100)	1 (100)	–	–	–
Age group							
18–29	133 (98.5)	122 (90.4)	74 (54.8)	25 (18.5)	5 (3.7)	1 (0.7)	5 (3.7)
30–39	62 (95.4)	58 (89.2)	45 (69.2)	26 (40.0)	3 (4.6)	3 (4.6)	3 (4.6)
40–49	39 (90.6)	36 (83.7)	26 (60.5)	12 (27.9)	1 (2.3)	4 (9.3)	1 (2.3)
50–59	26 (92.8)	27 (96.4)	20 (71.4)	12 (42.8)	1 (3.6)	1 (3.6)	1 (3.6)
60+	18 (100)	17 (94.4)	17 (94.4)	12 (66.7)	2 (11.1)	–	2 (11.1)

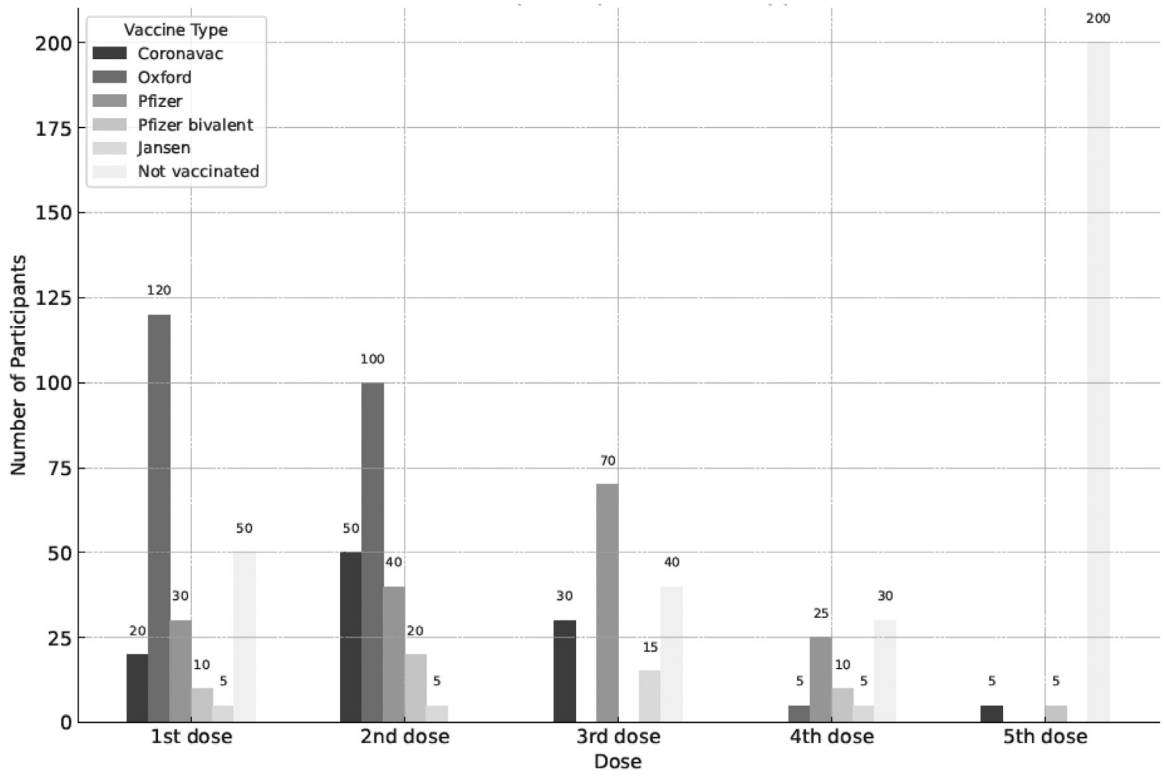

Figure 1 – Number of participants per vaccine type and dose.

rate ratio of the Poisson model was 0.99 (95% confidence interval: 0.93–1.07, p-value = 0.92).

During the study, 119 patients underwent RT-PCR testing for SARS-CoV-2. Of these, 52 were unvaccinated and 67 were vaccinated. Among the unvaccinated, four tested positive. They were hospitalized for vaso-occlusive crises (VOCr) but only one had respiratory symptoms and anosmia. They were discharged in three to five days. Among the vaccinated, two tested positive. They were also admitted due to VOCr and discharged after pain control.

There were two deaths during the pandemic, configuring a mortality rate of 0.7%: a 34-year-old man died due to liver failure on October 10, 2022. He tested positive for SARS-CoV-2 by RT-PCR before being vaccinated on April 20, 2021 and received two doses of the Coronavac vaccine on August 16 and September 23, 2021. The second was a 19-year-old woman who died due to ACS and septic shock on October 8, 2022. When she was admitted, she exhibited fatigue, fever, headache, and chest pain. No Testing for COVID-19 was done at this time. She had mild COVID-19 with a positive RT-PCR test 18 months before and received two doses of the Oxford/AstraZeneca vaccine on June 28 and October 4, 2021, one year before her death.

The SCD cohort had similar vaccination rates to the general population of Rio de Janeiro as seen in Figure 2, except for the fourth (p-value <0.01) and fifth (p-value <0.01) doses, which were lower. Up to the primary vaccination scheme of two doses, the vaccination pattern was the same for all age groups. From the first booster onwards, there was a reduction in the vaccination rate in the SCD population. The second booster was not considered due to insufficient time for evaluation.

Table 2 – Emergency room visits.

Period	Number of patients	Number of visits
Pre-pandemic	77	1020
Pandemic	88	1416
Post-pandemic	92	1286

Discussion

Despite the high reliance of SCD patients on emergency healthcare, particularly for VOCr, the number of emergency visits did not increase significantly during the COVID-19 pandemic. This may be attributed to robust antibody responses post-vaccination, as observed in previous studies.[9] SCD is associated with increased thromboplastic activity, characterized by heightened platelet activation, elevated markers of thrombin generation, and increased tissue factor expression, along with reduced levels of natural anticoagulants, even in the basal state and in the absence of acute events such as VOCr. However, it remains unclear whether this thromboplastic activity contributes to the pathophysiology of VOCr or, as is more widely accepted, merely reflects an acute-phase

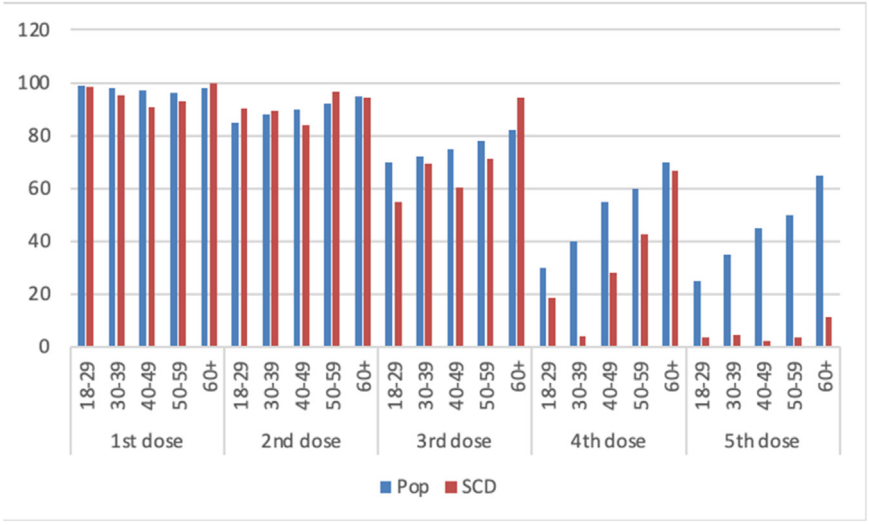

Figure 2 – Comparison of vaccination by age group between the sickle cell disease (SCD) cohort and the population of the city of Rio de Janeiro (Pop).

response to these events.[10,11] Notably, SARS-CoV-2 infection has been identified as a potent inducer of thrombosis, raising additional concerns in this already vulnerable population.[11] Despite these concerns, this study did not identify an increase in VOCr following COVID-19 vaccination, alleviating fears that vaccines might trigger vaso-occlusive events.[9] The possibility of VOCr triggered by mild or asymptomatic SARS-CoV-2 infection in patients with SCD cannot be entirely ruled out. This is a limitation of the current study, as most patients who presented to the emergency department with pain were not tested for COVID-19. One hundred and nineteen patients underwent PT-PCR testing for SARS-CoV-2 due to emergency admissions, as cohort wards were separated for patients with positive tests at that time. Only six tested positive (5%). All presented with VOCr but only one had respiratory symptoms related to COVID-19. These data are quite similar to those published by Konte et al., who performed RT-PCR tests for SARS-CoV-2 on 104 admissions for VOCr of 62 patients with SCD. Only five tested positive (4.8%), and only one had associated respiratory symptoms.[12] Thus, as discussed above, it is not possible to state whether the VOCr were triggered by COVID-19 or not. However, the low incidence of positive tests suggests that COVID-19 could have been at most an asymptomatic co-incidental infection.

The Brazilian National Immunization Program is a global reference, providing extensive free immunobiologicals to the population. SCD patients were included in the National Operational Plan for COVID-19 vaccination due to their compromised immunity resulting from functional hyposplenism and systemic vasculopathy.[5]

Vaccination adherence of individuals with SCD was comparable to the general population in Rio de Janeiro for the primary two-dose schedule. However, lower rates were observed for the fourth and fifth doses, likely due to the study period ending shortly after the rollout of bivalent vaccine in February 2023.[13] The most frequently used vaccine in the present cohort was the Oxford/AstraZeneca for the first two doses, followed by the Pfizer vaccine starting from the third dose. These were also the most used vaccines in Brazil in 2021.[14]

A study on vaccination intention assessment indicated that people with SCD, similar to other groups with chronic diseases, were less willing to get vaccinated, despite being vulnerable.[15,16] In contrast to studies on vaccine hesitancy, this study identified high adherence to the primary vaccination schedule, with a slight reduction for the first booster dose among younger individuals. Encouragement by healthcare professionals for individuals to get vaccinated, the widespread availability of vaccines to priority groups, and the fear of contracting and dying from COVID-19 were likely more influential than concerns over potential vaccine side effects.

The absence of vaccination in nine individuals who did not receive any vaccine dose might be explained by a combination of factors: fear of side effects, concerns over triggering VOCr and thrombosis, and, most notably, a disbelief in vaccines. These factors have been highlighted in other studies, such as one conducted in a treatment center in Saudi Arabia with 147 SCD patients, which showed that only 35% had been vaccinated, revealing high vaccine hesitancy. The main reason given by the lack of vaccination was the fear of developing brain clots and other side effects.[17,18] Despite not being vaccinated, only one patient who tested positive by RT-PCR exhibited mild symptoms of COVID-19 in September 2022, without requiring hospitalization. The other patients, despite frequently using the healthcare system, either did not contract the disease or it went unnoticed, due to the presence of non-specific symptoms, since some clinical manifestations of COVID-19 are also commonly seen in SCD.[4] Social isolation and herd immunity could explain the lack of illness or severe COVID-19 in this group.

COVID-19 vaccines have been shown to be safe and do not cause significant or more severe adverse effects in people with SCD, as evidenced by Han et al. Their work also observed that the patients who completed the primary vaccination regimen had a 70% reduction in the risk of SARS-CoV-2 infection before

the emergence of the Omicron variant.[19] In this study, 89% patients were fully vaccinated and one died of ACS that could not be dissociated from SARS-CoV-2 infection. No association was found between vaccination and sex, education, and age, possibly due to the sample size. Also, the adverse effects of the vaccines in this population were not evaluated. Nonetheless the adverse effects of COVID-19 vaccines are mostly mild, such as local pain, fever, fatigue, headache, muscle pain, chills, and diarrhea.[20] These symptoms are also commonly seen in SCD and often lead patients to seek emergency care.

Varelas et al.[21] studied the immune response after vaccination against Sars-Cov-2 in a group of SCD patients regarding the production of neutralizing antibodies (nAbs) and the increase in C5b-9 levels: nAbs >50% are highly protective. The study showed a satisfactory immune response, especially after the second dose, with 50% of the patients studied achieving nAb levels ≥50% and a significant increase in C5b-9 above baseline levels. As in the present sample, 89% completed the primary vaccination schedule: this may have been the reason for the low morbidity from COVID-19 and the low mortality rate of 0.7% (not related to COVID-19) in this group, when compared to the general population in Rio de Janeiro during the years 2020 (12.6%), 2021 (14.3%), 2022 (11.8%), and 2023 (11.2%).[22]

Both patients who died had received two doses of the COVID-19 vaccine approximately one year before their deaths. They presented with severe SCD complications, including liver failure and ACS with septic shock. While SARS-CoV-2 infection cannot be entirely ruled out – particularly given the limitations of RT-PCR testing – neither patient had been vaccinated against the Omicron variant. This underscores the potential role of waning immunity and the importance of timely booster doses, especially for vulnerable populations, such as individuals with SCD. Guo et al.[24] studied a cohort of patients who had recovered from COVID-19 to investigate the durability and cross-reactivity of immunological memory acquired from natural infection against SARS-CoV-2. They found that neutralizing antibodies continually declined but SARS-CoV-2-specific memory B-cell and T-cell responses were maintained for at least two years and the recall immune responses could limit viral replication and reduce disease severity after re-infection. Furthermore, humoral immunity was boosted against the prototype and Omicron sublineages in individuals who were infected by the prototype and who subsequently received the inactivated vaccine.[23,24] This was the case for the two patients who died: after having had COVID-19 they died about a year after being vaccinated.

It is true that the Omicron variant and its sublineages have posed an additional challenge due to their high potential for transmission/infection, immune evasion, and loss of vaccine efficacy as suggested in a propensity-matched analysis in morbidity and mortality of hospital-onset SARS-CoV-2 infections due to Omicron versus previous variants.[25] A systematic review on the efficacy of a second booster dose as a strategy to mitigate the effects of Omicron variants concluded that bivalent vaccines confer greater protection by restoring lost humoral immunity and also by stimulating cellular immunity.[26] The study suggests that the forth dose, or second booster, should be recommended for more vulnerable groups such as the elderly and immunocompromised individuals. COVID-19 vaccines have been included in the vaccination schedule of the Brazilian Ministry of Health, targeting children and priority groups, due to the risk of disease resurgence and the emergence of new variants.[27] As this study was concluded in August 2023 and the bivalent vaccine was only made available in February of the same year, there was insufficient time to assess whether the patients adhered to bivalent vaccine. Nevertheless, an important indicator of the vaccine effectivity in the study population was the absence of symptomatic COVID-19 cases in the emergency department after the emergence of the Omicron variant.

Despite providing valuable insights into the impact of COVID-19 and vaccination on patients with SCD, this study has imitations. COVID-19 was not systematically investigated in all patients as testing was primarily performed in those presenting with respiratory symptoms or requiring hospitalization. This may have led to an underestimation of asymptomatic or mild SARS-CoV-2 infections and their potential role as a trigger for VOCr. The lack of a systematic evaluation of adverse vaccine effects limits conclusions about vaccine safety in this cohort. Additionally, the small number of COVID-19-positive cases and the absence of severe outcomes or deaths directly attributable to COVID-19 make it challenging to establish definitive associations. Future studies with systematic SARS-CoV-2 testing, larger sample sizes, and prospective monitoring of clinical outcomes and immune responses are warranted to confirm these findings and further explore the relationship between COVID-19, vaccination and VOCr.

Conclusion

Contrary to initial expectations, the impact of the COVID-19 pandemic on patients with SCD was less severe than anticipated. Emergency department visits during the pandemic occurred primarily due to pain crises and SARS-CoV-2 infection did not appear to be a significant trigger for VOCr, severe COVID-19 outcomes, or deaths in this population. Instead, the virus may have acted as an incidental and often asymptomatic co-infection. High adherence to COVID-19 vaccination, comparable to that observed in the general population, likely played a key protective role. Interestingly, even unvaccinated individuals were not severely affected, raising questions about whether social isolation, herd immunity, or unidentified biological factors contributed to this. Further studies are needed to explore these possibilities. With COVID-19 vaccines now included in Brazil's 2024 vaccination schedule, it is crucial to address vaccine hesitancy among SCD patients and promote continued vaccination adherence as COVID-19 remains a vaccine-preventable disease.

Conflicts of interest

The authors declare no conflicts of interest.

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Original article

A common ground: an in silico assessment of the sources of intrinsic ex vivo resistance to venetoclax in acute myeloid leukemia

Brunno Gilberto Santos de Macedo [a], *Manuela Albuquerque de Melo* [a], *Diego Antonio Pereira-Martins* [b], *João Agostinho Machado-Neto* [a,c], *Fabiola Traina* [a,*]

[a] *Department of Medical Images, Hematology, and Oncology, University of São Paulo at Ribeirão Preto Medical School, Ribeirão Preto, São Paulo, Brazil*
[b] *Department of Hematology, University Medical Center Groningen, Groningen, The Netherlands*
[c] *Department of Pharmacology, University of São Paulo, São Paulo, São Paulo, Brazil*

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ABSTRACT

Venetoclax is a promising alternative for patients with acute myeloid leukemia who are considered unfit for conventional chemotherapy; however, its employment still faces challenges mostly related to drug resistance. Here, we provide further biological mechanisms underlying the previously described and potentially novel intrinsic sources of poor response to venetoclax departing from *ex vivo* response data. Acute myeloid leukemia data including *FLT3* mutation status, gene expression data, and *ex vivo* response data were extracted from the publicly available BeatAML 1.0 study database and aided sample categorization that supported differential gene expression analysis that, in turn, supported gene set enrichment analysis. CIBERSORTx-based bulk RNA sequencing deconvolution of BeatAML 1.0 data allowed us to categorize samples according to their cell type content. We observed that inflammation-related gene sets, such as cytokines and inflammatory response, NLRP3 inflammasome activation, and activation of adaptive immune response, were concordantly positively enriched across all the conditions reported to be associated with poor *ex vivo* venetoclax response, whereas samples from good *ex vivo* responders' mostly enriched gene sets related to mitochondrial activity, and early myeloid progenitor cell molecular programs. Besides the alternative reliance on *BCL2A1*, we highlight inflammation as a common element present across multiple sources of venetoclax *ex vivo* response modulation in acute myeloid leukemia samples. Hence, a potential key modulator for venetoclax response.

* Corresponding author: Department of Medical Images, Hematology, and Oncology, University of São Paulo at Ribeirão Preto Medical School, 3900 Bandeirantes Avenue, Ribeirão Preto, São Paulo CEP 14040-900, Brazil.
E-mail address: ftraina@fmrp.usp.br (F. Traina).

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Introduction

The growing understanding of the molecular diversity and pathogenesis of acute myeloid leukemia (AML) has not only contributed significantly to defining prognosis and guiding clinical decisions,[1,2] but has also opened up therapeutic avenues. These include opportunities for targeted treatments based on molecular profiles and, notably, the development of more tolerable strategies through the use of small molecule inhibitors.[2-4] The most promising and notable examples of Food and Drug Administration (FDA)-approved molecularly-oriented targeted approaches comprise FLT3, IDH1/2, and BCL-2 inhibition.[2-6]

In this context, the BCL-2 inhibitor venetoclax has drawn significant attention from the scientific community as a small molecule targeted therapy for AML. This strategy represents a promising alternative for patients who may not qualify for standard intensive induction chemotherapy regimens. Venetoclax is believed to target leukemic stem cells (LSCs) and their metabolic characteristics, which contribute to long-lasting remission.[7-10]

However, some of the significant mechanisms of resistance to venetoclax are mostly related to energy metabolism plasticity, and, most importantly, to the attenuation of *BCL2* dependency for survival.[6,9,11] This can be observed upon BCL2 downregulation, upregulation of additional BH3-family anti-apoptotic proteins such as BCL-XL, BCL-W, and MCL1, and even monocytic-like AML populations were observed to be inherently resistant and positively selected upon treatment with venetoclax.[2,5,6,9,11,12] Activation of other underlying parallel survival alterations such as gain of *FLT3*-ITD or *TP53* loss-of-function mutations are also reported as additional primary sources of resistance to venetoclax.[6] Another recently described mechanism of resistance to venetoclax encompasses the nicotinamide metabolism, prominently orchestrated by the enzyme nicotinamide phosphoribosyl transferase encoded by the *NAMPT* gene, in relapsed and refractory AML LSCs.[13]

Venetoclax exhibits restricted efficacy in relapsed or refractory AML, primarily owing to the presence of specific mechanisms.[9] Relapsed cells often depend on anti-apoptotic proteins other than BCL-2 or demonstrate increased metabolic adaptability to compensate for the disruption caused by BCL-2 inhibition. For example, they may increase fatty acid intake and activate alternative metabolic pathways such as mitochondrial fatty acid beta-oxidation to fuel the tricarboxylic acid cycle (TCA) and generate adenosine triphosphate.[9]

In the light of the diversity of these mechanisms, this study sought to unravel additional biological mechanisms underlying already described and novel sources of intrinsic and acquired poor response to venetoclax from an *ex vivo* screening perspective.

Methods

Data acquisition

Mutation status, gene expression (RNA sequencing), and *ex vivo* response data from primary AML bone marrow mononuclear cell samples were obtained from the BeatAML 1.0 functional genomic study cohort through the supplementary documentation in Tyner et al.[14], cBioPortal (cbioportal.org) repository, and the BeatAML 1.0 associated data viewer, Vizome (vizome.org). Along with transcriptional deconvolution data, the aforementioned data aided sample categorization for the following methods and statistical processing (Supplemental Figure 1).

Gene set enrichment analysis

The gene set enrichment analysis (RRID:SCR_003199) was performed from the gene expression logarithm fold change (logFC)-based genes pre-ranking. LogFC values were obtained from differential gene expression analyses using the *edgeR* (RRID:SCR_012802) and *limma* (RRID:SCR_010943) Bioconductor R packages under the *limma-voom* algorithm. The LogFC output of BH3 family genes —*BAD, BAK1, BAX, BBC3, BCL2, BCL2A1, BCL2L1, BCL2L11, BCL-W, BIM, BID, BOK*, and *PMAIP1* —was graphically represented through a heatmap built employing the *ComplexHeatmap* (RRID:SCR_017270) Bioconductor R package and clustered according to the Euclidean distance.

The pre-ranked genes also served as the input for the *fgsea* (RRID:SCR_020938) Bioconductor R package. The gene sets are obtainable from the Molecular Signature Database (MSigDB - gsea-msigdb.org).[15] The pre-ranked genes enrichment was submitted to 10,000 permutations under weighted enrichment statistics. The level of significance was pre-established at 5 % and adjusted to a false discovery rate of 25 %. This work employed the Gene Ontology: Biological Process (GOBP; 7751 gene sets) and the curated (C2; 7233 gene sets) collections of human gene sets that were loaded using the *qusage* Bioconductor R package. The gene sets were selected by convenience under the statistical significance and false discovery rate criteria and graphically represented using the *ComplexHeatmap* (RRID:SCR_017270) Bioconductor R 4.3.1 package.

Deconvolution analysis

The BeatAML 1.0 cohort's bulk RNAseq gene expression data was submitted to the CIBERSORTx tool (https://cibersortx.stanford.edu/).[16] The CIBERSORTx tool, in the current context, promoted bone marrow mononuclear cell gene expression signal deconvolution into the different cell populations that compose it and assigned a compartmental score to each type of cell population according to a reference to single-cell RNA sequencing data, in this case van Galen et al. (leukemia and primary healthy scRNAseq bone marrow samples).[17] The compartment score indicates which cell type is predominant within a sample, therefore its cellular composition regarding abundance; as an indirect measurement of how much of a particular cell type contributes to the total average gene expression signal. CIBERSORTx-derived deconvolution data from the BeatAML 1.0 cohort is available in the supplementary material of Zeng et al.[18]

Statistical analyses

All statistical analyses were performed using R programming language version 4.3.1 (R Core Team (2022) - https://www.R-

project.org/) (RRID:SCR_001905) and RStudio Integrated Development Environment (IDE) version 2023.03.0 + 386 (RStudio Team (2020) –http://www.rstudio.com/) (RRID:SCR_000432). The established level of significance (α) was 5 % for all the analyses. Contingency tables were analyzed using Fisher's exact test and the effect size was measured by the odds ratio (OR) and a 95 % confidence interval.

Results

Inflammation-related and mature blood cell-related gene sets are consistently enriched across the conditions associated with poor intrinsic venetoclax *ex vivo* response.

In both tested human gene set collections, conditions such as higher *NAMPT* and *MCL1* expression dichotomized by gene expression median, along with higher sample monocyte content, dichotomized according to CIBERSORTx score median value, displayed similarities to the intrinsic venetoclax poor response reference molecular signature (Figure 1). Conversely, the *FLT3*-ITD mutation and higher *BCL2* gene expression were molecular features associated with good *ex vivo* response to venetoclax (Figure 1). Inflammation-related biological processes such as inflammasome activation and cytokine production, macrophage activation and mature hematopoietic cells were consistently present in the molecular signature compatible with poor intrinsic *ex vivo* response to venetoclax (Figure 1). In contrast to this observation, gene sets related to mitochondrial activity, amino acid metabolism, and immature hematopoietic cells, including hematopoietic and LSCs, were found to be enriched within the molecular signature compatible with venetoclax sensitivity (Figure 1).

Acute myeloid leukemia samples with higher *MCL1* and *NAMPT* gene expressions, and higher monocytic cell content present increased likelihood of poor intrinsic *ex vivo* response to venetoclax.

The association analysis (Figure 2) presented higher *BCL2* expression as a factor which is strongly associated with good intrinsic *ex vivo* response to venetoclax (OR: 0.178; 95 % CI: 0.087–0.352). On the other hand, a higher *MCL1* expression made the samples over twice as likely to present poor intrinsic *ex vivo* response to venetoclax (OR: 2.28; 95 % CI: 1.202–4.405), a higher monocytic signature increases the likelihood by almost four times (OR: 3.906; 95 % CI: 2.012–7.752], and higher levels of *NAMPT* expression were associated with a poor likelihood of intrinsic *ex vivo* response to venetoclax (OR: 5.65; 95 % CI: 2.841–11.494). These results denote that our analyses are concordant with the reported venetoclax response modulation in current literature while offering an *ex vivo* perspective and a mathematical standpoint for the likelihood of intrinsic response.

BCL2A1 is a key player in differentially expressed BH3 family genes associated with poor intrinsic *ex vivo* response to venetoclax

The heatmap of differentially expressed genes (Figure 3) revealed a molecular signature for poor response strongly based on the *BCL2A1* and *BCL2* opposite gene expression behaviors in this set of samples. Conditions associated with poor response significantly upregulated *BCL2A1* while downregulated *BCL2*. Conversely, *FLT3*-ITD mutated samples were clustered along a condition widely described as a good response signature based mainly on *BCL2A1* downregulation.

Discussion

Due to its performance in early phases of clinical trials, in 2018, venetoclax had its approval by the FDA accelerated as long as it was combined with hypomethylating agents or low dose of cytarabine for AML patients who were over 75 years old and presenting comorbidities that forbid intensive chemotherapy.[3,6,19] The regular approval of venetoclax for newly-diagnosed untreated AML patients was granted by the FDA only 2 years later.

Considering its relatively brief regular approval time, it is important to further characterize and report sources of resistance and potential obstacles its employment might face. Assessing primary sources of resistance would provide a better decision-making capability while expanding the knowledge on mechanisms of drug resistance, how to address these mechanisms in order to circumvent these events, and expand the benefits of the drug.

In the BeatAML 1.0 cohort, we found that higher *NAMPT* and *MCL1* expressions and higher monocytic cell content were associated with poorer intrinsic response to venetoclax. These findings are consonant with the current literature.[12,13,20] In fact, these findings even complement observations in the current literature regarding *NAMPT*-mediated venetoclax resistance, not only making the gene a source of acquired resistance present in relapsed and refractory AML LSCs,[13] but also a prominent source of intrinsic *ex vivo* resistance to venetoclax.

In agreement with and complementing the findings of Zhao et al., we also observed that samples that enriched monocytes in their cellular composition were inherently resistant to venetoclax.[21] Although our data were not classified according to the French-American-British morphological classification, our gene set enrichment analysis revealed the presence of a gene set based on cluster 5 of AML samples of Valk et al., which are morphologically classified as myelomonocytic and monocytic leukemia.[22]

Waclawiczek et al. described that the current widely available AML therapy often spares cellular populations capable of evading it and driving relapse.[2] Our findings regarding cell population composition within studied samples and their response to venetoclax have shown that monocyte cells are enriched in samples from poor responders. In contrast, hematopoietic stem cell-like AML cells and early myeloid progenitors were enriched in good responders revealing two cell populations that are not substantially targeted by conventional chemotherapy but that present opposite sensitivity behaviors.

The enrichment of molecular signatures related to mitochondrial respiration and amino acid metabolism, along with the molecular signature compatible with early hematopoietic progenitors and LSCs, corroborates the metabolic behavior described as associated with de novo AML LSCs that are responsive to venetoclax.[9,23] The combination of venetoclax and azacytidine was described as inhibiting amino acid metabolism and impairing oxidative phosphorylation in LSC.[9,23]

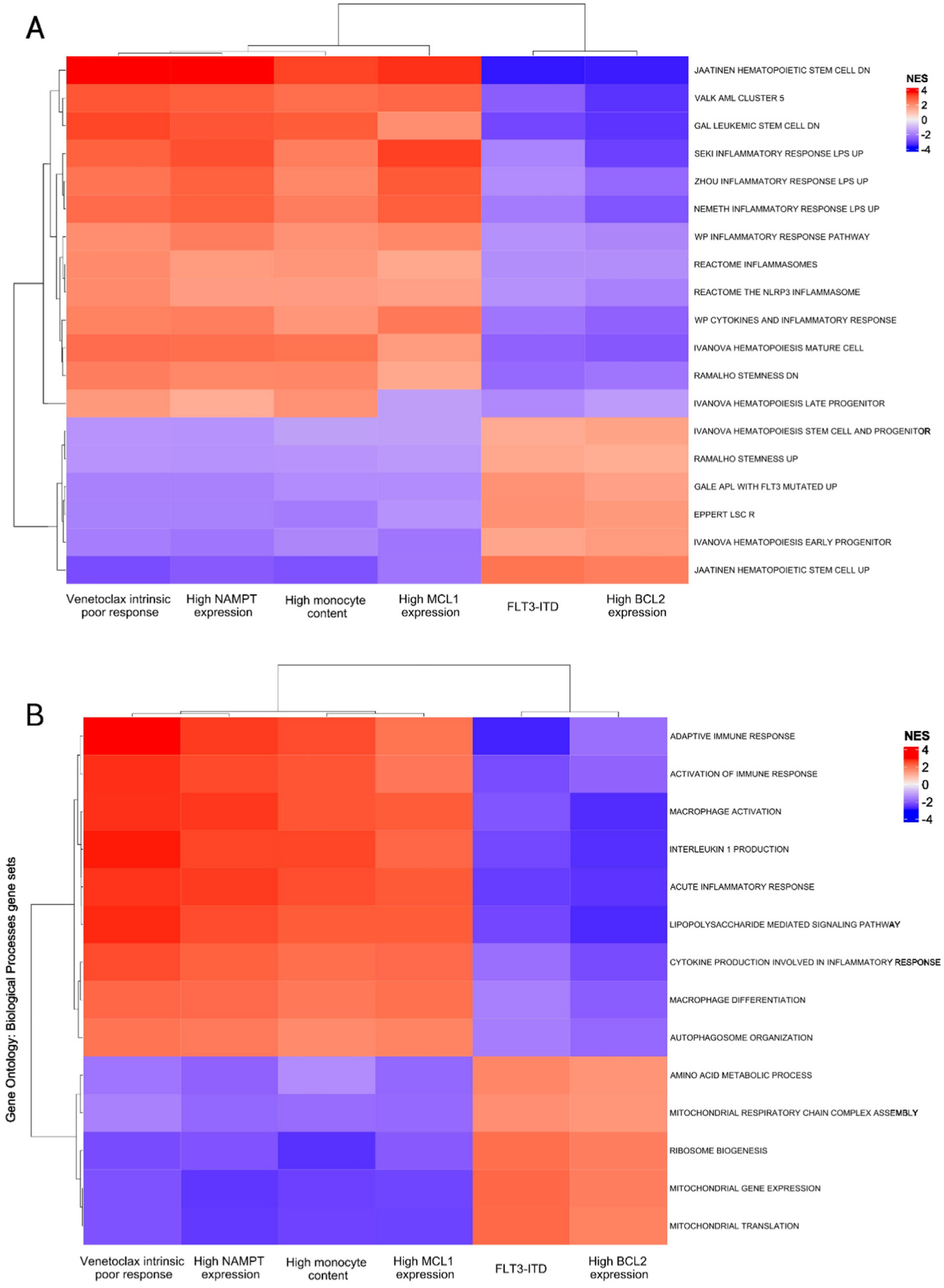

Figure 1 – Biological processes profiling regarding venetoclax response modulators according to gene set enrichment analysis In the columns, different conditions associated with intrinsic venetoclax response modulation as *NAMPT*, *MCL1*, and *BCL2* gene expression levels, AML samples monocytic cell content, the presence of the *FLT3*-ITD mutation, alongside intrinsic

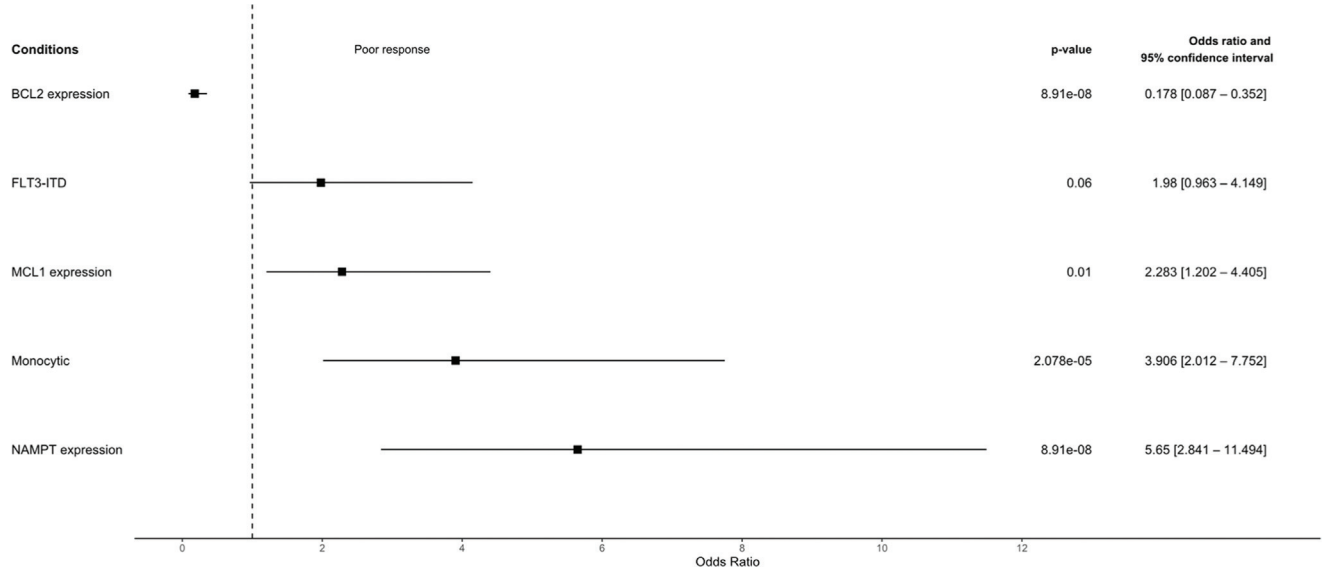

Figure 2 – The extent of association between Venetoclax *ex vivo* response and its reported sources of intrinsic resistance This forest plot displays the odds ratio and 95 % confidence interval (CI) for venetoclax *ex vivo* response across multiple reported sources of venetoclax response modulators, either promoting resistance or sensitivity. Each horizontal line represents a condition: 'gene expression' groups have the 'higher expression' group as reference; the *FLT3*-ITD mutation presence as reference; and monocytic content, higher monocytic signature as reference according to CIBERSORTx analysis of the samples. The OR and 95 % CI of each association are respectively represented as the square and the horizontal line on the plot. The vertical line intercepts the x-axis at an OR of 1 for which no association is observed.

Finding inflammation-related gene sets to be homogeneously enriched across different conditions reported as modulators of venetoclax response not only provides a potential biological process that consistently underlies venetoclax resistance across multiple conditions reported to promote intrinsic refractoriness, but also highlights a process currently poorly described as a basis for venetoclax resistance.

Currently, a single work directly states that inflammation is a promoter of venetoclax resistance in line with our findings. Wang et al. described that interferon-gamma (IFNγ) signaling was strongly correlated with venetoclax resistance, and treating primary AML cells with IFNγ increased their resistance to venetoclax, suggesting that IFNγ inhibition may be a potential strategy to bypass venetoclax resistance.[24]

It is also noteworthy that the patients considered ineligible for conventional chemotherapy that would benefit from venetoclax treatment are often under a tendency to maintain an increased chronic basal inflammatory state, mostly caused by age-related telomere shortening and associated comorbidities, that could, according to our data, mitigate the drug's efficacy.

This work also describes, for the first time, the effects of inflammasome activation over venetoclax response. The inflammasome was previously described as an enhancer of the fitness of AML cells,[25] and its main byproducts were credited to provide a beneficial microenvironment for the selection of leukemia cells at the expense of healthy hematopoietic cells.[25,26] We emphasize the activation process as a source of intrinsic venetoclax resistance.

Finally, our findings regarding the presence of *FLT3*-ITD were consistently associated with increased venetoclax sensitivity in contrast to those described by Liu et al.[6] Even though we failed to establish a likely association between the presence of *FLT3*-ITD and poor intrinsic *ex vivo* venetoclax response, the GSEA-based molecular signature of *FLT3*-ITD was not only constantly similar to *BCL2* upregulation, a biologically supported condition for good response to venetoclax, but also opposed to the molecular signatures related to venetoclax resistance.

Another piece of evidence that substantiates *FLT3*-ITD as a molecular entity linked to sensitivity to venetoclax is that *FLT3*-ITD samples presented a BH3 protein family differential gene expression profile very similar to *BCL2* high expression samples. Indeed, we observed that poor responders to venetoclax coordinately upregulated *BCL2A1* as an alternative antiapoptotic BH3 family protein to rely on. Differing from the observations of Pei et al., the monocytic population studied here was seemingly more reliant on the BCL2A1 protein instead of the MCL1 protein.[12] *BCL2A1* was also shown to increase venetoclax inhibitory concentration (IC_{50}) to 20-fold in AML cell models transduced with a lentivirus containing doxycycline-induced *BCL2A1*.[20]

venetoclax *ex vivo* response itself. In the rows, different gene sets from the curated human gene sets collection (A) and the Gene Ontology: Biological Processes collection (B). Each heatmap cell represents a normalized enrichment score (NES) value. Darker shades of red represent higher NES values, which means that this particular biological process or entity is related to the condition of interest. On the other hand, darker shades of blue stand for lower NES values, which means that a specific biological process or entity is more related to the condition of interest counterpart.

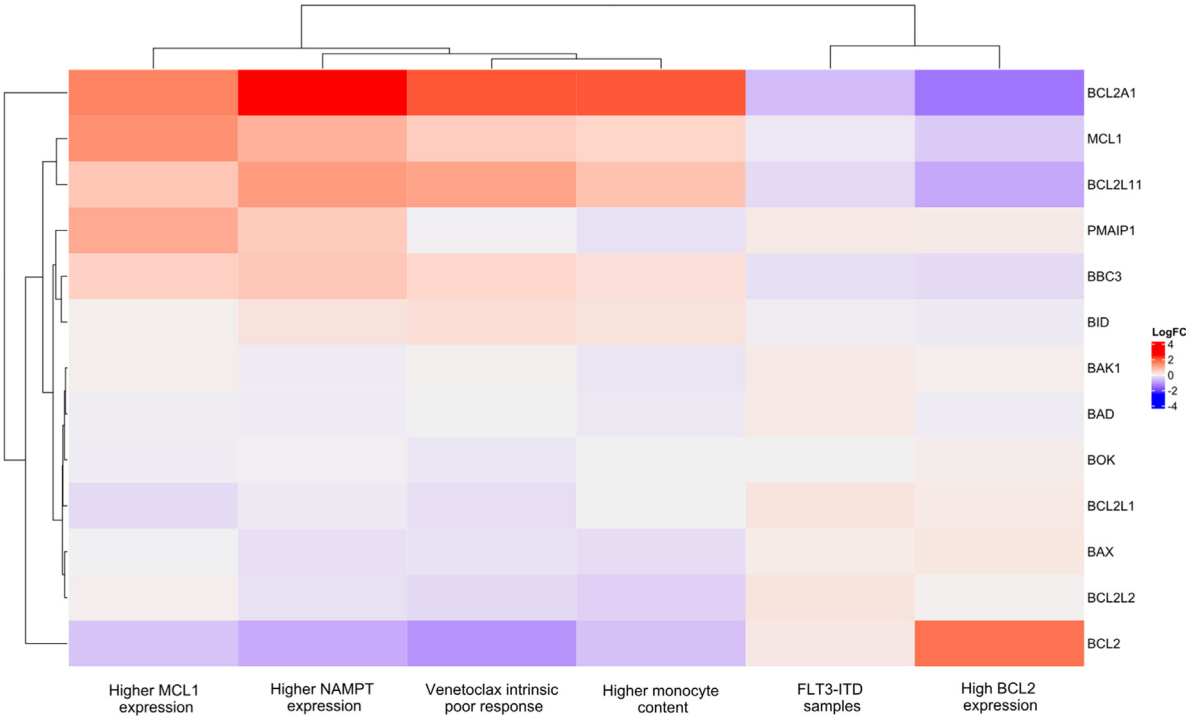

Figure 3 – Heatmap of the logarithm fold-change (LogFC) values on comparing modulators of venetoclax response. In the columns, BH3 family genes including apoptosis suppressors (*BCL2*, *BCL2A1*, *BCL2L1* or *BCL-XL*, and *BCL-W*), and activators (*BAD*, *BAK1*, *BAX*, *BBC3* a.k.a. *PUMA*, *BCL2L11* a.k.a. *BIM*, *BID*, *BOK*, and *PMAIP1* a.k.a. *NOXA*). In the rows, venetoclax response reported modulators as BCL2, MCL1, and NAMPT expression having higher expression as reference group for differential gene expression comparisons; sample monocytic content having higher monocytic content as reference group; *FLT3*-ITD mutation status having *FLT3*-ITD as reference group; and intrinsic *ex vivo* response to venetoclax having poor response as reference group. Each cell of the heatmap displays a LogFC value; the cells in shades of red represent upregulated genes in the reference groups, whereas the cells in shades of blue represent downregulated genes in the reference groups.

Conclusion

Taken together, this work offers a much-needed common ground across multiple response factors of venetoclax-based therapy. It highlights inflammation and, for the first time, identifies inflammasome activation as a potentially crucial biological process in venetoclax response, alongside sample cellular composition and developmental stage. This work also further solidifies *BCL2A1* as a relevant target to address venetoclax resistance, and emphasizes the importance of energy metabolism on the intrinsic venetoclax response. We acknowledge that a primary limitation of this work lies in the nature of the employed data, which is based on an *ex vivo* screening.

Conflicts of interest

The authors declare no potential 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.2025.103758.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Ocular graft-versus-host disease after allogeneic hematopoietic stem cell transplantation in a pediatric population

Cinthia Kim [a,*], Patricia Cabral Zacharias Serapicos [a], Cintia Monteiro Lustosa [b], Adriane da Silva Santos Ibanez [b], Victor Gottardello Zecchin [b], Lauro Augusto de Oliveira [a]

[a] Department of Ophthalmology and Visual Sciences, Federal University of São Paulo (UNIFESP), São Paulo, Brazil
[b] Pediatric Oncology Institute, GRAACC, Federal University of São Paulo (UNIFESP), São Paulo, Brazil

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ABSTRACT

Aim: To determine the prevalence of ocular graft-versus-host disease after allogeneic hematopoietic stem cell transplantation and to characterize the risk factors associated with its development in a pediatric population.
Methods: This retrospective chart review included 105 patients during a five-year period (2013–2017) from the Pediatric Oncology Institute (GRAACC-UNIFESP). The diagnosis of graft-versus-host disease was performed by the treating hematologist in conjunction with an ophthalmologist in accordance to National Institutes of Health (NIH) consensus criteria.
Results: Systemic graft-versus-host disease occurred in 44 of 105 (41.9%) patients, predominantly in males (54.5%) whereas ocular disease was diagnosed in seven (6.7%) of the patients. All the analyzed risk factors including diagnosis, type of conditioning regimen, use of radiotherapy in conditioning, donor sex, type and source of graft, human leukocyte antigen mismatch, and sex mismatch were not statistically significantly associated with the development of ocular disease, except for age. Ocular graft-versus-host disease patients presented a higher mean age compared to patients without ocular disease (p-value = 0.015).
Conclusion: Although less prevalent than in adults, ocular morbidity remains a concern in pediatric patients following allogeneic transplantation. Early diagnosis and regular ophthalmic follow-ups are recommended after the transplantation regardless of systemic graft-versus-host disease status.

* Corresponding author.
E-mail address: jiehck@gmail.com (C. Kim).
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Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is an established therapeutic modality for hematological neoplasms and non-neoplastic hematological disorders. The number of patients receiving this therapy has increased as a result of improvements in the assessment of the compatibility of human leukocyte antigens (HLA) in conjunction with advances in pre- and post-transplantation therapeutic regimens, as well as better supportive care. However, the occurrence of complications is increasing due to the longer survival of patients after transplantation [1].

Graft-versus-host disease (GvHD) is a common cause of morbidity and mortality after aHSCT. It is mediated primarily by donor T lymphocytes that recognize and target host antigens leading to inflammation and fibrosis of affected tissues. This condition usually occurs in the first three years after transplantation and can affect several organs such as skin, lung, liver, gastrointestinal tract, oral mucosa, the musculoskeletal system and eyes [1,2].

According to the National Institutes of Health (NIH) consensus in 2005, GvHD can be classified as acute or chronic. The chronic form is the most common affecting 30–70% of transplanted patients [3], however there are high concordance rates between both forms as the acute form is considered a strong predictor of chronic disease. Przepiorka et al. [4] reported that patients with acute GvHD had a higher risk of chronic disease and therefore the risk factors for acute GvHD can also be applied to the chronic form [1,4].

Ocular GvHD can occur in both forms, although it is more commonly seen in the chronic form [4]. Its incidence, of between 30% and 85%, is widely variable [1]. Ocular manifestations primarily affect the cornea, conjunctiva, lacrimal glands, eyelids and meibomian glands with dry eye syndrome (DES) or keratoconjunctivitis sicca being the most common manifestation in oGvHD [5].

Major symptoms of oGvHD are irritation, burning, pain, redness, foreign body sensation, excessive tearing, light sensitivity and visual haze. Clinical findings may include acute conjunctival inflammation, conjunctival hyperemia, chemosis, pseudomembranous epitheliopathy, filaments, painful erosions, cicatricial conjunctivitis, corneal opacification, corneal ulceration, and perforation. Disease severity is variable and can negatively affect quality of life and daily life activities [1,2,6].

The risk factors for oGvHD are not well understood but it is known that history of acute and severity of chronic GvHD, female donor, peripheral blood stem cell transplantation, conditioning regimen, and HLA mismatch are associated with increased development of ocular disease [1,2,5].

This study aims to determine the frequency of oGvHD after allo-HSCT and to identify risk factors associated with its development in a population of a pediatric oncology center.

Methods

This was a cross-sectional study based on a database review of allo-HSCT patients from January 2013 to December 2017 at the Pediatric Oncology Institute (GRAACC - Grupo de Apoio ao Adolescente e à Criança com Câncer), Federal University of São Paulo. During the study period, 108 patients underwent allo-HSCT. All medical records were reviewed, and clinical data were collected including demographic details, diagnosis, conditioning regime, source of graft, type of transplant, donor characteristics (sex, HLA matching), occurrence of acute or chronic systemic GvHD, and occurrence of oGvHD.

The diagnoses of systemic GvHD and oGvHD were performed by the treating hematologist in conjunction with an ophthalmologist in accordance to the 2005 and 2014 NIH consensus criteria (low Schirmer test values with a mean value of both eyes ≤5 mm at five minutes or a new onset of keratoconjunctivitis sicca by slit-lamp examination with mean values of 6–10 mm in the Schirmer test) [7].

The diagnoses of acute and chronic GvHD were based on clinical signs and symptoms, laboratory tests and whenever possible, on histopathologic findings of skin, oral mucosa and the gastrointestinal tract. The current consensus considers that clinical manifestations, and not the time to symptomatic onset after transplantation, determine whether the clinical syndrome of GvHD is considered acute or chronic [8].

Data were collected and presented in contingency tables. Continuous variables were compared using the Mann-Whitney test and categorical variables using the Fisher exact test. P-values of <0.05 were considered statistically significant. All analyses were achieved using the Stata v.14 computer program (College Station, Texas).

Results

A total of 108 pediatric patients were submitted to allo-HSCT and were regularly followed up at the Pediatric Oncology Institute - GRAACC - UNIFESP during the study period. All transplants were performed by the same hematology team supervised by the same physician. Of these patients, 105 survived longer than 90 days after allo-HSCT and were included in this study. Three patients died due to disease progression a few weeks after the transplant and were therefore excluded from the analysis. Patient demographics and transplant characteristics are summarized in Table 1.

The mean age of this population was 9.4 (standard deviation [SD]: 4.9) years and 63 (60%) were male. The most common indications for allo-HSCT were acute lymphoblastic leukemia (n = 38; 36.2%), followed by acute myeloid leukemia (n = 29; 27.6%), aplastic anemia (n = 15; 14.3%), and other reasons (n = 23; 21.9%).

Systemic GvHD occurred in 44 (41.9%) individuals in this study population with 24 (54.5%) being male. The mean age of those with systemic GvHD was 8.8 (SD: 5.3) years compared to those without GvHD (9.5; SD: 4.6 years; p-value = 0.491). Potential risk factors associated with the development of systemic GvHD were analyzed in this population. None of the risk factors analyzed were significantly associated with the development of systemic GvHD (Table 2).

A total of 40 (38.1%) individuals presented acute GvHD; the organs involved were skin in 36 (90.0%), liver in three (7.5%), and the gastrointestinal tract in eight (20.0%). Chronic GvHD occurred in 33 patients (31.4%); the organs involved were skin

Table 1 – Patient demographics and transplant characteristics.

Characteristic	n (%)
Total patients - n	105
Age (mean) – n (range)	9.4 (1.8–17.9)
Sex (male:female) – n (%)	63:42 (60:40)
Disease – n (%)	
ALL	38 (36)
AML	29 (28)
AA	15 (14)
Others	23 (22)
Stem cell source – n (%)	
Bone marrow	88 (84)
Peripheral blood	12 (11)
Cord blood	5 (5)
Donor sex (male:female) – n (%)	52:53 (50/50)
Donor and HLA histocompatibility – n %)	
Related identical donor	40 (38)
Mismatched related donor	10 (10)
Unrelated identical donor	35 (33)
Mismatched unrelated donor	20 (19)
Conditioning regimen (type) - n (%)	
Myeloablative	83 (79)
Reduced Intensity	22 (21)
Conditioning radiotherapy – n (%)	
Yes	54 (51)
No	51 (49)
Conditioning regimen – n (%)	
TBI + F + Cy	24 (23)
TBI + ETO	16 (15)
TBI + Cy	13 (12)
Bu + M	12 (11)
F + Cy + ATG	7 (7)
ALEM + F + M	7 (7)
Bu + M + ATG	6 (6)
Bu + Cy + M + ATG	4 (4)
Bu + Cy +M	4 (4)
Others	12 (11)
GvHD prophylaxis – n (%)	
CsA + MTX	40 (38)
CsA	31 (29)
Cy+ MMF + TAC	14 (13)
MTX + TAC	9 (9)
CsA + MMF	8 (8)
CsA + MMF + Cy	1 (1)
CsA	1 (1)
MTX	1 (1)

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AA, aplastic anemia; HLA, human leukocyte antigen; TBI, total body irradiation; FLU, fludarabine; CY, Cyclophosphamide; ETO, etoposide (Etoposide); BU, busulfan; M, melphalan; ATG, anti-thymocyte globulin; ALEM, alemtuzumab; CsA, cyclosporine (Cyclosporine capsules); MTX, methotrexate (Methotrexate); MMF, mycophenolate mofetil (CellCept); TAC, tacrolimus; GvHD, graft-versus-host disease.

(*n* = 16; 48.5%), liver (*n* = 7; 21.2%), lungs (*n* = 6; 18.2%), the gastrointestinal tract (*n* = 4, 12.1%), mouth (*n* = 11; 33.3%), and eyes (*n* = 7; 21.2%). Eighteen out of 33 subjects (54.5%) had chronic GvHD following acute GvHD, while 15 (45.5%) had chronic GvHD occurring *de novo*.

Ocular GvHD was diagnosed in seven patients with GvHD (7/105; 6.7%). There were no cases of acute ocular disease. The characteristics of these patients are summarized in Table 3.

Table 2 – Risk factors for systemic graft-versus-host disease (*n* = 105).

Risk factor	Systemic GvHD		p-value
	No (*n* = 61)	Yes (*n* = 44)	
Age, years – mean (SD)	9.5 (4.6)	8.8 (5.3)	0.491
Sex – n (%)			0.333
Male	39 (63.9)	24 (54.5)	
Female	22 (36.1)	20 (45.4)	
Diagnosis – n (%)			0.852
AAL	22 (36.1)	16 (36.3)	
AML	17 (27.9)	12 (27.3)	
AA	10 (16.4)	5 (11.4)	
Others	12 (19.6)	11 (25.0)	
Conditioning regiment – n (%)			0.704
Myeloablative	49 (80.3)	34 (77.3)	
Reduced intensity	12 (19.7)	10 (22.7)	
Radiotherapy conditioning – n (%)			0.587
No	31 (50.8)	20 (45.4)	
Yes	30 (49.2)	24 (54.6)	
Donor sex – n (%)			0.632
Male	29 (47.5)	23 (52.3)	
Female	32 (52.5)	21 (47.7)	
Source of graft – n (%)			0.862
Bone marrow	49 (87.5)	39 (88.6)	
Peripheral blood	7 (12.5)	5 (11.4)	
Type of transplant – n (%)			0.242
Related donor	29 (47.5)	26 (59.1)	
Unrelated donor	32 (52.5)	18 (40.9)	
HLA mismatch – n (%)			0.724
No	41 (67.2)	31 (70.4)	
Yes	20 (32.8)	13 (29.6)	
Sex mismatch – n (%)			0.912
No	27 (44.3)	19 (43.2)	
Yes	34 (55.7)	25 (56.8)	

GvHD, graft-versus-host disease; SD, standard deviation; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AA, aplastic anemia.

Ocular GvHD occurred in six (14.3%) cases with systemic GvHD compared to only one (1.6%) case among those without systemic GvHD (p-value = 0.016). Those who developed ocular GvHD were older than those who did not develop the condition (13.5 versus 8.9 years, respectively; p-value = 0.015). All other factors analyzed were not associated with oGVHD (Table 4).

Discussion

The incidence and risk factors associated with the development of oGvHD were analyzed in a single-institution chart review over a five-year period. The incidence of oGvHD in allo-HSCT patients varies widely from 30 to 70%, partly due to different diagnostic criteria. Approximately 40–90% of patients with systemic chronic GvHD will develop ocular disease which is usually diagnosed within two years of chronic GvHD diagnosis [1,5,9,10,11].

Several risk factors have been associated to systemic GvHD and oGvHD in adult patients. Previous history of acute GvHD is known to be a strong risk factor for chronic GvHD development

Table 3 – Ocular graft-versus-host disease patient characteristics.

Patient	1	2	3	4	5	6	7
Sex	M	F	M	M	F	M	M
Age	18	15	10	12	11	13	16
Diagnosis	AA	ALL	AML	AML	AML	AML	ALL
Conditioning regimen	Reduced intensity	Myeloablative	Myeloablative	Myeloablative	Myeloablative	Myeloablative	Myeloablative
Conditioning radiotherapy	No	Yes	No	No	No	No	Yes
Source of graft	Bone marrow	Bone marrow	Bone marrow	Bone marrow	Bone marrow	Bone marrow	Bone marrow
Donor and HLA histocompatibility	Related identical	Unrelated identical	Related identical	Related identical	Related identical	Unrelated identical	Unrelated identical
Onset of GvHD (days)	225	69	260	364	378	366	121
Slit lamp examination	Corneal Punctate epithelial erosions	Corneal Punctate epithelial erosions	Corneal punctate epithelial erosions	Corneal punctate epithelial erosions	Conjunctival hyperemia, corneal punctate epithelial erosions	Conjunctival hyperemia, Corneal punctate epithelial erosions	Corneal punctate epithelial erosions
Schirmer's test (mean value in mm)	5.5	4.5	5	5	0	7.5	2.5
Eye treatment	lubricant eye drops+ topical cyclosporine (Cyclosporine capsules)	lubricant eye drops+ topical cyclosporine (Cyclosporine capsules)	lubricant eye drops+ topical cyclosporine (Cyclosporine capsules)	lubricant eye drops+ topical cyclosporine (Cyclosporine capsules)	lubricant eye drops+ topical tacrolimus and steroid	lubricant eye drops+ topical steroid	lubricant eye drops
Immunosuppression after GVDH (PDN + CsA + MFM + TAC)	Yes (PDN + CsA)	Yes (PDN + TAC)	Yes (PDN + CsA + IM)	Yes (PDN + CsA)	Yes (PDN + CsA + MTX + IM)	No	Yes
Other organs involved	none	skin	skin, mouth, lungs	none	skin, lungs	skin	skin, mouth, liver, osteoarticular

M, male; F, female; AA, aplastic anemia; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; PDN, prednisone; CsA, cyclosporine (Cyclosporine capsules) TAC, tacrolimus; IM, imatinib; MTX, methotrexate (Methotrexate); MMF, mycophenolate mofetil (CellCept).

[12]. Other risk factors such as severity of systemic GvHD, use of peripheral blood stem cells, recipient age and donor-recipient sex mismatch have been shown to increase the risk of chronic GvHD [5,6,12,13]. However, these findings are mostly based on adult patients and rarely in pediatric populations. Recipient age has been identified as one of the major risk factors for chronic GvHD in several studies with younger patients being considered to have less risk [14–17].

Kondo et al. reported a lower incidence of chronic GvHD in a pediatric population (22%) when compared to studies in adult populations. They also reported that older patients (>10 years) had an increased risk of chronic GvHD [14]. Locatelli et al. [18] reported a similar low incidence of chronic GvHD in children (24%). Furthermore, Eisner et al. [19] reported a low incidence (29%) of chronic GvHD and found that recipient age was an important risk factor in pediatric patients, although this was not confirmed by multivariate analysis. Recipient age is therefore an important risk factor for chronic GvHD in children. In addition to recipient age, Kondo et al.[14] reported that acute GvHD, malignant disease, and a female donor to male recipient were identified as significant risk factors for chronic GvHD. In several reports, a female donor to male recipient was recognized as a risk factor for chronic GvHD based on the alloimmunization of the female donor [15,17]. In childhood hematopoietic stem cell transplantation (HSCT), however, HLA-identical siblings are rarely alloimmunized, and alloimmunized female donors would be considered non-identical. Therefore, it is unclear whether alloimmunization affects the development of chronic GvHD in childhood HSCT [14].

Reports on incidence rate and risk factors for oGvHD are poorly documented in the pediatric age group. Bradfield et al. reported an incidence of oGvHD in three of 93 patients (3.2%) who underwent transplantation [11]. Fahnehjelm et al. reported DES in 37 of 60 children (61.7%) who had undergone HSCT. They also found that DES was more common in female patients with malignant diseases, in male patients who underwent HSCT at older ages, and in patients who were exposed to repeated high trough levels of cyclosporine (Cyclosporine capsules) for immunosuppression. There were no associations between prolonged corticosteroid treatment, irradiation treatment or chronic GvHD with DES in the mentioned study. As previously reported in other studies, they also demonstrated that older recipients presented higher risk of developing oGvHD after allo-HSCT [20].

Suh et al. assessed ocular findings in 104 pediatric bone marrow recipients in a two-year follow-up. DES was found in 12.5%, cataracts in 23%, and fundus complications in 13.5% of the patients [21]. Accordingly, Bradifield et al. reported ocular complications in 20.3% of children who underwent organ and bone marrow transplantation (BMT) [11]. Cataracts were the most common ocular complication following the use of systemic steroids. The reported prevalence of cataracts in children has varied from 8.5% to 80% [11,21].

A high prevalence of DES, ranging from 17 to 44%, has been reported in studies of adult BMT. These studies also found a strong association between DES and the occurrence of acute GvHD. Suh et al. [21] found a lower prevalence of DES (12.5%) in a pediatric population. They also demonstrated associations between the occurrences of acute and chronic GvHD and DES (77% and 48% of DES patients, respectively).

Table 4 – Risk factors for ocular graft-versus-host disease (*n* = 105).

Risk factor	Ocular GvHD		p-value
	No (*n* = 98)	Yes (*n* = 7)	
Age, years – mean (SD)	8.9 (4.9)	13.5 (3.4)	0.015
Sex - n (%)			0.700
Male	58 (59.2)	5 (71.4)	
Female	40 (40.8)	2 (28.6)	
Diagnosis - n (%)			0.280
AAL	36 (36.7)	2 (28.6)	
AML	25 (25.5)	4 (57.1)	
AA	14 (14.3)	1 (14.3)	
Others	23 (23.5)	0 (0)	
Conditioning regiment - n (%)			0.547
Myeloablative	77 (78.6)	6 (85.7)	
Reduced intensity	21 (21.4)	1 (14.3)	
Radiotherapy conditioning - n (%)			0.711
No	47 (47.9)	4 (57.1)	
Yes	51 (52.0)	3 (42.9)	
Donor sex - n (%)			0.437
Male	50 (51.0)	2 (28.6)	
Female	48 (48.9)	5 (71.4)	
Source of graft - n (%)			0.603
Bone marrow	82 (88.2)	6 (85.7)	
Peripheral blood	11 (11.8)	1 (14.3)	
Type of transplant - n (%)			0.706
Related donor	52 (53.1)	3 (42.8)	
Unrelated donor	46 (46.9)	4 (57.2)	
HLA mismatch - n (%)			0.429
No	66 (67.3)	6 (85.7)	
Yes	32 (32.7)	1 (14.3)	
Sex mismatch - n (%)			0.463
No	44 (44.9)	2 (28.6)	
Yes	54 (55.1)	5 (71.4)	

SD, standard deviation; GvHD, graft-versus-host disease; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; AA, aplastic anemia.

In this study the prevalence of oGvHD was 6.7%, a lower prevalence compared to the general population but similar to other pediatric studies. In the current series, older patients presented a higher risk for oGvHD. None of other risk factors analyzed such as conditioning regime, source of graft, sex mismatch or malignant disease were associated with the development of oGvHD.

A lower prevalence of DES may be explained by the retrospective nature of the study and the fact that the evaluation of DES was less comprehensive than in prospective studies on adults. Mild cases of DES in preverbal children are more difficult to diagnose since children express subjective symptoms less frequently than adults. Worse yet, young children are harder to examine using the fluorescein staining and Schirmer tests. That said, the prevalence of DES may have been underestimated in this study. Ng et al. reported that 51.7% of pediatric BMT patients had tear abnormalities; none of these patients had dry eye symptoms despite a third of them having patchy fluorescein staining of the cornea [22].

Management of chronic oGvHD is essentially the same as for other types of severe dry eye disease, but understanding the status of the patient's systemic GvHD can influence the ocular treatment strategy. Therefore, multidisciplinary assessments with an ophthalmologist and hematologist-oncologist are important [23].

The supportive care goals for oGvHD involve lubrication, control of tear evaporation, control of tear drainage, epithelial support and reduction of ocular surface inflammation. The treatment needs to be matched to the particular mix of symptoms of each patient; the individual's systemic medications also should be taken into account [23]. The first steps in reducing the symptoms of decreased tearing are preservative-free artificial tears and punctal occlusion with silicone plugs or cautery. In case of severe or persistent epithelial damage, autologous serum eyedrops, which contain many growth factors and vitamins that support the healing and integrity of the ocular surface, can be prescribed. Topical therapies, such as corticosteroid or cyclosporine (Cyclosporine capsules) drops, can be effective and optimization of these therapies is warranted. Ocular care also consists of photoprotection along with regular evaluations for infection, cataract formation, and increased intraocular pressure. Regional care may include ocular ointments, a humidified environment, occlusive eye wear, moisture chamber eyeglasses, or gas permeable scleral contact lens for symptomatic relief [24].

It is important to follow transplant patients closely with serial Schirmer tests to assess the degree of wetting and to intervene early at the onset of ocular involvement even prior to the evolution of symptoms [24]. The Schirmer test without anesthesia may be difficult to perform and it is not recommended in younger children; an ophthalmologist's input may be needed for objective scoring in these children [25]. For children who are old enough to tolerate the procedure, routine Schirmer evaluations should be done to monitor tear production [24]. Chronic oGvHD might impact on the quality of life of the pediatric population especially those who have not even reached economically active age.

There is a lack of studies focused in oGvHD in children. A few reports from the United States [11,21], Japan [14] and Italy [26] have already reported their experience but no report on this issue in Brazil was found.

A few limitations should be mentioned in the current study. Due to the retrospective nature of the analysis, it was not possible to establish baseline ophthalmic examinations prior to allo-HSCT and to determine the potential influence of the conditioning regimen on the ocular surface. Diagnosis of oGvHD was based on Schirmer's test or subjective symptoms according to the NIH criteria (2005). Decreased corneal sensitivity may underestimate changes of the ocular surface and allow false negatives results. Less comprehensive and not always reliable information in preverbal children may also underestimate oGvHD prevalence.

It is recommended that a baseline ocular profile of tear dynamics and ocular surface parameters should be conducted before allo-HSCT as well as regular ophthalmological examinations after the procedure, rather than relying exclusively on the NIH criteria. This might enable the use of measurable clinical and objective parameters to avoid false negative cases. That said, a prospective study setting with a

baseline ocular surface work up before allo-HSCT and setting a disease (oGvHD) cutoff point using the metric parameters proposed by the International Chronic Ocular GvHD Consensus group [27] may provide more reliable data.

Conclusion

Although with a relatively lower prevalence compared to adults, oGvHD with its morbidities is a concern after allo-HSCT in pediatric patients. Regular ophthalmologic assessments following allo-HSCT are therefore recommended for early detection and treatment of these potentially problematic complications in pediatric patients.

Ethics

Ethics committee approval number: 014/11 – IOP, 0269/10 Federal University of São Paulo (UNIFESP).

Contribution of the author

All authors contributed significantly to the work reported in this paper. Cinthia Kim contributed to conceptualization, methodology, data curation, formal analysis, writing of the original draft, and review and editing of the manuscript. Patricia Serapicos, Cintia Lustosa e Adriane Ibanez provided resources and assited with data curation. Falvio Hirai was responsible for conceptualization, methodology, formal analysis, and manucript review and editing. Victor Zecchin contributed to conceptualization, resources, and data curation. Lauro Oliveira led the conceptualization, methodology, investigation, supervision, review and editing of the manuscript, and secured funding. All authors reviewed and approved the final version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Clinical and laboratorial characterization of a cohort of patients with hereditary platelet disorders in Brazil

Letícia Dalla Vecchia Grassi [a], *Erica Okazaki* [a], *Cynthia Rothschild* [a], *Paula Villaça* [a], *Fernanda Andrade Orsi* [a,b,*], *Bianca Stefanello* [a]

[a] *Hospital das Clínicas, Faculty of Medicine, University of São Paulo, São Paulo, São Paulo, Brazil*
[b] *School of Medical Sciences, University of Campinas, Campinas, São Paulo, Brazil*

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ABSTRACT

Introduction: Inherited platelet disorders are rare conditions characterized by altered platelet function and/or reduced platelet counts. Diagnosing these disorders is challenging and may result in delays, misdiagnosis, and inappropriate treatment. In low- and middle-income countries, data are scarce. Here, we describe a cohort of patients at a reference center in Brazil.

Methods: A descriptive analysis was conducted on patients followed at the Thrombosis and Hemostasis outpatient clinic of the Hospital das Clinicas, University of São Paulo, Brazil. Medical records of 857 patients with thrombocytopenia or bleeding disorders of unknown cause, evaluated between 1998 and 2023, were reviewed. Of these, 60 patients had a confirmed or suspected diagnosis of an inherited platelet disorder and were included in the study.

Results: Among the 60 patients, the majority were female (75 %), with a median age of 48 years. The suspicion of a platelet disorder was based on clinical presentation, family history, and laboratory findings. Overall, 65 % of the patients had abnormal platelet function, while 35 % presented with thrombocytopenia. A positive family history was reported in 62 % of those with low platelet counts and in 51 % of patients with platelet function abnormalities. Previous misdiagnoses included immune thrombocytopenia and von Willebrand disease. Overall, the bleeding phenotype was mild, with a median ISTH-BAT (International Society on Thrombosis and Haemostasis Bleeding Assessment Tool) score of 6. Patients with reduced platelet counts tended to have lower ISTH-BAT score.

Conclusions: Identifying inherited platelet disorders is essential for proper treatment and follow-up. This study emphasizes the need for careful assessment of family history, bleeding risk, platelet count, morphology, and function for diagnosis, particularly in low-resource settings without access to advanced genetic testing.

* Corresponding author at: Hematology, Hemotherapy and Cell Therapy Unit, Hospital das Clínicas, Faculty of Medicine, University of São Paulo, Av. Dr. Enéas Carvalho de Aguiar, 155. Cerqueira César, São Paulo, São Paulo, Brazil.
E-mail address: ferorsi@unicamp.br (F.A. Orsi).

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Introduction

Inherited platelet disorders (IPDs) encompass a diverse range of conditions characterized by altered platelet function (inherited platelet function disorders - IPFDs) and reduced platelet counts (inherited platelet number disorders - IPNDs).[1-3] These diseases are rare, with an estimated prevalence of 2–3 cases per 100,000 people.[3,4] Clinical presentations vary widely, with patients experiencing mild to severe bleeding episodes, sometimes accompanied by other findings such as decreased platelet counts, abnormal platelet morphology, syndromic features, or a predisposition to other systemic diseases.[5] Their pathophysiology is still being investigated. With advancing genetic mapping tools, many new entities are being identified. Today, 60 distinct IPDs and 75 related genes are recognized.[5,6]

Diagnosing IPDs is challenging and often leads to delays or misdiagnosis. Diagnostic assessments involve evaluating the location, triggers, severity of bleeding episodes, concurrent syndromic features, and relevant family history. Initial evaluations include examining platelet count and morphology. Additional tests, such as platelet aggregation, immunophenotyping, and electron microscopy, may be utilized to assess platelet function. Genetic mapping can aid in diagnosis by identifying mutations associated with inherited platelet disorders though its accessibility is often limited.[7,8]

Lack of knowledge about IPDs, combined with the heterogeneity of clinical manifestations and the difficulty of laboratory confirmation, can lead to delays in diagnosis and subsequently in treatment. Accurately identifying and characterizing these disorders is crucial for providing appropriate treatment, genetic counseling, and follow-up, especially considering their association with systemic diseases and neoplasias.[7,8]

Most of the existing data on these diseases is derived from registries in North America and Europe. In low- and middle-income countries, such as Brazil, there is a lack of data describing the characteristics of the population and the challenges faced in diagnosing this group of diseases, particularly in the context of the public health system with its limited resources.

In this context, this study describes a cohort of patients with IPDs followed at a reference center in Brazil. The objective is to contribute to the limited existing body of evidence on the clinical presentation of these diseases in low- and middle-income countries, with the ultimate goal of increasing awareness of IPDs and their diagnosis in these countries.

Methods

A retrospective descriptive analysis was conducted of a cohort of patients with suspected or confirmed IPD followed at the Thrombosis and Hemostasis outpatient clinic of the Hospital das Clinicas, Faculty of Medicine, University of São Paulo (HCFMUSP), Brazil.

The selection of patients for this study followed a three-step process. In the first step, all patients with a possible diagnosis of thrombocytopenia or platelet disorder were identified in the database of the Thrombosis and Hemostasis Outpatient Clinic at HCFMUSP. This database consists of a spreadsheet containing the records of all patients who had consulted with the team from 1998 to 2023, regardless of the diagnosis; details are added to the database during the first consultation. A broad search in this database was performed to minimize losses, given the rarity of hereditary platelet disorders. Specific filters were used to select records labeled with terms such as "thrombocytopenia" or "platelet dysfunction" or "unexplained bleeding" or "storage pool disease" or "Glanzmann syndrome" or "Bernard-Soulier syndrome" or "May-Hegglin anomaly." Through this process, 857 patients were identified for further chart review.

The second step consisted of a chart review, in which the medical records of each patient identified in the first step were individually evaluated to determine whether the diagnosis was accurate. This was critical because the database used in the first step is updated only once, during the patient's first consultation, and the documented diagnosis is based on a preliminary assessment. As a result, diagnoses may change in future medical visits. A total of 319 patients identified in the first step did not have a digital medical record because they were lost to follow-up before the 2010 record migration, and their physical records were no longer available. Another 217 patients identified in the first step had only one or two medical visits recorded in their digital charts, also due to loss of follow-up. Without being able to confirm their diagnosis, these cases had to be excluded, which resulted in the retention of 321 patients.

In the third step, all charts were reviewed for confirmed or suspected diagnosis of an inherited platelet disorder. Of the 321 patients with available medical records, 261 patients were further excluded because their final diagnosis indicated a different condition, most commonly idiopathic thrombocytopenia, which accounted for approximately 50 % of these exclusions (130 cases). Other patients were excluded because of acquired causes of platelet disorders, including those related to medication, infection, hypersplenism, or thrombocytopenia that resolved during the follow-up (131 cases).

Given the limited availability of certain diagnostic tests, such as electron microscopy and genetic mapping, the final cases were classified as suspected if patients had persistent thrombocytopenia since childhood without response to previous treatments, a suggestive family history, or increased bleeding associated with altered platelet aggregation and/or secretion tests. Figure 1 illustrates the study selection and reasons for exclusion.

This study was approved by the Research Ethics Committee of HCFMUSP (CAAE 67501923.7.0000.0068).

Results

The final cohort comprised 60 patients, 39 (65 %) with IPFDs and 21 (35 %) with IPNDs. There was a predominance of women (75 %), with a median age of 48 years. The racial distribution was 42 % white, 42 % mixed-race, and 16 % black, without differences between the subgroups (Table1).

The suspicion of IPDs was based on factors obtained from the clinical history and laboratory tests. In cases where patients exhibited thrombocytopenia (13/21 - 62 %), IPNDs were suspected due to a positive family history of

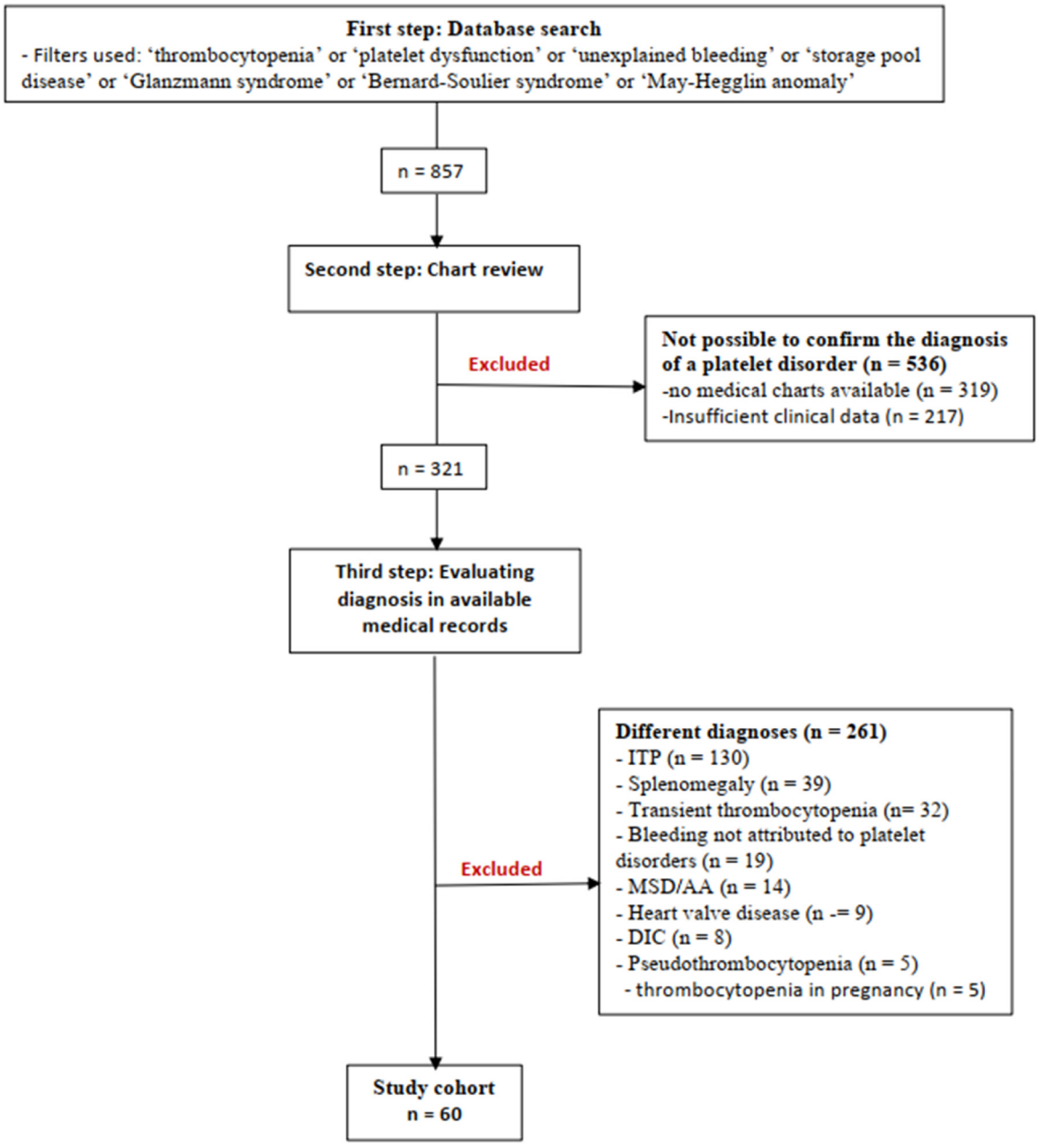

Figure 1 – Flowchart of the study illustrating patient selection and reasons for exclusion.

ITP: immune thrombocytopenia; MDS: Myelodysplastic syndrome; AA: aplastic anemia; DIC: disseminated intravascular coagulation.

thrombocytopenia or after the diagnosis of immune thrombocytopenia (ITP) was excluded. A diagnosis of ITP was excluded based on the observation of long-standing, stable thrombocytopenia in the absence of a response to previous therapeutic interventions. In the IPND cohort, eight patients (38 %) received treatment for ITP without response, including corticosteroids (eight patients - 38 %), immunoglobulin (one patient - 5 %), immunosuppressants (one patient - 5 %) and splenectomy (two patients; - 10 %).

Eight patients (15 %) had previously received different diagnoses. One patient, initially suspected of having ITP, was confirmed to have MYH9-related thrombocytopenia, two cases initially diagnosed with ITP were confirmed as Bernard-Soulier syndrome, three cases of ITP were reclassified as unspecified inherited thrombocytopenia, and two patients initially suspected as having von Willebrand disease were reclassified as unspecified inherited platelet function disorder.

In the IPFD Group, the family history was positive in 20 out of 39 patients (51 %). Since some had concomitant thrombocytopenia, two patients received treatment for ITP (6 %), including corticosteroids (one patient - 3 %), immunoglobulin (one patient - 3 %), immunosuppressants (one patient - 3 %) and

splenectomy (one patient - 3 %). In cases where patients exhibited excessive bleeding that was disproportionate to their platelet count, in addition to ruling out von Willebrand disease and other coagulopathies, platelet function was evaluated using aggregation tests, platelet immunophenotyping, and platelet secretion tests (lumiaggregometry). Abnormalities in these tests led to the suspicion or confirmation of IPFDs.

Nearly all patients (57 out of 60 patients - 95 %) faced some hemostatic challenge, including after dental extraction, or endoscopic or surgical procedures. Of a total of 51 women, 23 (51 %) had a previous pregnancy. There was a remarkable difference between the groups, with 12 out of 13 (92 %) IPND women reporting previous pregnancies, but only 11 out of 32 (34 %) IPFD women. The bleeding phenotype was assessed using the International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH BAT) score[9] giving a median score of 6 for the entire cohort (normal values are <4 for men and <6 for women). There was also a significant difference between subgroups: a median of 3.5 in the IPND Group and of 10 in the IPFD Group (Table 1).

In the entire cohort, six patients (10 %) presented with associated syndromic abnormalities: three in the IPND Group (15 %) and three in the IPFD Group (8 %). The observed features included: two cases of deafness associated with nephropathy, one case of growth delay and heart disease, one case of cognitive impairment, and two cases of skeletal malformations with cognitive impairment.

In the IPND Group, the median highest and lowest platelet counts were 108 $\times$ 10^9/L and 56 $\times$ 10^9/L, respectively. As expected, the IPFD Group had normal or slightly reduced platelet counts, with median highest and lowest platelet counts of 279 $\times$ 10^9/L and 132 $\times$ 10^9/L, respectively. The median mean platelet volume (MPV) was 11.5 fL for the entire cohort; 14.1 fL for the IPND Group, and 11.1 fL for the IPFD Group (Table 1).

Platelet immunophenotyping was performed in 27 patients (45 %), six patients (29 %) in the IPND Group and 21 patients (54 %) in the IPFD Group. Of the total number of patients who underwent immunophenotyping, 15 (55 %) exhibited abnormal results, all of whom were in the IPFD Group (patients with diagnosis of Glanzmann thrombasthenia and Bernard-Soulier syndrome). Platelet aggregation tests were conducted in 34 cases (57 %), with nine (43 %) in the IPND Group and 25 (64 %) in the IPFD Group. Seven patients (21 %) had normal results and 27 (79 %) had altered platelet aggregation, however, discordant results were noted in eight (30 %) of the altered tests. Lastly, platelet secretion tests were performed in 16 patients (27 %), with two (10 %) in the IPND Group and 14 (39 %) in the IPFD Group (Table 1).

Based on the tests performed, the current cohort consists of 20 patients with confirmed diagnoses (33 %) and 40 with suspected diagnoses (67 %) of IPDs. Of the 20 confirmed diagnoses, one case in the IPND Group had a MYH9 macrothrombocytopenia confirmed by a genetic panel, and 19 in the IPFD Group had Glanzmann thrombasthenia (13 cases), Bernard-Soulier syndrome (five cases) and storage pool disease (one case) confirmed by electron microscopy. Of the 40 suspected diagnoses, 20 patients had unspecified IPNDs and 20 had unspecified IPFDs (Table 2).

A total of 47 patients (78 %) received some treatment during follow-up; 43 % of the patients in the IPND Group and 97 % in the IPFD Group. The predominant treatments included the

Table 1 – Clinical and laboratory features of the inherited platelet disorder cohort.

Variable	Total (*n* = 60)	IPND (*n* = 21)	IPFD (*n* = 39)
Female - n (%)	45 (75)	13 (62)	32 (82)
Race - n (%)			
White	25 (42)	8 (38)	17 (44)
Black	10 (16)	2 (10)	8 (20)
Mixed race	25 (42)	11 (52)	14 (36)
ISTH BAT score[a] - median ± (IQR)	6.0 (3.0–11.0)	3.5 (1.8–4.3)	10.0 (6.0–13.0)
Highest platelet count during follow-up - x10^9/L median ± (IQR)	178 (105–316)	108 (85–125)	279 (195–369)
Lowest platelet count during follow-up - x10^9/L median ± (IQR)	90 (47–153)		
	56 (26–63)	132 (96–182)	
Highest MPV during follow-up (fL)[b] - median ± (IQR)	11.5 (10.4–14.3)	14.1 (13.4–16.0)	11.1 (10.2–11.7)
Platelet aggregation test - n (%)			
Patients with a platelet aggregation test result	34 (57)	9 (43)	25 (64)
Patients with abnormal platelet aggregation results	27 (79)	3 (33)	24 (96)
Consistent abnormalities[c]	19 (70)	2 (67)	17 (71)
Inconsistent abnormalities[c]	8 (30)	1 (33)	7 (29)
Platelet secretion assay - n (%)			
Patients with platelet secretion assay result	16 (27)	2 (10)	14 (39)
Patients with abnormal results	7 (44)	1 (50)	6 (43)
Immunophenotyping test - n (%)			
Patients with an immunophenotyping test result	27 (45)	6 (29)	21 (54)
Patients with abnormal immunophenotyping test results	15 (55)	0	15 (71)

IPND: inherited platelet number disorder; IPFD: inherited platelet function disorders; IQR: interquartile range; ISTH BAT: International Society on Thrombosis and Haemostasis Bleeding Assessment Tool; MPV: mean platelet volume.

[a] BAT score was available for 57 (95 %) IPD patients.
[b] The local MPV reference values range from 6.5 to 12.5 fl.
[c] For patients with more than one aggregation test, "consistent abnormalities" were considered when all the tests performed showed the same aggregation abnormality and "inconsistent abnormalities" when one or more results were discordant.

Table 2 – List of final diagnosis of the IPD cohort.

Diagnosis	
IPFD	n (%)
Storage pool disease	1 (2)
Glanzmann thrombasthenia	13 (22)
Bernard-Soulier syndrome	5 (8)
Unspecified inherited platelet function disorder[a]	20 (33)
IPND	n (%)
MYH9 macrothrombocytopenia	1 (2)
Unspecified inherited thrombocytopenia[b]	20 (33)

IPND: inherited platelet number disorder; IPFD: inherited platelet function disorders.

[a] Inherited platelet disorders were suspected, even in the absence of diagnostic testing, when the ISTH BAT score suggested a hemorrhagic phenotype and platelet aggregation or secretion was altered.

[b] inherited thrombocytopenia was suspected even when diagnostic tests were not available in cases of family history, altered platelet volume, persistent but stable long-term thrombocytopenia, and lack of response to immune thrombocytopenia treatment.

use of antifibrinolytics (73 %) and platelet transfusions (55 %) prior to surgical procedures or in cases of bleeding. In the IPFD Group, 90 % of patients received antifibrinolytic treatment and 77 % received platelet transfusions, while in the IPND Group, these numbers were 43 % and 14 %, respectively. Recombinant factor VIIa (FVIIa) was used in eight patients, all of whom had Glanzmann thrombasthenia.

Discussion

Initially described in 1948, IPDs are a rare group of diseases, with few cohorts described in the literature. Most studies show no association of sex with diagnoses, but some indicate a higher prevalence of IPDs among women.[10-12] In the current cohort, there was a predominance of women (75 %). A possible explanation is the greater frequency of hemostatic challenges that women face, such as menstruation and delivery, allowing increased bleeding tendencies to be identified. As for the distribution of ethnicity, this data is scarce in most publications. In the clinic of this study, there was a predominance of white and mixed-race patients with a smaller proportion of black patients. However, this ethnic distribution reflects the general Brazilian population.

The diagnosis of IPDs is challenging and probably most cases remain unidentified. Misdiagnoses are also common, often resulting in inadequate treatment.[13,14] Among patients with IPNDs, up to 30 % receive an incorrect diagnosis of ITP and are sometimes treated with prolonged corticosteroid therapy, immunosuppressants, and even splenectomy.[1,15]

A similar occurrence was observed in this cohort with 38 % of patients in the IPND Group having received prior treatment for ITP, primarily corticosteroids (38 %), but also including splenectomy (10 %). The IPFD Group, which sometimes presents with thrombocytopenia, had a smaller proportion of patients receiving inappropriate treatments for ITP (6 %), including one splenectomy (3 %). When thrombocytopenia is present, it is often challenging to differentiate IPDs from ITP.[13,14] This situation was found in the present cohort, as 10 % of the IPD patients were previously diagnosed with ITP. When increased bleeding occurs in the absence of thrombocytopenia, the main differential diagnosis was von Willebrand disease, with two changes in diagnosis during the follow-up.

A key factor in the investigation of IPDs is a family history of thrombocytopenia or increased bleeding, which was present in >50 % of both subgroups of the cohort. Similar data have been reported in other studies evaluating IPND,[13,16] with a lack of data in IPFD cohorts. This study reinforces the importance of having this information in cases of diagnostic suspicion of IPD. The initial division into IPND and IPFD can also help with diagnosis, it distinguishes the primary differences between the subgroups and guides subsequent steps in the diagnostic process.

The IPFD Group exhibited a severe hemorrhagic phenotype with a median ISTH BAT score of 10. Conversely, the IPND Group showed a normal ISTH BAT score with a median of 3.5 with the caveat that we classified Bernard-Soulier syndrome and Glanzmann thrombasthenia as IPFD. These data are consistent with the literature, as in the ISTH BAT score validation study for the IPD population, the results showed a median of 9 for the IPFD Group and of 2 for the IPND Group.[17] Comorbidities and syndromic features, although present and warranting evaluation, accounted for 10 % of the present sample. It is worth noting that only adult patients were evaluated in this study, and many syndromic patients often continue to be followed by pediatric and genetic teams.

As for laboratory propaedeutics, the platelet counts during follow-up were monitored, and thrombocytopenia was present in 25 % of patients with IPFD, however it was mild and disproportionate to the hemorrhagic phenotype. For the IPND Group, platelet size and morphology are important components of the diagnostic workup. In this cohort, 75 % of the IPND patients had altered MPV, in line with the association of these diseases with macro platelets and giant platelets, which is helpful for diagnosis.

Platelet aggregation was carried out on patients with no suspicion of Bernard-Soulier syndrome or Glanzmann's thrombasthenia, for whom direct immunophenotyping was chosen. Thus, of the 25 remaining cases of suspected IPFD, abnormal platelet aggregation consistently confirmed the diagnosis of platelet dysfunction in 17 (44 %). Secretion tests were altered in seven patients, detecting only one more whose aggregation test was inconsistent, but adding little information to the other six whose diagnosis was already confirmed with the aggregation test.

Mezanno et al. recommend the use of the platelet secretion assay as a first-line investigation for IPFD but highlight the lack of standardization as a major challenge to its implementation.[18] In scenarios where resources are limited, the findings here call into question the role of platelet secretion tests. When aggregation is consistent, secretion can add cost with marginal benefits to diagnostic capacity. It therefore seems reasonable to focus on standardizing the platelet aggregation test and controlling pre-analytical factors. In the hospital of this study, access to genetic mapping tools and electron microscopy is lacking; the immature platelet fraction (IPF) is still being implemented and was not used for this study.

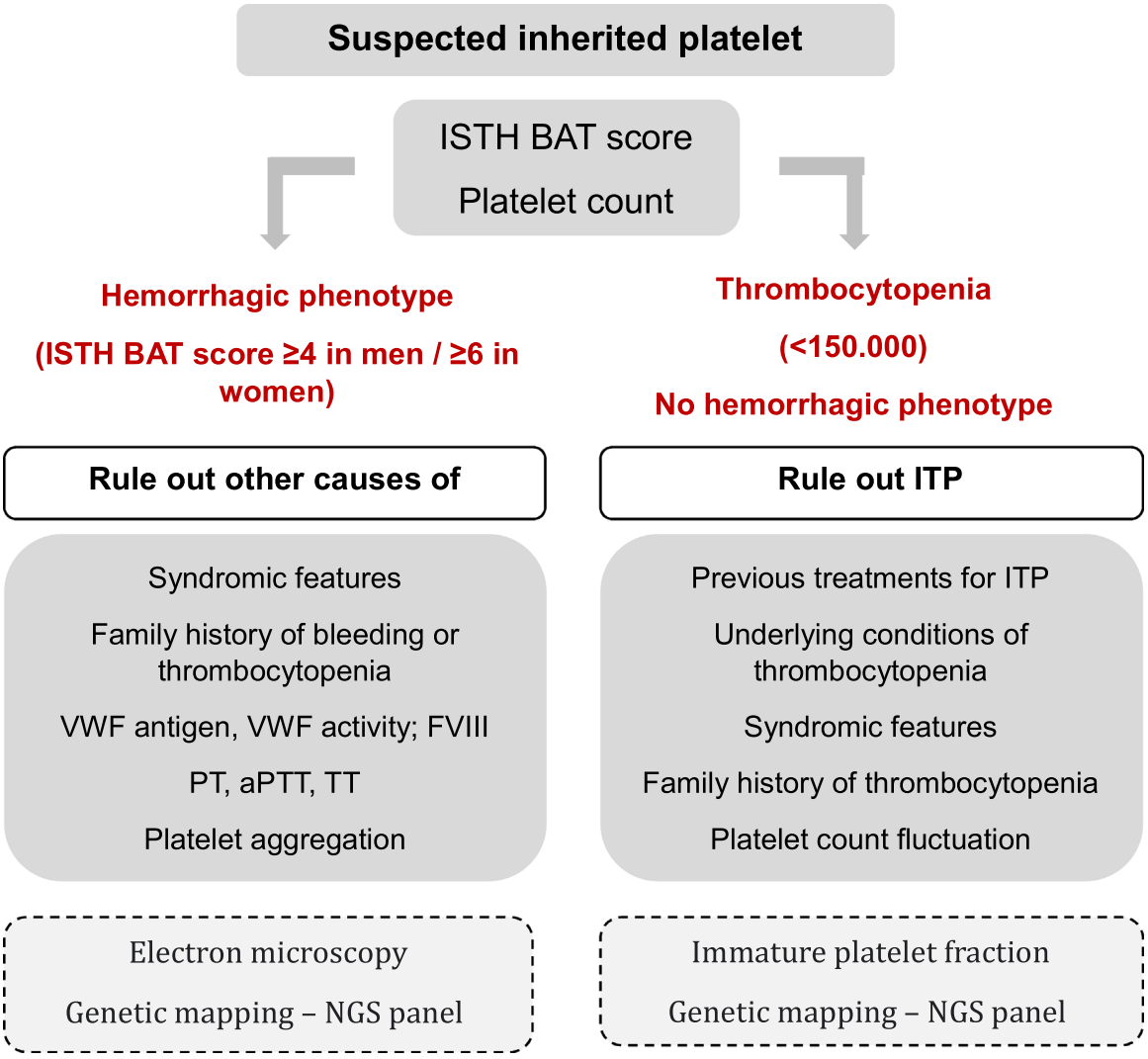

Figure 2 – Institutional work-up for the diagnosis of inherited platelet disorders

ISTH BAT: International Society on Thrombosis and Haemostasis bleeding assessment tool; ITP: immune thrombocytopenia; MPV: mean platelet volume; NGS panel: next-generation sequencing panel; VWF: von Willebrand factor; FVIII: factor VIII; PT: prothrombin time; aPTT: partial thromboplastin time; TT: thrombin time. Other possible tests that were not available in the institution at the time of this study have been highlighted in the dashed boxes.

Some limitations of this study should be acknowledged. First, it was not possible to obtain data on platelet morphology from this cohort due to the lack of standardized reporting at the institution. Second, there were challenges in interpreting platelet aggregation results, as the assays exhibited a 30% discordance rate, which compromised the diagnostic assessment. This variability was attributed to pre-analytical factors, which are frequently reported as sources of error in these assays. Third, most of the individuals identified in the first screening for patient selection had no further clinical data available due to loss of follow-up. In addition, many patients were excluded because of different diagnoses. This occurred because the approach to patient selection was highly inclusive, resulting in low specificity and giving rise to a large number of exclusions when the medical records were properly assessed. However, we believe that the loss of patients with IPD was minimal, as this is a rare, chronic, hereditary disease that generally requires continuous follow-up throughout life. Finally, we recognize that it is a challenge to diagnose IPDs without genetic evaluation, as two-thirds of our cohort still lack a confirmed diagnosis; however, this reflects the reality of most clinical services, particularly in low- and middle-income countries.

Given the available resources, the most important points used in the investigation of IPDs are highlighted and summarized in Figure 2, as an example of a diagnostic flow chart for these conditions.

Regarding management, the IPFD Group undergoes more therapeutic interventions, possibly due to a higher bleeding tendency,[19] similar to what was observed in this cohort. Platelet transfusions and the use of antifibrinolytics were frequent in this Group. On the other hand, IPNDs were treated less

frequently, with transfusion triggers depending on the platelet count.

Conclusion

Patients with IPDs may have a higher risk of bleeding, and the vast majority will be exposed to some hemostatic challenge during their lives. Identifying the correct condition makes it possible to provide appropriate treatment and follow-up. In scenarios with limited resources, confirmation of the diagnosis may not always be possible. Nevertheless, the documentation of critical elements of personal and family history, the assessment of bleeding risk, and the use of quantitative and qualitative platelet tests facilitate the identification of patients.

The present cohort serves as an example of the management of IPDs within a public healthcare system, devoid of genetic mapping exams. Moreover, the description of this cohort sheds light on the unique characteristics of these disorders within a Latin-American population.

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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Ethical approval

The study was reviewed and approved by the CEP HCFMUSP – Campus São Paulo, Brazil. Exemption from the consent form was granted due to the impossibility of contact with participants and the absence of potential harm.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Assessing the genetic profile of cytochrome P450 and glutathione S-transferases of patients diagnosed with acute myeloid leukemia

Gilmar de Andrade França [a,b], *Luciana Nardinelli* [a,b], *Ricardo Rodrigues Giorgi* [b], *Thiago Pagliarini* [b], *Otávio César Carvalho Guimarães Baiocchi* [d], *Elvira Deolinda Rodrigues Pereira Velloso* [a,b,c], *Wellington Fernandes da Silva Jr* [a,b,c], *Eduardo Magalhães Rego* [a,b,c], *Israel Bendit* [a,b,*]

[a] *Department of Hematology, Transfusion and Cell Therapy, University of Sao Paulo Medical School (HCFMUSP), Sao Paulo, SP, Brazil*

[b] *Laboratory of Medical Investigation in Pathogenesis and targeted therapy in Onco-Immuno-Hematology (LIM/31), Department of Hematology, Hospital das Clínicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, SP, Brazil*

[c] *Department of Hematology, Cancer Institute of Sao Paulo, University of Sao Paulo Medical School (ICESP), Sao Paulo, SP, Brazil*

[d] *Hospital Alemão Oswaldo Cruz, Sao Paulo, SP, Brazil*

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ABSTRACT

Objective: This study aimed to determine the frequency of genetic alterations as deletions and duplications in cytochrome P450 (*CYP450*) and glutathione S-transferases (*GST*) genes, as well as to investigate whether there is a relationship between these alterations and neutrophilic hematologic recovery in adult patients diagnosed with acute myeloid leukemia.

Method: DNA samples from 70 patients diagnosed with acute myeloid leukemia were evaluated using the Multiplex Ligation-dependent Probe Amplification technique. The presence or absence of polymorphisms was compared regarding the time to neutrophilic recovery (neutrophil count $\geq 1.0 \times 10^9$/L) using Kaplan-Meier curves, with the comparison between the curves being performed using the non-parametric log-rank test.

Results: The median age of the participants was 57 years, with a higher proportion of females (57.2%) and white individuals (61.4%)'. A total of 76 polymorphisms (*CYP450* + *GST*) were identified, comprising 38 deletions and 38 duplications. Kaplan-Meier curves revealed that the neutrophilic recovery time was longer for the group with polymorphisms (p-value = 0.0056).

* Corresponding author. Laboratory of Medical Investigation in Pathogenesis and targeted therapy in Onco-Immuno-Hematology (LIM/31), Department of Hematology, Hospital das Clínicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Avenida Dr Eneas de Carvalho Aguiar 155, primeiro andar, sala 30, São Paulo, São Paulo, Brazil CEP 05403-000.

E-mail address: isbendit@usp.br (I. Bendit).

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Conclusion: The study demonstrated that *CYP450* and *GST* genes are polymorphic, and these polymorphisms may lead to longer neutrophilic recovery after induction treatment of acute myeloid leukemia remission.

Introduction

The National Human Genome Research Institute (NHGRI) of the National Institutes of Health (NIH) of the United States of America defines pharmacogenomics as the area of medicine whose primary objective is to evaluate the relationship between the genetic profile and the variability in responses to drug therapy, and toxicities.[1,2] According to information from the National Cancer Institute (INCA), 11,540 new cases of leukemia are expected in the country in 2024 (www.gov.br/inca/pt-br/assuntos/cancer/tipos/leucemia).[3,4]

Among all leukemias, acute myeloid leukemia (AML) is the most frequent in the adult population, characterized by the disordered growth of immature blood cells called myeloblasts [5,6]. Various obstacles, like the need for early diagnosis, the biochemical characteristics of the disease itself, the emergency profile of starting treatment, and the difficulty of accessing new non-cytotoxic chemotherapy treatments, may influence therapeutic success.[7,8] However, among these challenges, the genotypic variability of patients in respect to the metabolism of drugs used to treat AML stands out. This variability can contribute to increases in toxicity of medications and influence the clinical outcome.[9,10]

With advancing precision medicine and personalized medicine, more and more studies in pharmacogenomics are being conducted.[11] These studies aim to unveil the genetic variability underlying the pharmacodynamics and pharmacokinetic response, which brings greater benefits and less harm to the patient.[12] Regarding harm, both adverse drug reactions (ADR) and drug resistance mechanisms can be cited, which can lead to failures and relapses during treatment.[13,14]

Cytochrome P450 (CYP450) is a superfamily of enzymes related to various reactions in the body, from the biosynthesis of steroids and fatty acids to the biotransformation of exogenous substances.[15]

In the human genome, 57 *CYP450* genes that encode functional proteins have been found, and grouped according to their homology into 18 families and 44 subfamilies. A significant number of copy number variations have been noted in these genes, which may influence the biotransformation of drugs.[16,17]

The main genes belonging to the large cytochrome P450 family are: *CYP1A1*, *CYP1A2*, *CYP2A6*, *CYP3A4*, *CYP3A5*, *CYP1B1*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP2D6*, and *CYP2E1*.[18]

The genes that encode the glutathione S-transferases (GST) enzymes present considerable polymorphisms depending on ethnic variations and can suffer both homozygous gene deletions, leading to loss of function, and heterozygous deletions or duplications, leading to loss or gain of function. Therefore, mutations can guide changes in the biotransformation of drugs.[19–21] The main genes belonging to this large family are *GSTM1* (μ), *GSTP1* (π), and *GSTT1* (θ).[22]

This study aims to determine the frequency of deletions and duplications in *CYP450* and *GST* genes in AML patients and their correlation with the neutrophilic recovery time (NRT) after remission induction treatment.

Material and methods

The present study evaluated the genetic profile of the CYP450 and GST genes. Eighty-three DNA samples were included in the study, but thirteen were excluded because they were inadequate for multiplex ligation-dependent probe amplification (MLPA) analysis. Genomic DNA was extracted from leukocytes using the QiaAmp DNA Blood Mini kit (Qiagen-Germany) following the manufacturer's instructions.

The quality of the DNA was monitored by a NanoDrop Spectrophotometer (Thermo Fisher Scientific, USA), and the working concentration of the DNA was adjusted to 10∼50 ng/μL.

This study met all established ethical principles. All patients participating in the study signed informed consent forms. DNA samples were used where the patient died, as approved by the Research Ethics Committee (CAAE 48,471,221.3.0000.0068).

Multiplex ligation dependent probe amplification (MLPA)

MLPA analysis was performed using the SALSA Probemix P128 CYP450 assay kit (MRC Holland, Netherlands) containing 52 MLPA probes with amplification products between 128 and 504 nucleotides. At least two probes are present for each of the target genes. Twelve-reference probes are included to detect autosomal chromosomal locations. The probes detect deletions or duplications in the *GSTM1*, *CYP1B1*, *CYP3A4*, *CYP3A5*, *CYP2C19*, *CYP2C9*, *CYP2E1*, *GSTP1*, *CYP1A2*, *CYP1A1*, *CYP2A6*, *CYP2B6*, *GSTT1* and *CYP2D6* genes.

Briefly, 250 ng of genomic DNA was hybridized to the probes following the manufacturer's protocol. Amplification was performed using the universal primers provided in the kit in a Veriti® Thermal Cycler (Applied Biosystems, USA). The cycling conditions were 30 s at 95 °C, 30 s at 60 °C, and 60 s at 72 °C for 35 cycles. For each run, three DNA samples were added from healthy individuals who did not present polymorphisms in the genes of interest according to previous analysis. Therefore, these samples were used as a control (reference) for comparative analyses. The polymerase chain reaction products were separated by capillary electrophoresis in the ABI 3130 automatic sequencer (Applied Biosystems, Foster City, USA). The peaks were analyzed using GeneMapper Software v4.0 (Applied

Biosystems, USA), and data were analyzed with the Coffalizer Software (MRC Holland, Netherlands). This software provides quality indexes for each reaction, and normalizes the MLPA data by comparing each sample with a set of control samples. Patients with polymorphisms were considered when the final proportion (FP) between reference probes and sample were FP = 0 (homozygous deletion), 0.40 < FP < 0.65 (heterozygous deletion), 1.30 < FP < 1.65 (duplication in heterozygosity), and 1.75 < FP < 2.15 (duplication in homozygosity). Samples that had 0.80 < FP < 1.20 were normal.

Analysis of neutrophil toxicity

Neutrophil toxicity was analyzed in 46 patients (65.7%). Minor neutrophil toxicity was characterized by a recovery time of up to 21 days to achieve $\geq 1.0 \times 10^9$/L neutrophils, while major toxicity takes longer than 21 days to recover. The recovery time of 21 days was chosen because it allows for the start of a new cycle of chemotherapy in most protocols for the treatment of AML. It is important to closely monitor neutrophil toxicity and take appropriate action to prevent major toxicity, which can significantly impact treatment outcomes.

Statistical analysis

The DNA samples extracted from patients with AML were done for convenience. Preliminarily, a descriptive statistical analysis of the genomic profile of deletions and duplications of the *CYP450* and *GST* genes was performed in patients diagnosed with AML. These findings are significant as they provide crucial insights into the genetic variations associated with AML. Categorical variables were analyzed for absolute and relative frequencies, while quantitative variables were assessed for median, interquartile range, and minimum and maximum values. Kaplan-Meier curves were constructed to compare the NRT of patients undergoing remission induction treatment and overall survival (OS). The differences in survival were estimated using the Kaplan-Meier method, and the differences between them and the cumulative rates of NRT were calculated using log-rank tests. The curves were compared using the non-parametric log-rank test, with a significance level of $\alpha = 0.05$.

Additionally, Fisher's exact test assessed the correlation between TNR and AML risk classifications based on cytogenetic and molecular biology. It determined if patients with unfavorable risk had a distinct NRT compared to those with favorable risk ($\alpha = 0.05$). The statistical analyses described above were performed using the statistical software R (version 4.3.0; https://www.r-project.org/) and RStudio (version 2022.12.0; https://www.rstudio.com/).

Results

Patient characteristics and polymorphisms presented

Tables 1 and 2 present the demographic data of the patients who participated in the study and the percentage of patients who presented or did not present polymorphisms according to self-declared ethnicity.

Table 1 – Demographic characteristics of 70 patients.

Characteristic		
Age (years)		57 (19–81)
		n (%)
Sex	Female	40 (57.2%)
	Male	30 (42.8%)
Ethnicity	White	43 (61.4%)
	Brown	19 (27.1%)
	Black	5 (7.1%)
	Not declare	3 (4.3%)

Table 2 – Polymorphism according to self-declared ethnicity.

	Polymorphisms	
Ethnicity	Present (n = 54)	Absent (n = 16)
White	32 (59.3%)	11 (68.8%)
Brown	18 (33.3%)	1 (6.3%)
Black	2 (3.7%)	3 (18.6%)
Not Declared	2 (3.7%)	1 (6.3%)

Table 3 – Type of genetic aberration according to the affected gene.

	Polymorphisms (n = 76)		
	CYP450	***GST***	**Total**
Deletion	14	24	38
Duplication	20	18	38
Total	34	42	76

Table 3 shows the frequency of polymorphisms observed in patients according to the type (deletion and duplication) and the affected gene family (*CYP450* and *GST*). A striking observation is that 76 occurrences of polymorphisms were verified in the 54 patients, with some patients presenting polymorphisms in more than one family of genes. Consistent duplications in *CYP450* and *GST*, deletions in *CYP450* and *GST*, deletions in *CYP450* and duplications in *GST*, and duplications in *CYP450* and deletions in *GST* were observed (Supplemental material Figures 1, 2 & 3). The following genes were analyzed: *GSTP1*, *CYP1A1*, *CYP1A2*, *CYP2B6*, *CYP2C9*, and *CYP2C19*. However, none of the patients had any polymorphisms in these genes.

Neutrophil recovery time

In Table 4, the urgency of this research is underlined as toxicity levels are presented according to the NRT concerning the presence or absence of polymorphisms. It is alarming to note that patients who presented polymorphisms had a median TNR higher than patients who did not.

The robust Fisher's exact test we applied to evaluate the NRT between groups with and without polymorphisms obtaining a p-value = 0.0287. This statistical significance

Table 4 – Neutrophil recovery time (NRT) according to the presence or absence of polymorphisms.

NRT	Polymorphisms (n = 46) Presence	Absence
NRT ≤21 days	0	6
NRT >21 days	20	20

further strengthens the research findings, instilling confidence in their validity.

Figure 1 shows the cumulative rate of NRT. The median NRT for patients with polymorphisms of *CYP450* and *GST* was 21 days, while the median NRT for the group without polymorphisms was 12 days (p-value = 0.0056).

Overall, the 9-year OS rate was assessed for 70 patients with a median follow-up time of 7.3 months. The estimated probabilities of OS were calculated and compared between patients with polymorphisms and those without. However, these two groups had no significant difference in the OS as shown in Figure 2.

Discussion

AML is a severe condition for which the standard treatment is cytotoxic chemotherapy. However, genetic factors may affect the metabolism of these drugs, leading to prolonged hematological toxicity. Immediate treatment by healthcare professionals is necessary to avoid life-threatening complications.[23,24]

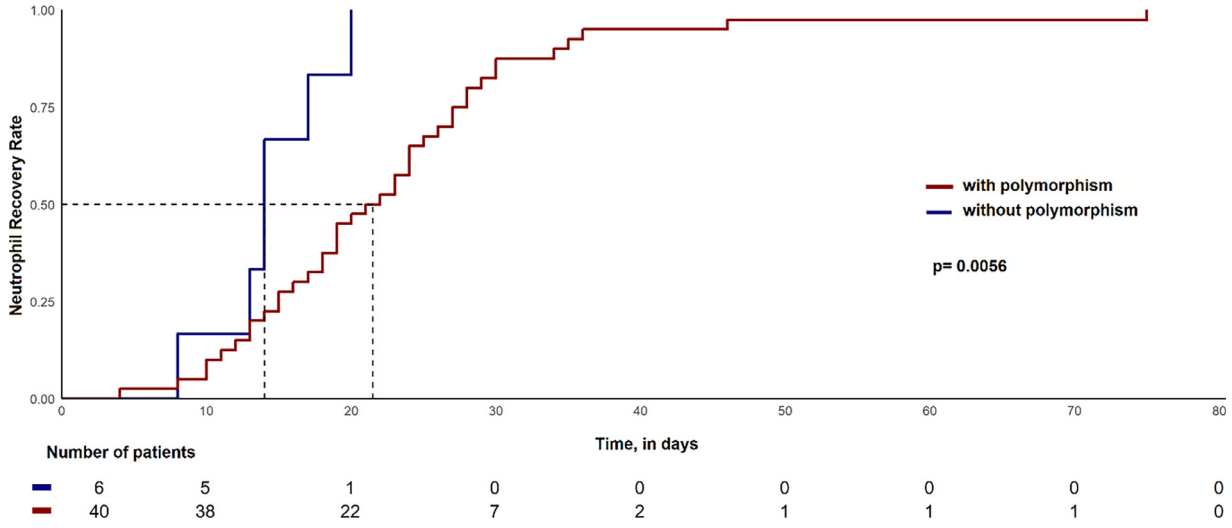

Figure 1 – Estimated incidence of neutrophil recovery time between the group presented or not polymorphisms in *CYP450* and *GST*.

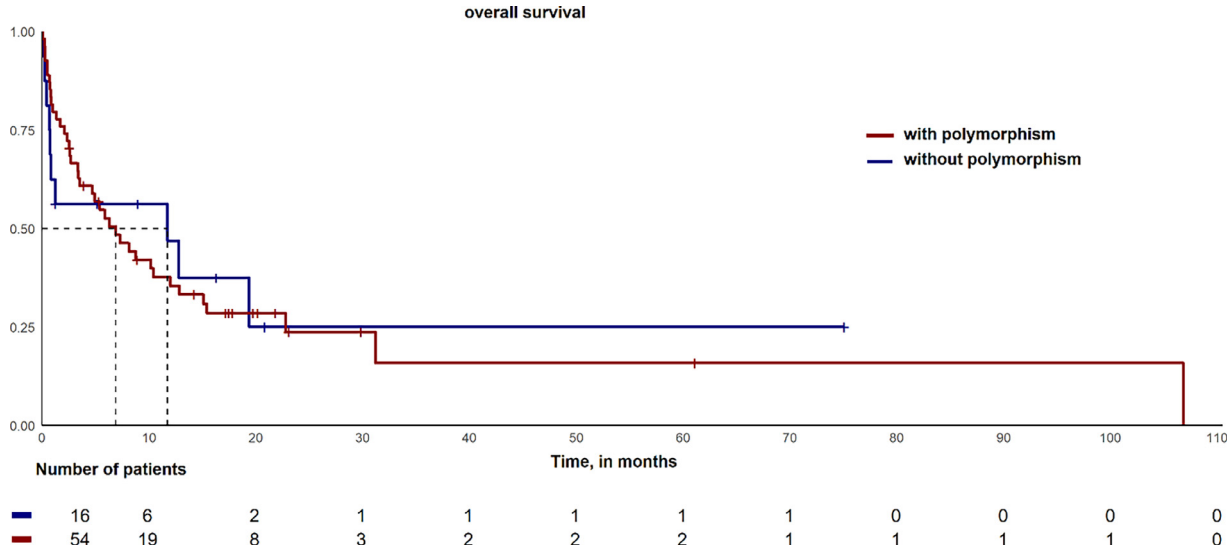

Figure 2 – Comparison of probabilities of Overall Survival between the groups with and without polymorphisms.

Previous studies have revealed that 43% of AML patients have a high mortality rate due to disease complications before they receive treatment. Studies have indicated that AML is more prevalent among white individuals and females The median age of patients is around 57 years.[25] It is essential to note that the ethnicities reported in the present study were self-reported by patients and gathered from medical records. This may lead to some information bias in the results. The prevalence in the present study of genetic abnormalities in the *CYP450* and *GST* genes between different ethnicities, namely white, brown, and black, yielded similar results to those published in the literature.[26,27] However, the white population had higher rates of *CYP450* and *GST* gene abnormalities (59.3%). Multiple genetic changes were found in the same gene family, an aspect that has also been reported previously in Brazilian population studies.[28] The *CYP2D6* gene had the highest percentage of polymorphisms among the genetic alterations in the *CYP450* family (47%), and the prevalence of deletion-type polymorphisms in *GST* genes was evident, which aligns with the findings of previous studies.[28]

Assessing the neutrophil count is crucial to evaluate the potential hematological toxicity following chemotherapy. A low count of neutrophils ($\leq 1.0 \times 10^9$/L) could indicate this toxicity. The time neutrophilic cells take to recover after chemotherapy can vary depending on the type of chemotherapy regimen. Consequently, this study investigated the effect of polymorphisms in CYP450 and GST on the NRT, defined as 21 days. In this study, the NRT could be determined effectively in only 65.7% of the 70 patients. Among these, 87% (40/46) had polymorphisms of the CYP450 and GST genes. The remaining 13% did not present any polymorphisms. Interestingly, the NRT was ≤21 days for these six patients. On the other hand, for patients with polymorphisms, 50% had NRTss equal to or <21 days, while the other 50% had NRTs greater than 21 days (Fisher exact test: p-value = 0.028). Specific polymorphisms of *CYP450* and *GST* may be related to drug metabolism, confirming published information. The patients who lacked genetic alterations in the *CYP450* and *GST* genes achieved neutrophil recovery faster than those with polymorphisms (Figure 1: *p*-value = 0.0056).

The OS rate of adult patients diagnosed with AML can vary based on various factors like the socioeconomic and cultural profiles, genetic mutations, and access to treatment. Additionally, neutrophilic toxicity during treatment can affect OS since it leads to immunosuppression and makes the patient more susceptible to bacterial, fungal, and viral infections. In this study, we found no significant statistical difference when comparing the OS rates of patients with and without any polymorphisms, as shown in Figure 2.

During this study, we faced some limitations that need to be addressed. Firstly, the series was limited due to the significant number of patients who were diagnosed with AML but died before starting induction treatment. One of the preponderant factors was the delay in reaching a reference center for diagnosis. Secondly, the MLPA technique used requires a good quality and quantity of DNA, free from contamination or degradation. Lastly, it is essential to note that MLPA does not detect point mutations or analyze the entire coding region of genes.

Conclusion

There is a need for more pharmacogenomic studies in Brazil, especially in the area of oncology and oncohematology. These studies can help understand how polymorphisms in genes related to drug metabolism can lead to medication toxicity in patients with these pathologies. AML is an emergency in terms of diagnosis and treatment due to its high mortality rate. This can cause significant myelotoxicity if the patient has a polymorphism in one of the genes responsible for drug biotransformation. In conclusion, pharmacogenomics is a crucial factor in pharmacotherapy and should be considered.

Conflicts of interest

The authors declare no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2025.103759.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Original article

Effect of fibrin on the expression of adhesion molecules (ICAM-1, ITGAV, and ITGB3) in unrestricted somatic stem cells

Sanaz Khaseb [a], *Mahdi Kohansal Vajari* [a,b], *Mina Soufi Zomorrod* [a], *Maryam Rezai Rad* [c], *Monireh Ajami* [d], *Mansoureh Ajami* [e], *Saba Sadeghpour* [f], *Amir Atashi* [g,*]

[a] *Faculty of Medical Sciences, Tarbiat Modares University (TMU), Tehran, Iran*
[b] *School of Allied Medical Sciences, Kerman University of Medical Sciences, Kerman, Iran*
[c] *Research Institute for Dental Sciences, Dental Research Center, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[d] *Faculty of Paramedical Sciences, Tehran Medical Sciences Branch, Islamic Azad University, Tehran, Iran*
[e] *School of Allied Medical Sciences, Shahroud University of Medical Sciences, Shahroud, Iran*
[f] *School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
[g] *Stem Cell and Tissue Engineering Research Center, Shahroud University of Medical Sciences, Shahroud, Iran*

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ABSTRACT

Background: Hematopoietic stem cell expansion relies on direct cell-cell interactions mediated by adhesion molecules, integrins, and cytokines. Unrestricted somatic stem cells have emerged as novel stromal cells supporting hematopoietic stem cell expansion in co-culture conditions via secretion of hematopoiesis-related cytokines and the expression of adhesion molecules. Previous research showed fibrin increased hematopoiesis-related gene expression in these cells. This study focused on the adhesive characteristics of unrestricted somatic stem cells on 3D fibrin scaffolds.

Methods: Unrestricted somatic stem cells were isolated from umbilical cord blood and characterized using flow cytometry and multilineage differentiation assays. Scanning electron microscopy and DAPI staining were employed to analyze cell attachment to fibrin. Viability on fibrin was assessed through MTT assays. Quantitative polymerase chain reaction was conducted to evaluate the expression of intercellular adhesion molecule 1 (ICAM-1), integrin subunit αv (ITGAV), and integrin subunit β3 (ITGB3) in cells cultured on 3D fibrin scaffolds.

Results: Cells were positive for CD73, CD105, and CD166 but negative for CD45. Alizarin red and Oil red O stains confirmed calcium deposition and lipid vacuoles. MTT assays revealed that fibrin positively impacts viability. ITGAV expression was significantly increased in cells cultured on fibrin compared to those cultured on plastic tissue culture plates (Control Group). Furthermore, ITGB3 expression showed no significant change in both groups, while ICAM-1 expression was downregulated in cells cultured on fibrin.

* Corresponding author.
E-mail address: atashia@shmu.ac.ir (A. Atashi).
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Conclusions: Our study revealed that fibrin has a positive impact on the expression of ITGAV, which plays a crucial role in direct cell-cell interactions affecting hematopoietic stem cell expansion.

Introduction

According to the European Society for Blood and Marrow Transplantation, hematopoietic stem cell transplantation (HSCT) is a potentially curative therapy for several life-threatening diseases including, solid tumors, immune disorders and hematological malignancies with acute myeloid leukemia being the most frequent indication for allogeneic HSCT, followed by acute lymphoblastic leukemia in Europe.[1] Although an HLA-matched sibling is the preferred donor, only approximately 30% of patients who could benefit from HSCT have such a donor available. One of the available options for tackling this issue is the manipulation of umbilical cord blood (UCB) -hematopoietic stem cells (HSCs) due to less stringent requirements for HLA matching.[2] However, despite all the advantageous aspects of UCB, its primary drawback is the low yield of HSCs in comparison to bone marrow (BM) or peripheral blood-mobilized HSCs. Consequently, this leads to complications including delayed hematological recovery, higher graft failure rates, and risk of infection.[3]

Great efforts have been dedicated to overcoming this limitation by expanding the number of HSCs both *in vivo* and *in vitro*. One of the applied methods is co-culture protocols developed for the expansion of UCB-HSC.[4] Mesenchymal stem cells (MSC), one of the cord blood cells used in co-culture, functions as a support for HSCs.[5] Different studies demonstrate that MSCs in NOD/SCID mice induce engraftment of UCB-derived $CD34^+$ cells.[6,7] In addition to MSCs, another UCB-derived cell termed unrestricted somatic stem cell (USSC) can also promote the expansion of HSCs.[8] This rare CD45-negative population grows adherently and can be expanded to 10^{15} cells without losing pluripotency.[9] Hashemi et al. used MSCs and USSCs as feeder layers to increase the population of UCB-$CD34^+$ cells for bone marrow transplantation.[10] Another study also reported that USSCs significantly supported the proliferation of HSCs in the bone marrow of NOD/SCID mice and showed no sign of tumorigenicity.[11] The possible underlying reason for the positive influence of USSCs on HSC proliferation is the production of hematopoiesis-supporting cytokines. Compared to MSCs, these cells produce significantly more hematopoiesis-related cytokines such as stem cell factor (SCF) thus making them a better candidate for stroma-driven *in vitro* expansion of UCB-HSCs.[8]

It is worth noting that the interaction of the HSCs with their micro-environmental constituents is another contributing factor to their expansion. For instance, the interaction of stromal cell−derived factor 1 (SDF−1) with CXCR4, a G−protein−coupled receptor, considerably affects HSC proliferation, survival, and differentiation.[12] Furthermore, direct cell-cell interactions mediated via various types of adhesion molecules play a crucial role in the fate of HSCs by affecting different mechanisms involved in self-renewal, differentiation, migration, quiescence, and apoptosis. Some of the most important adhesion molecules involved in HSC homing are integrins, selectins, N-Cadherin, notch receptors, CD44, esam1, cytohesin1, serum response factor (Srf), intercellular adhesion molecule 1 (ICAM-1), erythropoietin-producing hepatocellular (Eph) and ephrins as well as the SDF-1α/CXCR4 axis.[13] Integrin-αvβ3 plays a fundamental role in the maintenance of HSCs through interaction with thrombopoietin, a crucial cytokine for the activation of dormant HSCs.[14] Of note, ICAM-1 is essential for maintaining HSC quiescence and repopulation capacity in the niche, and in studies ICAM-1 deletion led to failure in the retention of HSCs in the bone marrow and changed the expression profile of stroma cell-derived factors.[15]

Three-dimensional culture systems are growing rapidly worldwide due to their ability to mimic tissue-like structures more efficiently compared to monolayer cultures particularly in cancer and stem cell research.[16] A study conducted by Kumbhar et al. demonstrated that the inhabitability of UCB-MSCs was improved using 3D scaffold-based cultures through proper adhesion and proliferation.[17] Furthermore, enhancement of the development and regulation of cellular signaling in stem cells using 3D cell platforms has also been reported.[18] Multiple studies acknowledge that 3D microenvironments can promote cell viability and direct cell adhesion,[19] proliferation,[20] differentiation,[21] and migration[22] via the regulated presentation of mechanical and biochemical cues. Among the most widely used scaffolds, fibrin gel is superior in various aspects, such as high seeding efficiency and uniform cell distribution. Additionally, it can be harvested from the patient's own blood and used as an autologous scaffold excluding the potential risk of unintended reaction or infection.[23]

Previous research[24] highlighted the favorable impact of fibrin on the increased expression of hematopoiesis-related genes in USSCs. In alignment with these findings, the present study focused on the expression of several adhesion molecule genes - ICAM-1, integrin subunit αv (ITGAV), and integrin subunit β3 (ITGB3) - in USSCs cultured on a 3D fibrin scaffold. This emphasis arises from the crucial role of direct cell-cell interactions in HSC expansion. Together, these studies provide a new perspective for further investigations into whether USSCs as stroma cells can effectively support HSC expansion in co-culture conditions on a 3D fibrin scaffold.

Materials and methods

Isolation and expansion of unrestricted somatic stem cells from umbilical cord blood

The procedures for the collection of human UCB units were performed after the informed consent of the

mothers, in accordance with the Ethics Committee of the Tarbiat Modares University (IR.MODARES.REC.1399.026). Experiments were performed with eight cord blood units. USSCs were isolated and cultivated according to the standardized protocol published by Kogler et al.[9] The mononuclear cell fraction was first separated from UCB using a hydroxyethyl starch buffer (Santa Cruz Biotechnology, Santa Cruz, CA; sc-215159) followed by centrifugation (400 g for 25 min) on a Ficoll density gradient (Panbiotech, Germany; density 1.077 g/cm^3; P04-60225). As a result, the solution inside the tube was divided into four distinct parts, serum, a layer of mononuclear cells, Ficoll, and red blood cells (RBCs). The separated mononuclear cells were plated out at 5-7×10^6 cells/mL in T25 culture flasks with Dulbecco's modified Eagle's medium (DMEM) - low glucose (Gibco, 31600-083) supplemented with 30% fetal bovine serum (FBS) (Gibco; 10270106), 10^{-7} M dexamethasone (SigmaAldrich; D4902), 2 mM glutamine (Sigma; G8540), 100 U/mL streptomycin (Gibco; 122-15140), and 100 mg/mL penicillin (Gibco). The cells were incubated at 37°C with 5% CO_2 in a fully humidified atmosphere. The culture medium was changed to DMEM supplemented with 10% FBS without dexamethasone after the appearance of adherent USSC colonies. The cells were split when confluency reached 80% by detaching the cells with 0.25% trypsin and re-plating them in a ratio of 1:3 under the previously described medium conditions.

Monoclonal antibodies for the immunophenotyping of unrestricted somatic stem cells

The immunophenotype of the USSC cultures in the 3th passage (5 μL for 10^6 cells) was investigated using the Attune NxT Flow cytometer. The following monoclonal antibodies were used: CD73-FITC (Biolegend, 344015), CD105-PE (Biolegend, USA; 323205), CD166-PE (Biolegend, USA; 343903), and CD45-FITC (Biolegend, USA; 304006).

Differentiation of unrestricted somatic stem cells into adipocytes and osteoblasts

The differentiation protocol was based on the Kogler protocol.[9] In the first stage, USSCs at the 3th passage were planted into six-well plates at a density of 5 x l0^3 cells/well. For osteoblasts to be induced, after reaching 80% confluency, the culture medium was replaced with osteogenic induction medium supplemented with 10% FBS (Gibco), 10 mM β−glycerol phosphate (Sigma Aldrich; 50020), 10^{-7} M dexamethasone (D2915), and 50 μg/mL ascorbic acid biphosphate (Sigma Aldrich; A8960). After 21 days of osteogenic stimulation, USSCs were fixed in 4% paraformaldehyde and stained with Alizarin Red (Sigma Aldrich; A5533) as an indication of osteoblast-typical calcification and functional competency of the differentiated cells. For induction of adipogenic differentiation, the same method was applied with the difference that the medium consisted of DMEM, 10% FBS, 250 nM dexamethasone, 60 nM insulin, 0.5 mM isobutyl− methylxanthine, and 0.2 mM indomethacin (all from Sigma−Aldrich) in order to stimulate adipogenesis. Moreover, for the detection of lipid vacuoles, Oil Red O staining was used on the 21st day. The images were captured via an inverted microscope using a 200x magnification.

Fibrin preparation

Utilizing a 3D scaffold for HSC expansion can mimic the bone marrow microenvironment, providing sufficient surface area for cell adhesion, as well as increased porosity to allow cell migration and nutrient exchange.[25] In contrast, 2D expansion strategies significantly reduce HSC proliferation.[26] Fabrication of fibrin gel was performed according to the method described by Soleimannejad et al.[27] The fibrinogen solution was prepared by dissolving 1.5 mg of fibrinogen (Sigma Aldrich; F3879) in 0.5 mL DMEM and transferred to a 24-well culture dish. Next, 50 μL of FBS and 15 μL of a thrombin solution (120 U/mL in 1 M sodium buffer; Sigma, USA; 1.12374) were added to the fibrinogen solution (3 mg/mL). To allow for the formation of a 3D network structure, the plate was incubated at 37°C for 1 hour.

Assessment of cell attachment

Scanning electron microscopy

To investigate the fibrin scaffold microstructure and cell-seeded fibrin gels, scanning electron microscopy (SEM) was used. After 12 hours of incubation, the specimen preparation for SEM analysis was according to the following procedure: in the first stage, the seeded cells on fibrin were fixed in 3% glutaraldehyde for 45 minutes at room temperature and rinsed twice in sterile phosphate-buffered saline (PBS). In the next step, the sample was kept at 4°C in PBS overnight and then dehydrated through a graded ethanol series solution (25%, 50%, 70%, 80%, 90%, and 100%). In the end, the prepared sample was dried and examined by SEM (XL30, Philips, Holland) under 1000x magnification.

4′,6-diamidino-2-phenylindole (DAPI) staining

USSCs were fixed in 4% paraformaldehyde for 20 minutes at 4° C. After paraformaldehyde removal, cells were incubated with DAPI (Sigma) for 30 minutes at room temperature in the dark. After three washes using PBS, the DAPI-stained nuclei were observed using a fluorescence microscope (Nikon TE-2000) under 100x magnification.

Assessment of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

In Both the Control Group (without fibrin) and the Experimental Group (with fibrin), USSCs were seeded at a concentration of 1×10^5 cells per well in a 48-well plate and were incubated (37°C and 5% CO_2) for 1, 3, and 5 days. After the incubation period, 10 μL of the MTT labeling reagent (final concentration 0.5 mg/mL; 475989) was added to each well. Thereafter, the MTT solution was removed, and prepared DMSO was added to sufficiently dissolve the formazan crystals. In the final stage, the absorbance for each well was measured at 570 nm optical density using an enzyme-linked immunosorbent assay (ELISA) reader (BIOTEK, ELX800, Germany).

Table 1 – Primer sequences used for quantitative polymerase chain reaction.

Homo sapiens	Primer	Sequence
ICAM1	ICAM1/F	GAAGGTGTATGAACTGAGCAATG
	ICAM1/R	TGGCAGCGTAGGGTAAGG
ITGAV	ITGAV/F	TCCGAAACAATGAAGCCTTAG
	ITGAV/R	GCACACTGAAACGAAGACC
ITGB3	ITGB3/F	AACCGTTACTGCCGTGAC
	ITGB3/R	GGACACTCTGGCTCTTCTAC

Gene expression analysis of unrestricted somatic stem cells by quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed to evaluate the expression of the *ICAM, ITGAV*, and *ITGβ3* genes. USSCs in both Control and Experimental Groups were seeded at a density of 2×10^4 cells per well into 24-well plates for 48 hours. Samples were then harvested and total RNAs were extracted using Trizol (Invitrogen: 15596018) according to the manufacturer's instructions. Subsequently, complementary DNA (cDNA) was synthesized using the cDNA Synthesis Kit (SMOBIO: RP1000) and qPCR was performed in an ABI StepOne PCR system (Applied Biosystems) over 40 cycles via SYBR Green Master Mix high ROX (Ampliqon, A325402). The primer sequences used for the qPCR are listed in (Table 1). The PCR products were run on 2% agarose gel electrophoresis and stained with ethidium bromide. *HPRT* was used as the housekeeping gene. Of note, all reactions were conducted in triplicate. Finally, relative gene expressions were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data were analyzed using Graph Pad Prism® software version 9.0 (GraphPad Software, USA). All data are presented as means ± standard deviation (SD). For the MTT assay, data were analyzed using two-way repeated-measures ANOVA to evaluate the effect of fibrin on cell viability over the specified days. For qPCR, the normality of the data was assessed using the Shapiro-Wilk test, which confirmed that the data followed a Gaussian distribution. Statistical analysis was then performed using two-way repeated-measures ANOVA. Data are normalized to the *HPRT* gene.

Results

Characterization of unrestricted somatic stem cells

In this study, USSCs were identified as adherent, spindle-shaped cells as the arrows indicate in Figure 1a. This figure shows USSCs cultured on plastic tissue culture plates (Control Group) for three days, with confluency levels of approximately 30%. Figure 1b shows the same cells on Day 8 with confluency levels reaching around 80%. Isolated USSCs at the 3th passage were analyzed using flow cytometry to assess cell surface markers. The results show a cell surface expression profile of CD73 (97.9%), CD105 (97.2%), and CD166 (93.8%), with negativity (0.2%) for CD45 markers (Figure 2). The percentages provided came directly from the acquisition for total cells. USSCs have the potential to differentiate into both osteogenic and adipogenic lineages as confirmed by positive staining. Specifically Figure 3a demonstrates differentiation toward osteogenesis, as indicated by Alizarin Red staining for calcium deposits, while Figure 3b shows differentiation toward adipogenesis, highlighted by Oil Red O staining for lipid vacuole, which are indicated by arrows in Figure 3.

Evaluation of cell attachment

As shown in Figure 4a, the images obtained by SEM analysis clearly show fibrin fibers with USSCs properly attached. In addition, Figure 4b demonstrates cells without fibrin networks. Fluorescence tracked DAPI-labeled USSCs

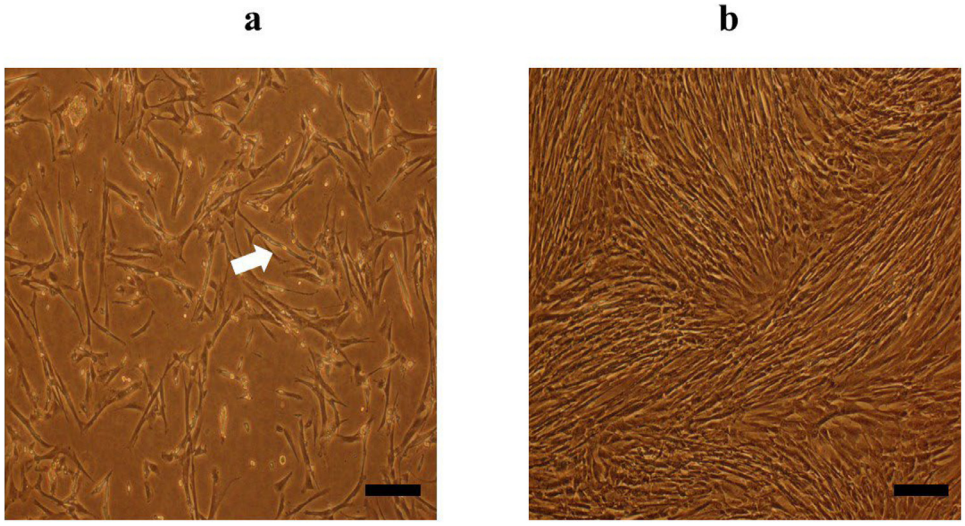

Figure 1 – Morphological characteristics of unrestricted somatic stem cells (USSCs). (a) Spindle-shaped morphology of USSCs under an optical microscope on Day 3 with approximately 30% confluency. (b) USSCs at 80% confluency on Day 8. Both images (a) and (b) are from the Control Group (magnification 100x, scale bar = 50 μm).

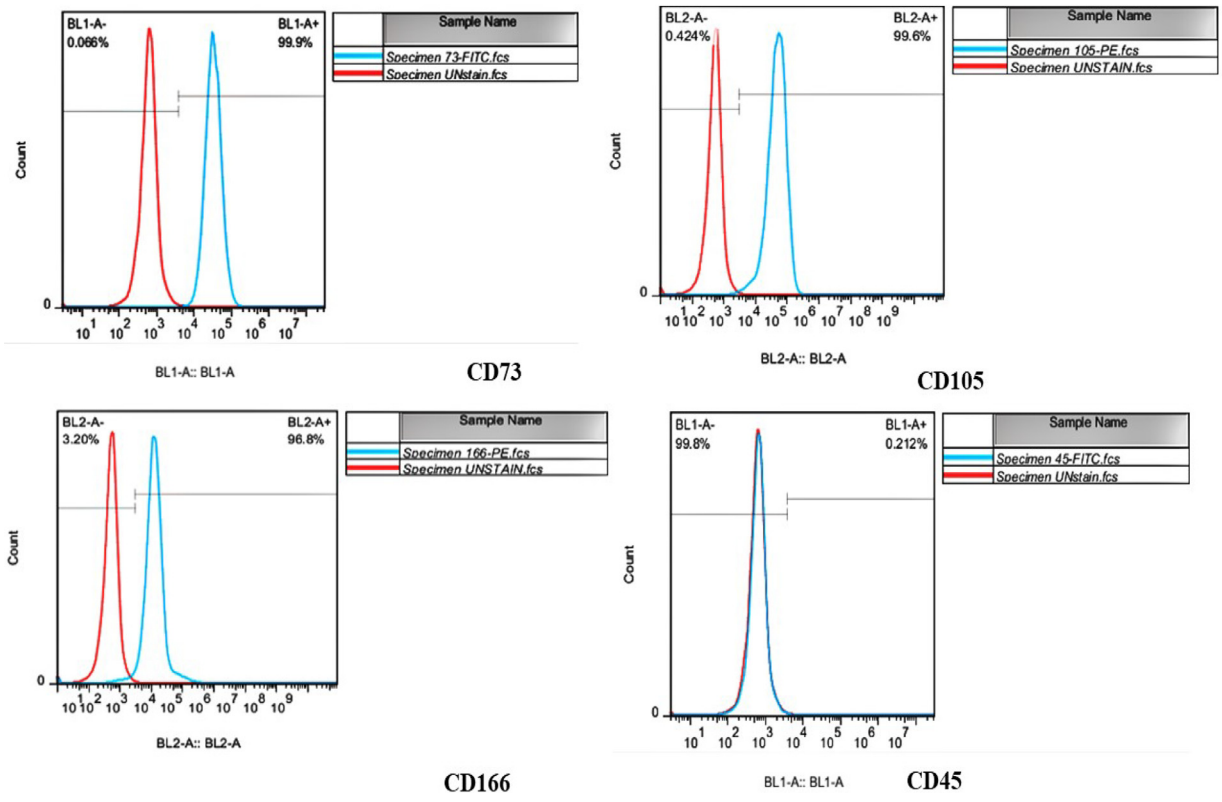

Figure 2 – Immunophenotypes of unrestricted somatic stem cells (USSCs). USSCs were positive for CD73, CD105, and CD166 but negative for CD45.

cultured on the fibrin scaffold are shown in Figure 4c. The blue color corresponds to viable nuclei, with more blue spots indicating a higher number of viable cells attached to fibrin. The light microscopy image in Figure 4d also shows USSCs attached to fibrin, representing the Study Group. Altogether, these three sets of data confirm the adhesion of cells to fibrin.

Evaluation of USSCs viability cultured on fibrin

Based on the results obtained from MTT assay, the proliferation of USSCs cultured on fibrin (Figure 5b) significantly increased compared to those cultured on plastic tissue culture plates (Control) on Day 1 (p-value = 0.0051), Day 3 (p-value = 0.0068), and Day 5 (p-value <0.0001).

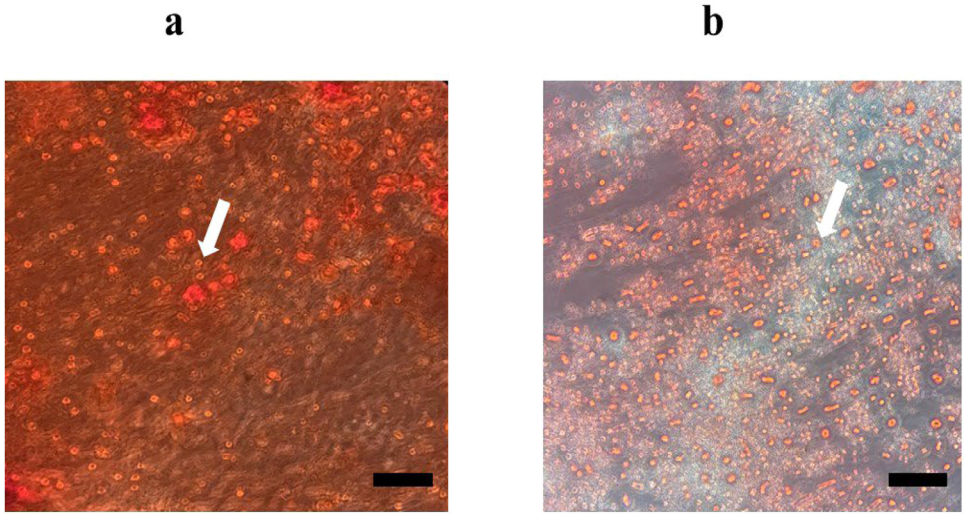

Figure 3 – Multilineage differentiation potential of unrestricted somatic stem cells (USSCs). (a) **After osteogenic induction, mineralized calcium nodules were detected using Alizarin Red staining (magnification 200x)** (b) **After adipogenic induction, lipid droplets were visualized by Oil Red O staining (magnification 200x).**

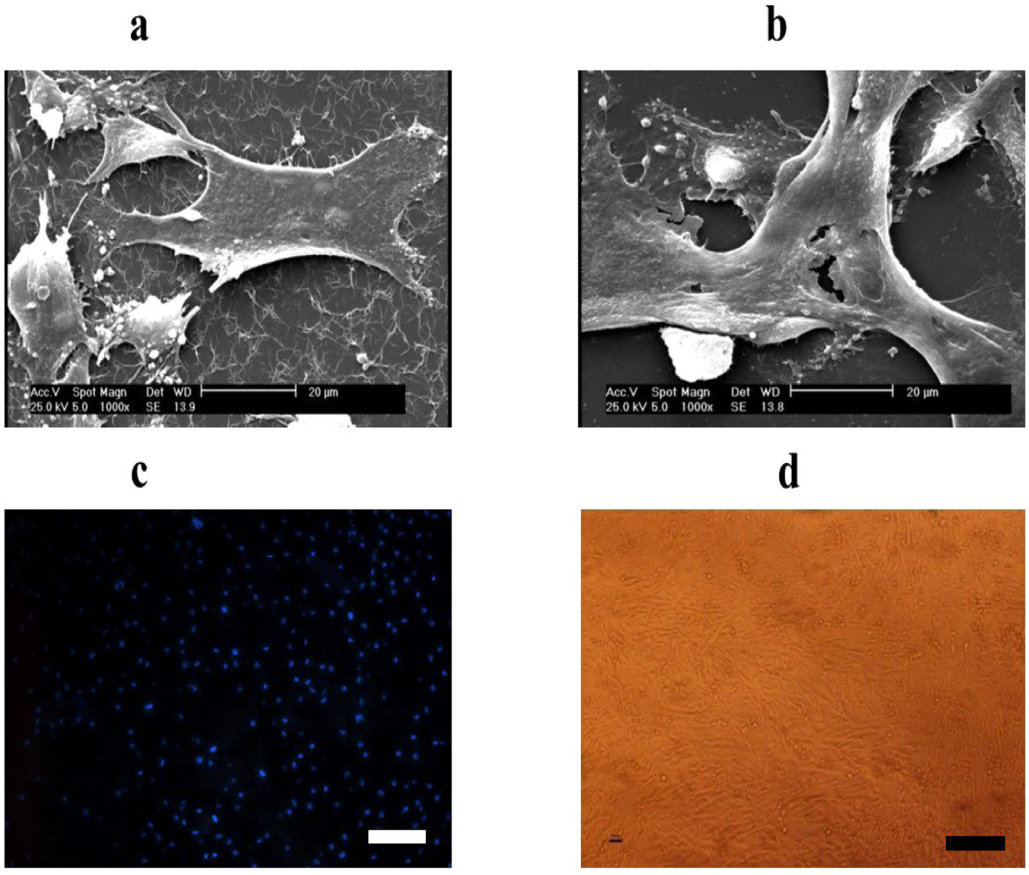

Figure 4 – Cell attachment on fibrin. (a) Scanning electron microscopy (SEM) image of unrestricted somatic stem cells (USSCs) with fibrin. (b) SEM image of USSCs without fibrin. (c) Viable nuclei (blue) were shown using DAPI staining under a fluorescence microscopy. (d) Light microscopy image of USSCs attached to fibrin (scale bar = 50 μm).

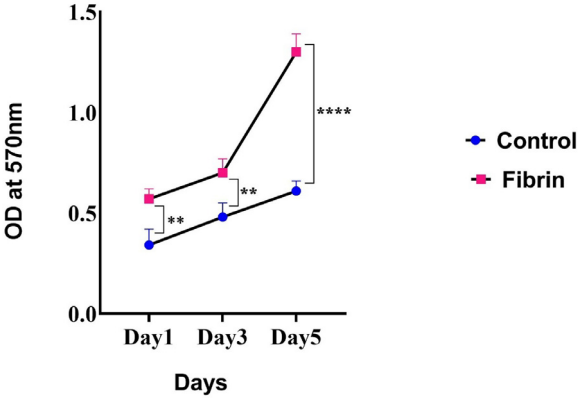

Figure 5 – Effect of fibrin on unrestricted somatic stem cell (USSC) viability. The MTT assay result showed that fibrin has a positive effect on the proliferation of USSCs cultured on fibrin scaffolds at Day 1, 3, and 5 of continuous culture compared to those cultured on plastic tissue culture plates (Control Group). **p-value = 0.0051, **p-value = 0.0068 and ****p-value < 0.0001 compared to control group.

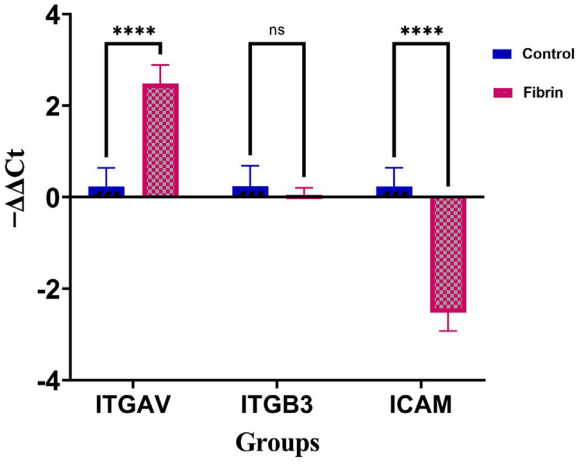

Figure 6 – Effect of fibrin on expression levels of *ICAM*, *ITGAV*, and *ITGB3* in unrestricted somatic stem cells (USSCs). Quantitative polymerase chain reaction analysis revealed a significant increase in the mRNA expression levels of ITGAV in the fibrin group. Notably, fibrin did not exert an effect on the mRNA expression of ITGB3 in USSCs. The expression level of ICAM was significantly decreased compared to those cultured on plastic tissue culture plate. Data are presented as means ± standard deviation (SD). NS, not significant; ****p-value < 0.0001 compared to Control Group (n = 3).

Expression of genes related to adhesion in unrestricted somatic stem cells cultured with fibrin

As shown in Figure 6, the expression of ITGAV was significantly higher in USSC cultured on fibrin compared to that cultured on plastic tissue culture plates (Control Group; p-value <0.0001). There was little difference in the expression of ITGB3 between the two groups (p-value = 0.2278). In contrast, the expression of ICAM-1 was downregulated in USSCs cultured on fibrin compared to those cultured on plastic tissue culture plates (p-value <0.0001). Information regarding the mean, SD and p-values for both groups is shown in Table S1.

Discussion

UCB-HSCs have many beneficial aspects, including noninvasive collection, greater capacity of expansion, and remarkable tolerance in respect to HLA matching in transplantation thus making them a potential therapeutic candidate for hematological disorders. Nevertheless, their insufficient amount may lead to complications such as delayed engraftment.[12] Therefore, finding a viable solution for the expansion of HSCs seems to be a promising area for future development. USSCs are potential candidates for stroma-driven *in vitro* expansion of $CD34^+$ cells from UCB to improve reconstitution and engraftment since they produce considerable amounts of functional hematopoiesis-supporting cytokines and are superior to MSCs in supporting the expansion of the UCB-HSCs.[8] Moreover, in a recent study by Chan et al., USSCs promoted a significant enhancement of $CD34^+$ cell homing to the bone marrow and spleen.[28] According to USSCs pluripotency and their expansion capacity into large quantities, they may serve as a global allogeneic stem cell source for various therapeutic options including transplantation, cellular therapy for tissue repair, and tissue regeneration.[9] Therefore, *in vitro* expansion of USSCs may hold the key to tackling the issue of an inadequate number of HSCs for these treatments.

The HSC fate decision is controlled through direct cell-cell interactions - mediated via different types of adhesion molecules, - cell-ECM interactions - mediated mostly via integrins, - or through soluble mediators like cytokines.[13] In other words, HSC adhesion to the substrate is assumed to be part of the natural process taking place in the HSC niche that regulates cell proliferation and differentiation.[29] There is also evidence that cell adhesion is a known indicator of cell expansion.[30,31] Integrins are one of the most important classes of the various types of adhesion molecules involved in the interaction of HSCs with their microenvironment.[13] A study conducted in 2003, revealed that MSCs express various integrins, including ITGAV, and ITGB3 as well as ICAM-1, suggesting a possible *in vivo* role for these cells in both hematopoietic and immune function.[32] Due to the ability of crosstalk between integrins and growth factor receptors through characteristic bidirectional signaling mechanisms, they can support cell proliferation and migration.[33] Specifically, $\alpha v\beta 1$ (VLA-5) along with $\alpha 4\beta 1$ (VLA-4) and $\alpha L\beta 2$ (LFA-1) play a crucial role in HSC adhesion to endothelial cells and their subsequent trans-endothelial migration toward the SDF-1α (CXCL12)-expressing stromal cells.[34] Wierenga et al.[35] reported a significant reduction in HSCs homing to bone marrow following the blocking of their $\alpha v\beta 1$ integrins before transplantation.

In a study conducted on different types of 3D biomaterial scaffolds, fibrin achieved the highest overall growth rate of $CD34^+$ HSCs, highest numbers of engraftment and multilineage differentiation, hence making it the most suitable option for *in vitro* expansion of UC-HSCs.[36] Furthermore, another study revealed that seeding cytotoxic stem cells in fibrin scaffolds considerably elevated the initial retention and significantly prolonged the persistence and efficacy of the cells in the post-surgical brain cancer glioblastoma resection cavity.[37] Moreover, fibrin also contributes to an increase in the expression of cytokines related to HSC proliferation, survival, and differentiation such as SCF and TPO.[24]

As expected, in this study the ITGAV expression was notably increased in USSCs cultured on fibrin compared to those cultured on plastic tissue culture plates which illustrates the positive impact of the fibrin scaffold on ITGAV levels. This result is in line with the findings of a previous study which highlighted the positive potential of fibrin on higher expressions of hematopoiesis genes such as SCF and TPO in USSCs cultured on fibrin.[24] Previous studies show that integrin $\alpha IIb\beta 3$ (CD41/CD61) probably plays a part in cell adhesion and cell surface-mediated signaling.[38] Nonetheless, in contrast to its counterpart, USSCs cultured on fibrin displayed no significant changes in the *ITGBβ3* gene expression in this study. Surprisingly, there was a decreasing trend observed in *ICAM1* expression. Referring to previously published data,[24] USSCs seeded on fibrin showed decreased levels of *Interleukin 6* (*IL-6*), which might be the underlying reason for the diminished *ICAM1* expression. Importantly, this reduction in mRNA level of *IL-6* was not attributable to the effect of fibrin and depended on atmospheric conditions (21% oxygen). Adjusting atmospheric conditions to 5% oxygen might result in the observation of positive effects on *IL-6* expression. Considering multiple studies indicating that *ICAM1* expression can be induced by *IL-6*, which can even promote its gene expression in endothelial cells and human osteosarcoma cells,[39,40] the lower amounts of *IL-6* in USSCs may contribute to the reduction in *ICAM1*. It can be concluded that improving the expression status of both genes is achievable through alterations in oxygen conditions.

Conclusions

This is the first time that the adhesive characteristics of USSCs on 3D fibrin scaffolds was studied. Taking together the findings of our previous and current study, in can be concluded that the fibrin scaffold demonstrates the potential to enhance the expression of various molecules such as SCF, TPO and ITGAV in USSCs. Therefore, in future studies, the co-culture of USSCs with HSCs on fibrin scaffolds looks promising due to the promoting effect of fibrin on the expressions of the aforementioned factors by USSCs. It is worth noting that according to previously mentioned studies, these adhesive and hematopoiesis factors play a fundamental role in HSC expansion and regulation in the bone marrow niche.

Conflicts of interest

None.

Contribution statement

SK: Data curation, Investigation, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **MKJ:** Data curation, Formal analysis, Methodology, Writing – original draft. **MSZ:** Investigation, Methodology, Writing – review & editing. **MRR:** Supervision, Methodology. **MoA, MaA:** Formal analysis, Validation, Writing – review & editing. **SS:** Data curation, Methodology. **AA:** Conceptualization, Investigation, Methodology, Project administration, Validation; Writing - review & editing.

Data availability statement

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

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Tarbiat Modares University of Medical Sciences (TUM), Tehran, Iran (Ethical code: IR.MODARES.REC.1399.026).

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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.2025.103827.

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Review article

Lack of association between the *TMPRSS6* gene polymorphism (rs855791) and anemia: a comprehensive meta-analysis

Jethendra Kumar Muruganantham, *Ramakrishnan Veerabathiran* *

Human Cytogenetics and Genomics Laboratory, Faculty of Allied Health Sciences, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Kelambakkam, Tamilnadu 603103, India

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ABSTRACT

Background: Anemia affects around 1.6 billion people worldwide and presents a significant challenge for healthcare providers. Despite the hemoglobin concentration being commonly used for diagnosis, identifying underlying causes remains challenging, particularly in vulnerable groups like children under five and pregnant women. Genetic factors, notably variations in the *TMPRSS6* gene, are implicated in iron deficiency anemia, yet the precise relationship with anemia remains unclear.

Methods: A thorough literature search was conducted across databases, including Embase, Google Scholar, and PubMed, focusing on studies investigating *TMPRSS6* gene polymorphisms and anemia. Thirteen eligible studies, comprising 2082 cases and 2684 controls, underwent meta-analysis using Review Manager 5.4 software. Various genetic models were assessed, including allelic, homozygous, heterozygous, dominant, and recessive, with no significant relationship found between the *TMPRSS6* rs855791 polymorphism and anemia.

Conclusion: This meta-analysis provides robust evidence suggesting no significant association between the *TMPRSS6* rs855791gene polymorphism and anemia. These findings underscore the complexity of genetic factors contributing to anemia and emphasize the importance of the further investigation to unravel the mechanisms underlying this relationship for improved diagnostic and therapeutic approaches.

Introduction

About 1.6 billion individuals worldwide are affected by anemia, a common hematological disorder encountered by general practitioners and hospital doctors.[1] Using the patient's hemoglobin (Hb) concentration to diagnose anemia is common practice, yet this information alone may not pinpoint the underlying disease responsible for the anemia.[2] Children

* Corresponding author at: Faculty of Allied Health Sciences, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Kelambakkam, 603103 Tamilnadu, India.
E-mail address: dr.ramakrishnan@care.edu.in (R. Veerabathiran).

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under five years old and pregnant and non-pregnant women aged 15–49 are considered the most vulnerable groups, with global prevalence estimates indicating rates of 47 %, 42 %, and 30 % for anemia, respectively.[3] Anemia is not a singular disease but a broad spectrum of pathological disorders. It is defined functionally and quantitatively as a state where there are insufficient erythrocytes (oxygen-carrying blood cells) in the bloodstream to meet metabolic demands. In clinical practice, the identification of anemia relies on measures such as Hb levels, hematocrit, or red blood cell count that falls below the expected norms adjusted for age and sex.[4] Several factors influence Hb levels, including smoking, residing at high altitudes, and dietary habits. Children in high-altitude regions can refer to normalized Hb value curves for accurate assessment. Moreover, individual genetic differences, as revealed by genome-wide association studies, contribute to variations in erythrocyte indices.[5] Iron deficiency or anemia in young children can lead to poor growth and failure to thrive, affecting their neurocognitive and behavioral development.[6] Historically, iron deficiency anemia (IDA) has been associated with environmental factors such as illness and nutrition. Recent studies suggest that genetic factors play a significant role in the development of IDA. Approximately 20–30 % of the variations in iron concentration may be due to genetic factors.[7] Common single nucleotide polymorphisms (SNPs) that have been replicated and shown to influence inter-individual variance in blood Hb levels are associated with various biological processes. These processes include Hb production, erythropoiesis (the production of red blood cells), and iron metabolism.[8] Genetic alterations in the gene coding for transferrin (*TF*), transmembrane serine protease 6 (*TMPRSS6*), and solute carrier family 40 member 1 (*SLC40A1*) are the primary sources of genetic diversity leading to iron deficiency. Among these genes, mutations in the *TMPRSS6* gene are frequently linked to reduced iron levels and hematological parameters, such as erythrocyte volumes and Hb concentrations.[9]

The *TMPRSS6* gene, predominantly expressed in the liver, plays a crucial role in regulating iron homeostasis by negatively modulating the synthesis of hepcidin, the master hormone governing iron levels in the body.[10] Matriptase-2 (MT-2) hinders the expression of hepcidin and is encoded by the *TMPRSS6* gene.[11] The MT-2 protein domain borders align precisely with the intron/exon junctions across all species in this gene located on chromosome 22, consisting of 18 exons and 17 intervening introns.[12] Several *TMPRSS6* SNPs were implicated as indicators of low blood indices, such as rs855791 and rs4820268.[13] The current meta-analysis seeks to elucidate the relationship between the *TMPRSS6* rs855791 variant and the occurrence of IDA.

Methodology

Literature search

PubMed, Google Scholar, and Embase were used to search for anemia-related articles with *TMPRSS6* gene, polymorphism, SNPs, and genetic variations as search criteria. The meta-analysis assessed relevant references with predetermined inclusion/exclusion criteria.

Inclusion and exclusion criteria

As a prerequisite to conducting an accurate meta-analysis, specific criteria were established to determine the suitability of relevant studies. The studies needed a case-control or similar design, focusing on the correlation between the *TMPRSS6* gene and anemia. The studies also had to deliver genotype and allelic frequency data, consistent 95 % confidence intervals (95 % CIs), and p-values for assessing the odds ratio (OR). The Newcastle-Ottawa Scale (NOS) was used to interpret the meta-analysis results. Any research that failed to meet these criteria or had insufficient data was disregarded.

Data extraction

Using the criteria, the researchers systematically gathered relevant papers and conducted data extraction in a standardized manner. A comprehensive examination of available publications was performed to retrieve details on allelic frequencies and genotypes for both case and control participants. In cases where genotypic data was insufficiently provided, it was derived from existing data, such as allelic frequencies. Studies that could not obtain meaningful data from both case and control groups were excluded. The extracted data from each study encompasses the Pubmed ID, study design, publication year, first author name, sample size, ethnicity, Hardy-Weinberg equilibrium (HWE) score, language, and other pertinent information. Figure 1 depicts a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart that describes how papers were selected and screened for meta-analysis.

Risk bias

The Cochrane Rob Tool 2 was utilized to thoroughly measure the methodological quality of the chosen studies, as depicted in Figure 2(a). In this representation, each study is described in a row and each column corresponds to a specific type of bias. The color assigned to each survey indicates the reviewer's evaluation of the bias risk associated with that particular type of analysis. Studies with a low bias risk are represented in green, while those with a high risk of bias are shown in red. Yellow indicates an unclear risk of bias. Overall, the findings suggest a significantly low bias risk for the selected studies, indicating that the research was conducted, executed, and documented to substantially minimize or eliminate potential bias or error.

Statistical analysis

The statistics we analyzed using Review Manager 5.4 software with a statistical consequence threshold of p-value <0.05 for each genetic variation. The chi-square-based Q statistic test was used to examine heterogeneity assumptions across previous research, measured by the I^2 metric value. In earlier studies, the random-effect model was used to evaluate the odds ratio and 95 % CI, creating a forest plot for ease of evaluation. Additionally, we used a funnel plot to scrutinize potential publication bias within the meta-analysis. The chromosomal interactions with the SNPs are represented by a

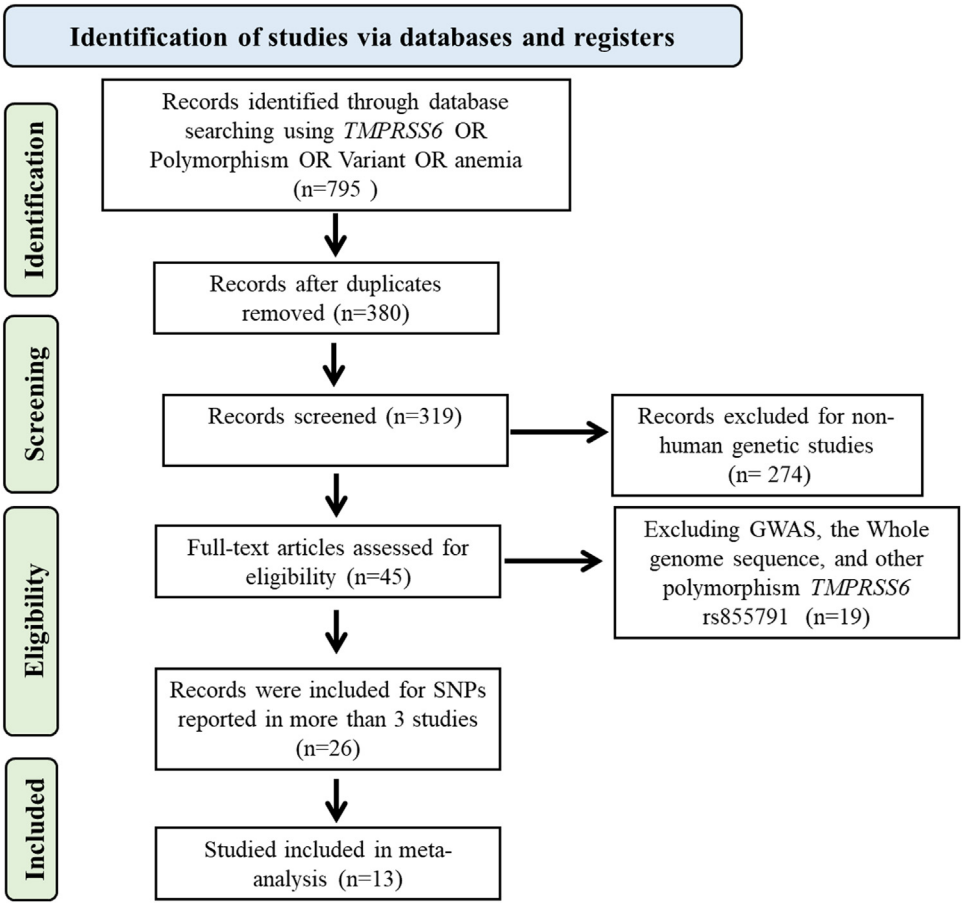

Figure 1 – Study selection of the *TMPRSS6* rs855791 gene polymorphism and anemia.

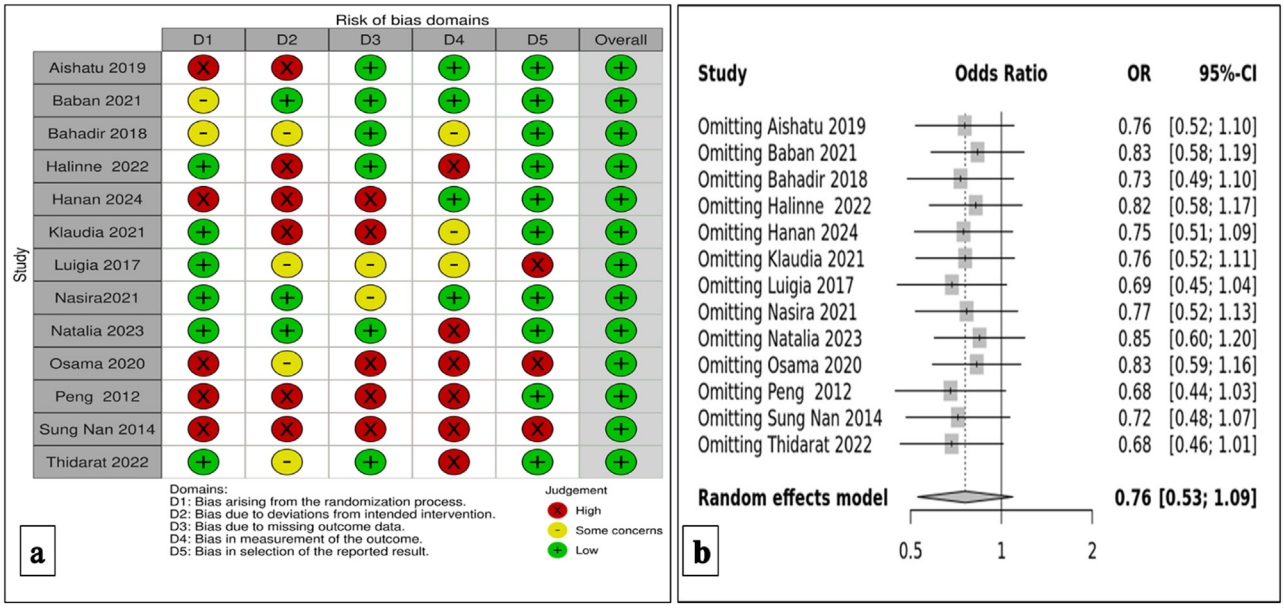

Figure 2 – (a): Risk of bias summary and graph for investigating the *TMPRSS6* rs855791 gene polymorphism (b): Sensitivity analysis of rs855791 for both cases and controls.

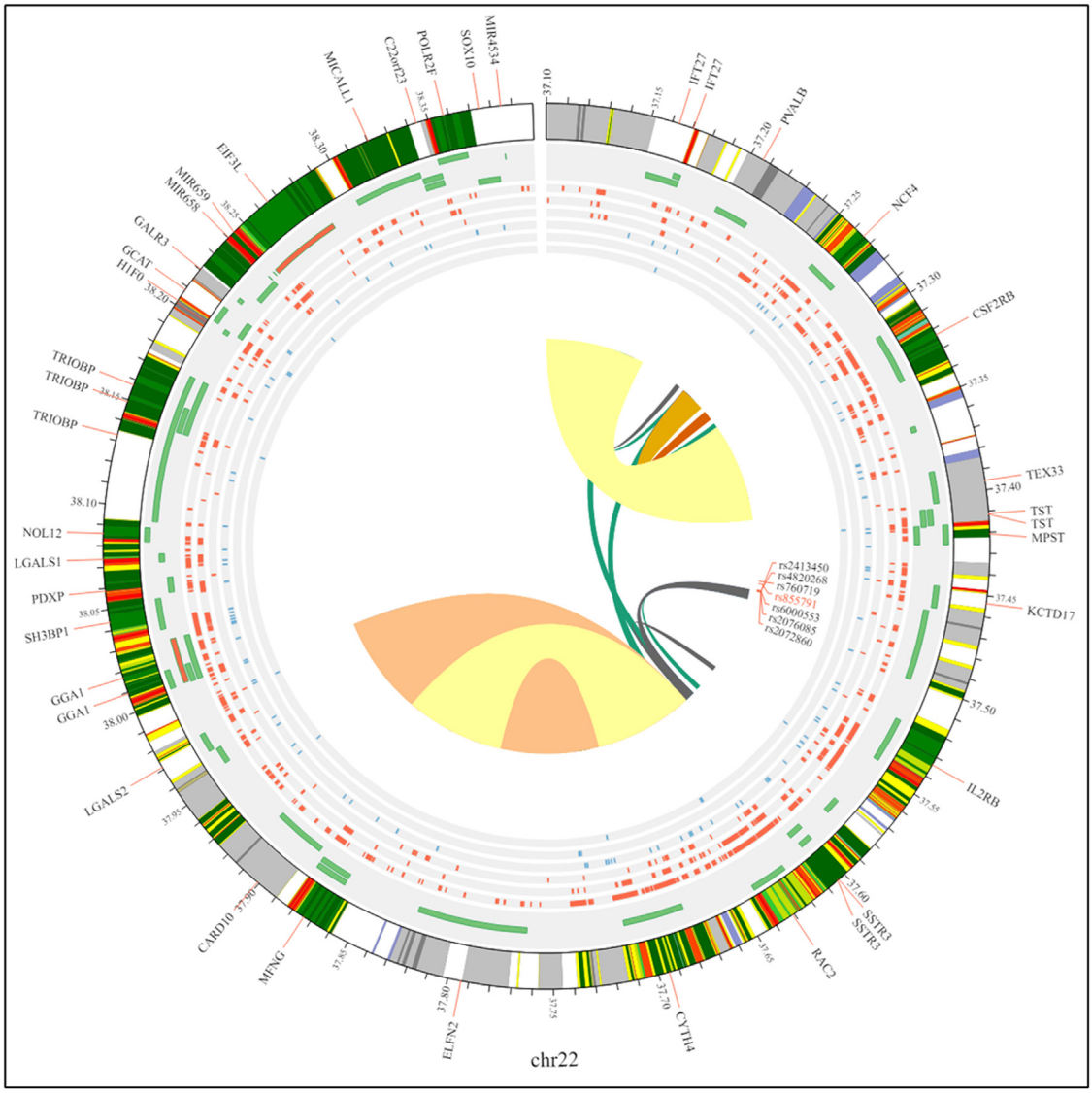

Figure 3 – Circos plot showing chromosomal interactions involving the rs855791 single nucleotide polymorphism.

Circos plot to visualize the complete data using the 3D SNP tool in Figure 3.

Results

Study characteristics

Figure 1 thoroughly illustrates the study selection and assessment process, which adheres to rigorous inclusion and exclusion criteria. At the outset, 795 publications were comprehensively gathered and meticulously evaluated to ensure their suitability. The inclusion of thirteen studies in this meta-analysis was based on careful assessment using the HWE score and NOS scale to ensure data accuracy and reliability.[14–26] The characteristics of the studies, including their NOS score, are presented, and detailed information concerning genotype distribution, allelic frequency, and HWE/chi-square values for the chosen polymorphisms are provided (Table S1).

Association between the TMPRSS6 variant with anemia

Random effects were used because the I^2 ≥50 % in the allelic model (I^2 = 79 %; OR = 0.81; 95 % CI: 0.64−1.02; p-value = 0.08), in the homozygous model (I^2 = 77 %; OR = 1.62; 95 % CI: 0.98 −2.68; p-value = 0.06), in the heterozygous model (I^2 = 57 %; OR = 0.87; 95 % CI: 0.63−1.18; p-value = 0.36), in the dominant model (I^2 = 72 %; OR = 1.34; 95 % CI: 0.95−1.90; p-value = 0.10), and in the recessive model (I^2 = 72 %; OR = 1.32; 95 % CI: 0.92 −1.90; p-value = 0.14). Overall, none of the five genetic models demonstrated any significant associations. All the data are shown using forest plots. To ascertain the sensitivity of the *TMPRSS6* rs855791 polymorphism, a comprehensive analysis was carried out, encompassing Begg's funnel plot and Egger's test. Figures 4 and S1 reveal no publication bias for the five genetic models in the data.

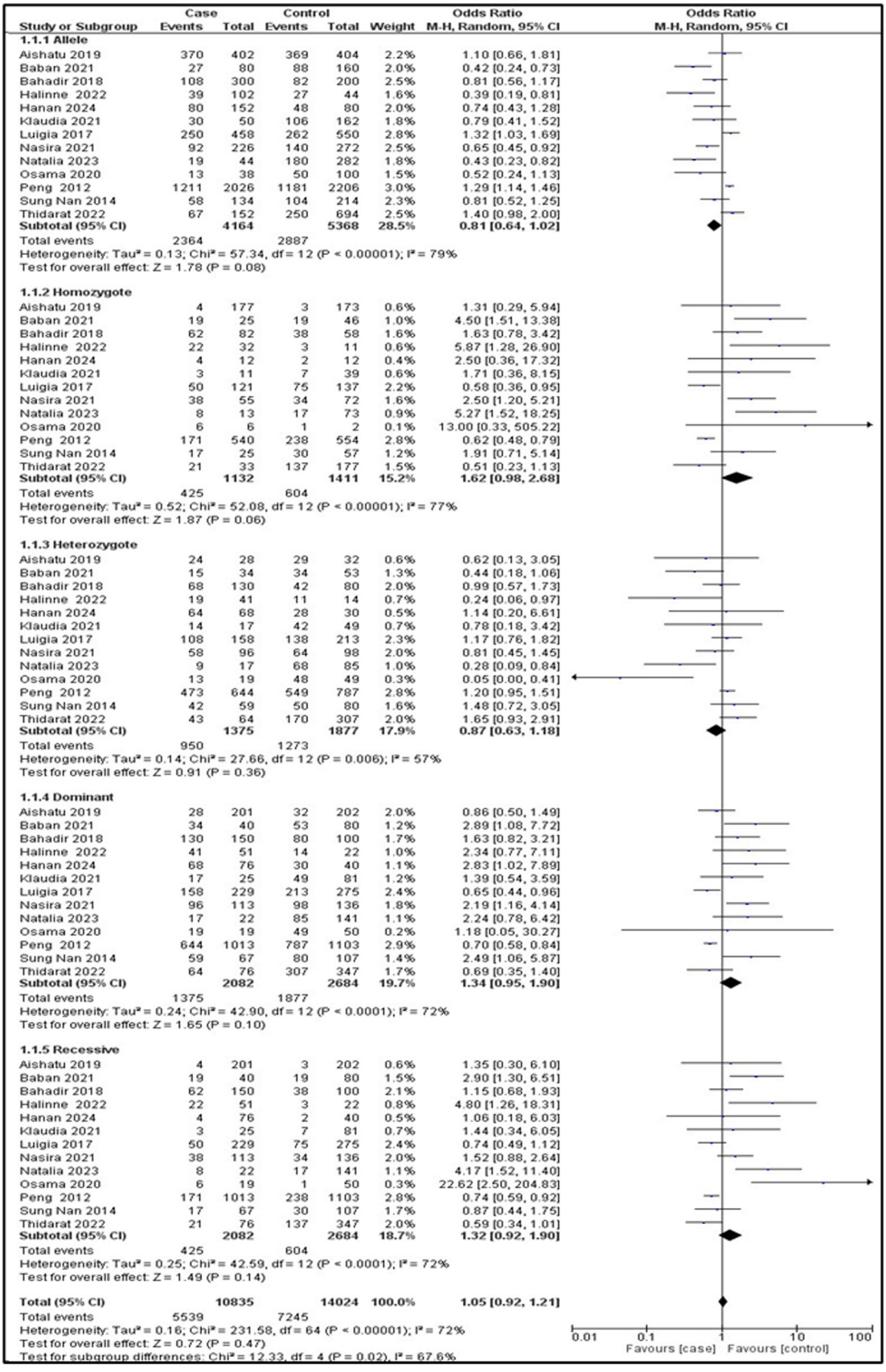

Figure 4 – Forest plot showing the association between the *TMPRSS6* rs855791 gene polymorphism and anemia in the genetic model.

An examination of sensitivity analysis

A sensitivity analysis for the *TMPRSS6* gene variation (rs855791) was performed, and the results in Figure 2(b) were similar. We, therefore, conclude that our findings are statistically significant.

Discussion

Genetic variations in DNA, particularly changes in SNPs, can influence how specific amino acid conversions in a protein affect the functions and activity of a gene.[27] Genetic variations in the *TMPRSS6* gene impact hematologic parameters and serum iron levels. Additionally, it was discovered that SNPs of the *TMPRSS6* gene were connected to quantitative changes in hematologic markers. Whether the relationship between the *TMPRSS6* gene variations and erythropoiesis relies on iron is unknown.[28] A study involving female university students in northern Saudi Arabia showed that the *TMPRSS6* polymorphism rs855791 significantly correlated with reduced iron levels. At the same time, rs2111833 did not show such a correlation.[14] The study provides relevant data concerning the genotype distribution of SNPs in the *TF*, *TMPRSS6*, and *HFE* genes, along with their potential association with the iron status and blood iron levels of pregnant Filipino women.[29] Data from Taiwan indicated that reproductive-age women diagnosed with IDA exhibited a reduced frequency of the *TMPRSS6* rs855791 CC genotype compared to women without the condition.[15] It is essential to acknowledge that IDA is a multifactorial condition influenced by various factors such as nutrition, socioeconomic status, gender, and age. These non-genetic factors are often more prevalent and could overshadow the genetic contributions, making it difficult to detect a direct correlation between *TMPRSS6* polymorphisms and anemia.[30]

Additionally, this study did not differentiate between IDA and iron refractory-iron deficiency anemia (IRIDA), a specific subtype of anemia that is more likely to be influenced by genetic factors such as *TMPRSS6* polymorphisms.[31] IRIDA, characterized by a poor response to oral iron therapy, has been directly linked to mutations in the *TMPRSS6* gene, with rs855791 being one of the polymorphisms of interest. Studies have shown that individuals with IRIDA are more likely to carry specific *TMPRSS6* variants, which disrupt iron regulation and lead to persistent anemia.[32]

This meta-analysis, which consisted of 2082 cases and 2684 controls, aimed to establish a correlation between anemia and *TMPRSS6* gene polymorphisms with a specific SNP. We tabulated the results using overall OR, a 95 % CI, and p-value. This study unequivocally found no evidence of a relationship between anemia and *TMPRSS6* (rs855791) in all genetic models. Therefore, our findings strongly suggest no potential link between the *TMPRSS6* rs855791 gene polymorphism and anemia. This study provides important insights into the lack of association between anemia and variations in the *TMPRSS6* gene. The results of this study differ from previous studies due to a larger sample size, diverse population coverage, and more rigorous methodologies, including comprehensive genetic model assessments and publication bias evaluations. These factors may have revealed a lack of association that smaller, less robust studies did not detect. Further research is needed to understand how variations in the *TMPRSS6* gene are connected to anemia. Additional studies with more diverse populations are required to explore other *TMPRSS6* polymorphisms, gene-gene interactions, and iron metabolism pathways. Longitudinal studies and functional assays could also provide deeper insights into the gene's role in anemia. Delving deeper could uncover vital information for better diagnostics and treatments. This study is a significant milestone in improving healthcare for individuals with IDA, as it enhances our understanding of this relationship.

Conclusion

This comprehensive meta-analysis examined the association between the *TMPRSS6* gene polymorphism (rs855791) and anemia, incorporating data from 13 studies comprising over 4700 participants. Contrary to previous hypotheses, our findings revealed no significant correlation between the *TMPRSS6* rs855791 gene variation and anemia across various genetic models. These results suggest that the *TMPRSS6* rs855791 polymorphism may not play a substantial part in predisposing individuals to anemia. However, given the multifactorial nature of anemia and the complexity of genetic influences, further investigation is warranted to elucidate the precise mechanisms underlying anemia development and identify additional genetic factors. Such insights are crucial for refining diagnostic methods and developing targeted therapeutic interventions, ultimately improving clinical management and outcomes for individuals affected by anemia.

Author contributions

JM wrote the contents, edited the figures and tables of this manuscript. RV designed the study, edited the contents of this manuscript, and approved the manuscript for submission. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest to report.

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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.2025.103737.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Review article

Anti-HLA antibody formation increases the chances of platelet refractoriness in platelet-transfused patients: a systematic review with meta-analysis

Luana Joana Barreto Cabral [a,b,c,*], *Daniela Pereira Lopes* [a,b], *Eduardo dos Santos Martins Filho* [c], *Rubenilson Caldas Valois* [d,e,g], *Paula Christine Amarantes Justino Oliveira* [f], *Patrícia Jeanne de Souza Mendonça-Mattos* [c,g]

[a] *Graduate Program in Multiprofessional Residency in Hemotherapy and Hematology, Pará State University (UEPA), Belém, Pará, Brazil*

[b] *Multiprofessional Residency in Hemotherapy and Hematology, Foundation Center of Hemotherapy and Hematology of Pará (HEMOPA Foundation), Belém, Pará, Brazil*

[c] *Laboratory of Immunogenetics, Foundation Center of Hemotherapy and Hematology of Pará (HEMOPA Foundation), Belém, Pará, Brazil*

[d] *Hemovigilance and Supervision Department, Foundation Center of Hemotherapy and Hematology of Pará (HEMOPA Foundation), Belém, Pará, Brazil*

[e] *Teaching Staff, Pará State University (UEPA), Belém, Pará, Brazil*

[f] *Technical Directory, Foundation Center of Hemotherapy and Hematology of Pará (HEMOPA Foundation), Belém, Pará, Brazil*

[g] *Preceptors of the State Program of Incentives to Qualification of Health Professionals – QUALIFICASAÚDE, Pará State University (UEPA), Belém, Pará, Brazil*

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ABSTRACT

Platelet refractoriness caused by alloimmunization to anti-HLA antibodies remains present in daily hemotherapy: the frequent need for platelet transfusions may influence the long-term survival of treated patients. This study aimed to perform a systematic review with meta-analysis to investigate the chances of anti-HLA antibody formation triggering immune-induced platelet refractoriness in platelet transfused individuals. By adopting Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria, a search was conducted of publications in online databases between 1976 and July 2022. The risk of bias in the studies was assessed according to the data quality assessment proposed by the 'A MeaSurement Tool to Assess systematic Reviews' (AMSTAR-2) tool. Meta-analysis was performed by evaluating the Forest and Funnel Plots. From 832 published articles, 50 were read in full with 14 studies being included in this systematic review. The forest plot showed a likely low heterogeneity (I^2: 12.3%; p-value = 0.32), and high odds ratio (174.57; confidence interval: 73.23–416.16) showing platelet refractoriness is triggered by anti-HLA alloantibodies. In this study, anti-HLA antibody formation contributed to an approximate

* Corresponding author.
E-mail address: luanajbcabral@gmail.com (L.J.B. Cabral).

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175-fold higher chance of triggering immune-induced platelet refractoriness. Some explanations about why some statistical differences were observed are offered by studies. This study demonstrates the need for developing policies to identify and monitor anti-HLA antibodies in patients, as well as for HLA matching, and makes some suggestions for future research to promote the prevention of patient sensitization due to platelet transfusions including the development of platelet refractoriness.

Introduction

In platelet concentrate transfusions, human leukocyte antigen (HLA) compatibility between recipients and donors is not mandatory during the pretransfusion testing phase.[1] Platelets have antigens from the ABH system, Lewis, P, I systems, Human platelet antigens (HPA), and Class I HLA antigens, but only ABO and HLA antigens and HPA are relevant for post-transfusion survival of platelets.[1,2] Because of this, the patient may receive antigens different from his genetic heritage at each transfusion and develop sensitization to anti-HLA antibodies.[3] This alloimmunization may trigger immune-induced platelet refractoriness, that is, an excessive consumption of platelet concentrates, without adequate therapeutic response, and with complications that can be fatal.[4]

Non-immunological causes that can trigger platelet refractoriness (60–70% of cases) include: disseminated intravascular coagulation (DIC); microangiopathic hemolytic anemia (MAHA); active bleeding; sepsis; fever; splenomegaly; graft-versus-host disease (GvHD); circulating immune-complexes; bone marrow transplantation; veno-occlusive disease; drug-induced thrombocytopenia (antithrombotics, infectious disease agents, histamine-receptor antagonists, analgesic agents, chemotherapeutic and immunosuppressant agents, cinchona alkaloids, platelet inhibitors, antirheumatic agents, sedatives and anticonvulsant agents, and diuretic agents); platelet dose/platelet quality due to the patient's blood volume, storage temperature, improper mode of agitation and pH, and platelet age.[5-7] However, 30–40% of cases are immune related including: HLA antibodies (80–90%), HPA antibodies (5–20%), HPA and HLA antibodies (5%), mismatched ABO antibodies, and platelet autoantibodies (e.g., platelet refractoriness related to an autoantibody to platelet glycoprotein).[5,8]

A recent prospective study of 3805 individuals pointed to pregnancy and platelet transfusion as the main risk factors for sensitization to anti-HLA antibodies: it was also observed that alloimmunization occurs mainly from platelet concentrate transfusions.[9] Thus, anti-HLA antibodies may be present in the serum of patients with a platelet concentrate transfusion history. These alloantibodies are mostly of the IgG type[4] and may contribute to the development of platelet refractoriness through the activation of the complement system by the classical pathway, causing the deposition of C4b and C3b and the formation of the membrane attack complex.[10]

Diagnosis can be by methods such as lymphocytotoxicity, enzyme immunoassay, platelet antigen immobilization using monoclonal antibodies, or flow cytometry. The gold standard to detect anti-HLA antibodies in blood samples is the flow cytometry technique (microspheres) called antibody reactivity panel, whose function is to identify anti-HLA antibodies, ensuring reliability, sensitivity, and specificity of the result.[4,11,12]

Thus, this review investigated the chances of anti-HLA antibody formation triggering immune-induced platelet refractoriness in platelet transfused individuals using a systematic review with meta-analysis in order to improve the statistical power of the research question.

Materials and methods

This systematic review was conducted according to the criteria of the International Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines.[13]

Data sources and research strategy

The PICO (population, intervention, comparison, outcome) strategy was used to formulate the following research question, "Is there evidence that, in thrombocytopenic patients, platelet transfusion with anti-HLA antibody formation increases the chances of immune-induced platelet refractoriness?" PICO was defined as: P (thrombocytopenic patients), I (platelet transfusion), C (anti-HLA antibody formation), and O (platelet refractoriness).[14] The Web of Science (Clarivate Analytics, Philadelphia, PA, USA), Scopus, PubMed, LILACS, BVS Brasil, EBSCOhost MEDLINE, and SciELO databases were used for the search with the descriptors and keywords shown in Table 1. Publications in Portuguese, Spanish, Italian, and English were considered with articles published from 1976 to July 24, 2022 being included.

Inclusion and exclusion criteria

The selection stage included articles that met the following criteria: investigation of anti-HLA antibodies in platelet-transfused patients; cross-sectional studies or randomized clinical trials, with or without transfusion reactions to platelets, in Portuguese, Spanish, Italian, and English. The following exclusion criteria were employed: research involving neonates, and bone marrow or organ transplant recipients, case studies, meeting abstracts, reference articles, letters, editorials, notes, news, abstracts and posters from conferences, symposia, and meetings, discussions, book chapters,

Table 1 – Databases consulted, search strategy used with the PICO methodology in four languages, and the number of articles found.

Search strategy			Database						
	AND	AND	AND	WOS	SCOPUS	PUBMED CENTRAL	LILACS	BVS BRAZIL	Medline (EBSCOhost)	SciELO Citation Index
("aplastic anemia "OR "myelodisplasic syndromes "OR "leukemias "OR "thrombocytopenic Patients" OR "thrombocytopenia" OR "anemia aplástica "OR "síndromes mielodisplásicas "OR "leucemias "OR "pacientes trombocitopênicos "OR "trombocitopenia "OR "síndromes mielodisplásicos "OR "pacientes trombocitopénicos "O "sindromi mielodisplasiche "OR "leucemie "OR "pazienti trombocitopenici")	(platelets OR platelet OR "platelet transfusion "OR plaquetas OR "transfusão de plaquetas "OR "transfusão de plaquetas "OR "transfusión de plaquetas "OR piastrine OR "trasfusione di piastrine")	("polytransfused platelets" OR "platelet polytransfused" OR " ineffective platelet transfusion" OR transfusion OR "plaquetas poli-transfundidas" OR "politransfusão de plaquetas" OR " transfusão de plaquetas ineficaz" OR transfusão OR "transfusión ineficaz de plaquetas" OR transfusión OR "piastrine politrasfuse" OR " trasfusione inefficace di piastrine" OR trasfusione)	("anti-HLA" OR "HLA antibodies" OR "anti-HLA antibodies" OR "Class I HLA" OR "HLA antibody" OR "anti-HLA" OR "anticorpos HLA" OR "anticorpos anti-HLA" OR "anticorpos HLA classe I" OR "anticorpo HLA" OR "anticuerpos anti-HLA" OR "Clase I HLA" OR "anticuerpo HLA" OR "anticorpi HLA" OR "anticorpi anti-HLA")	129	5	6	4	1	686	1

corrections, additions, duplications, experience reports, literature reviews, bibliographies, reprints, guidelines and retractions of publications.

Data selection and extraction

The following data were extracted from the databases: publication year, author, publication title, abstract, keywords, journal title, institution, and country. These data were entered into the Rayyan Systems Inc. - Intelligent Systematic Review system version 0.1.0.[15] A double-blind selection using the titles and abstracts of articles that met the inclusion criteria was made by two collaborators. A third reviewer resolved disagreements and doubts. Afterward, selected articles were read in full from the CAPES Portal Periódicos (CAFe access) and Google Scholar. Mendeley Reference Manager v. 2.76.0 was used to organize the selected articles.

Data quality assessment

As proposed by Ma et al., the best instrument for assessing the methodological quality of a systematic review is the 'A Measurement Tool to Assess Systematic Reviews' (AMSTAR-2) tool.[16] This instrument was developed and adapted from the Overview Quality Assessment Questionnaire (OQAQ), a checklist created by Sacks with the improved version was used for this paper.[17]

Literature bias assessment

The Checklist developed by the Joanna Briggs Institute Critical Appraisal Tools "Analytical Cross-Sectional Studies" was used for cross-sectional studies and the "Checklist for Randomized Controlled Trials" was used for one single study.[16,18] The risk of bias was considered high when positive responses were ≤49%; moderate from 50 to 69% and low risk when positive responses were ≥70%.[19]

Data synthesis and analysis

The RStudio v.4.2.2 Build 576 interface of the R-4.2.2 for Windows program was used for the meta-analysis, using the general package for meta-analysis. The Mantel-Haenszel statistical method was used for the binary variables, and odds ratios (ORs) were obtained, given the cross-sectional nature of most of the studies. Forest plot descriptive statistics were used to compare studies, where I^2 <30%, 30–60%, 61–75%, and >75% are suggestive of low, moderate, substantial, and considerable heterogeneity, respectively with the significance of this heterogeneity being agreed upon at a conservative level of p-value <0.01 [20,21]. The OR of the random or fixed (common) model was chosen. A funnel plot was also constructed to investigate the bias of all selected publications in this study.

Results

Table 1 shows the articles found in the different databases, the descriptors, and keywords. The PRISMA flowchart shows the entire data selection process and the number of articles included in this study (Figure 1). Of the 50 articles read in full, 33 papers were excluded for the reasons shown in Supplementary Table 1. Therefore, 14 papers (from 1976 to 2019) were included in this meta-analysis as listed in Supplementary Table 2. The risk of bias evaluated using the instruments developed by the Joanna Briggs Institute can be seen in Supplementary Tables 3 and 4. It was noted that the randomized study conducted by Hess et al. [22] presented a medium risk of bias with a percentage of 61.5%, while all cross-sectional studies presented a low risk of bias with percentages ranging from 87.5% to 100%.

From the articles initially selected, due to the impossibility of performing a meta-analysis, the publications that did not present data from refractory individuals and from which it was not possible to perform the calculations were excluded (Supplementary Table 5). Table 2 shows all studies included in the meta-analysis and presents the data entered in the R program. Table 3 shows the sum of the cases for each aspect of refractoriness to anti-HLA antibodies and the performance between refractory versus alloimmunized patients using the chi-square test with Yates correction (or Fisher's exact test) in OpenEpi v. 3.01 online software.[23] The test shows that the immunological aspect of developing refractoriness by anti-HLA antibody formation is not statistically significant compared to alloimmunized patients by non-immunological factors (p-value = 0.2708). Figure 2 shows the Forest Plot, which shows the low heterogeneity of the included studies (I^2: 12.3%; p-value = 0.32; OR: 174.57 confidence interval [CI]: 73.23 –416.16). The Funnel Plot shown in Figure 3 demonstrates the considerable symmetry in the distribution of studies (represented by dots). Therefore, it confirms the low heterogeneity and low biases of the studies. However, the study by Comont et al.[24] was outside the pyramid, even so the standard error ($1.0 < DP/\sqrt{n} < 2.0$) was similar to the others. As per the funnel plot, the papers by Wu et al.[25] ($DP/\sqrt{n} > 2.0$) and Peña et al.[26] ($DP/\sqrt{n} > 2.0$) had the highest standard errors relative to the total nevertheless, like the others, they remained within the statistical CI.

Data on the studies of Wu et al.[25], Murphy et al.[27], Godeau et al.[28], Novotny et al.[30], Bajpai et al.[31], Lin et al.[32], Pai et al.[33], Jackman et al.[34], Enein et al.[35], Kumawat et al.[36], Ramírez et al.[37], Hess et al.[22], Comont et al.[24], Peña et al.[26].

The Forest Plot in which the studies were included showed a probable low level of heterogeneity, represented by I^2 = 12.3% and p-value = 0.32. Therefore, the random or fixed model was adopted (OR: 174.57 (73.23–416.16).

The Funnel Plot showed considerable symmetry in the distribution of the studies (represented by the dots), starting from the central vertical line of the pyramid, and therefore confirms the low heterogeneity and, consequently, the low bias of the studies.

A new graphical analysis was performed, without the study by Comont et al.[24]. This showed a lesser heterogeneity (I^2 = 0; p-value = 0.55) and the permanence of a high OR (122.77; CI: 53.82–280.03) thereby causing the standard error of Wu et al.[25] and Peña et al.[26] to decrease (data not shown). However, since this new analysis confirmed the need to investigate the works of Comont et al.,[24] Wu et al.,[25] and Peña et al.,[26] Figure 2 and Figure 3 were considered for analysis.

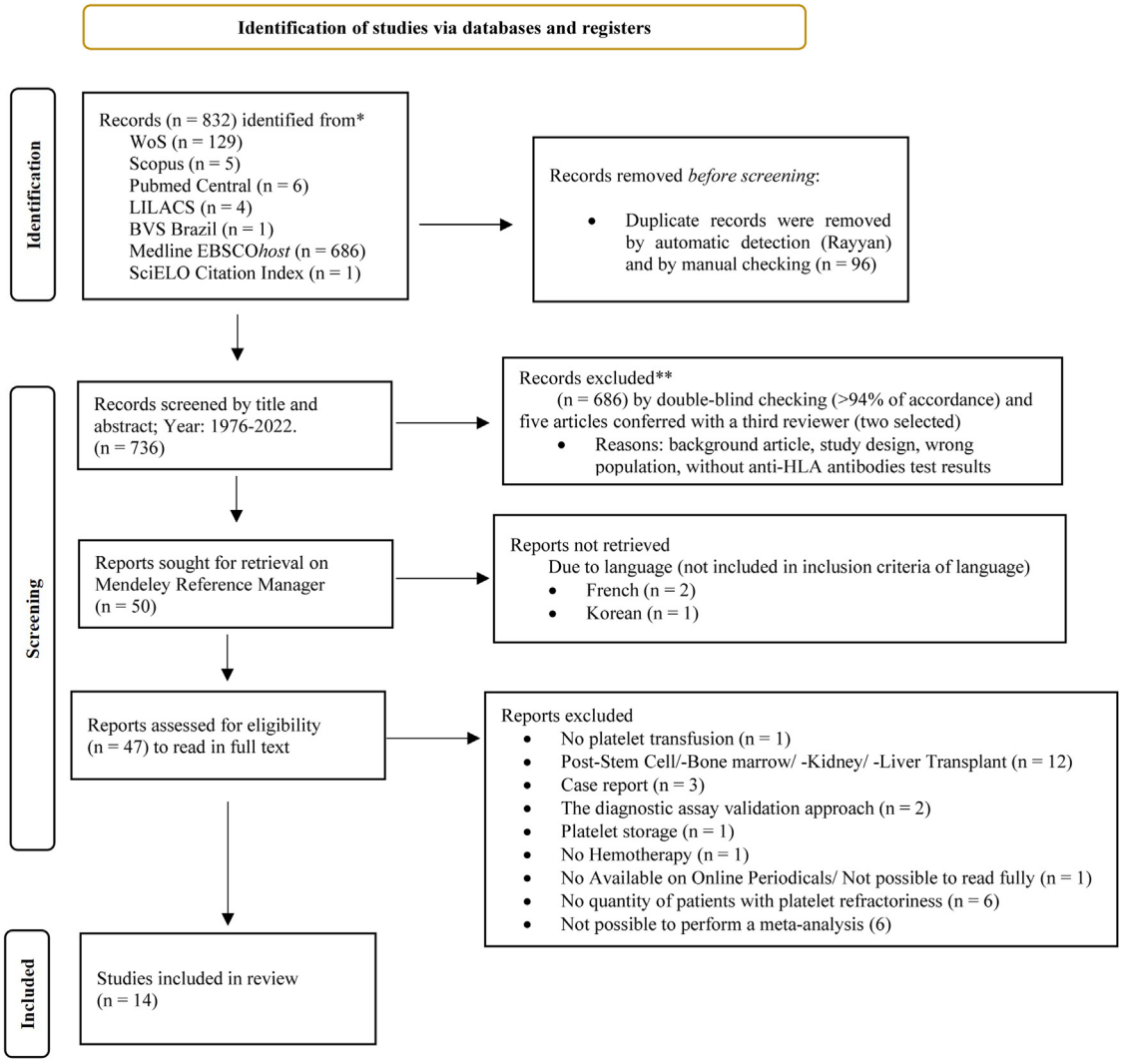

Figure 1 – PRISMA flow diagram for the systematic review.

Supplementary Table 2 shows all the included studies. In total, 2577 patients were investigated, and a prevalence of 12.92% (333/2577) of anti-HLA antibodies was found in refractory individuals.

Moreover, as frequent antibodies within the studied population were not one of the inclusion criteria in this study and the adopted methodologies have distinct analysis methods, only the studies by Wu et al.,[25] Pai et al.[33] and Peña et al.[26] showed anti-HLA antibodies specificities (Table 4). The most common anti-HLA antibodies found in the first study were A2 and X, the last named due to the incipient serological approach. The second study presented the following highly reactive public epitopes (shared by different HLA types): 145QRT, 65QIA, 62QE, 127 K, and 163EW. Also, a common private epitope was found: 151AHA (with HLA-A11 specificities). The third study mentioned only one patient (52 years old) with two pregnancies and a regimen of platelet transfusions at: a) Day 6: HLA-B7 and HLA-B81 were the most common antibodies with high mean fluorescence intensity values; and b) Day 22: stronger mean fluorescence intensity results for HLA-B7 and HLA-B81, followed by appearances of antibodies against HLA-A68, A69, A24, A2, A23, and B67.

Discussion

In the papers by Jackman et al.,[34] Enein et al.,[11] Kumawat et al.,[36] Hess et al.,[22] and Comont et al.,[24] there were more participants evaluated with platelet refractoriness than with anti-HLA antibody formation. One explanation for this is by understanding that platelet alloimmunization occurs from exposure to antigens present in the platelets of the donor and absent in the patient's platelets. This alloimmunization can occur through non-immunological mechanisms such as sepsis, fever, disseminated intravascular coagulation, drugs (amphotericin-B), hypersplenism, platelet consumption by hemorrhage, or by immunological mechanisms of HLA alloantibodies, ABO alloantibodies, platelet-specific alloantibodies, as well as autoantibodies.[38] However, in this review, the focus was on alloimmunization by the immunological mechanism of anti-HLA antibodies.

According to some papers, it would be justifiable that some patients formed anti-HLA antibodies that did not trigger platelet refractoriness. For this, one justification that exists in the literature is the description of transient anti-HLA

Table 2 – Quantitative data on the number of patients with the presence or absence of refractoriness and anti-HLA antibody formation in the 14 studies.

Author	Technique	Refractoriness[a]		Anti-HLA antibodies[a]		Total (n)	Rstudio program[b]			
		Present	Absent	Present	Absent		evtto	ntto	evcont	ncont
Wu et al.,[25]	LCY (One Lambda) and PA (Payton associates)	6	3	6	3	9	6	6	0	3
Murphy et al.,[27]	LCT (Mittal)	20	116	37	99	136	20	20	17	116
Godeau et al.,[28]	LCT (Mittal) and MAIPA (Kiefel et al.[29]	1	49	13	37	50	1	1	12	49
Novotny et al.[30]	LCT and PRA ≥ 2 0% and MAIPA (HLA w6/32)	31	133	48	116	164	31	31	17	133
Bajpai et al.[31]	LCT ≥ 2 0% (Terasaki e McClelland) and PSIFT	18	32	30	20	50	18	18	12	32
Lin et al.[32]	Flow Cytometry using donor platelet concentrates + kit FlowPRA™ (One Lambda)	31	13	28	16	44	28	31	0	13
Pai et al.[33]	Luminex Assay (LifeScreen, Tepnel Lifecodes Corporation, Stamford, CT)	19	54	23	50	73	19	19	4	54
Jackman et al.[34]	Luminex /LabScreen assay (One Lambda, Canoga Park, CA). Results with NGB. NGB > 10,8 (Class I HLA antibodies) and NGB > 6.9 (Class II HLA antibodies) were the cutoffs.	80	110	20	170	190	20	80	0	110
Enein et al.[35]	FlowPRA screening kit, (OneLambda Canoga Park, USA). And CDC	13	7	6	14	20	6	13	0	7
Kumawat et al.[36]	ELISA kit (Pakplus, GT diagnostic, USA) on three occasions (upon acceptance into the study, after 3 weeks or four transfusions, whichever occurred earlier, and at the end of 3 months)	21	9	18	12	30	18	21	0	9
Ramírez et al.[37]	Microlymphocytotoxicity for identification of HLA antibodies and Polyethylene glycol 6000 method for identification of circulating immune-complexes	14	57	26	45	71	14	14	12	57
Hess et al.[22]	CCI and PRA Class I HLA (FlowPRA Screening Kit, One Lambda Corp, Canoga Park, CA, USA);	102	614	40	776	816	40	102	0	614
Comont et al.[24]	Luminex/ LABScreen Mixed and Class I HLA Single Antigen (One Lambda). Confirmed the most reactive with LCA-CDC using a 60-cell panel.	41	856	31	866	897	31	41	0	856
Peña et al.[26]	Screening was performed with phenotyped beads from the LABScreen PRA (One Lambda Thermo Fisher; Luminex). If positive, single beads (LABScreen single antigen; One Lambda Thermo Fisher) were used. The strength of reactivity was reported as MFI. PRA was calculated using Fusion software (One Lambda Thermo Fisher). Overall, MFI≥1000 was considered positive, although standard reactivity was also considered. The percent of antibody reactivity panel calculation was determined by the online calculator optn.transplant.hrsa.gov/converge/resources/allocationcalculators.asp	7	20	7	20	27	7	7	0	20

CDC: complement-dependent cytotoxicity assay; ELISA: enzyme-linked immunosorbent assay; LCT: lymphocytotoxicity; MAIPA: monoclonal antibodies; MFI: mean fluorescence intensity; PRA: Panel Reactive Assay; NBG: normalized background.

[a]Number of refractory individuals and those who formed anti-HLA antibodies.

[b]In the RStudio program, the general package for meta-analysis package was adopted to create Forest plot and Funnel plot graphics. From the reasoning of the 2 × 2 contingency table: (I) evtto represents the number of individuals who formed anti-HLA antibodies and were refractory, (II) ntto the sum of refractory individuals who did or did not have anti-HLA antibody formation, (III) evcont the number of individuals who were not refractory but developed anti-HLA antibodies, and (IV) ncont the sum of non-refractory individuals who did or did not have anti-HLA antibody formation.

Table 3 – Results of chi-square test with yates correction (or fisher's exact test) between refractory versus alloimmunized patients with anti-HLA antibodies.

Refractoriness[a]		Anti-HLA antibodies[a]		Total (n)	OpenEpi v. 3.01		
Present n	Absent n	Present n	Absent n		Chi-square[b]	R	NR
404	2073	333	2244	2577	**anti-HLA+**	333	2073
					anti-HLA-	404	2244
Yates corrected chi-square (2-tail) p-value = 0.1662		**Fisher's exact (2-tail) p-value = 0.1659**		**Odds ratio 0.8922**	**Confidence interval [0.7627, 1.044[1]]**		

Note: [a]From reading the complete article, the number (n) of cases with or without refractoriness and those who formed or did not form anti-HLA antibodies were noted. [b]According to the 2 × 2 contingency table theory, the chi-square test was used considering as "disease" the refractoriness state and "exposure" anti-HLA antibody formation.

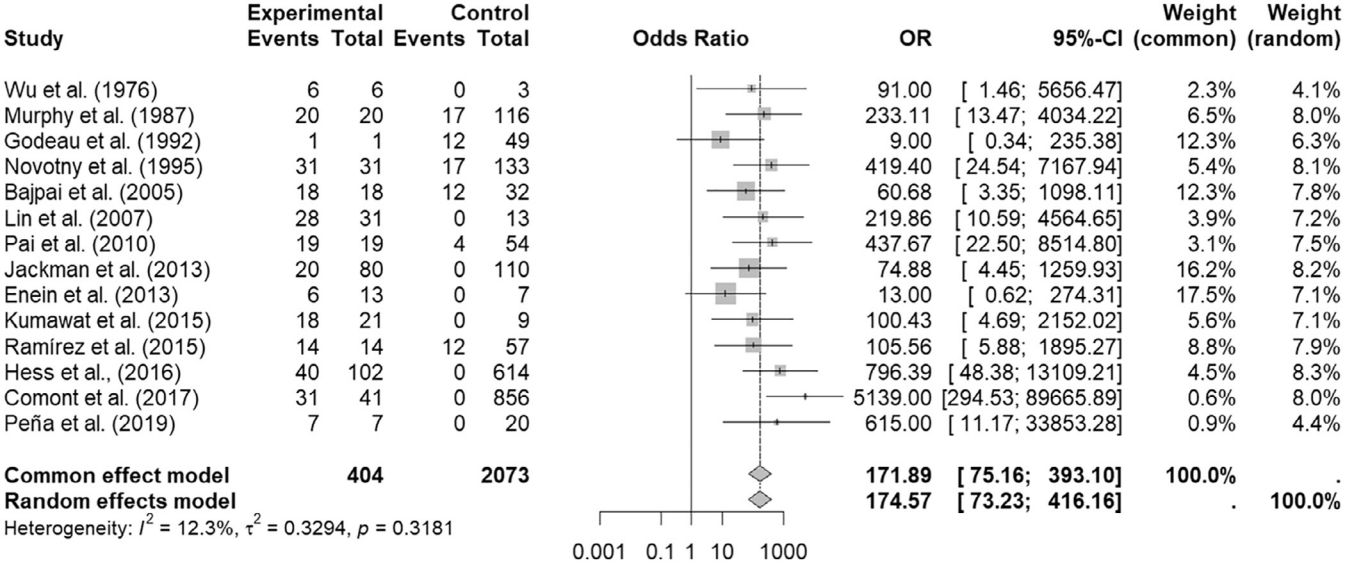

Figure 2 – The Forest Plot of the 14 papers presenting the odds Ratio data from the quantitative data of anti-HLA antibody formation and triggering platelet refractoriness.

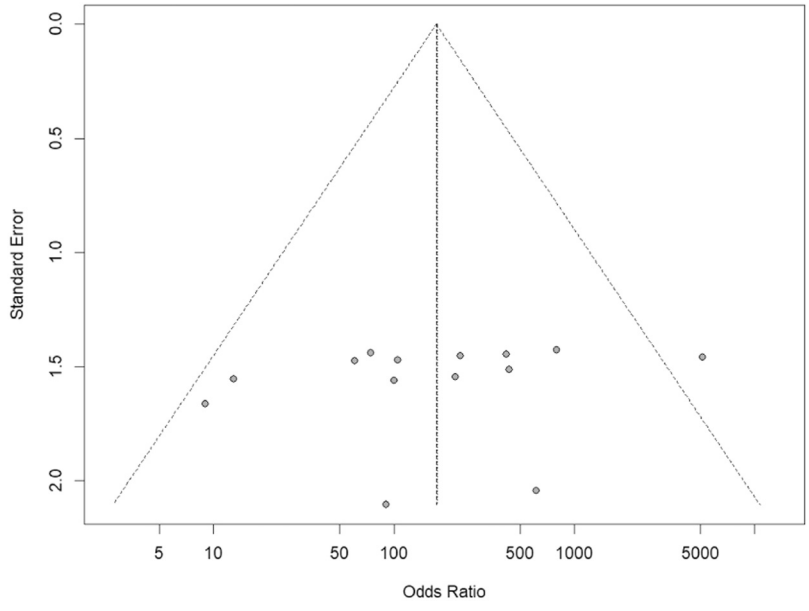

Figure 3 – The Funnel Plot of the 14 papers presenting the risk of biased data from the quantitative data of anti-HLA antibody formation and triggering platelet refractoriness.

Table 4 – Most common anti-HLA antibodies found in the studies.

Authors	Anti-HLA antibody
Wu et al.,[25]	A2 and X
Murphy et al.,[27]	Multispecific (it was not possible to discriminate)
Godeau et al.,[28]	Antibodies not specified, only said they were against Caucasian HLA antigens of cryopreserved lymphocytes
Novotny et al.,[30]	Antibodies not specified
Bajpai et al.,[31]	Antibodies not specified
Lin et al.,[32]	Common and rare HLA class I antigens, but not specified
Pai et al.,[33]	Highly reactive public epitopes: 145QRT, 65QIA, 62QE, 127 K, and 163EW. Most common private epitope found: 151AHA (with HLA-A11 specificities)
Jackman et al.,[34]	HLA Class I antigens, but not specified (samples from TRAP study included)
Enein et al.,[35]	HLA Class I and Class II antigens, but not specified
Kumawat et al.,[36]	HLA Class I antigens, but not specified. A significant association between HLA antibodies with (HPA)-5b/5b antibody (p-value = 0.033) associated with refractoriness was found
Ramírez et al.,[37]	Antibodies not specified
Hess et al.,[22]	HLA Class I antigens, but not specified
Comont et al.,[24]	HLA Class I antigens, but not specified
Peña et al.,[26]	From one patient (52 years old) with two remote pregnancies: a) Day 6 with routine PLT transfusions: HLA-B7 and HLA-B81; b) Day 22: mean fluorescence intensity stronger results of B7 and B81, followed by appearances of antibodies against A68, A69, A24, A2, A23 and B67

antibody formation, which disappeared after four weeks or did not persist under remission induction chemotherapy during leukemia treatment.[27,30]

Also, in this work, the stratification of two groups was considered, prospective and retrospective cross-sectional groups, because the authors stated that they understood that, for prospective studies, the OR can overestimate the chances of the outcomes [39], which may cause high statistical values. A situation that was very much present in the study of Comont et al.[24] (OR: 5139; CI: 294.53–89,665.89). Nevertheless, due to the low heterogeneity found (I^2 = 12.3%; p-value = 0.32) and the low number of studies that would result from stratification, the forest plot was generated with all 14 articles.

From the results of the meta-analysis, two studies deserve particular attention: Godeau et al.[28] (OR: 9; CI: 0.34–235.38), and Enein et al.[35] (OR: 13; CI: 0.62–274.31), because of their CIs reaching an OR <1 and Comont et al.[24] (OR: 5139; CI: 294.53 –89,665.89), because of the high OR and CI compared to the others.

The study by Comont et al.[24] was the one that reported the most medical treatments, and perhaps, that is why it had a high OR and CI and was outside the 95% CI of the funnel plot. Moreover, its sample population was much larger than the other studies (n = 856). The study by Godeau et al.[28] included patients who had not yet received platelet transfusions causing the CI to reach higher values than the other studies. In the Wu et al.,[25] study, one can suppose that the high standard error seen in this study might be due to the old methodology adopted, low participation in the research, and the absence of transfusions received by the participants before the beginning of the investigation.

The study by Enein et al.[35] found that the number of transfusions and the age do not influence the formation of anti-HLA antibodies that may explain the CI reaching an OR <1. In other words, the presence of anti-HLA antibodies may not have been triggered by multiple platelet transfusions. In addition, in this study, all were men, and it is well known that they are less exposed to alloimmunizations than women. The study by Peña et al.[26] presented a possible association with a non-hemolytic transfusion reaction and performed predictive calculations of anti-HLA antibody formation. Perhaps these features increased the standard error relative to the other studies that did not take this approach. Furthermore, in some studies such as those by Jackman et al. [34], Enein et al.,[11] Kumawat et al.,[36] Hess et al.,[22] and Comont et al.,[24] there were more participants evaluated with platelet refractoriness than with anti-HLA antibody formation. In only two studies, Wu et al.[25] and Peña et al.,[26] the numbers of participants who developed the antibodies and were platelet refractory were the same.

Thus, a high OR for anti-HLA antibody formation triggering immune-induced platelet refractoriness is presented (OR: 174.57; CI: 73.23–416.16), suggesting that anti-HLA antibody formation may contribute to a 175-fold greater chance of immune-induced platelet refractoriness. The studies by Bouquegneau et al.[40] (hazard ratio [HR]: 2.71; 95% CI: 1.98–3.72) and Kang et al.[41] (relative risk [RR]: 2.09; 95% CI: 1.53–2.86) pointed out that anti-HLA antibodies mediated by the complement system impaired survival and increased the risk of allograft rejection. Therefore, investigating the formation of anti-HLA antibodies is very relevant because it can assist in the follow-up and therapeutic strategy of individuals after transplantation and after transfusion.[42,43] Nevertheless, bone marrow or organ transplant recipients were not included in the selection of articles in this study because a prior allosensitization of anti-HLA antibodies could be added to sensitization by platelet donation.[44–46]

These studies do not inform which HLA antibodies were found, with only three studies by Wu et al.,[25] Pai et al.,[33] and Peña et al.[26] showing specificities of anti-HLA antibodies. The studies by Wu et al.[25] and Peña et al.[26] showed antibodies against the HLA-A*02 allelic group. As seen in allelefrequencies.net,[47] this is the most diverse allelic group present in all populations around the world, especially the following populations from the Americas: USA San Francisco Caucasian HLA (n = 220; allele frequency [AF]: 0.755), Mexico Zapotec

NA-DHS_7 (G) HLA (*n* = 20; AF: 0.7), Mexico Mixe NA-DHS_6 (G) HLA (*n* = 20; AF: 0.7), Ecuador Cayapa HLA (*n* = 183; AF: 0.762), and Bolivia/Peru Quechua NA-DHS_12 (G) HLA (*n* = 21; AF: 0.785), considering frequencies over 70%. On the other hand, populations for which there were no reports of its presence were: Colombia Kogi NA-DHS_17 (G) HLA (*n* = 15; AF: not reported), Brazil Vale do Ribeira Quilombos HLA (*n* = 144; AF: 0), India West Coast Parsi HLA (*n* = 50; AF: 0), Papua New Guinea Wosera Abelam HLA (*n* = 131; AF: 0), Papua New Guinea West Schrader Ranges Haruai HLA (*n* = 55; AF: 0), Papua New Guinea Madang HLA (*n* = 65; AF: 0), Papua New Guinea Karimui Plateau Pawaia HLA (*n* = 80; AF: 0). and Papua New Guinea East New Britain Rabaul HLA (*n* = 60; AF: 0).

In the study by Pai et al.,[33] antibodies against HLA-A*11 were detected. According to the allelefrequencies.net, this allelic group has a low distribution around the world and is only found in a few South and Southeast Asian countries (India, Myanmar, China, Vietnam, Malaysia, Thailand, Taiwan, and the Philippines). High expressions of this allele are found in Myanmar Kayar (*n* = 55; AF: 0.655). It is almost absent in the countries of sub-Saharan Africa (Natal Zulu, Burkina Faso, and Mozambique), North Africa (Morocco, Algeria, Tunisia, Sudan), and Australia.

From the point of view of clinical importance, HLA polymorphisms can generate a certain degree of susceptibility or be a protective factor against diseases since two of the most common mechanisms can affect the diversity of these alleles: 1. frequency-dependent selection, in which an individual with a rare allele may have a chance of survival in an epidemic and 2. heterozygous advantage, in which the individual may be better prepared to fight different pathogens by having a wide repertoire in the adaptive immune system, including Treg cells. Thus, HLA may play a direct role in predisposing to disease, or the polymorphism may be in linkage disequilibrium, and HLA acts as a marker.[48,49] For example, one study appointed greater HIV vaccine efficacy for participants who expressed HLA A*02.[50]

Taking everything into account, we can see that if there is a population with similar HLA proteins and these proteins are present on platelet surfaces, allosensitization of an immunological cause is somewhat predictable and can trigger platelet refractoriness and hinder the expected transfusion response. Therefore, when all hypotheses of non-immunological causes have been ruled out in the hemotherapy service, it is essential to investigate this immunological cause or integrate it into the daily transfusion service to prevent this from happening, especially in patients who are going to undergo multiple transfusions or who have a history of transplants, pregnancy, or previous transfusions.

In eight of the fourteen studies (Table 2), the most widely used methodology for detecting anti-HLA antibodies was the cell-based complement-dependent cytotoxicity assay (CDC), a technique introduced by Terasaki and McClelland in 1964.[51] The CDC is a technique based on cell lysis mediated by the binding of HLA molecules (expressed on the cell surface) to specific anti-HLA antibodies with subsequent activation of complement system proteins. This methodology, despite being low-cost, has some limiting factors such as different levels of expression of HLA antigens on the cell membrane, sensitivity to changes in reagents, incubation time or washing steps, and requires high cell viability and high purity, and may be susceptible to contamination with red blood cells, platelets or granulocytes.[52,53] There are also methods for detecting antibodies using solid-phase assays such as microtiter plates like the enzyme-linked immunosorbent assay, and microbead tests in a flow analyzer, based on Luminex®/FlowPRA™ technologies. The advantages of these methods are greater sensitivity, less subjectivity in interpreting the results, and the ability to identify antibody isotypes and detect complement-fixing and non-fixing antibodies. Some disadvantages are the high cost, reagent inconsistency, particularities of the cytometer, and the conformation of HLA epitopes that can change after purification.[52]

This review did not find biases in the studies as shown by the funnel plot, revealing a good selection and database search, i.e., with minimal inclusion of gray literature. The results were shown to have low heterogeneity. Furthermore, although the investigation of immune-induced refractoriness by the formation of anti-HLA antibodies in patients has existed since the 1950s,[54] several clinical studies are being carried out which have a high distinction in the quantitative sample population and techniques employed. Therefore, there is no methodological consensus on transfusion in the literature, and no systematic review with meta-analysis has been carried out with the current research question. In Brazil, the investigation of anti-HLA antibodies is not mandatory in pre-transfusion testing of platelet concentrates, unlike the investigation of antigens in red blood cell concentrates. This study, therefore, is concerned with individuals receiving massive platelet transfusions. Because of their underlying disease, they may be exposed to transfused platelet antigens and be sensitized with anti-HLA antibodies, potentially triggering immune-induced refractoriness with potentially life-threatening consequences.

Taking into account the reality closest to the authors (in Brazil), according to Consolidation Ordinance No 5, Annex IV (54). which deals with blood, components, and their derivatives, the mandatory pre-transfusion tests for platelet concentrates are: 1) ABO (direct and reverse) and RhD typing in the recipient's blood and 2) testing for irregular anti-erythrocyte antibodies in the recipient's blood. As can be seen, the research has an erythrocyte immunohematology approach only, but it is known that platelets have specific antigens (HPA), as well as HLA and ABO antigens. According to the same ordinance, "The pool of de-leukocytes platelet concentrates, obtained from whole blood, must contain $<5.0 \times 10^6$ leukocytes or each unit must contain $<0.83 \times 10^6$ leukocytes".[55,56]

The double-blind, prospective, randomized, multicenter study conducted by The Trial to Reduce Alloimmunization to Platelets Study Group[57] provided one of the first pieces of evidence on the equal effectiveness of different methods of platelet treatment by leukoreduction and ultraviolet B irradiation in preventing alloimmunization and refractoriness to platelet transfusions in patients with thrombocytopenia due to acute myeloid leukemia. This evidence has led to clinical implications and recommendations, currently applied in Brazil. This includes the use of leukoreduced and irradiated platelets in certain groups of patients as part of the transfusion protocol, intending to significantly reduce the development of

antibodies and alloimmune refractoriness when bags with filters are used to reduce the incidence of antibodies present in contaminated leukocytes found in the remaining plasma, as demonstrated by Brand et al.[58]. Therefore, despite this possibility of deleukocytation, the multi-institutional study TRAP also showed that 17%–20% of patients developed anti-HLA antibodies even after leukoreduction processes [57].

Duquesnoy et al. pointed out that platelets with cross-reactive HLA antigen compatibility can contribute to refractiveness, since even with HLA compatibility, unsatisfactory results can sometimes occur. This can be explained by the presence of non-HLA antigens, such as HPA.[59] Also, thrombocytopenic patients may not be able to undergo complete platelet phenotyping (including HPA) due to the insufficient number of samples. There is one caveat to these specific antigens (HPA): the genotype does not always correspond to the phenotype, especially in patients who are heterozygous for hereditary thrombopathies such as Glanzmann thrombasthenia and Bernard-Soulier syndrome. In other words, the patient may have a heterozygous genotype profile, but phenotypically have homozygosity, because one of the alleles is not expressed on the platelet surface.[60–62]

The first factor to be addressed that can contribute to the fact that this investigation of anti-HLA antibodies is not yet required as a pre-transfusion test is that it is known that HLA antigens are highly polymorphic, making it difficult to obtain several HLA-typed donors and thus provide HLA-compatible platelets for allosensitized patients. Secondly, it is important to remember that in these polymorphic HLA regions, there are epitopes shared between private and public antigens, so it is also necessary to analyze cross-reactivity, as this approach can help sustain an HLA-compatible platelet program, reducing the number of donors needed.[63] Thirdly, there is variable expression of HLA antigens on the surface of platelets. Fourthly, the satisfactory survival of platelets due to HLA compatibility is not absolute as pointed out by the studies selected in this systematic review, due to other immunological factors like HPA or non-immunological factors that were not covered here. Fifthly, the investigation of immune-induced platelet refractoriness or the consideration of its prevention should be carried out in partnership between erythrocyte immunohematology, platelet immunohematology, and immunogenetics laboratories. At the very least, this requires alignment between institutional management, a multi-professional team, the necessary equipment for the laboratory activity, and reproducible and applicable protocols to enable collaboration, sharing, and discussion of cases between the medical and scientific communities. Unfortunately, this entire organization is not yet present in Brazil or even globally, due to inter-laboratory and intra-laboratory differences, and the distinct availability of financial resources.

It is a practice with many obstacles, but with institutional, essentially staff support, theoretical and technical training, it is possible to do a good job as seen in some hospitals like Hospital das Clínicas de Porto Alegre (HCPA) where they demonstrated a good response with the use of a platelet protocol.[64] In this way, it will not only be possible to increase the survival of platelets in the individual but also to reduce transfusions (saving blood components that depend on the solidarity of blood donation and corroborate with the recent research on patient blood management), as well as minimizing hospitalizations in emergency rooms at transfusion agencies and reducing hospital stays when needed. Only the future will tell whether the program can be sustained through this entire support network. Those who already implanted it can be largely responsible for showing its importance and contributing to developing clinical procedures and public policies.

Suggestions for future research

To perform a systematic review on the impact of chemotherapies in reducing alloimmunization in platelet-transfused patients. To investigate platelet allocation for transfusions on reducing alloimmunization in thrombocytopenic patients, based on the different collection bags and special procedures performed. To analyze immune and non-immune causes of platelet-refractory patients in the local reality. Adopting detection techniques of immunological causes that can be used in the economic context and available human resources to elaborate a management protocol of platelet refractoriness. After the protocol for refractory individuals is adopted, verify the effects of platelet transfusions on survival rates.

Conclusion

This work shows that anti-HLA antibodies contribute to approximately 175-fold higher chances of triggering immune-induced platelet refractoriness. Therefore, it is interesting for hemotherapy services to investigate the existence of individuals with this condition in their local reality and with the available resources. Furthermore, this study demonstrates the need to develop public policies for identifying and monitoring anti-HLA antibodies in patients and to perform HLA matching to promote the prevention of sensitization of patients to platelet transfusions and the development of platelet refractoriness. Thus, diagnostic techniques may contribute to the excellent quality of transfusion services with the discovery and subsequent selection of more compatible platelets.

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Conflicts of interest

The authors declare no conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.htct.2025.103821.

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Review article

Optimization of hydroxyurea in sickle cell disease in Brazil

Clarisse Lobo [a], *Ana Cristina Silva-Pinto* [b], *Rodolfo Delfini Cançado* [c,*]

[a] *Instituto Estadual de Hematologia do Rio de Janeiro HEMORIO, Rio de Janeiro, RJ, Brazil*
[b] *Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil*
[c] *Faculdade de Ciências Médicas da Santa Casa de Sao Paulo, Sao Paulo, Brazil*

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ABSTRACT

Despite sickle cell disease (SCD) being a well-recognized and highly prevalent condition identified early through neonatal screening programs, it represents a substantial public health challenge due to high morbidity and premature mortality rates. Hydroxyurea (HU) is the only available disease-modifying therapy for SCD approved in Brazil. Indeed, its underutilization highlights the need for improved therapeutic strategies to enhance adherence and management of SCD. Innovative formulations of HU might favor treatment adherence and precise dosing. Thus, we aimed to describe HU's pharmacological characteristics, clinical efficacy, and tolerability, including dose escalation. Recent interventional and observational studies revealed the efficacy and safety of an innovative formulation: dispersible scored tablets of 100 mg and 1000 mg, allowing easier dose adjustments and, consequently, more precise dosing. The 100 mg tablets scored can be cut into two parts of 50 mg, and the 1000 mg tablets can be cut into four parts of 250 mg. The fractionating dose is possible due to the formulation technology that allows the tablet to be cut with a uniform amount of drug in each part. This new formulation of HU, suitable for children, may influence the prognosis of SDC, regardless of associated symptoms.

Introduction

Hemoglobinopathies, including sickle cell disease (SCD), are the most common inherited disorders worldwide.[1] Sickle cell disease (SCD) is a term that encompasses a variety of genetic disorders characterized by the presence of abnormal hemoglobin, primarily hemoglobin S (HbS).[2,3] The most prevalent genotype are: HbSS usually called sickle cell anemia (SCA), HbSC, and HbSβ-thalassemia subtypes characterized by mutations in the gene encoding the hemoglobin subunit β (HBB).[2,3] The most common form of SCD is sickle cell anemia (SCA), which occurs when an individual inherits two copies of the HbS gene (HbSS). In contrast, sickle cell disease with hemoglobin C (HbSC) occurs when an individual inherits one HbS gene and one hemoglobin C gene (HbC). The clinical manifestations of SCD can vary significantly between these genotypes, with SCA generally presenting more severe symptoms and complications compared to HbSC disease.[4]

* Corresponding author. Faculdade de Ciências Médicas da Santa Casa de Sao Paulo, Rua Conselheiro Brotero, 1486 CE P01232-010, Sao Paulo, SP, Brazil
E-mail address: rodolfo.cancado@gmail.com (R.D. Cançado).

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The burden of SCD particularly affects low-middle income countries (LMICs) from sub-Saharan Africa, Arab countries, the Mediterranean, the Indian subcontinent, the Caribbean, and South America, as well as African Americans and descendants of immigrants from these countries all over the world.[5,6] One of the highest incidences of SCD, however, is reported in Africa with an estimated global birth rate of 300,000 annually[6]. There are an estimated 90,000 to 100,000 individuals with SCD in the USA and 60,000 to 100,000 individuals living with SCD in Brazil.[6,7] Of note, SCA, the most common genotype and most severe variant of SCD, makes up >80% of all SCD cases, showing its dominance among sickle cell disorders.[8] Regarding mortality, significant death rates presents an alarming situation occur in LMICs where SCD is more prevalent.[9] SCA in children is an alarming situation due to high morbidity and mortality from vaso-occlusive crises, acute chest syndrome, and infections.[10–12] In Brazil, one of the countries most affected by the disease, Lobo et al. reported in a large sample of 1676 patients with SCD from a referral tertiary hospital (1998 to 2012), 281 deaths (mortality rate: 16.8%).[13] The most frequent causes of death were infection, SCA, overt stroke, organ damage, and sudden death during painful crises.[13] Populational-based data revealed 6553,132 deaths in Brazil from 2015 to 2019, identifying 3320 deaths from SCD (mean age: 32 years).[14] Also, an estimated annual economic burden of approximately 400 million USD was estimated from a reference center in Brazil.[15]

Despite SCD being a well-recognized and highly prevalent condition identified early through neonatal screening programs, it represents a substantial public health challenge due to high morbidity and premature mortality rates. Hydroxyurea (HU) is the only available disease-modifying therapy for SCD approved worldwide. Innovative formulations of HU as dispersible scored tablets might favor treatment adherence and precise dosification. Our objective was to detail an overview of pharmacological characteristics, clinical efficacy, safety profile, and current guidelines regarding the use of hydroxyurea in the management of SCD in Brazil.[16,17] Indeed, we update SCD treatment focused on HU and its innovative formulation in dispersible scored tablets. Treatment of SCD

The management of SCD patients may include using hydroxyurea (HU), folic acid, blood transfusion, iron chelation, antibiotic therapies, vaccination, hematopoietic stem cell transplantation (HSCT) and gene therapy.[1,2,18]

Hydroxyurea

HU is an inhibitor of ribonucleotide reductase with many beneficial effects for treating people with SCD, including increasing HbF concentration in red blood cells (RBC), improving nitric oxide metabolism, reducing red cell-endothelial interaction, and erythrocyte density.[1,2,25] Such disease-modifying effects have been shown to decrease vaso-occlusive crises (VOC), SCA, the number/length of hospitalizations, and the need for transfusions, noticeably reducing the mortality rate and improving overall survival.[1,2,18]

HU has a direct effect on the pathophysiological mechanisms of SCD, acting not only by increasing the synthesis of HbF but also by promoting a decrease in the number of neutrophils and erythrocyte adhesion molecules, thus directly contributing to the reduction of inflammatory phenomena and vaso-occlusion.[1,2] It was also observed that HU therapy is associated with an increase in intravascular and intraerythrocytic production of nitric oxide facilitating vasodilation, which represents another direct effect of the drug on the pathophysiological mechanisms of SCD.[1,2,25,26]

In clinical practice, the beneficial effects of HU are observed in the first weeks after its introduction.

The main benefits of HU scientifically proven are the elevation of HbF to a desirable level of 20% or more, with consequent prevention of sickling and a significant reduction in disease complications (morbidity) and, almost 50% in mortality.[19] Data shows that this result is usually achieved in patients who received escalating doses to a maximal tolerated dose (MTD).[20] In addition, it is cost-effective based on fewer hospitalizations, and positive impact on quality of life, physical and psychosocial, with oral formulation, which favors a better adherence to treatment.[21–24]

HU is still the most used disease-modifying therapy approved for SCD worldwide. Since 1996, HU has been released in the United States and Europe to treat SCA (the severe genotypes with more prominent anemia, hemolysis, and clinical complications). Of note, patients with "milder" genotypes (e.g., HbSC) were excluded from randomized clinical trials (RCTs) that were used to support the Food and Drugs Administration (FDA) approved. Due to the benefits of HU observed in adults, this drug is currently under approval for children older than 9 months, particularly SCD with severe manifestations of the disease in the United States and the United Kingdom.[25–27]

In Brazil, HU has been recommended for adults and children (> 2 years old) with a worse prognosis since 2002. In 2018, the Brazilian treatment protocol of SCD, “Protocolo Clinico e Diretrizes Terapêuticas (PCDT) de Anemia Falciforme” included using HU for children from 9 months of age considering situations of chronic organ damage and hemolysis as indications for drug use.[28] The inclusion of patients with SCD and 9 months old to treatment of HU should consider the following additional criteria: dactylitis (in the first year of life); Hb concentration lower than 7 g/dL (average of 3 values outside of an acute event); or leukocyte count higher than 20,000/mm^3 (average of 3 values outside of an acute event).[28] In 2024, the ”Consensus of the Brazilian Association of Hematology, Hemotherapy and Cellular Therapy (ABHH) and the Brazilian Ministry of Health” included HU treatment of children under two-years in the presence of HbSS, $HbSB^0$, HbSD Punjab and HbSB+ with <10% of Hb A, regardless any symptoms.[29] In 2024, the Brazilian treatment protocol of SCD, “Protocolo Clinico (PCDT) de Anemia Falciforme” was updated, according to these same criteria.

The Therapeutic Response Evaluation and Adherence Trial (TREAT) has shown that early initiation of HU, with personalized, pharmacokinetically guided dosing to optimize benefits and reduce toxicity, has the potential to be nearly curative. This approach leads to high levels and pancellular expression of Fetal hemoglobin (HbF) within red blood cells, resulting in a

significant reduction of clinical complications in most patients who adhere to the treatment.[30]

Pre-treatment considerations

Before initiating the treatment, it is recommended the following tests:

A complete blood count (CBC) that includes mean corpuscular volume (MCV), white blood cell (WBC) differential, reticulocyte count, and platelet count; hemoglobin (Hb) electrophoresis with HbF measurement (high-performance liquid chromatography HPLC, if available); a comprehensive metabolic profile that includes renal and liver function tests, and a pregnancy test for women of reproductive age. It is essential to highlight that even in patients with baseline elevation of HbF, it should not affect the decision to initiate HU therapy, and both males and females of reproductive age should be counseled regarding the need for contraception being HU a cytostatic drug which a potential teratogenic concerns.[1,2,25,26,28,29]

Initial dosing

According to international guidelines[27.23,24,] including the Brazilian[28,29], HU must be used orally at 15 to 35 mg/kg daily. For adults, the initial dose is 15 mg/kg/day, and 20 mg/kg/day for infants and children. The HU dose should be reduced to 5–10 mg/kg/day for adult patients with chronic kidney disease.[27-29]

Regarding its pharmacokinetics, HU is rapidly absorbed after oral administration, reaching a maximum plasma level between 20 and 30 min (fast absorbers) and 60 min (slow absorbers), with a $^1/_2$ time life of three to four hours. HU is metabolized in the liver and excreted via renal (80%).25·26With an initial once-daily dose for adults of 15 mg/kg/day, or children 20 mg/kg/day monitoring the number of leukocytes and platelets (CBC) every two weeks is recommended. 25 This initial dose should be increased by 5 mg/kg/day every 8 to 12 weeks as titration, with the goal being to reach the maximum tolerated dose (MTD). 25 Based on the published data, the MTD is the highest dose capable of promoting the most prominent improvement in the clinical and laboratory parameters, without the occurrence of hematological, hepatic (defined as an increase of twice the maximum reference value of transaminases), renal (increased urea and creatinine) or gastrointestinal toxicity. MTD should not exceed 35 mg/kg/day.[25,26]

Monitoring, dosage modification and response

It is advisable to monitor CBC with WBC differential and reticulocyte count every 4 weeks when adjusting dosage. The target absolute neutrophil count is ≥2000/μL. However, younger patients with lower baseline counts may safely tolerate counts down to 1250/μL and platelet counts ≥80,000/μL.[1,2,18,25,26,27,28,29]

If neutropenia or thrombocytopenia occurs, HU dosing should be suspended, and monitor CBC with WBC differential weekly. When blood counts recover, restart HU at 5 mg/kg/day lower than the previous dose.[1,2,18,25,26,28,29]

If escalation is warranted based on clinical and laboratory findings, increase by 5 mg/kg/day every 8 weeks until mild myelosuppression (absolute neutrophil count 2000/μL to 4000/μL) is achieved up to a maximum of 35 mg/kg/day.[1,2,18,25,26,28]

After a stable dose is determined, follow-up involves conducting blood counts with MCV, WBC differential, reticulocyte, platelet counts, and liver transaminase levels every 2–3 months. The HbF level should be checked every six months; however, its increase can vary significantly and does not always correlate with the clinical response. [1,2,18,25,26,28,29]

Monitor Hb, MCV, and HbF levels for evidence of consistent or progressive laboratory response. A clinical response to treatment with HU may take 3–6 months. Therefore, a 6-month trial on the MTD is required before considering discontinuation due to treatment failure, whether due to lack of adherence or failure to respond to therapy. [1,2,18,25,26,28,29]

A lack of increased MCV and/or HbF does not indicate discontinuing therapy. Hydroxyurea therapy should be continued during hospitalizations or illness. Patients should be constantly reminded that the effectiveness of HU depends on their adherence to daily dosing.[1,2,18,25,26,28,29]

Impact of HU on clinical outcome and mortality

Hydroxyurea has demonstrated effectiveness in reducing mortality and acute events in patients with HbSS. The Multicenter Study of Hydroxyurea Use found a 40% reduction in mortality among adults with HbSS and HbF >5.0 g/L treated with HU compared to those untreated.[26] In a 17-year prospective non-randomized study, HU therapy reduced mortality by 73%.[31] Similar results were seen in a Brazilian retrospective trial of children with SCD (ages 3–18).[32] In this study among 1760 patients, 267 received HU at an average dose of 20.8 mg/kg/day during a mean follow-up of 2 years. HU significantly increased Hb, HbF, and MCV levels while reducing leukocytes, neutrophils, platelets, and reticulocytes. The HU group had a 50% reduction in hospital admissions (0.4 to 0.2 events/year), a 60% decrease in hospital stay length (4 to 1.6 days), and a 35% reduction in emergency room visits. Survival was significantly higher in the HU group (99.5% vs. 94%, $p = 0.01$), with an 87% reduction in mortality risk (OR 0.13, 95% CI 0.02–0.99, $p = 0.049$). Adverse effects were mild; no severe neutropenia or thrombocytopenia was observed.[32] Another Brazilian single-center study showed a significant reduction in infectious episodes with HU treatment (1.03 to 0.5, $p = 0.047$).[33] The Baby Hug study, a multicenter randomized clinical trial performed in 13 centers in USA, included children aged 9–18 months with HbSS or Sβ0 thalassemia, randomized to 20 mg/kg/day of liquid HU ($n = 83$) or placebo ($n = 84$) for 2 years. Of note, the study sample was not selected for clinical severity. As the main findings, HU compared to placebo significantly reduced pain episodes (177 vs. 375, $p = 0.02$) and dactylitis (24 vs. 123, $p < 0.0001$) and showed trends toward lower rates of acute chest syndrome (ACS), hospitalization and transfusions. HU also increased Hb and HbF and lowered leukocyte counts. Toxicity was mild, mostly limited to moderate neutropenia.[34] The TWiTCH (Transfusions Changing to Hydroxyurea) was a non-inferiority, multicenter, open-label, phase 3 study, purposed to evaluate whether Hydroxyurea (HU) could serve as

an effective alternative to chronic red blood cell (pRBC) transfusions, which are the standard treatment, in preventing primary strokes in children with sickle cell anemia. The study involved 121 children aged 4 to 16 years, who had to have a transcranial Doppler (TCD) reading of ≥ 200 cm/s, have received pRBC transfusions for at least one year, and did not exhibit severe vasculopathy on magnetic resonance angiography (MRA). The participants were randomly assigned to two groups: one group continued receiving packed red blood cell (pRBC) therapy, which included 61 children, while the other group switched to hydroxyurea (HU) treatment at MTD, comprising 60 children. In the group that continued pRBC therapy, the average TCD velocity was 145 ± 21 cm/s, while in the group that switched to hydroxyurea (HU), it was 145 ± 26 cm/s. Children in the pRBC group maintained HbS levels below 30% and had an average HbF level of 25%, which was similar to the children in the HU group. The study was halted during the initial analysis after it was found that HU was not inferior to pRBC transfusions in preventing strokes. The maximum mean TCD velocity in the HU group was 143 ± 1.6 cm/s, compared to 138 cm/s in the pRBC group (95% confidence interval, 95% CI: 4.54, 0.10 − 8.98; non-inferiority $p = 8.82 \times 10\text{^}-16$).[35] Additionally, a trial in sub-Saharan Africa comparing fixed doses of HU (20 mg/kg/day) with the MTD (up to 30 mg/kg/day) demonstrated a 79% reduction in hospitalizations, a 70% decrease in transfusion rates, and reductions in acute chest syndrome (73%) and vaso-occlusive crises (57%), with similar toxicity across both groups.[36]

Hydroxyurea precision dosing

The HU formulation for SCD (Sickle Cell Disease) is available in both tablet and capsule forms, marketed under the trade names Siklos® and Tepev FF®. In Brazil and many other countries, only 500 mg capsules have been available, under off-label use, for adults and children in the recent past. However, when adjustments other than 500 mg or its multiples are necessary, the capsule formulation can pose several challenges. These include the need to manipulate the medication at home, the potential waste of leftover doses, and an increased risk of dosing errors due to the complexity of the dosing and dilution process.[1,2,23]

Particularly, the recent formulation of HU (Siklos®) is an innovative drug in water-dispersible scored tablets of 100 mg and 1000 mg, which allows dose adjustments for easier understanding of directions for patients or caregivers, thus favoring treatment adherence and precise dosification. The 100 mg scored tablets can be cut into two 50 mg parts, and the 1000 mg scored tablets can be cut into four parts of 250 mg each. The fractionating dose is possible due to the formulation technology that allows the tablet to be cut with a uniform amount of drug in each part.[37,38]

The European Sickle Cell Disease Cohort – Hydroxyurea (ESCORT-HU study) is a, prospective cohort multicenter study conducted in SCD patients treated with HU according to current clinical practice in Europe. Siklos® was administered to children and adults. The patients previously treated with the capsule formulation (500 mg) were switched to HU tablets and included in the study as non-naive patients. The other half of the patients enrolled in the ESCORT-HU started their HU treatment with the tablets (never users, naïve patients). Over 1906 participants aged 2 years and older (55% adults) with symptomatic SCD were enrolled, with a median follow-up of 45 months covering 7309 patient-years of observation. HU average doses were 20.6 mg/kg/day for children and 16.3 mg/kg/day for adults. statistically significant reductions were observed in VOC episodes lasting >48 h, acute chest syndrome, hospitalizations and blood transfusion rates within the first 12 months compared to the previous year.[38] The most common adverse effects were transient neutropenia and thrombocytopenia, with no new toxicity reported as shown in Table 1.

The subgroup of patients receiving HU treatment (N = 926) who transitioned to the dose-adjusted tablet (HU non-naïve) were compared to those for whom HU tablets were

Table 1 – Clinical and laboratory parameters of efficacy one year before and after the treatment of HU fractionable tablets in 1903 participants from the ESCORT-HU study according to age group.

Period of HU fractionable treatment	Age <18 years (*n* = 849)				Age >18 years (*n* = 1054)			
	Number	Previous year	After 1 year*	Change Pre-post (p value)	Number	Previous year	After 1 year*	Change Pre-post (p value)
N°. of VOC, mean (±SD)	682	1.6 (2.1)	0.9 (1.6)	−50% (<0.05)	907	1.8 (2.6)	0.9 (1.9)	−38% (<0.05)
N°. of ACS, mean (±SD)	708	0.3 (0.7)	0.1 (0.3)	−67% (<0.05)	940	0.3 (0.6)	0.1 (0.4)	−67% (<0.05)
N° of hospitalizations, mean (±SD)	681	1.7 (1.8)	0.93 (1.5)	−46% (<0.05)	930	1.3 (1.8)	0.7 (1.3)	−44% (<0.05)
Days of Hospitalization due to SCD, mean (±SD)	628	9.7 (12.1)	5.5 (10.1)	−46% (<0.05)	833	8.1 (13.4)	4.4 (10.8)	−43% (< 0.05)
No. of patients (%) with at least one blood transfusion	810	369 (45.6)	199 (24.6)	−21% (<0.001)	1024	400 (39.1)	177 (17.3)	−21.8% (<0.001)

Abbreviations: ACS, acute chest syndrome; HU, hydroxyurea; SCD, sickle cell disease; VOC, vaso-occlusive crises.
Adapted from Montalembert et al.38.
*After 1-year: Within 1-year after the HU fractionable tablet.

Table 2 – Number of clinical outcomes before and after one year of treatment with HU fractionable tablets in the subgroup of participants from the ESCORT-HU.

Previous treatment with another HU formulation	HU Non-naïve (N = 926)		HU Naïve (N = 976)	
	Previous year	After 1 year*	Previous year	After 1 year*
N° of VOC, Mean ± SD (95% CI)	2.79 ± 2.66 (2.68 to 2.89)	0.77 ± 1.37 (0.68 to 0.86)	2.96 ± 2.69 (2.79 to 3.12)	0.79 ± 1.94 (0.66 to 0.92)
N° of ACS, Mean ± SD (95% CI)	1.20 ± 0.53 (0.85 to 1.54)	0.11 ± 0.38 (0.08 to 0.13)	1.30 ± 0.81 (0.79 to 1.80)	0.07 ± 0.27 (0.05 to 0.09)
N° of hospitalizations, Mean ± SD (95% CI)	2.36 ± 1.85 (2.27 to 2.44)	0.73 ± 1.26 (0.64 to 0.81)	2.51 ± 1.63 (2.40 to 2.61)	0.67 ± 1.33 (0.58 to 0.75)

Abbreviations: ACS, acute chest syndrome; HU, hydroxyurea; SCD, sickle cell disease; VOC, vaso-occlusive crises; 95%CI, 95% confidence interval.
Adapted from Galactéros et al., 2022.37.
*After 1-year: Within 1-year after the HU fractionable tablet.

introduced during the ESCORT-HU study (HU naïve) (N = 976). In the subgroup of 926 patients, there was an increase in MCV from 86.15±14.37 to 94.72±16.39 after 12 months of Siklos use, which suggests an increase in adherence since these patients were already using HU (non-adjustable dose of 500 mg) in the previous year, it was also possible to observe in this group, a decrease in the number of VOC events from 2.68 to 2.89 (95%CI) to 0.68–0.86 (95%CI), a decrease in the number of acute chest syndrome (ASC) 0.85- 1.54 (95%CI) to 0.08–0.13 (95%CI) and in the number of hospitalizations from 2.27 to 2.44 (95%CI) to 0.64–0.81(95%CI). There was no increase in adverse events compared to HU non-naïve with HU naïve patients. These data favored medication adherence with the Siklos® adjusted daily dose, however, no specific analysis was performed [37]. The ESCORT-HU trial provides evidence about long term benefit and safety in a large group of participants enrolled. The clinical and laboratory outcomes with HU adjusted tablet (precision dosing: fractionable tablet) are shown in Tables 2 and 3.[37]

HU adverse effects and toxicity

Overall, the chronic use of HU is not related to severe adverse effects (AEs) such as death in the context of SCD, mainly SCA.[25,34,37,38,39] Common AEs reported by most studies (RCTs and observational) performed in adults and children with SCD included hematological symptoms (myelosuppression), gastrointestinal issues (nausea, diarrhea, constipation, anorexia), vasculitis toxicities, macrocytosis, onychomadesis, rash, hair loss, headache, dizziness, stomach pain, swelling, dry skin and nail pigmentation.[25,34,37,38,39] Neutropenia and thrombocytopenia, the most common effects observed with HU treatment, were reversed with temporary drug interruption, usually recovered within two weeks.[25,34,37,38,39] Particularly, the Baby-HUG trial (2011) performed in children with SCA, gastroenteritis and dactylitis occurred less frequently in those receiving hydroxyurea compared to placebo ($p < 0.001$). Other less frequent AEs, sepsis or bacteraemia occurred three times in those receiving HU and six times in the placebo group, but without statistical significance. Episodes of splenic sequestration were also equal in the two groups. Toxicity was limited to mild-to-moderate neutropenia (500–1250/mm^3), higher in the HU group than placebo.[34] In the RTC of Charache et al.[25] performed in adults with SCA, AEs were equally common in both placebo and active treatment groups. In the ESCORT-HU, real-world observational cohort performed with HU fractionable (scored/breakable) tablets, no reported differences between non-users (naïve) and previous users of HU capsules (non-naïve) regarding most common AEs incidence (<5% in both groups) were neutropenia, thrombocytopenia and dry skin.[37]

Other toxicities reported such as renal and hepatic toxicities are rare, and there is no evidence of increased cancer incidence with prolonged HU use. In males treated during childhood, no toxic effects on sperm tests were found, and spermatogenesis toxicity is not a significant concern in boys requiring HU treatment before puberty.[1,2,37,38,39]

Table 3 – Biological parameters before and after 12 and 24 months of the treatment with HU fractionable tablets in the subgroup of participants from the ESCORT-HU.

Previous treatment with another HU formulation		HU Non-naïve (N = 926)	HU Naïve (N = 976)
Hb (g/dl), mean ± SD	Baseline	9.01 ± 1.47	8.63 ± 1,60
	12 months	9.01 ± 1.56	9.01 ± 1.41
	24 months	9.04 ± 1.48	9.01 ± 1.46
HbF (g/dl), mean ± SD	Baseline	13.58 ± 9.44	7.12 ± 5.94
	12 months	16.24 ± 10.08	15.05 ± 9.67
	24 months	16.20 ± 9.80	15.34 ± 19.15
MCV (fl), mean ± SD	Baseline	86.15 ± 14.37	85.07 ± 12.34
	12 months	94.72 ± 16.39	89.55 ± 13.44
	24 months	95.31 ± 15.05	89.45 ± 12.91
Neutrophils (10^9/L), mean ± SD	Baseline	4.78 ± 2.44	5.54 ± 3.06
	12 months	4.41 ± 2.51	4.61 ± 2.98
	24 months	4.07 ± 2.01	4.23 ± 2.49

Adapted from Galactéros et al.37.

Barriers to the use of HU

Lobo et al., analyzed 1144 patients with SCD at HEMORIO (Rio de Janeiro), and observed that HU was prescribed to 40.5% of the children and 36.4% of adults.[32] Carneiro-Proietti et al. reported using HU in 29.3% of children (458 of 1104 patients) and 36.3% of adults (447 of 1044 patients).[40]

The attainment of optimal HU benefits requires selecting and maintaining the proper dose, which varies widely from one patient to the next. Inadequate HU dosing results in suboptimal clinical responses, poor medication adherence, and decreased utilization of HU as a disease-modifying and life-saving drug.[41]

HU is underutilized partly due to a lack of awareness of its benefits on the part of patients and providers, which compromises patient adherence; that is the primary reason why HU therapy is ineffective in children and adults with SCD, others reasons are: concerns regarding adverse events (i.e., myelosuppression), need for regular laboratory monitoring, uncertainties surrounding possible adverse effects on reproduction and fertility.[1,2,39,42]

Moreover, the new tablet formulation of 100 mg and 1000 mg scored tablets enables physicians to prescribe an accurate dose, as well as to perform dose escalation in 50 mg or 250 mg increments progressively and continuously until reaching the maximum tolerated dose. This approach is recommended and aligns with the pharmacodynamic features of hydroxyurea.[43]

To optimize HU treatment for both children and adults, it is crucial to address barriers related to its use, ensuring treatment is tailored to the patient's body weight and biological and clinical response. The introduction of fractionable (scored/breakable) tablet formulations, especially for children under 6–7 years of age, will facilitate appropriate dosing and may improve adherence.[37,38] This is because tablets allow for more precise dose adjustment, which is particularly important for patients who require individualized titration. The ability to tailor the dosage to each patient's specific needs enhances the effectiveness of the treatment and reduces the risk of under or over-dosing. Moreover, the convenience and ease of administering tablets including the benefits of dissolving water if needed may contribute to better treatment adherence. Patients are more likely to consistently take their medication when the process is straightforward and manageable, especially in the context of chronic conditions like SCD, where long-term adherence is critical. Improved adherence, in turn, positively impacts clinical outcomes, as patients are more likely to experience the full therapeutic benefits of the treatment. Therefore, the results of clinical studies suggest that tablet formulation can be a key factor in enhancing adherence, in the observed therapeutic success. Additional studies about the adherence of HU in fractionable (scored/breakable) tablets are needed since no specific evaluation was performed in the ESCORT-HU trial.[37,38]

Conclusion

Hydroxyurea (HU) has significantly altered the natural history of SCD globally, reducing mortality rates and improving overall survival. Despite its cost-effectiveness, efficacy and safety, HU should not be overshadowed by new SCD treatments. In Brazil, although HU is available through the Unified Health System (SUS), it is often administered in suboptimal doses with poor adherence, leading to high mortality rates, particularly among children. The introduction of new technologies for precise dosing and improved adherence is timely. However, ongoing efforts are needed to raise awareness among prescribers, patients, and parents, and to ensure greater commitment from governments, manufacturers, and society to guarantee HU's availability and accessibility for all SCD patients.

Conflicts of interest

CL is a consultant from EMS, Masters Speciality Pharma, Global Blood Therapeutics (GBT), Agios, Pfizer, and Novartis. ORCID: 0000–0002–4262–5792, **ACSP** is a consultant from EMS, Masters Speciality Pharma, Global Blood Therapeutics (GBT), Chiesi Farmacêutica S.p.A., and Novartis. ORCID: 0000–0002–41,042,296, **RDC** is a consultant from Pfizer, CSL Vifor, Masters Speciality Pharma, Global Blood Therapeutics (GBT), and Novartis. ORCID: 0000–0003–32,693,780

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Review article

Iron overload is not the same everywhere: Particularities of iron-metabolism gene mutations in Brazil and a proposal for the investigation and management of iron overload in this population

Paula de Melo Campos *, *Ana Carolina Toreli*, *Dulcinéia Martins de Albuquerque*, *Fernando Ferreira Costa*

Hemocentro, Universidade Estadual de Campinas - Unicamp, Campinas, São Paulo, Brazil

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ABSTRACT

There is no physiological mechanism for the excretion of iron in humans, and excess iron may lead to severe tissue damage if not adequately treated. Iron overload can be caused by genetic factors (hemochromatosis) or acquired conditions (e.g., ineffective erythropoiesis, transfusions, iatrogenic iron treatment, viral hepatitis, alcohol intake, severe liver disease, metabolic dysfunction), and, in many cases, by a conjunction of these factors. Historically, guidelines for the genetic investigation of patients with iron overload have been based on data obtained from Caucasian individuals in Europe and North America. However, due to the genetic heterogeneity of iron overload gene mutations worldwide, these recommendations might not be applicable to other ethnic groups. This study analyzed previously published genetic data obtained from Brazilian patients with iron overload and found a relevant but small prevalence of *HFE* C282Y/C282Y patients when compared to European populations, while mutations of the *TFR2*, *SCL40A1*, *HJV*, *HAMP*, *BMP6* and *SLC11A1* genes seem to be important. This study proposes an adapted algorithm for the investigation and management of iron overload in Brazil.

Introduction

Iron is essential for adequate functioning of the human body. The regulation of its amount in the organism is extremely important, since an excess of iron or iron deficiency has significant adverse effects on the human health. The regulation of the quantity of iron in the human organism is complex and involves the participation of multiple proteins and different biological steps that ultimately lead to the precise control of how much iron should be absorbed in the intestinal tract to keep iron at optimum levels [1,2].

There is no physiological mechanism for the excretion of iron in humans, thus iron that is absorbed in excess or infused during blood transfusion is deposited in different

* Corresponding author. Paula de Melo Campos, Carlos Chagas St, 480, Cidade Universitária Zeferino Vaz, Campinas, São Paulo, Brazil, 13083-878.
E-mail address: pmcampos@unicamp.br (P. de Melo Campos).

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tissues of the body, predominantly in the liver and spleen, and this abnormal deposition of iron, also known as iron overload (IO), leads to severe organ and tissue damage [1–3]. The multiple mechanisms responsible for causing IO are either hereditary or acquired and, in many cases, not yet completely understood. Possible mechanisms include genetic factors, disorders of red blood cells, transfusion, iatrogenic iron treatment, viral hepatitis, alcohol intake, severe liver disease, metabolic dysfunction (metabolic hyperferritinemia) and possibly a combination of two or more of these factors (Table 1) [1–4].

Diagnosing iron overload

The first suspicion of IO arises after a simple medical check-up with the observation of elevated serum ferritin levels, almost always the first iron biomarker to be detected. Starting with high serum levels of ferritin, the diagnosis of IO is very frequently not a simple procedure. Ferritin protein is present in a variety of cells, but predominantly in macrophages, with its main function being to store iron in a way that is safe for the cells [5]. The normal concentration of ferritin in serum is very low (30–200 μg/L in females and 30–300 μg/L in males) [5]. Ferritin in plasma is encountered as apoferritin and its function is still not clear. The main problem with the measurement of ferritin is that a number of relatively common clinical conditions may lead to a variable degree of increase in ferritin levels, but without actual IO [3]. These conditions include any kind of inflammatory disorder, diabetes, acute or chronic liver disease, obesity, regular alcohol intake and metabolic syndrome [3]. Thus, the majority of unselected patients with elevated ferritin levels do not have IO. In fact, the first problem regarding the diagnosis of IO is how to identify, among patients with high ferritin levels, those who have IO irrespective of the cause of this condition, thereby differentiating them from those with no IO [6].

The initial diagnostic approach for a patient with a significant increase in ferritin levels verified by at least two measurements (at least 30 days apart) carried out in a reliable laboratory is to estimate transferrin saturation (TS). The presence of hyperferritinemia in the context of normal TS is associated with IO in a very small group of patients [7]. It is important to emphasize that elevated ferritin and concomitantly elevated TS are associated with IO in about 90 % of cases [5]. Thus, it may be assumed that high serum ferritin and high TS (>45 %) is almost always associated with IO. Alternatively, it is possible to assume that high ferritin and normal or reduced TS is not IO, with very rare exceptions. Transferrin is the protein that safely transports iron in blood.

Table 1 – Co-factors associated with iron overload.

Viral hepatitis
Alcohol intake
Fatty liver disease
Hematological disorders (ineffective hematopoiesis)
Insulin resistance
Poorly controlled diabetes mellitus
Metabolic dysfunction
Exogenous (transfusions, iatrogenic iron reposition)

TS, indicated as a percentage, can be estimated easily by the ratio between serum iron and total iron-binding capacity. Alternatively, TS can be calculated by the ratio between serum iron concentration and serum transferrin concentration (this ratio should be multiplied by a correction factor of 1.42) [5]. Usually, the normal range for TS in different populations varies from 25% to 45 %. A TS higher than 45 % has been defined as elevated and indicates individuals with probable IO [2,7]. Of note, since the TS is the ratio between serum iron and total iron-binding capacity, expressed as a percentage, TS might be elevated by causes that reduce transferrin levels, such as cirrhosis and dyserythropoiesis, in the absence of IO, which should be take **into** account when analyzing patients [3]. Notably, in patients with hemochromatosis, the elevation of TS occurs significantly earlier than the increase in serum ferritin [8].

Although IO should be considered highly probable, if there are significant increases in both ferritin and TS, an absolute diagnosis of IO can only be made with evidence of higher iron deposits, mainly in the liver [3]. Historically, liver biopsy was used to detect elevated iron deposits, with the advantage of identifying iron distribution in liver cells. However, liver biopsy is a very invasive procedure, and it is no longer routinely carried out for the identification of IO. Currently, the most practical way to estimate liver iron concentration is by T2 magnetic resonance imaging (MRI) [2]. The concomitant findings of elevated serum ferritin levels, TS higher than 45 % and increased liver iron storage estimated by T2 MRI (or, in exceptional cases, liver biopsy) can confirm IO, without doubt, irrespective of the cause.

Investigation of iron overload

Once IO is identified, the next step is the investigation of the etiology of the iron excess, which is sometimes difficult and complex. In addition, there is some confusion and lack of uniformization in terms of nomenclature and the classification of these disorders. One possible approach to differential diagnoses of IO is presented in Table 2.

Table 2 – Diagnosis of iron overload (IO) based on the probable etiology.

Category	Condition
Hereditary	• Hemochromatosis • Aceruloplasminemia (low TS) • Ferroportin disease (normal or low TS) • Hereditary hematological disorders (with or without anemia) with iron overload (ineffective erythropoiesis) – with or without transfusion • Iron metabolism gene mutations associated with porphyria cutanea tarda
Acquired	• Acquired hematological disorders (with or without anemia) with iron overload (ineffective erythropoiesis) – with or without transfusion • Metabolic hyperferritinemia • Excess iron intake, oral or infusion (iatrogenic, chronic dialysis) • Severe liver dysfunction • Excessive and prolonged alcohol consumption

Before starting an investigation into the abnormalities in the genes involved in the iron metabolism pathway, clinicians should investigate iatrogenic iron intake and previous blood transfusions as possible causes of IO, especially for individuals without a positive family history of IO [9]. In addition, they should identify patients with strong evidence of hematological disease that could lead to ineffective erythropoiesis and increased iron absorption (i.e., hemolytic anemias, myelodysplastic neoplasms, sideroblastic anemias, among others), even if they are not regularly transfused [9]. Given that some rare mutations in heterozygotes for beta thalassemia may show normal red blood cell parameters, in exceptional cases it is recommended to carry out the sequencing of the *β-globin* gene to exclude the association of beta-thalassemia trait and IO with other possible molecular abnormalities related to iron metabolism [10,11]. Porphyria cutanea tarda is also a genetic condition that may be associated with mutations in genes related to iron metabolism and with IO. Although the precise mechanism involved in this association is not completely understood, it is recommended to investigate IO in patients diagnosed with porphyria cutanea tarda [12].

Causes of acquired environmental risk factors for hepcidin deficiency, such as alcohol consumption and end-stage liver disease should also be evaluated [5]. Another possible condition, provisionally called metabolic hyperferritinemia, presents preserved hepcidin production and a total body iron that is generally normal, but in some cases, may present a slight or even moderate IO [13]. Although iron deposits are not very high in metabolic hyperferritinemia, excessive iron may lead to the formation of reactive oxygen species and subclinical inflammation, potentially worsening glucose and lipid metabolism, fibrogenesis and carcinogenesis [13].

In parallel with the evaluation described above, patients should be screened for mutations in the genes of the hepcidin-ferroportin axis, which can lead to a decrease in the production (or activity) of hepcidin [5] or, more rarely, a mutation in the ferroportin gene that results in resistance to its destruction by hepcidin. Recent data using next-generation sequencing for the whole genome to study genetic abnormalities in IO patients have shown that, besides the classic mutations of the *HFE* gene identified as responsible for IO in the majority of patients from Northern Europe and the United Kingdom (the *HFE* mutations), and mutations of *HJV, TFR2, SLC40A1* and *HAMP* genes (the well-known non-HFE mutations), other genes related to iron metabolism are also very probably involved in the pathogenesis of IO [4,14,15], including for example *BMP-6* [16].

Following the suggestion made by a group of experts from the BIOIRON society [8], we recommend the definition of hemochromatosis (HC) as quoted below:

"The term "hemochromatosis" should be reserved for a unique genetic clinical-pathological condition characterized by increased TS, increased serum ferritin, IO in the liver (but not in the spleen), with prevalent involvement of periportal hepatocytes with iron-spared Kupffer cells, and signs and/or symptoms associated with IO. The panelists also emphasized that the term "hemochromatosis" itself implies an IO of genetic origin, which is why they would recommend avoiding the unnecessary use of qualifiers such as "hereditary", "genetic", or "primary". Indeed, genetic defects in the hepcidin/ferroportin regulatory axis (caused by variants in hepcidin regulators, the hepcidin gene itself, or in ferroportin) are responsible for inadequate production or activity of hepcidin or lack of hepcidin responsiveness of ferroportin" [5].

The major problem with the classic classification of HC (Table 3) is its limitation for the inclusion of all the new possible variants that are being described. In addition, the controversial inclusion of ferroportin disease as a subtype of hemochromatosis should also be mentioned.

The new classification proposed by the BIOIRON Society is more flexible and comprehensive, since it allows the inclusion of patients with digenic mutations and HFE/non-HFE compound heterozygosity. Moreover, it allows the inclusion of patients with newly described mutations pending confirmation or not yet identified as causing IO, potentially indicating a provisional diagnosis, as described in Table 4. We strongly recommend the use of this new classification.

Since the discovery that mutations of the *HFE* gene could lead to IO [17], the analysis of *HFE* mutations has been the mainstay for the investigation of hereditary IO in the general population. Evidence shows that C282Y homozygosity

Table 3 – Former classification of hemochromatosis.

Classification	Gene involved and location	Inheritance	TS	Other clinical features
Type 1	*HFE* (homeostatic iron regulator)	AR	Increased	Adult-onset; more severe in males; highly variable clinical expression, with predominant liver damage and arthritis
Type 2A	*HJV* (hemojuvelin)	AR	Increased	Earlier onset (e.g., <30 years old); similar severity in both sexes; prevalent cardiac and endocrine involvement
Type 2B	*HAMP* (hepcidin)	AR	Increased	Earlier onset (e.g., <30 years old); similar severity in both sexes; prevalent cardiac and endocrine involvement
Type 3	*TFR2* (transferrin receptor 2)	AR	Increased	Very rare (look for parental consanguinity); clinically similar to Type 1, with an earlier onset
Type 4A	*SLC40A1* (ferroportin)	AD	Low-normal	Adult-onset; IO in the spleen; mild anemia; possible low tolerance to venesection
Type 4B	*SLC40A1* (ferroportin)	AD	Increased	Very rare; in general, clinically similar to Type 1, but more severe/early onset forms are reported

Modified from Girelli et al. [5]
AD: autosomal dominant; AR: autosomal recessive; TS: transferrin saturation.

Table 4 – Novel classification of hemochromatosis – from the recommendations of the BIOIRON Society [5].

Novel classification	Molecular pattern	Note
HFE-related	C282Y homozygosity or compound heterozygosity of C282Y with other rare *HFE* pathogenic variants or *HFE* deletion	Low penetrance; consider presence of host-related or environmental cofactors for IO In subjects with other HFE genotypes (e.g., C282Y /H63D compound heterozygosity or *p.His63Asp* homozygosity) consider second-line genetic testing for rarer variants
Non-*HFE*-related	Rare pathogenic variants in "non-*HFE*" genes: • *HJV*-related • *HAMP*-related • *TFR2*-related • *SLC40A1* (GOF)-related	Potentially, mutations in any hepcidin-regulatory gene may be causative (the effects of novel mutations should be confirmed through functional and epidemiological studies) Molecular subtypes may be characterized only at specialized centers, but the diagnosis of non-*HFE* related HC is sufficient to start phlebotomies at nonspecialized centers
Digenic	Double heterozygosity and/or double homozygosity/heterozygosity for mutations in two different genes involved in iron metabolism (*HFE* and/or non-*HFE*)	More commonly, C282Y mutation in *HFE* gene might coexist with mutation in other genes; rarely, both mutations involve non-*HFE* genes
Molecularly undefined	Molecular characterization (still) not available after sequencing of known genes (provisional diagnosis)	Patients should be referred (or DNA should be sent) to specialized centers

predisposes to HC, whereas heterozygous C282Y and H63D, and compound C282Y/H63D heterozygosity are reported to be of much less pathological importance if not combined with additional genetic or secondary risk factors [2–4,14]. Of note, even homozygous C282Y individuals display a heterogeneous clinical presentation, varying from severe HC to a majority of subjects who may never develop symptoms of IO, showing that the natural history of HC relies on individual and environmental variables and not only on the genotype [4]. A recent publication evaluating the clinical penetrance of C282Y/C282Y among 2890 homozygotes from the UK Biobank showed that, by the age of 55 years, only 33.2 % of the men and 21.4 % of the women have a diagnosis of HC [18]. However, several analyses from the UK Biobank suggest that even C282Y/C282Y individuals without a diagnosis of HC may have serious consequences, possibly as a result of IO, even in the absence of clear clinical symptoms [19]. As examples, several recent publications have shown that homozygous C282Y/C282Y men with and without HC demonstrate a 24 % increase in death from any cause, when compared to a control population. In addition, homozygotes have a higher incidence of dementia, a six-fold higher risk of liver fibrosis and cirrhosis and a 10.5-fold higher risk of liver cancer, when compared to a control population [20–22].

Although the investigation of *HFE* mutations is widely recommended for the screening of IO, the prevalence of *HFE* polymorphisms is highly heterogeneous worldwide. While the C282Y heterozygous mutation is very prevalent in individuals of Northern European ancestry, it is very rare in those from Africa, the Middle East, Asia and Brazilian indigenous population [23]. In a large multiethnic cohort study performed in patients with IO living in Canada or the United States, the Hemochromatosis and IO Screening (HEIRS) study reported a 0.44 % prevalence of C282Y homozygosity in non-Hispanic whites, 0.11 % in Native Americans, 0.027 % in Hispanics, 0.014 % in black individuals, 0.012 % in Pacific Islanders and 0.000039 % in Asians [24].

Brazil is a country of continental dimensions, home to an admixed population. Historically, besides its native indigenous people, Brazil received a great number of immigrants from Western Europe, Africa, Japan and the Middle East [25], leading to regional ethnic particularities and to a very significant genetic heterogeneity. In a pioneer study, carried out in 227 Brazilian individuals from Campinas, the allelic frequency of the C282Y mutation was 1.4 % in the Caucasian population, 1.1 % in the African-derived population, 1.1 % in racially mixed normal controls and 0 % in the original populations (Parakanã Indians) [25]. In another report that included a population of 542 Brazilian healthy blood donors from the city of Sao Paulo, the frequencies of the C282Y and H63D alleles were 2.1 % and 13.6 %, respectively [26], which represent a low incidence, when compared to most European countries [3]. As a comparison, the prevalence of C282Y heterozygosity in European countries increases from the south to north, reaching up to 14.2 % in Ireland [27].

Considering the genetic specificities of the Brazilian population, the investigation of IO in this population is a challenge, since C282Y/C282Y homozygotes seem to represent a small proportion of patients, sharply contrasting with patients from Northern Europe and North America. This study aimed to review available literature related to HC in the Brazilian population and, based on these data, suggest a tentative adaptation of the most recent HC guidelines used worldwide for the specific context of the Brazilian population.

Method

A literature search of articles was performed in the following databases: PubMed, SciELO, Web of Science, ScienceDirect, Latin American and Caribbean Literature (LILACS) and SCOPUS. The search was independently carried out by two authors (P.M.C. and A.C.T.), using the following descriptors: ("iron" OR "hemochromatosis" OR "HFE" OR "hyperferritinemia") AND ("Brazil" OR "Brazilian"). The search period was April 2024.

All the articles were completely analyzed. Information regarding the characteristics of the cohort of each manuscript

and the incidence of mutations associated with iron physiology was collected.

Results and discussion

The search resulted in the identification of twelve articles reporting studies that were all performed in Brazilian institutions. The characteristics of the population studied in each manuscript and a summary of results are shown in Table 5. The prevalence of *HFE* mutations in healthy Brazilian subjects varied from 1.1–3.3 % for C282Y mutations, and from 7.5 –13.6 % for H63D. The only study that evaluated the prevalence of *HFE* mutations in an indigenous population from Brazil, found no mutations of the *HFE* gene [25]. Of Brazilian subjects with definitive evidence of IO (increased ferritin and TS), the prevalences of *HFE* mutations were 13–53 % and 0 –15 % for the C282Y/C282Y and C282Y/H63D genotypes, respectively. Only three studies evaluated additional mutations related to iron metabolism: (a) Bittencourt et al. [28] found no mutations of the *TFR2* and *SCL40A1* genes in a cohort of 19 patients; (b) Santos et al. [29] described

Table 5 – Prevalence of mutations in iron overload-related genes in different Brazilian populations.

Study	Population	Method	*HFE* C282Y	*HFE* H63D	*HFE* C282Y/ H63D	*TFR2*	Additional mutations
Agostinho, M. F. [25]	Healthy volunteers: *n* = 227	PCR-RFLP	1.4 % White 1.1 % blacks 1.1 % racially mixed 0 % Amerindians	16.3 % White 7.5 % blacks 9.8 % racially mixed 0 % Amerindians	NP	NP	NP
Bittencourt P. L. [31]	Patients with IO: *n* = 15	PCR-RFLP	53 % *n* = 8 (282/282) 7 % (*n* = 1) (282/WT)	7 %, *n* = 1	0 %	NP	NP
Barbosa, K. V. B. D. [32]	Blood donors with IO: *n* = 10 (screened from 1039 healthy blood donors)	PCR-RFLP	10 % *n* = 1 (282/282)	20 % (63/WT), *n* = 2 10 % (63/63), *n* = 1	0 %	NP	NP
Cançado R. D. [33]	Patients with IO: *n* = 35	PCR-RFLP	14 % (282/282) 17 % (282/WT)	29 % (63/WT) 3 % (63/63)	11 %	NP	NP
Terada, C. T. [34]	Blood donors: *n* = 108	PCR-RFLP	2.2 %	NP	NP	NP	NP
Bittencourt P. L. [28]	Patients with IO: *n* = 19	Haemochromatosis StripAssay A	47 % (282/282)	11 %	5 %	0 %[a]	*SCL40A1*[a]: 0 %
Santos, P. C. et al. [26]	Blood donors: *n* = 542	PCR-RFLP	2.1 %	13.6 %	0.7 %	0 %[b]	NP
Santos, P. C. et al. [29]	Patients with IO: *n* = 51	Bidirectional DNA sequencing of HFE, *HJV*, *HAMP*, *TFR2* and *SLC40A1*	21.6 % (282/282) 7.8 % (282/WT)	21.6 % (63/WT) 3.8 % (63/63)	11.7 %	7.8 %	*HJV*: 5.8 % (*n* = 3) *HAMP*: 1.9 % (*n* = 1) *SLC40A1*: 1.9 % (*n* = 1)
Leão, G. D. R. [35]	Patients with hyperferritinemia: *n* = 299	PCR-RFLP	2.67 % (282/282) 4.35 % (282/WT)	31.44 % (63/WT) 8.03 % (63/63)	5.02 %	NP	NP
Alves, L. N. R. [36]	(a) Healthy volunteers: *n* = 120 (b) Patients with IO: *n* = 20	PCR-RFLP	(a) 0 % (282/282) 3.33 % (282/WT) (b) 5 % (282/282) 25 % (282/WT)	(a) 20.83 % (63/WT) 0.83 % (63/63) (b) 5 % (63/WT) 5 % (63/63)	(a) 0 % (b) 15 %	NP	NP
Kersting, N. [37]	Patients with hyperferritinemia: *n* = 214	PCR-RFLP	14.0 % (282/282)	7.9 % (63/63) 21.5 % (63/?)	11.8 %	NP	NP
Toreli et al. [30]	Patients with IO: *n* = 40	Exome sequencing of 20 genes implicated in iron physiology	13 % (282/282) 17.5 % (282/WT)	50 % (63/WT)	13 %	5 % (*n* = 2)	*HAMP*: 2.5 % (*n* = 1) *BMP6*: 12.5 % (*n* = 5) *SLC11A1*: 10 % (*n* = 4)

PCR-RFLP, polymerase chain reaction - restriction fragment length polymorphism analysis; IO, iron overload; WT, wildtype; NP, not performed.

[a] Only TfR2 E60X, M172K, Y250X, AVAQ594–597del, and SCL40A1 N144H and V162del mutations were analyzed.
[b] Only TFR2 Y250X (*n* = 212) and TFR2 Q690P (*n* = 516) were analyzed.

prevalences of mutations of the *TFR2* (7.8 %), *HJV* (5.8 %), HAMP (1.9 %), and *SLC40A1* (1.9 %) genes in 51 patients with IO; (c) Toreli et al. [30] analyzed 20 genes involved in iron physiology from whole exome results, and found mutations in the following genes: *TFR2* (5.0 %), *HAMP* (2.5 %), *BMP6* (12.5 %), and *SLC11A1* (10.0 %). Although the numbers of patients studied in all of these reports were small, the results strongly indicate that the percentage of patients in the Brazilian population with IO who are C282Y/C282Y homozygotes is probably <20 %. The studies also indicate that other genetic alterations (non-*HFE*), either in isolation or in combination with *HFE* mutations, may be important in the Brazilian population. Table 6 shows the results of the whole exome sequencing of 20 genes involved in iron metabolism observed in 40 patients with IO in Brazil [30]. In this cohort, C282Y/C282Y homozygotes represent only 13 % of the patients, whereas other mutations (some described for the first time) in genes related to iron metabolism were found, including the *BMP6, TRF2*, and *HAMP* genes [30].

Taking into account the genetic characteristics of the Brazilian population described above, some modifications to the 2022 European Association for the Study of the Liver (EASL) Clinical Practice Guidelines on Haemochromatosis[3] can be suggested in order to adapt the guidelines to the Brazilian context, as follows (Figure 1).

Proposal of a new algorithm for the investigation and management of iron overload in the Brazilian population

Who should be screened for iron overload?

The suspicion of IO is usually raised by the finding of elevated ferritin levels, defined as >300 mg/L in males and >200 mg/L in females [38]. However, hyperferritinemia may potentially be found in 5.9–19.0 % of healthy individuals [24]. Moreover, since ferritin is an acute-phase reactant protein and is released in the presence of inflammation and from necrotic or lysed cells, it is common to observe its elevation in individuals who do not have IO [3]. Therefore, it is essential to associate ferritin results with transferrin saturation (TS). A TS >45 % in men and women has been defined as elevated [3] indicating that individuals should be investigated for IO. Family members of patients diagnosed with HC and patients with increased liver iron, evident by liver biopsy or by MRI, should undergo biochemical and genetic testing [3,4].

Patients with high ferritin levels but with TS <45 % very probably do not have IO and, as already mentioned, other factors should be investigated to determine the cause of hyperferritinemia. Patients with high ferritin levels and with TS <45 % without a clear cause, should be observed at least annually with measurements of serum ferritin and TS.

An elevated serum ferritin in association with low TS may occur in three pathological states that might result in iron tissue deposition: metabolic hyperferritinemia, ferroportin disease and aceruloplasminemia [39]. Metabolic hyperferritinemia should be suspected when there is an insulin resistance syndrome with mild hepatic iron excess. Ferroportin disease (A-form) is characterized by predominant macrophage iron excess and absent or mild iron-related complications. Hereditary aceruloplasminemia is associated with major hepatocyte IO and diabetes mellitus, with the common finding of anemia and the presence of a neurological syndrome [39]. Once there is a suspicion of one of these conditions, targeted clinical and/or genetic investigation is mandatory, in conjunction with the investigation of tissue iron deposition by T2* MRI [3].

Identifying risk factors and comorbidities

Ferritin levels are often increased in chronic inflammatory conditions, liver disease, high alcohol consumption, obesity fatty liver, insulin resistance, poorly controlled diabetes mellitus, and metabolic dysfunction [13,40]. Unlike patients with HC, patients with metabolic hyperferritinemia have preserved production of hepcidin, and total iron body stores range from normal to a moderate level of IO [13]. Although iron levels in metabolic hyperferritinemia are not as high as those seen in HC, excess iron leads to the formation of reactive oxygen species and subclinical inflammation, worsening glucose and lipid metabolism, fibrogenesis and carcinogenesis [13].

In addition to the metabolic causes described above, active liver disease should also be evaluated in patients with evidence of IO. Alcoholic liver disease and hepatitis C infection may lead to IO and reticuloendothelial iron deposition [39]. Of note, iron removal by phlebotomy has been reported to improve the rate of response to interferon treatment in hepatitis C patients [41], although with the use of direct-acting antivirals, IO seems not to be a barrier to achieve response [42].

Finally, in patients diagnosed with IO, hematological diseases that lead to ineffective erythropoiesis and increased iron absorption (i.e., hemolytic anemias, myelodysplastic neoplasms) should be regularly investigated, even if they are not regularly transfused.

Genetic investigation of iron overload in Brazil

The classic *HFE* mutations, C282Y and H63D, should, of course, be investigated in patients suspected of IO, since *HFE* C282Y homozygosity can result in a potentially severe phenotype of HC, and the heterozygous phenotypes of C282Y/−, H63D/− and C282Y/H63D may also result in IO when combined with other genetic and environmental risk factors [2–4,14]. It is important to emphasize that the association of C282Y/H63D should not be classified as classic *HFE* hemochromatosis since this genotype has minimal or no clinical penetrance. In patients with this association (C282Y/H63D), mutations and deletions in *HFE* and non-*HFE* genes should be investigated. Furthermore, causes of liver disease should be investigated. Moreover, since the incidence of *HFE* mutations in Brazilian patients with HC is much lower than that observed in Northern European populations, investigation of other mutations in genes involved in iron metabolism should be considered for patients that do not harbor *HFE* mutations or who are not C282Y homozygotes. Taking into account previous Brazilian studies that evaluated non-*HFE* mutations in IO patients [28–30], the investigation of mutations of the *TFR2, SCL40A1, HJV, HAMP, BMP6* and *SLC11A1* genes might be valuable. However, it is very important to emphasize that, in the few available studies in Brazil, the number of identified patients with non-*HFE* mutations is quite low and in many patients with IO, no mutations were found. This strongly indicates that further studies in larger multicentric cohorts

Table 6 – Iron overload in Brazil.

Patient	Gender	Gene	Mutation		Comorbidity
1	M	*TfR2*	p.Arg752His	HET	Thalassemic trait
		HFE	p.His63Asp	HET	
		HBB	p.Gln40	HET	
2	M	*BMP6*	p.Pro95Ser	HET	Thalassemic trait
		HBB	p.Gln40	HET	
3	M	*CYBRD1*	p.Arg226HIS	HET	
		HEPH	p.Ala649Thr	HOM	
4	M	*HFE*	p.Cys282Tyr	HOM	
5	M	*HFE*	p.Cys282Tyr	HET	
		HFE	p.His63Asp	HET	
		SLC11A1	p.Pro234Arg	HET	
6	M	*HAMP*	c.−72C>*T*	HET	
7	M	*HFE*	p.Cys282Tyr	HET	
8	M	*HFE*	p.His63Asp	HET	
		HFE	p.Cys282Tyr	HET	
9	M	–	–	–	
10	M	*SLC11A1*	p.Arg397Cys	HET	
		HFE	p.His63Asp	HET	
11	F	*BMP6*	p.Arg257His	HET	Hepatic cirrhosis
12	M	*HEPH*	p.Ala649Thr	HOM	
		HFE	p.His63Asp	HET	
13	M	*HFE*	p.Cys282Tyr	HOM	
14	M	–	–	–	Hepatic cirrhosis
15	M	*HFE*	p.Cys282Tyr	HET	
16	F	*HFE*	p.Cys282Tyr	HET	Porphyria Cutanea
		HFE	p.His63Asp	HET	Tarda (PCT)
		UROD	p.Pro62Leu	HET	
17	M	*HAMP*	c.−72C>*T*	HET	
		HFE	p.His63Asp	HET	
18	M	*HFE*	p.His63Asp	HET	
19	M	*HFE*	p.His63Asp	HET	
20	M	*TFR2*	p.Arg752His	HET	HIV; Hepatitis C;
		HFE	p.His63Asp	HET	PCT; Hepatic cirrhosis
21	M	*HFE*	p.His63Asp	HET	Alcohol abuse
22	M	–	–	–	
23	M	*HFE*	p.His63Asp	HET	
24	M	–	–	–	
25	M	*HFE*	p.Cys282Tyr	HET	
26	M	*HFE*	p.His63Asp	HET	Hepatitis C
27	M	*HFE*	p.Cys282Tyr	HOM	
28	M	*HFE*	p.His63Asp	HET	
29	M	*AHSP*	p.Asn75Ile	HET	
30	M	*BMP6*	p.Arg257His p.Cys282Tyr	HET	
		HFE	p.His63Asp	HET	
		HFE		HET	
31	M	*FTH1*	p.Lys54Arg	HET	
		HFE	p.His63Asp	HOM	
32	M	*TFR2*	p.Glu491Glu	HET	
		HFE	p.His63Asp		
33	M	*BMP6*	p.Val394Met	HET	
34	M	*SCL11A1*	p.Pro231Leu	HET	
		HFE	p.His63Asp	HET	
35	F	*HFE*	p.His63Asp	HOM	
36	M	*BMP6*	p.Leu71Val	HET	
		HFE	p.Cys282Tyr	HET	
		HFE	p.His63Asp	HET	
37	M	*HFE*	p.Cys282Tyr	HOM	
		SLC11A1	p.Ala244Thr	HET	
		SLC11A1	p.Ala244Val	HET	
38	F	*HFE*	p.His63Asp	HET	PCT
39	M	*HFE*	p.Cys282Tyr	HOM	
40	M	*TF*	p.Arg343Trp	HET	PCT
		HFE	p.His63Asp	HET	
		UROD	p.Pro62Leu	HET	

M, male; F, female; HET, heterozygous mutation; HOM, homozygous mutation; HIV, human immunodeficiency virus.
Genes - *TfR2* (transferrin receptor 2), *HFE* (homeostatic iron regulator), *HBB* (hemoglobin subunit beta), *BMP6* (bone morphogenetic protein 6), *CYBRD1* (cytochrome b reductase 1), *HEPH* (hephaestin), *SLC11A1* (solute carrier family 11 member 1), *HAMP* (hepcidin antimicrobial peptide), *UROD* (uroporphyrinogen decarboxylase), *AHSP* (alpha hemoglobin stabilizing protein), *FTH1* (ferritin heavy chain 1), *TF* (transferrin).
Toreli et al. [30].

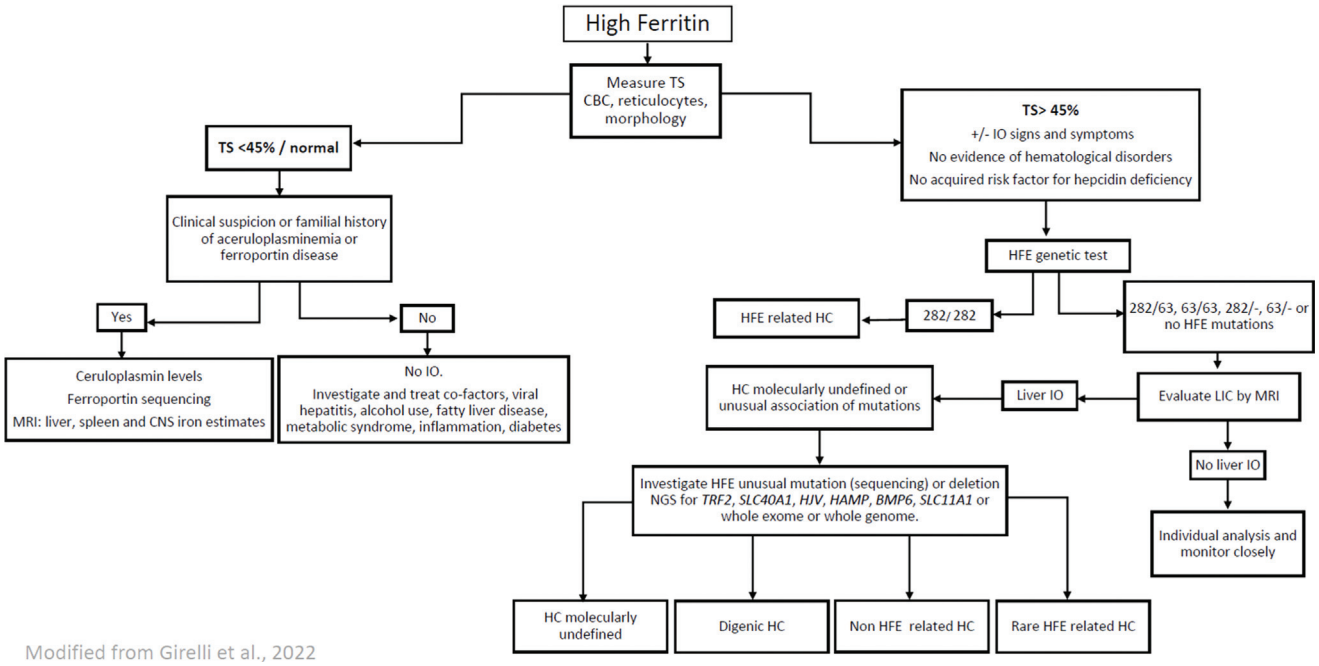

Figure 1 – Algorithm proposed for the diagnosis of haemochromatosis in Brazilian patients. TS, transferrin saturation; CBC, complete blood count; IO, iron overload; HC, hemochromatosis; LIC, liver iron concentration; MRI, magnetic resonance imaging; CNS, central nervous system

Genes, *HFE*, homeostatic iron regulator; *TfR2*, transferrin receptor 2; *SLC11A1*, solute carrier family 11 member 1; *HJV*, hemojuvelin BMP, co-receptor; *HAMP*, hepcidin antimicrobial peptide; *TFRC*, transferrin receptor; *BMP6*, bone morphogenetic protein 6; *BMP2*, bone morphogenetic protein 2; *SLC40A1*, solute carrier family 40 member.

should be carried out to fully clarify the mutation spectrum of IO in Brazil. If available, a broader study with next-generation sequencing, by whole exome or complete genome sequencing, should be carried out.

If a diagnosis of early-onset HC is suspected (within the second or third decades of life, family history, hypogonadotropic hypogonadism or unexplained heart failure), sequencing of the *HAMP* and *HJV* genes is recommended [39]. If there is clinical suspicion of hereditary aceruloplasminemia, ceruloplasmin levels should be evaluated [39].

As already mentioned, in some cases, the genes for porphyria cutanea tarda and the *β-globin* gene (or *HBB* gene) should be sequenced when clinically suspected.

Management of iron overload

If not adequately treated in an early phase, excess free iron may lead to progressive tissue damage, with subsequent cardiac failure, cirrhosis and endocrine disfunction [1]. Additionally, as already described, high serum levels of ferritin have been associated with the amount of hepatic lipid accumulation, the severity of insulin resistance and features of metabolic dysfunction [13]. For this reason, decisions require individualized clinical assessment and patients with evidence of IO should be treated appropriately even if a specific genetic mutation cannot be identified. Ideally, patients with suspected IO (high ferritin and high TS) should be evaluated for liver iron concentration at diagnosis using T2* MRI in order to estimate the amount of iron deposition and to guide treatment [2]. Patients diagnosed with early-onset forms of HC should also be evaluated for cardiac iron deposition by cardiac T2* MRI [3].

Patients with no (or minimal) symptoms, but with persistently increased levels of serum ferritin, TS higher than 45 % and increased liver iron estimated by MRI, should be treated by phlebotomy in order to prevent organ damage. The management of patients is determined by their phenotypic presentation and the presence of associated cofactors, not by the genotype alone [2,3,13]. Treatment of risk factors and comorbidities is mandatory [2]. Alcohol intake should be restricted [3]. The initiation and maintenance of a schedule of phlebotomies is determined by serum iron studies (ferritin and TS), and the MRI, if available [2].

During the initial phase, the performance of phlebotomies in the range of 400–500 mL, according to body weight, weekly or every two weeks has been proposed [38]. The main treatment goal is to lower ferritin levels, with a serum ferritin target of 50 μg/L, although, in real-life, levels up to 100 μg/L are acceptable, even in the induction phase. During the maintenance phase, one phlebotomy is performed every 1–4 months, depending on the patient's iron status [38], with the target serum ferritin being around 50–100 $\mu g/L^3$. Although the reduction of TS to <50 % should be considered as highly desirable, in some patients this reduction is slow and difficult to achieve. Thus, the real target to guide treatment must be ferritin levels and not TS. The clinical implications of the maintenance of high TS for long periods with concomitantly low ferritin levels are not yet well understood [43,44]. If hemoglobin concentrations are <12 g/dL, the volume or frequency

of phlebotomy should be decreased; if hemoglobin falls below 11 g/dL, phlebotomy should be discontinued until the causes of anemia can be assessed and adequately treated [3].

The role of iron depletion by phlebotomy in metabolically induced high ferritin levels remains an open question. In these patients, the maintenance of ferritin levels <50 μg/L does not seem to ameliorate metabolic endpoints (i.e., glucose control), nor transaminase levels or the liver fibrosis score [38]. However, if there is evidence of moderate or severe iron accumulation (defined by ferritin >1,000 ng/mL, or metabolic hyperferritinemia with a MRI estimative of >74 μmol/g of iron in the liver), phlebotomy should be considered, together with close monitoring of hemoglobin levels and adverse events [13] (using a cautious protocol of phlebotomy).

Since iron-chelating agents are not superior to therapeutic phlebotomy and usually cause adverse events, they are not routinely recommended for the treatment of IO in patients who are not anemic and who do not have other difficult issues regarding phlebotomy (inaccessible veins, needle phobia) [2,3]. However, iron-chelating agents can be used in association with phlebotomies in patients with severe IO, such as in cases of early-onset HC and cardiac iron deposition [3,38]. Proton pump inhibitors can also be an adjuvant to phlebotomy for some patients, since they reduce intestinal iron absorption [3]. Apparently, dietary heme or nonheme iron restrictions have no significant relationships with iron body content, and there is no strong evidence that dietary restrictions have an important role in IO treatment [45]. However, if the patient accepts the suggestion, black tea with meals and a vegetarian diet may be prescribed since these seem to be beneficial for some patients [46,47]. Erythrocytapheresis is very effective and can be considered where available to treat HC patients, however it is more expensive and less available than phlebotomy [38].

Special considerations regarding the Brazilian economic scenario

When guidelines are proposed, they must consider the best available evidence to help clinicians in taking the most appropriate decisions in specific clinical contexts. In the scenario of IO, advanced tools are certainly helpful for the diagnosis and management of this condition, particularly the complete genetic identification of mutations and quantification of tissue iron deposition by T2* MRI.

A frequent problem that many physicians may encounter in a country like Brazil, with limited resources and unequal access to technology in public health services is the impossibility to obtain an MRI estimate of liver iron or access to molecular diagnosis. Given the harmful consequences of untreated IO, as strongly suggested by published reports [43,44], it is recommended that patients with a very probable IO (elevated serum ferritin levels (>400 μg/L in men and >300 μg/L in women) for at least six months and TS higher than 45 %) should be treated with therapeutic phlebotomy targeting a serum ferritin of 50–100 μg/L, even if genetic investigation or MRI cannot be performed. We recognize that the numbers indicated here are somewhat arbitrary, but they are based on available evidence in the literature and also on the personal experience of several experts in the field.

The patients classified with "very probable IO" should be divided into two groups, the first with levels of ferritin higher than 1,000 μg/L and the second with ferritin between 400 μg/L and 1,000 μg/L. For those with ferritin higher than 1,000 μg/L and of course, high TS, there are a number of indications that liver damage secondary to IO may be very probable, and phlebotomy should be carried out following the traditional protocol, as described earlier. On the other hand, for patients who have ferritin levels of between 400 μg/L and 1,000 μg/L and elevated TS, the evidence supporting phlebotomies is weaker and these individuals should undergo careful individual analysis regarding their clinical status. However, a recent relevant study showed that patients with classic C282Y/C282Y *HFE*-related hemochromatosis with ferritin levels of between 300 μg/L and 1,000 μg/L demonstrated a significant clinical improvement when submitted to repeat phlebotomies. Thus, for individuals with intermediately elevated ferritin levels (between 400 μg/L and 1,000 μg/L), a specialized and carefully designed treatment protocol is proposed. Phlebotomies should be undertaken every three or four weeks. Hemoglobin levels must be monitored before each procedure and ferritin measured every 30 days. The treatment target should be ferritin of 100 μg/L. If Hb is ≤12 g/dL, the procedure should be stopped. After three or four phlebotomies the patient should be carefully reevaluated. After the target of 100 μg/L is reached, patients should be kept in the usual maintenance phase, with 2–4 phlebotomies per year, and checked for ferritin and TS every six months.

Conclusion

IO is a state in which an excess of free plasma iron leads to progressive cellular and tissue damage. The clinical phenotype relates to a conjunction of genetic mutations and co-factors, including comorbidities and environmental factors. The prevalence of mutations in iron physiology-related genes has a very important variation in different populations. In Brazil, the incidence of *HFE* mutations is much lower than that observed in the Northern European and North-American populations. Based on several reports in the literature, it is recommended that the genetic investigation of IO in Brazil be extended, and the investigation of mutations of the *TFR2, SCL40A1, HJV, HAMP, BMP6* and *SLC11A1* genes should be considered for individuals who are not homozygous for the C282Y mutation. It should be underscored that these proposals are still based on a limited sample population and future studies conducted in larger cohorts are important to strengthen these recommendations. The treatment decision requires an individualized clinical assessment and patients with evidence of IO should be treated appropriately, even if a specific genetic mutation cannot be investigated or identified.

Authors contribution

PMC performed the references selection, elaborated the tables and the figure, and wrote the manuscript. ACT performed the references selection, collected the genetic data from the references and reviewed the manuscript; DMA reviewed all the genetic data from the references, edited the tables and the figure and reviewed the manuscript; FFC conceived the format of the manuscript and wrote the text.

Conflicts of interest

The authors declare no conflicts of interest.

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Special article

Diagnosis and treatment of chronic lymphocytic leukemia: 2025 recommendations of the Brazilian Group of Chronic Lymphocytic Leukemia of the Brazilian Association of Hematology and Hemotherapy (ABHH)

Carlos Sérgio Chiattone [a,b], *Fernanda de Morais Marques* [a], *Valeria Buccheri* [c], *Mihoko Yamamoto* [d], *Sergio Costa Fortier* [a], *Maura Rosane Valerio Ikoma-Colturato* [e], *Nelson Hamerschlak* [f], *Vera Lucia de Piratininga Figueiredo* [g], *Talita Maira Bueno da Silveira* [b,h], *Abel Costa* [i], *Dani Laks* [j], *Rony Schaffel* [k], *Wolney Gois Barreto* [l], *Adriana Scheliga* [m], *Pedro Amoedo Fernandes* [d,n], *Samir Kanaan Nabhan* [o], *Rafael Dezen Gaiolla* [p], *Matheus Vescovi Gonçalves* [d,q], *Danielle Leão Cordeiro de Farias* [r], *Glaciano Ribeiro* [s], *Marcelo Pitombeira de Lacerda* [t], *Celso Arrais-Rodrigues* [a,d,u,*], *On behalf of the Brazilian Group of Chronic Lymphocytic Leukemia*

[a] *Brazilian Registry of CLL – Associação Brasileira de Hematologia e Hemoterapia, São Paulo, SP, Brazil*
[b] *FCM da Santa Casa de São Paulo, São Paulo, SP, Brazil; Hospital Samaritano Higienópolis, São Paulo, SP, Brazil*
[c] *ICESP - Faculdade de Medicina da USP, São Paulo, SP, Brazil*
[d] *Universidade Federal de Sao Paulo - UNIFESP/EPM, São Paulo, SP, Brazil*
[e] *Hospital Amaral Carvalho, Jaú, SP, Brazil*
[f] *Hospital Israelita Albert Einstein, São Paulo, SP, Brazil*
[g] *Hospital do Servidor Publico do Estado de Sao Paulo – IAMSPE, São Paulo, SP, Brazil*
[h] *AC Camargo Cancer Center, São Paulo, SP, Brazil*
[i] *Instituto D'Or de Pesquisa e Ensino, São Paulo, SP, Brazil*
[j] *Instituto de Hematologia, Porto Alegre, RS, Brazil*
[k] *Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[l] *Faculdade de Medicina do Unisalesiano, Araçatuba, SP, Brazil*
[m] *Grupo Oncoclínicas, Rio de Janeiro, RJ, Brazil*
[n] *Clínica AMO, Salvador, BA, Brazil*
[o] *Universidade Federal do Paraná, Curitiba, PR, Brazil*
[p] *Faculdade de Medicina de Botucatu (HC-FMB), Botucatu, SP, Brazil*
[q] *Grupo Fleury, São Paulo, SP, Brazil*
[r] *Beneficiência Portuguesa de São Paulo, São Paulo, SP, Brazil*
[s] *Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil*
[t] *Universidade da Região de Joinville - UNIVILLE, Joinville, Santa Catarina, SC, Brazil*
[u] *Hospital Nove de Julho - DASA, São Paulo, SP, Brazil*

* Corresponding author at: Rua Doutor Diogo de Faria, 824, Vila Clementino CEP, São Paulo, SP 04037-002, Brazil.
E-mail address: celsoarrais@gmail.com (C. Arrais-Rodrigues).

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ABSTRACT

Chronic lymphocytic leukemia, characterized by an accumulation of monoclonal B lymphocytes, is the most common adult leukemia. The disease predominantly affects older adults, with a significant proportion being asymptomatic at diagnosis. This manuscript provides a comprehensive review of chronic lymphocytic leukemia, including its epidemiology, clinical presentation, diagnostic criteria, and treatment strategies. Prognostic factors, particularly *IGHV* mutation status and chromosomal abnormalities, are discussed as critical determinants of disease behavior and treatment response. Recent advances in targeted therapies, such as Bruton's tyrosine kinase inhibitors (BTKi) and B-cell lymphoma 2 inhibitors (BCL-2i), have changed the treatment landscape by demonstrating superior efficacy to chemoimmunotherapy. However, disparities in access to care, particularly in low- and middle-income countries such as Brazil, highlight the need for equitable treatment approaches. The discussion of measurable residual disease (MRD) assessment for prognostication and treatment planning is also highlighted. This review highlights the need for continued research and integration of novel therapies to optimize patient outcomes in chronic lymphocytic leukemia.

Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults, accounting for approximately 30 % of all leukemias in this population. The median age at diagnosis is 71 years, with >95 % of patients over the age of 50. While genetic and environmental factors may play a role in its development, the etiology of CLL is still unknown. The lower incidence of CLL in individuals of Eastern descent and its higher incidence in family members (5–10 %) when compared to other mature B-cell neoplasms reinforce possible genetic components in the development of CLL.

A first version of the Recommendations of the Brazilian Group of CLL was published in 2016.[1] This updated second edition incorporates the latest therapeutic advancements, including novel targeted agents and combination regimens that have profoundly transformed the management landscape of CLL.

Clinical presentation of CLL

The clinical presentation of CLL at diagnosis is highly variable. Approximately 60 % of patients are asymptomatic, with the disease often detected during routine blood work. When symptomatic, patients often report vague symptoms such as fatigue or weakness. Lymphadenopathy is observed in approximately 80 % of cases during the course of the disease, particularly in more advanced stages, often involving the cervical, axillary and inguinal lymph nodes. Splenomegaly is generally mild to moderate and occurs in about 50 % of cases, while hepatomegaly is less common. Although uncommon at diagnosis, B symptoms may be present as the disease progresses, defined as unintentional weight loss of 10 % or more in the past six months, fever above 38 °C for two or more weeks without other evidence of infection, and night sweats for more than one month without infection.

Anemia and thrombocytopenia may be seen in 15–30 % of patients, typically due to bone marrow (BM) infiltration. However, autoimmune cytopenias such as autoimmune hemolytic anemia and autoimmune thrombocytopenia may be present.[2,3] Rarely, pure red cell aplasia and autoimmune granulocytopenia may be present. There is generally a good response to corticosteroids, but some patients require CLL-specific treatment for relapsed or refractory immune cytopenias. Other autoimmune manifestations are rarely seen in CLL patients, and may include myasthenia gravis, acquired von Willebrand disease and acquired angioedema The absolute lymphocyte count is highly variable both at diagnosis and over the course of the disease. Richter's transformation, formerly known as Richter's syndrome, is a condition that occurs when CLL transforms into an aggressive type of lymphoma, more commonly diffuse large B-cell lymphoma, or Hodgkin's lymphoma in a small subset of patients.[4] Richter's transformation can be suspected by the appearance of B symptoms, rapid enlargement of the lymph node group, and marked elevation of lactate dehydrogenase.

Central nervous system involvement in CLL

Central nervous system (CNS) involvement in CLL is rare but clinically significant, manifesting as confusion, cranial neuropathies, optic neuropathy, or cerebellar dysfunction. It can occur at any stage of the disease and may even be the first sign of progression requiring systemic treatment. In a recent analysis from the Brazilian Group of CLL, the most common presentations of CNS involvement were highlighted and relatively good outcomes were found, particularly with ibrutinib-based regimens.[5,6] Given its potential impact, any neurological symptoms in CLL patients should prompt a thorough CNS evaluation to guide timely intervention.

Infection is the most common complication of CLL and the leading cause of death over the course of the disease.[7] Both CLL itself and its treatment cause deficiencies in the cellular and humoral immune systems. Hypogammaglobulinemia is not uncommon and may worsen after CLL treatment. Bacterial infections are common even before treatment begins, with *Streptococcus pneumoniae* and *Haemophilus influenzae* being the most common pathogens. Response to vaccination varies and vaccination should be given early in the course of the disease for optimal results. Viral infections may also occur, with particular attention to herpes zoster reactivation. Hepatitis B and C virus reactivation may occur after treatment with immunosuppressive agents, including anti-CD20 antibodies. COVID-19 has also become an issue with a dismal clinical course in CLL patients, mostly during treatment and 6–12 months after anti-CD20 antibodies. Patients should be screened prior to initiation of therapy, and chronic hepatitis B virus carriers should be started on prophylactic antiviral therapy during CLL treatment, with entecavir being the drug of choice. The use of immunosuppressive agents such as corticosteroids, chemoimmunotherapy, and BTKi significantly increases the risk of opportunistic infections and invasive fungal diseases such as aspergillosis. Given the complexity of infection treatment and prevention in patients with CLL, it is advisable for the center to have an infectious disease specialist with expertise in oncohematology on staff.

Analysis of population-based data shows that patients with CLL have an increased risk of secondary cancers, with melanoma and squamous cell carcinoma of particular concern. They are also at higher risk for solid tumors, including colorectal, lung, kidney, thyroid and soft tissue sarcomas, than the general population. The occurrence of myeloid neoplasms was also elevated.

Diagnosis

CLL is diagnosed by the presence of monoclonal B lymphocytes with a specific immunophenotype ($CD5^+/CD23^+$) in the peripheral blood (PB) at a count greater than 5×10^9/L for more than 3 months.[8] Below this threshold, it is considered monoclonal B-cell lymphocytosis (MBL) with a CLL phenotype, which can be further classified as high-count MBL ($>0.5 \times 10^9$/L) or low-count MBL ($<0.5 \times 10^9$/L). Despite its correlation with CLL, MBL is considered a distinct entity due to its extremely low progression rate and asymptomatic nature, with clinical management consisting only of periodic surveillance.[9]

Although the majority of high-count MBL cases have favorable prognostic features (*IGHV*-mutated and low-risk genomics), an estimated 1–2 % of individuals with high-count MBL will develop CLL requiring treatment.[10]

Small lymphocytic lymphoma (SLL) differs from CLL in the absence of leukemia, i.e., white blood cell count $<5 \times 10^9$/L, but requires lymphadenopathy and/or splenomegaly and lymph node biopsy for diagnosis. CLL and SLL represent different clinical manifestations of the same disease, distinguished only by the primary site of involvement: CLL predominantly affects the blood and BM, while SLL is characterized by nodal involvement with limited or no circulating disease. Despite these differences, both entities share identical biological, genetic, and prognostic features and should be managed identically.

In CLL, lymphocytes have a dense nucleus and lack visible nucleoli. The presence of 15 % prolymphocytes indicates prolymphocytic progression of CLL. Gumprecht shadows are common. Typical immunophenotypic markers include $CD19^+$, $CD5^+$, $CD23^+$, $CD200^+$, and $CD43^+$, with weak expression of CD20, surface light chain ($sIg\kappa^+$ or $sIg\lambda^+$), and surface IgM, and weak or negative expression of CD79b, $CD22^+$, and $CD11c^+$, and absence of FMC7, CD10, and CD103. Historically, the Matutes scoring system based on five parameters ($CD5^+$, $CD23^+$, $FMC7^-$, weak CD22/CD79b, weak K/L) was widely used for CLL diagnosis.[11] Recently, standardized and internationally validated multicolor panels, including automated analysis, have gradually replaced its use.

Some cases of CLL exhibit atypical immunophenotypes, leading to diagnostic uncertainty. For example, high CD20 and FMC7 expressions have been associated with del(11q) and trisomy 12, while elevated IgM expression correlates with unmutated *IGHV* status and potential resistance to ibrutinib. However, despite this potential resistance mechanism, IgM expression is not currently used to guide treatment decisions. Nonetheless, monitoring IgM expression on CLL cells during ibrutinib treatment may serve as a biomarker for identifying the potential development of resistance.

Differential diagnosis

The main differential diagnosis is mantle cell lymphoma (MCL): $CD5^+$, but classically negative for CD23 and CD200 with strong expression of CD20 and immunoglobulins. The diagnosis of MCL is confirmed by FISH for t(11;14) or by immunohistochemistry for cyclin D1 or SOX11. Other B-cell lymphoproliferative disorders (BCLPD) may express $CD5^+$, but usually at low intensity. In cases of uncertainty, diagnostic confirmation by cytogenetic, molecular or immunohistochemical methods is required, depending on the clinical context.

Brazilian Group of CLL recommendations for diagnosis (mandatory)

Morphological evaluation of PB smear.

PB immunophenotyping is essential for the diagnosis of CLL, including differential diagnosis with other BCLPDs, starting with a screening panel to determine the nature of the disease. The recommended diagnostic markers for CLL are CD19, CD20, CD5, CD23, CD200, CD79b, and kappa and lambda light chains. Other markers such as CD43, CD81, and ROR-1 and/or prognostic markers such as CD38, CD49b, or CD305 may be included. Depending on the flow cytometer available in each laboratory, 4, 6, 8 or more color panel combinations can be used, provided the protocol has been validated between laboratories. The Euroflow panel is an internationally validated 8-color approach that adheres to these recommendations and has been routinely used.

Cases with diagnostic uncertainty on immunophenotyping may benefit from additional diagnostic measures.

BM biopsy and/or aspirate immunophenotyping are NOT recommended for the routine diagnosis of CLL, but may be considered in cases of cytopenias to rule out myelodysplastic syndrome in clinical trials or in cases of diagnostic uncertainty.

Imaging modalities (ultrasound, computed tomography, magnetic resonance imaging, positron emission tomography scan) are generally NOT indicated in the diagnosis or initial assessment of CLL.

Prognosis

First reported in the 1970s, the clinical staging systems (Rai and Binet, Table 1) are still widely used and are based on the assessment of nodal, splenic, and hepatic involvement, as well as cytopenias.[12,13] Cytopenia in CLL predicts poor prognosis, though its impact depends on etiology. In a Mayo Clinic cohort, autoimmune-related cytopenia showed significantly better survival (9.1 versus 4.4 years, *p*-value <0.001) compared to BM failure.[14]

Immunophenotypic markers such as CD38, CD49d, CD305, CCR6, CXCR5, and ZAP-70 do not outweigh the impact of clinical staging and assessment of *IGHV* mutational status and abnormalities involving *TP53* despite their association with poor prognosis and chromosomal abnormalities.[16,17] *IGHV* mutational status plays a critical role in prognosis: mutated *IGHV* is associated with a better prognosis and indolent course, while unmutated *IGHV* correlates with a more aggressive course.[18] However, testing is not always accessible due to its high cost. Beyond its prognostic value, immunogenetic analysis has identified stereotyped B cell receptor immunoglobulin subsets, which define distinct clinical and biological CLL subgroups, refining risk stratification.[19,20]

Chromosomal aberrations, preferably detected through PB cytogenetics, are also useful for prognosis, especially when multiple abnormalities are present, as in complex karyotype.[21] FISH detects aberrations in 80 % of cases, including del (13q14.1) (~55 %), trisomy 12 (10–20 %), del(11q22–23) (10–25 %), and del(17p) (5–10 %). Complex karyotype and del (17p) are associated with unfavorable prognosis and may influence the choice of treatment. Monoallelic *TP53* mutations detected by polymerase chain reaction (PCR) or next generation sequencing indicate poor prognosis and resistance to therapy.[22] *TP53* mutations and/or del(17p) are especially common in relapsed CLL and are associated with reduced overall survival.[23–25] Other somatic gene mutations, including *ATM, NOTCH1, SF3B1*, and *BIRC3*, have been identified as prognostic markers, but only *TP53* is consistently associated with therapy resistance and early relapse.

Finally, the CLL-IPI score[26,27] integrates genetic factors (*IGHV* mutation status, del(17p)/*TP53* mutation), clinical stage, age, and beta-2 microglobulin for prognostic assessment (Table 2). It is important to note that none of these factors indicates the need to start treatment in asymptomatic patients and that the prognosis associated with this staging and scoring system is related to an era of immunochemotherapy and has been modified with targeted therapies.

Table 2 – Risk groups in CLL according to the International Prognostic Index (CLL-IPI) Criteria.

(A) Multivariate analysis of independent predictors for survival in CLL-IPI

Variables	Risk factor	Relative risk	Score
Clinical stage	Binet B/C or Rai I-IV	1.6	1
Age	> 65 years	1.7	1
β2 microglobulin	> 3.5 mg/L	2	2
IgHV	Unmutated	2.6	2
Del17p and/or TP53 mutation	Deletion and/or mutation	4.2	4

(B) CLL-IPI risk groups according to overall survival

Score (no. of unfavorable factors)	5-Year Survival (95 % CI)
Low (0–1)	93.2 % (90.5–96.0)
Intermediate (2–3)	79.3 % (75.5–83.2)
High (4–6)	63.3 % (57.9–68.8)
Very High (7–10)	23.3 % (12.5–34.1)

Table 1 – Clinical stages and survival.

(A) Binet clinical stage (Binet et al.[13])

Stage	Risk	Characteristics (% of cases)	Median survival
A	Low	< 3 areas of lymphadenopathy without anemia or thrombocytopenia (63 %)	15 years
B	Intermediate	≥ 3 areas of lymphadenopathy[a] without anemia or thrombocytopenia (30 %)	5 years
C	High	Presence of anemia or thrombocytopenia (7 %)[b]	2 years

(B) Rai clinical stage (Rai et al.[12], Rai[15])

Stage	Risk	Characteristics	Median Survival (years)	Median Survival (years)
0	Low	Lymphocytosis	12.5	>13
I	Intermediate	Lymphocytosis + lymphadenopathy	8	7
II	Intermediate	Lymphocytosis + splenomegaly/hepatomegaly	6	
III	High	Lymphocytosis + anemia[c]	1.5	2
IV	High	Lymphocytosis + thrombocytopenia[c]	1.5	

[a] Bilateral cervical lymph nodes and Waldeyer's ring (one area), bilateral axillary (one area), bilateral inguinal (one area), palpable spleen and liver (one area each).
[b] Anemia: Hb <10 g/dL/Thrombocytopenia <100 × 10^9/L.
[c] Anemia: Hb <11 g/dL/Thrombocytopenia <100 × 10^9/L.

Recommendations of the Brazilian Group of CLL for prognostic stratification

In addition to clinical staging (Binet or Rai), the recommendations are to assess *IGHV* mutation status, and test for del(17p) by FISH, and TP53 mutation by PCR or next generation sequencing before initiating first-line treatment. If possible, detection of TP53 mutation and del (17p) deletion by FISH should be performed before starting each subsequent treatments because of the possibility of clonal selection after first-line treatment. There is no indication to repeat *IGHV* mutational status over the course of the disease.

Measurable residual disease assessment

Assessment of MRD has gained importance following evidence that deeper remissions correlate with longer progression-free survival (PFS) and the ability of new regimens with immunotherapy and targeted therapies to induce high rates of undetectable MRD. MRD assessment after three and six cycles of disease eradication regimens and three months post-treatment appears to be an important predictor of CLL treatment outcome.[28] As such, MRD assessment is increasingly being used in clinical trials in conjunction with traditional endpoints such as PFS and overall survival (OS).

MRD may also be an important predictor of outcome following hematopoietic stem cell transplantation. Both IgH-PCR and flow cytometry can be used to assess MRD.[29,30] It is recommended that validated methods according to the protocols of the European Research Initiative on CLL (ERIC) and the European Study Group on MRD Detection (EuroMRD) be used.[31] For both methods, the minimum sensitivity threshold of 1×10^{-4} ("MRD4") is a key endpoint in several studies due to its reproducibility at this detection level and its prognostic correlation. Higher sensitivity levels (i.e., 1×10^{-5} to 1×10^{-6}) can be achieved, but the clinical impact of lower thresholds is still being evaluated.

Flow cytometry is a widely used technique. The ERIC approach includes 6 or 8 markers (CD19/CD20/CD5/CD43/CD79b/CD81, with CD3 and CD22 for 8 colors). Sensitivity of 0.001 % (1×10^{-5}) can be achieved with the detection of a higher number of events (at least 2×10^{-6} events). Other validated panel options include the 10-color panel from MD Anderson with automated analysis capability, the 12-color panel validated by Euroflow, and the 14-color panel validated by Memorial Sloan Kettering Cancer Center. These panels can be used according to the infrastructure available in each laboratory.

PCR for IGH regions can be used according to validated protocols (see EuroMRD). Real time quantitative-PCR (RQ-PCR) achieves MRD4 with good correlation to flow cytometry. However, challenges include the need for a specific laboratory infrastructure and the necessity (and difficulty) of obtaining the initial diagnostic sample to develop individualized primers. Newer, more sensitive techniques, such as next generation sequencing, are still under development, validation and international standardization.

Both PB and BM can be used to assess MRD. BM is more likely to be positive than PB. Therefore, if PB is negative, BM assessment may be used depending on the treatment goals. However, recent clinical studies are increasingly abandoning BM MRD evaluation due to its lack of clinical relevance, despite its slightly higher sensitivity compared to PB.

It should be noted that studies modifying subsequent therapy after MRD^+ detection are still ongoing. Thus, despite its prognostic value, MRD assessment outside of clinical trials has no universal practical application as it does not guide treatment. The physician-patient relationship is critical to the assessment and interpretation of MRD outside of research contexts, as positive results may cause unnecessary distress. Furthermore, as demonstrated in the CAPTIVATE and FLAIR studies,[32,33] MRD may be negative even in the context of partial remission or complete remission with incomplete hematologic recovery (CRi). MRD kinetics may vary depending on the therapeutic strategy used and should be evaluated and interpreted in the specific context of each treatment. Therefore, MRD^+ is not synonymous with refractoriness, as patients may remain stable for years after treatment despite MRD^+. In some cases, MRD^+ may even become negative over time after treatment (e.g., the GLOW study).

Brazilian group of CLL recommendations for MRD assessment

Currently, the use of MRD assessment is NOT recommend in clinical practice. Routine MRD evaluation should be performed only in the context of research and clinical trials. In general, MRD assessment is conducted three months after completing therapeutic regimens aimed at eradicating leukemic clones (such as chemoimmunotherapy or venetoclax) and/or 12 months after hematopoietic stem cell transplantation.

The Brazilian Group of CLL recommends the use of standardized and validated protocols for MRD assessment, such as those established by ERIC and EuroMRD, considering the limit of detection (sensitivity) of the test.

Treatment

The treatment of CLL/SLL has evolved significantly in recent years with the introduction of novel agents with targeted mechanisms of action.[34] However, treatment indications remain those established by the International Workshop on CLL (iwCLL), mainly Binet C/Rai III/IV for patients with active and symptomatic disease. It is important to note that these recommendations have not changed with the introduction of targeted therapies (Table 3).

The isolated value of the absolute lymphocyte count, hypogammaglobulinemia, or monoclonal or oligoclonal paraproteinemia, should be interpreted in the context of a comprehensive clinical evaluation, rather than used as the sole indication to start treatment.

CLL and SLL should always be treated with the same therapeutic approach. SLL should not be treated as an indolent lymphoma because its natural history, treatment indications,

Table 3 – Treatment indications according to IWCLL Criteria (2018) and the Brazilian Group of CLL treatment indications.

Criteria	iwCLL treatment criteria (2018)	Brazilian CLL group treatment indications
Bone marrow failure	Progressive bone marrow failure with anemia (Hb < 10 g/dL) and/or thrombocytopenia (platelets < 100,000/mm^3)[a]	Progressive, symptomatic anemia and/or thrombocytopenia, persistent, excluding other causes
Splenomegaly	Massive (≥ 6 cm below the right costal margin), progressive, or symptomatic	Symptomatic splenomegaly
Lymphadenopathy	Lymph nodes ≥ 10 cm (longest diameter), progressive or symptomatic	Massive and symptomatic lymphadenopathy
Progressive lymphocytosis	Increase of ≥ 50 % in 2 months or lymphocyte doubling time (LDT) < 6 months[b,c]	–
Autoimmune complications	Anemia or thrombocytopenia with unsatisfactory response to corticosteroids	Autoimmune disease (anemia and/or thrombocytopenia) with inadequate response to corticosteroids or other treatments
Extranodal involvement	Symptomatic or functional extranodal involvement (skin, kidneys, lungs, CNS)	–
Constitutional symptoms	- Fever ≥ 38 °C for > 2 weeks without infection - Night sweats ≥ 1 month without infection - Weight loss ≥ 10 % in 6 months - Intense fatigue (ECOG ≥ 2)	- Significant unintended weight loss - Significant fatigue - Fever > 38.0 °C - Persistent night sweats (excluding other causes such as infection or neoplasms)

[a] Platelet values <100 × 10^9/L may remain stable for long periods without requiring treatment.
[b] Exclude infection or corticosteroid use as a cause of lymphocytosis.
[c] Only consider LDT for lymphocytosis ≥ 30,000/mm^3.

and responses to targeted therapies are identical to those of CLL. Consistent application of CLL treatment paradigms to SLL will ensure optimal patient outcomes and prevent undertreatment due to misclassification of disease behavior.

The Brazilian Group of CLL performed an analysis of 2511 patients from 41 centers. Of these, 1404 patients (56 %) met the iwCLL indication criteria (liberal criteria), while only 788 patients (31 %) met the more restrictive Brazilian Group of CLL criteria. These criteria establish different cut-offs for cytopenias (hemoglobin <9.5 g/dL and/or platelets <50,000/mm^3) and do not consider progressive lymphocytosis or disease-related symptoms for treatment initiation in the absence of cytopenias or symptomatic masses. Patients with liberal criteria had a better OS than those with restrictive criteria (85 % versus 68 %), suggesting that restrictive criteria are more predictive of prognosis than liberal criteria. Furthermore, among patients with liberal criteria, OS was significantly worse in treated patients (83 %) compared to untreated patients (97 %; *p*-value <0.0001), suggesting a possible detrimental effect of treatment in patients with borderline indications. The goal of this analysis was to suggest that treatment indication should only consider criteria that truly affect clinical outcomes and patient quality of life, thereby avoiding unnecessary treatments, costs, treatment-related toxicity, and potential interference with disease biology by selecting for more resistant clones (Table 3).

Asymptomatic disease

To date, there is no evidence to support treatment of CLL at the time of diagnosis in the absence of symptoms. For patients with asymptomatic disease (Rai 0, Binet A) or asymptomatic intermediate-risk disease (Rai I-II, Binet B), watchful waiting with clinical assessments and blood counts every three months, especially during the first year, is recommended. Those with stable disease may be followed at longer intervals, from six to even 12 months (Table 4).

Symptomatic disease

Proper assessment of symptomatic disease is critical to select the most appropriate treatment for each patient. In addition to disease stage and cytogenetic risk, the patient's physical condition and comorbidities must be considered. A useful tool in this context is the Cumulative Illness Rating Scale (CIRS), which allows patients to be ranked in terms of treatment suitability according to known comorbidities.[35] In clinical trials, patients with a CIRS score ≤6 and normal glomerular filtration rate (creatinine clearance >70 mL/min) are generally considered fit for more intensive treatments. It is important to note that age should not be used as a standalone marker of eligibility for CLL treatment, especially in the context of targeted therapies.

Chemotherapy/Chemo-immunotherapy

Monotherapy with alkylating agents such as chlorambucil has been a common choice for many decades and may still be an option, especially for very elderly patients or those in poor health and unsuitable for more aggressive treatments. Chlorambucil offers notable advantages such as lower cost, low toxicity and ease of oral administration. However, its main disadvantages include very low complete remission (CR) rates and the risk of long-term side effects such as myelodysplasia. Nowadays, when available, chlorambucil monotherapy is avoided in favor of its combination with anti-CD20 monoclonal antibodies, which leads to improved response rates and PFS probabilities.

Fludarabine, a purine analogue, has been extensively studied in CLL. In various studies, response rates to fludarabine monotherapy range from 63 to 73 %, with 7–40 % of patients

Table 4 – Treatment recommendations from the Brazilian CLL Group.

(A) First line

Binet A-B, Rai 0-II, asymptomatic		No treatment
Binet C ou Rai III-IV Symptomatic (restrictive criteria)	del(17p) ou TPP53 mutation	Ibrutinib / Acalabrutinib ± G / Zanubrutinib Venetoclax + G / Venetoclax + Ibrutinib
	Go go Fit – IGVH mutated	Venetoclax + G / Venetoclax + Ibrutinib Ibrutinib / Acalabrutinib ± G / Zanubrutinib FCR or BR (< 65)
	Go go Fit – IGVH unmutated	Ibrutinib / Acalabrutinib ± G / Zanubrutinib Venetoclax + G / Venetoclax + Ibrutinib
	Slow go Unfit – IGVH mutated	Venetoclax + G / Venetoclax + Ibrutinib Ibrutinib / Acalabrutinib ± G / Zanubrutinib Chlorambucil + G
	Slow go Unfit – IGVH unmutated	Venetoclax + G / Venetoclax + Ibrutinib Ibrutinib / Acalabrutinib ± G / Zanubrutinib

* G: obinutuzumab, R: rituximabe, FCR: fludarabine, cyclophosphamide, rituximabe, BR: bendamustine, rituximabe.

(B) Further lines (≥2nd line)

Asymptomatic relapse		No treatment
Symptomatic relapse (restrictive criteria)	Go go Fit	Venetoclax + rituximab Ibrutinib / Acalabrutinib / Zanubrutinib Consider allogeneic transplantation or CAR-T cell after failing both iBTK and iBCL2 Consider Pirtobrutinib as bridging before transplant
	Slow go Unfit	Venetoclax + rituximab Ibrutinib / Acalabrutinib / Zanubrutinib Consider Pirtobrutinib after failing both iBTK and iBCL2

* iBTK: Bruton's tyrosine kinase inhibitor, B-cell lymphoma 2 (Bcl-2) inhibitor.

achieving CR. Combinations of fludarabine with cyclophosphamide (FC) have demonstrated better overall response rates (74–94 %) and CR rates (23–38 %) compared to other regimens in the pre-rituximab era, which marked the beginning of combination therapy.[36]

The introduction of chemoimmunotherapy further improved outcomes in the frontline setting. Studies such as CLL8[37] demonstrated the superiority of the fludarabine, cyclophosphamide and rituximab (FCR) regimen over FC, providing higher response rates and prolonged PFS without increasing toxicity or the risk of infection. Long-term follow-up of the FCR arm showed prolonged OS, and in *IGHV*-mutated patients without del(17p), a survival plateau was observed, suggesting the potential for cure. This finding has been corroborated by other studies in FCR-treated patients.[38,39] The FCR combination became the treatment of choice for patients eligible for intensive therapy. However, it is important to note that FCR is associated with an increased risk of myelodysplastic syndrome and acute myeloid leukemia compared to targeted therapies. Given the high prevalence of CLL in elderly patients, an FCR-Lite regimen was developed to reduce toxicity while maintaining efficacy.[40]

The CLL11 trial also showed promising results with the combination of obinutuzumab and chlorambucil, with a response rate of 78.4 % and a CR rate of 20.7 % in patients ineligible for fludarabine-based treatment.[41] Obinutuzumab achieved better response rates than chlorambucil monotherapy and the rituximab/chlorambucil combination. It is

important to note that obinutuzumab-related infusion reactions occur in approximately 65 % of subjects during the first cycle, with 21 % of these reactions being Grade 3 or 4, leading to discontinuation in 7 % of patients.

In a clinical trial, bendamustine, an alkylating agent with purine analog properties, was compared to chlorambucil and achieved better response rates (68 %) with a CR of 31 % and PFS of 21.6 months.[42] The CLL10 trial showed that rituximab with a bendamustine dose of 90 mg/m^2 in the first-line setting resulted in response rates similar to FCR at 97 %, but with fewer CRs (31 %).

Currently, FCR is an appropriate option for patients ≤65 years of age with creatinine clearance >70 mL/min, mutated *IGHV*, and without *TP53* alterations or complex karyotypes, when targeted therapies with or without anti-CD20 antibodies are not available, mostly in limited access scenarios. This fixed-duration therapy can produce durable remissions, some lasting more than 10 years, justifying its continued use as first-line therapy. Patients with mutant *IGHV* aged >65 years or ≤65 years with comorbidities (CIRS >6 and <12) can receive the bendamustine/rituximab regimen (BR) or chlorambucil with an anti-CD20 agent (obinutuzumab is the most active).

Targeted therapies

Over the past decade, therapies targeting the B-cell receptor (BCR) or the anti-apoptotic protein B-cell lymphoma 2 (BCL-2) have profoundly transformed the treatment of CLL. These therapies include both continuous and fixed-duration regimens, all of which have demonstrated superiority over chemoimmunotherapy. This shift follows the FDA approvals of the covalent BTKi ibrutinib in 2014 and the BCL-2 inhibitor (BCL-2i) venetoclax in 2016.

Two second-generation covalent BTKis, acalabrutinib and zanubrutinib, have shown improved safety profiles compared to ibrutinib, with potentially lower toxicity, and were approved by the FDA in 2019 and 2023, respectively. With the availability of these agents, it has become clear that, in addition to clinical and molecular characteristics, other factors, such as specific comorbidities, concomitant medications, and therapy-related risks, should be considered when selecting the optimal first-line treatment for each patient.

For subsequent lines of treatment, it is crucial to assess the response or lack of response to prior therapy, duration of response, the development of resistance to a specific agent, or the occurrence of toxicity that prevents continuation, as well as the presence of *TP53* mutations or del(17p).

Covalent *Bruton's tyrosine kinase inhibitors*

Through irreversibly binding to the cysteine residue (C481) in Bruton's tyrosine kinase (BTK) domain and inhibiting its enzymatic activity, covalent BTKi interferes with B-cell receptor signaling, affecting adhesion, migration, proliferation and cell survival, resulting in redistribution of CLL cells from secondary lymphoid organs to the PB, reducing lymphadenopathy and splenomegaly, with an expected transient increase in PB lymphocytes over the first weeks or months of treatment. Over time, lymphocytosis decreases due to deprivation of survival signals from lymphoid tissues and a direct pro-apoptotic effect. Despite favorable long-term outcomes, monotherapy with covalent BTKi does not induce deep molecular responses, with low rates of undetectable MRD. Ibrutinib was the first in this class to enter clinical trials and currently has the most extensive data of any available BTKi, particularly in high-risk patients. The pivotal phase 1b/2 study PCYC-1102/1103 in heavily pretreated and treatment-naive patients aged ≥65 years compared two doses of ibrutinib (420 mg versus 840 mg) with identical overall response rates of 71 % in both groups. This established 420 mg/day as the standard dose for CLL/SLL. In patients with del(17p), the response rate was similar at 68 %, highlighting the efficacy of ibrutinib in this poor prognostic group. These initial results have been confirmed in three additional studies.

The RESONATE study (PCYC-1112) in relapsed/refractory CLL/SLL [86 % high-risk alterations (del17p/*TP53* mutation), del(11q) and/or unmutated *IGHV*] showed significant improvements in PFS and OS with ibrutinib compared to anti-CD20 ofatumumab. With a median follow-up of 6 years, the median PFS remained significantly longer in the ibrutinib arm with continued OS benefit.[43] The RESONATE-2 study (PCYC-1115/1116) in treatment-naive patients ≥65 years of age without del(17p) showed an OS benefit with a PFS rate of 70 % with ibrutinib versus 12 % with chlorambucil.[44] Recent data show good tolerability of this agent, with 42 % of patients on continuous ibrutinib after 7 years of follow-up.[45] Finally, the RESONATE-17 trial[46] confirmed the efficacy of ibrutinib in previously treated patients with a median age of 64 years and del(17p). The 24-month PFS rate was 63 %, and 75 % of patients were alive at 2 years. The most common reasons for discontinuation were disease progression in 24 % of patients and adverse events with unacceptable toxicity (mainly arrythmias and infections) or death in 17 % of patients.

The multicenter Phase 3 ILLUMINATE study[47] in previously untreated CLL/SLL patients aged >65 years or ≤65 years with comorbidities randomized patients to continuous oral ibrutinib plus obinutuzumab (IO) or chlorambucil plus obinutuzumab (CBL+O). At a median follow-up of 31.3 months, the median PFS was not achieved in the IO arm and was 19.0 months in the CBL+O arm, with 30-month PFS estimates of 79 % with IO and 31 % with CBL+O. The most common Grade 3 or 4 adverse events in both arms were neutropenia and thrombocytopenia. Serious adverse events occurred in 58 % of patients treated with IO and 35 % of patients treated with CBL +O.

The ALLIANCE 202 trial compared ibrutinib ± rituximab with BR in elderly patients with previously untreated CLL.[48] PFS at 2 years was 74 % with BR and 87 % with ibrutinib monotherapy. No significant difference in PFS was observed between the ibrutinib + R and ibrutinib monotherapy groups. The PFS benefit of ibrutinib over BR was seen in all cytogenetic subgroups, with del(17p) being the most prominent. PFS differences were maintained at 4 years of follow up.

Two studies compared ibrutinib ± rituximab with FCR. The Phase 3 E1912 study enrolled treatment-naive patients ≤70 years of age without high-risk genetic alterations. Three-year results showed that continuous ibrutinib plus rituximab was associated with improved PFS and OS versus FCR. However, the OS benefit is questioned by some experts because of

deaths unrelated to treatment or disease and because 31 % of FCR patients did not complete all six treatment cycles. The PFS benefit was more evident in patients with unmutated *IGVH*, with a PFS at 5 years of 75 % in the IR group versus 33 % in the FCR group. The incidence of Grade ≥3 adverse events was similar in both groups, while Grade ≥3 cytopenias and infectious complications were less common with IR than with FCR.

The open-label, multicenter, Phase 3 FLAIR trial,[32,33] in two of its four arms, compared 6 years of ibrutinib plus rituximab (IR) versus FCR as first-line treatment in patients with a median age of 62 years without del(17p). IR showed higher 5-year PFS rates compared to FCR, regardless of *IGHV* mutation status. No OS benefit was observed. Median PFS was not achieved in the IR arm and was 67 months in the FCR arm. In addition, at 3 years, 58 % of patients in the ibrutinib-venetoclax arm discontinued therapy due to undetectable MRD. After 5 years of ibrutinib-venetoclax therapy, 66 % of patients had undetectable MRD in BM and 93 % had undetectable MRD in PB.

In general, Grade 3 or greater adverse events were less common in the ibrutinib arms compared to the chemotherapy arms, and adverse events of any grade associated with ibrutinib were consistent across studies. The most common adverse events of any grade were diarrhea, hemorrhage, fatigue, nausea, cough, pyrexia, anemia, rash, thrombocytopenia, and neutropenia. The most common Grade ≥3 events were neutropenia, anemia, pneumonia, thrombocytopenia, hypertension, and diarrhea. Atrial fibrillation occurred in approximately 5–10 % of patients, and 3–8 % of patients developed Grade ≥3 atrial fibrillation. All adverse events should be managed according to institutional therapeutic measures, and multidisciplinary follow-up with a cardio-oncologist is recommended for cardiovascular events.

The introduction of the second-generation BTKis, acalabrutinib and zanubrutinib, provided additional treatment options for CLL in both first line and relapsed/refractory settings, demonstrating greater selectivity for BTK with fewer off-target effects. Follow-up data suggest a lower risk of cardiovascular events compared to ibrutinib.

Acalabrutinib monotherapy was evaluated in relapsed or refractory CLL in the ASCEND trial, which demonstrated its superiority over idelalisib-rituximab or BR, regardless of the presence of *TP53* alterations. The ELEVATE-TN study compared acalabrutinib ± obinutuzumab (Acala±O) with chlorambucil + obinutuzumab (CBL+O) in previously untreated CLL patients.[49] Median follow-up was 28.3 months with PFS rates of 93 %, 87 % and 47 % for Acala+O, Acala monotherapy and CBL+O, respectively. Median PFS was 22.6 months in the CBL+O arm and was not achieved in the Acala±O arms. There was no statistically significant difference in PFS between the Acala+O and Acala monotherapy arms and were beneficial for patients with *TP53* alterations. At the 5-year update, the PFS rate was 71 % for the Acala±O arm versus 18 % for CBL+O in patients with del(17p) and/or mutated *TP53*.

The ELEVATE-RR trial[50] compared acalabrutinib with ibrutinib in relapsed or refractory CLL patients with at least one high-risk genetic alteration (mutation and/or del(17p/*TP53*) or del(11q)). At a median follow-up of 40.9 months, acalabrutinib demonstrated non-inferiority to ibrutinib in terms of efficacy and was associated with lower rates of cardiovascular (hypertension and atrial fibrillation) and non-cardiac events (diarrhea, myalgia/arthralgia and bleeding), suggesting that greater BTK selectivity may reduce off-target effects, resulting in an improved clinical safety profile. However, acalabrutinib was associated with higher rates of headache and cough compared to ibrutinib.

The Phase 3 SEQUOIA study evaluated zanubrutinib in previously untreated CLL/SLL patients aged ≥ 65 years who were ineligible for FCR.[51] The patients were divided into two cohorts: Cohort A - patients without del(17p) randomized to receive zanubrutinib or BR; Cohort B (non-randomized) - patients with del(17) received zanubrutinib monotherapy. In cohort A, with a median follow-up of 26.2 months, the 24-month PFS rate was 85.5 % for zanubrutinib versus 69.5 % for BR. PFS was also superior in the zanubrutinib arm irrespective of *IGHV* status, with an acceptable safety profile. Zanubrutinib was compared to ibrutinib in patients with relapsed or refractory CLL in the Phase 3 ALPINE study.[52,53] Zanubrutinib was superior to ibrutinib with 2-year PFS rates of 78.4 % versus 65.9 % (p-value = 0.002); OS was not achieved in either treatment arm. The safety profile of zanubrutinib showed fewer serious adverse events and treatment discontinuations compared to ibrutinib. The incidence of Grade ≥3 hypertension was higher with zanubrutinib compared to ibrutinib, but the incidence of any grade atrial fibrillation was lower. Neutropenia occurred in 29 % of patients treated with zanubrutinib. To date, no clinical trial has directly compared acalabrutinib with zanubrutinib. Real-world evaluations of the efficacy and safety of second-generation covalent BTKi are ongoing, with preliminary data currently supporting the results of Phase 3 trials.

A recent matching-adjusted indirect comparison (MAIC) analysis[54] found that acalabrutinib and zanubrutinib have similar efficacy in relapsed or refractory CLL based on PFS. While adverse event rates were generally comparable, acalabrutinib showed lower rates of serious adverse events, Grade ≥3 hypertension, hemorrhage, and dose reductions. The strength of the study lies in its adherence to MAIC methodology and the minimal impact of matching on acalabrutinib outcomes, reflecting the similarity between ALPINE and ASCEND trials.

Non-covalent Bruton's tyrosine kinase inhibitors

Covalent BTKis have demonstrated high efficacy and, in many cases, long-term disease control. Because inhibition must be maintained indefinitely to achieve and maintain clinical response, there is a prolonged exposure period during which adverse events and the development of resistance to these agents may occur. Several mechanisms of resistance have been identified, many of which involve mutations in the *BTK* gene or related genes. The mutation at the C481S residue of *BTK* is the most common and occurs at the site where the covalent inhibitor binds to *BTK*, preventing this binding. Another mutation, almost always synchronic with *BTK* mutations, PLCG2, although less common, can activate alternative signaling pathways that bypass the need for BTK. In addition to *BTK* mutations, secondary mutations in other genes related

to the B cell receptor pathway or parallel pathways can contribute to the emergence of resistance.

Third-generation non-covalent BTKis have been developed to overcome resistance to covalent BTKis while exhibiting a favorable safety profile. Data show that the mutation at the C481S residue was successfully overcome by non-covalent BTKis, but others such as L528W (frequent after zanubrutinib) and T474I (frequent after acalabrutinib) are not.

Pirtobrutinib showed promising results in a Phase 1/2 study in 276 patients with previously treated CLL/SLL, with an overall response rate of 74 % and a median PFS of 19.4 months. Pirtobrutinib is currently being evaluated in the Phase 3 BRUIN CLL-321 study in populations with prior exposure to BTKis compared to BR or R-idelalisib. A total of 338 patients with a median age of 66 years were equally randomized to two arms, 50 % of patients were also previously treated with venetoclax, and high-risk features were ubiquitous in both arms. At 18 months of follow up, pirtobrutinib demonstrated superior median PFS with 14 months versus 8.7 months as assessed by an Independent Review Committee. No OS survival difference was observed with 73.4 % OS in the pirtobrutinib arm with a 76 % crossover rate from the Standard of care arm likely impacting these data. Although infections were more common in the pirtobrutinib arms, when these data were adjusted for drug exposure time, similar infection rates were documented in the pirtobrutinib arm. Adverse events of interest for pirtobrutinib were consistent with the BTKi class with fewer cases of atrial fibrillation and hypertension compared to cBTKi, but also with a shorter median exposure time.

Bruton's tyrosine kinase degraders

While currently approved BTKis, such as ibrutinib, work by reversibly or irreversibly binding to BTK to modulate its signaling activity, they do not eliminate the protein itself. Instead, they suppress BTK-mediated survival pathways in malignant B cells, leading to apoptosis.

In contrast, BTK degraders represent an emerging class of therapeutic agents that not only inhibit BTK function, but also actively induce its degradation via the proteasome. These agents use targeted protein degradation (TPD) technology, such as proteolysis-targeting chimeras (PROTACs), to recruit ubiquitin ligases that tag BTK for proteasomal degradation. By eliminating the BTK protein rather than merely inhibiting its activity, BTK degraders may overcome resistance mechanisms associated with BTKis, particularly mutations such as *BTK* C481S that confer resistance to covalent BTKis. However, early data suggest that some non-covalent BTKi resistant mutations, such as A428D, may also confer resistance to BTK degraders, highlighting the need for further investigation into their clinical utility.

Although BTK degraders are not yet approved for clinical use, early phase studies suggest that they may provide deeper and more sustained inhibition of BTK-driven signaling, potentially expanding treatment options for patients with relapsed or refractory CLL and other B-cell malignancies.

With the development of novel BTK-targeting strategies, another critical pathway in B-cell malignancies is the BCL-2-regulated apoptotic machinery, which is effectively targeted by BCL-2is such as venetoclax.

B-cell lymphoma 2 inhibitors

The B-cell lymphoma 2 (Bcl-2) family of proteins are key regulators of the apoptotic process. The Bcl-2 family includes pro-apoptotic and pro-survival proteins. Shifting the balance toward the latter is an established mechanism by which cancer cells evade apoptosis. Bcl-2, the founding member of this protein family, is encoded by the *BCL2* gene, first described in follicular lymphoma as a result of translocations involving chromosomes 14 and 18, leading to protein overexpression. Venetoclax is a BH3 mimetic that inhibits Bcl-2, promoting apoptosis by releasing pro-apoptotic proteins. It effectively suppresses the growth of Bcl-2-dependent tumors in vivo while sparing human platelets, unlike navitoclax, which was previously tested but did not reach the market due to dose-limiting thrombocytopenia. A single oral dose of venetoclax in three patients with refractory CLL resulted in tumor lysis within 24 h. To mitigate this risk, a stepwise dose escalation regimen was introduced, increasing weekly from 20 mg to 50 mg, 100 mg, 200 mg, and finally to 400 mg over 4–5 weeks. After completing the ramp-up phase, patients continued on 400 mg daily until disease progression or unacceptable toxicity occurred. In the pivotal Phase 1/2 clinical trial, 56 patients received venetoclax in one of eight dose groups ranging from 150 to 1200 mg per day.[55] In an expansion cohort, an additional 60 patients received venetoclax with progressive weekly dose escalation up to 400 mg per day. Most patients had received multiple prior therapies and 89 % had poor prognostic clinical or genetic features. Venetoclax was effective at all dose levels. Clinical tumor lysis syndrome occurred in three of 56 patients in the dose-escalation arm, with one death. After adjustments to the dose-escalation schedule, none of the 60 patients in the expansion cohort experienced clinical tumor lysis syndrome. No maximum tolerated dose was observed. Of the 116 patients who received venetoclax, 92 (79 %) responded. Response rates ranged from 71 to 79 % in patients with poor prognosis, including those with fludarabine resistance or del(17p) or unmutated *IGHV*. Complete remissions occurred in 20 % of patients, including 5 % of remissions with MRD negativity. The 15-month PFS estimate for the 400 mg dose group was 69 %. Another study was conducted in 107 patients with relapsed or refractory del(17p) CLL. At a median follow-up of 12.1 months, 85 patients (79.4 %) achieved investigator-driven CR. The most common Grade 3–4 adverse events were neutropenia (40 %), infection (20 %), anemia (18 %) and thrombocytopenia (15 %). Serious adverse events occurred in 55 % of patients, with the most common (≥5 % of patients) being fever and autoimmune hemolytic anemia (7 % each), pneumonia (6 %), and febrile neutropenia (5 %). Eleven patients in the study died within 30 days of the last dose of venetoclax, seven due to disease progression and four due to adverse events (none considered treatment-related). Together, the results of the two studies demonstrate that venetoclax monotherapy is active and well tolerated in patients with relapsed or refractory del(17p) CLL, providing a new therapeutic option for this population with a very poor prognosis.

The Phase 3 CLL14 trial, a multicenter, randomized, open-label study conducted at 196 research centers in 21 countries,[56] enrolled treatment-naïve CLL patients over 65 years of age and/or with comorbidities (CIRS score greater than 6). Patients were randomized to receive venetoclax (orally initiated on Day 22 of Cycle 1 [28-day cycles] with a 5-week escalation [20 mg, 50 mg, 100 mg, 200 mg, then 400 mg daily for 1 week], continuing at 400 mg daily until completion of cycle 12; combined with intravenous obinutuzumab for six cycles starting with 100 mg on Day 1 and 900 mg on Day 2 [or 1000 mg on Day 1], 1000 mg on Days 8 and 15 of Cycle 1, and then 1000 mg on Day 1 of Cycles 2 through 6) or chlorambucil with obinutuzumab (oral chlorambucil at 0. 5 mg/kg body weight on Days 1 and 15 of each cycle for 12 cycles in combination with the same obinutuzumab regimen). A total of 432 patients were randomized (venetoclax and obinutuzumab: n = 216; chlorambucil and obinutuzumab: n = 216). At a median follow-up of 76.4 months,[57] PFS remained superior in the venetoclax-obinutuzumab arm compared to the chlorambucil-obinutuzumab arm (median, 76.2 versus 36.4 months, p-value <0.0001). Similarly, time to next treatment (TTNT) was significantly longer (6-year TTNT: 65.2 % versus 37.1 %, respectively; p-value <0.0001). The most common Grade 3 or 4 adverse event in both groups was neutropenia (53 % in the venetoclax-obinutuzumab arm and 48 % in the chlorambucil-obinutuzumab arm). In an update of the study 2 years after completion of treatment, patients who received venetoclax-obinutuzumab continued to show a significant PFS benefit with no new evidence of associated adverse events, making it an excellent finite therapy option for this elderly and/or comorbid patient population.

In the Phase 3 GAIA (CLL13) clinical trial,[58] patients with CLL who were fit and had no *TP53* gene abnormalities were randomized in a 1:1:1 ratio to receive six cycles of chemotherapy: one to six cycles of chemoimmunotherapy (fludarabine-cyclophosphamide-rituximab or bendamustine-rituximab) or 12 cycles of venetoclax-rituximab, venetoclax-obinutuzumab or venetoclax-obinutuzumab-ibrutinib. Of the 926 patients randomized, 229 received chemoimmunotherapy, 237 received venetoclax-rituximab, 229 received venetoclax-obinutuzumab and 231 received venetoclax-obinutuzumab-ibrutinib. At Month 15, the proportion of patients with undetectable MRD was significantly higher in the venetoclax-obinutuzumab (86.5 %) and venetoclax-obinutuzumab-ibrutinib (92.2 %) arms compared to the chemoimmunotherapy (52 %) and venetoclax-rituximab (57 %) arms. The 3-year PFS was higher in the venetoclax-obinutuzumab (87.7 %) and venetoclax-obinutuzumab-ibrutinib (90.5 %) arms compared to the chemoimmunotherapy (75.5 %) and venetoclax-rituximab (80.8 %) arms. Grade 3 and 4 infections were more common with chemoimmunotherapy (18.5 %) and venetoclax-obinutuzumab-ibrutinib (21.2 %) than with venetoclax-rituximab (10.5 %) or venetoclax-obinutuzumab (13.2 %). The study suggests that, as in elderly patients, the combination of venetoclax and obinutuzumab appears to be the most effective finite therapy option, offering better response rates, survival, and safety. However, longer follow-up is needed to confirm these results.

The Phase 3 AMPLIFY trial (NCT03836261) evaluated acalabrutinib-venetoclax (AV) and acalabrutinib-venetoclax-obinutuzumab (AVO) versus chemoimmunotherapy (FCR or BR) in treatment-naive CLL patients without *TP53* aberrations.[53] At a median follow-up of 41 months, both AV and AVO significantly improved PFS compared to chemoimmunotherapy, with median PFS not reached in either acalabrutinib-containing arm. The 36-month PFS rates were 76.5 % (AV), 83.1 % (AVO) and 66.5 % (FCR/BR). Overall response rates were also higher with AV (92.8 %) and AVO (92.7 %) versus FCR/BR (75.2 %). Grade ≥3 neutropenia was the most common adverse event, and serious adverse events were most common in AVO-treated patients (38.4 %). These findings support acalabrutinib-based regimens as effective, chemotherapy-free alternatives in treatment-naive CLL, with AV offering a favorable safety/efficacy balance and AVO achieving the highest PFS but with more toxicity.

Comorbidities and patient preference

Selection of the optimal therapy for CLL continues to be guided by a personalized approach that considers both disease biology and patient-specific factors. Comorbidities play a critical role in treatment selection, as many patients with CLL are elderly and have cardiovascular, renal, or autoimmune diseases that may limit the use of certain therapies. For example, patients with a history of atrial fibrillation or bleeding disorders may not tolerate BTKis, particularly ibrutinib, while patients with renal impairment may require dose adjustments or alternative regimens to BCL-2is. In addition, patient preferences have a significant impact on treatment decisions, as considerations such as route of administration (oral versus intravenous), treatment duration (fixed duration versus continuous therapy) and side effect profiles affect adherence and quality of life. The increasing use of MRD-driven strategies also allows for more individualized treatment durations, allowing for discontinuation of therapy in patients who achieve deep remissions while maintaining durable disease control.[59]

Infectious complications in CLL

Given the profound immune dysregulation associated with CLL and the immunosuppressive effects of targeted therapies, infection prevention remains a cornerstone of patient management. Vaccination strategies have gained prominence, with strong recommendations for the administration of inactivated vaccines, including influenza, pneumococcal, and COVID-19 vaccines, to all patients with CLL, ideally prior to treatment initiation. While response rates to vaccines may be suboptimal due to underlying immune dysfunction, newer strategies such as booster doses and passive immunization with monoclonal antibodies against SARS-CoV-2 have shown promise in improving protection. In addition to vaccination, prophylactic antimicrobials, including antiviral agents (e.g., acyclovir for herpesvirus reactivation) and *Pneumocystis jirovecii* pneumonia (PJP) prophylaxis in selected patients receiving B-cell depleting therapies, remain essential to reduce infectious complications. Regular immunoglobulin replacement therapy is being considered for patients with recurrent infections and hypogammaglobulinemia, further highlighting the

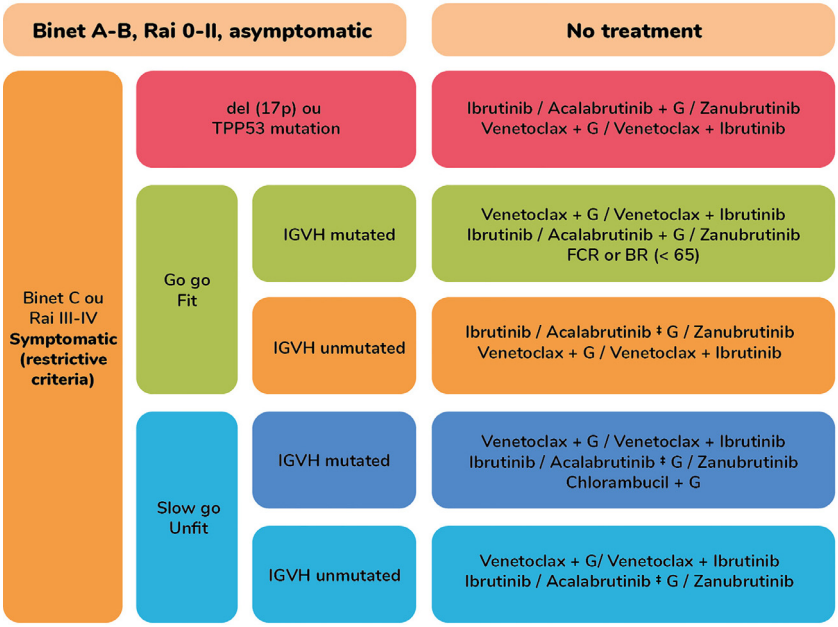

Figure 1 – Brazilian Group of CLL established treatment recommendations.

importance of a proactive, individualized approach to infection management in CLL.

Treatment in public or private centers in Brazil and other less-resourced countries

Due to systemic inequities, hematologists in Brazil face additional challenges in determining the best treatment regimen for CLL patients. Access to novel therapies is highly inequitable between public and private healthcare institutions, with significant implications for the efficacy and tolerability of CLL treatments.[60]

In the first published analysis of the Brazilian Group of CLL,[61] the median follow-up time of 1903 patients was 36 months (range: 3–155 months). Treatment-free survival at 3 and 5 years was 44 % and 32 %, respectively with advanced Binet staging showing a strong correlation with inferior survival.

Patients from public and private institutions were compared in an analysis of the Brazilian CLL Registry.[62] Of 3326 patients, 81 % were in public hospitals and 19 % in private hospitals. Public hospital patients were older (median age 66 years versus 63 years in private hospitals), had more advanced disease (44 % versus 33 % Binet B or C), and more frequently had elevated creatinine levels (18 % versus 10 %). Prognostic markers were evaluated more frequently in private hospitals: FISH for del(17p) (45 % versus 10 %), *IGHV* mutation (19 % versus 6 %), karyotype (24 % versus 12.5 %), and beta-2 microglobulin (47 % versus 32 %). The frequency of FISH-positive del(17p) was similar (10.5 % versus 9 %), as was the frequency of unmutated *IGHV* (50 % versus 56 %). Due to missing data, only 432 patients (13 %) were stratified by CLL-IPI: 175 (40 %) with low/intermediate scores and 257 (60 %) with high/very high scores.[62] High-risk CLL-IPI patients were more likely to be found in public hospitals (69 % versus 45 %).

Regarding treatment, chlorambucil or fludarabine was the most commonly used first-line therapy (chlorambucil: 41 %; fludarabine: 38 %). Anti-CD20 monoclonal antibodies were used in only 36 % of cases (rituximab: 32 %; obinutuzumab: 4 %). New agents were used in only 5 % of cases. Public hospitals were less likely to use fludarabine (36 % versus 48 %) and anti-CD20 monoclonal antibodies (26 % versus 75 %). Surprisingly, the majority of patients with del(17p) or *TP53* mutations (69 %) received chemoimmunotherapy as first-line therapy. Median follow-up was 39 months, and overall survival was 71 % at 5 years, which was worse in public hospitals (68 % versus 82 %). These data show significant differences between patients treated in public and private hospitals likely due to a more advanced initial presentation and lack of access to appropriate testing and therapies.[62]

In 2025, the Brazilian Group of CLL established treatment recommendations based on the accumulated evidence to date, which are presented in Figure 1.

Conclusions

CLL remains the most common form of leukemia in adults and presents complex clinical challenges due to its heterogeneous nature and variable response to treatment. Research highlights several key points:

1. Epidemiology and diagnosis: CLL typically affects older adults, with a significant proportion of patients asymptomatic at diagnosis. Identification of monoclonal B lymphocytes with specific immunophenotypic markers is critical for diagnosis, highlighting the importance of advanced diagnostic techniques in differentiating CLL from other B-cell malignancies.

2. Prognostic factors: Prognostic stratification remains critical, with *IGHV* mutation status and chromosomal abnormalities, mainly deletions involving *TP53*, serving as significant indicators of disease aggressiveness and treatment resistance. The integration of clinical staging systems with genetic profiling is essential to tailor treatment strategies.
3. Treatment advances: The introduction of targeted therapies, such as BTK inhibitors and BCL-2 inhibitors, has revolutionized the treatment of CLL. These therapies show superior efficacy compared to traditional chemotherapy, especially in high-risk populations. However, the need for careful patient selection and consideration of comorbidities is paramount to optimize outcomes and minimize treatment-related adverse effects.
4. Healthcare disparities: Analysis of access to care in Brazil highlights the disparities between public and private healthcare systems, revealing significant differences in patient demographics, treatment modalities and prognostic assessments. These disparities call for strategic interventions to improve access to effective therapies, especially for vulnerable populations.
5. Future directions: Ongoing research into measurable residual disease (MRD) assessment and novel therapeutic agents holds promise for further improving outcomes in CLL. Further development of treatment protocols and incorporation of MRD assessment into clinical practice may improve long-term survival and quality of life for patients.

In conclusion, a comprehensive understanding of the biological basis of CLL, coupled with advances in diagnostic and therapeutic approaches, is critical to improving patient care. Future efforts should focus on bridging gaps in care and optimizing treatment protocols to ensure equitable access to effective therapies for all CLL patients.

Conflicts of interest

The authors declare no conflicts of interest.

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Letter to the Editor

Hybrid histone deacetylase-kinase inhibitor potentiates venetoclax-induced cell death in chronic lymphocytic leukemia

Dear Editor,

Chronic lymphocytic leukemia (CLL) is characterized by the abnormal production of mature B lymphocytes in the blood, bone marrow, spleen, and lymphoid tissues. However, these cells are dysfunctional due to genomic alterations. CLL cells express functional B-cell receptors (BCRs) on their surface and can be classified into two subgroups based on somatic hypermutations in the variable regions of the *immunoglobulin heavy chain* (*IGHV*) genes. CLL patients with somatic mutations in the *IGHV* gene (M-CLL) generally show better survival rates than those with the unmutated *IGHV* gene (UM-CLL). Clinically, CLL typically presents with lymphocytosis, along with lymphadenopathy or cytopenias (anemia, thrombocytopenia, and neutropenia).[1] BCR signaling is essential in CLL, with Bruton's tyrosine kinase (BTK) playing a key role. BTK inhibitors (BTKis) block this signaling by binding to BTK, thereby hindering the proliferation and survival of both malignant and normal B cells. BTK is crucial for activating survival pathways such as nuclear factor kappa B (NFκB) and mitogen-activated protein kinase (MAPK).[2]

Over the past three decades, several drugs have been approved, including combination chemotherapies and immunotherapies, such as fludarabine, cyclophosphamide, and rituximab, as well as chlorambucil (CLB) combined with obinutuzumab. More recently, inhibitors targeting key pathways have emerged, such as ibrutinib (Bruton's tyrosine kinase inhibitor), idelalisib (PI3Kδ inhibitor), and venetoclax (BCL2 inhibitor).[1,3] Despite these diverse treatment options, genetic abnormalities associated with chemoresistance frequently arise in CLL patients, leading to the use of immunotherapy as a first-line treatment.[3] Additionally, resistance to fludarabine (flu-refractory) remains a major cause of treatment failure in CLL.[4] Therefore, the development of new therapeutic agents for CLL treatment is crucial.

Venetoclax (ABT-199/GDC-0199) is a highly selective BCL2 inhibitor that mimics the BH3 protein by competitively binding to the anti-apoptotic BCL2 protein. This action releases BAK and BAX, subsequently inducing apoptosis.[5] CLL cells exhibit constitutively high expression of BCL2, an anti-apoptotic protein that renders them resistant to cell death. This resistance contributes to the accumulation of long-lived, clonal lymphocytes characteristic of the disease.[6] This feature makes BCL2 inhibitors promising targets for chemotherapy. Venetoclax became a Food and Drug Administration (FDA)-approved standard treatment in June 2018 as a second-line therapy for CLL patients, demonstrating deep and durable responses, regardless of adverse prognostic features such as a 17p deletion.[7]

Histone modification modulates chromatin structure and gene expression, with abnormal histone acetylation linked to cancer development. The histone function is regulated by multiple post-translational modifications, including the reversible acetylation of ε-amino groups of histone's lysine. Histone acetylation is tightly controlled by a balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs).[8]

Vorinostat, the first FDA-approved HDAC inhibitor for lymphoma, is now also used clinically for other cancers.[9] Elevated HDAC activity in CLL B-cells is associated with shorter treatment-free and overall survival, serving as an independent prognostic marker for overall survival and refining the accuracy of established prognostic factors.[10] Preclinical studies show that depsipeptide (FR901228), suberoylanilide hydroxamic acid (SAHA or vorinostat), and chidamide inhibit cellular processes critical to CLL progression and chemoresistance by targeting HDAC activity.[11,12,13] Due to the lack of selectivity and toxicity associated with certain HDAC inhibitors, there is a pressing need for selective inhibitors targeting specific HDAC classes, underscoring the importance of studying novel HDAC inhibitors. However, some novel class I HDAC inhibitors tested in CLL patients as monotherapy presented limited clinical efficacy[14], suggesting that its combination

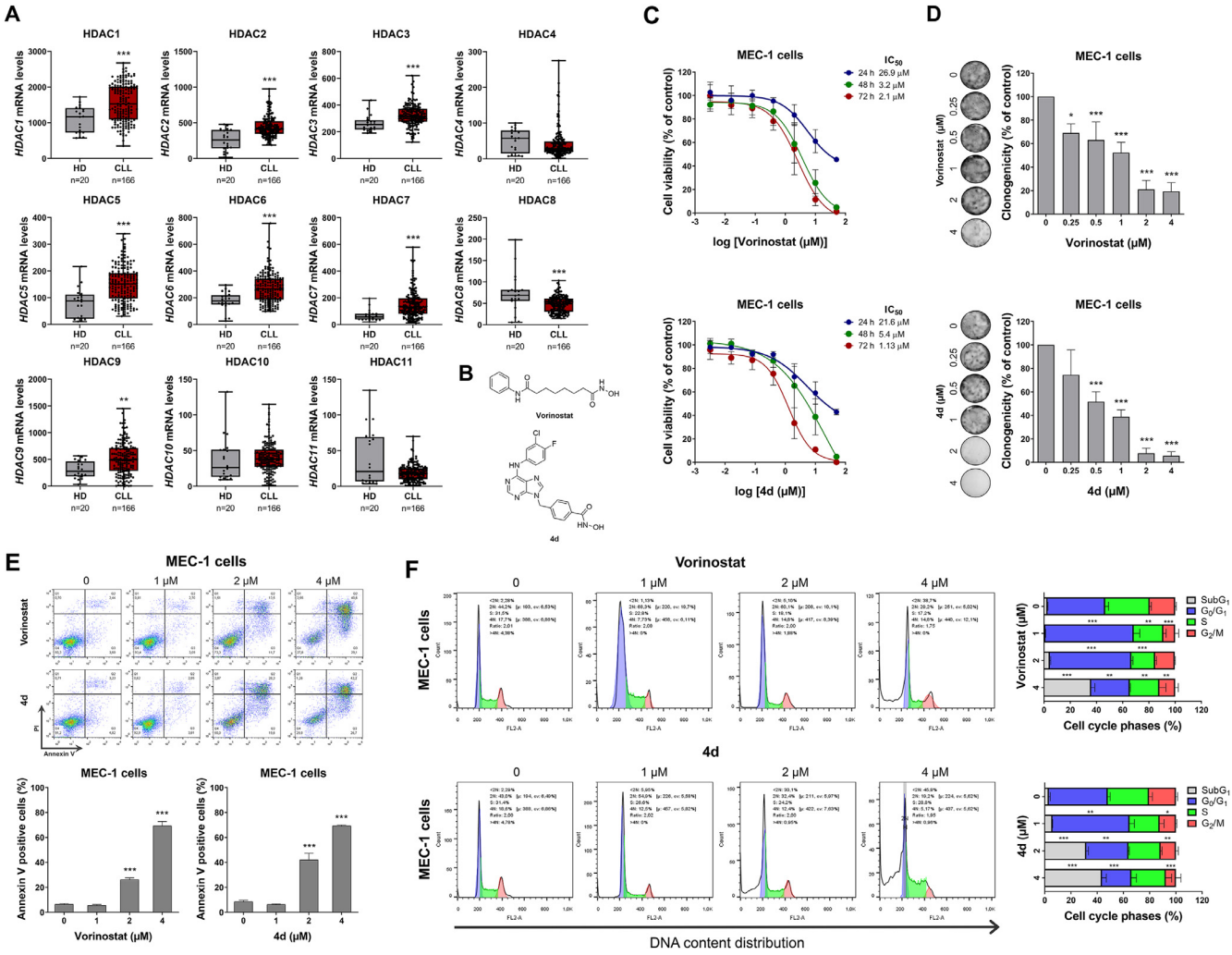

Figure 1 – Histone deacetylases (HDACs) are highly expressed and a potential druggable target in chronic lymphocytic leukemia.(A) The mRNA levels of HDACs (1-11) were measured in samples from healthy donors (n = 20) and CLL patients (n = 166) using data from the Amazonia! database (2008). Gene expression data on the Y-axis were derived from cDNA microarray analysis performed with Affymetrix HGU133 Plus 2.0 arrays. Datasets were cross-referenced using tumor-specific identification numbers, and the sample size for each group is indicated. **p-value <0.01 and ***p-value <0.0001; Mann-Whitney test. (B) The chemical structure of the HDAC inhibitors used are illustrated. (C) Dose- and time-dependent cytotoxicity was assessed using the methylthiazolyl tetrazolium (MTT) assay in MEC-1 cells treated with either vehicle or increasing concentrations of vorinostat or compound 4d (0.0032−50 μM) for 24, 48, or 72 h. Cell viability was quantified as a percentage relative to vehicle-treated controls. Results are expressed as the mean ± SD from at least three independent experiments. The IC_{50} values for each compound are provided in the corresponding Figure. (D) MEC-1 cells were cultured in semisolid medium with either vehicle or increasing concentrations of vorinostat or compound 4d. After 12 days, colonies containing viable cells were detected by adding MTT reagent. Representative colony images are shown, and bar graphs represent the mean ± SD from at least three independent experiments. Statistical significance was determined by ANOVA followed by Bonferroni post-test; *p-value <0.05, *p-value <0.001. (E) MEC-1 cells were labeled with APC-annexin V and propidium iodide (PI) after 48-h treatment with either vehicle or the specified concentrations of vorinostat or compound 4d. Representative dot plots are shown for each condition, with apoptotic cells (annexin V^+ cells) identified in the upper and lower right quadrants (Q2 + Q3). Bar graphs display the mean ± SD from at least three independent experiments. The p-values and cell line are indicated in the graphs; *p-value <0.0001; ANOVA followed by Bonferroni post-test. (F) Cell cycle phases were assessed by analyzing DNA content through propidium iodide staining and flow cytometry after treating MEC-1 cells with either vehicle, vorinostat, or compound 4d at the specified concentrations for 48 h. Representative histograms are shown for each condition, with bar graphs presenting the mean ± SD from at least three independent experiments. p-values and cell lines are indicated in the graphs; *p-value <0.05, **p-value <0.01, *p-value <0.001; ANOVA followed by Bonferroni post-test.

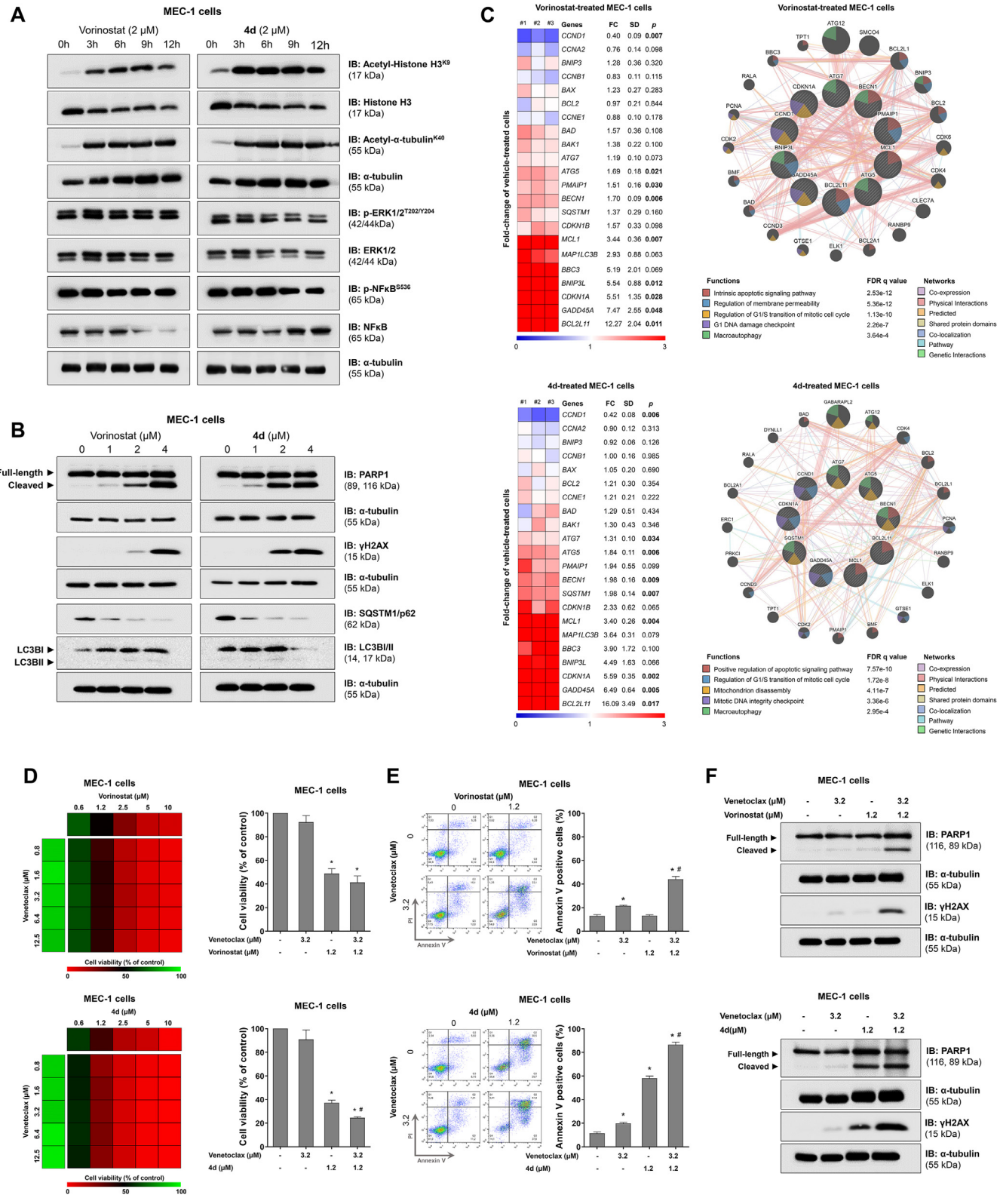

Figure 2 – 4d, a hybrid histone deacetylase (HDAC)-kinase inhibitor, shows greater efficacy in potentiating venetoclax-induced apoptosis in MEC-1 cells.(A) Western blot analysis was conducted to detect acetyl-histone H3 (K9), histone H3, acetyl-α-tubulin (K40), α-tubulin, p-ERK1/2, ERK1/2, p-NFkB, and NFkB in total cell extracts from MEC-1 cells treated with vehicle, vorinostat (2 μM), or compound 4d (2 μM) for 0, 3, 6, 9, or 12 h. (B) Western blot analysis was conducted to detect total and cleaved PARP1, γH2AX, SQSTM1/p62, and LC3BI/II in total cell extracts from MEC-1 cells treated with vehicle, vorinostat (1, 2, and 4 μM), or compound 4d (1, 2, and 4 μM) for 24 h. Membranes were subsequently reprobed with antibodies against total protein or α-tubulin as loading controls and developed using the SuperSignal™ West Dura Extended Duration Substrate system with a

with other therapies could be a strategy to improve efficacy, while avoiding undesirable side effects.

Anilino-purine-benzohydroxamate hybrids were synthesized as dual inhibitors targeting kinases and HDACs. Among these, compound 4d showed promising potency and specificity against leukemia and lymphoma. Notably, some of the identified kinase targets, including BTK, JAK2, and JAK3, are of particular interest in CLL.[15] In the present study, we characterized the cellular and molecular effects of 4d and evaluated its combination with venetoclax in a CLL cell model.

The mRNA expression data for HDACs from healthy donors (normal B cells; n = 20) and CLL patients (n = 103) were sourced from the publicly accessible AmaZonia! Database 2008.[16] MEC-1 cells were kindly provided by Prof. Rodrigo Alexandre Panepucci (Hemocenter of Ribeirão Preto, Brazil) and cultured according to the recommendations of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). Compound 4d was synthesized as previously described.[15] Vorinostat was obtained from Sigma-Aldrich (St. Louis, MO, USA). The structures of HDAC/kinase inhibitors are shown in Figure 1. Cellular and molecular assays were performed as previously described.[17] In summary, cell viability was assessed by MTT assay, clonogenic potential by colony formation assay in methylcellulose (MethoCult 4230; StemCell Technologies Inc., Vancouver, BC, Canada), apoptosis by annexin V/propidium iodide (PI) staining followed by flow cytometry, cell cycle analysis using PI staining was used for assess DNA content and flow cytometry, protein expression and activation by Western blot with specific antibodies (Supplementary Table 1), and gene expression by quantitative PCR with specific primers (Supplementary Table 2). Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software Inc.), with the Mann-Whitney test, analysis of variance (ANOVA) and Bonferroni post-test, or Student's t-test used as appropriate. A p-value <0.05 was considered statistically significant.

The mRNA levels of *HDAC1, HDAC2, HDAC3, HDAC5, HDAC6, HDAC7,* and *HDAC9* were elevated in CLL patients compared to healthy donors (all p-value <0.05), while *HDAC8* expression was lower in CLL patients (Figure 1A). In MEC-1 cells, treatment with vorinostat and 4d reduced cell viability in a dose- and time-dependent manner (Figure 1C). Similarly, these compounds reduced clonal growth in a concentration-dependent manner (Figure 1D) and induced apoptosis (Figure 1E). Both vorinostat and 4d caused cell cycle arrest in the G0/G1 phase at lower concentrations, indicating a cytostatic effect, while higher concentrations led to an increase in the sub-G1 cell population, indicating a cytotoxic effect.

On a molecular level, both compounds strongly induced acetylation of histone H3 and alpha-tubulin, suggesting the inhibition of class I and II HDACs. However, only compound 4d slightly reduced ERK1/2 and NFkB phosphorylation, likely reflecting its hybrid activity on kinases (Figure 2A). Furthermore, markers of cell death such as PARP1 cleavage and γH2AX, were more prominently induced by 4d. Both compounds activated autophagic flux, as shown by SQSTM1/p62 degradation and/or LC3B consumption (Figure 2B). Exploratory gene analysis involving cell cycle progression, DNA damage, apoptosis, and autophagy showed a similar profile of impacted cellular and molecular processes for both compounds (Figure 2C).

Finally, combination assays with venetoclax and either vorinostat or 4d highlighted the superiority of the HDAC-kinase hybrid inhibitor compared to vorinostat. Although both compounds enhanced venetoclax-induced apoptosis, 4d demonstrated greater efficacy in viability assays (Figure 2D), apoptosis induction (Figure 2E), and molecular analysis (Figure 2F).

In summary, CLL patients exhibit increased expression of various HDACs. Vorinostat and 4d reduced cell viability and induced apoptosis in a CLL cell model, with 4d showing higher efficacy in combination with venetoclax. Molecularly, both inhibited HDAC activity, and 4d had additional effects on ERK1/2 and NFkB pathways. These findings suggest that the hybrid compound 4d holds promise for more effective therapies in CLL, warranting further studies focused on its clinical potential and combination with BCL2 inhibitors.

G:Chemi XX6 imaging system. (C) The heatmap displays the gene expression profile of MEC-1 cells treated with vehicle, vorinostat (2 μM), or compound 4d (2 μM) for 24 h. Blue denotes reduced mRNA levels, while red denotes increased mRNA levels, normalized to vehicle-treated cells (n = 3). Fold-change (FC), standard deviation (SD), and p-values were calculated using Student's t-test. A gene network of vorinostat- or 4d-modulated genes was generated using the GeneMANIA database (https://genemania.org/). Genes with significant modulation are represented as crosshatched circles, while interacting genes added by the software are shown as non-crosshatched circles. The main biological interactions, associated functions, and false discovery rate (FDR) q-values are detailed in the Figure. (D) Dose-response cytotoxicity for the combinations of vorinostat plus venetoclax and compound 4d plus venetoclax was evaluated using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. MEC-1 cells were exposed to vehicle or increasing concentrations of venetoclax in combination with vorinostat or compound 4d alone or together for 48 h, as indicated. Cell viability values are expressed as a percentage relative to vehicle-treated controls, with bar graphs highlighting context-relevant combinations. *p-value <0.05, treatment versus vehicle; #p-value <0.05, monotherapy versus combination therapy; ANOVA with Bonferroni post-test. Data represents the mean from at least three independent experiments. (E) For cell death analysis, MEC-1 cells were labeled with APC-annexin V and propidium iodide (PI) after treatment with vehicle, venetoclax, vorinostat, or compound 4d alone or in combination for 48 h. Representative dot plots are shown for each condition, with the upper and lower right quadrants (Q2 + Q3) cumulatively representing the cell death population (annexin V$^+$ cells). Bar graphs display the mean ± SD from at least three independent experiments; *p-value <0.05, treatment versus vehicle; #p-value <0.05, monotherapy versus combination therapy; ANOVA with Bonferroni post-test. (F) Western blot analysis was conducted to detect total and cleaved PARP1, γH2AX and α-tubulin in total cell extracts from MEC-1 cells treated with vehicle, venetoclax, vorinostat, or compound 4d alone or in combination for 24 h.

Conflicts of interest

The authors declare no competing interests.

Acknowledgments

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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.2025.103757.

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Anali Del Milagro Bernabe Garnique [a], Jorge Antonio Elias Godoy Carlos [a], Natalia Sudan Parducci [a], Mauricio Temotheo Tavares [b,c,d], Karoline de Barros Waitman [b], Keli Lima [a,e], Leticia Veras Costa-Lotufo [a], Roberto Parise-Filho [b], João Agostinho Machado-Neto [a,*]

[a] *Department of Pharmacology, Institute of Biomedical Sciences, University of São Paulo, São Paulo, SP, Brazil*
[b] *Department of Pharmacy, Faculty of Pharmaceutical Science, University of São Paulo, São Paulo, SP, Brazil*
[c] *Department of Cancer Biology, Dana-Farber Cancer Institute, Boston, MA, United States*
[d] *Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, United States*
[e] *Laboratory of Medical Investigation in Pathogenesis and Targeted Therapy in Onco-Immuno-Hematology (LIM-31), Department of Internal Medicine, Hematology Division, Faculty of Medicine, University of São Paulo, São Paulo, SP, Brazil*

*Corresponding author. Department of Pharmacology, Institute of Biomedical Sciences of University of São Paulo, Av. Prof. Lineu Prestes, 1524, CEP 05508-900, São Paulo, SP, Brazil.
E-mail address: jamachadoneto@usp.br (J.A. Machado-Neto).

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Letter to the Editor

Evidence-based medicine during the COVID-19 pandemic: A hematologist's perspective

Dear Editor,

In December 2019, the first cases of a previously unknown pneumonia emerged in Wuhan, China. In January 2020, the causative agent was identified as a novel coronavirus, SARS-CoV-2.[1] The virus quickly spread worldwide, and in March 2020, the World Health Organization declared it a pandemic. The rest is history.

I vividly remember the first patient admitted to our hematology-oncology ward with COVID-19 pneumonia. She was a young woman with acute myeloid leukemia in remission after her first cycle of induction chemotherapy. She presented with fever and nasal congestion, which quickly progressed to dry cough and shortness of breath. Upon admission, she was tachypneic, experiencing mild respiratory distress, and her oxygen saturation was 86% on room air, requiring supplemental oxygen. A chest computed tomography scan revealed bilateral ground-glass opacities involving >75% of her lung parenchyma - a radiological pattern that would soon become the hallmark of COVID-19 pneumonia,[2] and a grim predictor of severe, potentially fatal, outcomes.

How do you apply the principles of evidence-based medicine (EBM) - the best available evidence, clinical expertise, and patient values - to guide decisions in managing a previously unknown disease? At the onset of the pandemic, no literature existed to inform clinical practice. By the end of 2020, however, nearly 95,000 articles on COVID-19 had flooded PubMed. Clinical experience had to be extrapolated from analogous conditions, while patient values were often reduced to a desperate plea: "Please, doctor, don't let me die."

In June 2020, amidst an overwhelming influx of poor-quality studies, a large randomized clinical trial demonstrated that dexamethasone reduced 28-day mortality in hospitalized COVID-19 patients requiring oxygen or mechanical ventilation compared to standard care.[3] Finally, there was evidence supporting a treatment that reduced mortality, utilizing an inexpensive, widely available, and well-known drug. Dexamethasone quickly became the global standard of care for these patients, likely saving thousands of lives at the pandemic's peak. One fundamental pillar of EBM - the best available evidence - was now accessible to guide clinical decisions. I could prescribe dexamethasone for my onco-hematologic patients with COVID-19 pneumonia to reduce their risk of death. Or could I?

Despite the trial's robustness and broad inclusion criteria, it did not include onco-hematologic patients. How applicable were the results to my patients, who were profoundly immunosuppressed due to their disease and treatments? Would initiating dexamethasone worsen their immunosuppression, exacerbating the viral infection or predisposing them to secondary infections and potentially fatal outcomes? While there was biological plausibility for both benefit and harm, high-quality evidence supported benefit. However, data on onco-hematologic patients - theoretically among the most vulnerable to increased immunosuppression - were lacking. With patients continuing to arrive, we could not wait for a trial specifically designed for hematologic malignancies. Decisions had to be made despite considerable uncertainty.

This scenario exemplifies an extreme application of the concept of external validity. It challenges the extent to which findings from a study's target population (general hospitalized COVID-19 patients) can be extrapolated to a distinct population (onco-hematologic patients with COVID-19). This process is neither statistical nor purely methodological; it is an intellectual exercise requiring specialized knowledge, clinical judgment, and decision-making in the face of uncertainty. Fully aware of the possibility of error, we decided to prescribe dexamethasone for our onco-hematologic patients hospitalized with COVID-19 pneumonia requiring oxygen support.

Time passed. We treated countless patients, celebrated successes, mourned losses, gathered data, and learned through practice. As vaccination campaigns took effect, hospital admissions declined, and cases generally became milder.[4] With growing experience, we reflected on our decisions. Had prescribing dexamethasone been the right choice?

In 2024, a real-world observational study titled "Dexamethasone Treatment for COVID-19 is Associated with

Increased Mortality in Patients with Hematologic Malignancies" was published.[5] For those unfamiliar with critical appraisal of evidence, this finding may have been alarming, raising concerns about how many patients may have been harmed by our decision. However, for those well-versed in EBM principles, the study reinforced a crucial lesson: randomized controlled trials (RCTs) remain the gold standard for evaluating interventions. Random allocation ensures comparable groups, isolating the intervention's effect. Observational studies, in contrast, frequently reflect clinician-driven treatment decisions.[6] In this case, sicker patients were more likely to receive dexamethasone, introducing confounding by indication - a scenario in which disease severity, rather than the intervention, determines the outcome.

To this day, we do not know whether dexamethasone helped, harmed, or had no effect on our patients. What we do know is that science - particularly through vaccines and a collective global effort - ultimately triumphed over the pandemic. During those challenging times, we made the best decisions we could with the information available, our clinical judgment, and an unwavering intent to help our patients.

Conflicts of interest

The author declares no conflicts of interest.

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Yung Gonzaga *
Instituto Nacional de Câncer (INCA), Rio de Janeiro, Rio de Janeiro, Brazil

*Corresponding author.
E-mail address: ygonzaga@inca.gov.br

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Letter to the Editor

Impact of the creation of a multidisciplinary amyloidosis study group in a public hospital of a developing Latin American country

To the editor:

Amyloidosis represents a diagnostic challenge due to low clinical suspicion and the technical difficulties involved in correctly typing amyloid. Recognition of the clinical picture is usually delayed, in part due to its multisystemic and nonspecific involvement. Patients may spend between seven months and more than five years visiting various specialists and receiving multiple incorrect diagnoses before obtaining the correct diagnosis.[1] Moreover, most treatments are expensive, which makes it a difficult disease to manage in developing countries.

In 2015 an alliance between cardiology and hematology specialists was made at our center in order to evaluate patients with suspected amyloidosis. In 2017, an amyloidosis study group was formally created, made up of specialists in hematology, cardiology, nephrology, neurology, dermatology, and pathology.

The aim of this study was to evaluate the impact of the creation of an Amyloidosis Multidisciplinary Study Group in a public center of a developing Latin American country.

An observational analytic ambispective cohort study was made. This study included all patients in the amyloidosis registry of our center (Hospital del Salvador, Santiago de Chile) diagnosed between 2005 and 2022. We divided the cohort into two groups - Period 1 (P1): patients diagnosed from 2005 to 2014 (before the Amyloidosis Study Group), and Period 2 (P2) from 2015 to 2022 (after establishing the Amyloidosis Study Group). Comparisons between the groups were performed using the *t*-test or Chi-square test. Overall survival (OS) was estimated using Kaplan Meier curves and comparisons were made by the Log Rank test. The analyzes were performed using the Statistical Package for Social Sciences (SPSS) computer program version 26.0. The study was approved by the local Ethics Committee.

Fifty-six patients with diagnosis of amyloidosis were included: 12 in P1 and 44 in P2 (Figure 1). The median ages were 63 and 66 years-old (p-value = 0.38) in P1 and P2, respectively and 67% versus 39% were male (p-value = 0.08). All cases were amyloid light-chain amyloidosis (i.e. primary amyloidosis - AL) in P1, while in P2 there were also two cases of secondary amyloidosis (AA) and two cases of hereditary transthyretin amyloidosis (ATTRm).

Analysis in regard to the availability of diagnostic and prognostic tools between P1 and P2, respectively was as follows: echocardiography was performed in 58% versus 93% of the patients (p-value < 0.001), the longitudinal strain was estimated in echocardiograms in 0% versus 61% (p-value < 0.001), cardiac magnetic resonance imaging (MRI) was performed in 0% versus 14% (p-value = 0.176), N-terminal pro-B-type natriuretic peptide (NT-proBNP) was evaluated in 8% versus 68% (p-value < 0.001), and the free light chain assay was performed in 25% versus 82% of the cases (p-value < 0.001).

No treatment based on bortezomib was prescribed in P1, and most patients were treated with a melphalan-prednisone regimen. In P2, 45% of patients were induced with a bortezomib (Bortezomib)-based regimen (p-value = 0.004). The early mortality rate was 67% in P1 and 30% in P2 (p-value = 0.020). The estimated five-year OS of the cohort in P1 was 16.7% versus 43.6% in P2 (p-value = 0.017 - Figure 2).

Since the creation of the group, the diagnosis of amyloidosis clearly improved with a better access to diagnostic and prognostic tools.

Amyloidosis is considered an orphan disease, which is chronically debilitating, serious, and life-threatening. Because of this, it must be addressed in a particular way. Worldwide, several measures have been proposed in this regard including: education, support for research, the possibility of entering in clinical trials, requesting equitable access to appropriate diagnosis and treatment, and the creation of multidisciplinary teams for its study. Our results prove that better management of these patients can be achieved, without necessarily meaning a large increase in the budget.

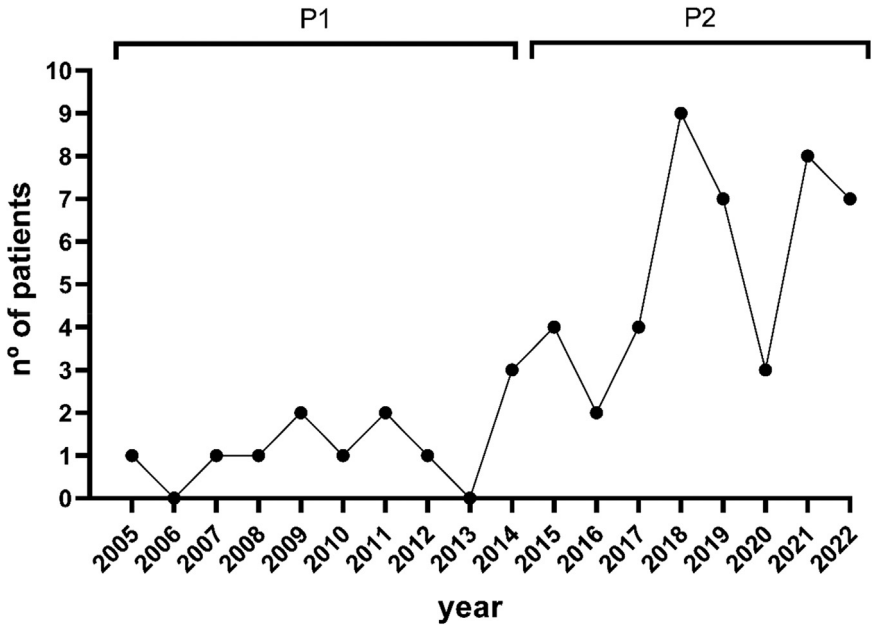
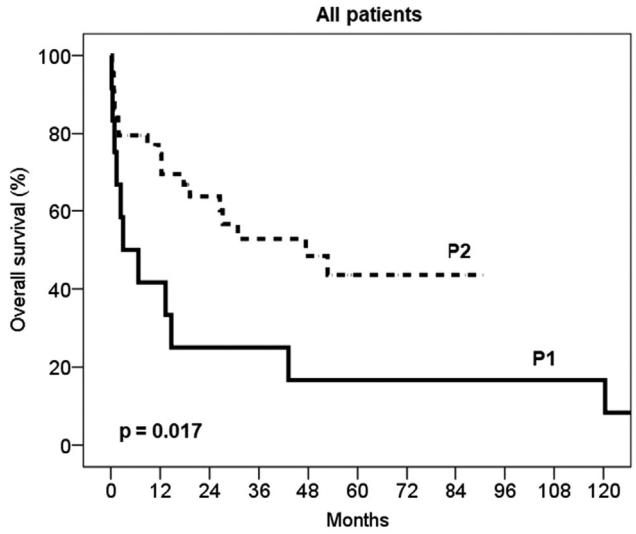


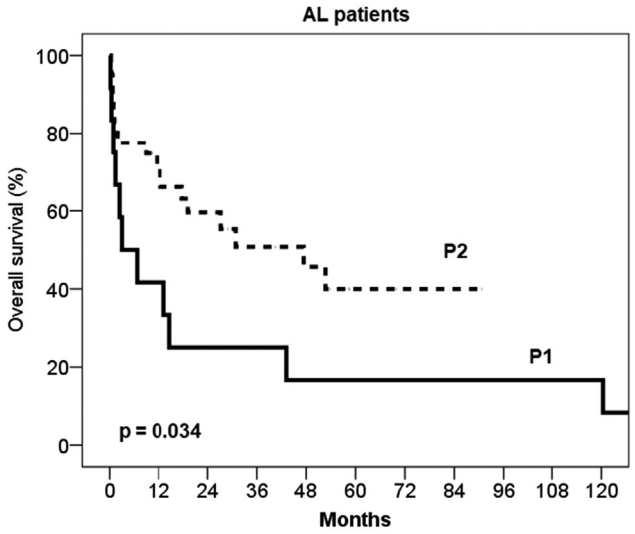
Figure 1 – Number of patients diagnosed with amyloidosis per year.

This group has focused mainly on constant education, both for specialists and non-specialist physicians. We have also managed to incorporate basic tests, such as echocardiography with longitudinal strain estimation, free light chain assays, and measurement of NT-ProBNP and troponin levels. More recently, access to cardiac MRI was added for selected patients. Moreover, since 2018 we can treat AL amyloidosis with bortezomib-based induction.

The most important result is that the diagnosis of amyloidosis improved progressively, except in 2020, which can be



All patients	Estimated cumulative overall survival		
Time	1 year	3 years	5 years
P1 (%)	41.7 ± 14.2	25.0 ± 12.5	16.7 ± 10.8
P2 (%)	74.5 ± 6.4	52.8 ± 8.5	43.6 ± 9.2



AL patients	Estimated cumulative overall survival		
Time	1 year	3 years	5 years
P1 (%)	41.7 ± 14.2	25.0 ± 12.5	16.7 ± 10.8
P2 (%)	71.9 ± 7.2	50.7 ± 9.1	39.9 ± 9.8

Figure 2 – Overall survival for the periods 2005–2014 (P1) and 2015–2022 (P2) for the whole cohort and for AL patients.

explained by the shutdown during the COVID-19 pandemic. This possibly means there has been an increased awareness of the disease in our center.

We were also able to diagnose other types of amyloidosis: two cases of AA and two of ATTRm. The latter were the first patients with ATTRm with a neurological phenotype diagnosed in our country.[2] This milestone was achieved thanks to the incorporation of immunohistochemistry for AA and genetic testing for suspected ATTRm. In P2, there was greater access to all relevant diagnostic and prognostic tools, although unfortunately this was not for all patients. This will improve in coming years, when we will have greater availability of these tests, including cardiac MRI. Wild-type transthyretin amyloidosis (ATTRwt) remains undiagnosed. In the future, one of the goals of our group is to incorporate pyrophosphate scintigraphy to our diagnostic arsenal according to the currently recommended non-invasive diagnostic algorithm.[3]

In regard to treatment, we experienced great improvements as, in 2018 we incorporated bortezomib to our treatment arsenal, as previously mentioned. We hope we soon have access to the anti-CD38 monoclonal antibody daratumumab, as daratumumab-CyBorD became standard of care for AL amyloidosis.[4] Access to disease-modifying therapy for ATTR has been restricted due to high costs and the absence of a national funding policy. Nevertheless, we recently started tafamidis treatment in an ATTRm patient with late-onset cardiovascular involvement.

We observed a relevant decrease in the early mortality rate and a better OS in P2, which we believe reflects the joint efforts with increased awareness, early diagnosis, and prompt treatment using improved therapeutic drugs.

Our study has several limitations, including its ambispective and unicentric nature, and the relatively low number of patients included. Nevertheless, it seems relevant to report that an improvement in both diagnosis and treatment is possible, even in poorer countries.

We are aware that there is still a long way to go to reach international standards. Our next step will be to start performing microdissection and mass spectrometry in biopsies for a better characterization, cardiac MRI and technetium-99 m pyrophosphate scintigraphy imaging, with the final goal of becoming a national reference center.

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Conflicts of interest

The authors declare no conflicts of interest.

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To all the members of the Amyloidosis Study Group of the Hospital del Salvador.

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Camila Peña [a,b,c,*], José Manuel Matamala [c,d,e,f], Cristián Vargas [g], Jaime Álvarez [h], Ricardo Valjalo [i], Fernando J. Verdugo [h]

[a] *Hematology Unit, Hospital del Salvador, Santiago, Chile*
[b] *Department of Internal Medicine, Faculty of Medicine, University of Chile, Santiago, Chile*
[c] *Center for Advance Clinical Research (CICA) Oriente, Faculty of Medicine, University of Chile, Santiago, Chile*
[d] *Translational Neurology and Neurophysiology Laboratory (NODO Lab), Faculty of Medicine, University of Chile, Santiago, Chile*
[e] *Department of Neurological Sciences, Faculty of Medicine, University of Chile, Santiago, Chile*
[f] *Biomedical Neuroscience Institute (BNI), Faculty of Medicine, University of Chile, Santiago, Chile*
[g] *Internal Medicine Service, Hospital del Salvador, Santiago Chile*
[h] *Cardiology Unit, Hospital del Salvador, Santiago, Chile*
[i] *Nephrology Unit, Hospital del Salvador, Santiago, Chile*

*Corresponding author.
E-mail address: camipena@gmail.com (C. Peña).

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HEMATOLOGY, TRANSFUSION AND CELL THERAPY

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Letter to the Editor

Problems with single platforms for CD34+ quantification: How aware are Brazilian hematologists and transplant specialists about them?

Dear Editor,

I will start with a short anecdote. At the last XXVIII Congress of the Brazilian Society of Bone Marrow Transplantation, held in August 2024, I met with a longtime friend, an experienced hematologist with decades of expertise in various aspects of CD34 cell research, including quantification, collection, and cryopreservation. During our conversations, I mentioned that after reviewing the entire scientific program of the event, I did not find a single roundtable, symposium, or lecture devoted to the discussion of CD34 cells. In effect, during the entire Congress, there was only one presentation of a single study that addressed the quantification of $CD34^+$ cells and its relationship with the success of leukapheresis. Considering the significance and centrality of CD34 cells in the execution of both autologous and allogeneic stem cell transplants, this observation was not just a curiosity – it was worrying, I told my friend. He agreed with me and said that, in fact, it would be advisable that at least a single symposium or roundtable should be devoted to the discussions on CD34 cells. "Yes," I replied, "especially because, although very precise for $CD34^+$ quantification, the modern single-platform templates that use microbeads for the enumeration of $CD34^+$ cells are not free from problems." Suddenly, he turned to me, his expression revealing a hint of surprise at what I had said, and asked, "What problems?"[1] His response immediately made me think about how much Brazilian hematologists and transplant specialists are aware of the problems involving the quantification $CD34^+$ cells.

Flow cytometry single-platform assays to enumerate $CD34^+$ hematopoietic stem cells ($CD34^+$ HSC) are the best methodology we currently have for the accurate and reliable determination of how many $CD34^+$ HSC there are in each leukapheresis product intended for transplantation. In effect, over the past two decades, the single-platform technique became the 'gold standard' strategy for the quantification of $CD34^+$ HSC for autologous and allogeneic hematopoietic stem cell transplantations (HSTC), surpassing the traditional International Society of Hematotherapy and Graft Engineering (ISHAGE)-based dual platform. As widely recognized, the principal advantage of the single-platform technique is its reduced variability as it excludes the need for white blood cell counts using automated hematology analysers [1,2].

Notwithstanding, single-platform assays are not without problems. In 2001, Bruno Brando et al. [3] described for the first time an uncanny phenomenon occurring with the single-platform method. The authors perceived that some of the microbeads present in the flow cytometry tube just vanished when phosphate-buffered saline (PBS)-diluted leukapheresis samples were vortexed before acquisition in the flow cytometer. As a result, the phenomenon generated artifactually high $CD34^+$ HSC counts. They concluded that, when microbeads were resuspended in saline media, the vortex agitation almost invariably induced what they called the 'vanishing counting bead' (VCB) phenomenon. Nevertheless, although worrying, the problem of VCB is easy to solve: the addition of small amounts of protein (1% bovine serum albumin or 10% human pooled plasma) completely prevents the phenomenon. In order to avoid the VCB, the authors then advised that sample suspensions containing microbeads for single-platform analysis be resuspended in media containing protein supplements [3]. This guarantees precise $CD34^+$ counting, preventing the realization of HSTC with a dose of $CD34^+$ HSC that is below ideal.

After briefly explaining these points to my friend, I started to wonder whether Brazilian laboratories involved in the quantification of $CD34^+$ HSC cells routinely supplement their samples with proteins and, furthermore, whether transplant physicians are aware of the possibility that the $CD34^+$ HSC report they receive from general laboratories may contain inaccuracies due to the occurrence of VCB. So, preliminarily, my first intention with this letter is to share these concerns with other hematologists and transplant colleagues. But the

[1] If the readers are at this moment asking themselves the same question, then a careful reading of this "letter to the editor" is of utmost importance.

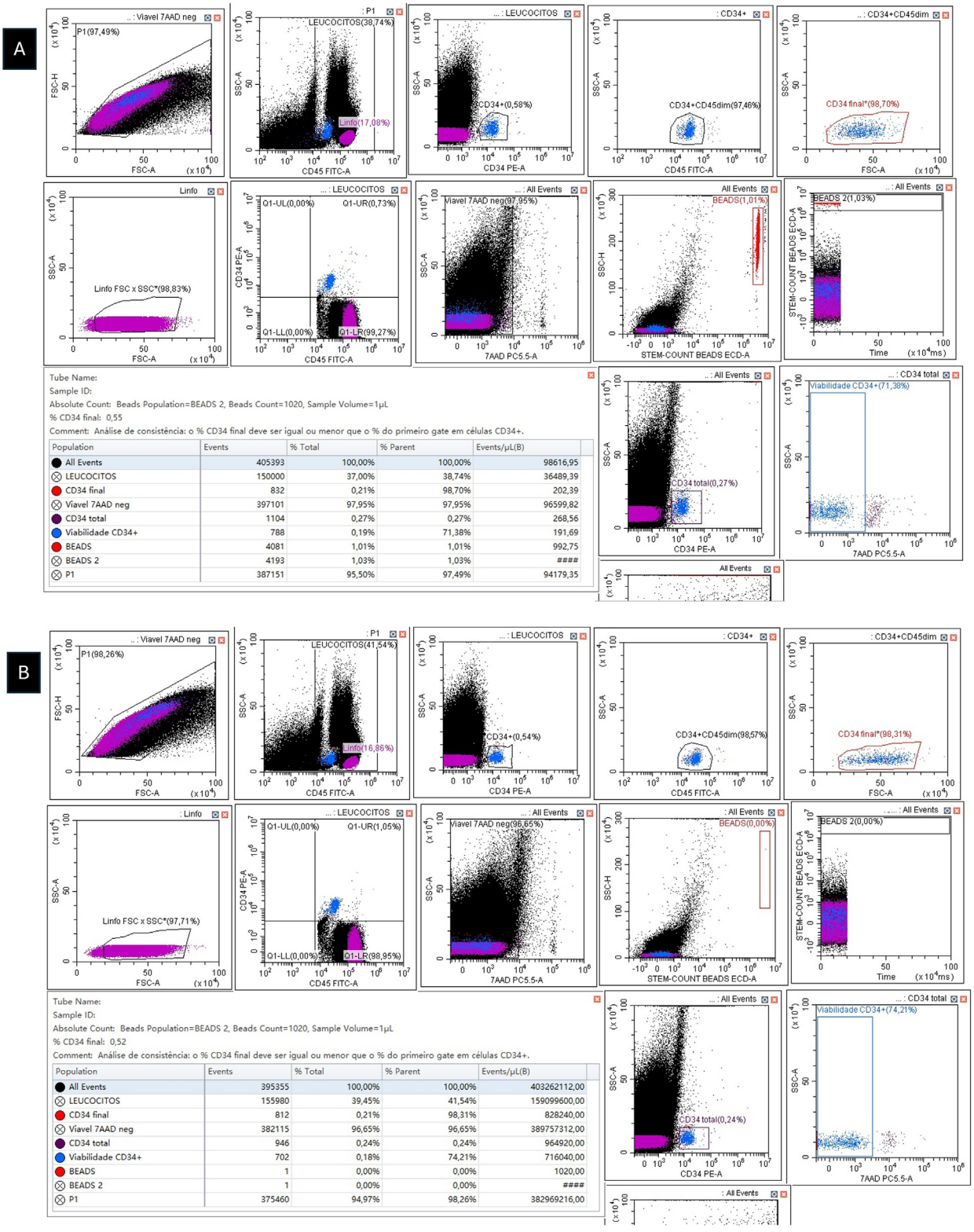

Figure 1 – A) Enumeration of viable CD34⁺ cells with the single-platform ISHAGE protocol (Stem-Kit) on a DxFLEX flow cytometer (3-laser and 13-color detection) (Beckman Coulter). Leukapheresis sample incubated with CD34 PE, CD45 FITC, and 7-AAD plus 2% human albumin (dilution factor = 7). Viable CD34⁺ = 1.416 cells/mm³. B) Enumeration of viable CD34⁺ cells with the dual-platform ISHAGE protocol (Stem-Kit) on a DxFLEX flow cytometer (3-laser and 13-color detection) (Beckman Coulter). Leukapheresis sample incubated with CD34 PE, CD45 FITC, and 7-AAD plus 2% human albumin (white blood cell count = 143.700/mm³). Viable CD34⁺ = 748 cells/mm³. The dual-platform assay showed that the single-platform overestimated the viable CD34⁺ count, confirming the protein-resistant VCB phenomenon. Notice that a simple way to suspect the phenomenon is to use what we call 'internal dual-platform' derived from the single-platform template. In practice, what we do is to compare the

issue is not so simple because, even if Brazilian laboratories are already adding proteins to avoid this problem, VCB- is like the Hydra from Greek mythology: it has many heads... or at least two heads.

In fact, we recently described a new problem with single-platform assays, a phenomenon we called 'protein-resistant VCB'. In this case, VCB occurs even in protein-supplemented samples [4,5]. Although still awaiting the exclusion of local confounding variables that could be impacting this phenomenon, it appears that protein-resistant VCB is a very real, albeit rare, phenomenon, whose presence greatly increases the complexity of single-platform analyses. Therefore, it is important that Brazilian flow cytometry laboratories and transplant centers check whether they have encountered cases of classic (non-protein resistant) and of protein-resistant VCB and share their experience with the scientific community. Until the phenomenon is better defined and, more importantly, until we figure out how to eliminate it, we recommend that, in the presence of protein-resistant VCB, the dual-platform assay should be used for determining $CD34^+$ HSC counts (Figure 1) [4,5].

Back to my friend. When I explained to him about the existence of VCB, he commented that he was unsure how many physicians in transplant centers and flow cytometry laboratories involved in $CD34^+$ cell quantification in Brazil were aware of this problem concerning single-platform assays. I told him that I had no idea either. I hope, with this letter, that Brazilian hematologists and transplant specialists become aware about the need to substantially increase their attention when dealing with $CD34^+$ HSC quantification platforms that use bead-based methods.

Conflicts of interest

The author declares no conflicts of interest.

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Daniel Mazza Matos [a,b,*]
[a] *Flow Cytometry Section, Cell Processing Center (CPC), Center of Hematology and Hemotherapy of Ceará (HEMOCE), Fortaleza, Ceará, Brazil*
[b] *Universidade de Fortaleza (UNIFOR), Fortaleza, Ceará, Brazil*

*Corresponding author. Center of Hematology and Hemotherapy of Ceará (HEMOCE), Avenida José Bastos, 3390, Fortaleza, CE, Brazil, 60431-086.
E-mail address: dmazza@alumni.usp.br

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value of viable $CD34^+$ cells/mm^3 of the single-platform method with the calculated value of viable $CD34^+$ cells/mm^3 of the 'dual platform' that is intrinsically present in single-platform studies. The formula is: Internal dual-platform = ($CD34^+$ events ÷ $CD45^+$ events) x WBC (mm^3)**. In this case, the internal dual-platform viable $CD34^+$ cell count was (832 ÷ 150.000) x 143.700 = 797 cells/mm^3. This result is quite consistent with that obtained in the dual-platform assay (for more details, see Matos DM.[5]).**

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Images in Clinical Hematology

Spinal cord leptomeningeal myelomatosis

Gustavo Kazuo Silva Yamada [a,*], *Guilherme Duffles* [b], *Carmino Antonio de Souza* [b], *Fabiano Reis* [a]

[a] *Faculdade de Ciências Médicas, Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil*
[b] *Universidade de Campinas (UNICAMP), Campinas, SP, Brazil*

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A 62-year-old man was admitted for investigation of a 3-month history of progressive lower back pain with hypoesthesia. He had been diagnosed with multiple myeloma 5 years before, treated with four cycles of CyBorD (cyclophosphamide, bortezomib (Bortezomib), dexamethasone) and pamidronate, followed by hematopoietic autologous stem-cell transplantation (conditioned with 200 mg/m^2 of melphalan) and maintenance chemotherapy with two cycles of CyBorD and isolated bortezomib (Bortezomib). In a regular medical follow-up, he had a very good partial response before admission. An examination showed paresthesia and hypoesthesia of lower limbs. Seric hemoglobin was 17.6 g/dL (normal reference [NR]: 14–18 g/dL), leukocytes of 8.23 x 10^3/μL (NR: 4.0 –10.0 x 10^3/μL) subdivided in 5.61 x 10^3/μL segmented neutrophils, 1.62 x 10^3/μL lymphocytes, 0.73 x 10^3/μL monocytes, 0.17 x 10^3/μL eosinophils and 0.04 x 10^3/μL basophils, without blasts, plasmocytes and other atypical cells. Magnetic resonance imaging (MRI) findings are shown in Figures 1 and 2. The imaging findings were consistent with leptomeningeal neoplasic infiltration, a condition called meningeosis myelomatosis,[1–3] as a recurrence of the multiple myeloma. A cerebrospinal fluid (CSF) analysis was performed, which demonstrated plasmocytes with atypical morphology: increased volume, loose chromatin and evident nucleoli, that in a differential count was consistent with clonal plasmocytes.[3,4] Meningeosis myelomatosis is a rare but an important differential diagnosis to consider in patients with new neurological symptoms after multiple myeloma treatment. MRI is essential to evaluate the patients,[1,5] with a CSF analysis being the gold standard for confirming the diagnosis.[1,4]

* Corresponding author at: Department of Radiology, Faculty of Medical Sciences, State University of Campinas (UNICAMP), Campinas, SP, Brazil.
E-mail address: gkazuo@unicamp.br (G.K.S. Yamada).
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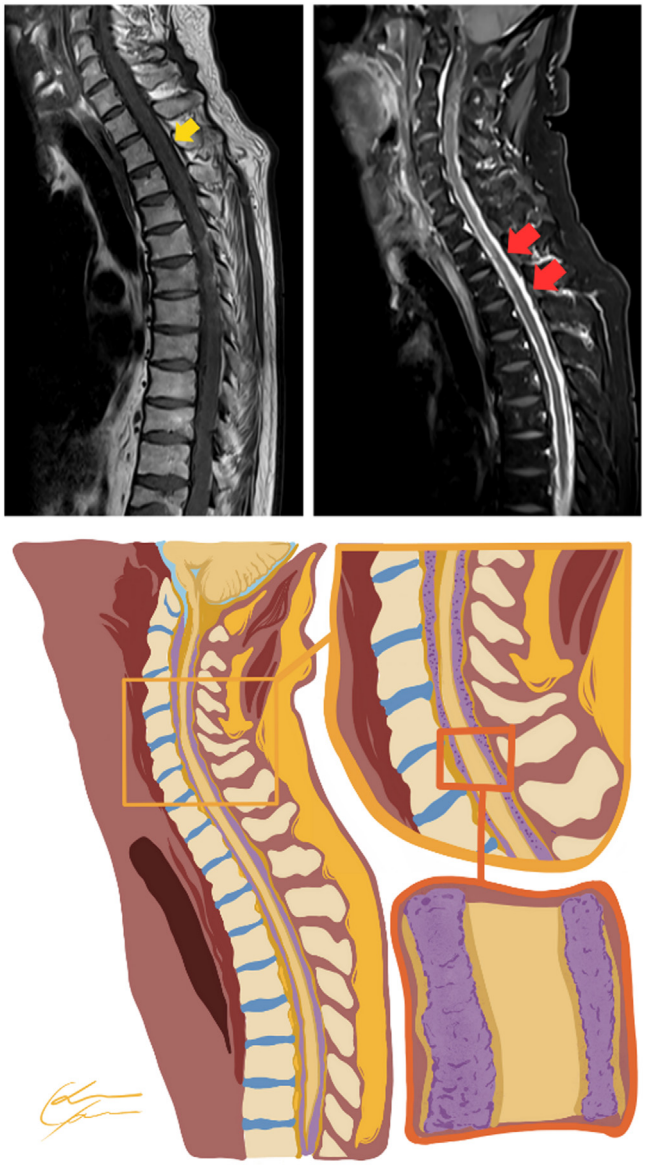

Figure 1 – Sagittal T1 before (top left) and after contrast (top right). Sequences with diffuse and thick leptomeningeal enhancement involving the spinal cord. At the bottom, a graphic representation of the magnetic resonance imaging (MRI) findings with infiltration of the leptomeninges around the spinal cord.

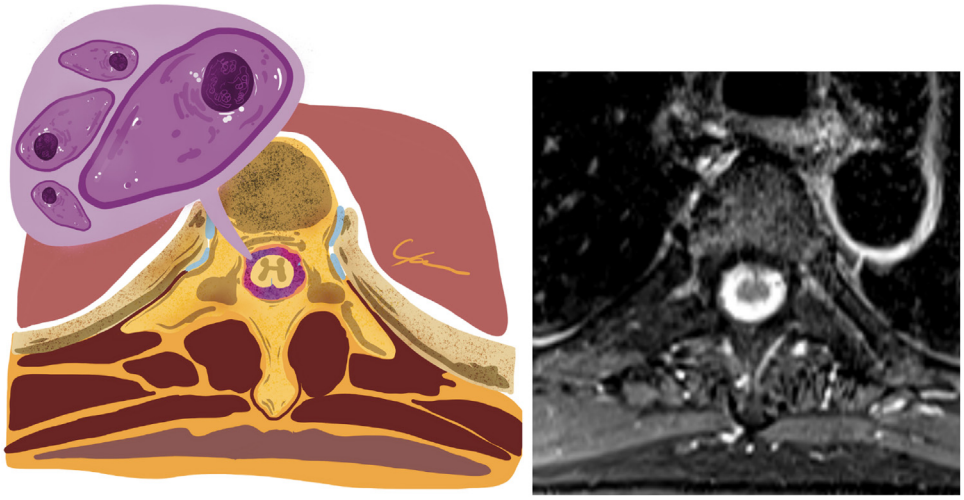

Figure 2 – Graphic representation (left) from the Axial T1 after contrast (right) magnetic resonance acquired in the same patient. The representation illustrates the leptomeningeal neoplasic infiltration enhanced by contrast in T1 sequences that was confirmed to be by plasmocytes with atypical morphology (enlarged cells, loose chromatin and evident nucleoli), a rare recurrence of multiple myeloma.

Conflicts of interest

The authors declare no conflicts of interest.

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