frontiers | Research Topics

Rising stars in hematology 2022

Edited by
Marcos De Lima, Paolo Fabrizio Caimi,
Mutlu Arat and Eleni Gavriilaki

Published in
Frontiers in Medicine
Frontiers in Oncology
Frontiers in Transplantation

ISSN 1664-8714
ISBN 978-2-8325-4080-0
DOI 10.3389/978-2-8325-4080-0

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Rising stars in hematology: 2022

Topic editors

Marcos De Lima — The Ohio State University, United States
Paolo Fabrizio Caimi — Cleveland Clinic, United States
Mutlu Arat — Istanbul Florence Nightingale Hospital, Türkiye
Eleni Gavriilaki — G. Papanikolaou General Hospital, Greece

Citation

De Lima, M., Caimi, P. F., Arat, M., Gavriilaki, E., eds. (2023). *Rising stars in hematology: 2022*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-4080-0

Table of contents

frontiers | Frontiers in Medicine

TYPE Editorial
PUBLISHED 22 November 2023
DOI 10.3389/fmed.2023.1334125

OPEN ACCESS

EDITED AND REVIEWED BY
Giancarlo Castaman,
University of Florence, Italy

*CORRESPONDENCE
Eleni Gavriilaki
✉ elenicelli@yahoo.gr

RECEIVED 06 November 2023
ACCEPTED 13 November 2023
PUBLISHED 22 November 2023

CITATION
Gavriilaki E, Lima Md, Caimi PF and Arat M (2023) Editorial: Rising stars in hematology: 2022. *Front. Med.* 10:1334125. doi: 10.3389/fmed.2023.1334125

Editorial: Rising stars in hematology: 2022

Eleni Gavriilaki[1]*, Marcos de Lima[2], Paolo Fabrizio Caimi[3] and Mutlu Arat[4]

[1]2nd Propedeutic Department of Internal Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece, [2]Department of Medicine, The Ohio State University, Columbus, OH, United States, [3]Department of Medicine, Case Western Reserve University, Cleveland, OH, United States, [4]Department of Medicine, Istanbul Florence Nightingale Hospital, Istanbul, Türkiye

KEYWORDS

hematology, rising stars, future, #CollectionSeries, review

Editorial on the Research Topic
Rising stars in hematology: 2022

Recognizing the future leaders of Hematology is fundamental to safeguarding tomorrow's driving force in innovation. Indeed, early career researchers have faced a significant and potential "long" impact from the pandemic (1, 2).

Therefore, this Research Topic aimed to showcase the high-quality work of internationally recognized researchers in the early stages of their careers. We highlighted research by leading scientists of the future across the entire breadth of Hematology, and present advances in theory, experiment and methodology with applications to compelling problems. Contributions to the Research Topic were by invitation only. All Rising Star researchers were suggested by established Editors within the Frontiers board in recognition of their influence on the future directions in their respective fields.

All articles submitted to us for this Research Topic underwent a rigorous peer review process. Ultimately, 11 articles were published.

(i) An interesting case report in hematology was presented by analysis of a heterozygous somatic BLNK mutation associated with T-cell LGL (large granular lymphocyte) leukemia and autoimmune diseases. Fouquet et al. highlighted the link between genotype and the unusual clinical phenotype.

(ii) Application of metagenomic next-generation sequencing (NGS) in the diagnosis of visceral leishmaniasis and its treatment evaluation was presented by a case report. Liang et al. indicated the feasibility of the NGS approach and the evaluation of its therapeutic effect.

(iii) A review article presented an update on emerging treatment strategies in rare anemias. Fattizzo and Motta covered a broad spectrum of novel and exciting treatment options in rare congenital and acquired anemias.

(iv) An original article reported an intelligent detection method for plasmodium based on self-supervised learning and attention mechanism. Fu et al. presented an artificial intelligence method of excellent performance for the diagnosis and potentially automatic detection of malaria in the future.

(v) A review article on immune thrombotic thrombocytopenic purpura (iTTP) provided up-to-date knowledge on long-term outcomes and survivorship. Selvakumar et al. focused

on epidemiology and potential mechanisms for adverse long-term sequelae of iTTP, best practices in survivorship care, and presented a research agenda for the future.

(vi) A mini review article discussed activation of APC-EPCR-PAR1 axis in sickle cell disease (SCD). Ramadas and Sparkenbaugh reported novel insights into the activation of PAR1 by APC and thrombin, the APC-EPCR-PAR1 axis, and their potential roles in SCD.

(vii) A review article focused on total marrow irradiation in hematopoietic stem cell transplantation for hematologic malignancies. Kerbauy et al. reviewed literatures on applying these novel techniques in autologous and allogeneic transplantation across different clinical entities.

(viii) A study protocol article presented the phase I/II trial of induced HLA-G+ regulatory T cells in patients undergoing allogeneic hematopoietic cell transplantation from an HLA-matched sibling donor. Lysandrou et al. described the study rationale and design, including patient screening, product manufacturing, infusion, and participant follow-up to data collection, management, and analysis.

(ix) A brief research report article highlighted "waitlist mortality" for myeloma patients with limited access to BCMA therapy. Ahmed et al. reported that many patients who were eligible for ide-cel were not able to secure a timely slot, with high mortality rates as a result.

(x) A mini review article explained the interactions of the fibrinolytic and innate immune systems. Whyte has introduced readers into the world of "thromboinflammation" or "immunothrombosis".

(xi) A review article delineated promises and challenges of a decentralized CAR T-cell manufacturing model. Shah et al. provided in-depth knowledge of the concept of point-of-care (POC) manufacturing or decentralized *in-house* production.

Considering the multi-thematic character of this Research Topic, our hope is to inspire researchers and physicians to continue their explorations into novel advances in Hematology.

Author contributions

EG: Writing – original draft. ML: Writing – review & editing. PC: Writing – review & editing. MA: Writing – review & editing.

Funding

The author(s) declare that no financial support was received for the research, authorship, and/or publication of this article.

Conflict of interest

EG has received honoraria from Alexion, Gilead, Sanofi, Sobi, and Omeros Pharmaceuticals.

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

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

References

1. Gavriilaki E, Maiques-Diaz A. "Long COVID-19" of researchers: what to do next? *Hemasphere.* (2022) 6:e673. doi: 10.1097/HS9.0000000000000673

2. Kabanova A, Gavriilaki E, Pelzer BW, Brunetti L, Maiques-Diaz A. Effect of the COVID-19 pandemic on laboratory and clinical research: a testimony and a call to action from researchers. *Hemasphere.* (2020) 4:e499. doi: 10.1097/HS9.0000000000000499

frontiers | Frontiers in Medicine

TYPE Case Report
PUBLISHED 16 November 2022
DOI 10.3389/fmed.2022.997161

OPEN ACCESS

EDITED BY
Mutlu Arat,
Istanbul Florence Nightingale Hospital, Turkey

REVIEWED BY
Anjali Mishra,
Sidney Kimmel Cancer Center, United States
Valentina Giudice,
University of Salerno, Italy
Vanessa Rebecca Gasparini,
University of Padua, Italy

*CORRESPONDENCE
Guillemette Fouquet
guillemette.fouquet@gmail.com

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 18 July 2022
ACCEPTED 11 October 2022
PUBLISHED 16 November 2022

CITATION
Fouquet G, Rossignol J, Ricard L, Guillem F, Couronné L, Asnafi V, Vavasseur M, Parisot M, Garcelon N, Rieux-Laucat F, Mekinian A and Hermine O (2022) BLNK mutation associated with T-cell LGL leukemia and autoimmune diseases: Case report in hematology. *Front. Med.* 9:997161. doi: 10.3389/fmed.2022.997161

BLNK mutation associated with T-cell LGL leukemia and autoimmune diseases: Case report in hematology

Guillemette Fouquet[1,2,3]*, Julien Rossignol[4], Laure Ricard[5], Flavia Guillem[2,6], Lucile Couronné[2], Vahid Asnafi[7], Manon Vavasseur[2], Mélanie Parisot[8], Nicolas Garcelon[2], Frédéric Rieux-Laucat[2], Arsène Mekinian[5,9] and Olivier Hermine[2,4,6]

[1]Hématologie Clinique, Centre Hospitalier Sud Francilien, Corbeil-Essonnes, France, [2]Imagine Institute, INSERM U1163, Université de Paris, Paris, France, [3]Institut Cochin, INSERM, Centre National de la Recherche Scientifique (CNRS), Université de Paris, Paris, France, [4]Department of Adult Hematology, Hôpital Universitaire Necker-Enfants Malades, Assistance Publique des Hôpitaux de Paris, Paris, France, [5]Department of Internal Medicine and Inflammatory Diseases, Hôpital Saint Antoine, Assistance Publique des Hôpitaux de Paris, Sorbonne University, Paris, France, [6]Laboratory of Excellence GR-ex, Paris, France, [7]Institut Necker-Enfants Malades (INEM), INSERM U1151, Université de Paris, and Laboratory of Onco-Hematology, Hôpital Universitaire Necker-Enfants Malades, Assistance Publique des Hôpitaux de Paris, Paris, France, [8]Genomics Core Facility, Institut Imagine-Structure Fédérative de Recherche Necker, INSERM U1163, Paris, France, [9]Immunopathology/Biotherapy Department (DHU i2B), UMR 7211, Sorbonne University, Paris, France

We present the case of a female patient with a heterozygous somatic BLNK mutation, a T-cell LGL (large granular lymphocyte) leukemia, and multiple autoimmune diseases. Although this mutation seems uncommon especially in this kind of clinical observation, it could represent a new mechanism for autoimmune diseases associated with LGL leukemia. The patient developed several autoimmune diseases: pure red blood cell apalsia, thyroiditis, oophoritis, and alopecia areata. She also presented a T-cell LGL leukemia which required treatment with corticosteroids and cyclophosphamide, with good efficacy. Interestingly, she had no notable infectious history. The erythroblastopenia also resolved, the alopecia evolves by flare-ups, and the patient is still under hormonal supplementation for thyroiditis and oophoritis. We wanted to try to understand the unusual clinical picture presented by this patient. We therefore performed whole-genome sequencing, identifying a heterozygous somatic BLNK mutation. Her total gamma globulin level was slightly decreased. Regarding the lymphocyte subpopulations, she presented a B-cell deficiency with increased autoreactive B-cells and a CD4+ and Treg deficiency. This B-cell deficiency persisted after complete remission of erythroblastopenia and LGL leukemia. We propose that the persistent B-cell deficiency linked to the BLNK mutation can explain her clinical phenotype.

We believe that BLNK could be a new gene of interest in autoimmune diseases, which could warrant further explorations and may lead to a better understanding of these pathologies.

KEYWORDS

B-cell linker, large granular lymphocyte leukemia, autoimmune diseases, regulatory B-cells, regulatory T-cells (T reg), case report

Introduction

Large granular lymphocyte leukemia is a rare chronic mature lymphoproliferative disorder of either T-cell or NK lineage characterized by a clonal expansion of LGLs-resistant to activation-induced cell death. The exact initiation process of LGL leukemia is still unknown; however, the involvement of autoimmune and inflammatory processes in the pathology is generally accepted. LGL leukemia can indeed be associated with various autoimmune diseases—primarily rheumatoid arthritis, autoimmune thyroiditis, or lupus erythematosus (1). In these cases, LGL leukemia can be seen as a consequence of the autoimmune disease or as a secondary lymphoproliferative disease.

STAT3 mutations are the most common mutations to date in LGL leukemia and can be found in 30–40% of cases (2). More recently, many mutations have been described in this rare disease, especially affecting the JAK/STAT pathway such as mutations in STAT5b (3–6). However, LGL leukemia remains a clinically and genetically heterogeneous disease, and genomic analysis could allow a better understanding of the molecular mechanisms of the LGL leukemia and of the associated autoimmune diseases, and the identification of new treatments.

Patient information

The patient was born on 1974. She was diagnosed with ovarian failure with early menopause revealing autoimmune oophoritis in 1990 (16 years old), for which she receives hormone replacement. She presented alopecia areata in the following years, treated by topical corticosteroids. She was then treated in hematology as described below, for autoimmune pure red cell aplasia and LGL leukemia in 2011 (37 years old). In 2015, she developed hypothyroidism linked to Hashimoto's thyroiditis (41 years old), treated by hormone replacement.

Abbreviations: BCR, B-cell receptor; BLNK, B-cell linker protein (synonyms: SLP-65, BASH, BCA); Breg, regulatory B-cell; KO, knockout; LGL, large granular lymphocyte; NK-LGL, NK-cell LGL leukemia; PRCA, pure red cell aplasia; T-LGL, T-cell LGL leukemia; Treg, regulatory T-cell; WES, whole exome sequencing.

She had no relevant infectious history. She has no other significant medical nor psychosocial history.

There is no particular pathology in her family, nor consanguinity.

Clinical findings

The patient first consulted in hematology at 37 years old. She presented with anemia (hemoglobin 8.3 g/dL) and neutropenia (neutrophils 0.7 G/L).

The blood smear showed circulating large granular lymphocytes, and immunophenotyping and molecular biology confirmed T-cell LGL leukemia. Bone marrow analysis revealed pure red blood cell aplasia (PRCA).

Timeline

Timeline of the clinical case is presented Figure 1.

Diagnostic assessment

Immunophenotyping and protein electrophoresis

The blood count revealed a total lymphocyte count of 1,400/mm^3. The blood smear showed large granular T-cells. At diagnosis, immunophenotyping confirmed an excess of large granular T-cells (700/mm^3), along with B-cell lymphopenia (CD19$^+$ cells: 24/mm^3; normal range: 169–271), and a moderate decrease in CD4$^+$ T-cells (CD4$^+$ cells: 387/mm^3; normal range: 460–1,232). The LGL T-cells were CD2+, CD3+, CD5low, CD7low, CD4-, CD8+, CD57+, CD16+, CD56-, CD62L-. Clonality assessment found a restricted expression of Vb3.1, and molecular biology confirmed monoclonality.

Her total gamma globulin level was 6.9 g/L (normal range: 8–13.5), IgG 4.8 g/L (normal range: 6.65–12.78), IgA 1.6 g/L (normal range: 0.7–3.45), and IgM 0.4 g/L (normal range: 0.5–2.09).

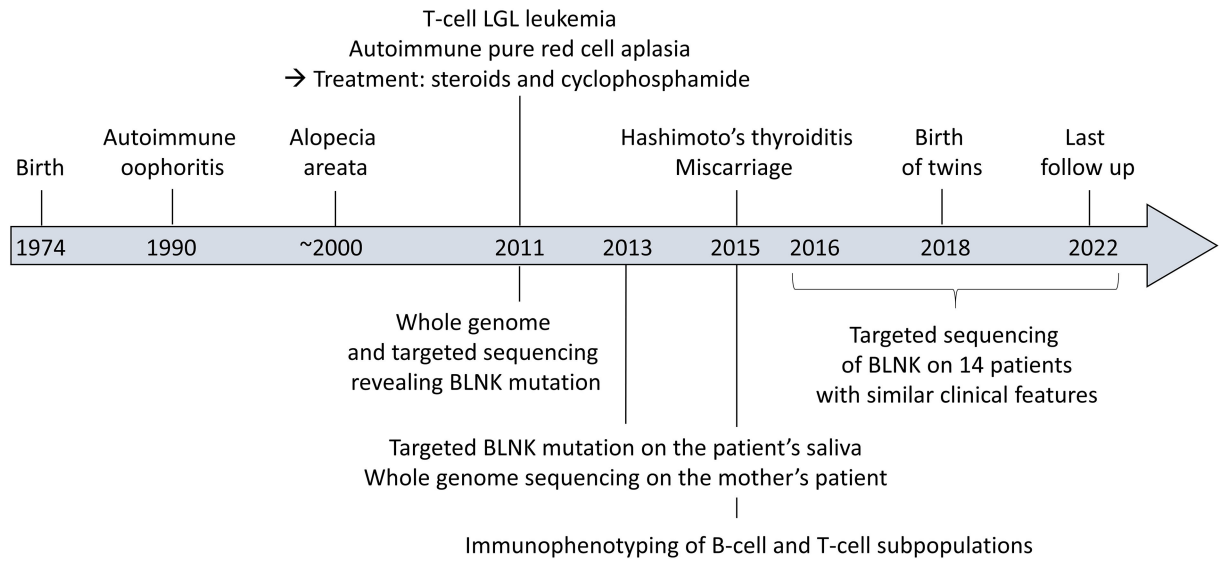

FIGURE 1
Timeline of the clinical case.

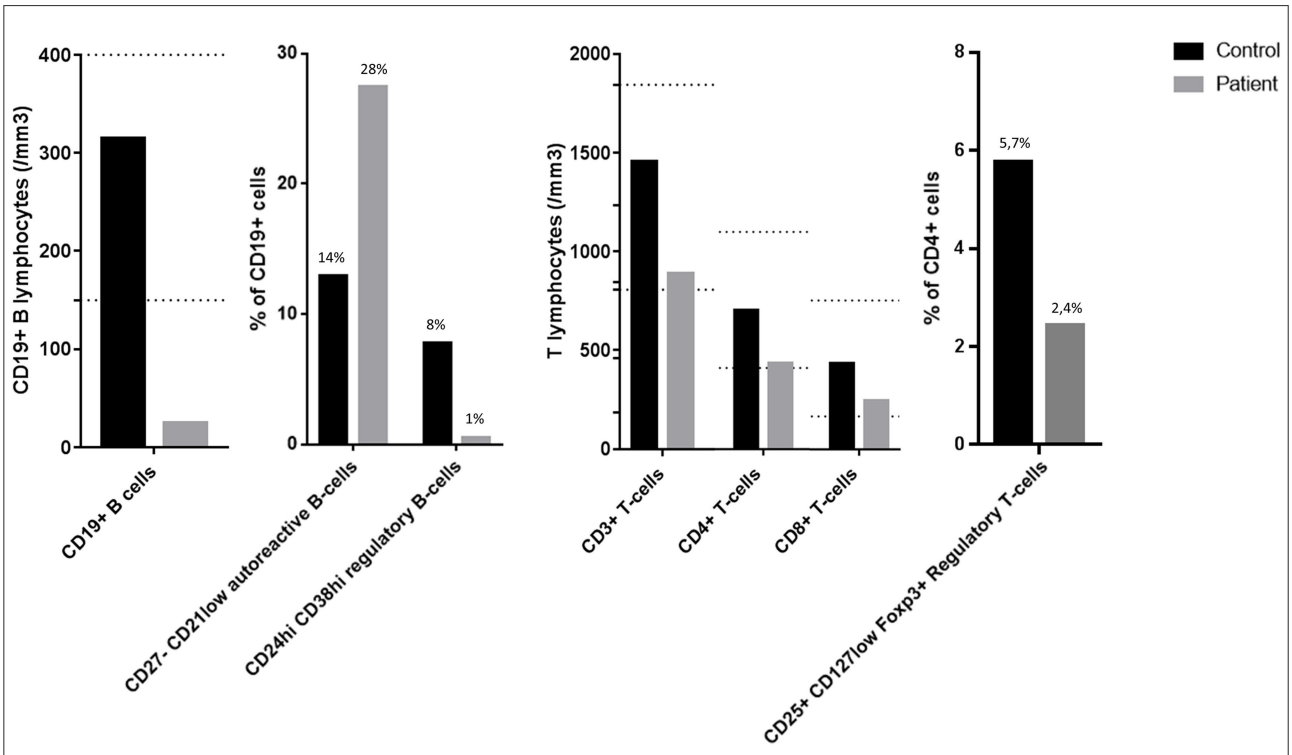

FIGURE 2
Flow cytometry analyses of B-cell and T-cell subpopulations. The dotted lines represent the normal range of the laboratory: CD19+ B lymphocytes 169–271/mm^3; CD3+ T lymphocytes 807–1844/mm^3; CD4$^+$ T lymphocytes 460–1232/mm^3; CD8$^+$ T lymphocytes 187–844/mm^3.

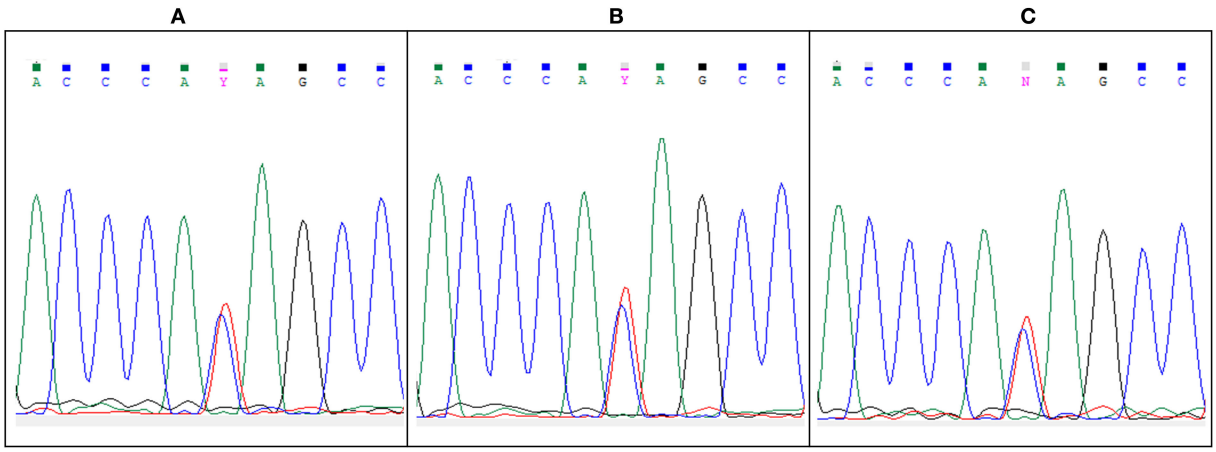

FIGURE 3
Heterozygous C > T BLNK mutation revealed by Sanger sequencing of BLNK in **(A)** whole blood at diagnosis, and in **(B)** T-cells and **(C)** non–T-cell PBMCs at remission.

Immunophenotyping of B-cell and T-cell subpopulations

Of note, these B- and T-cell analyses were performed while the patient was in complete remission of PRCA and LGL leukemia, without any immunosuppressive therapy.

Analysis of B-cell and T-cell subpopulations revealed persistent and profound B-cell lymphopenia with conserved distribution of various B-cell subpopulations (memory, transitional, and naive B-cells), along with an increased proportion of $CD27^{-}CD21^{low}$ B-cells, previously described as autoreactive B-cells (7). Breg analyses showed a reduction in $CD19^{+}CD24^{hi}CD38^{hi}$ cells but no significant drop in other Breg populations (i.e., IL10 producing B-cells, $CD24^{hi}CD27^{+}$ (B10) and $CD27^{int}CD38^{hi}$ plasmablasts) (8). T-cell population analyses only showed a moderate $CD4^{+}$ and Treg deficiency (Figure 2).

Unfortunately, the patient's B-cell lymphopenia made functional exploration of the B-cell and T-cell populations inconclusive.

Flow cytometry gating strategies are presented in Supplementary Figure 1.

Whole genome sequencing

This unusual association of multiple autoimmune diseases prompted us to perform whole-exome sequencing (WES) for this patient in order to search for any predisposing mutation. WES was performed on a blood sample, while the patient still presented circulating T LGL clonal cells. Analysis revealed the presence of a heterozygous nonsense mutation in the BLNK gene (c.1,102 C>T, p.Q368*, 47%) that yielded a premature STOP codon. The mutation was located in the BLNK C-terminal SH2 domain, which allows recruitment of BLNK to the BCR complex through its interaction with BTK.

No STAT3 mutation was found, neither by WES nor using targeted sequencing. No mutation was found in the JAK/STAT pathway by WES.

To understand the development of LGL leukemia in this patient, we performed comparative exome analysis of sorted T-cells ($CD3^{+}$) and B-cells ($CD19^{+}$). However, no mutation other than BLNK that could explain a proliferative advantage was found in T-cells. This could suggest that the T-LGL proliferation, even clonal, was reactive in this autoimmune context.

WES was also performed on a blood sample of the patient's mother: no BLNK mutation was found.

BLNK-targeted sequencing

Targeted BLNK sequencing on sorted medullary populations—T-cells ($CD3^{+}$), B-cells ($CD19^{+}$), monocytes ($CD14^{+}$), and others ($CD3^{-}$ $CD19^{-}$ $CD14^{-}$)—revealed the same heterozygous BLNK mutation in all cell groups.

The mutation was not found by targeted BLNK sequencing on the patient's saliva, demonstrating that it is a somatic and not a germline mutation. These findings are in accordance with a somatic mosaicism, the BLNK mutation presumably having occurred during the embryonic, fetal, or postnatal period. Such a mechanism has already been described in the context of lymphoproliferative autoimmune syndromes (9).

In line with this hypothesis, the heterozygous BLNK mutation was still present in sorted peripheral blood mononuclear cells—T-cells (CD3+) and others (CD3-)—after complete remission of the LGL leukemia (Figure 3).

TABLE 1 Characteristics of patients with BLNK-targeted sequencing.

	Age at LGL diagnosis	Sex	Type of LGL	Treatment of LGL	Associated autoimmune diseases	Treatment of autoimmune diseases	Source of DNA
1	38	F	T LGL	Methotrexate	Rheumatoid arthritis	Adalimumab, Rituximab, Abatacept, Tocilizumab	Blood
2	41	M	T LGL	No added treatment	Immune thrombocytopenia	Steroids, Immunoglobulins, Splenectomy, Rituximab Vincristine, Cyclophosphamide, Romiplostim	Bone marrow
3	54	F	T LGL	Cyclosporine Methotrexate	Autoimmune enteropathy	Local (intestinal) steroids	Blood
4	73	M	T LGL	Methotrexate	Rheumatoid arthritis, Immune thrombocytopenia	Rituximab, Leflunomide	Blood
5	87	F	T LGL	Methotrexate	Cold agglutinin disease	Steroids, Plasma exchange, Cyclophosphamide, Chloraminophene, Interferon, Vesanoid, Fludarabine, Rituximab	Blood
6	70	F	T LGL	Methotrexate Cyclosporine Cyclophosphamide	Vasculitis, Autoimmune hemolytic anemia	Colchicine, Hydroxychloroquine	Blood
7	58	F	T LGL	Cyclosporine	Rheumatoid arthritis	Salazopyrin, Methotrexate, Hydroxychloroquine, Steroids, Leflunomide	Bone marrow
8	33	M	NK LGL	Cyclophosphamide	Autoimmune hemolytic anemia	Steroids	Blood
9	61	F	T LGL	Methotrexate	Rheumatoid arthritis, Sjögren's syndrome, Pyoderma gangenosum	Steroids, Methotrexate	Blood
10	50	M	T LGL	No added treatment	Autoimmune hemolytic anemia	Steroids, Rituximab, Splenectomy	Blood
11	72	F	T LGL	No added treatment	Celiac disease	Steroids	Blood
12	79	M	T LGL	No added treatment	Rheumatoid arthritis	Steroids, Hydroxychloroquine	Blood
13	36	M	T LGL	Steroids	Autoimmune hemolytic anemia, Immune thrombocytopenia	Steroids, Hydroxychloroquine, Immunoglobulins, Rituximab, Splenectomy	Blood
14	58	F	T LGL	Methotrexate	Inflammatory rheumatism	Steroids, Methotrexate	Blood

10.3389/fmed.2022.997161

BLNK-targeted sequencing in patients with similar clinical manifestations

In order to investigate whether this acquired BLNK mutation could be present in other patients, we performed targeted sequencing of BLNK on 14 patients presenting similar clinical features: clonal LGL and autoimmune disease(s). Characteristics of patients are presented in Table 1. DNA samples were available from the Hôpital Universitaire Necker-Enfants Malades LGL leukemia cohort and a computerized database (Dr Warehouse; N. Garcelon) (10). No BLNK mutation was found in this 14 patients cohort.

To note, no BLNK mutation was found in a PRCA cohort at Imagine Institute (data not shown).

Therapeutic intervention

The patient was treated for PRCA and LGL leukemia: she received corticosteroids at a dose of 1 mg/kg/day for 4 weeks for PRCA, before gradual decrease over 4 additional weeks. As the LGL leukemia was associated with neutropenia and an autoimmune disease, the patient also received, at the same time, cyclophosphamide (100 mg/day orally) over 6 months.

This treatment resulted in a rapid complete remission of PRCA and concomitant disappearance of the LGL clone in <6 months.

Follow-up and outcomes

The patient is still in complete remission of PRCA and LGL leukemia.

Her alopecia improved after dermatologic treatment including topical corticosteroids, but evolves by flare-ups.

She is still receiving hormone replacement for her thyroiditis and oophoritis. She had a miscarriage but then gave birth to twins after receiving an oocyte donation. She and her children were doing well at the last follow-up.

Discussion

BLNK is an adaptor protein expressed in B-cells and macrophages that plays a major role in BCR signaling. Mutations affecting the pre-BCR signaling pathway result in severe B-cell differentiation blockades at the pre-B1 to pre-B2 cell transition, leading to primary B-cell immunodeficiencies characterized by the total or near-total absence of circulating B-cells, severe hypo- or agammaglobulinemia, and recurrent bacterial infections (11). BLNK KO mice display stymied pre-B-cell development and reduced numbers of mature B-cells, in addition to autoimmune diseases associated with lower B-cell and Treg percentages (12).

In humans, the function of BLNK in B-cells seems non-redundant (13). A few cases of homozygous BLNK mutations have been described, all leading to near-total absence of circulating B-cells and severe hypo- or agammaglobulinemia (11, 13–16). A dysfunction of BLNK has also been associated with autoimmune diseases, such as multiple sclerosis (17, 18), or rheumatoid arthritis which is known to be associated with LGL leukemia (19).

As far as we know, this is the first report about a symptomatic heterozygous BLNK mutation. Our patient presented an association of multiple autoimmune disorders, whose pathogenic mechanism is not fully understood but seems to involve B-cells—as is usually the case in other idiopathic autoimmune conditions and BLNK KO mice.

Her mutation leads to a premature STOP codon and is located in the BLNK C-terminal SH2 domain, which allows recruitment of BLNK to the BCR complex through its interaction with BTK. These data imply that the mutation necessarily impacts BLNK function. Unfortunately, the patient presented with a profound B-cell lymphopenia which made our functional assays inconclusive, but is consistent with a BLNK defect leading to an altered B lymphopoiesis. In proportion, all populations were preserved including Bregs, but there was a relative increase in autoreactive B-cells. As described elsewhere, an alteration of BLNK could lead to a dysregulation of B-cells with activation and selection of autoreactive B clones responsible for autoimmune manifestations (20). As her mutation appears to be somatic but is not expected to give a proliferative advantage, we believe that it may have occurred early in development. Since the BLNK alteration involves the pre-B stage, there could be a negative selection defect either centrally, leading to an enrichment of autoreactive B-cells, or peripherally with abnormal B-cells surviving in the periphery by escaping the GC cycle. However, further work is needed to resolve this very interesting issue.

She also presented with T-LGL, which might be reactive to the autoimmune diseases and chronic excess of cytokines production (such as IL-15) since BLNK is not expressed in T-cells. Alternatively, we cannot rule out that invalidated BLNK B-cells may interact with T-cells and induce their proliferation. In agreement with this hypothesis, it has recently been demonstrated that the anti-CD20 monoclonal antibody rituximab, a specific anti–B-cell drug, is somewhat effective in T-LGL associated with rheumatoid arthritis (21).

In conclusion, here we have presented the case of a female patient with a heterozygous somatic BLNK mutation, T-LGL, and multiple autoimmune diseases, and we propose that persistent B-cell deficiency linked to the BLNK mutation primarily explains her clinical phenotype.

Patient perspective and informed consent

The treatment for PRCA and LGL leukemia was well tolerated. The patient is doing well today. The most painful problem for her was her ovarian failure, but she was very happy to give birth to two children thanks to an oocyte donation.

She gave informed consent for all analyses performed and for this manuscript, and repeatedly mentioned her enthusiasm for helping research.

Data availability statement

The datasets presented in this article are not readily available because of ethical and privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

JR and OH cared for the patient. GF, JR, LR, FG, LC, AM, FR-L, NG, and OH conceived and planned the experiments. GF, LR, FG, MV, and MP performed the experiments. NG provided the computerized database used to select the 14 patients cohort. VA provided the samples for the 14 patients cohort. GF, JR, AM, and OH contributed to analysis, interpretation of the results, and wrote the original manuscript. GF, FR-L, and OH revised the manuscript. All authors contributed to the article and approved the submitted version.

Funding

GF was supported by the Institut National du Cancer (INCA); and JR, through a PhD fellowship from the French Ministry of Higher Education and Research. Funding for whole genome sequencing came from the GR-Ex Laboratory of Excellence (ANR-11-LABX-0051) and the Imagine Institute. The Imagine Institute and Labex GR-Ex are funded by the Investissements d'Avenir program of the French National Research Agency (ANR-10-IAHU-01 and ANR-11-IDEX-0005-02, respectively). Publication fees were provided by Imagine Institute.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.997161/full#supplementary-material

SUPPLEMENTAL FIGURE 1
Flow cytometry gating strategies for B-cell and T-cell analyses.

References

1. Lamy T, Moignet A, Loughran TP. LGL leukemia: from pathogenesis to treatment. *Blood.* (2017) 129:1082–94. doi: 10.1182/blood-2016-08-692590

2. Teramo A, Barilà G, Calabretto G, Ercolin C, Lamy T, Moignet A, et al. STAT3 mutation impacts biological and clinical features of T-LGL leukemia. *Oncotarget.* (2017) 8:61876–89. doi: 10.18632/oncotarget.18711

3. Muñoz-García N, Jara-Acevedo M, Caldas C, Bárcena P, López A, Puig N, et al. STAT3 and STAT5B mutations in T/NK-cell chronic lymphoproliferative disorders of large granular lymphocytes (LGL): association with disease features. *Cancers.* (2020) 12:3508. doi: 10.3390/cancers12123508

4. Moosic KB, Paila U, Olson KC, Dziewulska K, Wang TT, Xing JC, et al. Genomics of LGL leukemia and select other rare leukemia/lymphomas. *Best Pract Res Clin Haematol.* (2019) 32:196–206. doi: 10.1016/j.beha.2019.06.003

5. Cheon H, Xing JC, Moosic KB, Ung J, Chan VW, Chung DS, et al. Genomic landscape of TCR$\alpha\beta$ and TCR$\gamma\delta$ T-large granular lymphocyte leukemia. *Blood.* (2022) 139:3058–72. doi: 10.1182/blood.2021013164

6. Savola P, Bhattacharya D, Huuhtanen J. The spectrum of somatic mutations in large granular lymphocyte leukemia, rheumatoid arthritis, and Felty's syndrome. *Semin Hematol.* (2022) 59:123–30. doi: 10.1053/j.seminhematol.2022.07.004

7. Isnardi I, Ng YS, Menard L, Meyers G, Saadoun D, Srdanovic I, et al. Complement receptor 2/CD21- human naive B-cells contain mostly autoreactive unresponsive clones. *Blood.* (2010) 115:5026–36. doi: 10.1182/blood-2009-09-243071

8. Mauri C, Menon M. Human regulatory B-cells in health and disease: therapeutic potential. *J Clin Invest.* (2017) 127:772–9. doi: 10.1172/JCI85113

9. Holzelova E, Vonarbourg C, Stolzenberg MC, Arkwright PD, Selz F, Prieur AM, et al. Autoimmune lymphoproliferative syndrome with somatic Fas mutations. *N Engl J Med.* (2004) 351:1409–18. doi: 10.1056/NEJMoa040036

10. Garcelon N, Neuraz A, Salomon R, Faour H, Benoit V, Delapalme A, et al. A clinician friendly data warehouse oriented toward narrative reports: Dr. *Warehouse J Biomed Inform.* (2018) 80:52–63. doi: 10.1016/j.jbi.2018.02.019

11. Conley ME, Dobbs AK, Farmer DM, Kilic S, Paris K, Grigoriadou S, et al. Primary B-cell immunodeficiencies: comparisons and contrasts. *Annu Rev Immunol.* (2009) 27:199–227. doi: 10.1146/annurev.immunol.021908.132649

12. Jin G, Hamaguchi Y, Matsushita T, Hasegawa M, Le Huu D, Ishiura N, et al. B-cell linker protein expression contributes to controlling allergic and autoimmune diseases by mediating IL-10 production in regulatory B-cells. *J Allergy Clin Immunol.* (2013) 131:1674–82. doi: 10.1016/j.jaci.2013.01.044

13. Lagresle-Peyrou C, Millili M, Luce S, Boned A, Sadek H, Rouiller J, et al. The BLNK adaptor protein has a non-redundant role in human B-cell differentiation. *J Allergy Clin Immunol.* (2014) 134:145–54. doi: 10.1016/j.jaci.2013.12.1083

14. NaserEddin A, Shamriz O, Keller B, Alzyoud RM, Unger S, Fisch P, et al. Enteroviral infection in a patient with BLNK adaptor protein deficiency. *J Clin Immunol.* (2015) 35:356–60. doi: 10.1007/s10875-015-0164-2

15. Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, et al. An essential role for BLNK in human B-cell development. *Science.* (1999) 286:1954–7. doi: 10.1126/science.286.5446.1954

16. van Zelm MC, Geertsema C, Nieuwenhuis N, de Ridder D, Conley ME, Schiff C, et al. Gross deletions involving IGHM, BTK, or Artemis: a model for genomic lesions mediated by transposable elements. *Am J Hum Genet.* (2008) 82:320–32. doi: 10.1016/j.ajhg.2007.10.011

17. Turkoglu R, Yilmaz V, Ozdemir O, Akbayir E, Benbir G, Arsoy E, et al. Peripheral blood B-cell subset ratios and expression levels of B-cell-associated genes are altered in benign multiple sclerosis. *Mult Scler Relat Disord.* (2021) 52:103019. doi: 10.1016/j.msard.2021.103019

18. Marsh-Wakefield F, Juillard P, Ashhurst TM, Juillard A, Shinko D, Putri GH, et al. Peripheral B-cell dysregulation is associated with relapse after long-term quiescence in patients with multiple sclerosis. *Immunol Cell Biol.* (2022) 100:453–67. doi: 10.1111/imcb.12552

19. Liubchenko GA, Appleberry HC, Striebich CC, Franklin KE, Derber LA, Holers VM, et al. Rheumatoid arthritis is associated with signaling alterations in naturally occurring autoreactive B-lymphocytes. *J Autoimmun.* (2013) 40:111–21. doi: 10.1016/j.jaut.2012.09.001

20. Csomos K, Ujhazi B, Blazso P, Herrera JL, Tipton CM, Kawai T, et al. Partial RAG deficiency in humans induces dysregulated peripheral lymphocyte development and humoral tolerance defect with accumulation of T-bet(+) B-cells. *Nat Immunol.* (2022) 23:1256–72. doi: 10.1038/s41590-022-01271-6

21. Cornec D, Devauchelle-Pensec V, Jousse-Joulin S, Marhadour T, Ugo V, Berthou C, et al. Long-term remission of T-cell large granular lymphocyte leukemia associated with rheumatoid arthritis after rituximab therapy. *Blood.* (2013) 122:1583–6. doi: 10.1182/blood-2013-03-491464

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 09 January 2023
DOI 10.3389/fmed.2022.1097426

OPEN ACCESS

EDITED BY
Marcos De Lima,
The Ohio State University,
United States

REVIEWED BY
Guillemette Fouquet,
Centre Hospitalier Sud Francilien,
France
Mohamed A. Yassin,
Hamad Medical Corporation, Qatar

*CORRESPONDENCE
Bruno Fattizzo
✉ bruno.fattizzo@unimi.it

SPECIALTY SECTION
This article was submitted to Hematology,
a section of the journal
Frontiers in Medicine

RECEIVED 13 November 2022
ACCEPTED 14 December 2022
PUBLISHED 09 January 2023

CITATION
Fattizzo B and Motta I (2023) Rise of the planet of rare anemias: An update on emerging treatment strategies.
Front. Med. 9:1097426.
doi: 10.3389/fmed.2022.1097426

Rise of the planet of rare anemias: An update on emerging treatment strategies

Bruno Fattizzo[1,2]* and Irene Motta[3,4]

[1]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, SC Ematologia, Milan, Italy, [2]Department of Oncology and Hemato-Oncology, University of Milan, Milan, Italy, [3]Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, SC Medicina ad Indirizzo Metabolico, Milan, Italy, [4]Department of Clinical Sciences and Community Health, University of Milan, Milan, Italy

Therapeutic options for rare congenital (hemoglobinopathies, membrane and enzyme defects, congenital dyserythropoietic anemia) and acquired anemias [warm autoimmune hemolytic anemia (wAIHA), cold agglutinin disease CAD, paroxysmal nocturnal hemoglobinuria (PNH), and aplastic anemia (AA)] are rapidly expanding. The use of luspatercept, mitapivat and etavopivat in beta-thalassemia and pyruvate kinase deficiency (PKD) improves transfusion dependence, alleviating iron overload and long-term complications. Voxelotor, mitapivat, and etavopivat reduce vaso-occlusive crises in sickle cell disease (SCD). Gene therapy represents a fascinating approach, although patient selection, the toxicity of the conditioning regimens, and the possible long-term safety are still open issues. For acquired forms, wAIHA and CAD will soon benefit from targeted therapies beyond rituximab, including B-cell/plasma cell targeting agents (parsaclisib, rilzabrutinib, and isatuximab for wAIHA), complement inhibitors (pegcetacoplan and sutimlimab for CAD, ANX005 for wAIHA with complement activation), and inhibitors of extravascular hemolysis in the reticuloendothelial system (fostamatinib and FcRn inhibitors in wAIHA). PNH treatment is moving from the intravenous anti-C5 eculizumab to its long-term analog ravulizumab, and to subcutaneous and oral proximal inhibitors (anti-C3 pegcetacoplan, factor D and factor B inhibitors danicopan and iptacopan). These drugs have the potential to improve patient convenience and ameliorate residual anemia, although patient compliance becomes pivotal, and long-term safety requires further investigation. Finally, the addition of eltrombopag significantly ameliorated AA outcomes, and data regarding the alternative agent romiplostim are emerging. The accelerated evolution of treatment strategies will need further effort to identify the best candidate for each treatment in the precision medicine era.

KEYWORDS

beta-thalassemia, sickle cell disease, congenital hemolytic anemias, pyruvate kinase deficiency, autoimmune hemolytic anemia, cold agglutinin disease, paroxysmal nocturnal hemoglobinuria, aplastic anemia

1. Introduction

Rare anemias encompass several nosological entities, including inherited and acquired forms, which may present at various ages and with heterogeneous features. The former includes congenital defects of the number and structure of globin genes, as in alpha- and beta-thalassemia and sickle cell disease (SCD), as well as alterations of erythrocyte membrane or enzymes, as in congenital hemolytic anemias (CHAs). Acquired forms include immune-mediated destruction of erythrocytes [i.e., autoimmune hemolytic anemias (AIHAs)] or bone marrow precursors [i.e., aplastic anemia (AA)], and the very rare paroxysmal nocturnal hemoglobinuria (PNH). The rarity of these entities, along with the several clinical/laboratory overlaps, results in frequent misdiagnosis and delays in proper treatment. For ages, therapy of rare anemias has mainly relied on transfusion support for congenital forms and PNH, and traditional immunosuppressive treatments for acquired ones. In the last decade, a deeper understanding of physiopathology, particularly regarding the underlying molecular mechanisms, led to the development of several targeting agents. The rise of a new era of personalized medicine for rare anemia is ongoing, moving from supportive treatment to disease-modifying agents and the advent of gene therapy. This review will provide a snapshot of novel therapies for rare anemias to highlight the most recent advances in the field.

2. Congenital anemias

2.1. Update on hemoglobinopathies

Hemoglobinopathies, including thalassemia and SCD, are the most common monogenic diseases worldwide (1). Although conventional supportive treatment, including transfusion programs and iron chelation therapy, has been highly optimized, these strategies still encounter significant limitations leading to morbidity and mortality. New treatment approaches and novel therapies have been proposed (**Table 1**), some of which have the potential to change the natural history of the disorders. Although thalassemia and SCD carry a hemoglobin (Hb) chain defect, they have different pathophysiology and clinical complications (2, 3). Emerging therapies for beta-thalassemia aim to target α/β chain imbalance, ineffective erythropoiesis, and iron dysregulation and overload. In SCD the main targets are reducing the amount of Hb S (HbS), preventing red cell dehydration or sickling, endothelial adhesion, and oxidative stress (4) (**Figure 1**).

2.1.1. Thalassemias

Luspatercept is the first-in-class erythroid maturation agent approved by the Food and Drug Administration (FDA) and European Medicine Agency (EMA) for transfusion-dependent thalassemia (TDT). Luspatercept is a recombinant fusion protein that binds ligands of the transforming growth factor beta (TGF-β) superfamily, thus inhibiting SMAD2/3 signaling and promoting late-stage erythropoiesis. The phase III BELIEVE trial showed that a significantly greater percentage of patients receiving luspatercept achieved the primary endpoint of a ≥33% reduction in transfusion burden from baseline during weeks 13–24, with a reduction of ≥2 RBC units compared with placebo (5). Data from the 5-year open-label extension phase (NCT04064060), which is currently ongoing, showed that 125 patients (55.8%) completed 144 weeks of treatment with luspatercept (6). The main reasons for treatment discontinuation were patient withdrawal (23.7% luspatercept vs. 11.6% placebo) and adverse events (10.3 vs. 1.8%), which included headache, arthralgia, bone pain, dizziness, nausea, hypertension, jaw pain, and hyperuricemia. More than 75% of patients had a reduction in transfusion burden ≥33%, and 50% had a reduction ≥50%. The transfusion window for ≥50% responders increased by 9.88 days, with 12.1% of patients achieving transfusion independence ≥8 weeks (placebo 1.8%, $P = 0.0015$). Long-term luspatercept treatment resulted in a decreasing trend in liver iron concentration compared with baseline. No new safety concerns were reported, and the occurrence of treatment-emergent adverse events of special

TABLE 1 Novel drugs in patients with rare congenital anemias.

Disease	Drug	Phase/Status	Target
β-Thal	Luspatercept	FDA and EMA approved	Ineffective erythropoiesis
	Mitapivat	Phase 2	Pyruvate kinase activator
	LentiGlobin (BB305)	FDA and EMA approved	Gene therapy
	CTX001	Phase 3	Gene editing
	Vamifeport	Phase 2a	ferroportin inhibitor
	Sapablursen	Phase 2	TMPRSS6 inhibitor
SCD	Voxelotor (GBT440)	FDA and EMA approved	HbS polymerization
	Crizanlizumab	FDA and EMA approved	Vaso-occlusion
	Mitapivat	Phase 2/3	Pyruvate kinase activator
	Etavopivat	Phase 1	Pyruvate kinase activator
	L-glutamine	FDA approved/Phase 3	Substrate of NAD + synthetase
	LentiGlobin (BB305)	Phase 3	Gene therapy
	CTX001	Phase 3	Gene editing
PKD	Mitapivat	FDA and EMA approved	Pyruvate kinase activator
	RP-L301	Phase 1	Gene therapy

β-Thal, beta-thalassemia; SCD, sickle cell disease; PKD, pyruvate kinase deficiency; FDA, food and drug administration; EMA, European Medical Agency.

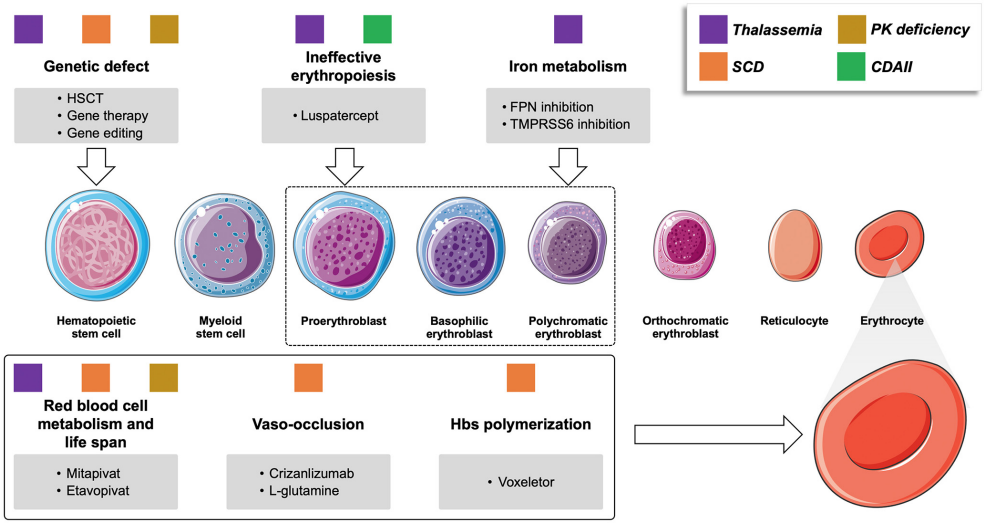

FIGURE 1
Novel drugs for rare congenital anemias and their targets. Colored squares represent the different conditions that may benefit of the various compounds under investigation. PK pyruvate kinase; CDAII congenital dyserythropoietic anemia type II; SCD sickle cell anemia; HSCT, hematopoietic stem cell transplant; PK, pyruvate kinase; FPN, ferroportin.

interest was comparable with previous reports (7). Recently, the results of the BEYOND trial of luspatercept in NTDT have been published, showing that 77% of 96 patients in the luspatercept group and none in the placebo group had an increase of at least 1·0 g/dL of Hb. Mitapivat, initially investigated in pyruvate kinase deficiency (PKD) (see dedicated paragraph) is currently under evaluation also in alpha and beta non-transfusion-dependent thalassemia (NTDT) (NCT03692052) (8). Sixteen out of twenty (80%) patients showed an increase in Hb $\geq$ 1.0 g/dL, along with improvements in markers of hemolysis and ineffective erythropoiesis. Long-term data on 17 patients with a median duration of treatment of 70.9 weeks showed that Hb improvements achieved in the core period were sustained as well as improvement of markers of hemolysis and ineffective erythropoiesis. The safety profile was consistent with that observed in the core period. Headache and back pain were reported in $\geq$15% of patients; however, none were grade $\geq$3 (8). Molecules targeting iron metabolism include ferroportin inhibitor vamifeport (VIT-2763) and those up-regulating hepatic hepcidin production through inhibition of transmembrane serine protease 6 (TMPRSS6). Vamifeport improved anemia and erythropoiesis in a mouse model of β-thalassemia (9). A phase IIa double-blind, randomized, placebo-controlled study with the primary endpoint of assessing the safety and tolerability of vamifeport compared to placebo in NTDT patients ł12 years has been completed, but the results have not been published yet (NCT04364269). Antisense oligonucleotides that inhibit TMPRSS6 have shown promising results in β-thalassemia mouse models by reducing the iron burden and improving ineffective erythropoiesis (10), and a clinical trials with sapablursen is currently ongoing (NCT04059406).

2.1.2. Sickle cell disease

For many years hydroxyurea has been the only pharmacological option for SCD patients, while more recently, a significant acceleration in potential treatment approaches has been observed. Indeed, EMA and FDA have recently approved two new compounds: voxelotor and crizanlizumab. Voxelotor is a Hb modulator with a good safety profile, which inhibits the polymerization of HbS stabilizing the hemoglobin in the oxygenated status. In the phase 3 trial, a significant increase in Hb levels and a decrease in markers of hemolysis were observed. However, no significant reduction in vaso occlusive crises (VOCs) was demonstrated (11). Crizanlizumab is a monoclonal antibody against P-selectin, an adhesion factor expressed by endothelium cells involved in the formation of aggregates between platelets and leukocytes, thus contributing to vessels occlusion in the microcirculation. Crizanlizumab reduced the rate of SCD–related pain crises compared to placebo, with a reduction of the annual rate of crises of 45.3% in the high-dose group (5 mg/kg). In the low-dose crizanlizumab group (2.5 mg/kg) a reduction of 32.6% compared with placebo was observed, although not statistically significant (12). Adverse events observed in at least 10% of patients in the crizanlizumab group were headache, back pain, nausea, arthralgia, pain in upper and lower limbs, urinary tract and upper respiratory infections, pyrexia, diarrhea, musculoskeletal pain, pruritus, vomiting, and chest pain. Interestingly, mitapivat is also under investigation in these patients (NCT05031780). The drug was generally well tolerated, a reduction of VOCs was

observed, together with an increase in Hb levels and a decrease in markers of hemolysis (13). Etavopivat, another selective activator of erythrocyte pyruvate kinase (PKR), increases PKR activity, resulting in decreased 2,3-DPG and increased ATP. Data from the Phase 1 study (NCT03815695) showed that etavopivat 400 mg once daily was generally well tolerated. Another molecule approved only by FDA for patients older than 5 years is L-glutamine, an amino acid used by the enzyme NAD + synthetase to produce NAD + from NADH, an essential cofactor in redox reactions whose requirement is increased in SCD. A phase 3 trial showed a significant reduction in VOCs and hospital visits in children and adults treated with L-glutamine compared to placebo (14). EMA did not approve the drug for efficacy concerns.

2.1.3. Gene therapy for beta-thalassemia and sickle cell disease

Allogeneic hematopoietic stem cell transplantation (HSCT) has been the only curative option for hemoglobinopathies for decades. Meanwhile, gene therapy and gene editing trials have been developed, leading to the approval in 2019 by EMA and in 2022 by FDA of the first additive gene therapy product betibeglogene autotemcel (LentiGlobin BB305) for TDT patients non-beta0/beta0. However, in March 2022, the European Commission withdrew the marketing authorization for betibeglogene autotemcel in the European Union as requested by the marketing authorization holder (bluebird bio) for commercial reasons. The same product is under investigation in SCD, with more than 30 patients treated, showing promising results (15). Of note, in 2021 a temporary suspension of the clinical trials and EMA license was announced because of a case of acute myeloid leukemia in a SCD patient treated with LentiGlobin BB305, which afterward was demonstrated as not associated with the vector (16). A gene-editing strategy that aims to reactivate HbF inhibiting BCL11A is CTX001, a CRISPR/Cas9-modified autologous HSCT product currently investigated in both TDT and SCD (17). Updated efficacy and safety data have been reported for the first 75 patients in the CLIMB THAL-111 (NCT03655678) and CLIMB SCD-121 (NCT03745287) trials, with a median follow-up of 12.3 and 9.6 months, respectively. CTX001 infusion led to the independence from transfusions in almost all patients with TDT (42/44 patients), with a sustained increase in HbF and thereby of total Hb levels (mean of >9 g/dL). All SCD patients (n = 31) no longer presented severe VOCs after CTX001 infusion with a mean HbF increase of ∼40% at month 4 and attainment of mean Hb levels >11 g/dL (18).

2.2. Update on congenital hemolytic anemias

Congenital hemolytic anemias are characterized by reduced lifespan and early destruction of erythrocytes. They encompass defects of the erythrocyte membrane proteins (hereditary spherocytosis, HS, hereditary elliptocytosis, HE, and hereditary stomatocytosis, Hst) and red cell enzymes metabolism (glucose-6-phosphate dehydrogenase, G6PD, and pyruvate kinase, PK), as well as alterations of erythrocyte precursors, resulting in defective erythropoiesis (congenital dyserythropoietic anemia, CDA) (19). Current management of CHAs mainly relies on transfusions, iron chelation, and splenectomy. The latter is highly effective in HS, less in PKD and CDA, and contraindicated in Hst due to the increased thrombotic risk. The greatest therapeutic advances for CHAs regard PKD (**Table 1**), including therapies that boost enzyme activity by activating PK and gene therapy (20).

2.2.1. Pyruvate kinase activators

Mitapivat (AG-348), is an oral allosteric activator of erythrocyte PK. In a pivotal phase 2 study (NCT02476916) (21) enrolling 52 adults with PKD not requiring transfusions, a Hb increase of more than 1 g/dL from the baseline was reported in 26 patients (50%, mean maximum Hb increase of 3,4 g/dL, range 1.1 + 5.8) with a favorable safety profile. Hb response occurred only in patients with at least one missense mutation of the PK gene (i.e., those with residual PK activity), highlighting the importance of assessing the underlying molecular defect. Further two phase 3 trials assessed mitapivat in PKD patients requiring or not transfusion support (ACTIVATE-T and ACTIVATE) studies (NCT03548220) (22). In the ACTIVATE-T open label study (NCT03559699) (23), 37% met the primary endpoint of >33% reduction in transfusion burden, and 6 (22%) became transfusion independent. In the ACTIVATE trial of mitapivat vs. placebo, 40% achieved a sustained Hb response vs. 0 patients in the placebo arm. Data from ACTIVATE and ACTIVATE-T confirmed the long-term reduction of transfusion need in both regularly and not-regularly transfused patients (24, 25) along with Hb normalization in a proportion of patients (26). Additionally, mitapivat was shown to improve ineffective erythropoiesis and iron overload (27). Furthermore, despite mitapivat effect on aromatase, bone mineral density remains stable during long-term treatment confirming a good safety profile (28). On these bases, two novel trials with mitapivat in regularly and not-regularly transfused children with PKD have been announced (29, 30). Finally, an elegant preclinical study showed that mitapivat induced similar Hb improvement and reticulocyte decrease as splenectomy in a murine model of HS, heralding its use even in this setting (31).

2.2.2. Gene therapy

In a murine model of PKD, transplantation of hematopoietic stem cells transfected with a lentiviral vector carrying PK gene restored normal glycolytic activity and erythropoiesis, and improved hemolysis. Gene therapy by lentiviral transduction of autologous stem cells and progenitor cells is under study in an open-label phase I trial (NCT04105166). Preliminary data on two adult splenectomized patients showed a Hb increase from

baseline in both, along with hemolytic markers improvement. Notably, no severe adverse events were reported.

2.3. Summary of congenital anemias

Red blood cell transfusions have been the only therapeutic option for TDT and the severe forms of SCD. However, during the last years, the approval of luspatercept for TDT, and voxelotor and crizanlizumab for SCD, have the potential to modify the current management of these patients. Gene therapy, approved for TDT by EMA and FDA, is available only in the US for market reasons. However, promising results from gene editing trials represent a potentially curative option for beta-thalassemia and SCD. Given the complex pathophysiology of these disorders and inter-patient variability, new drugs will likely be managed with a patient-tailored approach which could include a combination of different drugs according to the individual characteristics. The management of CHAs should be individualized considering the definite diagnosis (PKD vs. HS vs. Hst, etc.), different ages, comorbidities, and frequency of complications (gallstone, hemolytic and aplastic crises, and iron overload), harnessing the need for transfusions, iron chelation, splenectomy, and cholecystectomy. Splenectomy is less effective in PKD vs. HS and contraindicated in Hst. It is discouraged during the first 6 years of age since transfusion needs may spontaneously improve, and in elderly patients for infectious and thrombotic risks. The allosteric PK stimulator mitapivat is a promising new option for PKD, both alpha and beta-thalassemia, and SCD, and possibly for HS in the next future. Among PKD patients, responses in PKD are generally observed only in patients with at least one missense mutation, whilst those with more disruptive mutations represent an unmet clinical need. Gene therapy might be a chance in these cases, but the results are still preliminary and require further investigation.

3. Acquired anemias

3.1. Update on autoimmune hemolytic anemia

Autoimmune hemolytic anemia is a rare disease with an incidence of 0.8 to 3/100,000 people per year and is caused by an autoimmune attack against erythrocyte antigens (32). AIHA are classified as "warm" (wAIHA) or "cold" forms (CAD), according to the thermal amplitude of the autoantibody and basing on the direct anti-globulin test (IgG + or IgG plus C3d + in wAIHA vs. C3d + and cold agglutinin detection in CAD) (32). AIHA displays multifactorial pathogenesis, including genetic (association with congenital conditions and certain mutations), environmental (drugs, infections, including SARS-CoV-2, pollution, etc.), and miscellaneous factors (solid/hematologic neoplasms, systemic autoimmune diseases, etc.) contributing to tolerance breakdown. Several mechanisms, such as autoantibody production, complement activation, monocyte/macrophage phagocytosis, and bone marrow compensation, are implicated in extra-/intra-vascular hemolysis. Management is based on standard therapies that should be differentiated and sequenced according to AIHA type. wAIHA are treated with steroids frontline, followed by rituximab, an anti-CD20 monoclonal antibody, as second line. The latter is effective in about 70–80% of cases with a median duration of response of 18 months. wAIHA patients failing rituximab represent an unmet clinical need and may be subjected to splenectomy (if young with few comorbidities) or treated with cytotoxic immunosuppressants. Frontline rituximab is advised in CAD, since steroids are effective only at high unacceptable doses. The drug induces short-term partial responses in about 50–60% of cases, and relapsed ones are handled with transfusions and cytotoxic immunosuppressants (33, 34). Novel treatments (**Table 2**) mainly target autoantibody production by the B-cell/plasma-cell compartment or the final erythrocyte breakdown by either complement or reticuloendothelial systems (**Figure 2**) (35, 36).

3.1.1. B-cell and plasma cell inhibitors

These agents include B-cell targeting agents mainly used in secondary AIHA, such as oral B-cell receptor inhibitors parsaclisib (NCT03538041 and NCT05073458), ibrutinib (NCT03827603), and rilzabrutinib (NCT05002777) that are being studied in clinical trials. In a recent multicenter, phase 2, open-label study (NCT03538041) of parsaclisib in relapsed/refractory wAIHA and CAD, the primary endpoint was the overall response at any visit from week 6 to 12. Sixteen patients (64%) responded, and 8 (32%) achieved a CR, although some toxicities emerged including diarrhea, cytomegalovirus reactivation, and psoriasis), and 2 subjects discontinued treatment (37). The drug is now being evaluated in a randomized, controlled phase 3 trial in wAIHA (NCT05073458). Among plasma cell targeting agents, the proteasome inhibitor bortezomib and the anti-CD38 MoAb daratumumab are interesting agents (38, 39). Their efficacy is supported by several case reports/series and from a single phase 2 trial of bortezomib in CAD where a 30% overall response rate was registered with limited toxicity (40). The rationale is to eliminate long-lived plasma cells that do not express CD20 and may cause rituximab refractoriness. Isatuximab, another anti-CD38 MoAb is under investigation in wAIHA in phase 1 study (NCT04661033).

3.1.2. Complement inhibitors

Complement modulation is the most promising drug under study for CAD: sutimlimab, a monoclonal antibody against complement protein C1s, demonstrated a short time to response, rapid normalization of hemolysis, and good

TABLE 2 Novel drugs in patients with rare, acquired anemias.

Disease	Drug	Phase/Status	Target
wAIHA	Parsaclisib	Phase 3	PI3K inhibitor
	Ibrutinib/ Rilzabrutinib	Phase 2	BTK inhibitor
	Bortezomib	Phase 2/Case reports	Proteasome inhibitor
	Daratumumab/ Isatuximab	Case reports/Phase 1	Anti-CD38 MoAb
	Pegcetacoplan	Phase 2	C3 inhibitor
	ANX005	Phase 2	Anti-C1q MoAb
	Fostamatinib	Phase 3	SyK inhibitor
	Nipocalimab/RVT-1401	Phase 3/Phase 2	Anti-FcRn MoAb
CAD	Sutimlimab	FDA and EMA approved	Anti-C1s MoAb
	Pegcetacoplan	Phase 3	C3 inhibitor
PNH	Ravulizumab	FDA and EMA approved	Anti-C5 Moab
	Crovalimab	Phase 3	Anti-C5 Moab
	Pegcetacoplan	FDA and EMA approved	C3 inhibitor
	Iptacopan	Phase 3	Factor B inhibitor
	Danicopan	Phase 3	Factor D inhibitor
	Vemircopan	Phase 2	Factor D inhibitor
	BCX9930	Phase 2	Factor D inhibitor
AA	Eltrombopag	FDA and EMA approved	TPO-RA
	Romiplostim	Phase 2/3	TPO-RA

WAIHA, warm autoimmune hemolytic anemia; CAD, cold agglutinin disease; PNH, paroxysmal nocturnal hemoglobinuria; AA, aplastic anemia; MoAb, monoclonal antibody; PI3K, phosphoinositide 3-kinase; BTK, bruton tyrosine kinase; SyK, spleen tyrosine kinase; FcRn, neonatal Fc receptor; TPO-RA, thrombopoietin receptor agonist.

safety profile in two phase 3 trials (41, 42). Results were further updated, demonstrating long-lasting responses while on treatment (43) and reappearance of hemolysis in most patients discontinuing the drug in an 8 weeks washout periodv (44). In fact, since sutimlimab does not eliminate autoantibody production, long-term treatment is required to control hemolysis; additionally, it seems less effective on peripheral CAD-induced circulatory symptoms. Pegcetacoplan, a pegylated peptide that inhibits C3 (36), also showed good activity in a phase 2 trial in CAD and wAIHA with IgG + C DAT positivity, and a phase 3 study has been announced in CAD (NCT05096403). In wAIHA with complement activation, a novel C1q inhibitor ANX005 is also being developed (NCT04691570).

3.1.3. IgG-mediated hemolysis targeting agents

The reticuloendothelial system may be targeted by inhibiting the spleen tyrosine kinase with fostamatinib, which also blocks the B-cell receptor downstream pathway (35). The drug was effective in about 45% of patients in a phase 2 trial, with mainly hypertension and diarrhea as related toxicities, and is now in phase 3 studies in wAIHA (NCT02612558). Finally, the safety/efficacy of several inhibitors of the neonatal Fc receptor (FcRn), such as intravenous nipocalimab (NCT03075878), and subcutaneous RVT-1401 (NCT04253236), are under investigation. The FcRn is structurally homologous to the MHC Class I receptor family, is expressed by several cells, and is responsible for the salvage of IgG from catabolism. Blocking FcRn induces an increased clearance of IgG, including pathogenic IgG autoantibodies.

3.2. Update on bone marrow failure syndromes/paroxysmal nocturnal hemoglobinuria

Bone marrow failure syndromes include the PNH–AA spectrum (45, 46). PNH is due to the acquisition of a somatic mutation of PIGA gene by the hematopoietic stem cell (HSC). PIGA encodes a glycosyl phosphatidyl inositol (GPI) molecule that anchors several factors to cell membranes. The lack of CD-55 and CD-59 GPI-anchored proteins renders PNH erythrocytes susceptible to complement-mediated destruction resulting in intravascular hemolysis with anemia and thrombosis. The expansion of PIGA-mutated HSCs to reach a clinically significant clone size is thought to be partly due to an immune attack against PNH-negative HSCs. This T-cell-mediated autoimmune attack against BM precursors is typical of AA, which is associated with PNH in up to 60% of cases (47). Up to the early 2000s, PNH therapy was mainly supportive, and AA patients received immunosuppressive treatment with anti-thymocyte globulin and cyclosporine, or, if candidate, HSC transplant, with heterogeneous and mainly age-related efficacy. In the last 15 years, the treatment of PNH and AA has been revolutionized by the introduction of complement inhibitors in the former, and of the thrombopoietin receptor agonist eltrombopag in the latter (**Table 2**).

3.2.1. Complement inhibitors for paroxysmal nocturnal hemoglobinuria

The anti-C5 MoAb eculizumab was the first drug to reduce hemolysis, improve anemia, and abate thrombotic risk in PNH patients. The risk of Neisseria meningitidis infections mandated the vaccination with anti-Meningococcus ACYW135 and B before starting therapy, along with life-long education and monitoring of infectious risk. Additionally, the drug required fortnightly intravenous infusions, and up to 2/3 of cases had residual anemia due to persistent intravascular hemolysis, concomitant bone marrow failure, and development of extravascular hemolysis driven by C3 deposition on PNH erythrocytes (48, 49). In the last decade, the long-half-life

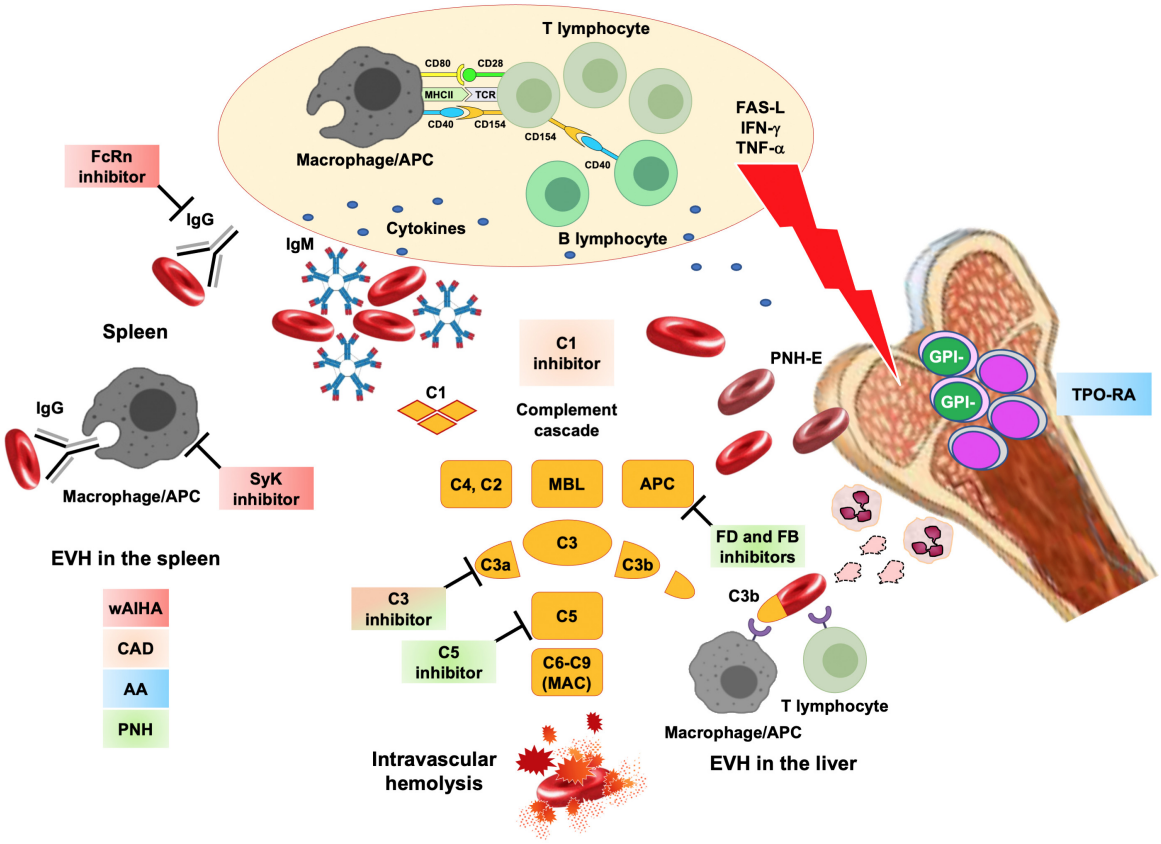

FIGURE 2
Novel drugs for rare acquired anemias and their targets. Acquired anemias encompass autoimmune hemolytic anemias, where hemolysis is due to autoantibodies produced after a tolerance break with altered B-, T- cells and antigen presenting cells (APC) crosstalk and production of several cytokines. In warm forms (wAIHA), IgG autoantibodies cause extravascular hemolysis (EVH) in the spleen. These processes may be targeted by neonatal Fc receptor inhibitors (FcRn that clear the autoantibodies from the circulation) and spleen tyrosine kinase (SyK) inhibitors (which inhibits phagocytosis). In cold agglutinin disease (CAD), IgM activate the classical complement cascade and cause C3d mediated extravascular hemolysis in the liver and minor C5 mediated intravascular hemolysis. This may be targeted by complement inhibitors (particularly C1 and C3 inhibitors). Even in wAIHA complement activation may occur and complement inhibitors are under study. Aplastic anemia (AA) is due to a T-cell attack to hematopoietic stem cells, through exposure/release of mediators such as FAS, interferon gamma (IFN) and tumor necrosis factor alpha (TNF). Thrombopoietin receptor agonists (TPO–RA) are effective, along with standard immunosuppressors, to restore hematopoiesis. After immune attack to bone marrow precursors, stem cell that acquired PIG-mutation and are glycophosphatidylinositol (GPI-) negative, may be spared and may expand in a paroxysmal nocturnal hemoglobinuria (PNH) clone. PNH erythrocytes lack natural anti-complement molecules CD55 and CD59 and are destroyed intravascularly by complement cascade (mainly through homeostatic alternative pathway activation). Along with already approved C5 inhibitors, novel drugs include C3 inhibitors, Factor B and Factor D (FB, FD). Colored squares represent the different conditions that may benefit of the various compounds under investigation. FAS-L, FAS ligand; IFN, interferon; TNF, tumor necrosis factor; macrophage/APC, antigen presenting cell; APC, alternative complement pathway.

anti-C5 ravulizumab has been studied and shown not inferior to eculizumab in two phase 3 trials in PNH naïve or previously exposed to eculizumab (50, 51) and was recently approved. Administered every 8 weeks, the drug has the potential to stabilize hematologic response and better control breakthrough hemolytic episodes. Another promising anti-C5, currently in phase 3 investigation, is crovalimab (52). It is administered subcutaneously every 4 weeks, is well tolerated, and has a different target from ecu/ravu, thus being active on the Asian C5 polymorphism. The development of drug-target-drug immune-complexes should be surveilled during the switch from ecu/ravu to crovalimab, since it may cause immunologic reactions that tend to resolve over time (53). Pegcetacoplan, previously mentioned for CAD, is a C3 inhibitor that reduced C3-mediated extravascular hemolysis and alleviated anemia and transfusion dependence in PNH patients who were suboptimal responders to eculizumab (54). The drug is infused subcutaneously twice a week and is now approved for the frontline treatment of PNH patients in the US and those anemics after at least 3 months of anti-C5 treatment in Europe. More recently, several updates on the long-term safety and efficacy of pegcetacoplan have been presented, also highlighting an anti-thrombotic effect of the drug (55–58). Oral agents targeting factor B and D of the alternative

pathway represent a further innovation. Factor B inhibitor iptacopan, and factor D inhibitor danicopan have been shown to improve anemia and reduce hemolysis and transfusion needs in PNH patients who are suboptimal responders to anti-C5 in early phase trials (59, 60). The first is being developed as a single agent BID oral therapy, and a phase 3 trial is ongoing (NCT04558918); the second is a TID oral therapy given as add on to anti-C5. A more potent anti-D, vemircopan, administered as monotherapy once a day, is under study in naïve and suboptimal responders to C5 inhibition (NCT04170023). Another oral factor D inhibitor, BCX9930 is also being studied in naïve and previously treated PNH patients, and preliminary results are encouraging (NCT05116787 and NCT05116774). Notably, proximal inhibitors require vaccination with anti-Meningococcus, anti-Pneumococcus, and anti-Haemophilus before treatment start.

3.2.2. Eltrombopag in aplastic anemia

Ten years ago, the NIH group published the first results regarding eltrombopag efficacy in up to 40% of AA patients relapsed/refractory to immunosuppressive treatment (61). Since then, several real-world series confirmed the use of eltrombopag as a single agent at 150 mg day in this setting with trilinear improvement in some cases (62). The addition of eltrombopag to first-line immunosuppression further improved responses to >90% in more recent reports (63) and was superior to IST alone in a phase 3 randomized European trial (64), without increasing toxicity nor clonal evolution. Treatment schedules, particularly regarding the length of eltrombopag administration, the possibility of tapering and discontinuing the drug and to re-start it in case of relapse, deserve further investigation. The drug interferes with cation-containing foods, thus requiring fasting before and after administration. Asian groups are exploring the use of the alternative TPO-RA agent romiplostim. Preliminary results seem promising, with more than 80% response rates if used frontline in association with IST (65–67).

3.3. Summary of acquired anemias

Novel agents represent a basket of opportunities for wAIHA and CAD, while a gray zone of uncertainness remains for treating mixed and atypical AIHA forms. B-cell targeting small molecules and anti-plasma cell agents are promising, although response rates are still lower than those obtained with rituximab, and toxicities may be higher, deserving further investigation. The spleen tyrosine kinase inhibitor fostamatinib blocks phagocytosis by the reticuloendothelial system in wAIHA, and also modulates B-cell receptor activity, potentially reducing autoantibody production. Regarding complement inhibitors, they have high efficacy in CAD; they do not eliminate the autoantibody and should likely be administered indefinitely. Similarly, anti-FcRn agents increase the clearance of pathogenic autoantibodies in wAIHA, but autoantibody production is preserved, suggesting the need for combination therapy in the future. Additionally, the very short response time to these agents may be particularly helpful in severely anemic patients and acute crises.

Regarding PNH, its treatment is facing an era of expanding options with different routes of administration. The latter will likely improve patient convenience but also pose warnings on compliance (68). Proximal inhibitors (C3, factor B and D inhibitors) show dramatic efficacy on extravascular hemolysis and in improving residual anemia while on anti-C5 agents. The "sparing" of a large PNH cell clone with these agents may, in turn, fuel severe hemolytic breakthroughs in case of complement-activating events such as infections, traumas, surgery, etc. The proper management of such pharmacodynamics breakthrough hemolysis is still unknown and will require further investigation. Other open issues include the efficacy of these novel agents in preventing thrombotic episodes, and their long-term safety, particularly regarding infections. While eculizumab has been proven safe and is indicated in the case of pregnancy, no data are available for novel agents. Finally, the introduction of eltrombopag improved the treatment of AA, particularly in the setting of relapse/refractory patients and in the elderly, given the good safety profile and the absence of kidney toxicity. Its use frontline is supported by convincing evidence, but it is still not licensed in Europe, and the timing of administration and the possibility of clonal evolution deserves further investigation. From a patient perspective, the interference with cations containing food should be considered, and future strategies, including romiplostim, and possibly the new TPO-RA agent avatrombopag that has no food interference, require further exploration.

4. Red blood cell transfusions in the current era

Supportive treatment with RBC transfusions remains the mainstay for the management of anemia in the acute setting of both congenital and acquired forms, as well as chronically in patients with TDT. The thresholds of Hb levels are highly heterogeneous across centers and should be carefully weighed on patient age, comorbidities, and disease type. For instance, during infancy, patients with TDT require chronic support to allow development and to avoid extramedullary erythropoiesis and skeletal deformities, whilst in CHAs, transfusions are seldom required on a regular basis. Furthermore, in PKD, the augmented levels of 2,3 diphosphoglyceric acid increase oxygen release to the tissues, thus improving anemia tolerance (19, 20). The relevant issue of alloimmunization should be considered both in poly transfused patients with congenital forms and in those with acquired autoimmune ones, where the risk is higher due to disease-related immune dysfunction (32). In the current era, the advances in phenotyping and genotyping of patients

and blood donors have markedly improved unit matching, thus abating alloimmunization and transfusion reactions.

5. Conclusion

Therapeutic options for rare anemias are rapidly expanding and continue to ameliorate disease outcomes with reduction of transfusion need, VOCs, and iron overload in hemoglobinopathies and CHAs and improvement of hemolysis and anemia in AIHA, PNH, and AA. Interestingly, compounds designed for a specific disorder have been considered beneficial also for other anemias in a sort of repurposing process with potentially lower overall development costs and shorter development timelines. Importantly, these compounds may also improve patient convenience. On the other hand, the accelerated evolution of treatment strategies will need a further effort to identify the best candidate for each treatment in the precision medicine era. Non-responders to novel therapies are often disregarded in clinical trials and predictors of response are only seldomly explored (i.e., presence of disruptive genotype in PKD). They represent an unmet need for further development in this area. Finally, as more and more agents become available, costs are also rising for the national health systems and would require careful consideration within regulatory and clinical communities.

Author contributions

Both authors equally contributed to the conceiving, writing, revision of the manuscript, and approved the submitted version.

Funding

For APC covering, funding from the Ministry of Health, Current Research 2021 were used.

Acknowledgments

We thank Luigi Ghilardini for his help in designing the figures of the manuscript. We also thank Wilma Barcellini and Maria Domenica Cappellini for their unvaluable mentorship during clinical and academic studies.

Conflict of interest

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

Publisher's note

References

1. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ.* (2008) 86:480–7. doi: 10.2471/BLT.06.036673

2. Taher AT, Musallam KM, Cappellini MD. β-Thalassemias. *N Engl J Med.* (2021) 384:727–43. doi: 10.1056/NEJMra2021838

3. Piel FB, Steinberg MH, Rees DC. Sickle cell disease. *N Engl J Med.* (2017) 376:1561–73. doi: 10.1056/NEJMra1510865

4. Cappellini MD, Marcon A, Fattizzo B, Motta I. Innovative treatments for rare anemias. *Hemasphere.* (2021) 5:e576. doi: 10.1097/HS9.0000000000000576

5. Cappellini MD, Viprakasit V, Taher AT, Georgiev P, Kuo KHM, Coates T, et al. A phase 3 trial of luspatercept in patients with transfusion-dependent β-thalassemia. *N Engl J Med.* (2020) 382:1219–31. doi: 10.1056/NEJMoa1910182

6. Cappellini MD, Taher AT, Porter JB, Kuo KHM, Coates TD, Voskaridou E, et al. S270: longer-term analysis of efficacy of luspatercept versus placebo in patients with transfusion-dependent β-thalassemia enrolled in the believe study. *Hemasphere.* (2022) 6:171–2. doi: 10.1097/01.HS9.0000843972.88850.ba

7. Viprakasit V, Cappellini MD, Porter JB, Kuo KHM, Coates TD, Voskaridou E, et al. P1518: long-term safety results of the believe study of luspatercept in adults with βeta-thalassemia. *Hemasphere.* (2022) 6:1399–400. doi: 10.1097/01.HS9.0000848928.08455.02

8. Kuo KHM, Layton DM, Lal A, Al-Samkari H, Bhatia J, Kosinski PA, et al. P1522: long-term efficacy and safety of the oral pyruvate kinase activator mitapivat in adults with non–transfusion-dependent alpha- or β-thalassemia. *Hemasphere.* (2022) 6:1403–4. doi: 10.1097/01.HS9.0000848944.56958.95

9. Nyffenegger N, Flace A, Doucerain C, Dürrenberger F, Manolova V. The oral ferroportin inhibitor VIT-2763 improves erythropoiesis without interfering with iron chelation therapy in a mouse model of β-thalassemia. *Int J Mol Sci.* (2021) 22:873. doi: 10.3390/ijms22020873

10. Casu C, Aghajan M, Oikonomidou PR, Guo S, Monia BP, Rivella S. Combination of Tmprss6- ASO and the iron chelator deferiprone improves erythropoiesis and reduces iron overload in a mouse model of β-thalassemia intermedia. *Haematologica.* (2016) 101:e8–11. doi: 10.3324/haematol.2015.133348

11. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A. A Phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med.* (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

12. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med.* (2017) 376:429–39. doi: 10.1056/NEJMoa1611770

13. van Dijk MJ, Rab MAE, van Oirschot BA, Bos J, Derichs C, Rijneveld AW, et al. Safety and efficacy of mitapivat, an oral pyruvate kinase activator, in sickle

cell disease: a phase 2, open-label study. *Am J Hematol.* (2022) 97:E226–9. doi: 10.1002/ajh.26554

14. Niihara Y, Miller ST, Kanter J, Lanzkron S, Smith WR, Hsu LL, et al. A phase 3 Trial of l-glutamine in sickle cell disease. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med.* (2018) 379:226–35. doi: 10.1056/NEJMoa1715971

15. Kanter J, Walters MC, Krishnamurti L, Mapara MY, Kwiatkowski JL, Rifkin-Zenenberg S, et al. Biologic and clinical efficacy of lentiglobin for sickle cell disease. *N Engl J Med.* (2022) 386:617–28. doi: 10.1056/NEJMoa2117175

16. Goyal S, Tisdale J, Schmidt M, Kanter J, Jaroscak J, Whitney D, et al. Acute myeloid leukemia case after gene therapy for sickle cell disease. *N Engl J Med.* (2022) 386:138–47. doi: 10.1056/NEJMoa2109167

17. Frangoul H, Altshuler D, Cappellini MD, Chen YS, Domm J, Eustace BK. CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. *N Engl J Med.* (2021) 384:252–60. doi: 10.1056/NEJMoa2031054

18. Locatelli F, Frangoul H, Corbacioglu S, de la Fuente J, Wall D, Capellini MD, et al. Efficacy and safety of a single dose of CTX001 for transfusion dependent beta-thalassemia and severe sickle cell diseare. *Paper Presented at the Annual Meeting of the European Hematology Association*. Hague: The European Hematology Association (2022).

19. Fattizzo B, Giannotta JA, Cecchi N, Barcellini W. Confounding factors in the diagnosis and clinical course of rare congenital hemolytic anemias. *Orphanet J Rare Dis.* (2021) 16:415. doi: 10.1186/s13023-021-02036-4

20. Fattizzo B, Cavallaro F, Marcello A, Vercellati C, Barcellini W. Pyruvate kinase deficiency: current challenges and future prospects. *J Blood Med.* (2022) 13:461–71. doi: 10.2147/JBM.S353907

21. Grace RF, Rose C, Layton DM, Galacteros F, Barcellini W, Morton DH, et al. Safety and efficacy of mitapivat in pyruvate kinase deficiency. *N Engl J Med.* (2019) 381:933–44. doi: 10.1056/NEJMoa1902678

22. Al-Samkari H, Galacteros F, Glenthoj A, Rothman JA, Andres O, Grace RF, et al. Mitapivat versus placebo for pyruvate kinase deficiency. *N Engl J Med.* (2022) 386:1432–42. doi: 10.1056/NEJMoa2116634

23. Glenthoj A, van Beers EJ, Al-Samkari H, Viprakasit V, Kuo KHM, Galacteros F, et al. Mitapivat in adult patients with pyruvate kinase deficiency receiving regular transfusions (ACTIVATE-T): a multicentre, open-label, single-arm, phase 3 trial. *Lancet Haematol.* (2022) 9:e724–32. doi: 10.1016/S2352-3026(22)00214-9

24. Grace RF, Glenthøj A, Barcellini W, Verhovsek M, Rothman JA, Morado Arias M, et al. P1545: durability of hemoglobin response and reduction in transfusion burden is maintained over time in patients with pyruvate kinase deficiency treated with mitapivat in a long-term extension study. *Hemasphere.* (2022) 6:1426–7. doi: 10.1097/01.HS9.0000849036.11558.e5

25. Al-Samkari H, Morales-Arias J, Xu R, Beynon V, Grace RF. P1543: mitapivat decreases the need for transfusions secondary to poorly tolerated anemia and acute events compared to placebo in patients with pyruvate kinase deficiency who are not regularly transfused. *Hemasphere.* (2022) 6:1424–5. doi: 10.1097/01.HS9.0000849028.24492.c6

26. Barcellini W, Grace RF, Al-Samkari H, Glenthøj A, Rothman JA, Arias M, et al. P1548: long-term treatment with oral mitapivat is associated with normalization of hemoglobin levels in patients with pyruvate kinase deficiency. *Hemasphere.* (2022) 6:1429–30. doi: 10.1097/01.HS9.0000849048.44424.2a

27. van Beers EJ, Al-Samkari H, Grace RF, Barcellini W, Glenthøj A, Judge MP, et al. P1565: mitapivat improves ineffective erythropoiesis and reduces iron overload in patients with pyruvate kinase deficiency. *Hemasphere.* (2022) 6:1446–7. doi: 10.1097/01.HS9.0000849116.94935.15

28. Al-Samkari H, Grace RF, Glenthøj A, Andres O, Barcellini W, Galactéros F, et al. P1544: bone mineral density remains stable in pyruvate kinase deficiency patients receiving long-term treatment with mitapivat. *Hemasphere.* (2022) 6:1425–6. doi: 10.1097/01.HS9.0000849032.75798.50

29. Grace RF, Tyler PD, Little M, Kosinski PA, Beynon V. P1546: activate-kidst: mitapivat in children with pyruvate kinase deficiency who are regularly transfused. *Hemasphere.* (2022) 6:1427–8. doi: 10.1097/01.HS9.0000849040.78582.c7

30. Grace RF, Tyler PD, Little M, Kosinski PA, Beynon V. P1547: activate-kids: mitapivat in children with pyruvate kinase deficiency who are not regularly transfused. *Hemasphere.* (2022) 6:1428–9. doi: 10.1097/01.HS9.0000849044.32404.85

31. Matte A, Recchiuti A, Federti E, Kosinski PA, Riccardi V, Iatcenko I, et al. S273: evidence of noninferiority of mitapivat versus splenectomy in murine hereditary spherocytosis. *Hemasphere.* (2022) 6:174–5. doi: 10.1097/01.HS9.0000843984.82229.aa

32. Fattizzo B, Barcellini W. Autoimmune hemolytic anemia: causes and consequences. *Expert Rev Clin Immunol.* (2022) 18:731–45. doi: 10.1080/1744666X.2022.2089115

33. Berentsen S, Barcellini W. Autoimmune hemolytic anemias. *N Engl J Med.* (2021) 385:1407–19. doi: 10.1056/NEJMra2033982

34. Barcellini W, Zaninoni A, Giannotta JA, Fattizzo B. New insights in autoimmune hemolytic anemia: from pathogenesis to therapy stage 1. *J Clin Med.* (2020) 9:3859. doi: 10.3390/jcm9123859

35. Fattizzo B, Barcellini W. New therapies for the treatment of warm autoimmune hemolytic anemia. *Transfus Med Rev.* (2022) 36:175–80. doi: 10.1016/j.tmrv.2022.08.001

36. Barcellini W, Fattizzo B. The changing landscape of autoimmune hemolytic anemia. *Front Immunol.* (2020) 11:946. doi: 10.3389/fimmu.2020.00946

37. Barcellini W, Murakhovskaya I, Terriou L, Pane F, Patriarca A, Butler K, et al. S286: long-term efficacy and safety results from an ongoing open-label phase 2 study of parsaclisib for the treatment of autoimmune hemolytic anemia (AIHA). *Hemasphere.* (2022) 6:187–8. doi: 10.1097/01.HS9.0000844036.02998.15

38. Pasquale R, Giannotta JA, Barcellini W, Fattizzo B. Bortezomib in autoimmune hemolytic anemia and beyond. *Ther Adv Hematol.* (2021) 12:20406207211046428. doi: 10.1177/20406207211046428

39. Zaninoni A, Giannotta JA, Galli A, Artuso R, Bianchi P, Malcovati L, et al. The immunomodulatory effect and clinical efficacy of daratumumab in a patient with cold agglutinin disease. *Front Immunol.* (2021) 12:649441. doi: 10.3389/fimmu.2021.649441

40. Rossi G, Gramegna D, Paoloni F, Fattizzo B, Binda F, D'Adda M, et al. Short course of bortezomib in anemic patients with relapsed cold agglutinin disease: a phase 2 prospective gimema study. *Blood.* (2018) 132:547–50. doi: 10.1182/blood-2018-03-835413

41. Roth A, Barcellini W, D'Sa S, Miyakawa Y, Broome CM, Michel M, et al. Sutimlimab in cold agglutinin disease. *N Engl J Med.* (2021) 384:1323–34. doi: 10.1056/NEJMoa2027760

42. Roth A, Berentsen S, Barcellini W, D'Sa S, Jilma B, Michel M. Sutimlimab in patients with cold agglutinin disease: results of the randomized placebo-controlled phase 3 cadenza trial. *Blood.* (2022) 140:980–91. doi: 10.1182/blood.2021014955

43. Roth A, Barcellini W, D'Sa S, Miyakawa Y, Broome CM, Michel M, et al. Complement C1s inhibition with sutimlimab results in durable response in cold agglutinin disease: cardinal study 1-year interim follow-up results. *Haematologica.* (2022) 107:1698–702. doi: 10.3324/haematol.2021.279812

44. Röth A, Barcellini W, D'Sa S, Miyakawa Y, Broome CM, Michel M, et al. P1538: inhibition of complement c1s with sutimlimab in patients with cold agglutinin disease (cad): results following 9-week washout period in phase 3 cardinal study (NCT03347396). *Hemasphere.* (2022) 6:1419–20. doi: 10.1097/01.HS9.0000849008.10618.c0

45. Young NS. Aplastic anemia. *N Engl J Med.* (2018) 379:1643–56. doi: 10.1056/NEJMra1413485

46. Brodsky RA. How i treat paroxysmal nocturnal hemoglobinuria. *Blood.* (2021) 137:1304–9. doi: 10.1182/blood.2019003812

47. Fattizzo B, Ireland R, Dunlop A, Yallop D, Kassam S, Large J, et al. Clinical and prognostic significance of small paroxysmal nocturnal hemoglobinuria clones in myelodysplastic syndrome and aplastic anemia. *Leukemia.* (2021) 35:3223–31. doi: 10.1038/s41375-021-01190-9

48. Fattizzo B, Kulasekararaj AG. Second-generation C5 inhibitors for paroxysmal nocturnal hemoglobinuria. *BioDrugs.* (2020) 34:149–58. doi: 10.1007/s40259-019-00401-1

49. Risitano AM, Marotta S, Ricci P, Marano L, Frieri C, Cacace F, et al. Anti-complement treatment for paroxysmal nocturnal hemoglobinuria: time for proximal complement inhibition? A position paper from the SAAWP of the EBMT. *Front Immunol.* (2019) 10:1157. doi: 10.3389/fimmu.2019.01157

50. Lee JW, Sicre F, de Fontbrune L, Wong L, Lee V, Pessoa S, et al. Ravulizumab (ALXN1210) vs eculizumab in adult patients with PNH naive to complement inhibitors: the 301 study. *Blood.* (2019) 133:530–9. doi: 10.1182/blood-2018-09-876136

51. Kulasekararaj AG, Hill A, Rottinghaus ST, Langemeijer S, Wells R, Gonzalez-Fernandez FA, et al. Ravulizumab (ALXN1210) vs eculizumab in C5-inhibitor-experienced adult patients with PNH: the 302 study. *Blood.* (2019) 133:540–9. doi: 10.1182/blood-2018-09-876805

52. Roth A, Nishimura JI, Nagy Z, Gaal-Weisinger J, Panse J, Yoon SS, et al. The complement C5 inhibitor crovalimab in paroxysmal nocturnal hemoglobinuria. *Blood.* (2020) 135:912–20. doi: 10.1182/blood.2019003399

53. Röth A, Egyed M, Ichikawa S, Ito Y, Kim JS, Nagy Z, et al. P830: crovalimab maintains clinical benefit over long-term treatment in patients with paroxysmal nocturnal hemoglobinuria: results from the composer trial open-label extension. *Hemasphere.* (2022) 6:724–5. doi: 10.1097/01.HS9.0000846204.59341.a5

54. Hillmen P, Szer J, Weitz I, Roth A, Hochsmann B, Panse J, et al. Pegcetacoplan versus eculizumab in paroxysmal nocturnal hemoglobinuria. *N Engl J Med.* (2021) 384:1028–37. doi: 10.1056/NEJMoa2029073

55. Wong RSM. Safety and efficacy of pegcetacoplan in paroxysmal nocturnal hemoglobinuria. *Ther Adv Hematol.* (2022) 13:20406207221114673. doi: 10.1177/20406207221114673

56. Shah S, Chamlagain R, Musalman ZH, Raj Adhikari Y, Chhetri S, Paudel S, et al. Pegcetacoplan in paroxysmal nocturnal hemoglobinuria: a systematic review on efficacy and safety. *Res Pract Thromb Haemost.* (2022) 6:e12781. doi: 10.1002/rth2.12781

57. Wong R, Al-Adhami M, Savage J, Horneff R, Yeh M, Dumagay T, et al. P838: pegcetacoplan rapidly stabilizes complement inhibitor naïve patients with paroxysmal nocturnal hemoglobinuria experiencing hemolysis with acute hemoglobin decreases; prince trial post hoc analysis. *Hemasphere.* (2022) 6:732–3. doi: 10.1097/01.HS9.0000846236.11666.3d

58. Weitz I, Al-Adhami M, Min J, Persson E, Yeh M, Savage J, et al. P839: patients with paroxysmal nocturnal hemoglobinuria treated with pegcetacoplan show improvements in d-dimer normalization and decrease in incidence of thrombosis. *Hemasphere.* (2022) 6:733–4. doi: 10.1097/01.HS9.0000846240.50953.6f

59. Risitano AM, Roth A, Soret J, Frieri C, de Fontbrune FS, Marano L, et al. Addition of iptacopan, an oral factor B inhibitor, to eculizumab in patients with paroxysmal nocturnal haemoglobinuria and active haemolysis: an open-label, single-arm, phase 2, proof-of-concept trial. *Lancet Haematol.* (2021) 8:e344–54. doi: 10.1016/S2352-3026(21)00028-4

60. Kulasekararaj AG, Risitano AM, Maciejewski JP, Notaro R, Browett P, Lee JW, et al. Phase 2 study of danicopan in patients with paroxysmal nocturnal hemoglobinuria with an inadequate response to eculizumab. *Blood.* (2021) 138:1928–38. doi: 10.1182/blood.2021011388

61. Olnes MJ, Scheinberg P, Calvo KR, Desmond R, Tang Y, Dumitriu B, et al. Eltrombopag and improved hematopoiesis in refractory aplastic anemia. *N Engl J Med.* (2012) 367:11–9. doi: 10.1056/NEJMoa1200931

62. Fattizzo B, Levati G, Cassin R, Barcellini W. Eltrombopag in immune thrombocytopenia, aplastic anemia, and myelodysplastic syndrome: from megakaryopoiesis to immunomodulation. *Drugs.* (2019) 79:1305–19. doi: 10.1007/s40265-019-01159-0

63. Townsley DM, Scheinberg P, Winkler T, Desmond R, Dumitriu B, Rios O, et al. Eltrombopag added to standard immunosuppression for aplastic anemia. *N Engl J Med.* (2017) 376:1540–50. doi: 10.1056/NEJMoa1613878

64. Peffault de Latour R, Kulasekararaj A, Iacobelli S, Terwel SR, Cook R, Griffin M, et al. Eltrombopag added to immunosuppression in severe aplastic anemia. *N Engl J Med.* (2022) 386:11–23. doi: 10.1056/NEJMoa2109965

65. Jang JH, Tomiyama Y, Miyazaki K, Nagafuji K, Usuki K, Uoshima N, et al. Efficacy and safety of romiplostim in refractory aplastic anaemia: a phase II/III, multicentre, open-label study. *Br J Haematol.* (2021) 192:190–9. doi: 10.1111/bjh.17190

66. Lee JW, Lee SE, Jung CW, Park S, Keta H, Park SK, et al. Romiplostim in patients with refractory aplastic anaemia previously treated with immunosuppressive therapy: a dose-finding and long-term treatment phase 2 trial. *Lancet Haematol.* (2019) 6:e562–72. doi: 10.1016/S2352-3026(19)30153-X

67. Yamazaki H, Lee JW, Jang JH, Sawa M, Kizaki M, Tomiyama Y, et al. P827: efficacy and safety of romiplostim added to immunosuppressive therapy as a first-line treatment in patients with aplastic anemia: a phase 2/3 clinical trial. *Hemasphere.* (2022) 6:721–2. doi: 10.1097/01.HS9.0000846192.06541.42

68. Gavriilaki E, de Latour RP, Risitano AM. Advancing therapeutic complement inhibition in hematologic diseases: PNH and beyond. *Blood.* (2022) 139:3571–82. doi: 10.1182/blood.2021012860

frontiers | Frontiers in Medicine

TYPE Case Report
PUBLISHED 13 January 2023
DOI 10.3389/fmed.2022.1044043

OPEN ACCESS

EDITED BY
Eleni Gavriilaki,
G. Papanikolaou General Hospital, Greece

REVIEWED BY
Niloofar Deravi,
Shahid Beheshti University of Medical Sciences, Iran
Christos Demosthenous,
G. Papanikolaou General Hospital, Greece

*CORRESPONDENCE
Feng Chen
✉ cfch2004@sina.com

†These authors have contributed equally to this work and share first authorship

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 14 September 2022
ACCEPTED 13 December 2022
PUBLISHED 13 January 2023

CITATION
Liang Q, Liang X, Hong D, Fang Y, Tang L, Mu J, Tan X and Chen F (2023) Case report: Application of metagenomic next-generation sequencing in the diagnosis of visceral leishmaniasis and its treatment evaluation. *Front. Med.* 9:1044043. doi: 10.3389/fmed.2022.1044043

Case report: Application of metagenomic next-generation sequencing in the diagnosis of visceral leishmaniasis and its treatment evaluation

Qiuping Liang[1†], Xiaogong Liang[2†], Dengwei Hong[3], Yuan Fang[3], Lanlan Tang[1], Jiao Mu[1], Xiaoli Tan[3] and Feng Chen[1*]

[1]Department of Respiratory and Critical Care Medicine, The Third People's Hospital of Chengdu, Chengdu, China, [2]Department of Hematopathology, Mianyang Central Hospital, Mianyang, China, [3]Genoxor Medical Science and Technology Inc., Shanghai, China

Visceral leishmaniasis is a vector-borne infection by the *Leishmania* spp., a parasite. Although the overall incidence of visceral leishmaniasis is low, the disease still occurs frequently in some high-risk areas. In our study, two patients were admitted to the hospital with an unprovoked and recurrent high fever, and the condition was not improved after antibiotics administration. Meanwhile, bone marrow aspiration smears failed to find out any pathogen. Finally, *Leishmania*-specific nucleic acid sequences were successfully detected in the peripheral blood of two patients through metagenomic next-generation sequencing (mNGS), which was further confirmed by bone marrow smear microscopy and antibody tests. After targeted treatment for visceral leishmaniasis in the patients, mNGS reported a decrease in the reads number of *Leishmania* sequence. The results indicate the feasibility of mNGS in detecting *Leishmania* spp. in peripheral blood samples. Its therapeutic effect evaluation may be achieved through a comparative analysis of the number of reads before and after the treatment.

KEYWORDS

visceral leishmaniasis, mNGS, *Leishmania*, treatment efficacy, case

Introduction

Leishmaniasis is a vector-borne infection caused by protozoan parasites of the genus *Leishmania*, and is transmitted through the bite of female *Phlebotomus Sandflies* (1–3). There are several forms of leishmaniasis in China, wherein visceral leishmaniasis cases predominate, whose main causative agents are *Leishmania donovani* (*L. donovani*) and *Leishmania infantum* (4). According to the 2011 Chinese official record, the annual incidence of visceral leishmaniasis is very low, i.e., only 0.03/100,000, with an overwhelming majority of cases reported from sites of endemicity in the western and northwestern regions of China (5). Due to the low incidence, visceral leishmaniasis is among the most neglected infectious diseases. However, it is also the most severe form, with a poor prognosis and a high fatality rate. So far, early diagnosis and treatment are essential in controlling visceral leishmaniasis.

Due to the low incidence of visceral leishmaniasis and atypical symptoms in some patients, its diagnostic procedure is usually not straightforward. It is generally made by combining clinical signs with parasitological and serological tests. Chronic fever, hepatosplenomegaly, and pancytopenia are the primary classical manifestations of visceral leishmaniasis (6), varying from the host's immune status, the parasite, and immunoinflammatory responses (7). Microscopic examination of aspirate smears in bone marrow, lymph nodes, and spleen is the most reliable diagnostic method for visceral leishmaniasis. In general, splenic aspirate shows the highest diagnostic value (with specificity and sensitivity of more than 90%), followed by bone marrow (with sensitivity in the range of 53 and 86%) and lymph nodes (with sensitivity ranging from 53 to 65%) (8). The low sensitivity of bone marrow cytomorphology limits its application in accurately identifying or excluding suspicious patients. The serological examination based on the rK39 antigen is the most rapid and widely used in China. However, specific antibody testing may not be arranged timely for patients with atypical symptoms due to the low incidence of visceral leishmaniasis.

Recently, next-generation sequencing (NGS) technology has been applied to the etiological diagnosis of infectious diseases, known as metagenomic next-generation sequencing (mNGS) (9). Due to its unbiased and hypothesis-free characteristics, mNGS has emerged as a vital method in identifying complex microorganisms to culture and diagnosing infectious diseases with low incidence. As recently reported, mNGS allows for *Leishmania* detection and early diagnosis of visceral leishmaniasis, improving the prognosis of the patients (10). In this case report, two patients with fever of unknown origin were enrolled, with some abnormal items in their physical examinations, including blood biochemistry, liver function, and blood routine. However, bone marrow aspiration smears failed to find any pathogen until *mNGS identified Leishmania* in the peripheral blood samples of these two patients. Then, *Leishmania* was found in the bone marrow aspiration smears and further confirmed with the antibody tests. Visceral leishmaniasis was diagnosed, and antimonials were used as the conventional therapy. After the symptoms disappeared, mNGS was performed again to assess the treatment effect by comparing the number of reads before and after the treatment. The detailed histories of both patients were as follows.

Case description

Case 1:

On January 9, 2021, a 52-year-old female patient who lives in Jiuzhaigou in the Sichuan province of China was admitted to another hospital because of an unprovoked high fever (Table 1). The body temperature reached 39.6°C, accompanied by coughing, sputum expectoration, tiredness, anhelation, chilly, and shivering. A mass of rash was seen on the face, chest, and groin. She was checked by ultrasound in urinary system, brain magnetic resonance imaging (MRI) scan, and chest computed tomography (CT), but no significant abnormality was seen. Based on the above symptoms, antibiotics ceftriaxone sodium and clindamycin were used for anti-infection, and methylprednisolone was administered as an anti-asthmatic drug.

On January 11, 2021, the concentration of folic acid (FOL), vitamin B12 (VB12), and serum ferritin (SF) was determined, with the level of SF on the high side. On January 13, 2021, the bone marrow aspiration smear showed a low proliferation rate of nucleated cells, a downside of granulocyte/erythrocyte ratio, and some erythroblasts presented with abnormal morphology. In addition, a significant reduction of iron content in the bone marrow was seen, and the platelet production by megakaryocytes was poor. Scattered and clustered platelets could be seen in the bone marrow. After administering drugs for a week, she recovered from symptoms like coughing, sputum expectoration, tiredness, and shortness of breath. However, the fever was not on the mend. On January 14, 2021, the results of the blood routine indicated decreased levels of white blood cells (WBC), red blood cells (RBC), hemoglobin (HGB), and platelet (PLT). The timeline of diagnosis and treatment of case 1 is demonstrated in Table 2.

On January 15, 2021, she was admitted to our hospital (The Third People's Hospital of Chengdu) and confirmed a history of hypertension, diabetes, and anemia, together with thalassemia in her daughter. Based on the medical history and physical examination results, the patient was tentatively diagnosed with "a fever pending for diagnosis, with moderate anemia, chronic nephritis, hypertension of grade 2, and type 2 diabetes." Chest CT showed splenomegaly, and the abdominal B-mode ultrasound revealed hepatosplenomegaly. After admission, the patient was administered piperacillin sodium/tazobactam sodium and moxifloxacin for anti-infection, oseltamivir for anti-influenza, folic acid tablets, and vitamin B12 as the hematopoietic raw materials, and clexane for preventive anticoagulant.

On January 19, 2021, the bone marrow aspiration smear showed active bone marrow hyperplasia and some dysplastic changes (>10%) in erythroid cells. The patient's fever had not been remitted. On January 20 and 21, no significant abnormality was seen in the bone marrow chromosome karyotyping and gene mutation analysis in myeloid blood diseases.

On January 24, 2021, peripheral blood mNGS was performed, and 11,552 reads of *Leishmania* genus-specific sequence were detected in microbial cell-free deoxyribonucleic acid (mcfDNA), and 7,509 reads were detected in microbial cell-free ribonucleic acid (mcfRNA) (Table 3), with a genome

TABLE 1 Demographics and clinical presentations of the 2 cases.

Items	Case 1	Case 2
Gender	Female	Male
Age, y	52	55
Past medical history	Anemia, history of blood transfusion	Pancreatitis, septicopyemia
Occupation	Unemployed	Farmer
Family history	Thalassemia	None
History of the epidemic area	Sichuan	Sichuan, Xinjiang
Chief complaints	Recurrent fever for 7 d, up to 39.6°C	Recurrent fever for 1 m, up to 41°C
Accompanying symptoms	Cough, expectoration, fatigue, anhelation, chilly, shiver	Shiver
Clinical signs	Hepatosplenomegaly, pancytopenia	Hepatosplenomegaly

coverage of 2.7% (Figure 1). The sequencing data were deposited in the database under the Sequence Read Archive (SRA) accession number PRJNA850821. On January 26, the RK39 antibody test showed a positive result. After repeated examinations on the bone marrow aspiration smears, *Leishmania* was found (Supplementary Figure 1). Finally, the patient was diagnosed with visceral leishmaniasis, sepsis, moderate anemia, rash, leukopenia, thrombocytopenia, grade 2 hypertension, and type 2 diabetes mellitus.

On January 26, 2021, a sodium stibogluconate regimen (6 ml/vial, course of treatment for 14 days, D1 3 ml antimonials & 7 ml glucose and sodium chloride injection iv, D2–D14 6 ml antimonials & 4 ml glucose and sodium chloride injection iv) was administrated, and her fever was remitted on that day. On March 2, 2021, the decreased WBC had been resolved, but splenomegaly remained. On March 7, the peripheral blood was collected again for mNGS detection, and 73 reads of the *leishmania*-specific genus sequence were detected in mcfDNA, but none was seen in mcfRNA (Table 3). Meanwhile, the rash on the face, chest, and groin disappeared utterly.

Case 2:

On January 25, 2021, a 55-year-old man was admitted to our hospital (Mianyang Central Hospital) with recurrent fever, a body temperature of 39.6°C, and acute febrile symptoms. One month before admission, the patient developed a fever of unknown origin, with the highest body temperature of 41°C, accompanied by a shiver. Before admission, he had been treated with piperacillin sodium/tazobactam in the other hospital, but without any improvement. Upon entry, he told a history of pancreatitis and septicemia. He denied any family history. On January 25, 2021, the results of the blood routine revealed reduced levels of WBC, RBC, HGB, and PLT. Hepatosplenomegaly was observed by using the abdominal color ultrasound. Increased amylase and lipase levels were noticed, which are the markers of pancreatitis. A positive result was noted in the Epstein-Barr virus (EBV), but no other blood or respiratory tract pathogen was detected. The timeline of diagnosis and treatment of case 2 is shown in Table 4.

In the following 14 days after the admission (from January 25, 2021, to February 6, 2021), blood routine examinations were performed, and the low levels of WBC, RBC, HGB, and PLT were all continued. On February 7, 2021, active bone marrow hyperplasia and hemophagocytosis were seen in bone marrow aspiration smears, but no pathogenic microorganism was found. On February 9, 2021, peripheral blood mNGS was performed, and 606 reads of *Leishmania* genus-specific sequence and 15 reads of *L. donovani*-specific sequence were detected in mcfDNA, accounting for 0.2% of genome coverage (Figure 2). The sequencing data were deposited in the database under the SRA accession number PRJNA850821.

On February 10, 2021, the bone marrow aspiration smear was re-examined, and ten suspected *Leishmania* were seen (Supplementary Figure 2). On the same day, a positive outcome of the rK39 test confirmed *Leishmania*'s existence. The final diagnosis was visceral leishmaniasis, hemophagocytic syndrome, sepsis, liver dysfunction, electrolyte imbalance, hypoproteinemia, coagulopathy, moderate anemia, thrombocytopenia, and leukopenia. Anti-leishmaniasis treatment with sodium stibogluconate (0.6 g, im, qd, course of treatment for 3 weeks) was adopted, and the fever was entirely resolved. On February 25, no *Leishmania* was found in the bone marrow aspiration smear. On February 25, 2021, the results of the analysis on gene mutation in hemophagocytic syndrome manifested no disease-causing mutation, suggesting that this disease is not present in genetic forms in the patient. On March 12, 2021, the WBC count returned to normal, and the levels of HGB and PLT were primarily increased. On March 14, 2021, peripheral blood was collected again for mNGS detection, and no *Leishmania* genus-specific or

TABLE 2 Timeline of diagnosis and treatment in the case 1.

Timeline	Physical examination items	Medical results	Diagnosis and treatment
2021-1-9	Urinary ultrasound Brain MRI scan Chest CT	Urate crystal in bilateral renal Slight ischemic lacuna in the brain No abnormality seen	Treatment: Ceftriaxone sodium, clindamycin, methylprednisolone
2021-1-11	Anemia detection	FOL 16.62 ng/ml VB12 343.7 ng/ml SF 614.8 ng/ml	
2021-1-13	Bone marrow aspiration smear 24 h urine protein quantitation Blood clotting assays	Low proliferation rate of nucleated cells 563.8 mg/24 h Fibrinogen 4.59 g/L D-dimer 3.86 mg/L	
2021-1-14	Blood routine	WBC 3.3 × 10^9/L RBC 2.88 × 10^{12}/L HGB 63 g/L PLT 50 × 10^9/L	
2021-1-15	Urine routine Blood routine CRP PCT IL-6 Blood pathogens detection Respiratory tract pathogens detection Coombs test Polyspecific antibody Chest CT scan Abdominal B-mode ultrasound	Occult blood 10 (±) RBC/ul WBC 3.43 × 10^9/L HGB 66 g/L PLT 61 × 10^9/L ESR 99 mm/H 106.96 mg/L 0.41 ng/ml 164.3 pg/ml Negative Weakly positive Parainfluenza Virus Types 1-2-3 IgM antibody Positive Positive Splenomegaly Hepatosplenomegaly	Tentative diagnosis: Fever, with a moderate anemia, chronic nephritis, hypertension of grade 2, and type 2 diabetes Treatment: Tazocin, moxifloxacin, oseltamivir, FOL Tablets, VB12, clexane
2021-1-19	Bone marrow aspiration smear	Active bone marrow hyperplasia, dysplastic changes in erythroid cells	
2021-1-20	Bone marrow chromosome karyotyping	No abnormality seen	
2021-1-21	Analysis of gene mutation in myeloid blood diseases	No pathogenic mutation found	
2021-1-24	Peripheral blood mNGS detection	*Leishmania* genus-specific sequence	
2021-1-26	rK39 test	Positive	Diagnose: Visceral leishmaniasis Treatment: Sodium stibogluconate regimen for 14 days
2021-3-2	Abdominal color ultrasound Blood routine	Splenomegaly WBC 4.22 × 10^9/L HGB 75g/L PLT 136 × 10^9/L	
2021-3-8	Peripheral blood mNGS detection	*Leishmania* genus in mcfDNA	

MRI, magnetic resonance imaging; CT, computed tomography; FOL, folic acid; VB12, vitamin B12; SF, serum ferritin; mNGS, metagenomic next-generation sequencing; WBC white blood cell; RBC, red blood cell; HGB, hemoglobin; PLT, platelet; CRP C-reactive protein; PCT, procalcitonin; IL-6, interleukin-6; ESR, erythrocyte sedimentation rate.

L. donovani-specific sequence was detected in the mcfDNA nor mcfRNA.

Discussion

Visceral leishmaniasis is an endemic disease, and a history of residence or stays in endemic areas is an essential criterion in its diagnosis (5). The statistical data surveyed from 2004 to 2019 and released by the Chinese Center for Disease Control and Prevention indicated that visceral leishmaniasis is mainly prevalent in five provinces in China, including Xinjiang (2,351 cases), Gansu (1,607 cases), Sichuan (594 cases), Shaanxi (139 cases), and Shanxi (142 cases). These cases account for 96.89% (4,833/4,988) of the nationally reported cases during the same period. In this study, case 1 is a native of Sichuan province and claimed no ecdemic travel history, and can be identified as a local case in Sichuan. For case 2, the source of infection is unclear because he lived in Sichuan province and had a history of Xinjiang residence before the

TABLE 3 Data and information of mNGS detection.

Cases	Date	Test items	Total reads number	Non-human reads number	Leishmania sequence reads		Relative abundance
					Genus	Species	
Case 1	2021-1-24	CfDNA	19,608,044	177,919	11,552	/	100%
	2021-1-24	CfRNA	31,691,424	433,348	7,509	/	100%
	2021-3-8	CfDNA	33,464,222	284,827	73	/	100%
	2021-3-8	CfRNA	24,055,354	206,251	0	/	100%
Case 2	2021-2-9	CfDNA	20,469,766	848,480	606	15	100%
	2021-3-7	CfDNA	55,851,536	563,416	8	8	4.54%
	2021-3-14	CfDNA	24,341,788	161,652	0	0	/
	2021-3-14	CfRNA	19,744,882	129,835	0	0	/

CfDNA, cell-free deoxyribonucleic acid; CfRNA, cell-free ribonucleic acid.

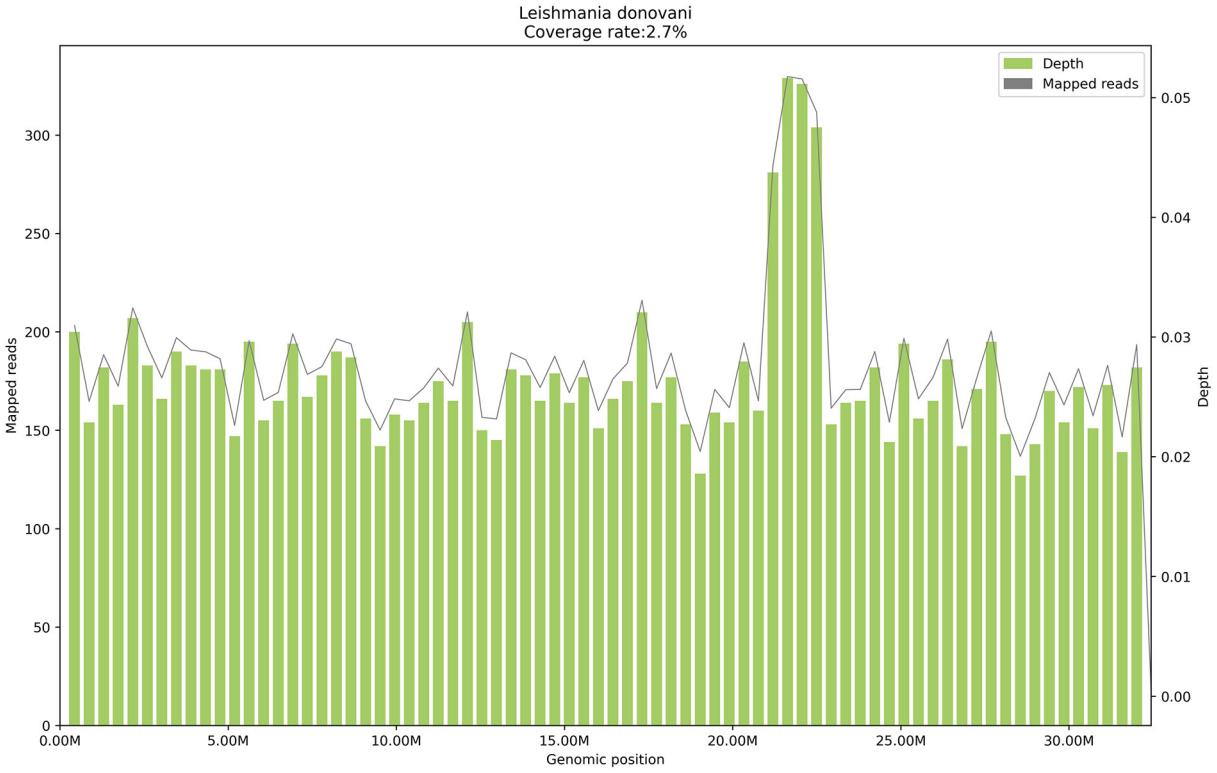

FIGURE 1
The genome coverage map of *Leishmania donovani* detected by mNGS in case 1. The genome coverage of *Leishmania donovani* in the peripheral blood of case 1 is 2.7%.

onset of symptoms, which are all endemic areas of visceral leishmaniasis in China (11). This two-case report highlights the necessity to strengthen the surveillance and control of this infectious disease in its epidemic areas, despite its low incidence in China.

Owing to the wide range of non-specific clinical symptoms, patients with leishmaniasis frequently could not be diagnosed using conventional methods. For example, in seven patients whose blood mNGS findings pointed to *Leishmania* infection and were finally diagnosed with leishmaniasis, only three individuals tested positive for rK39, and two had *Leishmania* amastigotes identified in their bone marrow (12). In the two cases of this study, bone marrow aspirate smears failed to find *Leishmania* before mNGS detection, because of short

TABLE 4 Timeline of diagnosis and treatment in the case 2.

Timeline	Physical examination items	Medical results	Diagnosis and treatment
2021-1-25	Blood routine	WBC 2.94 × 10^9/L RBC 2.68 × 10^{12}/L HGB 80 g/L PLT 209 × 10^9/L	Tentative diagnosis: Fever, suspected infection or hemophagocytic syndrome
	Abdominal color ultrasound	Hepatosplenomegaly
	ESR	24 mm/H
	PCT	0.499 μg/L
	Amylase	149 U/L
	Lipase	210 U/L
	Blood pathogens detection	Negative
	Respiratory tract pathogens	Negative
	EBV	Positive
	Tumor marker	CA125 36.92 U/mL CA19-9 35.67 U/mL NSE 13.02 μg/L
	Blood culture	Negative
2021-1-27	Blood routine	WBC 2.24 × 10^9/L RBC 2.49 × 10^{12}/L HGB 75 g/L
	PCT	PLT 112 × 10^9/L 4.61 μg/L
2021-1-30	Blood routine	WBC 3.03 × 10^9/L RBC 2.87 × 10^{12}/L HGB 87 g/L PLT 102 × 10^9/L
	PCT	1.05 μg/L
	Amylase	139 U/L
	Lipase	215 U/L
	FOL	4.4 ng/ml
	VB12	439 ng/ml
	SF	>40,000 ng/ml
2021-2-4	Blood routine	WBC 1.76 × 10^9/L RBC 2.6 × 10^{12}/L HGB 78 g/L PLT 84 × 10^9/L
	Amylase	189 U/L
	Lipase	182 U/L
2021-2-6	Blood routine	WBC 5.78 × 10^9/L RBC 2.5 × 10^{12}/L HGB 75 g/L PLT 78 × 10^9/L
	PCT	0.186 μg/L
	Amylase	169 U/L
	Lipase	222 U/L
	SF	>40,000 ng/ml
2021-2-7	Bone marrow aspiration smear	Active bone marrow hyperplasia, hemophagocytosis
2021-2-9	Peripheral blood mNGS detection	*Leishmania donovani*
2021-2-10	Bone marrow aspiration smear	Suspected *Leishmania*	Diagnosis: Visceral leishmaniasis, hemophagocytic syndrome Treatment: sodium stibogluconate for 3 weeks
	rK39 test	Positive
	Blood routine	WBC 1.8 × 10^9/L RBC 2.4 ×10^{12}/L HGB 71 g/L PLT 85 ×10^9/L
	PCT	0.184 μg/L
2021-2-25	Analysis of gene mutation in hemophagocytic syndrome	No pathogenic mutation found

(Continued)

TABLE 4 (Continued)

Timeline	Physical examination items	Medical results	Diagnosis and treatment
2021-3-5	Blood routine	WBC 3.65 × 10^9/L RBC 2.89 × 10^{12}/L HGB 95 g/L PLT 240 × 10^9/L	
2021-3-7	Peripheral blood mNGS detection	*Leishmania*	
2021-3-12	Blood routine SF	WBC 5.97 × 10^9/L RBC 3.2 × 10^{12}/L HGB 104 g/L PLT 342 ×10^9/L 2,756.38 ng/ml	
2021-3-14	Peripheral blood mNGS detection	None	

EBV, Epstein-Barr virus.

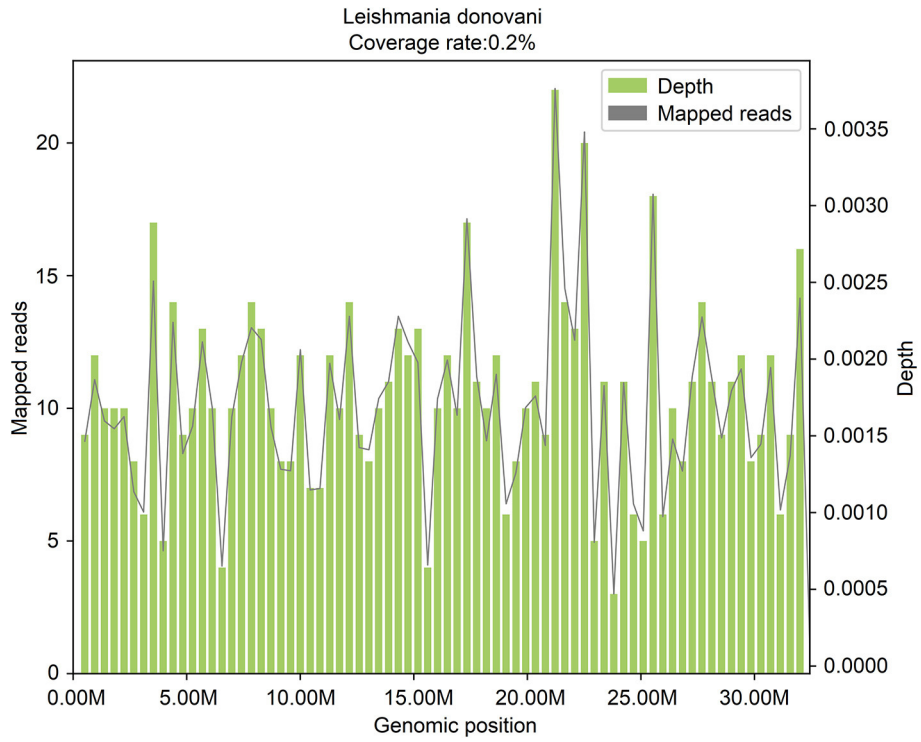

FIGURE 2
The genome coverage map of *Leishmania donovani* detected by mNGS in case 2. The genome coverage of *Leishmania donovani* in the peripheral blood of case 2 is 0.2%.

of knowledge on this parasite and experience of visceral leishmaniasis diagnosis, especially in the local hospital. Due to a history of anemia in case 1, together with the thalassemia in her daughter, she was suspected of hematological system diseases like leukemia. Infection was also considered because of the physical examination and relevant laboratory abnormalities. Before and after admission to our hospital, case 1 was administered different antibiotics for anti-infection, but the fever had not been remitted. In addition, no sufficient evidence was found to show a myeloid blood disease. Worse still, no pathogen was seen in the bone marrow aspiration smear. Until the *Leishmania* reported by mNGS detection, it was also found in the bone marrow aspiration smear after repeated and careful examinations.

Case 2 suffered from underdiagnosis severely, who had been hospitalized in different hospitals for nearly 1 month and spent massively. Before admitting to our hospital, he had been administered various antibiotics, such as piperacillin sodium/tazobactam, but without any improvement. He was suspected of infection or hemophagocytic syndrome after

admission to our hospital. But no blood or respiratory tract pathogenic microorganism was detected. Within 14 days, the bone marrow aspiration smear was performed three times but showed no abnormality. On day 16, peripheral blood mNGS was arranged, and *Leishmania* genus- and *L. donovani*-specific sequences were reported. According to the result of mNGS, we re-examined the bone marrow smear and found ten suspected *Leishmania*, which was confirmed by the positive outcome of the rK39 antibody test. Finally, he was diagnosed with visceral leishmaniasis and hemophagocytic syndrome, was treated timely, and discharged from the hospital with drugs, and the outcome was so satisfactory. Then, analysis of gene mutation in hemophagocytic syndrome found no disease-causing mutation. Therefore, hemophagocytic syndrome is more likely to be associated with the infection of *Leishmania*.

Furthermore, mNGS is available for *Leishmania* detection not only in bone marrow aspirate (13, 14), especially for quick and accurate diagnosis in patients with suspected leishmaniasis (14, 15), but also in the easy-to-obtain peripheral blood. Sequencing readings of *L. infantum* and *L. donovani* also have been identified by mNGS using peripheral blood (16). During the continuous reproduction process of *Leishmania* in the blood circulation system, the nucleic acid released into the peripheral blood forms mcfDNA and mcfRNA along with apoptosis occurring, which is the basis for the detection of *Leishmania* nucleic acid in the peripheral blood by mNGS (17). Even if the pathogen has been killed, its mcfDNA and mcfRNA may still exist in the peripheral blood for a while, and mcfRNA will be degraded faster because of its shorter half-life. Therefore, mcfRNA is superior to mcfDNA in monitoring the number of reads to evaluate the treatment efficacy of sodium stibogluconate, which is commonly used to inhibit *L. donovani* in the treatment of visceral leishmaniasis (18). Our mNGS reports indicated a marked decrease in the reads number of the *Leishmania*-specific sequence in the two patients' peripheral blood after a period of sodium stibogluconate treatment.

In this study, we proved the feasibility of mNGS in detecting causative pathogens and diagnosing visceral leishmaniasis from peripheral blood samples, dramatically avoiding misdiagnosis or underdiagnosis. Changes in the number of reads of mcfDNA and mcfRNA sequences yielded in mNGS can be taken as evidence of effective anthelmintic therapy. Finally, the relatively high cost of mNGS is the main shortcoming that limits its sample size and hinders its wide use in clinical. Once this challenge is addressed, further studies with a more extensive sampling size are more likely to validate *Leishmania* detection's reliability *via* mNGS in blood samples.

Data availability statement

The datasets presented in this article are not readily available because of ethical/privacy restrictions. Requests to access the datasets should be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

QL and XL performed the study concept and design, manuscript review, and revision. FC and DH contributed to the data acquisition and analysis. YF and LT performed development and methodology and writing. XT and JM provided data acquisition, analysis, and interpretation. All authors contributed to the article and approved the submitted version.

Funding

This study was approved by the grants from the Chengdu Medical Research Project (2021142).

Acknowledgments

We thank the efforts and contributions of the reported patients and all the clinical staff in this study.

Conflict of interest

Authors DH, YF, and XT were employed by Genoxor Medical Science and Technology Inc.

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

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.1044043/full#supplementary-material

SUPPLEMENTARY FIGURE 1
A macrophage infected with amastigotes in leishmaniasis case 1. Bone marrow aspiration shows the *Leishmania* spp amastigotes (arrow) in case 1. Original magnification ×50.

SUPPLEMENTARY FIGURE 2
A macrophage infected with amastigotes in leishmaniasis case 2. Bone marrow aspiration shows the *Leishmania* spp amastigotes (arrow) in case 2. Original magnification ×40.

References

1. Natera S, Machuca C, Padron-Nieves M, Romero A, Diaz E, Ponte-Sucre A. *Leishmania* Spp.: proficiency of drug-resistant parasites. *Int J Antimicrob Agents.* (2007) 29:637–42. doi: 10.1016/j.ijantimicag.2007.01.004

2. Kaye P, Scott P. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol.* (2011) 9:604–15. doi: 10.1038/nrmicro2608

3. Bates PA. Transmission of leishmania metacyclic promastigotes by phlebotomine sand flies. *Int J Parasitol.* (2007) 37:1097–106. doi: 10.1016/j.ijpara.2007.04.003

4. Diseases EBCJI. Expert consensus on diagnosis and treatment of leishmania infection in China. *Chin J Infect Dis.* (2017) 35:6. doi: 10.3760/cma.j.issn.1000-6680.2017.10.001

5. Lun ZR, Wu MS, Chen YF, Wang JY, Zhou XN, Liao LF, et al. Visceral Leishmaniasis in China: an endemic disease under control. *Clin Microbiol Rev.* (2015) 28:987–1004. doi: 10.1128/CMR.00080-14

6. van Griensven J, Diro E. Visceral leishmaniasis. *Infect Dis Clin North Am.* (2012) 26:309–22. doi: 10.1016/j.idc.2012.03.005

7. Elmahallawy EK, Sampedro Martinez A, Rodriguez-Granger J, Hoyos-Mallecot Y, Agil A, Navarro Mari JM, et al. Diagnosis of leishmaniasis. *J Infect Dev Ctries.* (2014) 8:961–72. doi: 10.3855/jidc.4310

8. Mondal S, Bhattacharya P, Ali N. Current diagnosis and treatment of visceral leishmaniasis. *Expert Rev Anti Infect Ther.* (2010) 8:919–44. doi: 10.1586/eri.10.78

9. Chiu CY, Miller SA. Clinical metagenomics. *Nat Rev Genet.* (2019) 20:341–55. doi: 10.1038/s41576-019-0113-7

10. Gao H, Wang J, Zhang S, Li T. A case report of two kala-azar cases in China diagnosed by metagenomic next-generation sequencing. *Front Med.* (2022) 9:922894. doi: 10.3389/fmed.2022.922894

11. Zheng C, Wang L, Li Y, Zhou XN. Visceral leishmaniasis in northwest China from 2004 to 2018: a spatio-temporal analysis. *Infect Dis Poverty.* (2020) 9:165. doi: 10.1186/s40249-020-00782-4

12. Han N, Yu J, Wang M, Ma Y, Yan L, Tang H. The value of metagenomic next-generation sequencing in leishmaniasis diagnosis: a case series and literature review. *Open Forum Infect Dis.* (2022) 9:ofac511. doi: 10.1093/ofid/ofac511

13. Williams E, Isles NS, Seemann T, Kilpatrick T, Grigg A, Leroi M, et al. Case report: confirmation by metagenomic sequencing of visceral leishmaniasis in an immunosuppressed returned traveler. *Am J Trop Med Hyg.* (2020) 103:1930–3. doi: 10.4269/ajtmh.19-0841

14. Zhang HC, Zhang QR, Ai JW, Cui P, Wu HL, Zhang WH, et al. The role of bone marrow metagenomics next-generation sequencing to differential diagnosis among visceral leishmaniasis, histoplasmosis, and talaromycosis marneffei. *Int J Lab Hematol.* (2020) 42:e52–e4. doi: 10.1111/ijlh.13103

15. Chen H, Fan C, Gao H, Yin Y, Wang X, Zhang Y, et al. Leishmaniasis diagnosis via metagenomic next-generation sequencing. *Front Cell Infect Microbiol.* (2020) 10:528884. doi: 10.3389/fcimb.2020.528884

16. Wang C, Li A, Shi Q, Yu Z. Metagenomic next-generation sequencing clinches diagnosis of leishmaniasis. *Lancet.* (2021) 397:1213. doi: 10.1016/S0140-6736(21)00352-4

17. Han D, Li R, Shi J, Tan P, Zhang R, Li J. Liquid biopsy for infectious diseases: a focus on microbial cell-free DNA sequencing. *Theranostics.* (2020) 10:5501–13. doi: 10.7150/thno.45554

18. Sinha R, Roychoudhury J, Palit P, Ali N. Cationic liposomal sodium stibogluconate (Ssg), a potent therapeutic tool for treatment of infection by ssg-sensitive and -resistant leishmania donovani. *Antimicrob Agents Chemother.* (2015) 59:344–55. doi: 10.1128/AAC.03305-14

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 28 February 2023
DOI 10.3389/fmed.2023.1137019

OPEN ACCESS

EDITED BY
Eleni Gavriilaki,
G. Papanikolaou General Hospital, Greece

REVIEWED BY
Camila Masias,
Baptist Health South Florida, United States
Giuseppe Lassandro,
University of Bari Aldo Moro, Italy

*CORRESPONDENCE
Shruti Chaturvedi
✉ schatur3@jhmi.edu

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 03 January 2023
ACCEPTED 03 February 2023
PUBLISHED 28 February 2023

CITATION
Selvakumar S, Liu A and Chaturvedi S (2023) Immune thrombotic thrombocytopenic purpura: Spotlight on long-term outcomes and survivorship.
Front. Med. 10:1137019.
doi: 10.3389/fmed.2023.1137019

Immune thrombotic thrombocytopenic purpura: Spotlight on long-term outcomes and survivorship

Sruthi Selvakumar[1], Angela Liu[2] and Shruti Chaturvedi[3]*

[1]Dr. Kiran C. Patel College of Allopathic Medicine, Nova Southeastern University, Fort Lauderdale, FL, United States, [2]Division of Hematology and Oncology, Mount Sinai School of Medicine, New York, NY, United States, [3]Division of Hematology, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, United States

Advances in diagnosis and treatment have dramatically improved survival of acute immune thrombotic thrombocytopenic purpura (iTTP) and iTTP has evolved from an acute fatal condition to a chronic relapsing disorder. In addition to the risk of iTTP relapse, iTTP survivors are at risk of multiple adverse health outcomes including higher than expected rates of all-cause mortality, increased rates of stroke and other cardiovascular disease, and higher rates of morbidities such as obesity, hypertension, and autoimmune disorders. iTTP survivors also report neurocognitive impairment, depression, and reduced quality of life. Women with iTTP are at risk for recurrent iTTP, preeclampsia, and other maternal and fetal complications in subsequent pregnancies. ADAMTS13 activity during clinical remission has emerged as an important targetable risk factor for iTTP relapse and other outcomes including stroke and all-cause mortality. This review summarizes current literature regarding the epidemiology and potential mechanisms for adverse long-term sequelae of iTTP, outlines current best practices in iTTP survivorship care, and highlights a research agenda to improve long-term iTTP outcomes.

KEYWORDS

thrombotic microangiopathy, thrombotic thrombocytopenic purpura, ADAMTS13, survivorship, rare disease

Introduction

Immune thrombotic thrombocytopenic purpura (iTTP) is a rare hematologic disorder characterized by episodes of microvascular thrombosis and ischemic organ damage (1). iTTP is caused by an autoantibody-mediated deficiency of ADAMTS13, a von Willebrand factor-cleaving protease that results in circulating high molecular weight multimers of von Willebrand factor that cause platelet aggregation and systemic microvascular thrombi (2). Untreated iTTP is almost universally fatal; however, treatment with plasma exchange (PEX) and immunosuppression has reduced mortality in acute iTTP from >90% to < 5–10% (3–5). Recent therapeutic advances, including Rituximab (6–10) and caplacizumab (11), have further improved outcomes of acute iTTP. Since most patients now survive acute iTTP, late complications and survivorship issues have emerged as an important clinical and research

focus with the potential to improve outcomes for the growing number of individuals living with this rare disorder (**Figure 1**) (12, 13). Until recently, iTTP was viewed primarily as an acute condition, and survivors were expected to return to their previous level of health except for a 30–50% risk of relapse (14). However, recent studies suggest that iTTP survivors experience a plethora of adverse long-term health outcomes following recovery (12). These range from higher than expected rates of all-cause mortality and increased rates of chronic morbidities such as hypertension (15), obesity (12), cardiovascular disease including stroke (16–18), renal injury (19), and autoimmune disease (20) as well as neurocognitive impairment (21, 22), depression (22, 23), and reduced quality of life (**Figure 2**) (24). Women with iTTP may also experience recurrent iTTP as well as other maternal and fetal complications in subsequent pregnancies (25, 26).

With this paradigm shift of approaching iTTP as a chronic disorder, the long-term sequelae of iTTP, and their underlying mechanisms and risk factors are an active area of investigation. This review summarizes current literature investigating long-term outcomes after recovery from acute iTTP and current best practices in iTTP survivorship care.

iTTP relapse

Relapse risk

Despite successful management of most acute episodes, iTTP remains a chronic relapsing disorder. Relapse rates ranging from 30% to over 50% have been reported, and each relapse comes with the risk of significant morbidity and mortality (27–31). While most relapses occur within the first 2 years after the initial episode, relapses have been reported even after a decade (29–31). Notably, the increasing use of rituximab with the first presentation of iTTP likely delays relapse, which may then occur later in the disease course (30, 32). Multiple risk factors for relapse have been identified, including younger age, male sex, presenting in iTTP relapse (versus a *de novo* episode), non-O blood group, and Black race (30–32). However, these are generally non-modifiable risk factors.

Reduced ADAMTS13 activity during clinical remission has been identified as currently the most reliable, and importantly targetable, risk factor for relapse (33, 34). Jin et al. analyzed 157 serial measures (in 24 patients) of ADAMTS13 activity and ADAMTS13 IgG antibodies and reported that while ADAMTS13 antibody levels were not significant predictors of relapse, lower ADAMTS13 activity ($p = 0.03$) and younger age ($p = 0.02$) were significant risk factors for TTP relapse and particularly sensitive and specific for relapse in the next 90 days (34). Peyvandi et al. reported significantly lower levels of ADAMTS13 activity (12% vs. 41%, $p = 0.007$) and antigen (36 vs. 58%, $p = 0.003$) among patients with recurrent TTP compared to patients with no recurrence, and severely low ADAMTS13 levels (<10%) were associated with a higher risk of recurrence (OR: 2.9, 95% CI: 1.3–6.8, $p = 0.01$). This study additionally reported that the presence of ADAMTS13 antibodies increased the likelihood of TTP recurrence (OR: 3.1, 95% CI: 1.4–7.3, $p = 0.006$) (33). A more recent multi-institutional study also demonstrated similar findings, identifying both ADAMTS13 activity $\leq$ 20% and high anti-ADAMTS-13 titers as independent risk factors for iTTP relapse (35). Recognizing the significance of reduced remission ADAMTS13 as a predictor of relapse, the International Working Group in iTTP recently updated its outcome definitions to include ADAMTS13 relapse, defined as ADAMTS13 activity < 20% during clinical remission (normal platelet count and no symptoms of iTTP) (36).

Relapse prevention

Recent studies have explored the use of rituximab to promote swifter recovery of ADAMTS13 activity, decrease ADAMTS13 antibodies, and subsequently decrease relapse rates. Early observational studies showed that patients with refractory or relapsed iTTP treated with rituximab had remarkably low rates of relapse (0–19%) (7–10). Subsequently, a phase II trial with matched historical controls showed that early use of rituximab reduced the 2-year relapse rate from 57 to 10% (28), which led to wider adoption of upfront rituximab for the treatment of acute iTTP, which is endorsed by the ISTH iTTP treatment guidelines (37). Most recently "preemptive" rituximab is often used to prevent clinical relapse in patients in clinical remission but with ADAMTS13 relapse. This is largely based on observational data from the French iTTP registry in which 92 patients with ADAMTS13 < 10% in clinical remission were preemptively treated with rituximab (38). The median cumulative relapse rate decreased from 0.33 (IQR 0.23–0.66) episodes per year before rituximab therapy to 0 (IQR 0–1.32) episodes per year after treatment ($P < 0.001$) (38). Compared with 23 historical controls with ADAMTS13 < 10% who did not receive preemptive rituximab, the relapse rate was much lower in the rituximab treated patients (15 vs. 74% $P < 0.001$) (38). Our practice is to monitor ADAMTS13 activity every 3 months during clinical remission for most patients, and offer preemptive rituximab if ADAMTS13 activity is <20–30% (13). The decision for preemptive rituximab is frequently influenced by a prior history of relapse; we recommend this most strongly for patients who have already had iTTP relapses since they are at much greater risk for relapse and most likely to benefit from preemptive rituximab. Some patients with a single episode of acute iTTP elect for observation alone. The monitoring and treatment plan can be further individualized. For example, patients with a single iTTP episode may opt for less frequent follow up at every 6–12 months starting at 2–3 years after the index episode (13); however, patients with a history of relapses or those that have needed repeated courses of preemptive rituximab will likely benefit from continued close follow up.

A large retrospective study from the United States Thrombotic Microangiopathy Registry that included 645 participants reported that Black patients had higher relapse rates, and the relapse free survival prolonging benefit of rituximab was also reduced in Black participants, particularly those with relapsing iTTP (32). Thus, Black patients may require closer monitoring, earlier retreatment, and consideration of alternative immunosuppressive therapies. Overall, about 15% of patients may be refractory to rituximab or may have only a transient response (38). In some of these cases, retreatment with an anti-CD20 directed therapy may be effective but comes with increased risk of adverse events and uncertain efficacy (39). Cyclosporine A has shown excellent efficacy in improving ADAMTS13 activity in patients

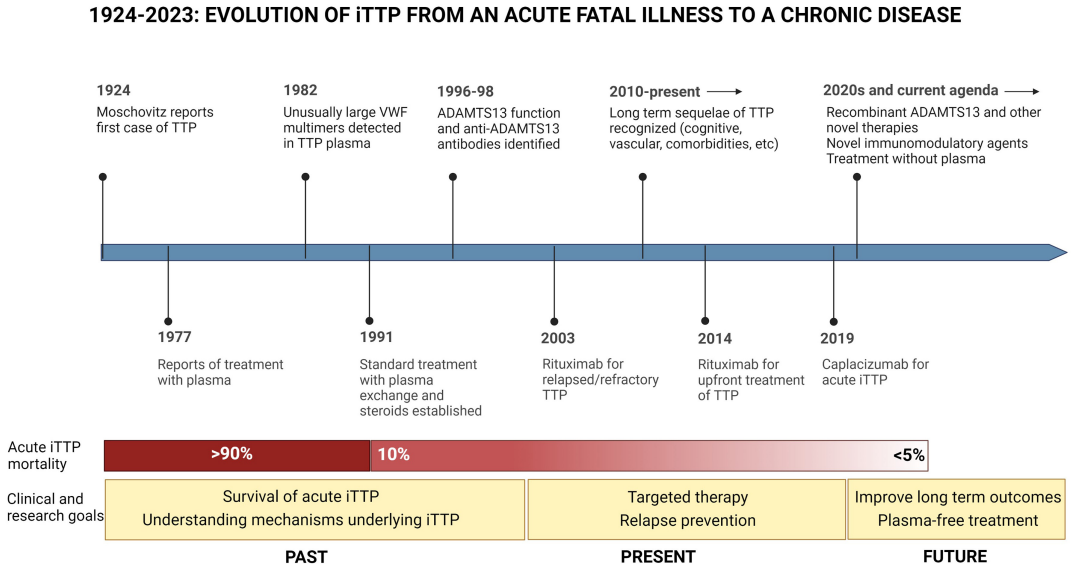

FIGURE 1
Evolution of iTTP from an acute fatal illness to a chronic disease. Since iTTP was described in 1924, the pathophysiology of iTTP has been elucidated, and advances in treatment have reduced mortality from acute iTTP episodes from over 90 to less than 5% per episode. In this landscape, the clinical and research agenda has also evolved toward a focus on issues of survivorship, and further advancements in therapy including the development of novel targeted therapies targeting immune response as well as VWF-platelet microthrombi, and steps toward treating iTTP without plasma.

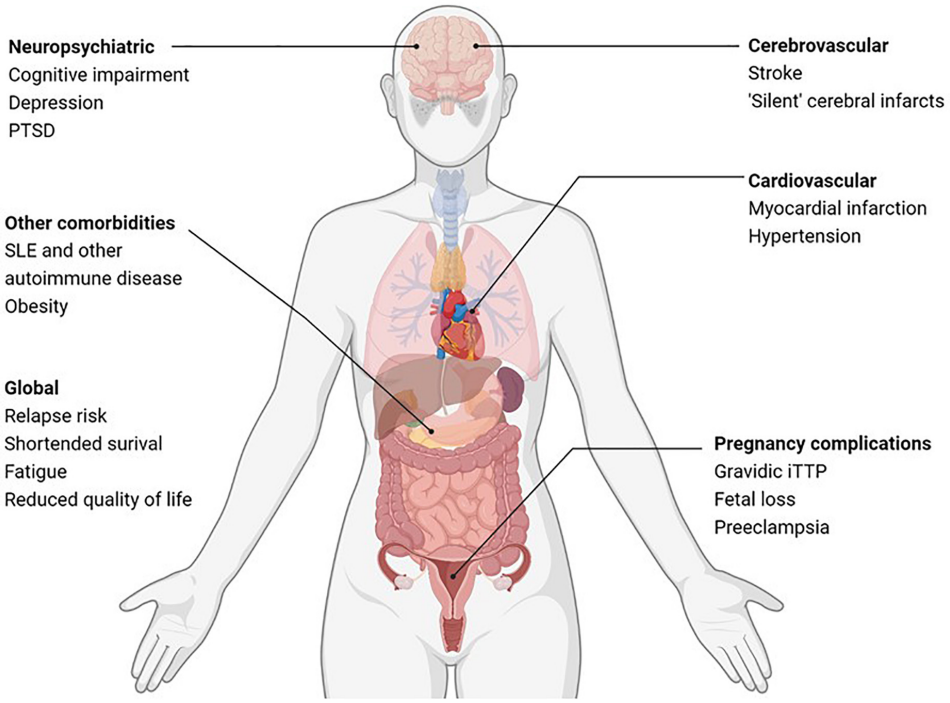

FIGURE 2
Long term complications of immune TTP.

refractory to rituximab (40). Plasma cell directed therapies such as bortezomib and daratumumab have also shown promise in iTTP refractory to rituximab (41–44). Other immunosuppressive therapies such as cyclophosphamide and even splenectomy have been used with success in rituximab refractory cases (45, 46). The optimal immunosuppressive regimen after rituximab failure has not been established and remains a critical unmet need in iTTP care.

Shortened overall survival

Immune thrombotic thrombocytopenic purpura survivors are at a higher risk of premature death compared with age-, sex-, and race-matched control populations. Moreover, since most patients will survive acute iTTP, late complications, such as cardiovascular disease, are the leading cause of mortality and morbidity (12, 15, 18). In an analysis of 57 iTTP survivors from the Oklahoma iTTP registry, 19% had died over a median follow-up of 7.8 years, which was significantly higher than expected based on age- and sex-matched U.S. or Oklahoma reference populations (12, 15). Only 18% of deaths were associated with iTTP relapse and cardiovascular and cerebrovascular complications accounted for the majority (64%) of deaths (15). Subsequently, a 222 patient cohort from the Johns Hopkins University and Ohio State University also demonstrated higher all-cause mortality in iTTP survivors compared to age and sex-matched reference populations with cardiovascular disease (27.6%, 8 of 29) and iTTP relapse (27.6%, 8 of 29) being the leading causes of death (18). Several studies have identified male sex, the number of iTTP episodes, and increasing age as risk factors for early mortality among iTTP survivors (12, 18). The French iTTP registry also reported that male sex, diabetes, tobacco use, malignancy, hypertension, cerebrovascular events, dementia, and COPD were risk factors for 1-year mortality among older iTTP survivors (47). Along with the finding that cardiovascular disease is a leading cause of death, these findings suggest that it is likely that comorbidities such as obesity, hypertension, and autoimmune disease may contribute to lower survival in TTP survivors. Indeed, TTP survivors have a higher prevalence of obesity (BMI $>$ 30), particularly morbid obesity (BMI $>$ 40), as well as higher rates of hypertension compared to the U.S. reference population (12), and these traditional cardiovascular risk factors are well-recognized as predictors of all-cause and cardiovascular mortality (48). The combined Johns Hopkins and Ohio State data found a trend toward increased mortality with lower ADAMTS13 activity during clinical remission (18). Lending credence to this association is an observation from the Rotterdam study, a population-based study in the Netherlands, which shows that lower ADAMTS13 activity is a risk factor for cardiovascular death in the general population (49).

Cardiovascular disease and stroke

Cardiovascular disease is a leading cause of mortality and complications among iTTP survivors (12, 17, 18). The risk of stroke during clinical remission is increased nearly fivefold compared with age and sex matched control population (13.1 vs. 22.6%) and this risk is strongly associated with incomplete ADAMTS13 recovery during clinical remission (16). A subsequent study found that major adverse cardiovascular events (stroke, non-fatal and fatal MI, and cardiac revascularization) occurred in 29% of iTTP survivors over a median follow up of 7.6 years with the first event occurring at a mean age 10–20 years younger than in the US reference population (17). Stroke was more common than myocardial infarction, similar to the pattern seen in congenital TTP cohorts, suggesting that the brain may be particularly vulnerable to iTTP-related thromboembolic events. Black race and diabetes mellitus were associated with these adverse cardiovascular events though a clear association with remission ADAMTS13 was not seen, likely due to limited statistical power (17).

In large population-based cohorts from the Netherlands, lower ADAMTS13 activity has emerged as a risk factor for coronary heart disease, stroke, and all cause and cardiovascular mortality (49–51). The hypothesized mechanism is that the lower ADAMTS13 levels leads to an accumulation of larger, more procoagulant von Willebrand Factor multimers that promote platelet activation (52), complement activation (53, 54), and accelerated atherosclerosis (50, 51, 55, 56). This may be of particular relevance among iTTP survivors who may not fully recover ADAMTS13 activity even during clinical remission (called partial ADAMTS13 remission) (36, 49). Comorbidities associated with increased cardiovascular risk such as hypertension, obesity, and autoimmune diseases are also more prevalent in iTTP survivors (12, 15). It is likely that multiple factors including traditional cardiovascular risk factors, ischemic events during acute iTTP and reduced ADAMTS13 activity all contribute to the risk of adverse cardiovascular events among iTTP survivors (16–18). Until specific strategies to mitigate cardiovascular risk in iTTP are developed, aggressive screening and management of cardiovascular risk factors is suggested, and the use of antiplatelet therapies such as aspirin may be considered especially in patients with other vascular risk factors. Current treatment strategies target ADAMTS13 activity $>$ 20% to prevent relapse. It is possible, but as yet unproven, whether targeting higher levels will improve outcomes such as stroke and mortality, and optimal ADAMTS13 targets still need to be established.

Other morbidities

Compared to general population controls, iTTP survivors have a higher rate of comorbidities such as hypertension (15), obesity (15), autoimmune disorders (15), and depression, which can contribute to cardiovascular disease and mortality as well as impaired quality of life. For example, in the Oklahoma iTTP registry, the prevalence of hypertension (40 vs. 23%; P = 0.013) and major depression (19 vs. 6%; P = 0.005) and was greater than expected values from the US and Oklahoma reference populations. The rate of obesity, particularly morbid obesity, was also higher in iTTP patients. Similarly high rates of hypertension, obesity, and autoimmune disease were reported from other US cohorts (4, 17), and a French study comparing 36 elderly TTP survivors ($\geq$65 years) with 127 age-matched controls demonstrated a higher prevalence of ischemic heart disease, stroke, osteoporosis, autoimmune disorders, and hypertension among the aging TTP patients (57). Autoimmune disorders, particularly systemic lupus erythematosus (SLE), commonly coexist with iTTP. In follow up of patients from the Oklahoma iTTP registry, the period prevalence of SLE was 12% (5 of 43), which was significantly increased compared to the U.S. population estimates (0.3%, p $<$ 0.001) (15). The combined Johns Hopkins and Ohio State Cohort reported a 11% prevalence of SLE with 8.8% having other autoimmune disorders, and data from French Reference Center registry showed that 21.4% (56 of 261) patients developed autoimmune conditions in association with iTTP, of which 30.4% developed the autoimmune condition(s) during an average 22-month follow-up period (20).

The factors driving the higher prevalence of these comorbidities are incompletely elucidated and likely complex. iTTP is associated with depression, which is an independent risk factor for obesity (58), and may increase cardiovascular morbidity (59). Obesity in turn is a risk factor for hypertension, cardiovascular disease, and mortality (60, 61). iTTP more commonly affects women and Black people, the same patient demographic susceptible to autoimmune conditions like SLE (62, 63). Moreover, race is not a purely biological construct and is often a surrogate for economic status, education, access to resources, and other social determinants of health that have huge impacts on the risk of developing chronic disease, as well as overall survival (64, 65).

Cognitive impairment

Chronic cognitive difficulties, especially issues with memory and concentration, are a frequently reported concern among iTTP survivors (21, 22, 66–69). A study that evaluated neurocognitive function in 24 iTTP survivors reported that 88% (21 of 24) performed below expectations in at least 1 of 11 domains, and 17% (4 of 24) participants scored below expectations in 6–8 of the 11 domains. Compared to healthy controls, the participants' median scores were significantly lower in 4 of 11 cognitive domains–complex attention and concentration skills, information processing speed, language generation, and memory (21). Cataland et al. reported that 63% (17 of 27) of the study participants were classified as having cognitive impairment, specifically in the visual learning and memory domains. Additionally, neurocognitive function among patients whose most recent TTP episode occurred within 1 year of the study (73%) was significantly more impaired compared to cognitive functioning in survivors whose last episode was more than 1 year prior (31%, $p = 0.035$) (68). Finally, a recent prospective study of neurocognitive function in iTTP survivors also found that over half had cognitive impairments, most commonly affecting executive function, attention and processing speed (70).

Multiple possible etiologies for iTTP associated cognitive impairment have been proposed. Comorbid depression has been suggested as a cause based on an association of depression severity with worse cognitive performance in a European study that used self-reported cognitive performance as an outcome measure (22); however, Han et al. did not find an association of depressive symptoms among iTTP survivors in the Oklahoma registry (23). The pattern of cognitive impairments seen in iTTP is similar to that seen in patients with hypertension (71), sickle cell disease (72, 73), and other vascular disorders (74), suggesting that it could be due to acquired diffuse, subcortical microvascular lesions. In support of this hypothesis, Cataland et al. observed small and large vessel ischemic changes indicative of silent cerebral infarcts on brain MRI in 39% (9 of 23) of TTP survivors who were otherwise clinically stable and without apparently neurological symptoms, thus suggesting a higher prevalence of persistent sub-clinical neurological injury following recovery (68). A recent prospective study found that silent cerebral infarction, defined as magnetic resonance imaging (MRI) evidence of brain ischemic infarction without a corresponding neuro-deficit, was present in 50% of iTTP patients in clinical remission and was strongly associated with the presence of cognitive impairment, particularly major cognitive impairment (70). Given that silent cerebral infarctions are a risk factor for both cognitive impairment (75) and stroke (76) in the general population, it is likely that neurocognitive deficits and stroke in TTP survivors are associated with silent infarctions in the brain. Ongoing studies to establish the prevalence and incidence of silent cerebral infarcts, stroke, and cognitive impairment among TTP survivors will identify an opportunity for early interventions aimed at reducing the incidence of sub-clinical neurologic injury (**Figure 3**).

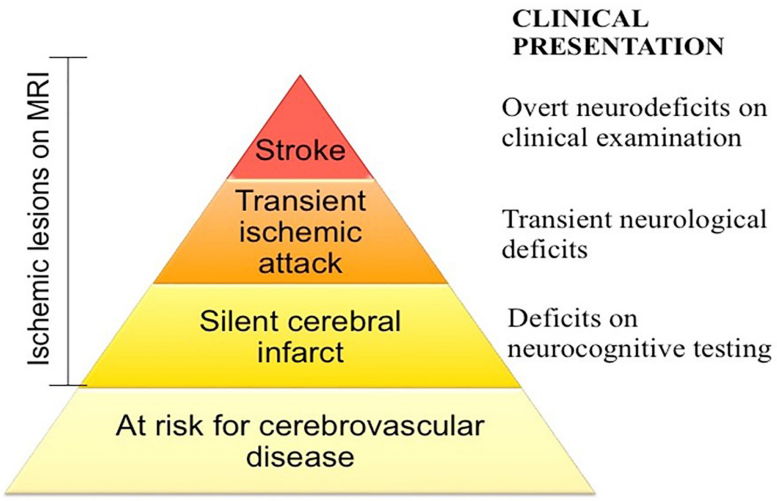

FIGURE 3
Spectrum of cerebrovascular disease in iTTP. iTTP survivors not only experience stroke at disproportionately high rates, but also exhibit evidence of silent cerebral infarction, defined as an ischemic lesion of brain magnetic resonance imaging that is not accompanied by a neurologic deficit attributable to that location of the brain, which may be associated with cognitive deficits as well. Screening for and addressing cardiovascular risk factors in at risk patients may mitigate some of the long term neurologic morbidity of iTTP.

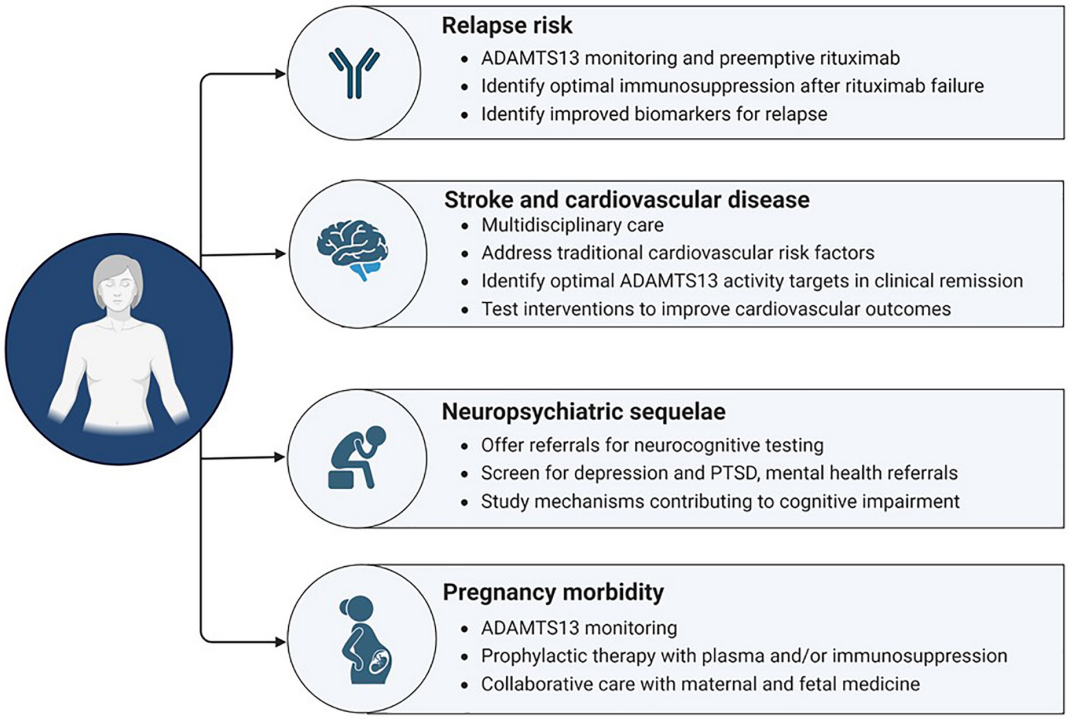

FIGURE 4
Opportunities to mitigate risk and improve long term outcomes in iTTP.

Mental health and quality of life

Multiple studies have reported a very high prevalence of depressive symptoms in patients who have recovered from acute iTTP. The exact estimate varies depending upon sampling and methodology. For example, investigators from the Oklahoma registry reported a 19% point prevalence of depression in a cross sectional sample (15), but nearly 59% of patients screened positive for depression at some point during an 11 year period (23). An European observational cohort study reported that 68% (74 of 104) of iTTP survivors scored positive for depression, a significantly higher prevalence compared to the controls ($p < 0.001$), and the severity of depression did not significantly vary between a 1-year period. This study also noted a positive correlation between the survey scores for clinically relevant depression and cognitive impairment ($p < 0.001$) (22). A survey-based study conducted through the Answering TTP foundation reported that 80.8% (169 of 209) of respondents reported at least mild depressive symptoms (77). In addition, 35.1% (81 of 231) of iTTP survivors who responded to the study met the criteria for post-traumatic stress disorder (77). In this study, 21% of participants reported that they were unemployed for reasons that they attributed to iTTP sequelae (77). While this study was likely affected by responder bias that could inflate estimates, these numbers are sobering and highlight the social and economic impact of the mental health sequelae of iTTP (69).

Following recovery, iTTP survivors have described persistent difficulties with endurance, concentration, and memory, which may contribute to long-term deficits in quality of life. Lewis et al. documented that that iTTP survivors in the Oklahoma cohort scored significantly lower than the US population across all eight domains of the SF-36 questionnaire when the initial SF-36 assessment was administered 6–24 months following iTTP recovery (24). These findings were supported by Cataland et al., who reported that the aggregate mental and physical component scores of iTTP patients were significantly lower than the age- and gender-matched US norms (68).

Pregnancy outcomes in women with a history of TTP

It is well established that pregnancy may trigger both initial and recurring episodes of congenital or immune TTP (25, 78–80). Regardless of whether the first iTTP episode is associated with pregnancy, pregnancy after a diagnosis of iTTP carries significant risk to both mother and fetus. Rates of recurrent iTTP during pregnancy range from 20% to 65%, and live birth rates range from 30% to 80% (12, 26, 80, 81). These widely varying rates are heavily influenced by the nature of the cohort (all patients followed prospectively versus high risk cases referred to tertiary care centers) and are much improved in cohorts that underwent ADAMTS13 activity monitoring and prophylactic therapy with plasma exchange when ADAMTS13 levels drop below 10–20% (81, 82). This is consistent with reports that ADAMTS13 activity at the onset of pregnancy is a predictor of relapse during pregnancy (81–84). In addition to the risk of relapse and miscarriage, upto a third of pregnancies in women with iTTP are complicated by preeclampsia, which is much higher than the 2–3% rate of preeclampsia reported in the general population (85–88). The association between iTTP and preeclampsia is likely multifactorial. Preeclampsia has been linked with reduced ADAMTS13 activity,

likely due to the combination of pregnancy-related reduction in ADAMTS13, the risk of vWF-mediated placental microthrombosis in the setting of reduced protease activity, and the inflammation-induced inhibition of ADAMTS13 proteolytic activity (89–92). The prevalence of iTTP among Black women, who have a higher risk of hypertensive disorders of pregnancy, and the increased incidence of hypertension, which is an independent risk factor of preeclampsia, among iTTP survivors may also contribute to the increased risk of preeclampsia observed even after iTTP recovery (80, 89, 90). Though we do not have prospective studies to inform best practices for managing subsequent pregnancy in women with iTTP, these data support ADAMTS13 monitoring prior to and during pregnancy and the use of rituximab to improve ADAMTS13 activity prior to pregnancy (93).

For pregnant individuals with a history of iTTP, monitoring of platelet counts and ADAMTS13 activity is recommended at least once per trimester although our approach is to check monthly. Recurrent iTTP during pregnancy should be treated with plasma exchange and corticosteroids; rituximab may be deferred until after pregnancy given the potential effects on the fetus. If ADAMTS13 activity drops to 10–20% in the absence of thrombocytopenia and other signs of microangiopathy, low dose corticosteroids may be considered in an attempt to increase ADAMTS13 activity. If the ADAMTS13 activity drops below 10%, prophylactic plasma exchange may be considered (82). We prefer to avoid rituximab and other immunosuppressive medications in pregnancy due to concerns of safety. Low dose aspirin therapy as preeclampsia prophylaxis may also be considered (94, 95).

Conclusion and future directions

Advances in the management of TTP have dramatically improved outcomes of acute iTTP episodes, and TTP is now appropriately treated as a chronic, relapsing disorder. Other adverse health outcomes in iTTP survivors include increased mortality, high rates of cardiovascular disease, cognitive impairment, and poor mental health outcomes. In addition to traditional cardiovascular risk factors, recent data suggests that low ADAMTS13 activity is a risk factor for some of these outcomes, particularly stroke. Recent research also suggests that Black patients, who represent the majority of patients with iTTP in the United States, are at higher risk of relapse and adverse cardiovascular outcomes (17, 32). Additional research is required to understand the risk factors and mechanisms underlying these complications, to establish strategies for screening, and to identify interventions to improve outcomes that can be tested in clinical trials (Figure 4). We anticipate that the more widespread use of rituximab for first episodes of iTTP and for preemptive therapy may reduce the burden of some vascular complications to the extent that they are mediated by ADAMTS13 deficiency, although other factors are also likely to be involved. The role of recombinant ADAMTS13 in acute iTTP as well as in remission remains to be defined. As for all ultra-rare disorders, this will require multicenter studies, with national and international collaboration, and collaboration with patient advocacy groups that have facilitated rare disease research.

The future of iTTP will be focused on optimizing outcomes in survivors.

Author contributions

SS prepared the first draft of the manuscript. AL drafted the parts of the manuscript. SC drafted the parts of the manuscript and critically reviewed and edited the entire manuscript. All authors read and approved the submitted manuscript.

Funding

SC was supported by National Institutes of Health (NIH), Heart, Lung, and Blood Institute grant K99HL150594 and an ASH Scholar Award.

Conflict of interest

SC has participated on advisory boards for Sanofi Genzyme, Takeda, Alexion, Sobi, and UCB.

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

Publisher's note

References

1. Rizzo C, Rizzo S, Scirè E, Di Bona D, Ingrassia C, Franco G, et al. Thrombotic thrombocytopenic purpura: a review of the literature in the light of our experience with plasma exchange. *Blood Transfus.* (2012) 10:521–32.

2. Saha M, McDaniel J, Zheng X. Thrombotic thrombocytopenic purpura: pathogenesis, diagnosis and potential novel therapeutics. *J Thromb Haemost.* (2017) 15:1889–900. doi: 10.1111/jth.13764

3. Bell W, Braine H, Ness P, Kickler T. Improved survival in thrombotic thrombocytopenic purpura-hemolytic uremic syndrome. Clinical experience in 108 patients. *N Engl J Med.* (1991) 325:398–403. doi: 10.1056/NEJM199108083250605

4. Chaturvedi S, Abbas H, McCrae K. Increased morbidity during long-term follow-up of survivors of thrombotic thrombocytopenic purpura. *Am J Hematol.* (2015) 90:E208. doi: 10.1002/ajh.24138

5. Rock GA, Shumak KH, Buskard NA, Blanchette VS, Kelton JG, Nair RC, et al. Comparison of plasma exchange with plasma infusion in the treatment of thrombotic thrombocytopenic purpura. Canadian apheresis study group. *N Engl J Med.* (1991) 325:393–7. doi: 10.1056/NEJM199108083250604

6. Bresin E, Gastoldi S, Daina E, Belotti D, Pogliani E, Perseghin P, et al. Rituximab as pre-emptive treatment in patients with thrombotic thrombocytopenic purpura and evidence of anti-ADAMTS13 autoantibodies. *Thromb Haemost.* (2009) 101:233–8. doi: 10.1160/TH07-12-0753

7. Dane K, Chaturvedi S. Beyond plasma exchange: novel therapies for thrombotic thrombocytopenic purpura. *Hematol Am Soc Hematol Educ Program.* (2018) 2018:539–47. doi: 10.1182/asheducation-2018.1.539

8. Hie M, Gay J, Galicier L, Provôt F, Presne C, Poullin P, et al. Preemptive rituximab infusions after remission efficiently prevent relapses in acquired thrombotic thrombocytopenic purpura. *Blood.* (2014) 124:204–10. doi: 10.1182/blood-2014-01-550244

9. Lim W, Vesely S, George J. The role of rituximab in the management of patients with acquired thrombotic thrombocytopenic purpura. *Blood.* (2015) 125:1526–31. doi: 10.1182/blood-2014-10-559211

10. Page E, Kremer Hovinga J, Terrell D, Vesely S, George J. Rituximab reduces risk for relapse in patients with thrombotic thrombocytopenic purpura. *Blood.* (2016) 127:3092–4. doi: 10.1182/blood-2016-03-703827

11. Peyvandi F, Scully M, Hovinga JA, Cataland S, Knöbl P, Wu H, et al. Caplacizumab for acquired thrombotic thrombocytopenic purpura. *N Engl J Med.* (2016) 374:511–22. doi: 10.1056/NEJMoa1505533

12. George JN. TTP: long-term outcomes following recovery. *Hematol Am Soc Hematol Educ Program.* (2018) 2018:548–52. doi: 10.1182/asheducation-2018.1.548

13. Akwaa F, Antun A, Cataland S. How I treat immune-mediated thrombotic thrombocytopenic purpura after hospital discharge. *Blood.* (2022) 140:438–44. doi: 10.1182/blood.2021014514

14. Page E, Kremer Hovinga J, Terrell D, Vesely S, George J. Thrombotic thrombocytopenic purpura: diagnostic criteria, clinical features, and long-term outcomes from 1995 through 2015. *Blood Adv.* (2017) 1:590–600. doi: 10.1182/bloodadvances.2017005124

15. Deford C, Reese J, Schwartz L, Perdue J, Kremer Hovinga J, Lämmle B, et al. Multiple major morbidities and increased mortality during long-term follow-up after recovery from thrombotic thrombocytopenic purpura. *Blood.* (2013) 122:2023–29; quiz 2142. doi: 10.1182/blood-2013-04-496752

16. Upreti H, Kasmani J, Dane K, Braunstein EM, Streiff MB, Shanbhag S, et al. Reduced ADAMTS13 activity during TTP remission is associated with stroke in TTP survivors. *Blood.* (2019) 134:1037–45. doi: 10.1182/blood.2019001056

17. Brodsky MA, Sukumar S, Selvakumar S, Yanek L, Hussain S, Mazepa MA, et al. Major adverse cardiovascular events in survivors of immune-mediated thrombotic thrombocytopenic purpura. *Am J Hematol.* (2021) 96:1587–94. doi: 10.1002/ajh.26341

18. Sukumar S, Brodsky M, Hussain S, Yanek L, Moliterno A, Brodsky R, et al. Cardiovascular disease is a leading cause of mortality among TTP survivors in clinical remission. *Blood Adv.* (2022) 6:1264–70. doi: 10.1182/bloodadvances.2020004169

19. Tsai H. The kidney in thrombotic thrombocytopenic purpura. *Minerva Med.* (2007) 98:731–47.

20. Roriz M, Landais M, Desprez J, Barbet C, Azoulay E, Galicier L, et al. Risk factors for autoimmune diseases development after thrombotic thrombocytopenic purpura. *Medicine.* (2015) 94:e1598. doi: 10.1097/MD.0000000000001598

21. Kennedy AS, Lewis QF, Scott JG, Hovinga JA, Lämmle B, Terrell DR, et al. Cognitive deficits after recovery from thrombotic thrombocytopenic purpura. *Transfusion.* (2009) 49:1092–101. doi: 10.1111/j.1537-2995.2009.02101.x

22. Falter T, Schmitt V, Herold S, Weyer V, Auer C v, Wagner S, et al. Depression and cognitive deficits as long-term consequences of thrombotic thrombocytopenic purpura. *Transfusion.* (2017) 57:1152–62. doi: 10.1111/trf.14060

23. Han B, Page EE, Stewart LM, Deford CC, Scott JG, Schwartz LH, et al. Depression and cognitive impairment following recovery from thrombotic thrombocytopenic purpura. *Am J Hematol.* (2015) 90:709–14. doi: 10.1002/ajh.24060

24. Lewis Q, Lanneau M, Mathias S, Terrell D, Vesely S, George J. Long-term deficits in health-related quality of life after recovery from thrombotic thrombocytopenic purpura. *Transfusion.* (2009) 49:118–24. doi: 10.1111/j.1537-2995.2008.01938.x

25. Vesely S. Life after acquired thrombotic thrombocytopenic purpura: morbidity, mortality, and risks during pregnancy. *J Thromb Haemost.* (2015) 13(Suppl. 1):S216–22. doi: 10.1111/jth.12953

26. Jiang Y, McIntosh JJ, Reese JA, Deford CC, Hovinga JA, Lämmle B, et al. Pregnancy outcomes following recovery from acquired thrombotic thrombocytopenic purpura. *Blood.* (2014) 123:1674–80. doi: 10.1182/blood-2013-11-538900

27. Kremer Hovinga J, Vesely S, Terrell D, Lammle B, George J. Survival and relapse in patients with thrombotic thrombocytopenic purpura. *Blood.* (2010) 115:1500–11; quiz 1662. doi: 10.1182/blood-2009-09-243790

28. Scully M, McDonald V, Cavenagh J, Hunt BJ, Longair I, Cohen H, et al. A phase 2 study of the safety and efficacy of rituximab with plasma exchange in acute acquired thrombotic thrombocytopenic purpura. *Blood.* (2011) 118:1746–53. doi: 10.1182/blood-2011-03-341131

29. Shumak K, Rock G, Nair R. Late relapses in patients successfully treated for thrombotic thrombocytopenic purpura. Canadian apheresis group. *Ann Intern Med.* (1995) 122:569–72. doi: 10.7326/0003-4819-122-8-199504150-00002

30. Sun L, Mack J, Li A, Ryu J, Upadhyay VA, Uhl L, et al. Predictors of relapse and efficacy of rituximab in immune thrombotic thrombocytopenic purpura. *Blood Adv.* (2019) 3:1512–8. doi: 10.1182/bloodadvances.2019031039

31. Zhan H, Streiff M, King K, Segal J. Thrombotic thrombocytopenic purpura at the Johns Hopkins hospital from 1992 to 2008: clinical outcomes and risk factors for relapse. *Transfusion.* (2010) 50:868–74. doi: 10.1111/j.1537-2995.2009.02528.x

32. Chaturvedi S, Antun AG, Farland AM, Woods R, Metjian A, Park YA, et al. Race, rituximab, and relapse in TTP. *Blood.* (2022) 140:1335–44. doi: 10.1182/blood.2022016640

33. Peyvandi F, Lavoretano S, Palla R, Feys HB, Vanhoorelbeke K, Battaglioli T, et al. ADAMTS13 and anti-ADAMTS13 antibodies as markers for recurrence of acquired thrombotic thrombocytopenic purpura during remission. *Haematologica.* (2008) 93:232–9. doi: 10.3324/haematol.11739

34. Jin M, Casper TC, Cataland SR, Kennedy MS, Lin S, Li YJ, et al. Relationship between ADAMTS13 activity in clinical remission and the risk of TTP relapse. *Br J Haematol.* (2008) 141:651–8. doi: 10.1111/j.1365-2141.2008.07107.x

35. Schieppati F, Russo L, Marchetti M, Barcella L, Cefis M, Gomez-Rosas P, et al. Low levels of ADAMTS-13 with high anti-ADAMTS-13 antibodies during remission of immune-mediated thrombotic thrombocytopenic purpura highly predict for disease relapse: a multi-institutional study. *Am J Hematol.* (2020) 95:953–9. doi: 10.1002/ajh.25845

36. Cuker A, Cataland SR, Coppo P, Rubia J d, Friedman KD, George JN, et al. Redefining outcomes in immune TTP: an international working group consensus report. *Blood.* (2021) 137:1855–61. doi: 10.1182/blood.2020009150

37. Zheng XL, Vesely SK, Cataland SR, Coppo P, Geldziler B, Iorio A, et al. ISTH guidelines for treatment of thrombotic thrombocytopenic purpura. *J Thromb Haemost.* (2020) 18:2496–502. doi: 10.1111/jth.15010

38. Jestin M, Benhamou Y, Schelpe A, Roose E, Provôt F, Galicier L, et al. Preemptive rituximab prevents long-term relapses in immune-mediated thrombotic thrombocytopenic purpura. *Blood.* (2018) 132:2143–53. doi: 10.1182/blood-2018-04-840090

39. Barba C, Peyre M, Galicier L, Cathebras P, Ranta D, Malot S, et al. Intensive rituximab regimen in immune-mediated thrombotic thrombocytopenic purpura can circumvent unresponsiveness to standard rituximab treatment. *Br J Haematol.* (2021) 192:e21–5. doi: 10.1111/bjh.17170

40. Comparon C, Galicier L, Rebibou J, Coppo P, Benhamou Y. Preemptive cyclosporin A in immune-mediated thrombotic thrombocytopenic purpura. *Eur J Haematol.* (2022) 110:157–60. doi: 10.1111/ejh.13886

41. Yin J, Tian H, Kong D, Li Y, Gu C, Wang Z, et al. Bortezomib, a promising alternative for patients with refractory or relapsed thrombotic thrombocytopenic purpura after rituximab treatment. *Br J Haematol.* (2022) 199:619–22. doi: 10.1111/bjh.18430

42. Shortt J, Oh D, Opat S. ADAMTS13 antibody depletion by bortezomib in thrombotic thrombocytopenic purpura. *N Engl J Med.* (2013) 368:90–2. doi: 10.1056/NEJMc1213206

43. Mazepa M, Raval J, Moll S, Ma A, Park Y. Bortezomib induces clinical remission and reduction of ADAMTS13 inhibitory antibodies in relapsed refractory idiopathic thrombotic thrombocytopenic purpura. *Br J Haematol.* (2014) 164:900–2. doi: 10.1111/bjh.12696

44. Berg J v, Hovinga JA, Pfleger C, Hegemann I, Stehle G, Holbro A, et al. Daratumumab for immune thrombotic thrombocytopenic purpura. *Blood Adv.* (2022) 6:993–7. doi: 10.1182/bloodadvances.2021005124

45. Aqui N, Stein S, Konkle B, Abrams C, Strobl F. Role of splenectomy in patients with refractory or relapsed thrombotic thrombocytopenic purpura. *J Clin Apher.* (2003) 18:51–4. doi: 10.1002/jca.10053

46. Beloncle F, Buffet M, Coindre J, Munoz-Bongrand N, Malot S, Pène F, et al. Splenectomy and/or cyclophosphamide as salvage therapies in thrombotic thrombocytopenic purpura: the French TMA reference center experience. *Transfusion.* (2012) 52:2436–44. doi: 10.1111/j.1537-2995.2012.03578.x

47. Prevel R, Roubaud-Baudron C, Gourlain S, Jamme M, Peres K, Benhamou Y, et al. Immune thrombotic thrombocytopenic purpura in older patients: prognosis and long-term survival. *Blood.* (2019) 134:2209–17. doi: 10.1182/blood.2019000748

48. Abdelaal M, le Roux C, Docherty N. Morbidity and mortality associated with obesity. *Ann Transl Med.* (2017) 5:161. doi: 10.21037/atm.2017.03.107

49. Sonneveld MA, Maat MP, Portegies ML, Kavousi M, Hofman A, Turecek PL, et al. Low ADAMTS13 activity is associated with an increased risk of ischemic stroke. *Blood.* (2015) 126:2739–46. doi: 10.1182/blood-2015-05-643338

50. Sonneveld MA, Kavousi M, Ikram MA, Hofman A, Ochoa OL, Turecek PL, et al. Low ADAMTS-13 activity and the risk of coronary heart disease – a prospective cohort study: the Rotterdam study. *J Thromb Haemost.* (2016) 14:2114–20. doi: 10.1111/jth.13479

51. Sonneveld MA, Franco OH, Ikram MA, Hofman A, Kavousi M, Maat MP, et al. Von Willebrand factor, ADAMTS13, and the risk of mortality: the Rotterdam

study. *Arterioscler Thromb Vasc Biol.* (2016) 36:2446–51. doi: 10.1161/ATVBAHA.116.308225

52. Federici A, Bader R, Pagani S, Colibretti M, De Marco L, Mannucci P. Binding of von Willebrand factor to glycoproteins Ib and IIb/IIIa complex: affinity is related to multimeric size. *Br J Haematol.* (1989) 73:93–9. doi: 10.1111/j.1365-2141.1989.tb00226.x

53. Noone DG, Riedl M, Pluthero FG, Bowman ML, Liszewski MK, Lu L, et al. Von Willebrand factor regulates complement on endothelial cells. *Kidney Int.* (2016) 90:123–34. doi: 10.1016/j.kint.2016.03.023

54. Turner N, Nolasco L, Nolasco J, Sartain S, Moake J. Thrombotic microangiopathies and the linkage between von Willebrand factor and the alternative complement pathway. *Semin Thromb Hemost.* (2014) 40:544–50. doi: 10.1055/s-0034-1383547

55. Jin S, Tohyama J, Bauer R, Cao N, Rader D, Zheng X. Genetic ablation of Adamts13 gene dramatically accelerates the formation of early atherosclerosis in a murine model. *Arterioscler Thromb Vasc Biol.* (2012) 32:1817–23. doi: 10.1161/ATVBAHA.112.247262

56. Gandhi C, Khan M, Lentz S, Chauhan A. ADAMTS13 reduces vascular inflammation and the development of early atherosclerosis in mice. *Blood.* (2012) 119:2385–91. doi: 10.1182/blood-2011-09-376202

57. Agosti P, Mancini I, Gianniello F, Bucciarelli P, Artoni A, Ferrari B, et al. Prevalence of the age-related diseases in older patients with acquired thrombotic thrombocytopenic purpura. *Eur J Intern Med.* (2020) 75:79–83. doi: 10.1016/j.ejim.2020.01.024

58. Luppino FS, Wit LM, Bouvy PF, Stijnen T, Cuijpers P, Penninx BW, et al. Overweight, obesity, and depression: a systematic review and meta-analysis of longitudinal studies. *Arch Gen Psychiatry.* (2010) 67:220–9. doi: 10.1001/archgenpsychiatry.2010.2

59. Hare D, Toukhsati S, Johansson P, Jaarsma T. Depression and cardiovascular disease: a clinical review. *Eur Heart J.* (2014) 35:1365–72. doi: 10.1093/eurheartj/eht462

60. Collaboration PS, Whitlock G, Lewington S, Sherliker P, Clarke R, Emberson J, et al. Body-mass index and cause-specific mortality in 900 000 adults: collaborative analyses of 57 prospective studies. *Lancet.* (2009) 373:1083–96. doi: 10.1016/S0140-6736(09)60318-4

61. Flegal K, Kit B, Orpana H, Graubard B. Association of all-cause mortality with overweight and obesity using standard body mass index categories: a systematic review and meta-analysis. *JAMA.* (2013) 309:71–82. doi: 10.1001/jama.2012.113905

62. Martino S, Jamme M, Deligny C, Busson M, Loiseau P, Azoulay E, et al. Thrombotic thrombocytopenic purpura in black people: impact of ethnicity on survival and genetic risk factors. *PLoS One.* (2016) 11:e0156679. doi: 10.1371/journal.pone.0156679

63. Feldman CH, Hiraki LT, Liu J, Fischer MA, Solomon DH, Alarcón GS, et al. Epidemiology and sociodemographics of systemic lupus erythematosus and lupus nephritis among US adults with Medicaid coverage, 2000-2004. *Arthritis Rheum.* (2013) 65:753–63. doi: 10.1002/art.37795

64. Javed Z, Maqsood MH, Yahya T, Amin Z, Acquah I, Valero-Elizondo J, et al. Race, racism, and cardiovascular health: applying a social determinants of health framework to racial/ethnic disparities in cardiovascular disease. *Circ Cardiovasc Qual Outcomes.* (2022) 15:e007917. doi: 10.1161/CIRCOUTCOMES.121.007917

65. Devareddy A, Sarraju A, Rodriguez F. Health disparities across the continuum of ASCVD risk. *Curr Cardiol Rep.* (2022) 24:1129–37. doi: 10.1007/s11886-022-01736-y

66. Howard MA, Duvall D, Terrell DR, Christopher AT, Thomas I, Holloway N, et al. A support group for patients who have recovered from thrombotic thrombocytopenic purpura-hemolytic uremic syndrome (TTP-HUS): the six-year experience of the Oklahoma TTP-HUS study group. *J Clin Apher.* (2003) 18:16–20. doi: 10.1002/jca.10045

67. George J, Terrell D, Swisher K, Vesely S. Lessons learned from the Oklahoma thrombotic thrombocytopenic purpura-hemolytic uremic syndrome registry. *J Clin Apher.* (2008) 23:129–37. doi: 10.1002/jca.20169

68. Cataland SR, Scully MA, Paskavitz J, Maruff P, Witkoff L, Jin M, et al. Evidence of persistent neurologic injury following thrombotic thrombocytopenic purpura. *Am J Hematol.* (2011) 86:87–9. doi: 10.1002/ajh.21881

69. Kelley R, Cheney M, Martin C, Cataland S, Quick L, Keller S, et al. Health following recovery from immune thrombotic thrombocytopenic purpura: the patient's perspective. *Blood Adv.* (2022). doi: 10.1182/bloodadvances.2022008342 [Epub ahead of print].

70. Yu J, Brown J, Salzberg E, Wei A, Gerber G, Pan X, et al. Silent cerebral infarction during immune TTP remission – prevalence, predictors and impact on cognition. *Blood.* (2022) 140(Suppl. 1):338–40. doi: 10.1182/blood-2022-167054

71. Elias M, Wolf P, D'Agostino R, Cobb J, White L. Untreated blood pressure level is inversely related to cognitive functioning: the Framingham study. *Am J Epidemiol.* (1993) 138:353–64. doi: 10.1093/oxfordjournals.aje.a116868

72. Kral M, Brown R, Hynd G. Neuropsychological aspects of pediatric sickle cell disease. *Neuropsychol Rev.* (2001) 11:179–96. doi: 10.1023/A:1012901124088

73. Prussien K, Jordan L, DeBaun M, Compas B. Cognitive function in sickle cell disease across domains, cerebral infarct status, and the lifespan: a meta-analysis. *J Pediatr Psychol.* (2019) 44:948–58. doi: 10.1093/jpepsy/jsz031

74. O'Brien JT, Erkinjuntti T, Reisberg B, Roman G, Sawada T, Pantoni L, et al. Vascular cognitive impairment. *Lancet Neurol.* (2003) 2:89–98. doi: 10.1016/S1474-4422(03)00305-3

75. Sigurdsson S, Aspelund T, Kjartansson O, Gudmundsson EF, Jonsdottir MK, Eiriksdottir G, et al. Incidence of brain infarcts, cognitive change, and risk of dementia in the general population: the AGES-reykjavik study (age gene/environment susceptibility-reykjavik study). *Stroke.* (2017) 48:2353–60. doi: 10.1161/STROKEAHA.117.017357

76. Gupta A, Giambrone AE, Gialdini G, Finn C, Delgado D, Gutierrez J, et al. Silent brain infarction and risk of future stroke: a systematic review and meta-analysis. *Stroke.* (2016) 47:719–25. doi: 10.1161/STROKEAHA.115.011889

77. Chaturvedi S, Oluwole O, Cataland S, McCrae K. Post-traumatic stress disorder and depression in survivors of thrombotic thrombocytopenic purpura. *Thromb Res.* (2017) 151:51–6. doi: 10.1016/j.thromres.2017.01.003

78. Ridolfi R, Bell W. Thrombotic thrombocytopenic purpura. Report of 25 cases and review of the literature. *Medicine.* (1981) 60:413–28. doi: 10.1097/00005792-198111000-00003

79. Scully M, Yarranton H, Liesner R, Cavenagh J, Hunt B, Benjamin S, et al. Regional UK TTP registry: correlation with laboratory ADAMTS 13 analysis and clinical features. *Br J Haematol.* (2008) 142:819–26. doi: 10.1111/j.1365-2141.2008.07276.x

80. Brown J, Potugari B, Mazepa MA, Kohli R, Moliterno AR, Brodsky RA, et al. Maternal and fetal outcomes of pregnancy occurring after a diagnosis of immune-mediated thrombotic thrombocytopenic purpura. *Ann Hematol.* (2022) 101:2159–67. doi: 10.1007/s00277-022-04936-2

81. Ferrari B, Maino A, Lotta LA, Artoni A, Pontiggia S, Trisolini SM, et al. Pregnancy complications in acquired thrombotic thrombocytopenic purpura: a case-control study. *Orphanet J Rare Dis.* (2014) 9:193. doi: 10.1186/s13023-014-0193-6

82. Scully M, Thomas M, Underwood M, Watson H, Langley K, Camilleri RS, et al. Thrombotic thrombocytopenic purpura and pregnancy: presentation, management, and subsequent pregnancy outcomes. *Blood.* (2014) 124:211–9. doi: 10.1182/blood-2014-02-553131

83. Ducloy-Bouthors A, Caron C, Subtil D, Provot F, Tournoys A, Wibau B, et al. Thrombotic thrombocytopenic purpura: medical and biological monitoring of six pregnancies. *Eur J Obstet Gynecol Reprod Biol.* (2003) 111:146–52. doi: 10.1016/S0301-2115(03)00199-4

84. Raman R, Yang S, Wu H, Cataland S. ADAMTS13 activity and the risk of thrombotic thrombocytopenic purpura relapse in pregnancy. *Br J Haematol.* (2011) 153:277–9. doi: 10.1111/j.1365-2141.2010.08558.x

85. Wallis A, Saftlas A, Hsia J, Atrash H. Secular trends in the rates of preeclampsia, eclampsia, and gestational hypertension, United States, 1987-2004. *Am J Hypertens.* (2008) 21:521–6. doi: 10.1038/ajh.2008.20

86. Kuklina E, Ayala C, Callaghan W. Hypertensive disorders and severe obstetric morbidity in the United States. *Obstet Gynecol.* (2009) 113:1299–306. doi: 10.1097/AOG.0b013e3181a45b25

87. Lo J, Mission J, Caughey A. Hypertensive disease of pregnancy and maternal mortality. *Curr Opin Obstet Gynecol.* (2013) 25:124–32. doi: 10.1097/GCO.0b013e32835e0ef5

88. Lisonkova S, Joseph K. Incidence of preeclampsia: risk factors and outcomes associated with early- versus late-onset disease. *Am J Obstet Gynecol.* (2013) 209:544.e1–12. doi: 10.1016/j.ajog.2013.08.019

89. Stepanian A, Cohen-Moatti M, Sanglier T, Legendre P, Ameziane N, Tsatsaris V, et al. Von Willebrand factor and ADAMTS13: a candidate couple for preeclampsia pathophysiology. *Arterioscler Thromb Vasc Biol.* (2011) 31:1703–9. doi: 10.1161/ATVBAHA.111.223610

90. Aref S, Goda H. Increased VWF antigen levels and decreased ADAMTS13 activity in preeclampsia. *Hematology.* (2013) 18:237–41. doi: 10.1179/1607845412Y.0000000070

91. Bernardo A, Ball C, Nolasco L, Moake J, Dong J. Effects of inflammatory cytokines on the release and cleavage of the endothelial cell-derived ultralarge von Willebrand factor multimers under flow. *Blood.* (2004) 104:100–6. doi: 10.1182/blood-2004-01-0107

92. Feys H, Canciani M, Peyvandi F, Deckmyn H, Vanhoorelbeke K, Mannucci P. ADAMTS13 activity to antigen ratio in physiological and pathological conditions associated with an increased risk of thrombosis. *Br J Haematol.* (2007) 138:534–40. doi: 10.1111/j.1365-2141.2007.06688.x

93. Scully M, Hunt B, Benjamin S, Liesner R, Rose P, Peyvandi F, et al. Guidelines on the diagnosis and management of thrombotic thrombocytopenic purpura and other thrombotic microangiopathies. *Br J Haematol.* (2012) 158:323–35. doi: 10.1111/j.1365-2141.2012.09167.x

94. Henderson J, Vesco K, Senger C, Thomas R, Redmond N. Aspirin use to prevent preeclampsia and related morbidity and mortality: updated evidence report and systematic review for the US preventive services task force. *JAMA.* (2021) 326:1192–206. doi: 10.1001/jama.2021.8551

95. Duley L, Meher S, Hunter K, Seidler A, Askie L. Antiplatelet agents for preventing pre-eclampsia and its complications. *Cochrane Database Syst Rev.* (2019) 2019:CD004659. doi: 10.1002/14651858.CD004659.pub3

frontiers | Frontiers in Medicine

TYPE Review
PUBLISHED 20 April 2023
DOI 10.3389/fmed.2023.1155954

OPEN ACCESS

EDITED BY
Marcos De Lima,
The Ohio State University, United States

REVIEWED BY
Luca Castagna,
Azienda Ospedaliera Ospedali Riuniti Villa Sofia Cervello, Italy
Guillemette Fouquet,
Centre Hospitalier Sud Francilien, France

*CORRESPONDENCE
Mariana Nassif Kerbauy
✉ mari.kerbauy@gmail.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Hematology, a section of the journal Frontiers in Medicine

RECEIVED 01 February 2023
ACCEPTED 23 March 2023
PUBLISHED 20 April 2023

CITATION
Kerbauy MN, Arcuri LJ, Favareto SL, de Rezende ACP and Hamerschlak N (2023) Total marrow irradiation in hematopoietic stem cell transplantation for hematologic malignancies. *Front. Med.* 10:1155954. doi: 10.3389/fmed.2023.1155954

Total marrow irradiation in hematopoietic stem cell transplantation for hematologic malignancies

Mariana Nassif Kerbauy[1*†], Leonardo Javier Arcuri[2†], Sergio Leonardo Favareto[3], Ana Carolina Pires de Rezende[3] and Nelson Hamerschlak[1]

[1]Department of Hematology and Bone Marrow Transplantation, Hospital Israelita Albert Einstein, São Paulo, Brazil, [2]Academic Research Organization, Hospital Israelita Albert Einstein, São Paulo, Brazil, [3]Department of Radiation Oncology, Hospital Israelita Albert Einstein, São Paulo, Brazil

Total body irradiation (TBI) has been an essential component of the conditioning regimen in hematopoietic cell transplantation for many years. However, higher doses of TBI reduce disease relapse at the expense of more significant toxicities. Therefore, total marrow irradiation and total marrow and lymphoid irradiation have been developed to deliver organ-sparing targeted radiotherapy. Data from different studies show that TMI and TMLI can be safely administered in escalating doses in association with different chemotherapy conditioning regimen protocols, in situations with unmet needs, such as multiple myeloma, high-risk hematologic malignancies, relapsed or refractory leukemias, and elderly or frail patients, with low rates of transplant-related mortality. We reviewed the literature on applying TMI and TMLI techniques in autologous and allogeneic hematopoietic stem cell transplantation in different clinical situations.

KEYWORDS

hematopoietic stem cell transplant, bone marrow transplantation, total body irradiation, total marrow irradiation, total marrow and lymphoid irradiation, targeted marrow irradiation, conditioning regimen, multiple myeloma

Introduction

Total body irradiation (TBI) has been an essential component of the conditioning regimen in hematopoietic cell transplantation (HCT) for many years. In patients with malignant diseases, the conditioning regimen has two main objectives: (1) to reduce tumor burden and disease relapse after transplant and (2) to provide sufficient immunoablation to prevent rejection. TBI has been widely used due to its immunosuppressive profile and effectiveness against leukemias and lymphomas, even in sanctuary sites such as the brain and testes (1). Higher doses of TBI reduce disease relapse at the expense of more significant toxicities, especially in the lungs, liver, bowel, thyroid, and gonads. It also leads to impaired growth and development in children, fertility issues, secondary malignancies, and increased transplant-related deaths (2). To decrease organ toxicity, fractionated TBI was an effective strategy due to a higher proportion of intact repair mechanisms retained in normal tissues as opposed to cancer cells (3), and hyperfractionated TBI and partial lung shielding have reduced fatal interstitial pneumonitis from 50% in the single-dose regimen to 18%, with 100% engraftment rate (4).

The need for reducing TBI toxicity to critical organs while increasing doses to selected targets through technical optimization has emerged as an essential need for the bone marrow transplant team and radiation oncologists. Total marrow irradiation (TMI) and total marrow and lymphoid irradiation (TMLI) were developed to deliver organ-sparing targeted radiotherapy using intensity-modulated radiation therapy (IMRT). The term TMI is usually used if the target is the bone and bone marrow, and TMLI adds the spleen and major lymph node chain. As a result, radiation to structures such as the lenses, oral cavity, thyroid, parotid glands, esophagus, lungs, heart, liver, stomach, kidneys, urinary bladder, genitalia, and small bowel are usually minimized.

Total marrow irradiation was usually used in autologous HCT for multiple myeloma and TMLI in allogeneic HCT (5). Data from different studies show that TMI and TMLI can be safely delivered at an escalated dose in association with different chemotherapy conditioning regimen protocols. Our objective is to review literature data regarding conditioning regimes and the application of TMI techniques in autologous and allogeneic TMI. Thus, we performed a search in PubMed with the following strategy: ("Total marrow"[Title]) OR ("Targeted marrow"[Title]) OR ("Targeted-marrow"[Title]) OR (TMI[Title] AND transplant*[Title]).

TMI treatment planning

The main targets for TMI planning purposes are bones and bone marrow. For TMLI, the spleen and major lymph node chains are also included. Depending on the institution's protocol, the liver, brain, and testes may also be included. There are several protocols with different radiation dose prescriptions, ranging from 6 to 20 Gy (5, 6). The irradiation protocol starts with the simulation, outline of targets and organs at risk (OAR), planning (dose calculation) and, finally, the delivery of the treatment.

A computed tomography (CT) scan with 5–10 mm is performed for the simulation. Patients usually stay in the supine position, with immobilization devices such as vacuum bags and/or thermoplastic masks. In addition, 4D CT scans of the chest and abdomen may be utilized to account for any organ movement during respiration (5).

The contouring begins with defining the target volumes and the avoidance structures. First, the clinical target volume (CTV) is defined as the spleen and entire bony skeleton, excluding the mandible. After that, anisotropic margins to account for possible uncertainties in beam alignment, patient positioning, organ motion, and organ deformations are generated to create the planning target volume (PTV). Avoidance structures, also known as organs at risk, include lenses, parotid glands, oral cavity, thyroid, esophagus, lungs, heart, liver, stomach, kidneys, small bowel, urinary bladder, and genitalia (6).

The treatment may be delivered by a regular linear accelerator (LINAC) or TomoTherapy® (combines intensity-modulated radiation therapy (IMRT) with a spiral delivery pattern). For LINAC, the planning is generated using 4–5 isocentric volumetric modulated arc therapy techniques to the upper body. The lower extremities are planned and treated with junctioned AP-PA fields (7, 8). Volumetric imaging (image-guided radiation therapy – IGRT) is used for daily patient setup (5).

TMI in autologous HCT

Total marrow irradiation in autologous conditioning regimen for multiple myeloma was pioneered by Einsele et al. (9). Eighty-nine patients with stage II–III multiple myeloma, *de novo* or pretreated, received TMI 9 Gy, 12 mg/kg oral busulfan (equivalent to 9.6 mg/kg intravenous busulfan), and cyclophosphamide 120 mg/kg in patients. Three patients had sinusoidal obstruction syndrome (SOS). Transplant-related mortality was as low as 2%. Median progression-free survival was 36 months for patients with *de novo* MM. Complete response increased from 2% to 44% following chemoradiotherapy (Table 1). In the current days, the use of intravenous busulfan may attenuate the incidence of SOS.

Cailleteau et al. (10) reported the results of a phase I trial of melphalan 140 mg/m2 combined with TMI before autologous HCT for patients in their first relapse. Thirteen patients were included. Four dose levels were explored: 8 Gy, 10 Gy, 12 Gy, and 14 Gy. The dose administered to the lungs was systematically below 8 Gy. Maximum tolerated dose was not reached, and the rate of acute toxicity was low. Pre-TMI rate of CR was 15% and the post-TMI rate of CR was 31%, showing that TMI increased the depth of response in these patients without increasing toxicity. Patel et al. (13) combined up to 9 Gy of TMI with melphalan 200 mg/m^2 in 12 patients with relapsed myeloma. The maximum tolerated dose was not achieved, there was no grade 4 non-hematologic toxicity, and median overall survival was around 3 years. Although feasible, maximal doses of TMI and melphalan, when combined, have yet to be established.

Ladbury et al. (11) reported the results of a unicentric protocol that included a second autologous HCT with single-agent TMI (doses: 10–18 Gy) after a first transplant based on melphalan 200 mg/m^2. Thirty patients received the second HCT based on TMI. The maximum tolerated dose was 16 Gy. In a long follow-up (12.3 years among survivors), the progression-free survival at 10 years was 20%. There were five cases of secondary malignancies and an additional five cases of non-melanoma skin cancers. Somlo et al. (15) also tested a second high-dose therapy based on TMI (doses: 10–18 cGy) in 22 patients. The maximum tolerated dose was also 16 Gy. In a quite similar strategy, Giebel et al. (12) published the results of a tandem HCT strategy with 12 Gy in the first course and melphalan 200 mg/m^2 in the second one. Fifty patients were included. Five-year progression-free survival was 55%. The authors showed the anti-myeloma activity of TMI monotherapy since VGPR increased from 46% to 74% after the first transplant and 86% after the second transplant. The rate of complete response changed from 10% before the first auto-HCT to 42% after tandem transplantation. Non-hematological complications were infrequent and only 14% of patients had mucositis grades 2–4. Both studies have shown that the maximal dose of TMI, when used as a single agent, is 16 Gy.

Lin et al. (14) tried to compare the results of autologous HCT for multiple myeloma with TMI 8 Gy combined with melphalan 140 mg/m2 with single-agent melphalan 200 mg/m2, but the small number of patients hampered further analyses.

Most of these reports do not represent the current practice in multiple myeloma, and these strategies should be tested in the context of highly active induction regimens and maintenance therapy.

TABLE 1 Clinical results of TMI in autologous transplants for multiple myeloma.

Author, year	Study type	*N*	Drugs	TMI dose	% Relapsed disease	Complete response	VGPR
Cailleteau et al. (10)	Phase I	13	Melphalan 140 mg/m2	Max 14 Gy	100%	31%	69%
Ladbury et al. (11)	Phase I/II	54	-	MTD 16 Gy	0%	48%	70%
Giebel et al. (12)	Phase II	50	-	12 Gy	0%	42%	NA
Patel et al. (13)	Phase I	12	Melphalan 200 mg/m2	Max 9 Gy	100%	NA	73%
Lin et al. (14)	Randomized	3	Melphalan 140 mg/m2	8 Gy	0%	-	-
Somlo et al. (15)	Phase I	25	-	MTD 16 Gy	0%	23%	73%
Einsele et al. (9)	Phase I/II	89	Busulfan 12 mg/kg Cyclophosphamide 120 mg/kg	9 Gy	0%	44%	56%

TMI, total marrow irradiation; VGPR, very good partial response; MTD, maximum tolerated dose.

TMI in matched-related and unrelated HCT

The safety and efficacy of TMI and TMLI as part of the conditioning regimen in allogeneic HCT have been evaluated in different publications over the years (Table 2).

Ali et al. (6) evaluated RIC conditioning with busulfan (2 days) and fludarabine with TMI 6 Gy in 26 patients with high-risk hematologic malignancies not eligible for myeloablative transplantation. In this publication, the median age was 64 years, and 73% had active or measurable residual disease at transplantation. They included 18 matched-unrelated donors, five matched sibling donors, and three haploidentical donors. All patients engrafted neutrophils. The 1-year overall survival was 65%, the 1-year TRM was 4%, and the 1-year cumulative incidence of relapse was 43%, demonstrating a feasible intensification of RIC conditioning with TMI in medically frail patients with high-risk disease. These results show that low-dose TMI combined with a RIC conditioning regimen leads to a high engraftment rate. The relapse rate was relatively high, although the frequency of active disease prior to HCT was also high.

Reduced-intensity conditioning (RIC) may provide reasonable disease control in frail patients with high-risk hematologic malignancies undergoing allogeneic HCT. Wong et al. (28), Rosenthal et al. (24), and Jensen et al. (20) prospectively studied the association of a RIC conditioning regimen with melphalan and fludarabine associated with TMLI 12 Gy. Wong et al. evaluated toxicities in eight patients with hematologic malignancies with this conditioning regimen, and grades 2–3 nausea, vomiting, mucositis, and diarrhea were observed, with no grade 4 non-hematologic toxicity. Rosenthal et al. (24) included 33 patients over 50 years or with compromised organ function and showed the median time to neutrophil engraftment of 14 days, 1-year OS of 75%, 2-year NRM of 25%, and 21% deaths due to progressive disease. Jensen et al. evaluated the clinical outcomes of 61 patients with a median age of 55 years and 5-year OS, NRM, and relapse were 44%, 33%, and 26%, respectively (20).

Ogawa et al. (16) tested myeloablative conditioning consisting of cyclophosphamide 60 mg/kg/day for 2 days and TMLI with doses of 14, 16, and 18 Gy for 3 days in 3+3 design in nine patients with acute lymphoblastic leukemia or chronic myeloblastic leukemia with unrelated donors. All patients achieved neutrophil engraftment at a median of 19 days. No patient showed dose-limiting toxicities, and 1-year overall survival was 83.3%. There were three disease relapses and no documented TRM. Stein et al. (21) evaluated TMLI with a dose ranging from 12 to 20 Gy in 51 patients with active relapsed/refractory leukemia undergoing HCT with matched-related or unrelated donors, combined with cyclophosphamide and etoposide. The maximum tolerated dose was 20 Gy. All patients had neutrophil engraftment at a median of 15 days. In this high-risk population, 88% of patients achieved complete response at D+30. With a median follow-up of 24.6 months, 33 patients relapsed. The 1-year OS was 55.5%, and the 1-year TRM was 3.9%. Hui et al. (22) evaluated TMI in a phase I dose-escalation trial with 12 patients who received conditioning therapy with cyclophosphamide and fludarabine in conjunction with TMI at 15 Gy and 18 Gy (in 3 Gy/fractions) while maintaining TBI dose to vital organs at <13.2 Gy. The median time for neutrophil recovery was 26 days. The 1-year OS was 42%, 1-year relapse was 36%, and 1-year TRM was 42%. Although excessive specific organ toxicity was not found, the authors decided to suspend enrollment in the 18 Gy arm due to 50% transplant-related mortality. In summary, these studies show that higher doses of TMI combined with cyclophosphamide-based chemotherapy are feasible and that the maximum tolerated dose of TMI might not have been reached.

In a phase I trial published by Patel et al. (23), TMI 3 Gy, 6 Gy, 9 Gy, and 12 Gy were combined with myeloablative fludarabine and busulfan in 14 patients with high-risk hematologic disease undergoing HCT with HLA-matched related, unrelated, or mismatched donor using peripheral blood grafts. All patients engrafted promptly at a median of 15 days. Extrahematologic toxicities were limited to grades 1–2. With a median follow-up of 3 years, the OS was 50%, with four deaths caused by transplantation-related complications and three due to relapse.

Wong et al. (25) presented 2 phase I trials of a combination of TMI with higher-intensity chemotherapy in patients with advanced leukemias. The first trial consisted of a TMI dose of 12–15 Gy combined with etoposide (60 mg/kg) and cyclophosphamide (100 mg/Kg). The median age of the 12 included patients was 33 years, with a median follow-up of 14.7 months, and five patients

TABLE 2 Clinical results of TMI/TMLI in matched-donor allogeneic transplants.

Author, year	Type of study	*N*	Disease	Disease status at transplant	Conditioning regimen	TMI dose	Lymphoid irradiation	TRM	OS	Relapse
Ogawa et al. (16)	Prospective phase I trial	9	ALL and CMML	Complete response	Cyclophosphamide and TMLI	14–18Gy	Yes	0	1 y:83%	33,3% (3 cases)
Ali et al. (6)	Prospective phase I trial	23	Hematological malignancies	73% had active or measurable residual disease at transplant	Busulfan (2 days) and fludarabine	6Gy	No	1y:4%	1y 65%	1y: 43%
Stein et al. (17)	Prospective phase II trial	18	AML	Complete response and (MRD)-negative	TMLI and PTCy	20Gy	Yes	2y:0%	2y:86.7%	2y:16.7%
Haraldsson et al. (18)	Prospective observational study	37	Hematological malignancies	Complete and partial response	TMI-based	12 Gy	No	-	1y GRFS: 67.5%	-
Shi et al. (19)	Retrospective study	61	Hematological malignancies	20/ patients with refractory leukemia	TMI/ TMLI hypo-fractionation	10 Gy	Yes	2 y:5%	2 y:74.7%	27%
Jensen et al. (20)	Prospective study	61	Hematological malignancies	Complete and partial response and active disease	Fludarabine, Melphalan, TMLI	12 Gy	Yes	5 y:41%	5 y:33%	5 y:26%
Stein et al. (21)	Prospective phase I study	51	Acute leukemia	relapsed/refractory	Cyclophosphamide and etoposide, TMLI	12–20 Gy	Yes	1y:3.9%	1y:55.5%	64%
Hui et al. (22)	Prospective phase I study	12	Acute leukemia and prolymphocytic leukemia	Complete and partial response and active disease	Cyclophosphamide and fludarabine, TMI	15 Gy and 18 Gy	No	1 y:42%	1 Y:42%	1 y:36%
Patel et al. (23)	Prospective phase I study	14	High-risk hematologic malignancies	Complete response and active disease	Busulfan and fludarabine, TMI	3–12 Gy	No	3 y:28%	3 y:50%	
Rosenthal et al. (24)	Prospective Phase 1/2 study	33	Advanced hematologic disease	Complete and partial response and active disease	Fludarabine, Melphalan, TMLI	12 Gy	Yes	2 y:25%	1 y:75%	21%
Wong et al. (25)	Prospective phase I study	12	Advanced AML/ALL	Induction failure and first relapse	TMI, etoposide and cyclophosphamide	12–15 Gy	No	16%	50%	33.3%
Wong et al. (25)	Prospective phase I study	20	Advanced AML/ALL	Induction failure, first and second relapse	Busulfan and etoposide, TMI	12–13.5Gy	No	40%	25%	35%

ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CMML, chronic myelomonocytic leukemia; TMLI, total marrow and lymphoid irradiation; TMI, total marrow irradiation; TRM, transplant-related mortality; OS, overall survival; PTCy, post-transplant cyclophosphamide; GRFS, graft-vs.-host disease-relapse-free survival.

10.3389/fmed.2023.1155954

remained in CR, and no dose-limiting toxicity was achieved. The second trial consisted of TMI 12–15.5 Gy associated with busulfan (4 days) and etoposide (30 mg/kg). They included 20 patients with a median age of 41 years. Grade 4 dose-limiting toxicities were seen at 13.5 Gy (stomatitis and hepatotoxicity) (25). Therefore, doses of TMI combined with higher-intensity, busulfan-based chemotherapy, probably should be limited to 12 Gy.

Haraldsson et al. (18) compared 37 patients who underwent allogeneic transplants and received TMI to a retrospective control of 33 patients that had received TBI. Dose distribution for TMI patients was kept to a minimum in kidneys, heart, bowel bag, and liver. The 1-year GVHD-free, relapse-free survival (GRFS) was 67.5% for TMI and 39.4% for TBI ($p = 0.03$), and this difference was higher in patients receiving MUD HCT ($p < 0.01$). No significant differences in neutrophil recovery, overall survival (OS), transplant-related mortality (TRM), or relapse were found. Therefore, compared with TBI, TMI may offer superior results in terms of GRFS. A recent study compared dosimetric changes in irradiation received by the target volume and organs at risk between TBI and TMI plans. The authors theoretically calculated TMI plans for 35 patients who had already received TBI conditioning. In this analysis, TMI significantly reduced the dose in organs at risk, with mean dose reduction in the liver by 49% and spleen by 55–59%, and achieved the prescribed dose in the target volume (29).

Shi et al. (19) evaluated 61 patients with hematologic malignancies who underwent HLA-matched related or unrelated HCT with peripheral blood stem cells. In eight patients, the conditioning regimen consisted of TMI, and in 53 patients, TMLI. Patients received 8 Gy in the bone marrow in a single day treatment and 10 Gy in the involved field in two fractions a day associated with GVHD prophylaxis with tacrolimus and sirolimus. None of the patients had grades 3–4 non-hematologic toxicities. The 2-year OS was 74.7%, 2-year TRM was 5%, and the relapse rate was 27%, demonstrating that the hypo-fractionation TMI/TMLI scheme may be an alternative in this scenario.

Graft-vs.-host disease (GVHD) is a significant cause of morbidity and mortality after allogeneic stem cell transplantation, and comparisons between haploidentical HCT with post-transplant cyclophosphamide (PTCy) and unrelated donor (URD) HCT have shown comparable overall survival with lower incidences GVHD with PTCy. Therefore, in recent years, PTCy has been expanded to matched transplants. Stein et al. (17) have recently published a study testing TMLI at 20 Gy with PTCy for patients with acute myeloid leukemia in the first or second complete response undergoing matched donor allogeneic HCT. The patient safety lead-in segment followed a 3+3 dose expansion cohort of up to 12 additional patients. PTCy was administered 50 mg/kg on days +3 and +4 after infusion, and tacrolimus was given until day +90 and then tapered. All patients were engrafted. The authors demonstrated the feasibility of a chemotherapy-free conditioning regimen with a 2-year OS of 86.7%, 2-year TRM of 0%, 2-year relapse of 16.7%, and GRFS rate of 59.3% after a median follow-up of 24.5 months.

Another recent study evaluated 50 patients with hematologic malignancies who underwent conditioning with fludarabine, thiotepa, cyclophosphamide, and TMLI total dose of 13.5 Gy and TLI of 11.5 Gy. They included 11 matched-related donors and 39 haploidentical donors. Patients received donor regulatory T cells and conventional T cells before infusion, followed by CD34+ selected grafts on day 0, with no pharmaceutical immunosuppressive therapy. Eighteen patients (36%) had grade II acute GVHD, and the mean TMLI dose to the whole intestine was 7.1 Gy (30).

Extramedullary (EM) relapse in patients receiving TMI or TMLI is a concern in allogeneic HCT. Kim et al. (31) evaluated 101 patients enrolled in TMLI trials between 2006 and 2012, with total radiation doses ranging from 12 to 15 Gy. In this population, EM relapse occurred in 13 patients (12.9%) and bone marrow relapse in 25.7%, comparable to published results with regimens including TBI. Of the 13 patients, seven had the extramedullary disease before HCT, which was the only significant predictor of subsequent EM disease (31).

As TMI decreases radiation in organs at risk and allows dosage escalation to improve oncologic outcomes, subsequent malignant neoplasms (SMNs) are another concern. Han et al. (32) retrospectively compared the estimated radiation-induced, organ-specific, secondary solid-tumor rates in 20 patients who received TMI (10 patients received 12 Gy and 10 patients 20 Gy with 12 Gy to the brain and liver) to a generated conventional TBI treatment plan with a prescription dose of 12 Gy and showed TMI could significantly reduce overall radiation-induced secondary solid-tumor. Another recent publication compared SMN in patients submitted to TBI (171 patients) or TMI-based conditioning regimens (171 patients) to a 12–20 Gy dose. TMI patients received higher radiation doses (16 Gy vs. 13.2 Gy), with no significant difference in the risk of SMN in the two cohorts (nine patients in the TBI group and 10 in the TMI group), and no patients developed a subsequent hematologic malignancy (33).

TMI in haploidentical HCT

Using the PTCy platform has revolutionized haploidentical HCT with low rates of GVHD and TRM. However, relapse remains a concern in high-risk patients. In this context, TMI and TMLI may be an alternative to reduce toxicities without hampering disease control (Table 3). A recent phase I trial has been published, including 31 patients with high-risk leukemias or myelodysplastic syndrome. The conditioning regimen was based on TMLI with increasing doses from 12 to 20 cGy associated with fludarabine and cyclophosphamide. PTCy with tacrolimus and mycophenolate mofetil was the GVHD prophylaxis. All patients achieved neutrophil recovery at a median of 17 days. In addition, the authors demonstrated a 2-year NRM of 13%, a cumulative incidence of grade II to IV acute GVHD at day 100 of 52%, and acute GVHD grades III to IV of 6%. In the group of patients who received recommended TMLI dose at 20 Gy, 1-year overall survival was 83% and 1-year relapse of 17% (26), demonstrating the feasibility and safety of TMLI combined with PTCy.

Sarina et al. (27) evaluated TMLI 2 Gy instead of TBI 2 Gy associated with a non-myeloablative (NMA) conditioning regimen with cyclophosphamide, fludarabine in patients who underwent haploidentical transplantation with PTCy, calcineurin inhibitor,

10.3389/fmed.2023.1155954

TABLE 3 Clinical results of TMI/TMLI in haploidentical allogenic transplants.

Author, year	Type of study	N	Disease	Disease Status at transplante	Conditioning regimen	TMI dose	Lymphoid irradiation	TRM	OS	Relapse
Al Malki et al. (26)	Prospective phase I study	31	leukemias or myelodysplastic syndrome	Complete and partial response and active disease	Fludarabine, cyclophosphamide and TMLI and PTCy	12–20Gy	Yes	2y:13%	1y:83% (20Gy)	1y:17% (20Gy)
Ali et al. (6)*	Prospective phase I trial	3	Hematological malignancies	73% had active or measurable residual disease at transplant	Busulfan (2 days) and fludarabine, TMI and PTCy	6Gy	No	1y:4%	1y 65%	1y: 43%
Sarina et al. (27)	Retrospective study	59	HL, NHL, CLL/PLL	Complete and partial response and active disease	Fludarabine, cyclophosphamide and TMLI and PTCy	2Gy	Yes	2y:23%	2y:63%	2y:23%

*Data of haploidentical were not presented separately.

TMLI, total marrow and lymphoid irradiation; TMI, total marrow irradiation; TRM, transplant-related mortality; OS, overall survival; PTCy, Post-transplant cyclophosphamide; HL, Hodgkin lymphoma; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia.

and mycophenolate mofetil. Fifty-nine patients were included in TMLI and 37 in the TBI group, and, in multivariable analysis, TMLI did not influence OS, TRM, or PFS compared with TBI. This finding is relatively expected since TBI 2 Gy is seldom toxic.

Ali et al. (6) have included three haploidentical transplants in the cohort of patients with high-risk hematologic malignancies submitted to RIC Bu/Flu conditioning associated with TMI. These patients received tacrolimus, MMF, and PTCy, and the authors showed the feasibility of this association in frail patients, although the low number of patients hampers further conclusions.

In summary, these studies show that TMLI and TMI in doses up to 20 Gy are feasible with PTCy and RIC or NMA conditioning regimens. To the best of our knowledge, there is no data on TMLI or TMI combined with myeloablative doses of chemotherapy and PTCy.

Discussion

The data reviewed herein show that TMI and TMLI are feasible and safe and may reduce TBI toxicities associated with different chemotherapy protocols. Furthermore, it can be delivered to other populations, but only a few prospective trials have been published so far, with small samples restricted to phase I or II clinical trials.

In the autologous HCT scenario, some trials with multiple myeloma patients have been done. Multiple myeloma is caused by the abnormal proliferation of plasma cells and accounts for about 15% of all hematological malignancies (34). Over the last 20 years, autologous HCT has been considered the standard therapy for transplant-eligible patients with multiple myeloma, as it improves progression-free survival compared to schemes without HCT, even with new therapies in recent years (35, 36). The most frequently used conditioning regimen is melphalan 200 mg/m^2, but different schemes have been evaluated to increase response rates and progression-free survival. Since multiple myeloma is highly sensitive to radiotherapy, some studies have evaluated the combination of chemotherapy with total body irradiation (TBI), but with no benefit in overall survival because of increased toxicities (37, 38). In this scenario, TMI was evaluated in association with different chemotherapy protocols or as a second HCT and showed that high doses of TMI may be delivered upfront or in the relapse setting. However, as the treatment of multiple myeloma has been revolutionized with new treatments in recent years, these strategies should be tested in the context of highly active induction and maintenance regimens.

In the allogeneic HCT scenario, higher intensities of the conditioning regimens reduce the chance of relapse of the underlying disease (39), at the expense of increased transplant-related mortality, including regimens with TBI (40).

For elderly patients or patients with comorbidities, different reduced-intensity conditioning protocols have been increasingly used, and a conditioning regimen that changes the balance point with lower transplant-related mortality and higher cure rates is desirable. Therefore, TMI and TMLI may be an alternative to these frailer patients. Their feasibility and safety have been demonstrated, with engraftment rates similar to those found with other conditioning regimes, even in haploidentical HCT and associated with PTCy. In addition, the relapse rates of these RIC

regimens with high doses of TMI are encouraging. On the other hand, high doses of busulfan combined with TMI may enhance the antileukemic effect of the conditioning regimen and be an option for younger patients with high-risk leukemia.

The technological gap limits the widespread use of TMI and TMLI. Technical issues in precise radiotherapy are challenging, and manual contouring of targets may take long periods (41). To increase clinical experience and the number of clinical trials with this strategy, it is necessary to develop uniform planning and treatment guidelines in radiation oncology departments and a collaborative effort between radiation oncology and the hematology team. In addition to that, the optimum chemotherapy association for each population still needs to be defined, and comparative trials with other conditioning regimen strategies are needed (42).

Conclusion

In conclusion, TBI has been widely used in HCT at the expense of more significant toxicities. TMI or TMLI addresses the need to reduce the toxicity of TBI to critical organs while increasing the dose to selected targets through technical optimization. This strategy has been used safely and effectively in autologous HCT for multiple myeloma and allogeneic HCT for hematologic malignancies at escalating doses in combination with different chemotherapy regimen protocols. The main benefits of this technique are the possibility of intensifying the conditioning regimen, which leads to a reduced chance of relapse, without increasing transplant toxicity and transplant-related mortality. The main limitations are the availability and training of staff in this technique, long periods for manual contouring of targets, and lack of randomized studies compared with other conditioning regimens. In clinical practice, this technique can be used in association with non-myeloablative conditioning regimens in transplants of unfit or elderly patients due to its low toxicity, reducing relapse rates, including haploidentical transplantation, and also in association with myeloablative conditioning in transplants of young patients with high-risk hematological malignancies.

Author contributions

MK and LA did the review structure, literature search, data discussion, tables, and manuscript preparation. SF and AR performed the summary of radiotherapy findings. NH participated in the design of this study and performed critical review of the manuscript. All authors provided critical review of the manuscript and approved the submitted version.

Conflict of interest

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Gyurkocza B, Sandmaier BM. Conditioning regimens for hematopoietic cell transplantation: one size does not fit all. *Blood.* (2014) 124:344–53. doi: 10.1182/blood-2014-02-514778

2. Clift RA, Buckner CD, Appelbaum FR, Sullivan KM, Storb R, Thomas ED. Long-term follow-up of a randomized trial of two irradiation regimens for patients receiving allogeneic marrow transplants during first remission of acute myeloid leukemia. *Blood.* (1998) 92:1455–6. doi: 10.1182/blood.V92.4.1455

3. Peters LJ, Withers HR, Cundiff JH, Dicke KA. Radiobiological considerations in the use of total-body irradiation for bone-marrow transplantation. *Radiology.* (1979) 131:243–7. doi: 10.1148/131.1.243

4. Shank B, O'Reilly RJ, Cunningham I, Kernan N, Yaholom J, Brochstein J, et al. Total body irradiation for bone marrow transplantation: the memorial Sloan-Kettering cancer center experience. *Radiotherapy Oncol.* (1990) 18:68–81. doi: 10.1016/0167-8140(90)90180-5

5. Wong JYC, Liu A, Han C, Dandapani S, Schultheiss T, Palmer J, et al. Total marrow irradiation (TMI): addressing an unmet need in hematopoietic cell transplantation—A single institution experience review. *Front Oncol.* (2022) 12:1003908. doi: 10.3389/fonc.2022.1003908

6. Ali N, Sharma AA, de Rezende ACP. Targeted marrow irradiation intensification of reduced-intensity fludarabine/busulfan conditioning for allogeneic hematopoietic stem cell transplantation. *Transplantat Cell Therapy*. (2022) 28:370. doi: 10.1016/j.jtct.2022.04.001

7. Aydogan B, Mundt AJ, Roeske JC. Linac-based intensity modulated total marrow irradiation (IM-TMI). *Technol Cancer Res Treat.* (2006) 5:513–9. doi: 10.1177/153303460600500508

8. Aydogan B, Yeginer M, Kavak GO, Fan J, Radosevich JA, Gwe-Ya K. Total marrow irradiation with rapidArc volumetric arc therapy. *Int J Rad Oncol Biol Physics.* (2011) 81:592–9. doi: 10.1016/j.ijrobp.2010.11.035

9. Einsele H, Bamberg M, Budach W. A new conditioning regimen involving total marrow irradiation, busulfan, and cyclophosphamide followed by autologous PBSCT in patients with advanced multiple myeloma. *Bone Marrow Transplant.* (2003) 32:593–9. doi: 10.1038/sj.bmt.1704192

10. Cailleteau A, Maingon P, Choquet S. Phase 1 study of the combination of escalated total marrow irradiation using helical tomotherapy and fixed high-dose melphalan (140 mg/m^2) followed by autologous stem cell transplantation at first relapse in multiple myeloma. *Int J Rad Oncol Biol Physics*. Published online September 2022:S0360301622033946. doi: 10.1016/j.ijrobp.2022.09.069

11. Ladbury C, Somlo G, Dagis A. Long-Term follow-up of multiple myeloma patients treated with tandem autologous transplantation following melphalan and upon recovery, total marrow irradiation. *Transplant Cell Therapy*. (2022) 28:367–9. doi: 10.1016/j.jtct.2022.04.022

12. Giebel S, Sobczyk-Kruszelnicka M, Blamek S, Saduś-Wojciechowska M, Najda J, Czerw T, et al. Tandem autologous hematopoietic cell transplantation with sequential use of total marrow irradiation and high-dose melphalan in multiple myeloma. *Bone Marrow Transplant.* (2021) 56:1297–304. doi: 10.1038/s41409-020-01181-x

13. Patel P, Oh AL, Koshy M. A phase 1 trial of autologous stem cell transplantation conditioned with melphalan 200 mg/m ^{2} and total marrow irradiation (TMI) in patients with relapsed/refractory multiple myeloma. *Leuk Lymphoma.* (2018) 59:1666–71. doi: 10.1080/10428194.2017.1390231

14. Lin SC, Hsieh PY, Shueng PW, Tien HJ, Wang LY, Hsieh CH. Total marrow irradiation as part of autologous stem cell transplantation for asian patients with multiple myeloma. *Biomed Res Int.* (2013) 2013:1–7. doi: 10.1155/2013/321762

15. Somlo G, Spielberger R, Frankel P, Karanes C, Krishnan A, Parker P, et al. Total marrow irradiation: a new ablative regimen as part of tandem autologous stem cell transplantation for patients with multiple myeloma. *Clin Cancer Res.* (2011) 17:174–82. doi: 10.1158/1078-0432.CCR-10-1912

16. Ogawa H, Konishi T, Najima Y. Phase I trial of myeloablative conditioning with 3-day total marrow and lymphoid irradiation for leukemia. *Cancer Sci.* (2022) 3:cas.15611. doi: 10.1111/cas.15611

17. Stein AS, Malki MMA, Yang D, Palmer JM, Tsai N-C, Aldoss I, et al. Total marrow and lymphoid irradiation with post-transplantation cyclophosphamide for patients with AML in remission. *Transplant Cell Therapy.* (2022) 28:368. doi: 10.1016/j.jtct.2022.03.025

18. Haraldsson A, Wichert S, Engström PE, Lenhoff S, Turkiewicz D, Warsi S, et al. Organ sparing total marrow irradiation compared to total body irradiation prior to allogeneic stem cell transplantation. *Eur J Haematol.* (2021) 107:393–407. doi: 10.1111/ejh.13675

19. Shi L, Lu X, Deng D, Yang L, Zhao H, Shen J, et al. The safety and efficacy of a novel hypo-fractionated total marrow and lymphoid irradiation before allogeneic stem cell transplantation for lymphoma and acute leukemia. *Clin Translat Rad Oncol.* (2021) 26:42–6. doi: 10.1016/j.ctro.2020.11.004

20. Jensen LG, Stiller T, Wong JYC, Palmer J, Stein A, Rosenthal J. Total marrow lymphoid irradiation/fludarabine/melphalan conditioning for allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* (2018) 24:301–7. doi: 10.1016/j.bbmt.2017.09.019

21. Stein A, Palmer J, Tsai NC. Phase I trial of total marrow and lymphoid irradiation transplantation conditioning in patients with relapsed/refractory acute leukemia. *Biol Blood Marrow Transplant.* (2017) 23:618–24. doi: 10.1016/j.bbmt.2017.01.067

22. Hui S, Brunstein C, Takahashi Y, DeFor T, Holtan SG, Bachanova V, et al. Dose escalation of total marrow irradiation in high-risk patients undergoing allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant.* (2017) 23:1110–6. doi: 10.1016/j.bbmt.2017.04.002

23. Patel P, Aydogan B, Koshy M, Mahmud D, Oh A, Saraf SL, et al. Combination of linear accelerator–based intensity-modulated total marrow irradiation and myeloablative fludarabine/busulfan: a phase I study. *Biol Blood Marrow Transplant.* (2014) 20:2034–41. doi: 10.1016/j.bbmt.2014.09.005

24. Rosenthal J, Wong J, Stein A, et al. Phase 1/2 trial of total marrow and lymph node irradiation to augment reduced-intensity transplantation for advanced hematologic malignancies. *Blood.* (2011) 117:309–15. doi: 10.1182/blood-2010-06-288357

25. Wong JYC, Forman S, Somlo G, Rosenthal J, Liu A, Schultheiss T, et al. Dose escalation of total marrow irradiation with concurrent chemotherapy in patients with advanced acute leukemia undergoing allogeneic hematopoietic cell transplantation. *Int J Rad Oncol Biol Physics.* (2013) 85:148–56. doi: 10.1016/j.ijrobp.2012.03.033

26. Malki MMA, Palmer J, Tsai NC, Mokhtari S, Hui S, Tsai W, et al. Total marrow and lymphoid irradiation as conditioning in haploidentical transplant with posttransplant cyclophosphamide. *Blood Advances.* (2022) 6:4098–106. doi: 10.1182/bloodadvances.2022007264

27. Sarina B, Mancosu P, Navarria P, Bramanti S, Mariotti J, De Philippis C, et al. Nonmyeloablative conditioning regimen including low-dose total marrow/lymphoid irradiation before haploidentical transplantation with post-transplantation cyclophosphamide in patients with advanced lymphoproliferative diseases. *Transplant Cell Therapy.* (2021) 27:492. doi: 10.1016/j.jtct.2021.03.013

28. Wong JYC, Rosenthal J, Liu A, Schultheiss T, Forman S, Somlo G. Image-guided total-marrow irradiation using helical tomotherapy in patients with multiple myeloma and acute leukemia undergoing hematopoietic cell transplantation. *Int J Rad Oncol Biol Physics.* (2009) 73:273–9. doi: 10.1016/j.ijrobp.2008.04.071

29. Köksal M, Kersting L, Schoroth F, Garbe S, Koch D, Scafa D, et al. Total marrow irradiation versus total body irradiation using intensity-modulated helical tomotherapy. *J Cancer Res Clin Oncol.* (2023) 6:2. doi: 10.1007/s00432-022-04565-2

30. Saldi S, Fulcheri CPL, Zucchetti C, Abdelhamid AMH, Carotti A, Pierini A, et al. Impact of total marrow/lymphoid irradiation dose to the intestine on graft-versus-host disease in allogeneic hematopoietic stem cell transplantation for hematologic malignancies. *Front Oncol.* (2022) 12:1035375. doi: 10.3389/fonc.2022.1035375

31. Kim JH, Stein A, Tsai N, Schultheiss TE, Palmer J, Liu A, et al. Extramedullary relapse following total marrow and lymphoid irradiation in patients undergoing allogeneic hematopoietic cell transplantation. *Int J Rad Oncol Biol Physics.* (2014) 89:75–81. doi: 10.1016/j.ijrobp.2014.01.036

32. Han C, Liu A, Wong JYC. Estimation of radiation-induced, organ-specific, secondary solid-tumor occurrence rates with total body irradiation and total marrow irradiation treatments. *Pract Radiat Oncol.* (2020) 10:e406–14. doi: 10.1016/j.prro.2020.03.006

33. Ladbury C, Armenian S, Bosworth A, He T, Wong FL, Dandapani S, et al. Risk of subsequent malignant neoplasms following hematopoietic stem cell transplantation with total body irradiation or total marrow irradiation: insights from early follow-up. *Transplant Cell Therapy.* (2022) 28:860. doi: 10.1016/j.jtct.2022.09.013

34. Siegel RL, Miller KD, Fuchs HE, Jemal A. Cancer statistics, 2021. *CA A Cancer J Clin.* (2021) 71:7–33. doi: 10.3322/caac.21654

35. Attal M, Lauwers-Cances V, Hulin C. Lenalidomide, bortezomib, and dexamethasone with transplantation for myeloma. *N Engl J Med.* (2017) 376:1311–20. doi: 10.1056/NEJMoa1611750

36. Richardson PG, Jacobus SJ, Weller EA, Hassoun H, Lonial S, Raje NS, et al. Triplet therapy, transplantation, and maintenance until progression in myeloma. *N Engl J Med.* (2022) 387:132–47. doi: 10.1056/NEJMoa2204925

37. Moreau P. Comparison of 200 mg/m2 melphalan and 8 Gy total body irradiation plus 140 mg/m2 melphalan as conditioning regimens for peripheral blood stem cell transplantation in patients with newly diagnosed multiple myeloma: final analysis of the Intergroupe Francophone du Myelome 9502 randomized trial. *Blood.* (2002) 99:731–5. doi: 10.1182/blood.V99.3.731

38. Blade J. High-dose therapy intensification compared with continued standard chemotherapy in multiple myeloma patients responding to the initial chemotherapy: long-term results from a prospective randomized trial from the Spanish cooperative group PETHEMA. *Blood.* (2005) 106:3755–9. doi: 10.1182/blood-2005-03-1301

39. Scott BL, Pasquini MC, Logan BR. Myeloablative vs. reduced-intensity hematopoietic cell transplantation for acute myeloid leukemia and myelodysplastic syndromes. *JCO.* (2017) 35:1154–61. doi: 10.1200/JCO.2016.70.7091

40. Clift RA, Buckner CD, Appelbaum FR, Bearman SI, Petersen FB, Fisher LD, et al. Allogeneic marrow transplantation in patients with acute myeloid leukemia in first remission: a randomized trial of two irradiation regimens. *Blood.* (1990) 76:1867–71.

41. Schultheiss TE, Wong J, Liu A, Olivera G, Somlo G. Image-guided total marrow and total lymphatic irradiation using helical tomotherapy. *Int J Rad Oncol Biol Physics.* (2007) 67:1259–67. doi: 10.1016/j.ijrobp.2006.10.047

42. Wong JYC, Filippi AR, Scorsetti M, Hui S, Muren LP, Mancosu P. Total marrow and total lymphoid irradiation in bone marrow transplantation for acute leukaemia. *Lancet Oncol.* (2020) 21:e477–87. doi: 10.1016/S1470-2045(20)30342-9

frontiers | Frontiers in Medicine

TYPE Study Protocol
PUBLISHED 19 May 2023
DOI 10.3389/fmed.2023.1166871

OPEN ACCESS

EDITED BY
Mutlu Arat,
Istanbul Florence Nightingale Hospital, Türkiye

REVIEWED BY
Tomomi Toubai,
Yamagata University, Japan
Antonia Mueller,
Medical University of Vienna, Austria
Robert Zeiser,
University of Freiburg, Germany

*CORRESPONDENCE
Alexandros Spyridonidis
✉ spyridonidis@upatras.gr

†These authors share first authorship

‡These authors share senior authorship

RECEIVED 15 February 2023
ACCEPTED 19 April 2023
PUBLISHED 19 May 2023

CITATION
Lysandrou M, Kefala D, Christofi P, Savvopoulos N, Papayanni PG, Theodorellou R, Sagiadinou E, Zacharioudaki V, Moukouli M, Tsokanas D, Karavalakis G, Liga M, Stavrinos K, Papadopoulou A, Yannaki E and Spyridonidis A (2023) Study protocol: Phase I/II trial of induced HLA-G⁺ regulatory T cells in patients undergoing allogeneic hematopoietic cell transplantation from an HLA-matched sibling donor.
Front. Med. 10:1166871.
doi: 10.3389/fmed.2023.1166871

Study protocol: Phase I/II trial of induced HLA-G⁺ regulatory T cells in patients undergoing allogeneic hematopoietic cell transplantation from an HLA-matched sibling donor

Memnon Lysandrou[1†], Dionysia Kefala[1†], Panayiota Christofi[1,2], Nikolaos Savvopoulos[1], Penelope Georgia Papayanni[2], Rodanthy Theodorellou[3], Eleftheria Sagiadinou[1], Vassiliki Zacharioudaki[1], Maria Moukouli[3], Dimitrios Tsokanas[1], Georgios Karavalakis[2], Maria Liga[1], Konstantinos Stavrinos[3], Anastasia Papadopoulou[2], Evangelia Yannaki[2‡] and Alexandros Spyridonidis[1*‡]

[1]Bone Marrow Transplantation Unit and Institute of Cell Therapy, University of Patras, Rio, Greece, [2]Gene and Cell Therapy Center, Hematopoietic Cell Transplantation Unit, Hematology Department, "George Papanikolaou" Hospital, Thessaloniki, Greece, [3]Pharmassist Ltd., Athens, Greece

Regulatory T-cell (Treg) immunotherapy has emerged as a promising and highly effective strategy to combat graft-versus-host disease (GvHD) after allogeneic hematopoietic cell transplantation (allo-HCT). Both naturally occurring Treg and induced Treg populations have been successfully evaluated in trials illustrating the feasibility, safety, and efficacy required for clinical translation. Using a non-mobilized leukapheresis, we have developed a good manufacturing practice (GMP)-compatible induced Treg product, termed iG-Tregs, that is enriched in cells expressing the potent immunosuppressive human leucocyte antigen-G molecule (HLA-G⁺). To assess the safety and the maximum tolerable dose (MTD) of iG-Tregs, we conduct a phase I–II, two-center, interventional, dose escalation (3+3 design), open-label study in adult patients undergoing allo-HCT from an HLA-matched sibling donor, which serves also as the donor for iG-Treg manufacturing. Herein, we present the clinical protocol with a detailed description of the study rationale and design as well as thoroughly explain every step from patient screening, product manufacturing, infusion, and participant follow-up to data collection, management, and analysis (registered EUDRACT-2021-006367-26).

KEYWORDS

allogeneic hematopoietic cell transplantation, graft versus host disease, adoptive cellular immunotherapy, regulatory T cells, HLA-G, hypomethylating agents, advanced therapeutic medicinal products (ATMPs)

Background and rationale

Allogeneic hematopoietic stem cell transplantation (allo-HCT) has become a standard treatment for patients with marrow failure syndromes and hematologic malignancies such as acute leukemia. More than 20.000 allo-HCTs are performed every year in Europe with the numbers increasing (1). During allo-HCT, the patient is prepared with a conditioning regimen (e.g., chemotherapy and radiotherapy) to eliminate existing hematopoiesis and immune system followed by the administration of hematopoietic stem cells harvested from the donor. The graft will in turn engraft, proliferate, and finally reconstitute hematopoiesis and lymphopoiesis in the recipient (2). The therapeutic potential of this high-risk intervention relies on the ability of the engrafting immune system to mount a highly effective, alloreactive immune response against leukemia, termed the graft-versus-leukemia effect. However, this treatment is often accompanied by the occurrence of graft-versus-host disease (GvHD), a common life-threatening complication in which donor T cells attack the host's normal tissues, which can occur early (acute, aGvHD) or later (chronic, cGvHD) after allo-HCT. GvHD can occur despite aggressive prophylaxis even when the donor is a matched HLA-identical sibling (3). Despite groundbreaking discoveries in GvHD pathobiology and advances in our understanding of the underlying mechanisms of this disease, conventional immunosuppressive pharmacotherapy remains the mainstay of GvHD prophylaxis and treatment. In particular, GvHD prophylaxis relies on the administration of calcineurin inhibitors, anti-T-lymphocyte agents, methotrexate, mycophenolate mofetil, or more recently post-transplantation cyclophosphamide. Acute and chronic GvHD treatment depends mostly on corticosteroids. Only during the past few years have other drugs gained approval as second-line treatment, such as Janus kinase 1/2 (JAK1/2), Bruton's tyrosine kinase (BTK), or Rho-associated coiled-coil-containing protein kinase-2 (ROCK2) inhibitors, and real-life data for their efficacy need to be collected. GvHD and its currently available immunosuppressive treatment still pose the principal cause of post-transplant impairment of quality of life, morbidity (35–50%), and mortality (20–30%) after allo-HSCT, and in particular, patients with refractory GvHD have a dismal prognosis (4). Subsequently, the necessity for the development of novel preventive and therapeutic strategies is warranted.

A very promising alternative strategy against GvHD is immunotherapy using the adoptive transfer of T cells with regulatory properties (Tregs) as a living drug aiming to avoid prolonged pharmacological immunosuppression. There is convincing evidence in pre-clinical models and promising data from early-phase clinical trials that immunotherapy with naturally occurring Tregs (nTregs) is feasible, safe, and can suppress exuberant immune activation (5–9). However, their low numbers in the periphery and the lack of specific cell surface markers for efficient purification challenge the clinical translation of nTregs. We and other researchers attempt to overcome these hurdles by developing protocols for the *ex vivo* generation of stable induced Treg (iTreg) products of a defined phenotype that can be easily manufactured for clinical purposes. Some of these products have proceeded to phase I/II clinical studies showing the feasibility and safety of this approach with encouraging results (10, 11). Our approach aspired to mimic the physiological mechanism of the successful immune tolerance transpiring during pregnancy, where the human leukocyte antigen-G (HLA-G), a well-known immunoregulatory molecule, is expressed in the placenta, thereby protecting the "semi-allogeneic" fetus from maternal immune rejection (12). As the HLA-G gene is epigenetically repressed after prenatal life and the methylation status of the HLA-G promoter regulates its transcriptional activity, we showed in small-scale *in vitro* experiments that exposure of human peripheral T cells to hypomethylating agents (azacitidine or decitabine) induces a *de novo* and stable expression of HLA-G, in turn, converting them to Tregs with *in vitro* immunosuppressive functions (13, 14). Subsequently, we developed and validated the manufacturing process of an HLA-G$^+$ regulatory T-cell-enriched product, termed iG-Tregs, that exerts its suppressive function through the HLA-G using a clinical scale and good manufacturing practice (GMP)-grade protocol (15). iG-Tregs can be consistently and robustly produced and display suppressive properties with a favorable safety profile both *in vitro* and *in vivo* (manuscript in preparation). Herein, we present the protocol of the ongoing phase I/II clinical study for the evaluation of iG-Tregs against GvHD in the context of HLA-matched sibling donor allo-HCT.

Study setting

This is a phase I–II, two-center, interventional, dose escalation, open-label study of iG-Tregs as GvHD prophylaxis in adult patients undergoing HLA-matched sibling donor HCT (Group A). The study includes a dose escalation phase I cohort to define the maximum tolerable dose (MTD) and an extension phase II cohort at the selected MTD. The primary objective is to assess the safety of *ex vivo* generated iG-Tregs in adult patients undergoing HLA-matched sibling donor HCT and the secondary objective is to assess the clinical efficacy in preventing GVHD. Patient enrollment will also be enabled for an ancillary study for the clinical evaluation of iG-Tregs in the treatment of patients with cGvHD refractory to at least two lines of treatment (Group B; Supplementary Data).

The clinical study will be conducted at the bone marrow transplantation (BMT) unit of the University General Hospital of Patras (UGHP), Rio, Greece, and HCT Unit (HCTU), "George Papanicolaou" Hospital, Thessaloniki, Greece, which are both equipped with GMP facilities and allo-HCTs are routinely performed according to the Joint Accreditation Committee ISCT-Europe & EBMT (JACIE) standards (16). The current protocol has received approval from competent authorities (National Organization for Medicines: IS 129-22, National Ethics Committee: 181/22) and is registered in the European Union Drug Regulating Authorities Clinical Trials (EUDRACT) database (2021-006367-26).

Study population

Recruitment of patients focuses on adults (>16 years of age) who undergo an allo-HCT from an HLA-matched sibling donor. The sibling donor will provide the starting material for iG-Treg manufacturing. All patients will be evaluated for eligibility after allo-HCT, along with the availability and eligibility of the sibling donor for leukapheresis at day (d) 30 after allo-HCT. The sample size is determined based on the "3 + 3" design (17). Briefly, patients will receive the iG-Tregs in cohorts of three (Cohort 1: 0.1×10^6 iG-Tregs/kg; Cohort 2: 0.5×10^6 iG-Tregs/kg; and Cohort 3: 1.5×10^6 iG-Tregs/kg), in a dose escalation fashion if no dose-limiting toxicity (DLT) is documented. The occurrence of DLT in one patient leads to the extension of the cohort to a total of six patients, whereas the presence of DLT in more than one patient of a given cohort denotes that the MTD has been surpassed. During phase

II, the enrollment of an additional eight patients and infusion of the MTD or best available dose (Cohort 4) will take place to collect further information concerning the safety profile (Figure 1). Collectively, a maximum of 26 patients could be enrolled in this study.

Patient inclusion criteria

1. Previous HLA-matched sibling allo-HCT at least 30 days before.
2. The following criteria must be fulfilled at the initial assessment and on the day of the iG-Treg infusion:
   a. Performance status: Karnofsky ≥80%.
   b. Adequate hematopoiesis and organ function.
   c. Negative pregnancy test (female patients).
3. Ability to understand and willingness to sign an informed consent form.

Additional inclusion criteria for the Group B patients enrolled in the ancillary study can be found in the Supplementary Data.

Patient exclusion criteria

1. History of a GVHD grade ≥ II [according to the MAGIC criteria (18)] or administration of any first-line systematic treatment against aGvHD.
2. Patients with evidence of residual disease during the final assessment.
3. Active serious infections not responding to treatment.
4. HBV-, HCV-, or HIV-positive patients.
5. Administration of any investigation drug/product ≤28 days prior to iG-Treg infusion.

Donor inclusion criteria

1. The HLA-matched sibling donor who donated the graft for the allo-HCT of the corresponding patient will be assessed and fulfill the requirements according to institution procedures and JACIE standards to undergo leukapheresis (donor lymphocytes) without prior granulocyte colony-stimulating factor (G-CSF) infusion.
2. Age between 16 and 75 years.
3. Body weight > 40 kg and in good general health.
4. The donor must be able to understand and be willing to sign an informed consent form.
5. Negative pregnancy test (female donors).

Study timeline

Allo-HCT

The day of graft infusion is defined as d0. Succeeding the allo-HCT, the patient serially follows carefully planned visits through the screening, follow-up, and extended follow-up periods (Figure 1).

Screening period

Visit-2 (week 4 post-allo-HCT)—Patient and donor eligibility

During this visit, the patient and the sibling donor are checked for inclusion eligibility. The consent form is signed and collected.

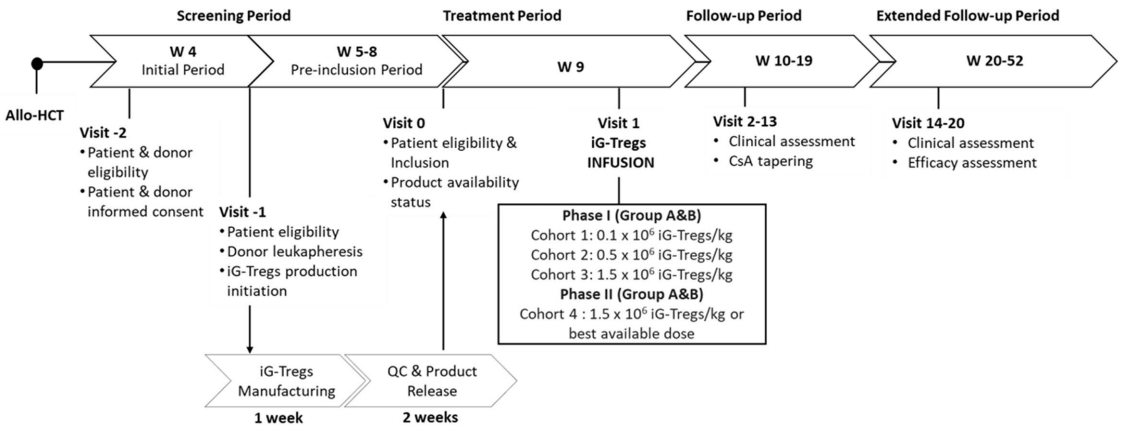

FIGURE 1
Participant timeline of the iG-Tregs phase I/II clinical trial (Group A). The day of the HLA-matched sibling allo-HCT defines the beginning of the study timeline. The screening period starts 4weeks (W) following the transplant where the eligibility of the patient and donor is assessed, and informed consent is given. At W5, the sibling donor undergoes leukapheresis, and iG-Tregs production is initiated, which requires 1week for manufacturing and 2weeks for quality control (QC) and product release. At W5, the patient is re-evaluated for eligibility, and if the product is released and available, the patient is included. Upon inclusion, iG-Tregs infusion takes place within 1week (W9) at a dose according to the current recruiting cohort. During the subsequent follow-up period (W10-19), the patient is continually assessed, and cyclosporine (CsA) tapering is allowed from W13 onwards. Finally, for the extended follow-up period (W20-52) patient clinical evaluation continues and the efficacy of iG-Tregs is assessed. Allo-HCT, allogeneic hematopoietic cell transplantation; Group A refers to the patients recruited for the main study for GvHD prophylaxis; Group B refers to the patients recruited for the ancillary study for chronic GvHD treatment.

TABLE 1 Quality control testing of the final iG-Tregs product.

Quality control test	Method	Criteria	Purpose
Sterility	Cultures for aerobic and anaerobic bacteria	Negative at week 7	Final release
	Cultures for fungi	Negative at week 8	
Mycoplasm	Biochemical assay	Negative	Final release
Endotoxin levels	LAL test	<5.0 EU/kg	Final release
Immunophenotype	Flow Cytometry	CD3 + ≥ 80%, alive HLA-G+ cells ≥10%	Product manufacturing
Cell number	Cell counting using hemocytometer	Cell Number ≥ 0,1*10^6 /kg	Product manufacturing
Viability	Trypan Blue staining	Viable cells ≥50%	Product manufacturing

Visit-1 (week 5 post-allo-HCT)—donor leukapheresis

The patient is screened to meet all eligibility criteria, and the sibling donor undergoes leukapheresis for the initiation of the iG-Tregs manufacturing. The donor undergoes steady-state leukapheresis according to institutional guidelines for less than 1 h yielding at least 60 mL of the leukapheresis product.

iG-Tregs manufacturing period (weeks 5–8 post-allo-HCT)

The leukapheresis product will be transferred to the GMP Unit within 24 h in order to generate the final iG-Treg product, according to a GMP-compliant protocol (15). Briefly, T cells are enriched from peripheral blood mononuclear cells, then activated for 3 days using an anti-CD3/CD28 activation agent (GMP TransAct, Miltenyi), and finally undergo hypomethylation treatment with decitabine (Dacogen, Janssen) in the presence of interleukin-2 (Miltenyi) for an additional 3–4 days. The final product is thoroughly washed four times with 10% human serum albumin in Hank's balanced salt solution prior to the final formulation (weeks 5–6). The final cell product will first undergo quality control (QC) testing to assess cell numbers, viability, and HLA-G^+ content, and the transplant center will be informed of product manufacturing completion. Each iG-Tregs product comprises a unique batch of cells intended for a specific patient. The appropriate cell dose depending on the cohort is cryopreserved. Finally, the product goes through rigorous QC testing for the final release (weeks 6–8; Table 1).

Visit 0 (week 8)—patient inclusion

The patient is reassessed to meet all eligibility criteria, and final product availability is confirmed. If the criteria are met, the patient is scheduled to receive the infusion of iG-Tregs within a week.

Treatment period

Visit 1 (week 9 post-allo-HCT)—iG-Tregs infusion

Thawing and intravenous infusion of iG-Tregs are performed bedside by an experienced physician. Prior to infusion, the patient will receive antihistaminic prophylaxis (dimetindene). Patients are closely monitored (pulse oximetry and vital signs) for 1 h post-infusion. If any toxicity and/or adverse event (AE, graded according to CTCAEv.4) occurs, this will be recorded and managed appropriately. Participants will be instructed to avoid steroids up to 1 week after the iG-Tregs infusion.

Follow-up period

Visits 2–13 (weeks 10–19 post-allo-HCT)

At each weekly visit, the patient is evaluated for any AE and the occurrence of aGvHD. It is emphasized that there are no drug restrictions regarding supportive care, or other treatment to prevent disease relapse while steroids should be avoided for at least up to 1 week following iG-Tregs administration. From week 13 and onward, should the patient's status allow, a cyclosporine (CsA) tapering of a 25% weekly dose reduction can be applied until complete cessation.

Extended follow-up period

Visits 14–20 (weeks 20–52 post-allo-HCT)

The patient will be evaluated at predefined time intervals according to the investigational sites' clinical practice protocol and the observed clinical course.

Outcomes

The current clinical trial aims to define the safety, tolerability, and MTD of the iG-Tregs (within a timeframe of 90 days following the infusion) as well as collect data regarding its clinical efficacy (within a time frame of 52 weeks following allo-HCT). The primary outcomes of this study include the (1) incidence of infusion toxicity (within 1 h after the infusion), (2) additional toxicities that may occur related to iG-Tregs infusion (e.g., the occurrence of exacerbation of GvHD, infections, and disease relapse), and (3) AEs occurring during the first 3 weeks following iG-Treg infusion, which will be accounted for the assessment of the safety profile and tolerability of each dose during the dose escalation phase.

The secondary outcomes of this study include the (1) incidence and severity of GvHD, (2) day of CsA cessation, and (3) treatment failure (includes the diagnosis of GvHD, inability to cease CsA administration until d + 150 following allo-HCT, and disease relapse).

Additional secondary outcomes for patients (Group B) enrolled in the ancillary study can be found in the Supplementary Data.

Interruption and early termination of the study

The sponsor reserves the right to suspend enrollment or terminate the study at any time as defined in the clinical study agreement for

reasons including, but not limited to, insufficient data collection, low participant enrollment rate, achievement of full enrollment, conditions imposed by the regulatory authorities, non-compliance with the clinical trial protocol, or medical reasons.

Statistical methods

Given the small numbers, events will be summarized using descriptive statistics, such as frequencies and proportions. GVHD and relapse rates will be estimated as cumulative incidence curves, death in remission as a competing risk for relapse, and death without GVHD as a competing risk for GVHD. Estimates of overall survival (OS) and relapse-free survival (RFS) will be obtained by the method of Kaplan–Meier. Differences between subgroups will be compared using the Fisher exact test for categorical data and the Mann–Whitney U-test for continuous data. Statistical significance was based on $p < 0.05$.

Data collection, management, and monitoring

All required clinical data of this trial will be collected on standardized patient follow-up forms. Confidentiality will be maintained in accordance with current clinical research principles and the General Data Protection Regulation (GDPR), and participants' personal information will be in a pseudonymous format. The study will be monitored by the clinical trial quality assurance company (Contract Research Organization, CRO). Data will be examined for protocol compliance and accuracy against source documents.

Research studies

Additional research studies will be conducted in conjunction with the clinical assessment of enrolled patients. Specifically, extensive patient immunophenotyping using flow cytometry, and iG-Tregs evaluation for effector cytokine production, alloreactivity *in vitro* and *in vivo,* and immune suppressive function both *in vitro* and *in vivo* will be performed. Moreover, released products will undergo T-cell receptor (TCR) repertoire next-generation sequencing (NGS)-based analysis for further characterization and *in vivo* tracking. Finally, serum cytokine levels will be monitored at multiple time points following product infusion.

Discussion

The present protocol aims to assess the clinical translation of a novel iTreg product targeting GvHD post-allo-HCT. Several groups have sought to prevent potentially lethal GvHD by employing adoptive immunotherapies either based on nTregs or iTregs (6–8, 10, 11). Despite the differences in product manufacturing and trial design, previous studies have paved the way for such clinical applications with promising results (19).

Throughout these protocols variability in donor selection, which have been either matched siblings (6, 11), haploidentical (8), or mismatched donors (10) is apparent. Moreover, for nTregs, these approaches have relied mostly on fresh isolation and administration (6, 8) while iTreg products have both been infused as fresh (11) or cryopreserved (10). As far as manufacturing is concerned, fresh isolation of nTregs requires specialized equipment for immunomagnetic isolation and/or flow sorting, whereas iTregs demand laborious time-consuming manufacturing with extensive QC for release. Treg products are commonly diverse in content with the dominant regulatory population varying per patient batch. For example, initial approaches with $CD4^+CD25^+$ selection were accompanied by a high proportion of activated conventional T cells, which were finally co-infused along with the intended nTreg population (8). Overall, even though these trials have recruited low numbers of patients, a phase III trial is currently ongoing, exhibiting encouraging results (7). In our study, the iG-Tregs product is manufactured consistently through a short, simple, and robust protocol (15). Notably, iG-Tregs contain a distinct regulatory population characterized by the surface expression of HLA-G, which enables easy *in vivo* tracking. Subsequently, the presence of HLA-G apart from mediating the suppressive function can serve as a selection marker for in-depth analysis to unravel novel modes of action. This, in turn, will shed light on which mechanism holds clinical relevance and ultimately lead to process refinement and targeted cell engineering.

Up to date, most completed and ongoing trials employing Treg immunotherapies for GvHD in the setting of allo-HCT have focused on prevention. These strategies depicted the production feasibility and safety of these cell therapies by determining the MTD at phase I studies. Common ground for these trials lies upon the infusion of the final product during the peri-transplantation period (day −4 to day 0 of allo-HCT), with the aim of promoting proper immune reconstitution. In our study, product infusion takes place at week 9 post-allo-HCT and not during the peri-transplantation period. Following MTD determination, we shall assess clinical efficacy through the ability to taper and finally cease CsA administration at an earlier time point in contrast to others, where GvHD occurrence was the main parameter. Moreover, previous work has described the effect of immunosuppressive prophylaxis—targeting calcineurin and the mammalian target of the rapamycin (mTOR) pathway—on the function of nTregs (20, 21). Hence, we cannot exclude the possibility of CsA interfering with the function of iG-Tregs, something which we are currently evaluating *in vitro* and *in vivo*. Finally, in an ancillary study, we will evaluate the safety and applicability of iG-Tregs immunotherapy as a third-line treatment for cGvHD.

Collectively, the ongoing phase I/II clinical trial of iG-Tregs constitutes an innovative approach of iTreg immunotherapy in patients undergoing allo-HCT from an HLA-matched sibling donor.

Dissemination policy

The trial has been registered in the EUDRACT registry prior to the inclusion of the first participant to meet the regulatory requirements (EUDRACT-2021-006367-26). After the conclusion and final analysis of the trial data, results will be submitted to a peer-reviewed medical scientific journal.

Ethics statement

The clinical trial is conducted in accordance with the ethical principles for medical research referred to in the Declaration of Helsinki by the World Medical Association and all applicable Greek and European laws and regulations regarding clinical drug trials, including the Principles of Good Clinical Practice (GCPs). Approval was obtained by jurisdictional ethics committees (National Ethics Committee and Institutional Review Boards of UGHP, University of Patras, and George Papanikolaou Hospital) and the Greek National Organization for Medicines (IS 129-22). The Investigator will submit and, where necessary, obtain approval from the aforementioned authorities for all material amendments to the original approved documents.

Author contributions

AS: principal investigator. EY and AS: primary investigators of centers. MLy, RT, MM, KS, EY, and AS: clinical protocol design. MLy, DK, PC, NS, PP, and AP: manufacturing processes. ES, VZ, DT, GK, and MLi: clinical study procedures. MLy, DK, PC, NS, PP, RT, ES, VZ, MM, DT, GK, MLi, KS, AP, EY, and AS: project planning. MLy, DK, and AS: manuscript preparation—initial draft. All authors contributed to the article and approved the submitted version.

Funding

This research has been co-financed by the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship, and Innovation, under the call "RESEARCH—CREATE—INNOVATE (project code: T2EDK–02437)."

Acknowledgments

We would like to thank Maria Tsima, Georgia Tzirou, and Anastasia Christodoulou for their administrative support. We would like to express our gratitude to the charitable organization "Choose Life" for its generous and continuous support.

Conflict of interest

RT, MM, and KS were employed by Pharmassist Ltd.

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1166871/full#supplementary-material

References

1. Passweg JR, Baldomero H, Chabannon C, Basak GW, de la Cámara R, Corbacioglu S, et al. Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplant*. (2021) 56:1651–64. doi: 10.1038/s41409-021-01227-8

2. Singh AK, McGuirk JP. Allogeneic stem cell transplantation: a historical and scientific overview. *Cancer Res*. (2016) 76:6445–51. doi: 10.1158/0008-5472.CAN-16-1311

3. Zeiser R, Blazar BR. Acute graft-versus-host disease-biologic process, prevention, and therapy. *N Engl J Med*. (2017) 377:2167–79. doi: 10.1056/NEJMra1609337

4. Zeiser R, Lee SJ. Three US Food and Drug Administration-approved therapies for chronic GVHD. *Blood*. (2022) 139:1642–5. doi: 10.1182/blood.2021014448

5. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, Strober S, et al. CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. *Nat Med*. (2003) 9:1144–50. doi: 10.1038/nm915

6. Meyer EH, Laport G, Xie BJ, MacDonald K, Heydari K, Sahaf B, et al. Transplantation of donor grafts with defined ratio of conventional and regulatory T cells in HLA-matched recipients. *JCI Insight*. (2019) 4(10):e127244. doi: 10.1172/jci.insight.127244

7. Oliai C, Hoeg RT, Pavlova A, Gandhi A, Muffly L, Mehta RS, et al. Precision-engineered cell therapy Orca-T demonstrates high relapse-free survival at 1 year while reducing graft-versus-host disease and toxicity. *Blood*. (2022) 140:654–6. doi: 10.1182/blood-2022-165654

8. di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, Bonifacio E, et al. Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. *Blood*. (2011) 117:3921–8. doi: 10.1182/blood-2010-10-311894

9. Martelli MF, di Ianni M, Ruggeri L, Falzetti F, Carotti A, Terenzi A, et al. HLA-haploidentical transplantation with regulatory and conventional T-cell adoptive immunotherapy prevents acute leukemia relapse. *Blood*. (2014) 124:638–44. doi: 10.1182/blood-2014-03-564401

10. Chen PP, Cepika AM, Agarwal-Hashmi R, Saini G, Uyeda MJ, Louis DM, et al. Alloantigen-specific type 1 regulatory T cells suppress through CTLA-4 and PD-1 pathways and persist long-term in patients. *Sci Transl Med*. (2021) 13:eabf5264. doi: 10.1126/scitranslmed.abf5264

11. MacMillan ML, Hippen KL, McKenna DH, Kadidlo D, Sumstad D, DeFor TE, et al. First-in-human phase 1 trial of induced regulatory T cells for graft-versus-host disease prophylaxis in HLA-matched siblings. *Blood Adv*. (2021) 5:1425–36. doi: 10.1182/bloodadvances.2020003219

12. Carosella ED, Favier B, Rouas-Freiss N, Moreau P, LeMaoult J. Beyond the increasing complexity of the immunomodulatory HLA-G molecule. *Blood*. (2008) 111:4862–70. doi: 10.1182/blood-2007-12-127662

13. Moreau P, Mouillot G, Rousseau P, Marcou C, Dausset J, Carosella ED. HLA-G gene repression is reversed by demethylation. *Proc Natl Acad Sci U S A*. (2003) 100:1191–6. doi: 10.1073/pnas.0337539100

14. Stamou P, Marioli D, Patmanidi AL, Sgourou A, Vittoraki A, Theofani E, et al. Simple in vitro generation of human leukocyte antigen-G-expressing T-regulatory cells through pharmacological hypomethylation for adoptive cellular immunotherapy against graft-versus-host disease. *Cytotherapy*. (2017) 19:521–30. doi: 10.1016/j.jcyt.2017.01.004

15. Kefala D, Lysandrou M, Christofi P, Pieridou C, Papayanni PG, Savvopoulos N, et al. Immunotherapy: pre-clinical development of a decitabine-induced regulatory HLAG+CD4+-T cell-enriched cell product (IG-TREG) against graft-vs-host-disease. *Cytotherapy*. (2022) 24:S118–9. doi: 10.1016/S1465-3249(22)00322-X

16. JACIE Accreditation|EBMT. (2018) Available at: https://www.ebmt.org/jacie-accreditation

17. Storer BE. Design and analysis of phase I clinical trials. *Biometrics*. (1989) 45:925–37. doi: 10.2307/2531693

18. Harris AC, Young R, Devine S, Hogan WJ, Ayuk F, Bunworasate U, et al. International, multicenter standardization of acute graft-versus-host disease clinical data collection: a report from the Mount Sinai acute GVHD international consortium. *Biol Blood Marrow Transplant*. (2016) 22:4–10. doi: 10.1016/j.bbmt.2015.09.001

19. Hefazi M, Bolivar-Wagers S, Blazar BR. Regulatory T cell therapy of graft-versus-host disease: advances and challenges. *Int J Mol Sci*. (2021) 22:9676. doi: 10.3390/ijms22189676

20. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J, et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood*. (2006) 108:390–9. doi: 10.1182/blood-2006-01-0329

21. Zeiser R, Leveson-Gower DB, Zambricki EA, Kambham N, Beilhack A, Loh J, et al. Differential impact of mammalian target of rapamycin inhibition on CD4+CD25+Foxp3+ regulatory T cells compared with conventional CD4+ T cells. *Blood*. (2008) 111:453–62. doi: 10.1182/blood-2007-06-094482

frontiers | Frontiers in Medicine

TYPE Mini Review
PUBLISHED 02 June 2023
DOI 10.3389/fmed.2023.1212201

OPEN ACCESS

EDITED BY
Eleni Gavriilaki,
G. Papanikolaou General Hospital, Greece

REVIEWED BY
Oonagh Shannon,
Malmö University, Sweden

*CORRESPONDENCE
Claire S. Whyte
✉ c.s.whyte@abdn.ac.uk

RECEIVED 25 April 2023
ACCEPTED 17 May 2023
PUBLISHED 02 June 2023

CITATION
Whyte CS (2023) All tangled up: interactions of the fibrinolytic and innate immune systems. *Front. Med.* 10:1212201. doi: 10.3389/fmed.2023.1212201

All tangled up: interactions of the fibrinolytic and innate immune systems

Claire S. Whyte*

Aberdeen Cardiovascular and Diabetes Centre, The Institute of Medical Sciences, School of Medicine, Medical Sciences and Nutrition, University of Aberdeen, Aberdeen, United Kingdom

The hemostatic and innate immune system are intertwined processes. Inflammation within the vasculature promotes thrombus development, whilst fibrin forms part of the innate immune response to trap invading pathogens. The awareness of these interlinked process has resulted in the coining of the terms "thromboinflammation" and "immunothrombosis." Once a thrombus is formed it is up to the fibrinolytic system to resolve these clots and remove them from the vasculature. Immune cells contain an arsenal of fibrinolytic regulators and plasmin, the central fibrinolytic enzyme. The fibrinolytic proteins in turn have diverse roles in immunoregulation. Here, the intricate relationship between the fibrinolytic and innate immune system will be discussed.

KEYWORDS

fibrinolysis, innate immune, infection, plasminogen, thrombosis

Introduction

Over the last two decades there is increasing awareness of immunothrombosis, where components of the immune system promote coagulation to limit the action of invading pathogens (1). Whilst thromboinflammation describes the inflammatory process induced by pathogens leading to platelet-neutrophil and platelet-monocyte interactions and endothelial dysfunction that promote a prothrombotic environment (2, 3). Activation of monocytes and neutrophils induces release of tissue factor (TF) promoting the extrinsic coagulation pathway, whilst intrinsic coagulation is triggered by binding of factor XII (FXII) to neutrophils (4). Additionally, activated neutrophils degranulate and expel their nuclear and cytoplasmic content to form neutrophil extracellular traps (NETs) during the neutrophil death process, NETosis (4). NETs act as a surface for assembly of procoagulant proteins including TF, FXII and von Willebrand factor (5). Furthermore, released neutrophil elastase cleaves tissue factor pathway inhibitor, thereby dampening the anticoagulant effect and contributing to fibrin persistence (6). The fibrinolytic system is responsible for limiting ongoing fibrin formation and degrading the fibrin meshwork to resolve thrombi.

Fibrinolysis

Plasmin, the central enzyme responsible for fibrin degradation is formed after cleavage of Arg_{561}-Val_{562} of the zymogen form, plasminogen, via plasminogen activators (**Figure 1**). The primary physiological activators are tissue plasminogen activator (tPA) and urokinase (uPA). Efficient tPA-mediated plasminogen activation requires binding of both proteins to fibrin

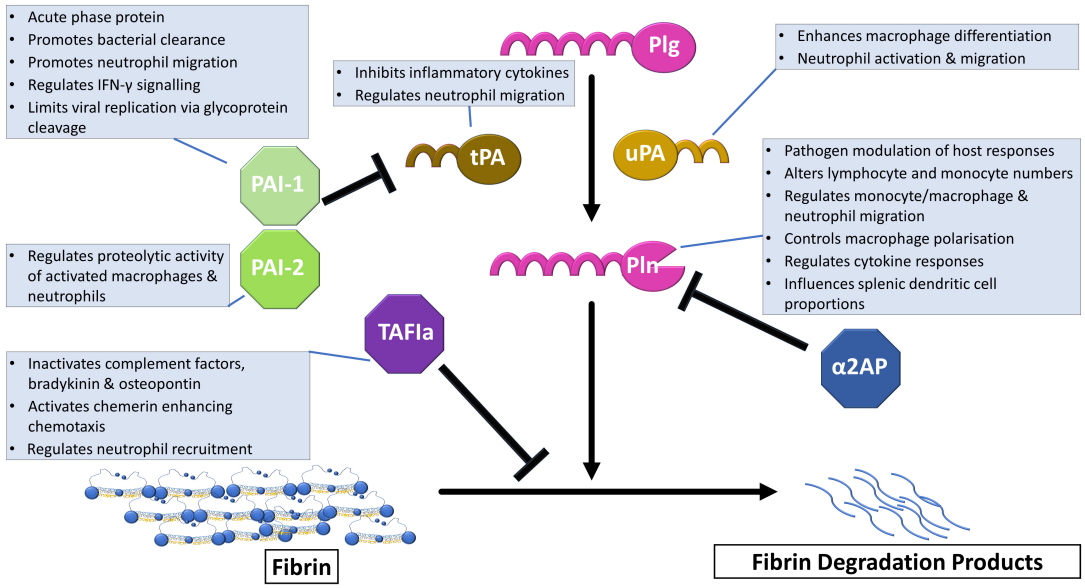

FIGURE 1
The fibrinolytic system in immune regulation. Plasminogen (Plg) is converted to the active enzyme plasmin (Pln) after cleavage by tissue plasminogen activator (tPA) or urokinase (uPA). This step is regulated by plasminogen activator inhibitor-1 (PAI-1), which is the primary physiological inhibitor, and plasminogen activator-2 (PAI-2). The active enzyme, plasmin, cleaves crosslinked fibrin into fibrin degradation products that can be cleared from the circulation. Alpha2-antiplasmin (α2AP) directly inhibits plasmin by forming a non-covalent complex. Activated thrombin activatable fibrinolysis inhibitor (TAFIa) exerts its effects by removal of C-terminal lysine required for plasminogen binding to fibrin. Boxes detail functions of the fibrinolytic proteins in immune regulation.

or cellular surfaces. uPA-mediated activation can occur in solution, although it can be localized to cellular surfaces via urokinase plasminogen activator receptor (uPAR) (7). The fibrinolytic system is normally tightly regulated by various inhibitors. Plasminogen activation is primarily regulated by plasminogen activator inhibitor-1 (PAI-1) which forms a 1:1 complex with the activators (8). Plasminogen activator inhibitor-2 (PAI-2) is not as efficient an inhibitor as PAI-1 but does function in uPA-mediated extracellular activity (9). The principal plasmin inhibitor is the serine protease inhibitor (SERPIN), α2-antiplasmin (α2AP) which forms a non-covalent complex with the active enzyme (10). Crosslinking of α2AP to fibrin by active transglutaminase factor XIII (FXIIIa) enhances the ability of this SERPIN to inhibit plasmin (11). Thrombin activatable fibrinolysis inhibitor (TAFI) further acts as a fibrinolytic break by removing C-terminal lysines from fibrin which are required for the binding of plasminogen and tPA.

Pathogen hijacking of the fibrinolytic system

Invading pathogens take advantage of the fibrinolytic system, activating plasminogen in order to remove the confines of fibrin and extracellular matrix barriers and to evade the innate immune system (12). Indeed, certain strains of bacteria can produce plasminogen activators. Beta hemolytic strains of *Streptococci* possess streptokinase which induces non-proteolytic plasminogen activation by causing a conformational change that exposes the catalytic site and this complex can hydrolytically activate other plasminogen molecules (13). *Staphylococcus aureus* produces staphylokinase which also non-proteolytically activates plasminogen by forming a complex which generates plasmin (14). Staphylokinase is considered to be fibrin specific and in the absence of fibrin it is susceptible to inhibition by α2AP (15). Whilst *Yersinia pestis*, are able to proteolytically activate plasminogen and scuPA by the membrane protein Pla (16). Plasminogen contributes to lethality of *Y. pestis*, promoting spread of the bacteria and dampening immune cell recruitment to sites of infection [reviewed in (17)].

Additionally, a plethora of plasminogen binding proteins (e.g. α-enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PAM) exist on bacteria, fungal pathogens, protozoan and helminth parasites (12, 18, 19). Bacteria utilize plasminogen to remove fibrin barriers and enable invasion through extracellular matrices both directly and indirectly by activating matrix metalloproteases (17, 19–21). Additionally, plasmin-mediated cleavage of members of the complement system and immunoglobulin facilitates immune evasion of some strains of bacteria (22, 23). Bacteria also use plasminogen as a molecular linker to enable interaction with host cells (23).

Binding of plasminogen to *Cryptococcus neoformans* may facilitate the ability of this fungal pathogen to cross the blood brain barrier (24). It has been suggested that the affinity for plasminogen binding could reflect the observed strain differences in virulence of *C. neoformans* (24). However, plasminogen may not function in promoting virulence of all fungal pathogens. Although *Candida albicans* binds plasminogen and can cleave fibrin when in the presence of exogenous plasminogen activators, this binding does not affect virulence or endothelial damage and therefore the *in vivo* significance is not known (18). Multiple species of helminth parasites possess plasminogen binding proteins that

facilitate their invasion and immune evasion (19). Protozoans are also considered to use plasminogen to support their host invasion but binding varies with morphotype and age for *Leishmania mexicana* (25). As not all pathogens can endogenously activate the zymogen they therefore require interaction with host plasminogen activators (18).

Immune cells as sources of fibrinolytic proteins

Plasminogen activation is enhanced by assembly of plasminogen and its activators on fibrin or cellular surfaces (26–29) which also protect plasmin from inhibition by α_2AP (30–32). Plasminogen receptors are found on endothelial cells, platelets, monocytes, macrophages and neutrophils [reviewed in (33)]. The multitude of plasminogen receptors have the common feature of availability of C-terminal basic residues (33). This includes binding proteins that lack a transmembrane protein (e.g., α-enolase and histone 2B), transmembrane proteins that require proteolysis to expose the C-terminal basic residue (e.g., integrins $\alpha_{IIb}\beta_3$ and $\alpha_M\beta_2$) and Plg-R_{KT} a transmembrane protein synthesized with a C-terminal lysine residue (33, 34).

Plg-R_{KT} was first identified on the surface of monocytes and macrophages and co-localizes with uPAR (35) and facilitates plasminogen activation by tPA (35) and uPA (36). Monocyte-derived uPA is required for incorporation of these cells into thrombi for efficient thrombus resolution (37). Although uPA is the predominant plasminogen activator in monocytes, stimulation with lipopolysaccharide (LPS), interferon-γ (IFN-γ) interleukin-4 (IL-4) all induce tPA secretion (38). Monocytes also express PAI-1 and are a major source of PAI-2 (39). Intracellular and secreted PAI-2 can be induced by stimulation of monocytes with thrombin and LPS (39, 40). Presence of PAI-2 in arterial and venous thrombi, presumed to be from monocytes, inhibits uPA-mediated lysis (41, 42). Both PAI-1 and PAI-2 are decreased by targeted upregulation of uPA which enhances fibrinolysis induced by monocyte-derived macrophages (43).

Thrombin activatable fibrinolysis inhibitor is also expressed by monocytes and macrophages with the level of expression being dependent on the activation status (44). Stimulation of macrophages with IL-4 downregulates TAFI expression whilst the proinflammatory stimuli IFN-γ and LPS has no effect (44). Additionally, monocytes and macrophages contain cellular FXIII-A (45, 46) which is trafficked to the membrane in association with golgi vesicles (47). IL-4 and IL-10-induced externalization of FXIII-A on monocytes stabilizes thrombi against degradation (48).

Polymorphonuclear leukocytes, assumed to be neutrophils, participate in endogenous thrombus lysis, mainly mediated by uPA with small contributions from tPA, elastase and cathepsin G (49). More recently neutrophils and their ability to form NETs have gained attention for their antifibrinolytic function. NETs consist of extruded nuclear and cytoplasmic content including histones, DNA strands and granular proteins including neutrophil elastase (4). The presence of DNA, histones and NETs inhibits plasminogen activation *in vitro* which can be reversed by degrading the chromatin with DNase (50, 51). Targeting DNA *in vivo* limits DVT growth in mice (52) and enhances tPA-mediated *ex vivo* thrombolysis of thrombi obtained from acute ischemic stroke patients (53, 54).

Alongside their role in promoting coagulation, platelets also regulate fibrinolysis and form part of the innate immune response. These anucleate cell fragments are packaged with granular content required for these multifaceted functions. Activated platelets expose P-selectin which facilitates interaction with the P-selectin glycoprotein ligand-1 (PSGL1) expressed on leukocytes and endothelial cells. Platelet-leukocyte interactions also occur via CD40-CD40L. These interactions allow platelets to direct leukocytes to sites of inflammation and propagate the inflammatory process (55, 56).

Platelet-rich thrombi are more resistant to lysis than erythrocyte-rich thrombi (57, 58) and platelets have largely been considered to be antifibrinolytic. Platelets are a major pool of circulating PAI-1 which is contained within the α-granules (59). Model thrombi formed at high shear rates contain elevated PAI-1 and lower tPA and plasminogen (60). This is consistent with the greater abundance of PAI-1 in platelet dense arterial thrombi compared to venous thrombi (61, 62). Platelet-derived PAI-1 is retained on activated platelet membranes, localizing to the platelet "cap" or "body" on phosphatidylserine (PS)-exposing procoagulant platelets or centrally over spread platelets (63, 64). This platelet-derived PAI-1 is functional in conferring resistance to lysis (63).

Additional anti-fibrinolytic factors contained within platelet α-granules include TAFI (65, 66), PN-1 (67), and α_2AP (68, 69) which can downregulate fibrinolysis. The role of α_2AP in maintaining thrombus stability may be limited as addition of circulating platelet concentration to α2AP-depeleted plasma does not protect against degradation (70). However, platelets contain a cytoplasmic pool of FXIII-A which crosslinks high molecular weight γ-dimers, α-polymers and α_2AP-fibrin (71–75). Platelets retain externalized cellular FXIII-A in the "cap" region stabilizing thrombi against lysis due to crosslinking of α_2AP (70). FXIII-A is also observed in platelet microparticles translocated via intracellular signaling that is calcium-independent (76).

In contrast to this, platelets support fibrinolytic activity through binding and exposure of plasminogen (28, 64, 77). Strong platelet stimulation facilitates plasminogen binding by fibrin-dependent and fibrin-independent mechanisms (64, 78). Plg-R_{KT} accounts for binding of approximately 40% platelet-derived plasminogen (28). Plasminogen activators also localize to the platelet surface with tPA binding being fibrinogen-dependent (65). Single chain uPA is activated on the platelet surface in a mechanism of reciprocal activation with plasminogen (77).

Platelet dense granules contain polyphosphate (polyP), a biomolecule which functions in modulation of coagulation and inflammation (79). PolyP delays fibrin polymerization altering clot structure (80). The knotted fibrin structure downregulates tPA and plasminogen binding thereby inhibiting tPA-mediated fibrinolysis (81). The effect on uPA-mediated plasminogen activation may depend on the contribution of other proteins as polyP accelerates activation in a purified system (82) whilst inhibits it in a plasma-based system (83). FXII has close structural homology to tPA and uPA and as such can function as a plasminogen activator. PolyP auto activates FXII to active single chain FXII (84) which facilitates plasminogen activation (85). Platelet-derive polyP could

therefore have differential roles in thrombus resolution and cellular proteolytic process depending on the surrounding environment.

During vascular insult, many of the innate cell immune responses require interaction with the endothelium. Endothelial cells are the main source of circulating tPA, and secretion occurs via both constitutive and regulated mechanisms (86). Both plasminogen and tPA can bind to endothelial cells and therefore have the potential to generate plasmin (87). Endothelial cells also secrete uPA which bind to the cell surface uPAR (88). Additionally, endothelial cells produce the fibrinolytic inhibitors PAI-1 (89, 90), PAI-2 (91), and TAFI (92) which are upregulated in response to inflammatory cytokines.

Interaction of innate immune cells within the thrombus environment could influence resolution and stability. In pulmonary thrombi, rolling neutrophils rip membrane fragments from PS-exposing platelets facilitating formation of neutrophil macroaggregates (93). It is interesting to speculate that this could act to deliver platelet-derived fibrinolytic proteins within these aggregates and may facilitate platelet-neutrophil fibrinolytic crosstalk.

The role of the fibrinolytic system in immunomodulation

Fibrinolytic proteins have a multitude of roles outside of their primary function of fibrin degradation including regulating the immune response. PAI-1 is an acute phase protein that is upregulated in response to injury, infection and inflammation (90, 94, 95) (**Figure 1**). Upregulation of PAI-1 is considered to be a protective mechanism important for early immune responses against bacterial pathogens, including *Haemophilus influenzae* (96), *Pseudomonas aeruginosa* (97). PAI-1 promotes bacterial clearance and limits inflammation (96). Downregulation of PAI-1 by *Streptococcus pneumoniae* pneumolysin is associated with increased mortality which can be reversed by administering recombinant PAI-1, protecting against alveolar haemorrhage (98).

Plasminogen activator inhibitor-1 facilitates neutrophil migration and its inhibition or deletion reduces influx at the site of injury in response to *Pseudomonas aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* infections (97, 99, 100). PAI-1 regulates IFN- γ in response to LPS and *Staphylococcal enterotoxin B* (101) in a mechanism independent of the plasminogen activators. PAI-1 may also have a protective role in viral infections, due to inhibition of proteases required for glycoprotein cleavage, therefore limiting viral replication (102).

TAFIa modulates inflammation by removal of C-terminal arginine or lysine residues from C3a, C5a, bradykinin osteopontin and chenerin (103–105) (**Figure 1**). Cleavage of C5a by TAFI is protective in inflammatory models of LPS induced acute lung injury (106), bronchial asthma (107), and rheumatoid arthritis (108). The development of post-traumatic sepsis is associated with a reduction in TAFI and increased C5a (109). Additionally, TAFI-deficient mice display enhanced neutrophil recruitment and tumor necrosis factor-α (TNF-α) and IL-6 levels in the peritoneum after *Escherichia coli* induced abdominal sepsis (110). This was independent of its antifibrinolytic function (110). In contrast to this, in *Pseudomonas aeruginosa*-induced sepsis, TAFI inhibition potentiates the effects of the antibiotic, ceftazidime and reduces organ dysfunction (111).

Plasmin(ogen) has multifaceted roles in the regulation of proinflammatory processes [reviewed in (20)]. Plasminogen is required for efficient recruitment of monocytes and lymphocytes in response to inflammation (112) and promotes macrophage phagocytosis and migration (113, 114) (**Figure 1**). Deficiency of plasminogen alters the expression of phagocytic genes (113). Whilst the fibrinolytic activity of plasmin is required for macrophage migration in experimental peritonitis (114). Interestingly, the absence of fibrinogen or the integrin $\alpha_M\beta_2$ reverses the requirement for plasminogen suggesting fibrinolytic activity is required to remove the physical restraint of macrophages by fibrin(ogen) (114). Plg-$R_{KT,}$ is upregulated during differentiation of monocytes to macrophages (35) and drives polarization to an M2-like macrophage phenotype (115). Additionally, dendritic cell phagocytosis is enhanced by plasmin which maintains these cells in an immature phenotype an reduces migration to the lymph nodes (116).

Plasminogen activators modulate the innate immune response, in mechanisms both dependent and independent of their fibrinolytic action. In a *Escherichia coli*-induced sepsis model, tPA deficiency caused increased bacterial loads, reduced neutrophil migration and was associated with increased mortality by a plasmin-independent mechanism (117) (**Figure 1**). Consistent with this, enzymatically inactive tPA blocks LPS induced increase in proinflammatory cytokines such as TNF-α, and IL-6 via low density lipoprotein receptor-related protein-1 (LRP1) and *N*-methyl-D-aspartic acid receptor (NMDA-R) (118, 119). However, in an ischemia/reperfusion model, tPA-mediated plasmin activity was required for neutrophil transmigration and disruption of endothelial junctions which allows further recruitment of neutrophils (120). Plasmin does not directly activate neutrophils and recruitment of these cells requires mast cell activation and leukotriene generation (120). Whilst in a stroke model, tPA-mediated plasmin generation decreased lymphocyte and monocyte counts, elevated IL-10 and TNF-α and altered splenic dendritic cell proportions (121).

Urokinase enhances monocyte differentiation into macrophages (122) and promotes neutrophil activation and migration (123). The uPA receptor, uPAR facilitates neutrophil migration in response to LPS-induced peritonitis, but this was not observed with *Escherichia coli* or in a polymicrobial sepsis model suggesting a compensatory mechanism may occur (124, 125). The function of uPAR on neutrophil migration is independent of its role in plasminogen activation and requires toll-like receptor signaling (125). Deficiency of uPAR promotes proinflammatory cytokines and macrophage polarization towards M1 phenotype and reduced phagocytosis in an experimental colitis model (126).

The varying roles of the fibrinolytic system in immunomodulation highlights the complex interactions which must be carefully balanced so as not exacerbate the inflammatory response and promote a prothrombotic environment.

Dysregulation of fibrinolysis

Fibrinogen is an acute phase protein that dramatically increases during infection due to enhanced hepatic synthesis (127).

Fibrin films form on the outside of blood clots which limit bacterial infiltration (128). However, aberrant fibrin accumulation contributes to development of a prothrombotic environment. During acute bacterial or viral infections, thrombotic complications can arise including deep vein thrombosis (DVT) and pulmonary embolisms (PE) (129, 130), acute myocardial infarction (AMI) (131, 132) and strokes (132). Thrombotic events occurring after infections affect various organ systems including respiratory, urinary and oral (133). The risk of thrombosis is higher in the first weeks succeeding infection and falls gradually after the initial infection (129). Consistent with a prothrombotic response to infection, seasonal variability in occurrence of AMI has been observed (134). The underlying mechanisms of the prothrombotic state are not fully understood. However, derailment of the fibrinolytic system is often a contributing factor to this.

Sepsis, a life-threatening response to infection, leads to tissue and organ damage and has a mortality rate of approximately 30%, although this is higher with older age or pre-existing conditions (135). As a result of the inflammatory state development of disseminated intravascular coagulation (DIC) can occur. This causes systemic dysregulation of coagulation and fibrinolysis resulting in depletion of coagulation factors and platelets and hemorrhaging. Platelet count is associated with severity, being significantly reduced with development of septic shock (136).

Plasmin(ogen) has a protective role in sepsis and levels are reduced with disease severity (137). However, a hypofibrinolytic state predominates in sepsis, largely due to elevated levels of PAI-1. Indeed, PAI-1 is a potential biomarker of disease severity and predictor of mortality (138). Initially, increased tPA and plasmin generation may predominate peaking at 2 h at which point TNF-α induces a steep increase in PAI-1 (139). Patients with the PAI-1 polymorphism 4G/5G, which is associated with elevated PAI-1 levels, are at increased risk of mortality from sepsis (140, 141). NETs may contribute to the elevated PAI-1 in sepsis as PAI-1 is downregulated in petidylarginine deiminase-4 (PAD-4) deficient mice which are unable to form NETs (142). NETs further contribute to a hypofibrinolytic state in sepsis due to the presence of cell-free DNA, an effect that can be overcome by DNase (143).

Hypofibrinolysis in sepsis may be further precipitated by other antifibrinolytic proteins. PAI-2 is not normally detected in healthy neutrophils but in patients with sepsis significant levels are present (144). Activation of TAFI could also be a contributing factor to the development of sepsis DIC (145). Interestingly, the TAFI Thr325 Ile/Ile single nucleotide polymorphism, which has increased antifibrinolytic potential, is associated with increased risk of contracting meningococcal disease and risk of mortality (146).

Acute respiratory distress syndrome (ARDS) is a hyperinflammatory condition that occurs in response to infection characterized by heightened alveolar-capillary permeability leading to extrusion of plasma proteins and inflammatory cytokines. This results in enhanced leukocytes and platelets recruitment to the lung microvasculature (147–149). Respiratory dysfunction and right heart failure develops, confounded by fibrin deposits which are observed in the air spaces and lung parenchyma due to the procoagulant environment along with hyaline-membranes and fibrosis (150–153).

Fibrin persistence is exacerbated by the inflammatory environment which promotes an imbalance in the fibrinolytic factors. Of note, PAI-1 synthesis is upregulated by several proinflammatory cytokines. Elevated levels of PAI-1 are observed with respiratory infections including influenza (154), severe acute respiratory syndrome coronavirus (SARS-CoV) (155) and SARS-CoV2 which downregulates fibrinolytic activity (156). Elevated PAI-1 is associated with worsening disease severity after SARS-CoV2 infection (156, 157). IL-6 induces an upregulation in PAI-1 gene expression and plasma levels of both PAI-1 and tPA (158–160). In endothelial cells, trans-signaling by IL-6 causes a circular amplification of IL-6 as well as IL-8, MCP-1 and PAI-1 synthesis (161). Additionally, endothelial cells release PAI-1 in response to the acute phase reactant, C-reactive protein (CRP) (150, 162, 163). Levels of uPA antigen are unaffected in ARDs but the heightened levels of PAI-1 cause a downregulation in fibrinolytic activity in the bronchoalveolar space (150).

Therapeutic potential of targeting the fibrinolytic pathway

The appropriation of fibrinolytic system by pathogens to evade the host immune response and the varied function of fibrinolytic proteins in immunomodulation makes them potential therapeutic targets. Plasmin(ogen) binding and subsequent proteolytic activity are inhibited by lysine analogues. Lysine analogues therefore have potential in modulating the proinflammatory and immunosuppressive properties of plasmin. One such lysine analogue, epsilon aminocaproic acid (εACA), has been shown to reduce experimental Group B streptococcus meningitis and neonatal mortality rates (164). Whilst tranexamic acid (TXA), has shown promise at reducing rates of post-surgical infection (165). Furthermore, plasmin inhibition by aprotinin, εACA or TXA reduces neutrophil recruitment and may have potential to ischemia-reperfusion reduced injury (166).

On the other hand, when aberrant fibrin(ogen) develops during infection, promoting fibrinolysis is desirable. The use of recombinant tPA as an adjuvant therapy in a small retrospective study of infective endocarditis facilitated clearance of fibrin rich vegetations that encase the bacteria (167). In trauma or sepsis induced ARDS, uPA and streptokinase, were beneficial producing a significant improvement in PaO_2 (168, 169). Coronavirus disease-19 (COVID-19) is caused by infection with severe acute respiratory SARS-CoV2. Severely ill patients with COVID-19 are prone to thrombosis and can develop ARDS, sepsis and multiorgan failure. Thrombolytic therapy has therefore garnered interest for treatment in severely ill COVID patients (170, 171). Initial studies indicate that tPA improves PaO_2/FiO_2 ratio, however, larger studies are required to establish treatment regimens and the safety profile (172, 173). Targeting the inflammatory response also has potential to correct fibrinolytic dysregulation. Indeed, blocking IL-6 with Tocilizumab decreases PAI-1 levels and this was found to be beneficial in SARS-CoV2 infection and is a recommended therapy in ICU patients (161, 174).

Summary

The fibrinolytic and innate immune systems work in concert to protect from infection and inflammation and to regulate thrombus resolution. Derailment of one system therefore influences the other. Invading pathogens take advantage of plasminogen and its activators to evade protective immune responses. Whilst immune cells are a source of fibrinolytic proteins and act as a surface for their assembly and function in thrombus resolution. The fibrinolytic system participates in host immune responses, however, dysregulation can precipitate in aberrant fibrin distribution or impede immune cell function. There is much still to learn on the interplay between the fibrinolytic and innate immune systems. Improved understanding of these intricacies could lead to development of more targeted immunothrombolytic or immunomodulating therapies.

Author contributions

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

Funding

CW was supported by British Heart Foundation (PG/20/17/35050).

Conflict of interest

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

Publisher's note

References

1. Engelmann B, Massberg S. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol.* (2013) 13:34–45.

2. Martinod K, Deppermann C. Immunothrombosis and thromboinflammation in host defense and disease. *Platelets.* (2021) 32:314–24.

3. Bonaventura A, Vecchi A, Dagna L, Martinod K, Dixon D, Tassell BW, et al. Endothelial dysfunction and immunothrombosis as key pathogenic mechanisms in COVID-19. *Nat Rev Immunol.* (2021) 21:319–29. doi: 10.1038/s41577-021-00536-9

4. Brinkmann V, Reichard U, Goosmann C. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5.

5. Stark K, Massberg S. Interplay between inflammation and thrombosis in cardiovascular pathology. *Nat Rev Cardiol.* (2021) 18:666–82.

6. Massberg S, Grahl L, Bruehl M, Manukyan D, Pfeiler S, Goosmann C, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med.* (2010) 16:887–96.

7. Stoppelli M, Corti A, Soffientini A, Cassani G, Blasi F, Assoian R. Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. *Proc Natl Acad Sci USA.* (1985) 82:4939–43. doi: 10.1073/pnas.82.15.4939

8. Kruithof E, Tran-Thang C, Ransijn A, Bachmann F. Demonstration of a fast-acting inhibitor of plasminogen activators in human plasma. *Blood.* (1984) 64:907–13.

9. Thorsen S, Philips M, Selmer J, Lecander I, Astedt B. Kinetics of inhibition of tissue-type and urokinase-type plasminogen activator by plasminogen-activator inhibitor type 1 and type 2. *Eur J Biochem.* (1988) 175:33–9.

10. Lu B, Sofian T, Law R, Coughlin P, Horvath A. Contribution of conserved lysine residues in the alpha2-antiplasmin C terminus to plasmin binding and inhibition. *J Biol Chem.* (2011) 286:24544–52. doi: 10.1074/jbc.M111.229013

11. Sakata Y, Aoki N. Significance of cross-linking of alpha 2-plasmin inhibitor to fibrin in inhibition of fibrinolysis and in hemostasis. *J Clin Invest.* (1982) 69:536–42.

12. Peetermans M, Vanassche T, Liesenborghs L, Lijnen R, Verhamme P. Bacterial pathogens activate plasminogen to breach tissue barriers and escape from innate immunity. *Crit Rev Microbiol.* (2016) 42:866–82. doi: 10.3109/1040841X.2015.1080214

13. Wang X, Lin X, Loy J, Tang J, Zhang X. Crystal structure of the catalytic domain of human plasmin complexed with streptokinase. *Science.* (1998) 281:1662–5.

14. Collen D, Schlott B, Engelborghs Y, Hoef BV, Hartmann M, Lijnen HR, et al. On the mechanism of the activation of human plasminogen by recombinant staphylokinase. *J Biol Chem.* (1993) 268:8284–9.

15. Collen D. Staphylokinase: a potent, uniquely fibrin-selective thrombolytic agent. *Nat Med.* (1998) 4:279–84. doi: 10.1038/nm0398-279

16. Järvinen H, Laakkonen L, Haiko J, Johansson T, Juuti K, Suomalainen M, et al. Human single-chain urokinase is activated by the omptins PgtE of *Salmonella enterica* and Pla of *Yersinia pestis* despite mutations of active site residues. *Mol Microbiol.* (2013) 89:507–17. doi: 10.1111/mmi.12293

17. Degen J, Bugge T, Goguen J. Fibrin and fibrinolysis in infection and host defense. *J Thromb Haemost.* (2007) 5:24–31.

18. Crowe J, Sievwright I, Auld G, Moore N, Gow N, Booth N. Candida albicans binds human plasminogen: identification of eight plasminogen-binding proteins. *Mol Microbiol.* (2003) 47:1637–51. doi: 10.1046/j.1365-2958.2003.03390.x

19. Ayon-Nunez D, Fragoso G, Bobes R, Laclette J. Plasminogen-binding proteins as an evasion mechanism of the host's innate immunity in infectious diseases. *Biosci Rep.* (2018) 38:5. doi: 10.1042/BSR20180705

20. Medcalf R, Keragala C. Fibrinolysis: a primordial system linked to the immune response. *Int J Mol Sci.* (2021) 22:7. doi: 10.3390/ijms22073406

21. Bhattacharya S, Ploplis V, Castellino F. Bacterial plasminogen receptors utilize host plasminogen system for effective invasion and dissemination. *J Biomed Biotechnol.* (2012) 2012:482096. doi: 10.1155/2012/482096

22. Castiblanco-Valencia MM, Fraga TR, Pagotto AH, Serrano SM, Abreu PA, Barbosa AS, et al. Plasmin cleaves fibrinogen and the human complement proteins C3b and C5 in the presence of *Leptospira interrogans* proteins: a new role of LigA and LigB in invasion and complement immune evasion. *Immunobiology.* (2016) 221:679–89. doi: 10.1016/j.imbio.2016.01.001

23. Sanderson-Smith M, De Oliveira D, Ranson M, McArthur J. Bacterial plasminogen receptors: mediators of a multifaceted relationship. *J Biomed Biotechnol.* (2012) 2012:272148. doi: 10.1155/2012/272148

24. Stie J, Bruni G, Fox D. Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One.* (2009) 4:e5780. doi: 10.1371/journal.pone.0005780

25. Calcagno M, Avilan L, Colasante C, Berrueta L, Salmen S. Interaction of different *Leishmania mexicana* morphotypes with plasminogen. *Parasitol Res.* (2002) 88:972–8. doi: 10.1007/s00436-002-0688-2

26. Hoylaerts M, Rijken D, Lijnen H, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role fibrin. *J Biol Chem.* (1982) 257:2912–9.

27. Urano T, Castellino F, Suzuki Y. Regulation of plasminogen activation on cell surfaces and fibrin. *J Thromb Haemost.* (2018) 16:1487–97.

28. Whyte CS, Morrow GB, Baik N, Booth NA, Jalal MM, Parmer RJ, et al. Exposure of plasminogen and a novel plasminogen receptor, Plg-RKT, on activated human and murine platelets. *Blood.* (2021) 137:248–57. doi: 10.1182/blood.2020007263

29. Stricker R, Wong D, Shiu D, Reyes P, Shuman M. Activation of plasminogen by tissue plasminogen activator on normal and thrombasthenic platelets: effects on surface proteins and platelet aggregation. *Blood.* (1986) 68:275–80.

30. Hall S, Humphries J, Gonias S. Inhibition of cell surface receptor-bound plasmin by alpha 2-antiplasmin and alpha 2-macroglobulin. *J Biol Chem.* (1991) 266:12329–36.

31. Lucas M, Fretto L, McKee P. The binding of human plasminogen to fibrin and fibrinogen. *J Biol Chem.* (1983) 258:4249–56.

32. Plow E, Freaney D, Plescia J, Miles L. The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *J Cell Biol.* (1986) 103:2411–20.

33. Plow E, Doeuvre L, Das R. So many plasminogen receptors: why? *J Biomed Biotechnol.* (2012) 2012:141806.

34. Miles L, Ny L, Wilczynska M, Shen Y, Ny T, Parmer R. Plasminogen receptors and fibrinolysis. *Int J Mol Sci.* (2021) 22:4.

35. Andronicos NM, Chen EI, Baik N, Bai H, Parmer C, Kiosses WB, et al. Proteomics-based discovery of a novel, structurally unique, and developmentally regulated plasminogen receptor, Plg-RKT, a major regulator of cell surface plasminogen activation. *Blood.* (2010) 115:1319–30. doi: 10.1182/blood-2008-11-188938

36. Lighvani S, Baik N, Diggs J, Khaldoyanidi S, Parmer R, Miles L. Regulation of macrophage migration by a novel plasminogen receptor Plg-R KT. *Blood.* (2011) 118:5622–30. doi: 10.1182/blood-2011-03-344242

37. Singh I, Burnand KG, Collins M, Luttun A, Collen D, Boelhouwer B, et al. Failure of thrombus to resolve in urokinase-type plasminogen activator gene-knockout mice: rescue by normal bone marrow-derived cells. *Circulation.* (2003) 107:869–75. doi: 10.1161/01.cir.0000050149.22928.39

38. Hart P, Burgess D, Vitti G, Hamilton J. Interleukin-4 stimulates human monocytes to produce tissue-type plasminogen activator. *Blood.* (1989) 74:1222–5.

39. Ritchie H, Jamieson A, Booth N. Thrombin modulates synthesis of plasminogen activator inhibitor type 2 by human peripheral blood monocytes. *Blood.* (1995) 86:3428–35.

40. Ritchie H, Booth N. Secretion of plasminogen activator inhibitor 2 by human peripheral blood monocytes occurs via an endoplasmic reticulum-golgi-independent pathway. *Exp Cell Res.* (1998) 242:439–50. doi: 10.1006/excr.1998.4118

41. Ritchie H, Robbie L, Kinghorn S, Exley R, Booth N. Monocyte plasminogen activator inhibitor 2 (PAI-2) inhibits u-PA-mediated fibrin clot lysis and is cross-linked to fibrin. *Thromb Haemost.* (1999) 81:96–103.

42. Siefert SA, Chabasse C, Mukhopadhyay S, Hoofnagle MH, Strickland DK, Sarkar R, et al. Enhanced venous thrombus resolution in plasminogen activator inhibitor type-2 deficient mice. *J Thromb Haemost.* (2014) 12:1706–16. doi: 10.1111/jth.12657

43. Humphries J, Gossage JA, Modarai B, Burnand KG, Sisson TH, Murdoch C, et al. Monocyte urokinase-type plasminogen activator up-regulation reduces thrombus size in a model of venous thrombosis. *J Vasc Surg.* (2009) 50:1127–34. doi: 10.1016/j.jvs.2009.06.047

44. Claesen K, De Loose J, Van Wielendaele P. ProCPU is expressed by (primary) human monocytes and macrophages and expression differs between states of differentiation and activation. *Int J Mol Sci.* (2023) 24:4. doi: 10.3390/ijms24043725

45. Henriksson P, Becker S, Lynch G, McDonagh J. Identification of intracellular factor XIII in human monocytes and macrophages. *J Clin Invest.* (1985) 76:528–34.

46. Muszbek L, Adany R, Szegedi G, Polgar J, Kavai M. Factor XIII of blood coagulation in human monocytes. *Thromb Res.* (1985) 37:401–10.

47. Cordell P, Kile B, Standeven K, Josefsson E, Pease R, Grant P. Association of coagulation factor XIII-a with golgi proteins within monocyte-macrophages: implications for subcellular trafficking and secretion. *Blood.* (2010) 115:2674–81. doi: 10.1182/blood-2009-08-231316

48. Alshehri F, Whyte C, Tuncay A, Williams M, Wilson H, Mutch N. Monocytes expose factor XIII-a and stabilize thrombi against fibrinolytic degradation. *Int J Mol Sci.* (2021) 22:12. doi: 10.3390/ijms22126591

49. Moir E, Booth N, Bennett B, Robbie L. Polymorphonuclear leucocytes mediate endogenous thrombus lysis via a u-PA-dependent mechanism. *Br J Haematol.* (2001) 113:72–80. doi: 10.1046/j.1365-2141.2001.02696.x

50. Varj I, Longstaff C, Szab L, Farkas ÁZ, Varga-Szab V, Tanka-Salamon A, et al. DNA, histones and neutrophil extracellular traps exert anti-fibrinolytic effects in a plasma environment. *Thromb Haemost.* (2015) 113:1289–98. doi: 10.1160/TH14-08-0669

51. Longstaff C, Varj I, Sótonyi P, Szab L, Krumrey M, Hoell A, et al. Mechanical stability and fibrinolytic resistance of clots containing fibrin. DNA, and histones. *J Biol Chem.* (2013) 288:6946–56. doi: 10.1074/jbc.M112.404301

52. Brühl M-, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice in vivo. *J Exp Med.* (2012) 209:819–35. doi: 10.1084/jem.20112322

53. Ducroux C, Meglio L, Loyau S, Delbosc S, Boisseau W, Deschildre C, et al. Thrombus neutrophil extracellular traps content impair tPA-induced thrombolysis in acute ischemic stroke. *Stroke.* (2018) 49:754–7.

54. Laridan E, Denorme F, Desender L, François O, Andersson T, Deckmyn H, et al. Neutrophil extracellular traps in ischemic stroke thrombi. *Ann Neurol.* (2017) 82:223–32.

55. Sreeramkumar V, Adrover J, Ballesteros I, Cuartero MI, Rossaint J, Bilbao I, et al. Neutrophils scan for activated platelets to initiate inflammation. *Science.* (2014) 346:1234–8.

56. Zuchtriegel G, Uhl B, Puhr-Westerheide D, Pörnbacher M, Lauber K, Krombach F, et al. Platelets guide leukocytes to their sites of extravasation. *PLoS Biol.* (2016) 14:e1002459. doi: 10.1371/journal.pbio.1002459

57. Jang IK, Gold HK, Ziskind AA, Fallon JT, Holt RE, Leinbach RC, et al. Differential sensitivity of erythrocyte-rich and platelet-rich arterial thrombi to lysis with recombinant tissue-type plasminogen activator. A possible explanation for resistance to coronary thrombolysis. *Circulation.* (1989) 79:920–8. doi: 10.1161/01.cir.79.4.920

58. Tomkins A, Schleicher N, Murtha L, Kaps M, Levi C, Nedelmann M, et al. Platelet rich clots are resistant to lysis by thrombolytic therapy in a rat model of embolic stroke. *Exp Transl Stroke Med.* (2015) 7:2. doi: 10.1186/s13231-014-0014-y

59. Booth N, Simpson A, Croll A, Bennett B, MacGregor I. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *Br J Haematol.* (1988) 70:327–33.

60. Whyte C, Mostefai H, Baeten K. Role of shear stress and tPA concentration in the fibrinolytic potential of thrombi. *Int J Mol Sci.* (2021) 22:4. doi: 10.3390/ijms22042115

61. Booth N, Robbie L, Croll A, Bennett B. Lysis of platelet-rich thrombi: the role of PAI-1. *Ann NY Acad Sci.* (1992) 667:70–80.

62. Robbie L, Bennett B, Croll A, Brown P, Booth N. Proteins of the fibrinolytic system in human thrombi. *Thromb Haemost.* (1996) 75:127–33.

63. Morrow G, Whyte C, Mutch N. Functional plasminogen activator inhibitor 1 is retained on the activated platelet membrane following platelet activation. *Haematologica.* (2020) 105:2824–33. doi: 10.3324/haematol.2019.230367

64. Whyte CS, Swieringa F, Mastenbroek TG, Lionikiene AS, Lanc MD, Meijden PE, et al. Plasminogen associates with phosphatidylserine-exposing platelets and contributes to thrombus lysis under flow. *Blood.* (2015) 125:2568–78. doi: 10.1182/blood-2014-09-599480

65. Ni R, Neves M, Wu C. Activated thrombin-activatable fibrinolysis inhibitor (TAFIa) attenuates fibrin-dependent plasmin generation on thrombin-activated platelets. *J Thromb Haemost.* (2020) 18:2364–76. doi: 10.1111/jth.14950

66. Schadinger S, Lin J, Garand M, Boffa M. Secretion and antifibrinolytic function of thrombin-activatable fibrinolysis inhibitor from human platelets. *J Thromb Haemost.* (2010) 8:2523–9.

67. Boulaftali Y, Ho-Tin-Noe B, Pena A, Loyau S, Venisse L, François D, et al. Platelet protease nexin-1, a serpin that strongly influences fibrinolysis and thrombolysis. *Circulation.* (2011) 123:1326–34. doi: 10.1161/CIRCULATIONAHA.110.000885

68. Plow E, Collen D. The presence and release of alpha 2-antiplasmin from human platelets. *Blood.* (1981) 58:1069–74.

69. Gogstad G, Stormorken H, Solum N. Platelet alpha 2-antiplasmin is located in the platelet alpha-granules. *Thromb Res.* (1983) 31:387–90. doi: 10.1016/0049-3848(83)90339-0

70. Mitchell J, Lionikiene A, Fraser S, Whyte C, Booth N, Mutch N. Functional factor XIII-a is exposed on the stimulated platelet surface. *Blood.* (2014) 124:3982–90. doi: 10.1182/blood-2014-06-583070

71. Reed G, Matsueda G, Haber E. Fibrin-fibrin and alpha 2-antiplasmin-fibrin cross-linking by platelet factor XIII increases the resistance of platelet clots to fibrinolysis. *Trans Assoc Am Physicians.* (1991) 104:21–8.

72. Reed G, Matsueda G, Haber E. Platelet factor XIII increases the fibrinolytic resistance of platelet-rich clots by accelerating the crosslinking of alpha 2-antiplasmin to fibrin. *Thromb Haemost.* (1992) 68:315–20.

73. Hevessy Z, Haramura G, Boda Z, Udvardy M, Muszbek L. Promotion of the crosslinking of fibrin and alpha 2-antiplasmin by platelets. *Thromb Haemost.* (1996) 75:161–7.

74. Francis C, Marder V. Rapid formation of large molecular weight alpha-polymers in cross-linked fibrin induced by high factor XIII concentrations. Role of platelet factor XIII. *J Clin Invest.* (1987) 80:1459–65. doi: 10.1172/JCI113226

75. Rubens F, Perry D, Hatton M, Bishop P, Packham M, Kinlough-Rathbone R. Platelet accumulation on fibrin-coated polyethylene: role of platelet activation and factor XIII. *Thromb Haemost.* (1995) 73:850–6.

76. Somodi L, Beke Debreceni I, Kis G. Activation mechanism dependent surface exposure of cellular factor XIII on activated platelets and platelet microparticles. *J Thromb Haemost.* (2022) 20:1223–35. doi: 10.1111/jth.15668

77. Baeten K, Richard M, Kanse S, Mutch N, Degen J, Booth N. Activation of single-chain urokinase-type plasminogen activator by platelet-associated plasminogen: a mechanism for stimulation of fibrinolysis by platelets. *J Thromb Haemost.* (2010) 8:1313–22.

78. Miles L, Plow E. Binding and activation of plasminogen on the platelet surface. *J Biol Chem.* (1985) 260:4303–11.

79. Muller F, Mutch N, Schenk W. Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. *Cell.* (2009) 139:1143–56.

80. Whyte C, Chernysh I, Domingues M. Polyphosphate delays fibrin polymerisation and alters the mechanical properties of the fibrin network. *Thromb Haemost.* (2016) 116:897–903. doi: 10.1160/TH16-01-0062

81. Mutch N, Engel R, de Willige S, Philippou H, Ariens R. Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin. *Blood.* (2010) 115:3980–8. doi: 10.1182/blood-2009-11-254029

82. Whyte C, Mutch N. uPA-mediated plasminogen activation is enhanced by polyphosphate. *Haematologica.* (2021) 106:522–31. doi: 10.3324/haematol.2019.237966

83. Smith S, Mutch N, Baskar D, Rohloff P, Docampo R, Morrissey J. Polyphosphate modulates blood coagulation and fibrinolysis. *Proc Natl Acad Sci USA.* (2006) 103:903–8.

84. Engel R, Brain C, Paget J, Lionikiene A, Mutch N. Single-chain factor XII exhibits activity when complexed to polyphosphate. *J Thromb Haemost.* (2014) 12:1513–22. doi: 10.1111/jth.12663

85. Mitchell J, Lionikiene A, Georgiev G. Polyphosphate colocalizes with factor XII on platelet-bound fibrin and augments its plasminogen activator activity. *Blood.* (2016) 128:2834–45. doi: 10.1182/blood-2015-10-673285

86. Emeis J, Eijnden-Schrauwen Y, Hoogen C, de Priester W, Westmuckett A, Lupu F. An endothelial storage granule for tissue-type plasminogen activator. *J Cell Biol.* (1997) 139:245–56.

87. Miles L, Parmer R. Plasminogen receptors: the first quarter century. *Semin Thromb Hemost.* (2013) 39:329–37. doi: 10.1055/s-0033-1334483

88. Quax P, Grimbergen J, Lansink M. Binding of human urokinase-type plasminogen activator to its receptor: residues involved in species specificity and binding. *Arterioscler Thromb Vasc Biol.* (1998) 18:693–701.

89. van Mourik J, Lawrence D, Loskutoff D. Purification of an inhibitor of plasminogen activator (antiactivator) synthesized by endothelial cells. *J Biol Chem.* (1984) 259:14914–21.

90. Morrow G, Whyte C, Mutch NJ. A serpin with a finger in many PAIs: PAI-1's central function in thromboinflammation and cardiovascular disease. *Front Cardiovasc Med.* (2021) 8:653655. doi: 10.3389/fcvm.2021.653655

91. Boncela J, Przygodzka P, Papiewska-Pajak I, Wyroba E, Cierniewski C. Association of plasminogen activator inhibitor type 2 (PAI-2) with proteasome within endothelial cells activated with inflammatory stimuli. *J Biol Chem.* (2011) 286:43164–71. doi: 10.1074/jbc.M111.245647

92. Lin J, Garand M, Zagorac B. Identification of human thrombin-activatable fibrinolysis inhibitor in vascular and inflammatory cells. *Thromb Haemost.* (2011) 105:999–1009.

93. Yuan Y, Alwis I, Wu M. Neutrophil macroaggregates promote widespread pulmonary thrombosis after gut ischemia. *Sci Transl Med.* (2017) 9:409. doi: 10.1126/scitranslmed.aam5861

94. Shetty S, Padijnayayveetil J, Tucker T, Stankowska D, Idell S. The fibrinolytic system and the regulation of lung epithelial cell proteolysis, signaling, and cellular viability. *Am J Physiol Lung Cell Mol Physiol.* (2008) 295:L967–75. doi: 10.1152/ajplung.90349.2008

95. Levi M, Poll T. Coagulation in patients with severe sepsis. *Semin Thromb Hemost.* (2015) 41:9–15.

96. Lim J, Woo C, Li J. Critical role of type 1 plasminogen activator inhibitor (PAI-1) in early host defense against nontypeable *Haemophilus influenzae* (NTHi) infection. *Biochem Biophys Res Commun.* (2011) 414:67–72. doi: 10.1016/j.bbrc.2011.09.023

97. Goolaerts A, Lafargue M, Song Y. PAI-1 is an essential component of the pulmonary host response during *Pseudomonas aeruginosa* pneumonia in mice. *Thorax.* (2011) 66:788–96. doi: 10.1136/thx.2010.155788

98. Lim J, Stirling B, Derry J. Tumor suppressor CYLD regulates acute lung injury in lethal *Streptococcus pneumoniae* infections. *Immunity.* (2007) 27:349–60. doi: 10.1016/j.immuni.2007.07.011

99. Roelofs J, Teske G, Bonta P. Plasminogen activator inhibitor-1 regulates neutrophil influx during acute pyelonephritis. *Kidney Int.* (2009) 75:52–9. doi: 10.1038/ki.2008.454

100. Renckens R, Roelofs J, Bonta P. Plasminogen activator inhibitor type 1 is protective during severe Gram-negative pneumonia. *Blood.* (2007) 109:1593–601. doi: 10.1182/blood-2006-05-025197

101. Renckens R, Pater J, van der Poll T. Plasminogen activator inhibitor type-1-deficient mice have an enhanced IFN-gamma response to lipopolysaccharide and staphylococcal enterotoxin B. *J Immunol.* (2006) 177:8171–6. doi: 10.4049/jimmunol.177.11.8171

102. Dittmann M, Hoffmann H, Scull M. A serpin shapes the extracellular environment to prevent influenza a virus maturation. *Cell.* (2015) 160:631–43. doi: 10.1016/j.cell.2015.01.040

103. Campbell W, Lazoura E, Okada N, Okada H. Inactivation of C3a and C5a octapeptides by carboxypeptidase R and carboxypeptidase N. *Microbiol Immunol.* (2002) 46:131–4. doi: 10.1111/j.1348-0421.2002.tb02669.x

104. Plug T, Meijers J. Structure-function relationships in thrombin-activatable fibrinolysis inhibitor. *J Thromb Haemost.* (2016) 14:633–44.

105. Shinohara T, Sakurada C, Suzuki T. Pro-carboxypeptidase R cleaves bradykinin following activation. *Int Arch Allergy Immunol.* (1994) 103:400–4. doi: 10.1159/000236661

106. Naito M, Taguchi O, Kobayashi T. Thrombin-activatable fibrinolysis inhibitor protects against acute lung injury by inhibiting the complement system. *Am J Respir Cell Mol Biol.* (2013) 49:646–53. doi: 10.1165/rcmb.2012-0454OC

107. Fujiwara A, Taguchi O, Takagi T. Role of thrombin-activatable fibrinolysis inhibitor in allergic bronchial asthma. *Lung.* (2012) 190:189–98. doi: 10.1007/s00408-011-9337-9

108. Song J, Hwang I, Cho K. Plasma carboxypeptidase B downregulates inflammatory responses in autoimmune arthritis. *J Clin Invest.* (2011) 121:3517–27. doi: 10.1172/JCI46387

109. Vollrath J, Marzi I, Herminghaus A, Lustenberger T, Relja B. Post-traumatic sepsis is associated with increased C5a and decreased TAFI levels. *J Clin Med.* (2020) 9:4. doi: 10.3390/jcm9041230

110. Renckens R, Roelofs J, Horst S. Absence of thrombin-activatable fibrinolysis inhibitor protects against sepsis-induced liver injury in mice. *J Immunol.* (2005) 175:6764–71. doi: 10.4049/jimmunol.175.10.6764

111. Muto Y, Suzuki K, Iida H. EF6265, a novel inhibitor of activated thrombin-activatable fibrinolysis inhibitor, protects against sepsis-induced organ dysfunction in rats. *Crit Care Med.* (2009) 37:1744–9. doi: 10.1097/CCM.0b013e31819ffc14

112. Ploplis V, French E, Carmeliet P, Collen D, Plow E. Plasminogen deficiency differentially affects recruitment of inflammatory cell populations in mice. *Blood.* (1998) 91:2005–9.

113. Das R, Ganapathy S, Settle M, Plow E. Plasminogen promotes macrophage phagocytosis in mice. *Blood.* (2014) 124:679–88.

114. Silva L, Lum A, Tran C. Plasmin-mediated fibrinolysis enables macrophage migration in a murine model of inflammation. *Blood.* (2019) 134:291–303. doi: 10.1182/blood.2018874859

115. Vago J, Sugimoto M, Lima K. Plasminogen and the plasminogen receptor, Plg-R(KT), regulate macrophage phenotypic, and functional changes. *Front Immunol.* (2019) 10:1458. doi: 10.3389/fimmu.2019.01458

116. Borg R, Samson A, Au A. Dendritic cell-mediated phagocytosis but not immune activation is enhanced by plasmin. *PLoS One.* (2015) 10:7. doi: 10.1371/journal.pone.0131216

117. Renckens R, Roelofs J, Florquin S. Endogenous tissue-type plasminogen activator is protective during *Escherichia coli*-induced abdominal sepsis in mice. *J Immunol.* (2006) 177:1189–96. doi: 10.4049/jimmunol.177.2.1189

118. Mantuano E, Azmoon P, Brifault C. Tissue-type plasminogen activator regulates macrophage activation and innate immunity. *Blood.* (2017) 130:1364–74.

119. Mantuano E, Brifault C, Lam M, Azmoon P, Gilder A, Gonias SL. LDL receptor-related protein-1 regulates NFkappaB and microRNA-155 in macrophages to control the inflammatory response. *Proc Natl Acad Sci USA.* (2016) 113:1369–74. doi: 10.1073/pnas.1515480113

120. Uhl B, Zuchtriegel G, Puhr-Westerheide D. Tissue plasminogen activator promotes postischemic neutrophil recruitment via its proteolytic and nonproteolytic properties. *Arterioscler Thromb Vasc Biol.* (2014) 34:1495–504. doi: 10.1161/ATVBAHA.114.303721

121. Draxler D, Lee F, Ho H, Keragala C, Medcalf R, Niego B. t-PA suppresses the immune response and aggravates neurological deficit in a murine model of ischemic stroke. *Front Immunol.* (2019) 10:591. doi: 10.3389/fimmu.2019.00591

122. Paland N, Aharoni S, Fuhrman B. Urokinase-type plasminogen activator (uPA) modulates monocyte-to-macrophage differentiation and prevents Ox-LDL-induced macrophage apoptosis. *Atherosclerosis.* (2013) 231:29–38. doi: 10.1016/j.atherosclerosis.2013.08.016

123. Abraham E, Gyetko M, Kuhn K. Urokinase-type plasminogen activator potentiates lipopolysaccharide-induced neutrophil activation. *J Immunol.* (2003) 170:5644–51.

124. Renckens R, Roelofs J, Florquin S, van der Poll T. Urokinase-type plasminogen activator receptor plays a role in neutrophil migration during lipopolysaccharide-induced peritoneal inflammation but not during *Escherichia coli*-induced peritonitis. *J Infect Dis.* (2006) 193:522–30. doi: 10.1086/499601

125. Kiyan Y, Tkachuk S, Rong S. TLR4 response to LPS is reinforced by urokinase receptor. *Front Immunol.* (2020) 11:573550. doi: 10.3389/fimmu.2020.573550

126. Genua M, D'Alessio S, Cibella J. The urokinase plasminogen activator receptor (uPAR) controls macrophage phagocytosis in intestinal inflammation. *Gut.* (2015) 64:589–600. doi: 10.1136/gutjnl-2013-305933

127. Dowton S, Colten H. Acute phase reactants in inflammation and infection. *Semin Hematol.* (1988) 25:84–90.

128. Macrae F, Duval C, Papareddy P. A fibrin biofilm covers blood clots and protects from microbial invasion. *J Clin Invest.* (2018) 128:3356–68. doi: 10.1172/JCI98734

129. Smeeth L, Cook C, Thomas S, Hall A, Hubbard R, Vallance P. Risk of deep vein thrombosis and pulmonary embolism after acute infection in a community setting. *Lancet.* (2006) 367:1075–9. doi: 10.1016/S0140-6736(06)68474-2

130. Lozinguez O, Arnaud E, Belec L. Demonstration of an association between Chlamydia pneumoniae infection and venous thromboembolic disease. *Thromb Haemost.* (2000) 83:887–91.

131. Meier C, Jick S, Derby L, Vasilakis C, Jick H. Acute respiratory-tract infections and risk of first-time acute myocardial infarction. *Lancet.* (1998) 351:1467–71.

132. Smeeth L, Thomas S, Hall A, Hubbard R, Farrington P, Vallance P. Risk of myocardial infarction and stroke after acute infection or vaccination. *N Engl J Med.* (2004) 351:2611–8.

133. Beristain-Covarrubias N, Perez-Toledo M, Thomas M, Henderson I, Watson S, Cunningham A. Understanding infection-induced thrombosis: lessons learned from animal models. *Front Immunol.* (2019) 10:2569. doi: 10.3389/fimmu.2019.02569

134. Meier C. Seasonal variations in the incidence of acute myocardial infarction. *J Am Coll Cardiol.* (1998) 32:2103–4.

135. Angus D, Linde-Zwirble W, Lidicker J, Clermont G, Carcillo J, Pinsky M. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. *Crit Care Med.* (2001) 29:1303–10.

136. Mavrommatis A, Theodoridis T, Orfanidou A, Roussos C, Christopoulou-Kokkinou V, Zakynthinos S. Coagulation system and platelets are fully activated in uncomplicated sepsis. *Crit Care Med.* (2000) 28:451–7. doi: 10.1097/00003246-200002000-00027

137. Vago J, Brito L, Teixeira L. The role of plasmin/plasminogen in experimental polymicrobial sepsis. *Blood.* (2020) 136:16–7.

138. Tipoe T, Wu W, Chung L. Plasminogen activator inhibitor 1 for predicting sepsis severity and mortality outcomes: a systematic review and meta-analysis. *Front Immunol.* (2018) 9:1218. doi: 10.3389/fimmu.2018.01218

139. Biemond B, Levi M, Ten Cate H. Plasminogen activator and plasminogen activator inhibitor I release during experimental endotoxaemia in chimpanzees: effect of interventions in the cytokine and coagulation cascades. *Clin Sci.* (1995) 88:587–94. doi: 10.1042/cs0880587

140. Li L, Nie W, Zhou H, Yuan W, Li W, Huang W. Association between plasminogen activator inhibitor-1 -675 4G/5G polymorphism and sepsis: a meta-analysis. *PLoS One.* (2013) 8:e54883. doi: 10.1080/15513815.2020.1775736

141. Hermans P, Hibberd M, Booy R. 4G/5G promoter polymorphism in the plasminogen-activator-inhibitor-1 gene and outcome of meningococcal disease. *Meningococcal Res Group Lancet.* (1999) 354:556–60.

142. McDonald B, Davis R, Kim S. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood.* (2017) 129:1357–67.

143. Gould T, Vu T, Stafford A. Cell-free DNA modulates clot structure and impairs fibrinolysis in sepsis. *Arterioscler Thromb Vasc Biol.* (2015) 35:2544–53. doi: 10.1161/ATVBAHA.115.306035

144. Haj M, Robbie L, Adey G, Bennett B. Inhibitors of plasminogen activator in neutrophils and mononuclear cells from septic patients. *Thromb Haemost.* (1995) 74:1528–32.

145. Emonts M, de Bruijne E, Guimaraes A. Thrombin-activatable fibrinolysis inhibitor is associated with severity and outcome of severe meningococcal infection in children. *J Thromb Haemost.* (2008) 6:268–76. doi: 10.1111/j.1538-7836.2007.02841.x

146. Kremer Hovinga J, Franco R, Zago M, Ten Cate H, Westendorp R, Reitsma PH. A functional single nucleotide polymorphism in the thrombin-activatable fibrinolysis inhibitor (TAFI) gene associates with outcome of meningococcal disease. *J Thromb Haemost.* (2004) 2:54–7.

147. Martin C, Papazian L, Payan M, Saux P, Gouin F. Pulmonary fibrosis correlates with outcome in adult respiratory distress syndrome. A study in mechanically ventilated patients. *Chest.* (1995) 107:196–200.

148. Moine P, McIntyre R, Schwartz M. NF-kappaB regulatory mechanisms in alveolar macrophages from patients with acute respiratory distress syndrome. *Shock.* (2000) 13:85–91.

149. Huang X, Xiu H, Zhang S, Zhang G. The role of macrophages in the pathogenesis of ALI/ARDS. *Mediators Inflamm.* (2018) 2018:1264913.

150. Bertozzi P, Astedt B, Zenzius L. Depressed bronchoalveolar urokinase activity in patients with adult respiratory distress syndrome. *N Engl J Med.* (1990) 322:890–7.

151. Bone R, Francis P, Pierce A. Intravascular coagulation associated with the adult respiratory distress syndrome. *Am J Med.* (1976) 61:585–9.

152. Ware L. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. *Semin Respir Crit Care Med.* (2006) 27:337–49.

153. Ware L, Matthay M. The acute respiratory distress syndrome. *N Engl J Med.* (2000) 342:1334–49.

154. Keller T, van der Sluijs K, de Kruif M. Effects on coagulation and fibrinolysis induced by influenza in mice with a reduced capacity to generate activated protein C and a deficiency in plasminogen activator inhibitor type 1. *Circ Res.* (2006) 99:1261–9. doi: 10.1161/01.RES.0000250834.29108.1a

155. Gralinski L, Bankhead A, Jeng S. Mechanisms of severe acute respiratory syndrome coronavirus-induced acute lung injury. *mBio.* (2013) 4:4.

156. Whyte C, Simpson M, Morrow G. The suboptimal fibrinolytic response in COVID-19 is dictated by high PAI-1. *J Thromb Haemost.* (2022) 20:2394–406.

157. Juneja G, Castelo M, Yeh C. Biomarkers of coagulation, endothelial function, and fibrinolysis in critically ill patients with COVID-19: a single-center prospective longitudinal study. *J Thromb Haemost.* (2021) 19:1546–57. doi: 10.1111/jth.15327

158. Mestries J, Kruithof E, Gascon M, Herodin F, Agay D, Ythier A. In vivo modulation of coagulation and fibrinolysis by recombinant glycosylated human interleukin-6 in baboons. *Eur Cytokine Netw.* (1994) 5:275–81.

159. Kruithof E. Regulation of plasminogen activator inhibitor type 1 gene expression by inflammatory mediators and statins. *Thromb Haemost.* (2008) 100:969–75.

160. Kruithof E, Mestries J, Gascon M, Ythier A. The coagulation and fibrinolytic responses of baboons after in vivo thrombin generation–effect of interleukin 6. *Thromb Haemost.* (1997) 77:905–10.

161. Kang S, Tanaka T, Inoue H. IL-6 trans-signaling induces plasminogen activator inhibitor-1 from vascular endothelial cells in cytokine release syndrome. *Proc Natl Acad Sci USA.* (2020) 117:22351–6. doi: 10.1073/pnas.2010229117

162. Prabhakaran P, Ware L, White K, Cross M, Matthay M, Olman M. Elevated levels of plasminogen activator inhibitor-1 in pulmonary edema fluid are associated with mortality in acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2003) 285:L20–8.

163. Ware L, Matthay M, Parsons P. Pathogenetic and prognostic significance of altered coagulation and fibrinolysis in acute lung injury/acute respiratory distress syndrome. *Crit Care Med.* (2007) 35:1821–8. doi: 10.1097/01.CCM.0000221922.08878.49

164. Magalhaes V, Andrade E, Alves J. Group B *Streptococcus hijacks* the host plasminogen system to promote brain endothelial cell invasion. *PLoS One.* (2013) 8:e63244. doi: 10.1371/journal.pone.0063244

165. Draxler D, Yep K, Hanafi G. Tranexamic acid modulates the immune response and reduces postsurgical infection rates. *Blood Adv.* (2019) 3:1598–609. doi: 10.1182/bloodadvances.2019000092

166. Reichel C, Lerchenberger M, Uhl B. Plasmin inhibitors prevent leukocyte accumulation and remodeling events in the postischemic microvasculature. *PLoS One.* (2011) 6:e17229. doi: 10.1371/journal.pone.0017229

167. Levitas A, Krymko H, Richardson J, Zalzstein E, Ioffe V. Recombinant tissue plasminogen activator as a novel treatment option for infective endocarditis: a retrospective clinical study in 32 children. *Cardiol Young.* (2016) 26:110–5. doi: 10.1017/S104795111400273X

168. Hardaway R. A brief overview of acute respiratory distress syndrome. *World J Surg.* (2006) 30:1829–34.

169. Hardaway R, Harke H, Tyroch A, Williams C, Vazquez Y, Krause G. Treatment of severe acute respiratory distress syndrome: a final report on a phase I study. *Am Surg.* (2001) 67:377–82.

170. Whyte C, Morrow G, Mitchell J, Chowdary P, Mutch N. Fibrinolytic abnormalities in acute respiratory distress syndrome (ARDS) and versatility of thrombolytic drugs to treat COVID-19. *J Thromb Haemost.* (2020) 18:1548–55. doi: 10.1111/jth.14872

171. Moore H, Barrett C, Moore E. Is there a role for tissue plasminogen activator as a novel treatment for refractory COVID-19 associated acute respiratory distress syndrome? *J Trauma Acute Care Surg.* (2020) 88:713–4. doi: 10.1097/TA.0000000000002694

172. Wang J, Hajizadeh N, Moore E. Tissue plasminogen activator (tPA) treatment for COVID-19 associated acute respiratory distress syndrome (ARDS): a case series. *J Thromb Haemost.* (2020) 18:1752–5.

173. Barrett C, Moore H, Moore E, Wang J, Hajizadeh N, Biffl W, et al. Study of alteplase for respiratory failure in SARS-CoV-2 COVID-19: a vanguard multicenter, rapidly adaptive, pragmatic, randomized controlled trial. *Chest.* (2022) 161:710–27. doi: 10.1016/j.chest.2021.09.024

174. Wise J. Covid-19: critically ill patients treated with arthritis drug tocilizumab show improved outcomes, researchers report. *BMJ.* (2020) 371:m4530. doi: 10.1136/bmj.m4530

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 29 June 2023
DOI 10.3389/fmed.2023.1117192

OPEN ACCESS

EDITED BY
Marcos De Lima,
The Ohio State University, United States

REVIEWED BY
Rubul Kumar Bania,
North-Eastern Hill University, India
Leila Zaki,
Tarbiat Modares University, Iran

*CORRESPONDENCE
Linkai Luo
✉ luolk@xmu.edu.cn
Wei Huang
✉ hz884698@gmail.com
Qi Zhang
✉ 524042405@qq.com

†These authors have contributed equally to this work

RECEIVED 06 December 2022
ACCEPTED 23 May 2023
PUBLISHED 29 June 2023

CITATION
Fu M, Wu K, Li Y, Luo L, Huang W and Zhang Q (2023) An intelligent detection method for plasmodium based on self-supervised learning and attention mechanism. *Front. Med.* 10:1117192. doi: 10.3389/fmed.2023.1117192

An intelligent detection method for plasmodium based on self-supervised learning and attention mechanism

Min Fu[1], Kai Wu[2†], Yuxuan Li[3†], Linkai Luo[1*], Wei Huang[4*] and Qi Zhang[5*]

[1]School of Aerospace Engineering, Xiamen University, Xiamen, China, [2]Department of Schistosomiasis and Endemic Diseases, Wuhan Center for Disease Control and Prevention, Wuhan, China, [3]School of Art and Design, Wuhan University of Technology, Wuhan, China, [4]Department of Orthopaedics, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China, [5]Wuhan Jinyintan Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan, China

Background: Malaria remains a severe life-threatening disease caused by plasmodium parasites. Microscopy is widely used for malaria diagnosis. However, it relies heavily on the skills and experience of inspectors. Due to low-level medical services and the lack of skilled inspectors, misdiagnoses are frequently made in some areas.

Methods: In recent years, many successful applications of CNN models have been reported. Unlike images in the ImageNet, the image of plasmodium only has a tiny defect area with a large amount of information. In addition, the dataset is extremely unbalanced: the number of positive samples is much less than that of negative samples. This paper proposes a classification network by combining attention mechanism and ResNeSt for plasmodium detection and using self-supervised learning to pre-train the network. First, the positive samples were adopted to pre-train the network. Then, attention modules were taken to highlight the feature area. To support current and future research, we also constructed a plasmodium dataset with Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, and Plasmodium malaria and non-Plasmodium. Through self-supervised learning, a large amount of unlabeled data is used to mine the representational features, thus improving the feature extraction capability of the model and achieving higher accuracy, while saving the physician's labeling time and improving the classification accuracy.

Results: The experiments show that our model exhibits an excellent performance and that the test accuracy, sensitivity, and specificity attain 97.8%, 96.5%, and 98.9%, respectively.

Conclusion: The AI classification method proposed in this paper can effectively assist clinicians in the diagnosis and provide a basis for the automatic detection of malaria parasites in the future.

KEYWORDS

deep learning, plasmodium parasites, self-supervised learning, attention mechanism, automatic detecting system

1. Introduction

Malaria is a parasitosis transmitted by the bite of female anopheles. It is one of three major public health problems across the world. According to the WHO Malaria Report (1), the year 2020 witnessed 241 million malaria cases worldwide, including over 627,000 death cases. The vast majority of the cases were from Africa and Southeast Asia. Malaria was extensively distributed across Mainland China. Even at the beginning of this century, hundreds of thousands of malaria cases were discovered each year (2). With the efficacious control of malaria, the number of patients decreased year by year, and no local malaria cases had been reported by 2017 (3). The laboratory diagnosis of malaria mainly includes microscopy, rapid diagnostic tests for plasmodium antigen (RDT) and nucleic acid detection. Microscopic examination is the gold standard for malaria diagnosis thanks to its advantages of simple operation, rapidness, low cost, etc. However, the accuracy of microscopic examination depends heavily on the personal skills and experience of inspectors. With the decrease in malaria cases, microscopic examination has gradually become an inevitable trend in terms of detecting plasmodium. Over the past years, the incidence of imported malaria has gradually built up, and missed or wrong detections have been detected in microscopic examination (4). Moreover, microscopic examination requires the inspectors to have high-level skills and rich experience, leading to great differences in the detection results between different regions and different people. It is especially serious in areas with weak medical and health foundations.

There have been some works using convolutional neural networks to detect malaria parasites. Rajaraman et al. (5) evaluated the performance of deep learning-based pretrained CNN models as feature extractors in classifying infected and non-infected cells where the region of interest (ROI) was obtained by the Laplacian of Gaussian (LoG). They achieved a positive predictive value (PPV) of 94.44% with a sensitivity value of 96.20% which indicates the superiority of the pre-trained ResNet-50 (6). Peñas et al. (7) built a system that can detect the presence of malaria parasites and identify the type of parasite species in blood samples. In this study, a series of morphological transformations were implemented prior to the segmentation with connected components analysis and the classification with CNN. They showed an accuracy of 92.40% for parasite detection and 87.90% for the identification of parasite types. Liang et al. (6) designed a new CNN model with 17 layers for the classification of blood cells. The average classification accuracy of the CNN model was 97.37%, while the sensitivity, specificity and precision also reached the level of 97%. It suggested that the proposed CNN model is superior to the transfer learning model with pre-trained AlexNet. With the advent of convolutional neural networks (CNNs), better performance has been realized in image classification, object detection, attention prediction, and so on. The detection results with CNNs are not correlated with regions or inspectors. The algorithm is expected to diagnose plasmodium with CNNs. In our research, we introduced a CNN model with spatial attention modules and channel attention modules to diagnose plasmodium. Besides, data enhancement was employed to eliminate color influence. The experiments show that our algorithm achieves excellent performance.

2. Related works

Abhik Paul (7) developed three convolutional neural network (CNN) models for predicting the occurrence of malaria from images of red blood cells. Infected parasitic red blood cells and uninfected parasitic red blood cells. Finally, in the three settings, the proposed CNN setup-1, with a kernel size of 3 × 3 and a pool size of 2 × 2, achieved an accuracy of 96%.

Rahman et al. (8) transformed a malaria parasite object detection dataset into a classification dataset, making it the largest malaria classification dataset (63,645 cells), and evaluated the performance of several state-of-the-art deep neural network architectures pretrained on both natural and medical images on this new dataset. We provide deeper insights into the influence of synthetic images for the class imbalance problem in the malaria diagnosis context.

Madhu G et al. (9) developed an Imperative Dynamic routing mechanism with fully trained capsule networks for malaria classification. This model identifies the presence of malaria parasites by classifying thin blood smears containing samples of parasitized and healthy erythrocytes. Such proposed model has been evaluated and compared with novel machine vision models that evolved over a decade such as VGG, ResNet, DenseNet, and MobileNet. Through the assistance of the proposed capsule network, the problems in previous research have been well addressed.

The above studies reflect that AI performs well in detecting and classifying plasmodium images. Nevertheless, it only carries out classification on the cropped thin blood smear image that is not practical in the real world. The detection and classification of a single picture have no clinical significance. We extracted a whole pathological slice and then divided it into small pictures for detection so that we could diagnose whether a person has malaria instead of just offering a picture that displays diagnostic outcomes, which is of great help to assist clinicians in making judgments.

3. Methods

In this section, a plasmodium classification method predicated on ResNeSt (10) is put forward. Since malaria plasmodium appeared anywhere in the picture, we proposed two methods to improve our training accuracy, respectively, during self-supervised learning in pre-training and supervised learning in training:

1. We utilized the self-supervised method during the pre-training stage. First, we selected pictures without malaria plasmodium; then we obtained the cells' location *via* segmentation; finally, we masked the cells and area at the same time instead of only masking the area during training for the purpose of enhancing training accuracy. Self-supervised learning by masking regions of cells allows the network to learn the features better.
2. Since malaria parasites showed up in any position. Also, Plasmodium staining is essentially the same, meaning that the features under certain color channels are the most distinct, we acquired better features through channel attention modules and spatial attention modules in the stage of supervised learning.

3.1. Datasets

The images of blood slices were retrospectively collected from the Department of Schistosomiasis and Endemic Diseases, Wuhan City Center between January 1, 2015 and February 29, 2020. They were labeled into two categories: confirmed plasmodium and non-plasmodium. The ages of all enrolled patients ranged from 18 to 75. Identifiable personal information, such as the names of patients, the names of hospitals, etc., was removed. The consecutive images of blood slices for each patient were selected for image recognition. A patient was classified as "plasmodium" as long as an image was identified with plasmodium. Otherwise, he was classified as "non-plasmodium."

The images were labeled by three experts with more than 10 years of clinical experience. A total of 30,033 blood slice images with 100 patients were employed to train our model. These images were randomized to the training set and validation set at a ratio of 3:1. Another test set with 12,784 blood slice images was exploited to independently evaluate the performance of our model. Table 1 details the basic information of the datasets.

3.2. Image enhancement

The color difference that occurs in Pathological images often influences the performance of classification. We applied image enhancement in SimCLR (11) to scale the dataset and eliminate the influence of color. First, a random patch of images was selected and resized to 224×224 with a random horizontal flip, followed by a color distortion, consisting of a random sequence of brightness, contrast, saturation, hue adjustments, and an optional grayscale conversion. Then, Gaussian blur and solarization were applied to the patches.

3.3. Image embedding by self-supervised learning

In deep learning, it is a frequent problem that there is not sufficient labeled data. The size of our labeled dataset is still inadequate for the detection of plasmodium. Considering the high cost of manually labelling data, we adopted BYOL (12), a self-supervised learning approach, to alleviate the problem of insufficient labeled data. BYOL is a powerful self-supervised learning method and does not need any negative samples. It only requires that similar samples have similar representations.

An architecture of BYOL is detailed in Figure 1. There are two neural networks: an online network and a target network. The two networks share the same structure. The θ and ξ are the parameters of the online network and the target network, respectively. The θ was updated by minimizing a similarity loss between $q_\theta(z_\theta)$ and $\mathrm{sg}(z_\xi)$ where sg means stop-gradient, while the ξ was updated by an exponential moving average of θ. After the training, the image embedding in the online network was utilized as the representation. BYOL has the advantages of high training efficiency and robustness to systematic deviation.

For malaria images, the cost of labelling is extremely high in that there are fewer doctors diagnosing malaria now. Thus, we only employed positive pictures for the self-supervised training of the model.

In the training, the images were rotated, color transformed, and subjected to different random cropping to get different positive sample pairs, and then input to the two networks. Since malaria parasites usually show up in cells, the pictures of malaria parasites are usually composed of cells. Therefore, when we masked the image, we first obtained the location of the cells through image segmentation. In the process of masking, we chose the masked cells, which is equivalent to improving the data set and distribution of positive samples with a view to promoting the efficiency of pre-training. We trained the online network to predict the target network representation of the same image under various enhanced views. Meanwhile, we used the slow moving average of the online network to update the target network. Furthermore, we kept the online network as our pre-training network subsequent to convergence.

TABLE 1 The basic information of the datasets.

	Training and validation set	Test set
Plasmodium	19,976	8,897
Non-plasmodium	10,057	3,887

3.4. Channel attention module and spatial attention module

Figure 2 gives a plasmodium image and the corresponding heat map. From Figure 2, we find that there are noticeable differences between different channels (Figure 3). Thus, we can use the channel attention mechanism (13) to improve the classification accuracy of plasmodium. Figure 4 provides a channel attention module that can be formulated as

$$\begin{aligned} \mathrm{M_c}(\mathrm{F}) &= \sigma\left(\mathrm{MLP}\left(\mathrm{avgPoolO}(\mathrm{F})\right)\right) \\ &+ \sigma\left(\mathrm{MLP}\left(\mathrm{maxPool}(\mathrm{F})\right)\right) \end{aligned} \tag{1}$$

The plasmodium randomly appears anywhere in the image. Therefore, the spatial attention mechanism (14) also applies to the classification of plasmodium. Different from channel attention, spatial attention focuses on 'where' the informative part lies, which is complementary to channel attention (15). To compute spatial attention, we first conducted average-pooling and max-pooling operations along the channel axis and concatenated them to generate an efficient feature descriptor. Data show that it is efficient to apply pooling operation along the channel axis in highlighting informative regions. Figure 5 displays a spatial attention module that can be described by

$$\begin{aligned} \mathrm{M_s}(\mathrm{F}) &= \sigma\left(\mathrm{f}^{7\times7}\left(\left[\mathrm{AvgPool}(\mathrm{F});\mathrm{MaxPool}(\mathrm{F})\right]\right)\right) \\ &= \sigma\left(\mathrm{f}^{7\times7}\left(\left[\mathrm{F}^{\mathrm{s}}_{\mathrm{avg}};\mathrm{F}^{\mathrm{s}}_{\mathrm{max}}\right]\right)\right) \end{aligned} \tag{2}$$

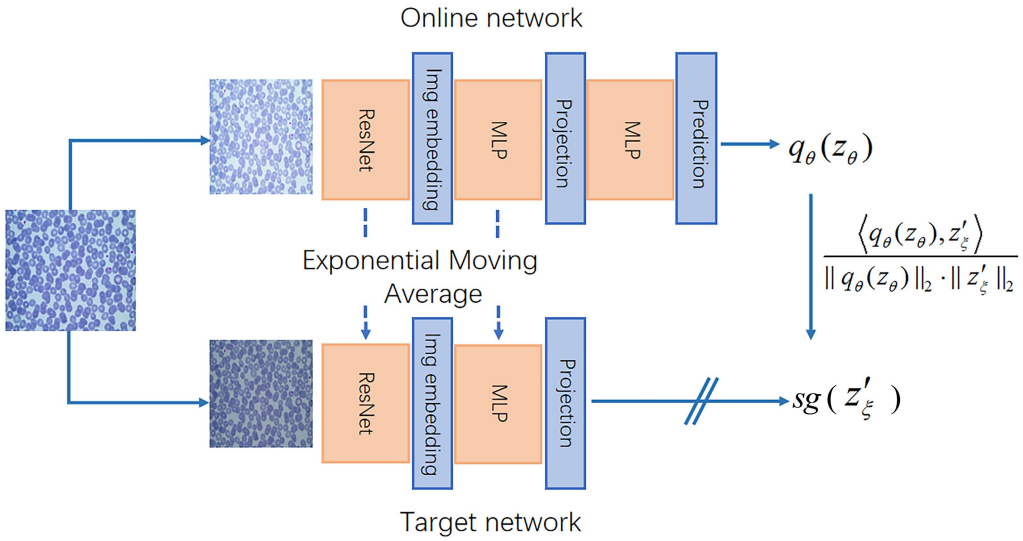

FIGURE 1
BYOL's architecture. BYOL minimizes a similarity loss between $q_\theta(z_\theta)$ and sg(z_ξ), where θ are the trained weights, ξ are an exponential moving average of θ and sg(z_ξ) means stop-gradient. At the end of training, everything but f_θ was discarded, and y_θ was used as the image representation.

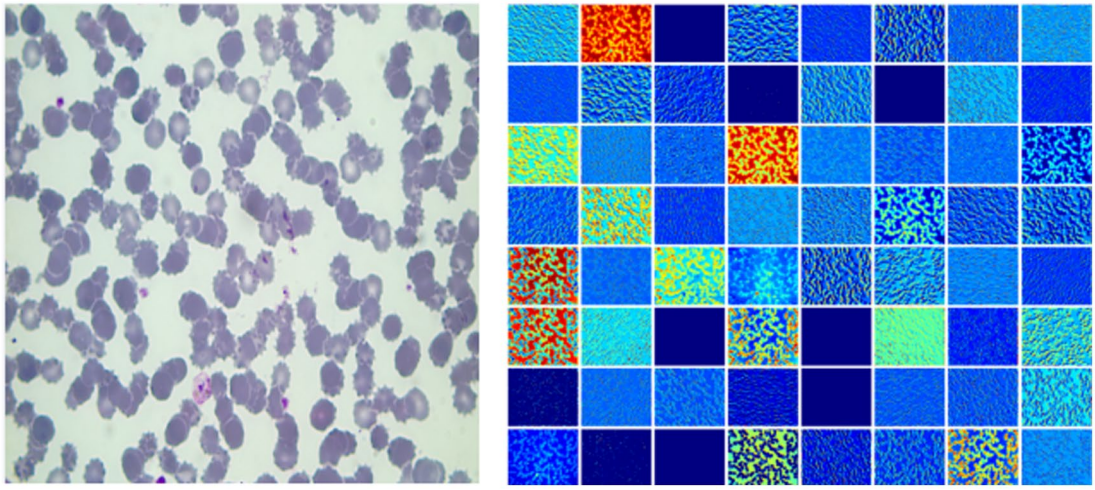

FIGURE 2
The left image is a plasmodium image, and the right image is the heat map.

4. Experiments

4.1. Training of the model

The training set was employed to construct the AI model, whereas the validation set was harnessed to assess the accuracy of classification performance of the established model.

With the application of the PyTorch platform, we adopted the ResNeSt-50 architecture pretrained and the BYOL to develop our AI algorithm. The retraining contained initializing the convolutional layers with loaded pretrained weights and updating the neural network to recognize plasmodium and non-plasmodium. The network structure was kept unchanged in this study. The weights of the last fully connected layer and the last three convolutional layers were tuned. After 50 epochs (iterations through the entire dataset), the training was terminated if no further improvements in accuracy or cross-entropy loss could be observed. The schematic diagram of our algorithm is exhibited in Figure 6.

4.2. The experimental results

During training and validation processes, accuracy and cross-entropy against the iteration step are plotted in Figure 7. Confusion matrix of the AI framework during the validation process is also displayed in Figure 8. The classification results of the test set are listed in Table 2. Given Figure 7 and Table 2, we can see that our training algorithm works and obtains the highest test accuracy of 97.8% compared to the other algorithms (Table 3).

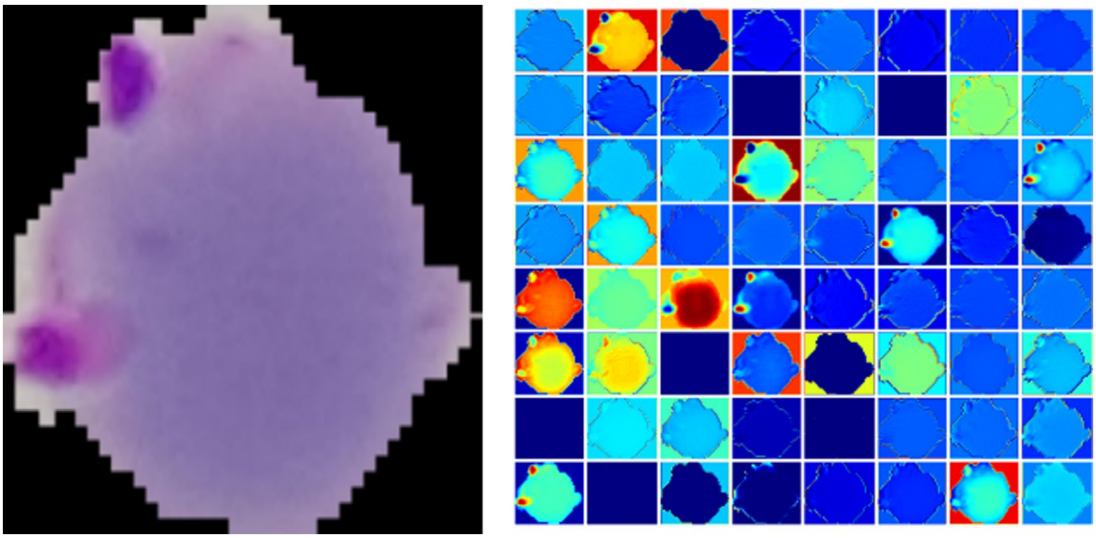

FIGURE 3
The left is a plasmodium image, and the right is a heat map.

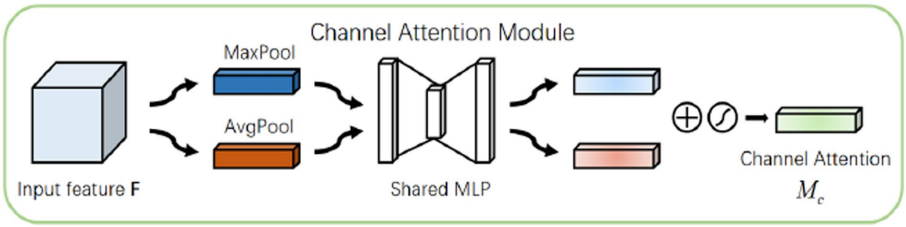

FIGURE 4
A channel attention block.

TABLE 2 The diagnostic performances of the different algorithms on the test set.

Algorithms	Accuracy (%)	Sensitivity (%)	Specificity (%)
ResNeSt	85.7	84.7	87.7
ResNeSt+Image Preprocessing	87.2	86.6	88.3
ResNeSt+Image Preprocessing+BYOL	90.5	89.7	92.1
ResNeSt+ Image Preprocessing+BYOL+Spatial Attention	92.7	91.9	94.2
ResNeSt+ Image Preprocessing+BYOL+Spatial Attentino+Channel Attention	95.8	94.9	97.4
ResNeSt+ Image Preprocessing+BYOL+Spatial Attentino+Channel Attention	97.6	96.4	98.7
ResNeSt+ Image Preprocessing+OurBYOL+Spatial Attentino+Channel Attention	**97.8**	96.5	98.9

5. Discussion

Malaria is a parasitic disease that seriously threatens human health. It primarily prevails in underdeveloped Africa, Southeast Asia, Oceania and Latin America where there is not enough capacity for detection. Detection is the key to the treatment of malaria cases and the control of infection sources. Microscopic examination and test paper are two commonly used detection methods with advantages such as rapidness, simplicity and low cost. The use of test paper is accompanied by the problems of false positive and false negative, and the antigen still exists in the blood for a long time even after the patient is cured. Therefore, microscopic examination is still regarded

TABLE 3 A comparison between the AI algorithm and radiologist.

Object	Accuracy (%)
Physicians ($N = 5$)	90
AI modal	99

The experiment used the same dataset and all five physicians had more than 10 years of diagnostic experience.

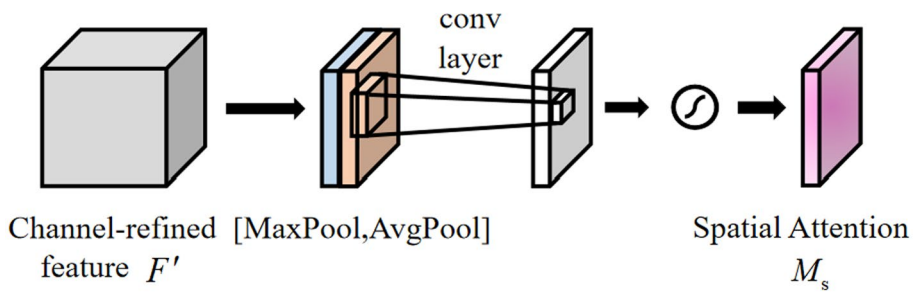

FIGURE 5
Spatial attention module.

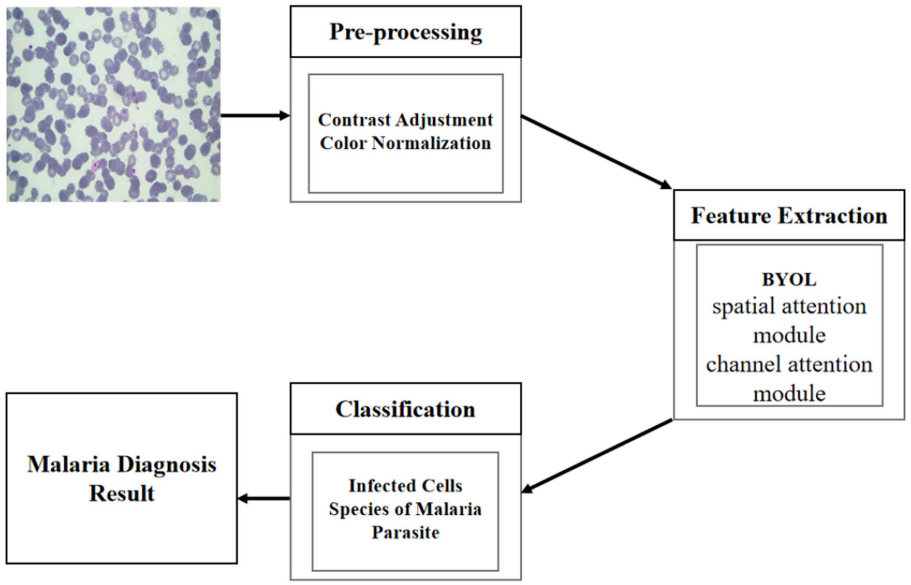

FIGURE 6
A schematic diagram of the algorithm.

as the gold standard of malaria diagnosis. Notwithstanding, the outcome of microscopic examination largely depends on personal experience and skill levels.

6. Conclusion

The classification method of thin blood membranes of AI based on the self-supervision and attention mechanism is a training model founded on the whole pathological section, which can better enable clinicians to make decisions. The brightness, contrast, saturation, and hue of most Plasmodium images are in a similar range due to staining, so the channel attention mechanism is used to improve the feature extraction ability of the model, and the spatial attention mechanism is used for the feature that Plasmodium can appear in various areas of the images. By using the channel attention and spatial attention, the feature extraction ability of the model is enhanced; at the same time, the feature extraction ability of the model is also improved by using self-supervised learning to learn unlabeled data in response to the difficulty of image labeling and the difficulty of labeling. In terms of classification accuracy, it exceeds the average level of existing human doctors and paves the way for the future automatic technology of malaria detection.

7. Prospect

Our article has done a series of studies on Plasmodium classification and proposed a novel classification algorithm for Plasmodium, but there is a lack of research on the location detection and Plasmodium segmentation. So, we will conduct the following studies: 1. Subsequently, we will continue to study the image detection algorithm to get the position of Plasmodium on the picture to allow medical person to do better analysis. 2. Image segmentation of the Plasmodium picture to get the Plasmodium shape, so that the doctor can make the determination of the worm type.

Translated with www.DeepL.com/Translator (free version).

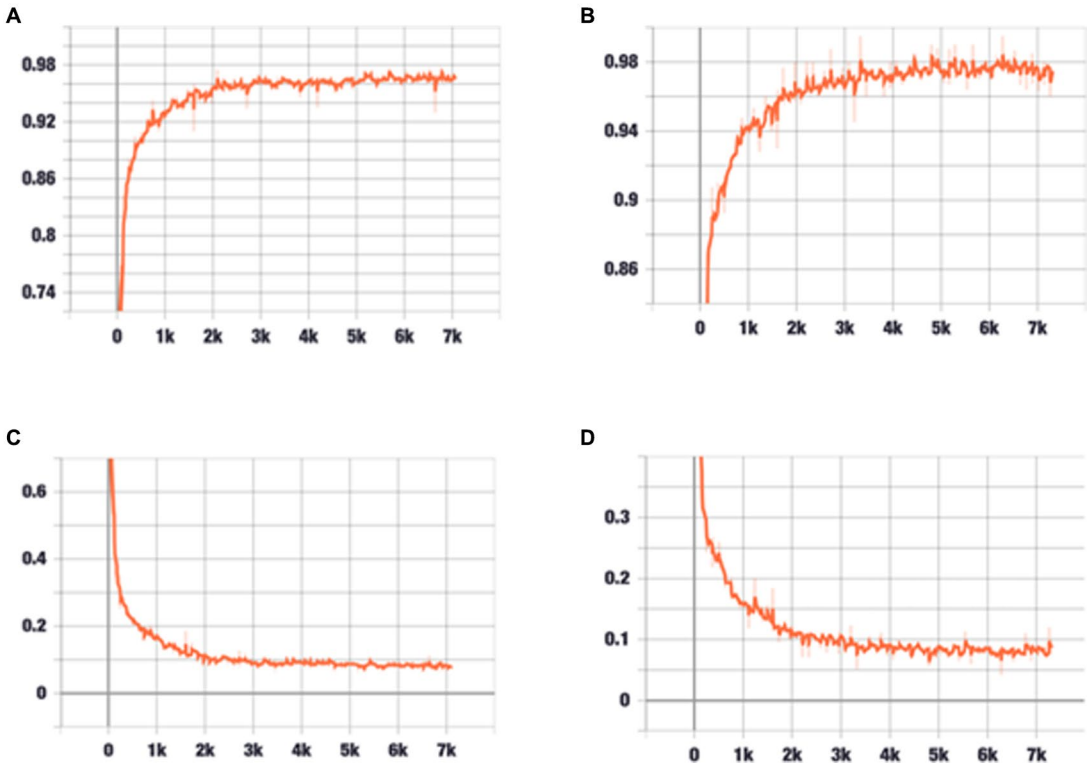

FIGURE 7
The performance of the model during training and validation. **(A)** Classification accuracy is plotted against training epochs. **(B)** The categorical cross-entropy loss is shown as a function of training epochs for the binary classification problem. **(C)** Classification accuracy is plotted against validation epochs. **(D)** The categorical cross-entropy loss is shown as a function of validation epochs for the binary classification problem. The graph shows that after about 1,000 rounds of training, the increase in accuracy rate slows down, and after about 2,000 rounds of training, the accuracy rate stabilizes at about 98%.

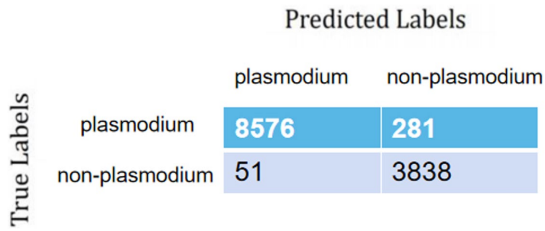

FIGURE 8
Confusion matrix of the AI framework.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: Contact first author for dataset use in deep learning. Requests to access these datasets should be directed to narcissist_fm@163.com.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of Wuhan Center for Disease Prevention and Control. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MF and KW: study concept and design. QZ and WH: acquisition of data. YL: image preprocessing. MF and LL: analysis and interpretation of data. MF and YL: drafting of the manuscript. LL and KW: critical revision of the manuscript for important intellectual content. All authors contributed to the article and approved the submitted version.

Funding

This study was supported by the Foundation for General Project of Health Commission of Hubei Province from 2021 to 2022 (Grant Number: WJ2021M024).

Acknowledgments

The authors are very grateful to the patients who enrolled in the study and provided the histological images.

Conflict of interest

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

Publisher's note

References

1. WHO. *World Malaria Report 2021*. Geneva: World Health Organization (2021).

2. Jun F, Shui-sen Z. From control to elimination: the historical retrospect of malaria control and prevention in China. *Chinese J Parasitol Parasit Dis*. (2019) 37:505–13.

3. Jun F, Li Z, Zhigui X, Ning X. Malaria elimination in China: an eminent milestone in the anti-malaria campaign and challenges in the post-elimination stage. *Chinese J Parasitol Parasit Dis*. (2021) 39:421–8. doi: 10.12140/j.issn.1000-7423.2021.04.001

4. Xie Y, Wu K, Cheng W, Jiang T, Yao Y, Xu M, et al. Molecular epidemiological surveillance of Africa and Asia imported malaria in Wuhan, Central China: comparison of diagnostic tools during 2011-2018. *Malar J*. (2020) 19:321. doi: 10.1186/s12936-020-03387-2

5. Rajaraman S, Antani SK, Poostchi M, Silamut K, Hossain MA, Maude RJ, et al. Pre-trained convolutional neural networks as feature extractors toward improved malaria parasite detection in thin blood smear images. *PeerJ*. (2018) 6:e4568. doi: 10.7717/peerj.4568

6. Liang Z, Powell A, Ersoy I, Poostchi M, Silamut K, Palaniappan K, et al. CNN-based image analysis for malaria diagnosis 2016 IEEE International Conference on Bioinformatics and Biomedicine (BIBM). IEEE, (2016).

7. Penas K, Rivera PT, Naval PC. Malaria parasite detection and species identification on thin blood smears using a convolutional neural network. IEEE/ACM International Conference on Connected Health: Applications. ACM (2017).

8. Rahman A, Zunair H, Reme TR, Rahman MS, Mahdy MRC. A comparative analysis of deep learning architectures on high variation malaria parasite classification dataset. *Tissue Cell*. (2021) 69:101473. doi: 10.1016/j.tice.2020.101473

9. Madhu G, Govardhan A, Srinivas BS, Sahoo KS, Jhanjhi NZ, Vardhan KS, et al. Imperative dynamic routing between capsules network for malaria classification. *Comput Mater Con*. (2021) 68:903–19. doi: 10.32604/cmc.2021.016114

10. Zhang H, Wu C, Zhang Z, Zhu Y, Lin H, Zhang Z, et al. Resnest: split-attention networks. Proceedings of the IEEE/CVF Conference on Computer Vision and Pattern Recognition (2022).

11. Chen T, Kornblith S, Norouzi M, Hinton G. A simple framework for contrastive learning of visual representations. International conference on machine learning, PMLR (2020).

12. Grill J-B, Strub F, Altché F, Tallec C, Richemond P, Buchatskaya E, et al. Bootstrap your own latent-a new approach to self-supervised learning. *Adv Neural Inf Proces Syst*. (2020) 33:21271–84.

13. Hu Jie, Shen Li, Sun Gang. Squeeze-and-excitation networks. Proceedings of the IEEE Conference on Computer Vision and Pattern Recognition (2018).

14. Woo S, Park J, Lee JY, Kweon IS Cbam: convolutional block attention module. Proceedings of the European Conference on Computer Vision (ECCV) (2018).

15. Gopakumar G, Swetha M, Siva GS, Subrahmanyam GR. (2016). Automatic detection of Malaria infected RBCs from a focus stack of bright field microscope slide images. Tenth Indian Conference on Computer Vision, ACM, 1–7.

frontiers | Frontiers in Medicine

TYPE Mini Review
PUBLISHED 11 July 2023
DOI 10.3389/fmed.2023.1141020

OPEN ACCESS

EDITED BY
Marcos De Lima,
The Ohio State University, United States

REVIEWED BY
Prasenjit Guchhait,
Regional Centre for Biotechnology (RCB), India

*CORRESPONDENCE
Erica M. Sparkenbaugh
✉ Erica_sparkenbaugh@med.unc.edu

RECEIVED 09 January 2023
ACCEPTED 26 June 2023
PUBLISHED 11 July 2023

CITATION
Ramadas N and Sparkenbaugh EM (2023) The APC-EPCR-PAR1 axis in sickle cell disease. *Front. Med.* 10:1141020. doi: 10.3389/fmed.2023.1141020

The APC-EPCR-PAR1 axis in sickle cell disease

Nirupama Ramadas[1] and Erica M. Sparkenbaugh[1,2]*

[1]Department of Medicine, Blood Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [2]Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

Sickle Cell Disease (SCD) is a group of inherited hemoglobinopathies. Sickle cell anemia (SCA) is caused by a homozygous mutation in the β-globin generating sickle hemoglobin (HbS). Deoxygenation leads to pathologic polymerization of HbS and sickling of erythrocytes. The two predominant pathologies of SCD are hemolytic anemia and vaso-occlusive episodes (VOE), along with sequelae of complications including acute chest syndrome, hepatopathy, nephropathy, pulmonary hypertension, venous thromboembolism, and stroke. SCD is associated with endothelial activation due to the release of danger-associated molecular patterns (DAMPs) such as heme, recurrent ischemia–reperfusion injury, and chronic thrombin generation and inflammation. Endothelial cell activation is mediated, in part, by thrombin-dependent activation of protease-activated receptor 1 (PAR1), a G protein coupled receptor that plays a role in platelet activation, endothelial permeability, inflammation, and cytotoxicity. PAR1 can also be activated by activated protein C (APC), which promotes endothelial barrier protection and cytoprotective signaling. Notably, the APC system is dysregulated in SCD. This mini-review will discuss activation of PAR1 by APC and thrombin, the APC-EPCR-PAR1 axis, and their potential roles in SCD.

KEYWORDS

sickle cell disease, vaso-occlusion, inflammation, sickle cell anemia, protease activated receptor 1 (PAR1), endothelial protein C receptor (EPCR), activated protein C (APC), thrombin

Introduction

Sickle cell disease

Sickle cell disease (SCD) is the most common inherited hemoglobinopathy worldwide. More than 300,000 babies are born with SCD annually and this rate is expected to increase over the next 30 years (1). The majority of cases are concentrated in sub-Saharan Africa and southern Asia, where sickle cell trait provides protection from malaria (2). Sickle Cell Anemia (SCA) is caused by a single nucleotide mutation in the gene for beta (β) globin. Normal hemoglobin is a tetramer of two α and two β subunits, each of which contain a heme molecule, and is the critical oxygen carrying protein in red blood cells (RBCs). In SCA, an A to T transversion in the sixth codon results in the substitution of a valine (Val) for a glutamine (Glu). This hydrophobic valine residue confers an adhesive property to HbS (comprised of two α and two β^S subunits); thus, when it becomes deoxygenated it forms rigid polymers in RBCs (Figure 1). This results in the sickling of RBCs, causing hemolysis and anemia. Hemolysis releases HbS and free heme into the circulation, acting as danger associated molecular patterns (DAMPs) to activate the endothelium and leukocytes. The activated endothelium upregulates adhesion molecules

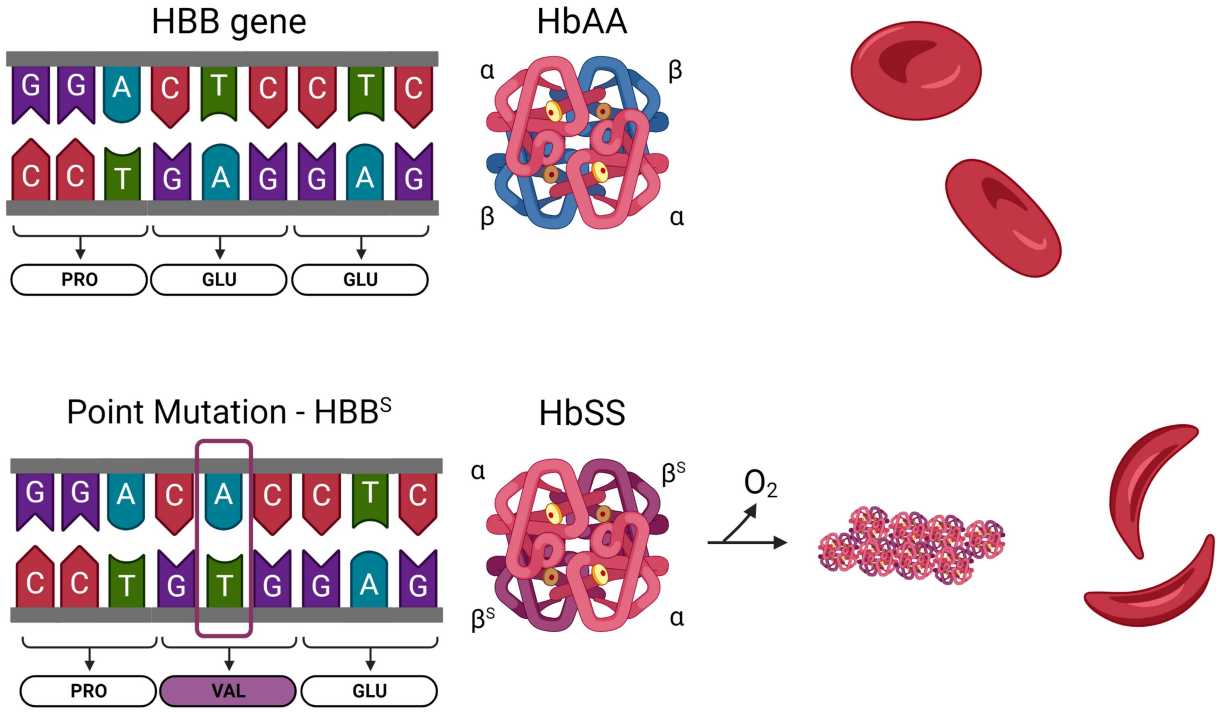

FIGURE 1
Sickle cell anemia is caused by a single nucleotide mutation in the *HBB* gene. Normal hemoglobin (HbAA) is formed from two α-globin and two β-globin subunits. The HBB gene encodes β-globin. In SCD, an adenine to thymine substitution changes the 6th codon of the mature protein from a glutamine to a valine. Sickle hemoglobin (HbSS) contains two α-globin and two β^S-globin. Upon deoxygenation, the presence of a hydrophobic valine residue in HbSS causes the molecules to polymerize, leading to stiffening of the RBC.

E-selectin, P-selectin, vascular cell adhesion molecule (VCAM), intercellular adhesion molecule (ICAM), and von Willebrand Factor (VWF). Activated neutrophils, platelets, and sickled RBCs form multicellular aggregates in the circulation, which adhere to the circulating endothelium, leading to vaso-occlusive episodes (VOE). These two primary pathologies, hemolytic anemia and VOE, are accompanied by sequelae of acute and chronic complications such as acute painful crises (3), chronic pain, stroke (4–8), venous thrombosis and pulmonary embolism (9, 10), pulmonary hypertension (11), acute chest syndrome (12), sickle nephropathy (13, 14), among others. More comprehensive reviews of SCD and its complications can be found elsewhere (3, 15).

Current treatments for SCD

Despite the significant global burden of SCA, there are only four FDA-approved drugs currently available to patients: hydroxyurea, L-glutamine, crizanlizumab, and Voxelotor (16). Hydroxyurea inhibits HbS polymerization and sickling by increasing production of fetal Hb (HbF) (17). L-glutamine reduces oxidative stress (18). Crizanlizumab inhibits P-selectin-dependent sRBC-endothelial interactions (19). Voxelotor changes the affinity of HbS for oxygen and inhibits hemoglobin polymerization (20). These therapies modestly limit the severity and frequency of VOC (17–19). Many SCA patients also routinely undergo whole blood transfusions and red blood cell exchange therapy (3, 21). Curative options such as hematopoietic stem cell transplantation (HSCT) and gene therapy (GT) are also being investigated (22). Allogeneic HSCT has been performed in approximately 2,000 patients in the past 30 years. A recent meta-analysis revealed that HSCT reduces the incidence of VOE, but it also identified risks including graft-versus host disease, graft failure, mortality, and secondary malignancies (23). The GT strategies currently being evaluated are correction of the HbS mutation, gene transfer to overexpress HbA in hematopoietic stem cells, and knockdown of BCL11a, the negative regulator of HbF, to increase HbF production and prevent sickling (3, 22–24). Although these therapies are promising, the FDA may require long-term follow up of 10–15 years after gene therapy to evaluate safety risks before they will be approved for clinical use (25). HSCT and GT carry significant costs and medical resources, and may not be feasible in low-resource countries where SCA is most prevalent (22). Recent GT trials have also been paused due to unexpected toxicity (26). The limited range of approved drugs for SCA, combined with an ageing population facing severe clinical complications, highlights the need to investigate new treatment options that are accessible and effective. Several drugs targeting downstream events are currently being evaluated in Phase II and III clinical trials, including anti-sickling agents, anti-inflammatory agents, and anticoagulants (3, 15).

Coagulation activation in SCA

A hallmark of SCA is activation of coagulation (27–33). Tissue factor (TF) is the primary initiator of extrinsic coagulation and is not normally expressed on intravascular cells. In SCA, TF expression is

upregulated on leukocytes and endothelial cells (34–37). TF is a transmembrane protein and obligate cofactor for coagulation factor VIIa (FVIIa), activating factor X (FX) to FXa, which converts prothrombin to thrombin. Thrombin cleaves fibrinogen into fibrin, leading to clot formation. We and others have shown that TF (4, 5, 37, 38), FXa (6, 39), thrombin (39, 40), and fibrin(ogen) (41, 42) contribute to inflammation, cardiovascular dysfunction, vascular congestion, nephropathy, and microvascular stasis (43) in mouse models of SCA. In addition to its prothrombotic role, thrombin can induce signaling through Protease Activated Receptor-1 (PAR1).

Protease activated receptors

Protease activated receptors (PARs) are a family of G-protein coupled receptors (GPCR) consisting of PAR1, PAR2, PAR3, and PAR4. PARs share a conserved mechanism of irreversible activation by proteolytic cleavage of specific amino acid residues on the extracellular N-terminus. This results in the exposure of a novel N-terminal peptide, or tethered ligand, which binds to extracellular loop 2 and induces a conformational change in the GPCR to signal through intracellular G proteins (44). PARs are subject to proteolysis by multiple proteases at different amino acid residues on the N-terminus, resulting in activation of different signaling pathways and outputs (45). The focus of this review is PAR1, which was first identified as the main thrombin receptor on platelets (46–48), triggering activation and aggregation of platelets that is critical for both hemostasis and thrombosis (49). Importantly, PAR1 is also expressed on leukocytes and endothelial cells. Thrombin cleaves PAR1 at arginine 41 (R41) (50), generating a tethered ligand that binds to a conserved sequence in extracellular loop 2 (51). This enables the C-terminus to engage with Gαq and $G\alpha_{12/13}$ which leads to inflammation, endothelial barrier permeability, and cytotoxicity (52, 53) (Figure 2). Thrombin/PAR1 activation of Gαq and $G\alpha_{12/13}$ upregulates inflammatory cytokines [interleukin-1 (IL-1), IL-6, and tumor necrosis factor α (TNFα)] and endothelial adhesion molecules [E-selectin, P-selectin, intracellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1)] via activation of MAPK and NFκB. G-protein mediated Ca^{2+} signaling promotes the release of Weibel Palade bodies to the endothelial surface, increasing P-selectin and von Willebrand Factor (VWF) release, thus increasing adhesion. These signaling pathways also cause apoptosis, through caspase activation, and endothelial barrier permeability, by modulating the cytoskeleton and disrupting tight junctions (54). Thrombin/PAR1 signaling is rapid, transient, and irreversible, due to the proteolytic cleavage of the protein. Signal termination is mediated by endocytosis of the receptor in clathrin-coated pits, and lysosomal degradation (55). Matrix metalloproteases MMP-1 and MMP-13 also cleave PAR1 at aspartate 39 (D39) and serine 42 (S42), respectively, with signaling outputs similar to thrombin (53).

Interestingly, PAR1 is also cleaved by activated protein C (APC), although with lower affinity than thrombin (56). The zymogen protein C binds to endothelial protein C receptor (EPCR), a type I transmembrane protein that binds the Gla domain of protein C and APC (57). On endothelial cells, the majority of EPCR is found in caveolin-1 (Cav1)-positive lipid rafts, where it colocalizes with PAR1 (58), which is required for cytoprotective signaling (59, 60). When bound to EPCR, protein C is cleaved by thrombomodulin-bound thrombin to generate the active serine protease APC, which cleaves PAR1 at Arg46 and activates multiple signaling pathways. APC/PAR1 signaling recruits and phosphorylates β arrestin-2, which activates Rac1, inhibits NFκB, and increases the barrier integrity of the endothelium (59). β arrestin-2 also activates sphingosine kinase 1 (SphK1), which converts the lipid messenger sphingosine into sphingosine-1-phosphate (S1P). In turn, S1P activates Sphingosine-1-phosphate receptor-1 (S1PR1), which signals through Gi/Akt and disheveled 2 (Dvl2) for anti-apoptotic and anti-inflammatory signaling (61, 62). aPC/PAR1-induced β-arrestin 2 also phosphorylates ERK1/2 (61). APC thus promotes anti-inflammatory and cytoprotective signaling on ECs and preserves endothelial barrier integrity (60, 63–65) (Figure 2). APC's cytoprotective and anti-inflammatory activity is not limited to ECs, as it can also modulate activation of monocytes, macrophages, and neutrophils, but has no effect on platelets (66). It is expressed on leukocytes (67–69), keratinocytes (70), vascular smooth muscle cells (71), cardiomyocytes (72), and neurons (73, 74), The opposing effects of PAR1 activation by thrombin and APC is a classic example of biased agonist signaling (65, 75), well described for other G-protein-coupled receptors including the neurokinin 1, angiotensin II type 1A, parathyroid hormone 1, μ opioid, and D2 dopamine receptors (76).

The dysregulated protein C system in SCA

APC is an important natural anticoagulant (77), in addition to its critical anti-inflammatory and cytoprotective role on the endothelium (78). Zymogen protein C is a glycoprotein produced by the liver. When activated by thrombin to its serine protease form APC, it irreversibly inactivates FVa and FVIIIa by proteolysis at arginine residues (79). Anticoagulant APC activity requires its cofactor protein S, a glycoprotein that binds negatively charged phospholipid membranes via its Gla domain (78). Dysfunction or deficiencies in the protein C—protein S system are associated with venous thrombosis (80–82). It is well-documented that individuals with SCD have deficiencies in both the antigen and activity levels of protein C and protein S (29, 83–89), and that they are further decreased during crisis (90). Protein C and protein S levels negatively correlate with markers of coagulation activation (88). Although deficiencies in this system have not been linked to VOC (29), lower levels of protein C and S are associated with a higher incidence of stroke in children and adolescents with SCA (87, 89, 91, 92). The low levels of protein C and S are likely caused by multiple factors common in SCA, including decreased synthesis due to liver disease (93), consumption due to chronic activation of coagulation, and binding to phosphatidylserine-positive sickled RBCs (94).

Decreased EPCR expression and EPCR shedding occurs in inflammatory bowel disease (95), malaria (96), diabetes (97), lupus, cardiovascular ischemia–reperfusion injury (98), and endotoxemia (99). EPCR shedding is mediated by pro-inflammatory cytokines and proteases such as TNFα converting enzyme (TACE), A Disintegrin and Metalloproteinase-10 (ADAM-10) and ADAM-17. Interestingly, EPCR shedding has been observed in individuals and mice with SCD (100, 101), and EPCR-positive microparticles are found in the circulation of individuals with SCD (90). A recent abstract described loss of EPCR expression in the kidney vasculature and presence of soluble EPCR in the urine of aged sickle mice, a phenomenon that

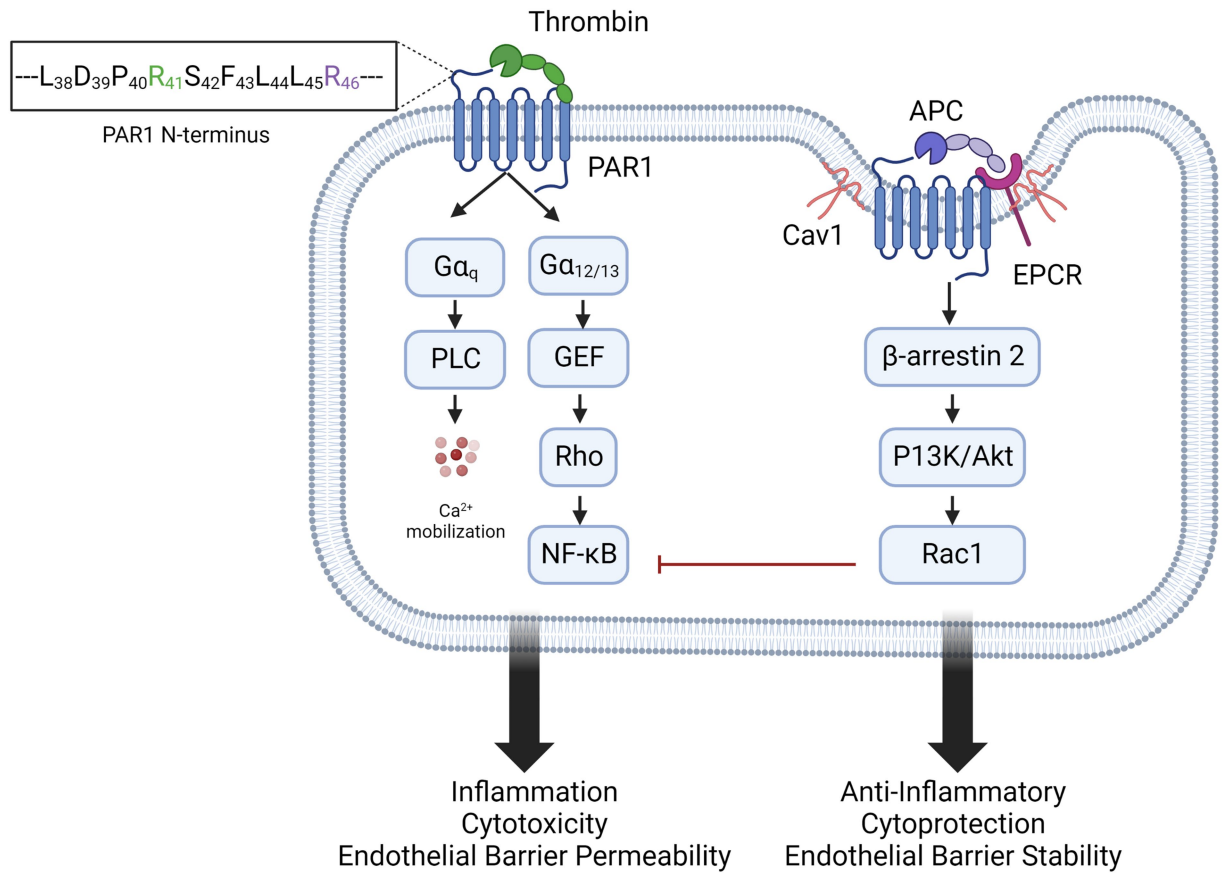

FIGURE 2
Biased agonism of PAR1 by thrombin and APC. Thrombin activation of PAR1, by cleavage at arginine 41 (R41) favors activation of Gαq and Gα12/13 signaling pathways. This results in inflammation, endothelial activation and barrier permeability, and cytotoxicity. In caveolin 1 (Cav1)-positive lipid rafts, PAR1 colocalizes with endothelial protein C receptor (EPCR). Activated protein C (APC) binds EPCR and activates PAR1 by cleavage of arginine 46 (R46), recruiting β-arrestin and inducing anti-inflammatory and endothelial stabilizing signaling.

could also be triggered in young sickle mice by infusion of a low dose of heme to mimic an acute sickling event (102).

Together, these observations describe dysfunction in regulation of the vascular endothelium. In SCD, the decreased availability of the natural anticoagulant APC and its cofactor protein S, along with diminished presence of endothelial EPCR could result in reduced cytoprotective APC/PAR1 signaling. Moreover, in a disease setting characterized by chronic thrombin generation, this imbalance might favor detrimental thrombin/PAR1 signaling. This imbalance could contribute to endothelial barrier dysfunction and vascular inflammation in SCD.

The role of PAR1 in SCA

The endothelium is chronically activated in SCA due to hemolysis (103), which contributes to the pathogenesis of vaso-occlusive events (VOE). Increased expression of adhesion molecules P-selectin and E-selectin on the endothelial surface promotes interaction with P-selectin glycoprotein ligand-1 (PSGL-1) and Cd11b/Cd18 (Mac1) on leukocytes, respectively (104). This event recruits sRBCs and platelets to form multicellular aggregates that drive vascular stasis and ultimately occlusion (105, 106). *In vitro* studies have demonstrated that activation of PAR1 with either thrombin or PAR1 agonist peptide drives the interactions between sickle RBCs and endothelial cells. This was found to be dependent on the release of P-selectin and von Willebrand Factor (VWF) from endothelial Weibel-Palade bodies (107, 108). Infusion of PAR1 agonist peptide caused rolling adhesion of sRBCs to the vascular endothelium that was p-selectin dependent in sickle mice, suggesting a role of PAR1 in vascular stasis (109). We also investigated the thrombin/PAR1 axis in sickle mice at steady state. To determine the role of PAR1, we transplanted sickle bone marrow (BM^{SS}) in $PAR1^{-/-}$ mice. Endothelial PAR1 deficiency did not affect the increased levels of thrombin generation (thrombin anti-thrombin complexes, TAT), systemic inflammation (IL-6), endothelial activation (sVCAM) or neutrophil recruitment in the lung vasculature. Interestingly thrombin inhibition with dabigatran reduced TAT, IL-6 and neutrophil recruitment to the organs (39). Agreeing with these results, Arumugam and colleagues found that decreasing expression of prothrombin reduces inflammation, vascular congestion, and improves survival of sickle mice (40). One possible interpretation of these data is that thrombin plays a role in endothelial activation and inflammation in SCD independent of PAR1, at least at steady state. An alternative hypothesis is that in BM^{SS} $PAR1^{-/-}$ mice, the lack of PAR1 also prevents beneficial APC/PAR1 signaling. Indeed, it has been shown that administration of APC to sickle mice can attenuate

thrombus formation in the cerebral microvasculature (101), indicating that APC is beneficial in SCD.

Since it is known that thrombin/PAR1 signaling contributes to endothelial P-selectin expression and sickle RBC adhesion, we also evaluated the role of this pathway in microvascular stasis. Using a dorsal skinfold chamber to evaluate blood flow in the skin microvasculature, we found that inhibition of PAR1 with the irreversible orthosteric antagonist vorapaxar protected sickle mice from heme-induced microvascular stasis (43). Similar results were obtained in BM^{SS} $PAR1^{-/-}$ mice, which also had significantly less endothelial P-selectin and VWF expression in lung tissue after heme treatment (43). These data suggest that thrombin/PAR1 activation might play a role in the cell–cell interactions that lead to VOC (43, 109), and that APC/PAR1 signaling can be beneficial (101), in SCD. Future studies should be aimed at determining the role of PAR1 in other acute and chronic complications of SCD, including stroke, thrombosis, and acute chest syndrome.

Current therapeutic strategies to target PAR1

Most PAR1 antagonists were designed to attenuate thrombin-mediated platelet activation and reduce thrombosis. Vorapaxar is an orally available antagonist that binds the extracellular pocket of PAR1 irreversibly and with high affinity. It effectively blocks PAR1 activation by both thrombin and APC. In clinical trials, administration of vorapaxar in combination with dual antiplatelet therapy improved cardiovascular outcomes but increased the risk of bleeding, especially in patients with a history of stroke (110). Thus, its use is counter-indicated in SCD patients. Recombinant APC (Xigris) was tested in pre-clinical models of sepsis, but had limited success and also increased the risk of bleeding in larger clinical trials (45). A signaling-selective variant of APC with limited anti-coagulant activity, 3K3A-APC, is also being tested for the treatment of stroke and amyotrophic lateral sclerosis (NCT02222714 and NCT05039268).

Another option for targeting PAR1 are small molecules. Q94 is an allosteric modulator that is thought to act at the intracellular face of PAR1. Although it inhibits PAR1-dependent platelet activation, it has limited efficacy on endothelial PAR1 signaling (111). Pepducins are a family of PAR1 modulators; they are biomimetic lipidated peptides that can enter the cell and target the intracellular loops of a receptor (112–114). One pepducin, PZ-128, is currently being tested in clinical trials for coronary artery disease (114) and has a promising safety profile. Parmodulins are small molecule allosteric modulators of PAR1, which bind the intracellular C-terminus and recruit β arrestin (115). They not only block thrombin-dependent PAR1 signaling; they can actually induce APC-like cytoprotective and anti-inflammatory signaling. Parmodulins have been shown to have significant anti-thrombotic and anti-inflammatory effects in mouse models of venous thrombosis (116, 117), neurologic diseases (115), virus (118), and diabetes (119).

Conclusion

The APC-EPCR-PAR1 axis is plays an important role in maintaining vascular endothelial homeostasis, and several studies described herein suggest that this pathway is dysfunctional in SCA. We speculate that chronic thrombin generation which can activate detrimental PAR1 signaling, paired with decreased APC/PAR1 signaling due to APC consumption and EPCR shedding, might play a role in the activated vascular endothelium in SCD.

Author contributions

NR and ES wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by NHLBI R01HL155193.

Conflict of interest

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

Publisher's note

References

1. Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. *Lancet*. (2013) 381:142–51. doi: 10.1016/S0140-6736(12)61229-X

2. Cyrklaff M, Sanchez CP, Kilian N, Bisseye C, Simpore J, Frischknecht F, et al. Hemoglobins S and C interfere with actin remodeling in plasmodium falciparum-infected erythrocytes. *Science*. (2011) 334:1283–6. doi: 10.1126/science.1213775

3. Kavanagh PL, Fasipe TA, Wun T. Sickle cell disease: a review. *JAMA*. (2022) 328:57–68. doi: 10.1001/jama.2022.10233

4. Francis RB. Large-vessel occlusion in sickle cell disease: pathogenesis, clinical consequences, and therapeutic implications. *Med Hypotheses*. (1991) 35:88–95. doi: 10.1016/0306-9877(91)90029-X

5. Prengler M, Pavlakis SG, Prohovnik I, Adams RJ. Sickle cell disease: the neurological complications. *Ann Neurol*. (2002) 51:543–52. doi: 10.1002/ana.10192

6. Adams RJ. Big strokes in small persons. *Arch Neurol*. (2007) 64:1567–74. doi: 10.1001/archneur.64.11.1567

7. Strouse JJ, Lanzkron S, Urrutia V. The epidemiology, evaluation and treatment of stroke in adults with sickle cell disease. *Expert Rev Hematol*. (2011) 4:597–606. doi: 10.1586/ehm.11.61

8. DeBaun MR, Jordan LC, King AA, Schatz J, Vichinsky E, Fox CK, et al. American Society of Hematology 2020 guidelines for sickle cell disease: prevention, diagnosis, and treatment of cerebrovascular disease in children and adults. *Blood Adv*. (2020) 4:1554–88. doi: 10.1182/bloodadvances.2019001142

9. Shet AS, Wun T. How I diagnose and treat venous thromboembolism in sickle cell disease. *Blood*. (2018) 132:1761–9. doi: 10.1182/blood-2018-03-822593

10. Lizarralde-Iragorri MA, Shet AS. Sickle cell disease: a paradigm for venous thrombosis pathophysiology. *Int J Mol Sci*. (2020) 21:5279. doi: 10.3390/ijms21155279

11. Vichinsky EP. Pulmonary hypertension in sickle cell disease. *N Engl J Med*. (2004) 350:857–9. doi: 10.1056/NEJMp038250

12. Vichinsky EP, Styles LA, Colangelo LH, Wright EC, Castro O, Nickerson B. Acute chest syndrome in sickle cell disease: clinical presentation and course. Cooperative study of sickle cell disease. *Blood*. (1997) 89:1787–92. doi: 10.1182/blood.V89.5.1787

13. Ataga KI, Orringer EP. Renal abnormalities in sickle cell disease. *Am J Hematol*. (2000) 63:205–11. doi: 10.1002/(SICI)1096-8652(200004)63:4<205::AID-AJH8>3.0.CO;2-8

14. Nath KA, Vercellotti GM. Renal functional decline in sickle cell disease and trait. *J Am Soc Nephrol*. (2020) 31:236–8. doi: 10.1681/ASN.2019121291

15. Kato GJ, Piel FB, Reid CD, Gaston MH, Ohene-Frempong K, Krishnamurti L, et al. Sickle cell disease. *Nat Rev Dis Primers*. (2018) 4:18010. doi: 10.1038/nrdp.2018.10

16. Ballas SK. The evolving Pharmacotherapeutic landscape for the treatment of sickle cell disease. *Mediterr J Hematol Infect Dis*. (2020) 12:e2020010. doi: 10.4084/mjhid.2020.010

17. Sethy S, Panda T, Jena RK. Beneficial effect of low fixed dose of hydroxyurea in vaso-occlusive crisis and transfusion requirements in adult HbSS patients: a prospective study in a tertiary care center. *Indian J Hematol Blood Transfus*. (2018) 34:294–8. doi: 10.1007/s12288-017-0869-x

18. Niihara Y, Miller ST, Kanter J, Lanzkron S, Smith WR, Hsu LL, et al. A phase 3 trial of l-glutamine in sickle cell disease. *N Engl J Med*. (2018) 379:226–35. doi: 10.1056/NEJMoa1715971

19. Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, et al. Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*. (2016) 376:429–39. doi: 10.1056/NEJMoa1611770

20. Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, et al. A phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med*. (2019) 381:509–19. doi: 10.1056/NEJMoa1903212

21. Han H, Hensch L, Tubman VN. Indications for transfusion in the management of sickle cell disease. *Hematol Am Soc Hematol Educ Program*. (2021) 2021:696–703. doi: 10.1182/hematology.2021000307

22. Tanhehco YC, Nathu G, Vasovic LV. Development of curative therapies for sickle cell disease. *Front Med Lausanne*. (2022) 9:1055540. doi: 10.3389/fmed.2022.1055540

23. Rotin LE, Viswabandya A, Kumar R, Patriquin CJ, Kuo KHM. A systematic review comparing allogeneic hematopoietic stem cell transplant to gene therapy in sickle cell disease. *Hematology*. (2023) 28:2163357. doi: 10.1080/16078454.2022.2163357

24. Ghiaccio V, Chappell M, Rivella S, Breda L. Gene therapy for beta-hemoglobinopathies: milestones, new therapies and challenges. *Mol Diagn Ther*. (2019) 23:173–86. doi: 10.1007/s40291-019-00383-4

25. Bailey AM, Arcidiacono J, Benton KA, Taraporewala Z, Winitsky S. United States Food and Drug Administration regulation of gene and cell therapies In: M Galli and M Serabian, editors. *Regulatory aspects of gene therapy and cell therapy products. Advances in experimental medicine and biology*. Cham: Springer (2015)

26. Quach D, Jiao B, Basu A, Bender MA, Hankins J, Ramsey S, et al. A landscape analysis and discussion of value of gene therapies for sickle cell disease. *Expert Rev Pharmacoecon Outcomes Res*. (2022) 22:891–911. doi: 10.1080/14737167.2022.2060823

27. Ataga KI, Key NS. Hypercoagulability in sickle cell disease: new approaches to an old problem. *Hematol Am Soc Hematol Educ Program*. (2007) 2007:91–6. doi: 10.1182/asheducation-2007.1.91

28. Leslie J, Langler D, Serjeant GR, Serjeant BE, Desai P, Gordon YB. Coagulation changes during the steady state in homozygous sickle-cell disease in Jamaica. *Br J Haematol*. (1975) 30:159–66. doi: 10.1111/j.1365-2141.1975.tb00530.x

29. Green D, Scott JP. Is sickle cell crisis a thrombotic event? *Am J Hematol*. (1986) 23:317–21. doi: 10.1002/ajh.2830230403

30. Stuart MJ, Setty BN. Hemostatic alterations in sickle cell disease: relationships to disease pathophysiology. *Pediatr Pathol Mol Med*. (2001) 20:27–46. doi: 10.1080/15513810109168816

31. Shah N, Thornburg C, Telen MJ, Ortel TL. Characterization of the hypercoagulable state in patients with sickle cell disease. *Thromb Res*. (2012) 130:e241–5. doi: 10.1016/j.thromres.2012.08.307

32. Sparkenbaugh E, Pawlinski R. Prothrombotic aspects of sickle cell disease. *J Thromb Haemost*. (2017) 15:1307–16. doi: 10.1111/jth.13717

33. Faes C, Sparkenbaugh EM, Pawlinski R. Hypercoagulable state in sickle cell disease. *Clin Hemorheol Microcirc*. (2018) 68:301–18. doi: 10.3233/CH-189013

34. Solovey A, Gui L, Key NS, Hebbel RP. Tissue factor expression by endothelial cells in sickle cell anemia. *J Clin Invest*. (1998) 101:1899–904. doi: 10.1172/JCI1932

35. Belcher JD, Marker PH, Weber JP, Hebbel RP, Vercellotti GM. Activated monocytes in sickle cell disease: potential role in the activation of vascular endothelium and vaso-occlusion. *Blood*. (2000) 96:2451–9. doi: 10.1182/blood.V96.7.2451

36. Shet AS, Aras O, Gupta K, Hass MJ, Rausch DJ, Saba N, et al. Sickle blood contains tissue factor-positive microparticles derived from endothelial cells and monocytes. *Blood*. (2003) 102:2678–83. doi: 10.1182/blood-2003-03-0693

37. Chantrathammachart P, Mackman N, Sparkenbaugh E, Wang JG, Parise LV, Kirchhofer D, et al. Tissue factor promotes activation of coagulation and inflammation in a mouse model of sickle cell disease. *Blood*. (2012) 120:636–46. doi: 10.1182/blood-2012-04-424143

38. Sparkenbaugh E, Faes C, Noubouossie D, Kirchhofer D, Gruber A, Key N, et al. FXIIa differentially regulates thrombin generation during steady state and vaso-occlusive crisis in sickle cell mice. *Blood*. (2016) 128:162. doi: 10.1182/blood.V128.22.162.162

39. Sparkenbaugh EM, Chantrathammachart P, Mickelson J, van Ryn J, Hebbel RP, Monroe DM, et al. Differential contribution of FXa and thrombin to vascular inflammation in a mouse model of sickle cell disease. *Blood*. (2014) 123:1747–56. doi: 10.1182/blood-2013-08-523936

40. Arumugam PI, Mullins ES, Shanmukhappa SK, Monia BP, Loberg A, Shaw MA, et al. Genetic diminution of circulating prothrombin ameliorates multiorgan pathologies in sickle cell disease mice. *Blood*. (2015) 126:1844–55. doi: 10.1182/blood-2015-01-625707

41. Nasimuzzaman M, Arumugam PI, Mullins ES, James JM, Vanden Heuvel K, Narciso MG, et al. Elimination of the fibrinogen integrin alphaMbeta2-binding motif improves renal pathology in mice with sickle cell anemia. *Blood Adv*. (2019) 3:1519–32. doi: 10.1182/bloodadvances.2019032342

42. Narciso MG, Hoeting B, James JM, Vanden Heuvel K, Nasimuzzaman M. A mutant fibrinogen that is unable to form fibrin can improve renal phenotype in mice with sickle cell anemia. *eJHaem*. (2021) 2:462–5. doi: 10.1002/jha2.204

43. Sparkenbaugh EM, Chen C, Brzoska T, Nguyen J, Wang S, Vercellotti GM, et al. Thrombin activation of PAR-1 contributes to microvascular stasis in mouse models of sickle cell disease. *Blood*. (2020) 135:1783–7. doi: 10.1182/blood.2019003543

44. Coughlin SR. Thrombin signalling and protease-activated receptors. *Nature*. (2000) 407:258–64. doi: 10.1038/35025229

45. Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost*. (2005) 3:1800–14. doi: 10.1111/j.1538-7836.2005.01377.x

46. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cells*. (1991) 64:1057–68. doi: 10.1016/0092-8674(91)90261-V

47. Vu TK, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. *Nature*. (1991) 353:674–7. doi: 10.1038/353674a0

48. Brass LF, Vassallo RR, Belmonte E, Ahuja M, Cichowski K, Hoxie JA. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J Biol Chem*. (1992) 267:13795–8. doi: 10.1016/S0021-9258(19)49635-X

49. Brass LF. Thrombin and platelet activation. *Chest*. (2003) 124:18S–25S. doi: 10.1378/chest.124.3_suppl.18S

50. Schuepbach RA, Madon J, Ender M, Galli P, Riewald M. Protease-activated receptor-1 cleaved at R46 mediates cytoprotective effects. *J Thromb Haemost*. (2012) 10:1675–84. doi: 10.1111/j.1538-7836.2012.04825.x

51. Nanevicz T, Ishii M, Wang L, Chen M, Chen J, Turck CW, et al. Mechanisms of thrombin receptor agonist specificity: chimeric receptors and complementary mutations identify an agonist recognition site (∗). *J Biol Chem*. (1995) 270:21619–25. doi: 10.1074/jbc.270.37.21619

52. Han X, Nieman MT. The domino effect triggered by the tethered ligand of the protease activated receptors. *Thromb Res*. (2020) 196:87–98. doi: 10.1016/j.thromres.2020.08.004

53. Willis Fox O, Preston RJS. Molecular basis of protease-activated receptor 1 signaling diversity. *J Thromb Haemost*. (2020) 18:6–16. doi: 10.1111/jth.14643

54. Rezaie AR. Protease-activated receptor signalling by coagulation proteases in endothelial cells. *Thromb Haemost*. (2014) 112:876–82. doi: 10.1160/TH14-02-0167

55. Grimsey N, Lin H, Trejo J. Chapter twenty-two-endosomal signaling by protease-activated receptors In: PM Conn, editor. *Methods in enzymology*. Cambridge, MA: Academic Press (2014). 389–401.

56. Ludeman MJ, Kataoka H, Srinivasan Y, Esmon NL, Esmon CT, Coughlin SR. PAR1 cleavage and signaling in response to activated protein C and thrombin. *J Biol Chem*. (2005) 280:13122–8. doi: 10.1074/jbc.M410381200

57. Fukudome K, Esmon CT. Identification, cloning, and regulation of a novel endothelial cell protein C/activated protein C receptor. *J Biol Chem*. (1994) 269:26486–91. doi: 10.1016/S0021-9258(18)47220-1

58. Mohan Rao LV, Esmon CT, Pendurthi UR. Endothelial cell protein C receptor: a multiliganded and multifunctional receptor. *Blood*. (2014) 124:1553–62. doi: 10.1182/blood-2014-05-578328

59. Roy RV, Ardeshirylajimi A, Dinarvand P, Yang L, Rezaie AR. Occupancy of human EPCR by protein C induces beta-arrestin-2 biased PAR1 signaling by both APC and thrombin. *Blood*. (2016) 128:1884–93. doi: 10.1182/blood-2016-06-720581

60. Bae JS, Yang L, Rezaie AR. Receptors of the protein C activation and activated protein C signaling pathways are colocalized in lipid rafts of endothelial cells. *Proc Natl Acad Sci U S A*. (2007) 104:2867–72. doi: 10.1073/pnas.0611493104

61. Molinar-Inglis O, Birch CA, Nicholas D, Orduña-Castillo L, Cisneros-Aguirre M, Patwardhan A, et al. aPC/PAR1 confers endothelial anti-apoptotic activity via a discrete, β-arrestin-2-mediated SphK1-S1PR1-Akt signaling axis. *Proc Natl Acad Sci U S A*. (2021) 118:e2106623118. doi: 10.1073/pnas.2106623118

62. Soh UJK, Trejo J. Activated protein C promotes protease-activated receptor-1 cytoprotective signaling through β-arrestin and dishevelled-2 scaffolds. *Proc Natl Acad Sci U S A*. (2011) 108:E1372–80. doi: 10.1073/pnas.1112482108

63. Bae JS, Yang L, Manithody C, Rezaie AR. The ligand occupancy of endothelial protein C receptor switches the protease-activated receptor 1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. *Blood*. (2007) 110:3909–16. doi: 10.1182/blood-2007-06-096651

64. Mosnier LO, Sinha RK, Burnier L, Bouwens EA, Griffin JH. Biased agonism of protease-activated receptor 1 by activated protein C caused by noncanonical cleavage at Arg46. *Blood*. (2012) 120:5237–46. doi: 10.1182/blood-2012-08-452169

65. Griffin JH, Zlokovic BV, Mosnier LO. Activated protein C: biased for translation. *Blood*. (2015) 125:2898–907. doi: 10.1182/blood-2015-02-355974

66. Healy LD, Rigg RA, Griffin JH, McCarty OJT. Regulation of immune cell signaling by activated protein C. *J Leukoc Biol*. (2018) 103:1197–203. doi: 10.1002/JLB.3MIR0817-338R

67. Sturn DH, Kaneider NC, Feistritzer C, Djanani A, Fukudome K, Wiedermann CJ. Expression and function of the endothelial protein C receptor in human neutrophils. *Blood*. (2003) 102:1499–505. doi: 10.1182/blood-2002-12-3880

68. Feistritzer C, Sturn DH, Kaneider NC, Djanani A, Wiedermann CJ. Endothelial protein C receptor-dependent inhibition of human eosinophil chemotaxis by protein C. *J Allergy Clin Immunol*. (2003) 112:375–81. doi: 10.1067/mai.2003.1609

69. Galligan L, Livingstone W, Volkov Y, Hokamp K, Murphy C, Lawler M, et al. Characterization of protein C receptor expression in monocytes. *Br J Haematol*. (2001) 115:408–14. doi: 10.1046/j.1365-2141.2001.03187.x

70. Xue M, Campbell D, Sambrook PN, Fukudome K, Jackson CJ. Endothelial protein C receptor and protease-activated receptor-1 mediate induction of a wound-healing phenotype in human keratinocytes by activated protein C. *J Invest Dermatol*. (2005) 125:1279–85. doi: 10.1111/j.0022-202X.2005.23952.x

71. Bretschneider E, Uzonyi B, Weber AA, Fischer JW, Pape R, Lotzer K, et al. Human vascular smooth muscle cells express functionally active endothelial cell protein C receptor. *Circ Res*. (2007) 100:255–62. doi: 10.1161/01.RES.0000255685.06922.c7

72. Wang J, Yang L, Rezaie AR, Li J. Activated protein C protects against myocardial ischemic/reperfusion injury through AMP-activated protein kinase signaling. *J Thromb Haemost*. (2011) 9:1308–17. doi: 10.1111/j.1538-7836.2011.04331.x

73. Gleeson EM, O'Donnell JS, Preston RJ. The endothelial cell protein C receptor: cell surface conductor of cytoprotective coagulation factor signaling. *Cell Mol Life Sci*. (2012) 69:717–26. doi: 10.1007/s00018-011-0825-0

74. Gorbacheva L, Davidova O, Sokolova E, Ishiwata S, Pinelis V, Strukova S, et al. Endothelial protein C receptor is expressed in rat cortical and hippocampal neurons and is necessary for protective effect of activated protein C at glutamate excitotoxicity. *J Neurochem*. (2009) 111:967–75. doi: 10.1111/j.1471-4159.2009.06380.x

75. Zhao P, Metcalf M, Bunnett NW. Biased signaling of protease-activated receptors. *Front Endocrinol Lausanne*. (2014) 5:67. doi: 10.3389/fendo.2014.00067

76. Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat Rev Drug Discov*. (2010) 9:373–86. doi: 10.1038/nrd3024

77. Walker FJ, Sexton PW, Esmon CT. The inhibition of blood coagulation by activated protein C through the selective inactivation of activated factor V. *Biochim Biophys Acta*. (1979) 571:333–42. doi: 10.1016/0005-2744(79)90103-7

78. Leon G, Rehill AM, Preston RJS. The protein C pathways. *Curr Opin Hematol*. (2022) 29:251–8. doi: 10.1097/MOH.0000000000000726

79. Mosnier LO, Gale AJ, Yegneswaran S, Griffin JH. Activated protein C variants with normal cytoprotective but reduced anticoagulant activity. *Blood*. (2004) 104:1740–4. doi: 10.1182/blood-2004-01-0110

80. Schwarz HP, Fischer M, Hopmeier P, Batard MA, Griffin JH. Plasma protein S deficiency in familial thrombotic disease. *Blood*. (1984) 64:1297–300. doi: 10.1182/blood.V64.6.1297.1297

81. Griffin JH. Clinical studies of protein C. *Semin Thromb Hemost*. (1984) 10:162–6. doi: 10.1055/s-2007-1004419

82. Gladson CL, Scharrer I, Hach V, Beck KH, Griffin JH. The frequency of type I heterozygous protein S and protein C deficiency in 141 unrelated young patients with venous thrombosis. *Thromb Haemost*. (1988) 59:18–22. doi: 10.1055/s-0038-1642558

83. Francis RB. Protein S deficiency in sickle cell anemia. *J Lab Clin Med*. (1988) 111:571–6.

84. Karayalcin G, Lanzkowsky P. Plasma protein C levels in children with sickle cell disease. *Am J Pediatr Hematol Oncol*. (1989) 11:320–3.

85. Marfaing-Koka A, Boyer-Neumann C, Wolf M, Leroy-Matheron C, Cynober T, Tchernia G. Decreased protein S activity in sickle cell disease. *Nouv Rev Fr Hematol 1978*. (1993) 35:425–30.

86. Peters M, Plaat BE, ten Cate H, Wolters HJ, Weening RS, Brandjes DP. Enhanced thrombin generation in children with sickle cell disease. *Thromb Haemost*. (1994) 71:169–72.

87. Tam DA. Protein C and protein S activity in sickle cell disease and stroke. *J Child Neurol*. (1997) 12:19–21. doi: 10.1177/088307389701200103

88. Westerman MP, Green D, Gilman-Sachs A, Beaman K, Freels S, Boggio L, et al. Antiphospholipid antibodies, proteins C and S, and coagulation changes in sickle cell disease. *J Lab Clin Med*. (1999) 134:352–62. doi: 10.1016/S0022-2143(99)90149-X

89. Schnog JB, Mac Gillavry MR, van Zanten AP, Meijers JC, Rojer RA, Duits AJ, et al. Protein C and S and inflammation in sickle cell disease. *Am J Hematol*. (2004) 76:26–32. doi: 10.1002/ajh.20052

90. Piccin A, Eakins E, Murphy CV, Mod WGM, Mcmahon C, Smith O. Circulating microparticles, plasma protein C and free protein S levels in children with sickle cell Anemia. *Blood*. (2008) 112:1431. doi: 10.1182/blood.V112.11.1431.1431

91. Khanduri U, Gravell D, Christie BS, Al Lamki Z, Zachariah M, Cherian E. Reduced protein C levels--a contributory factor for stroke in sickle cell disease. *Thromb Haemost*. (1998) 79:879–80. doi: 10.1055/s-0037-1615083

92. el-Hazmi MAF, Warsy AS, Bahakim H. Blood proteins C and S in sickle cell disease. *Acta Haematol*. (1993) 90:114–9. doi: 10.1159/000204390

93. Wright JG, Malia R, Cooper P, Thomas P, Preston FE, Serjeant GR. Protein C and protein S in homozygous sickle cell disease: does hepatic dysfunction contribute to low levels? *Br J Haematol*. (1997) 98:627–31. doi: 10.1046/j.1365-2141.1997.2663083.x

94. Whelihan MF, Lim MY, Mooberry MJ, Piegore MG, Ilich A, Wogu A, et al. Thrombin generation and cell-dependent hypercoagulability in sickle cell disease. *J Thromb Haemost*. (2016) 14:1941–52. doi: 10.1111/jth.13416

95. Kondreddy V, Keshava S, Esmon CT, Pendurthi UR, Rao LVM. A critical role of endothelial cell protein C receptor in the intestinal homeostasis in experimental colitis. *Sci Rep*. (2020) 10:20569. doi: 10.1038/s41598-020-77502-3

96. Maknitikul S, Luplertlop N, Grau GER, Ampawong S. Dysregulation of pulmonary endothelial protein C receptor and thrombomodulin in severe falciparum malaria-associated ARDS relevant to hemozoin. *PLoS One*. (2017) 12:e0181674. doi: 10.1371/journal.pone.0181674

97. Lattenist L, Ochodnicky P, Ahdi M, Claessen N, Leemans JC, Satchell SC, et al. Renal endothelial protein C receptor expression and shedding during diabetic nephropathy. *J Thromb Haemost*. (2016) 14:1171–82. doi: 10.1111/jth.13315

98. Ren D, Fedorova J, Davitt K, Van Le TN, Griffin JH, Liaw PC, et al. Activated protein C strengthens cardiac tolerance to ischemic insults in aging. *Circ Res*. (2022) 130:252–72. doi: 10.1161/CIRCRESAHA.121.319044

99. Gu JM, Katsuura Y, Ferrell GL, Grammas P, Esmon CT. Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding in vivo. *Blood*. (2000) 95:1687–93. doi: 10.1182/blood.V95.5.1687.005k08_1687_1693

100. Guo Y, Uy T, Wandersee N, Scott JP, Weiler H, Holzhauer S, et al. The protein C pathway in human and murine sickle cell disease: alterations in protein C, Thrombomodulin (TM), and endothelial protein C receptor (EPCR) at baseline and during acute vaso-occlusion. *Blood*. (2008) 112:538–202. doi: 10.1182/blood.V112.11.538.538

101. Gavins FN, Russell J, Senchenkova EL, De Almeida PL, Damazo AS, Esmon CT, et al. Mechanisms of enhanced thrombus formation in cerebral microvessels of mice expressing hemoglobin-S. *Blood*. (2011) 117:4125–33. doi: 10.1182/blood-2010-08-301366

102. Hazra R, Chen Q, Crosby D, Lenhart D, Tan R, Kim K, et al. Heme induced progressive loss of endothelial protein C receptor promotes development of chronic kidney disease in sickle cell disorders. *Blood*. (2021) 138:188. doi: 10.1182/blood-2021-154027

103. Hebbel RP, Osarogiagbon R, Kaul D. The endothelial biology of sickle cell disease: inflammation and a chronic vasculopathy. *Microcirculation*. (2004) 11:129–51. doi: 10.1080/mic.11.2.129.151

104. Zhang D, Xu C, Manwani D, Frenette PS. Neutrophils, platelets, and inflammatory pathways at the nexus of sickle cell disease pathophysiology. *Blood*. (2016) 127:801–9. doi: 10.1182/blood-2015-09-618538

105. Chen G, Chang J, Zhang D, Pinho S, Jang JE, Frenette PS. Targeting Mac-1-mediated leukocyte-RBC interactions uncouples the benefits for acute vaso-occlusion and chronic organ damage. *Exp Hematol*. (2016) 44:940–6. doi: 10.1016/j.exphem.2016.06.252

106. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*. (2014) 123:377–90. doi: 10.1182/blood-2013-04-495887

107. Cleator JH, Zhu WQ, Vaughan DE, Hamm HE. Differential regulation of endothelial exocytosis of P-selectin and von Willebrand factor by protease-activated receptors and cAMP. *Blood*. (2006) 107:2736–44. doi: 10.1182/blood-2004-07-2698

108. Matsui NM, Borsig L, Rosen SD, Yaghmai M, Varki A, Embury SH. P-selectin mediates the adhesion of sickle erythrocytes to the endothelium. *Blood*. (2001) 98:1955–62. doi: 10.1182/blood.V98.6.1955

109. Embury SH, Matsui NM, Ramanujam S, Mayadas TN, Noguchi CT, Diwan BA, et al. The contribution of endothelial cell P-selectin to the microvascular flow of mouse sickle erythrocytes in vivo. *Blood*. (2004) 104:3378–85. doi: 10.1182/blood-2004-02-0713

110. Franchi F, Rollini F, Faz G, Rivas JR, Rivas A, Agarwal M, et al. Pharmacodynamic effects of Vorapaxar in prior myocardial infarction patients treated with potent Oral

P2Y(12) receptor inhibitors with and without aspirin: results of the VORA-PRATIC study. *J Am Heart Assoc*. (2020) 9:e015865. doi: 10.1161/JAHA.120.015865

111. Francis LRA, Millington-Burgess SL, Rahman T, Harper MT. Q94 is not a selective modulator of proteinase-activated receptor 1 (PAR1) in platelets. *Platelets*. (2022) 33:1090–5. doi: 10.1080/09537104.2022.2026911

112. Kuliopulos A, Covic L. Blocking receptors on the inside: pepducin-based intervention of PAR signaling and thrombosis. *Life Sci*. (2003) 74:255–62. doi: 10.1016/j.lfs.2003.09.012

113. Covic L, Kuliopulos A. Protease-activated receptor 1 as therapeutic target in breast, lung, and ovarian cancer: Pepducin approach. *Int J Mol Sci*. (2018) 19:2237. doi: 10.3390/ijms19082237

114. Kuliopulos A, Gurbel PA, Rade JJ, Kimmelstiel CD, Turner SE, Bliden KP, et al. PAR1 (protease-activated receptor 1) Pepducin therapy targeting myocardial necrosis in coronary artery disease and acute coronary syndrome patients undergoing cardiac catheterzation: a randomized, placebo-controlled, phase 2 study. *Arter Thromb Vasc Biol*. (2020) 40:2990–3003. doi: 10.1161/ATVBAHA.120.315168

115. Festoff BW, Dockendorff C. The evolving concept of neuro-Thromboinflammation for neurodegenerative disorders and Neurotrauma: a rationale for PAR1-targeting therapies. *Biomol Ther*. (2021) 11:11. doi: 10.3390/biom11111558

116. Aisiku O, Peters CG, De Ceunynck K, Ghosh CC, Dilks JR, Fustolo-Gunnink SF, et al. Parmodulins inhibit thrombus formation without inducing endothelial injury caused by vorapaxar. *Blood*. (2015) 125:1976–85. doi: 10.1182/blood-2014-09-599910

117. De Ceunynck K, Peters CG, Jain A, Higgins SJ, Aisiku O, Fitch-Tewfik JL, et al. PAR1 agonists stimulate APC-like endothelial cytoprotection and confer resistance to thromboinflammatory injury. *Proc Natl Acad Sci U S A*. (2018) 115:E982–91. doi: 10.1073/pnas.1718600115

118. Subramaniam S, Ogoti Y, Hernandez I, Zogg M, Botros F, Burns R, et al. A thrombin-PAR1/2 feedback loop amplifies thromboinflammatory endothelial responses to the viral RNA analogue poly(I:C). *Blood Adv*. (2021) 5:2760–74. doi: 10.1182/bloodadvances.2021004360

119. Al-Dabet MM, Shahzad K, Elwakiel A, Sulaj A, Kopf S, Bock F, et al. Reversal of the renal hyperglycemic memory in diabetic kidney disease by targeting sustained tubular p21 expression. *Nat Commun*. (2022) 13:5062. doi: 10.1038/s41467-022-32477-9

frontiers | Frontiers in Oncology

TYPE Brief Research Report
PUBLISHED 03 August 2023
DOI 10.3389/fonc.2023.1206715

OPEN ACCESS

EDITED BY
Marcos De Lima,
The Ohio State University, United States

REVIEWED BY
Saurabh Chhabra,
Mayo Clinic Arizona, United States
Muhammad Salman Faisal,
University at Buffalo, United States
Hans C. Lee,
University of Texas MD Anderson Cancer Center, United States

*CORRESPONDENCE
Nausheen Ahmed
nahmed5@kumc.edu

†These authors have contributed equally to this work

RECEIVED 16 April 2023
ACCEPTED 29 June 2023
PUBLISHED 03 August 2023

CITATION
Ahmed N, Wesson W, Mushtaq MU, Bansal R, AbdelHakim H, Bromert S, Appenfeller A, Ghazal BA, Singh A, Abhyankar S, Ganguly S, McGuirk J, Abdallah A-O and Shune L (2023) "Waitlist mortality" is high for myeloma patients with limited access to BCMA therapy. *Front. Oncol.* 13:1206715. doi: 10.3389/fonc.2023.1206715

"Waitlist mortality" is high for myeloma patients with limited access to BCMA therapy

Nausheen Ahmed[1*], William Wesson[2], Muhammad Umair Mushtaq[1], Rajat Bansal[1], Haitham AbdelHakim[1], Sarah Bromert[1], Allison Appenfeller[1], Batool Abu Ghazal[1], Anurag Singh[1], Sunil Abhyankar[1], Siddhartha Ganguly[1,3], Joseph McGuirk[1], Al-Ola Abdallah[1†] and Leyla Shune[1†]

[1]Division of Hematologic Malignancies & Cellular Therapeutics, University of Kansas Cancer Center, Westwood, KS, United States, [2]School of Medicine, University of Kansas, Kansas, KS, United States, [3]Mary and Ron Neal Cancer Center, Houston Methodist Hospital, Houston, TX, United States

Background: The first-in-class approved BCMA CAR-T therapy was idecabtagene vicleucel (ide-cel), approved in March 2021, for RRMM patients who progressed after 4 or more lines of therapy. Despite the promising outcomes, there were limited apheresis/production slots for ide-cel. We report outcomes of patients at our institution who were on the "waitlist" to receive ide-cel in 2021 and who could not secure a slot.

Methods: We conducted a retrospective review of RRMM patients evaluated at the University of Kansas Cancer Center for ide-cel from 3/2021-7/2021. A retrospective chart review was performed to determine patient and disease characteristics. Descriptive statistics were reported using medians for continuous variables. Survival analysis from initial consult was performed using Kaplan-Meier Survival estimator.

Results: Forty patients were eligible and were on the "waitlist" for CAR-T. The median follow-up was 14 months (2-25mo). Twenty-four patients (60%) secured a production slot and 16 (40%) did not. The median time from consult to collection was 38 days (8-703). The median time from collection to infusion was 42 days (34-132 days). The median overall survival was higher in the CAR-T group (NR vs 9 mo, p<0.001).

Conclusion(s): Many patients who were eligible for ide-cel were not able to secure a timely slot in 2021. Mortality was higher in this group, due to a lack of comparable alternatives. Increasing alternate options as well as improvement in manufacturing and access is an area of high importance to improve RRMM outcomes.

KEYWORDS

BCMA, ide-cel, access, production slot, waitlist, myeloma

Introduction

Relapsed refractory multiple myeloma (RRMM) has a poor prognosis, with overall survival of around 6 months for penta-refractory patients, refractory to conventional therapy including immunomodulatory drugs (IMiDs), proteasome inhibitors (PIs), and CD38-directed therapy (1).

B cell maturation antigen (BCMA) is a novel treatment target for multiple myeloma due to its highly selective expression on plasma cells (2). The first-in-class BCMA chimeric antigen receptor T cell therapy (CAR-T) was idecabtagene vicleucel (ide-cel), approved in March 2021, for RRMM patients who progressed after ≥ 4 lines of therapy based on the results of the pivotal phase I/II KarMMa trial data. All patients had to be exposed to prior PIs, IMiDs, and CD38 targeting therapy as part of the FDA label. The overall response rate (ORR) was 73% and median duration of response (DOR) of 11 months in responders, and 20 mo in patients who had a stringent complete response (3, 4). Ide-cel manufacturing starts with leukapheresis, shipping of the T-cell apheresis product to the manufacturing facility, *in vitro* expansion, and transduction with a lentivirus vector (5). Purification and quality check is conducted prior to the release of the product and shipping back to the treating facility.

Despite the advancement in 2021 with ide-cel approval, the commercial manufacturing system has limited capacity, with limited production slot allocation nationally, and long manufacture times of at least 4 weeks (5–8). Long manufacturing and turnaround time increases the risk of mortality and morbidity in RRMM patients with rapidly progressive disease and potential deterioration before CAR-T infusion. In the KarMMa trial, 12 (8.5%) of the 140 patients who received leukapheresis were not able to receive the infusion of ide-cel. Only one of these was due to manufacturing failure, and the rest were secondary to patient condition and disease progression (4). The rollout of production slots for ide-cel has been relatively limited nationally.

In this report, we examine the outcomes of patients evaluated at our center for ide-cel between March to July 2021.

Patients and methods

RRMM patients seen at the University of Kansas Cancer Center for ide-cel consultation between March 2021 to July 2021 were included. Slot availability and utilization from March to October 2021 were reported. Per institution policy, only those who met the KarMMa inclusion criteria were considered eligible for commercial CAR-T. Those that did not meet the inclusion criteria were not considered eligible. All patients considered eligible were refractory to the latest therapy. Factors such as patient fitness and comorbidities, availability of caregivers were taken into consideration. All eligible patients who were agreeable to CAR-T therapy were enrolled in the pharmaceutical company's cell therapy portal. A "waitlist" was maintained with all eligible candidates. These patients were then presented at our Myeloma CAR-T planning weekly meeting, and the most appropriate candidates were selected for each ide-cel slot available. We evaluated the waitlist and compared the group that could not secure a CAR-T production slot (non-CAR-T group) and another group that secured a slot (CAR-T group). Patients who secured a slot but did not receive the ide-cel infusion were included in the CAR-T group. High-risk cytogenetics were per the International Myeloma Working Group (IMWG) criteria and included t(4;14), del (17/17p), t(14;16), t(14;20), and gain (1q) (9). Penta-refractory was defined as being refractory to two IMiDs, two PIs, and one CD38-directed therapy per IMWG criteria. The time to collection was defined as the time from consultation for CAR-T to leukapheresis. The time to manufacture was defined as the time from leukapheresis to CAR-T infusion. Lines of therapy at consultation was determined from diagnosis to initial consultation for CAR-T. Additional therapies for patients on the waitlist who could not secure a CAR-T production slot was not reported as line of therapy at consult. Bridging therapy was considered as therapy between leukapheresis to CAR-T infusion. Per institutional guidelines, bridging was held 2 weeks prior to cell infusion. Overall survival for both groups was calculated from the initial ide-cel consultation date. A retrospective chart review was performed to determine patient and disease characteristics, subsequent lines of therapy in the non-CAR-T group, and bridging therapy in the CAR-T group. The study was approved by the local institutional review board and conducted in accordance with the Declaration of Helsinki.

Statistical analysis

Descriptive statistics were reported using medians for continuous variables. Comparisons of categorical variables were conducted using the Fisher's Exact Test. A comparison of medians was conducted using a Mann-Whitney U test. Survival analysis was performed utilizing the Kaplan-Meier Survival estimator. All outcomes used an *a priori* two-sided p-value of 0.05 for significance. All statistical analyses were conducted in JMP® (v15.1.0).

Results

Patient characteristics

Fifty-four patients were evaluated for ide-cel between March 2021 and July 2021. 14 patients (26%) were not eligible or chose not to pursue CAR-T therapy. There were 40 eligible patients who were included in further analysis. The median follow-up was 14 months (2-25mo). During this period, 16 (40%) were in the non-CAR-T group and could not secure a production slot while 24 (60%) patients secured a production slot (Figure 1). Table 1 summarizes

Abbreviations: IMiDs, Immunomodulatory drugs; PIs, Proteosome inhibitors; RRMM, relapsed refractory multiple myeloma; ORR, overall response rates; sCR, stringent complete response; BCMA, B cell maturation antigen; CAR-T, chimeric antigen receptor T cell; ide-cel, Idecabtagene vicleucel; cilta-cel, Ciltacabtagene Autocel; DOR, Duration of response; GMP, Good Manufacture Practice.

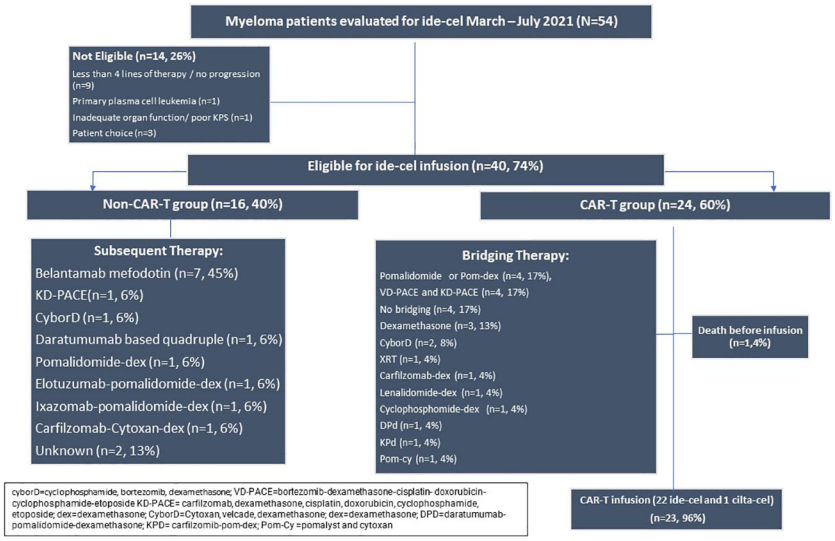

FIGURE 1
Consort of patients seen in consultation for standard of care ide-cel from March to July 2021.

the demographics of these two groups. The median age was 61 (43-82) years. The groups were similar in time since diagnosis, penta-refractory status, extramedullary disease, high risk cytogenetics, and exposure to prior BCMA therapy (belantamab mafodotin). There was a median of 2 production slots per month from March-October 2021 (range 0-9). All slots were utilized.

Non-CAR-T group

The alternate therapies in the sixteen patients who could not get CAR-T apheresis slots are listed in Figure 1. Belantamab mafodotin was the most frequent agent, used as monotherapy or in combination with dexamethasone (n=7, 45%).

CAR-T group

All patients in this group received leukapheresis for ide-cel except one patient who received leukapheresis for ciltacabtagene autocel (cilta-cel). The median time from the consult visit to the collection for the CAR-T group was 38 days (8-703). The median time from collection to infusion was 42 days (34-132). There were 5 patients who secured a CAR-T production slot > 4 months from initial consultation. These patients received between 1-3 additional lines of therapy after initial consultation before securing a production slot. Bridging therapies are listed in Figure 1. The most commonly used bridging regimens included pomalidomide monotherapy or in combination with dexamethasone (n=4, 17%), bortezomib or carfilzomib with dexamethasone-cisplatin-doxorubicin-cyclophosphamide-etoposide (VD-PACE or KD-PACE) (n=4, 17%) and dexamethasone only (n=3, 13%). Four patients (17%) received no bridging therapy.

Survival outcomes

The survival following CAR-T consult was lower among the non-CAR-T patients on the "waitlist" vs. the CAR-T group. With the median follow up of 14 mo, the mortality was 81% (13 patients) in the group that did not receive CAR-T, as depicted in Figure 2. Nine patients (38%) died in the ide-cel group due to progressive disease. One death occurred in a patient who was collected but did not receive an ide-cel infusion due to ongoing respiratory viral infection. The median OS was 9 mo in non-CAR-T group vs. NE in CAR-T group ($p<0.001$).

Discussion

Ide-cel launched in March 2021 as the first-in-class BCMA Car-T cell therapy in RRMM, and remained the only BCMA-CAR-T till 2/2022 (3, 10). It is available at authorized treatment centers, with 73 centers across the United States offering this therapy as of July 2022 (11). One of the main challenges faced by many of the centers delivering ide-cel has been the allocation of only a few production slots per month due to manufacturing capacity limitations. The median slots per month for our institution was 2 slot (0-9) in 2021 which resulted in a long time to collection for those on the waitlist. We note a survival advantage seen in the patients on the waitlist who were able to receive ide-cel compared to standard alternate therapies in 2021. This is reflected in other studies as well with both ide-cel and cilta-cel (12–15).

An additional challenge for those who secure a slot is the long manufacturing time. The median vein-to-vein time in the CAR-T patients was 42 days (range 34-132 days). This represented time to manufacture except for two patients who were delayed due to infection. This is consistent with the known manufacture time in KarMMa trial of around 4 weeks from apheresis to infusion (4).

TABLE 1 Patient and disease characteristics at consultation for CAR T.

Characteristics (n,%)	All patients (n=40)	Patients who Received Leukapheresis for BCMA CAR T (n=24)	Patients who could not receive leukapheresis for BCMA CAR T (n=16)	*p* value
Gender				
Male	26 (65)	16 (67)	10 (63)	1.000
Female	14 (35)	8 (33)	6 (38)	
Race				
Caucasian	28 (70)	19 (79)	9 (56)	0.126
African American	10 (25)	5 (21)	5 (31)	
Other	2 (5)	0 (0)	2 (13)	
Median Age at Consultation (Years, 25-75 Quartile)	61 (58-68)	61 (57-68)	61 (58-71)	0.709
Median Time from Diagnosis to Consult (Years, 25-75 Quartile)	5 (3-9)	6 (3-9)	4 (2-8)	0.192
Median Lines of Therapy at Consult (Number, 25-75 Quartile)	6 (5-8)	7 (5-8)	5 (5-7)	0.138
Myeloma Subtype				
IgG Kappa	12 (31)	8 (33)	4 (25)	0.925
IgG Lambda	8 (20)	5 (21)	3 (19)	
Light Chain (Lambda or Kappa)	7 (18)	4 (17)	3 (19)	
Other	13 (33)	7 (29)	6 (38)	
High-Risk Cytogenetics*	20 (50)	11 (46)	9 (56)	0.748
Extramedullary Disease**	12 (30)	7 (29)	5 (31)	1.000
Penta-refractory	32 (80)	21 (88)	11 (69)	0.229
Prior BCMA Therapy Exposure	10 (25)	7 (29)	3 (19)	0.711
Prior Autologous Transplant	30 (75)	20 (83)	10 (63)	0.159
Prior Allogeneic Transplant	3 (8)	3 (13)	0 (0)	0.255
Median Duration of Follow Up (Months, 25-75 Quartile)	14 (7-23)	22 (13-24)	8 (5-12)	**<0.001**
Median Time from Apheresis to Infusion (Days, 25-75 Quartile)	N/A	42 (37-49.5)	N/A	NC
6 Month Survival	31 (78)	22 (92)	9 (56)	**0.018**
Overall Survival (Months, 25-75 Quartile)	15 (9-NR)	NR (13-NR)	9 (5-12)	**<0.001**

*High risk Cytogenetics defined as t(4:14), t(14;16)t(14:20), gain 1q and 17p del. BCMA: B cell maturation antigen; **Defined as non-osseous extramedullary disease, BCMA=B cell maturation antigen.

One patient (5%) underwent leukapheresis but did not receive the infusion. Despite the long manufacture time, all other patients who underwent leukapheresis were able to receive the infusion. This appears to be better than the KarMMa data, where 12 (9%) patients did not receive the infusion after leukapheresis (4). Moreover, while manufacture failure was seen in 1% of patients in the KarMMa trial, we did not experience failure of manufacture in these patients in the real-world cohort (4). These observed differences observed may be secondary to limited sample size, differences in bridging strategies, and stringent patient selection adopted due to limited access.

The challenges with the production slot limitations and the long manufacture times has led to an ethical dilemma described by various institutions across the US (7). At our institution, we had stringent criteria for patient selection during the period evaluated. Only those patients who met the KarMMa inclusion criteria were considered. Even amongst these select patients, we had to choose the most appropriate candidates for the scarcely available slots. We had a weekly joint conference attended by the CAR-T and Plasma Cell Disorder teams at our institution to select appropriate patients, ideally who were fit, with a predictably slow pace of disease, with relatively low risk of morbidity

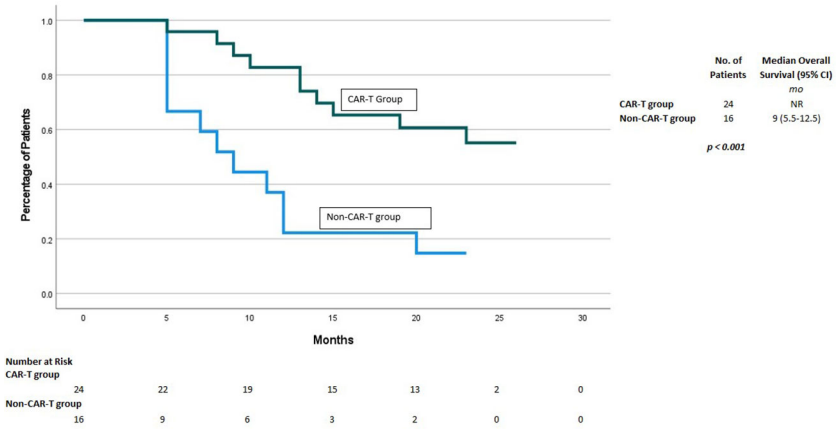

FIGURE 2
Overall Survival for CAR-T vs no CAR-T group.

and mortality during the manufacturing period. We took into consideration patient characteristics including performance status, cardiac function, creatinine clearance, comorbidities, and social support. We also had to take into consideration disease characteristics, such as lines of therapy, penta-refractory or triple-refractory status, number of transplants, prior BCMA therapy and clinical trial enrollment in last 24 months.

The RRMM field is dynamic and rapidly evolving with BCMA therapies. The approval of cilta-cel in February 2022 has increased the total slots per month, but opportunities for improvement exist (6). We describe in an editorial that the median number of production slots for BCMA CAR-T (including ide-cel and cilta-cel) increased at our institution in 2022 and 2023. In fact, in that report, we also now demonstrate that several production slots are not utilized, likely reflecting more readily available options with the approval of novel BCMA bispecifics such as teclistimab (16). Of note, belantamab is now withdrawn from the US market officially as of February 2023 (17). As BCMA CAR-T is being studied in earlier lines, and with various combinations, this novel class of therapy will likely be available for a broader patient population in the future (18, 19). Therefore, it is critical to prioritize research to improve manufacturing of ide-cel and other CAR-T products, and thereby increase utilization and access.

Several strategies are being proposed to counter the challenges in manufacturing and production seen with ide-cel as well as other autologous CAR-T products.

New CAR-T manufacturing platforms are being developed by numerous biotech companies that should allow for the scaling of manufacturing capacity while ensuring consistency in cell product properties (20, 21). These platforms too could be licensed to institutions once they receive full commercial approval to strengthen manufacturing. Decentralization and point of care manufacture at academic institutions is possible with at Good Manufacture Practice (GMP) – complaint closed automated systems which ensure reproducibility can improve supply chain and reduce manufacture time (22–24). Allogeneic BCMA CAR-T products are under investigation. These third-party products have the advantage of being readily available, "off-the-shelf", with no manufacturing requirements. Several allogeneic BCMA CAR-T cell therapies are in development, including CTX120, CYAD-211 (25–27).

Moreover, non-CAR-T alternatives for BCMA therapy are now available, which will help improve outcomes for those who cannot secure a CAR-T slot. Belantamab mafodotin was the only available first-in-class immunoconjugate targeting BCMA, showing single-agent activity in the phase 1 DREAMM-1 study available during the period that we conducted the study (28). Belantamab mafodotin was the most favored alternative for patients who did not have prompt access to CAR-T in our study population, however, it was withdrawn from the US market In November 2022 (29). Teclistimab is the first BCMA directed bispecific antibody T cell engager to be approved in December 2022 (30). Other promising novel BCMA and non-BCMA directed bispecific antibody T cell engagers are in development and will broaden the non-CAR-T options for RRMM patients (31, 32).

Limitations of our study include that it was a retrospective review conducted at a single center with limited sample size. Many patients were referred from outside health systems and received bridging therapy and alternate therapies at different health systems. Although we captured survival data, we were not accurately able to capture granular details on number of cycles of therapy, and disease responses to the alternate therapies. Moreover, since patients with more aggressive disease biology were less likely to be selected for CAR-T given long wait times and manufacture times, we acknowledge a natural selection bias in survival differences.

We conclude that access limitations to ide-cel production slots existed in 2021 and there was a high mortality rate for patients who are on the waitlist and not able to secure a timely production slot. The survival differences may reflect the fact that patient selection was stringent given challenges with production slots and manufacturing times. The landscape of access to BCMA therapy has been dynamic over the years with the availability of cilta-cel and now with BCMA bispecific antibody T-cell engagers such as teclistimab. Prioritizing research to optimize manufacturing time is urgently needed to facilitate prompt access to BCMA CAR-T for RRMM patients.

Data availability statement

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

Author contributions

NA and LS conceived the study. SB and BG contributed to data extraction. WW was responsible for writing the protocol and performing data analysis. NA and WW interpreted the results and wrote the initial draft. All authors contributed towards the final manuscript and provided feedback on the report. All authors contributed to the article and approved the submitted version.

Acknowledgments

University of Kansas Cancer Center Immune Effector Cell Therapy team.

Conflict of interest

Author NA: Advisory Board BMS, consultancy and institutional research funding from Kite. Author JM reports honoraria from Kite/Gilead, Juno Therapeutics, Allovir, Magenta Therapeutics, EcoR1 Capital, Janssen and BMS/Celgene. He receives research funding from Astellas Pharma, Bellicum Pharmaceuticals, Gamida Cell, Pluristem Therapeutics, Kite/Gilead and Allovir.

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

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Gandhi UH, Cornell RF, Lakshman A, Gahvari ZJ, McGehee E, Jagosky MH, et al. Outcomes of patients with multiple myeloma refractory to CD38-targeted monoclonal antibody therapy. *Leukemia* (2019) 33(9):2266–75. doi: 10.1038/s41375-019-0435-7

2. Friedman KM, Garrett TE, Evans JW, Horton HM, Latimer HJ, Seidel SL, et al. Effective targeting of multiple b-cell maturation antigen–expressing hematological malignances by anti-B-Cell maturation antigen chimeric antigen receptor T cells. *Hum Gene Ther* (2018) 29(5):585–601. doi: 10.1089/hum.2018.001

3. Sharma P, Kanapuru B, George B, Lin X, Xu Z, Bryan WW, et al. FDA Approval summary: idecabtagene vicleucel for relapsed or refractory multiple myeloma. *Clin Cancer Res* (2022) 28(9):1759–64. doi: 10.1158/1078-0432.CCR-21-3803

4. Munshi NC, Anderson LD, Shah N, Madduri D, Berdeja J, Lonial S, et al. Idecabtagene vicleucel in relapsed and refractory multiple myeloma. *N Engl J Med* (2021) 384(8):705–16. doi: 10.1056/NEJMoa2024850

5. Raje N, Berdeja J, Lin Y, Siegel D, Jagannath S, Madduri D, et al. Anti-BCMA CAR T-cell therapy bb2121 in relapsed or refractory multiple myeloma. *N Engl J Med* (2019) 380(18):1726–37. doi: 10.1056/NEJMoa1817226

6. Berdeja JG, Madduri D, Usmani SZ, Jakubowiak A, Agha M, Cohen AD, et al. Ciltacabtagene autoleucel, a b-cell maturation antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. *Lancet* (2021) 398(10297):314–24. doi: 10.1016/S0140-6736(21)00933-8

7. Kourelis T, Bansal R, Berdeja J, Siegel D, Patel K, Mailankody S, et al. Ethical challenges with multiple myeloma BCMA CAR-T slot allocation: a multi-institution experience. *Transplant Cell Ther* (2023) 29(4):255–58. doi: 10.1016/j.jtct.2023.01.012

8. Faiman B, Khouri J, Williams LS, Anwer F. Ethical challenges in CAR-T slot allocation. *Transplant Cell Therapy Off Publ Am Soc Transplant Cell Ther* (2023) 29 (4):215–6. doi: 10.1016/j.jtct.2023.03.003

9. Sonneveld P, Avet-Loiseau H, Lonial S, Usmani S, Siegel D, Anderson KC, et al. Treatment of multiple myeloma with high-risk cytogenetics: a consensus of the international myeloma working group. *Blood* (2016) 127(24):2955–62. doi: 10.1182/blood-2016-01-631200

10. Chekol Abebe E, Yibeltal Shiferaw M, Tadele Admasu F, Asmamaw Dejenie T. Ciltacabtagene autoleucel: The second anti-BCMA CAR T-cell therapeutic armamentarium of relapsed or refractory multiple myeloma. *Front Immunol* (2022) 13:991092. doi: 10.3389/fimmu.2022.991092

11. Available at: https://www.abecma.com/find-a-treatment-center/. Last Acccessed 7/23/23.

12. Costa LJ, Lin Y, Cornell RF, Martin T, Chhabra S, Usmani SZ, et al. Comparison of cilta-cel, an anti-BCMA CAR-T cell therapy, versus conventional treatment in patients with Relapsed/Refractory multiple myeloma. *Clin Lymphoma Myeloma Leukemia* (2022) 22(5):326–35. doi: 10.1016/j.clml.2021.10.013

13. Cohen YC, Cohen AD, Delforge M, Hillengass J, Goldschmidt H, Weisel K, et al. Efficacy and safety of ciltacabtagene autoleucel (Cilta-cel), a b-cell maturation antigen (BCMA)-directed chimeric antigen receptor (CAR) T-cell therapy, in lenalidomide-refractory patients with progressive multiple myeloma after 1-3 prior lines of therapy: updated results from CARTITUDE-2. *Blood* (2021) 138:3866. doi: 10.1182/blood-2021-146072

14. Raje NS, Siegel DS, Jagannath S, Lonial S, Munshi NC, Moreau P, et al. Idecabtagene vicleucel (ide-cel, bb2121) in relapsed and refractory multiple myeloma: analyses of high-risk subgroups in the KarMMa study. *Blood* (2020) 136:37–8. doi: 10.1182/blood-2020-134319

15. Al Hadidi S, Szabo A, Esselmann J, Hammons L, Hussain M, Ogunsesan Y, et al. Clinical outcome of patients with relapsed refractory multiple myeloma listed for BCMA directed commercial CAR-T therapy. *Bone Marrow Transplant* (2023) 58 (4):443–5. doi: 10.1038/s41409-022-01905-1

16. Ahmed N, Wesson W, Abdallah AO. Dawn of hope for improved access to BCMA therapy: chimeric antigen receptor T cell and bispecific products. *Transplant Cell Ther* (2023) 29(7):474–5. doi: 10.1016/j.jtct.2023.04.013

17. IMF. *Withdrawal of blenrep from the US market*. (2022). Available at: https://www.myeloma.org/news-events/withdrawal-blenrep-us-market.

18. Delforge M, Baz RC, Cavo M, Callander NS, Ghobadi A, Rodriguez-Otero P, et al. KarMMa-3: a phase 3 study of idecabtagene vicleucel (ide-cel, bb2121), a BCMA-directed CAR T cell therapy vs standard regimens in relapsed and refractory multiple myeloma. *Blood* (2020) 136:24–5. doi: 10.1182/blood-2020-137156

19. Agha ME, Cohen AD, Madduri D, Cohen YC, Delforge M, Hillengass J, et al. CARTITUDE-2: efficacy and safety of ciltacabtagene autoleucel (cilta-cel), a BCMA-directed CAR T-cell therapy, in patients with progressive multiple myeloma (MM) after one to three prior lines of therapy. *J Clin Oncol* (2021) 39(15_suppl):8013–3. doi: 10.1200/JCO.2021.39.15_suppl.8013

20. Alzubi J, Lock D, Rhiel M, Schmitz S, Wild S, Mussolino C, et al. Automated generation of gene-edited CAR T cells at clinical scale. *Mol Ther - Methods Clin Dev* (2021) 20:379–88. doi: 10.1016/j.omtm.2020.12.008

21. Bu D, Bennett P, Barton N, Bradshaw L, Pinon-Ortiz M, Li X, et al. Identification and development of PHE885: a novel and highly potent fully human anti-BCMA CAR-T manufactured with a novel T-charge TM platform for the treatment of multiple myeloma. *Blood* (2021) 138:2770. doi: 10.1182/blood-2021-148390

22. Abou-el-Enein M, Elsallab M, Feldman SA, Fesnak AD, Heslop HE, Marks P, et al. Scalable manufacturing of CAR T cells for cancer immunotherapy. *Blood Cancer Discov* (2021) 2(5):408–22. doi: 10.1158/2643-3230.BCD-21-0084

23. Otegbeye F. Bench at bedside models facilitate CAR-T cell supply chain. *Transplant Cell Therapy Off Publ Am Soc Transplant Cell Ther* (2022) 28(5):221–2. doi: 10.1016/j.jtct.2022.04.005

24. Jackson Z, Roe A, Sharma AA, Lopes F, Talla A, Kleinsorge-Block S, et al. Automated manufacture of autologous CD19 CAR-T cells for treatment of non-hodgkin lymphoma. *Front Immunol* (2020) 11:1941. doi: 10.3389/fimmu.2020.01941

25. Dar H, Henderson D, Padalia Z, Porras A, Mu D, Kyungah M, et al. Preclinical development of CTX120, an allogeneic CAR-T cell targeting bcma. *Blood* (2018) 132 (Supplement 1):1921–1. doi: 10.1182/blood-2018-99-116443

26. Al-Homsi AS, Anguille S, Brayer J, Deeren D, Meuleman N, Morgan G, et al. Clinical development of a non-Gene-Edited allogeneic bcma-targeting CAR T-cell product in relapsed or refractory multiple myeloma. *Blood* (2020) 136:27–8. doi: 10.1182/blood-2020-139516

27. Sommer C, Boldajipour B, Kuo TC, Bentley T, Sutton J, Chen A, et al. Preclinical evaluation of allogeneic CAR T cells targeting BCMA for the treatment of multiple myeloma. *Mol Ther* (2019) 27(6):1126–38. doi: 10.1016/j.ymthe.2019.04.001

28. Lonial S, Lee HC, Badros A, Trudel S, Nooka AK, Chari A, et al. Belantamab mafodotin for relapsed or refractory multiple myeloma (DREAMM-2): a two-arm, randomised, open-label, phase 2 study. *Lancet Oncol* (2020) 21(2):207–21. doi: 10.1016/S1470-2045(19)30788-0

29. *Withdrawal of blenrep from the US market*. (2022). Available at: https://www.myeloma.org/news-events/withdrawal-blenrep-us-market#:~:text=On%20November%2022%2C%202022%2C%20GSK%20announced%20it%20has,of%20the%20U.S%20Food%20and%20Drug%20administration%20%28FDA%29.

30. Usmani SZ, Garfall AL, van de Donk NW, Nahi H, San-Miguel JF, Oriol A, et al. Teclistamab, a b-cell maturation antigen× CD3 bispecific antibody, in patients with relapsed or refractory multiple myeloma (MajesTEC-1): a multicentre, open-label, single-arm, phase 1 study. *Lancet* (2021) 398(10301):665–74. doi: 10.1016/S0140-6736(21)01338-6

31. Krishnan AY, Minnema MC, Berdeja JG, Oriol A, van de Donk NWCJ, Rodriguez-Otero P, et al. Updated phase 1 results from MonumenTAL-1: first-in-Human study of talquetamab, a G protein-coupled receptor family c group 5 member d x CD3 bispecific antibody, in patients with Relapsed/Refractory multiple myeloma. *Blood* (2021) 138:158. doi: 10.1182/blood-2021-146868

32. Goldsmith SR, Streeter S, Covut F. Bispecific antibodies for the treatment of multiple myeloma. *Curr Hematol Malig Rep* (2022) 17(6):286–97. doi: 10.1007/s11899-022-00675-3

frontiers | Frontiers in Transplantation

TYPE Review
PUBLISHED 05 September 2023
DOI 10.3389/frtra.2023.1238535

OPEN ACCESS

EDITED BY
Roberto Passera,
University of Turin, Italy

REVIEWED BY
Mauro Di Ianni,
University of Studies G. d'Annunzio Chieti and Pescara, Italy
Claire Roddie,
University College London, United Kingdom

*CORRESPONDENCE
Evandro Bezerra
✉ evandro.bezerra@osumc.edu

†The authors have contributed equally to this work and share first authorship.

‡The authors have contributed equally to this work and share senior authorship.

RECEIVED 15 June 2023
ACCEPTED 21 August 2023
PUBLISHED 05 September 2023

CITATION
Shah M, Krull A, Odonnell L, de Lima MJ and Bezerra E (2023) Promises and challenges of a decentralized CAR T-cell manufacturing model. Front. Transplant. 2:1238535. doi: 10.3389/frtra.2023.1238535

Promises and challenges of a decentralized CAR T-cell manufacturing model

Manan Shah[1†], Ashley Krull[2†], Lynn Odonnell[3], Marcos J. de Lima[4‡] and Evandro Bezerra[4*†]

[1]Department of Hematology, the James Cancer Hospital and Solove Research Institute, Ohio State University, Columbus, OH, United States, [2]Department of Cell Therapy Manufacturing and Engineering, the James Cancer Hospital and Solove Research Institute, Ohio State University, Columbus, OH, United States, [3]Department of Hematology, Cellular Therapy Lab, the James Cancer Hospital and Solove Research Institute, Ohio State University, Columbus, OH, United States, [4]Department of Hematology, The James Cancer Hospital and Solove Research Institute, Ohio State University, Columbus, OH, United States

Autologous chimeric antigen receptor-modified T-cell (CAR T) products have demonstrated un-precedent efficacy in treating many relapsed/refractory B-cell and plasma cell malignancies, leading to multiple commercial products now in routine clinical use. These positive responses to CAR T therapy have spurred biotech and big pharma companies to evaluate innovative production methods to increase patient access while maintaining adequate quality control and profitability. Autologous cellular therapies are, by definition, manufactured as single patient batches, and demand has soared for manufacturing facilities compliant with current Good Manufacturing Practice (cGMP) regulations. The use of a centralized production model is straining finite resources even in developed countries in North America and the European Union, and patient access is not feasible for most of the developing world. The idea of having a more uniform availability of these cell therapy products promoted the concept of point-of-care (POC) manufacturing or decentralized *in-house* production. While this strategy can potentially decrease the cost of manufacturing, the challenge comes in maintaining the same quality as currently available centrally manufactured products due to the lack of standardized manufacturing techniques amongst institutions. However, academic medical institutions and biotech companies alike have forged ahead innovating and adopting new technologies to launch clinical trials of CAR T products produced exclusively in-house. Here we discuss POC production of CAR T products.

KEYWORDS

decentralized, CAR T, leukapheresis, chimeric, lentivirus

Limitations of current autologous chimeric antigen receptor T-cell manufacture model

Autologous chimeric antigen receptor-modified T-cell (CAR T) therapy has revolutionized the management of relapsed/refractory B-cell and plasma-cell malignancies. There are six different FDA approved autologous CAR T products manufactured by four different pharmaceutical companies (Table 1) (1–12). There are also more than 200 active CAR T clinical trials in the United States to optimize CAR T therapy efficacy, mitigate toxicities and broaden disease indications to additional hematologic malignancies, solid tumors, and even non-malignant diseases (13–18) The use of autologous CAR T therapy is expected to grow exponentially, but this growth will likely exacerbate two major current

TABLE 1 Commercially produced CAR-T cell products.

Product	Pharmaceutical company	Target/Year	Indications	FDA approved	UK/EU approved
Kymriah (Tisagenlecleucel)	Novartis	CD-19 (2017)	ALL, NHL	Yes	Yes
Yescarta (Axicabtagene)	Kite/Gilead	CD-19 (2017)	NHL, Follicular lymphoma	Yes	Yes
Tecartus (Brexucabtagene)	Kite/Gilead	CD-19 (2020)	ALL, Mantle cell	Yes	Yes
Breyanzi (Lisocabtagene)	BMS	CD-19 (2021)	NHL	Yes	Yes
Abecma (Idecabtagene)	BMS	BCMA (2021)	Multiple Myeloma	Yes	Yes
Carvykti (Ciltacebtegene)	Jansen	BCMA (2022)	Multiple myeloma	Yes	No

limitations of CAR T therapy: patient access and financial burden to the healthcare system (19–22).

Access is a major limitation even in large academic comprehensive cancer centers in the United States, where five out of the six FDA approved CAR T products were developed, and where the current commercial products are centrally manufactured. The access may be limited by the CAR T manufacturing capacity offered by pharmaceutical companies (23–25). Patients often wait up to 3 weeks for a "manufacturing slot", an allocated date when the company can receive the patient's autologous apheresis product to start manufacturing CAR T cells (26–29).

Another major access limitation is the prolonged manufacturing and release time that frequently ranges from 2 to 4 weeks. Candidates for CAR T therapy usually have aggressive diseases and are heavily pre-treated, and often may decline clinically and become ineligible while waiting to receive treatment (24, 25, 27). Manufacture delays can be attributed to the approved methods used for transduction and expansion of T-cells, mainly based on technology from when the products were initially developed over 10 years ago. Moreover, because of the centralized nature of the process, shipment, and cryopreservation are required adding more time to the clinically significant "vein-to-vein" time (time from leukapheresis to CAR T infusion) (28).

Lastly, the current costs of commercially manufactured CAR T products total nearly $500,000 USD, in addition to other clinical costs with medications, hospitalization, transfusions etc. Such costs present a major burden on the healthcare system of developed countries and, therefore, are prohibitive for most of the population in the developing world (21, 22, 28). There is an unmet need to optimize the current model of CAR T manufacture, so both the "vein-to-vein" time and costs are minimized. To this end, there are two approaches of large interest: use of off-the-shelf allogeneic CAR T and decentralized in-house autologous CAR T manufacture (30–33) Despite the enthusiasm for the development of off-the-shelf allogeneic CAR T seen in multiple ongoing trials, so far their efficacy and persistence have been limited by rejection, on top of significant donor variability leading to significant T-cell fitness differences (31, 34, 35).

Here, we review the decentralized in-house CAR T manufacturing model. We also discuss different platforms available for decentralized in-house manufacturing (**Table 2**) and the challenges associated with implementation of these procedures.

General concept of CAR T manufacturing

There are two different logistical approaches to CAR T manufacturing: (1) the standard centralized process where the product is manufactured in cell therapy laboratories controlled by the pharmaceutical company; (2) the decentralized method where CAR T products are manufactured in one or more cell therapy laboratories within the academic healthcare system to support clinical trials and eventually even commercial manufacturing. The outline of various steps involved is mentioned below (1, 3, 4, 5, 6, 12, 13, 15, 20, 24, 32, 36–38).

(1) **Cell collection:** Peripheral blood mononuclear cells (PBMCs) are collected through leukapheresis in an accredited healthcare facility. The cells are transported in a stringent cold chain system either at 2–8°C for fresh products or below −150°C for cryopreserved products (20, 36, 37).
(2) **CAR T manufacturing:** CAR T products are manufactured in accordance with Food and Drug Administration (FDA) current Good Manufacturing Practice (cGMP) regulations, following multiple steps, detailed below (39).

TABLE 2 Different place-of-care manufacturing.

Author	Production time	Indications	Manufacturing platform
Ortiz-Maldonado et al. (28)	8–11 days. Cryopreserved	Acute lymphoblastic leukemia, non-Hodgkin's lymphoma Chronic lymphocytic leukemia	ARI-0001 (locally produced vector) cells at a dose of $0.4–5 \times 10^6$ cells/kg. CliniMACS Prodigy©.
Maschan et al. (32)	8–12 days Fresh cells	Relapsed/refractory pediatric B-cell ALL and adult B-cell NHL. Phase I clinical trials in Moscow (Russia) and Cleveland (USA)	(Lentigen©) CliniMACS Prodigy©.
Palani et al. (30)	12 days	No patients received the product.	(Lentigen©) CliniMACS Prodigy©.
Kedmi et al. (24)	10 days Fresh cells	Adult patients with aggressive B-cell lymphoma.	Locally produced anti-CD19 retrovirus vector with a CD28 costimulatory domain.
Shah et al. (11)	14 days Fresh cells	Adult patients with B cell non-Hodgkin lymphoma or chronic lymphocytic leukemia	(Lentigen©) CAR T cells; CliniMACS Prodigy©.

(a) T-cell enrichment from peripheral blood mononuclear cells (PBMCs) and activation using CD3/CD28 antibodies (5, 6, 12, 40–46).
(b) Genetic engineering of the T-cells to induce CAR expression, most commonly through transduction with viral vectors (non-viral approaches are under investigation) (1, 3–6, 47–49).
(c) CAR T expansion and harvesting in the presence of specific cytokines (1, 3–6, 15, 42, 45).
(d) QC testing and release by the QA unit prior to distribution is critically important and typically takes 2–3 weeks. Fresh CAR T products typically must be infused within 48 h of harvest (19, 20, 50).

(3) **Administration:** After release from centralized manufacture, the cryopreserved product is shipped in temperature-monitored packaging to the healthcare facility where the patient will be treated. Prior to infusion of CAR T products, patients are subjected to a lymphodepleting agent over several days (1, 3–6).

CAR T manufacturing systems: open/manual vs. closed/automated

Traditionally, small-scale manufacturing procedures utilize open systems in which a bag, tube or culture vessel with cells is open to the environment, albeit in an aseptic manner. Such open systems are almost completely manual and, as such, require multiple highly skilled laboratory scientists and technologists working hands-on for several full days over the culture period. Compared to truly closed systems, open systems are also more susceptible to microbiologic contamination and human error. Product manipulation is conducted in a classified cleanroom. CAR T production also requires co-culture of the stimulated T-cells with a gene vector (frequently a replication deficient viral vector), which also must be handled in a controlled environment that prevents exposure of the technical personnel or the laboratory environment (42, 51–54).

Alternatively, closed/automated systems utilize platform devices (e.g., CliniMACS Prodigy©, Lonza Cocoon© and others) that enable the apheresis product to move through the multiple steps of CAR T manufacture (T-cell isolation, activation, transduction, and CAR T expansion) inside a single use, disposable kit that is not directly opened to the environment. Sample removal and reagent or media addition are accomplished through sterile tubing welders or aseptic access ports, thus maintaining what is known as a "functionally closed system". These automated systems may be operated in facilities with less stringent air classifications (i.e., ISO 8 or even unclassified space, as opposed to ISO 7) and mean significantly less hands-on time, and more consistent handling during manufacture. These advantages make automated manufacturing devices the preferred method for decentralized CAR T manufacture. It should be noted, however, that currently available devices are used for one CAR T product at a single time. To accommodate large patient volumes, centers would need to have multiple instruments with high price tags and requiring significant bench or floor space and implement well-controlled protocols for optimally timing the ending of one product's manufacture and apheresis collection and initiation of another product's run (13, 15, 23, 24, 30, 32, 38, 55, 56).

A third option is a hybrid semi-closed/semi-automatic system. Here, each individual step is automated, but done in separated devices with minimal manual product handling between steps/modules. This process can be facilitated by a robotic arm to minimize human error (57).

CAR T manufacturing location: centralized vs. decentralized

Centralized

All CAR T cell products approved by the FDA are manufactured centrally. The treating physician prescribes a specific FDA approved CAR T product and patient's PBMCs are shipped from the treating healthcare facility to the central manufacturing cell therapy laboratory. Often, pharmaceutical companies have a facility in North America and another in the European Union to supply the two main CAR T markets. After manufacture and cryopreservation, the product is shipped back to the treating healthcare facility (1, 3–6, 12). The main advantage of centralized manufacture is quality standardization that minimizes inter-product variability. The increased oversight and control afforded by a centralized manufacturing model was critical for commercialization of a labor-intense manual process (19, 20, 29). However, due to the personalized nature of autologous CAR T production, it is not possible to deliver a truly uniform CAR T product. There also remain many drawbacks with the centralized model as listed previously: long waiting time, patient access, and financial burden. Centrally manufactured CAR T products often spend more time in the QC/QA processes than in manufacturing (19, 20, 29).

Decentralized

In the decentralized model, the product is manufactured within or very near the same healthcare system where the patient will be treated. This model minimizes or eliminates the need for cryopreservation and improves timing and potentially costs. For instance, eliminating cryopreservation of the starting material can potentially influence cell quantity and quality, as the recovery and viability of PBMCs is often reduced after freezing and thawing compared with fresh apheresis products (24, 29, 30, 38, 58, 59). In the USA, the decentralized manufacturing model has been restricted to academic centers in the context of clinical trials. Initially, prior to CAR T FDA approvals, heavily manual POC manufacture was used exclusively for early phase, single center clinical trials at a select few institutions. Later, these groups transferred their technology and patents to biotechnology startups and large pharmaceutical companies that led the multi-center studies that adopted centralized manufacture strategies and resulted in the current FDA approvals (51–54).

The rapid development of bioreactors and other technologies is enabling a paradigm shift in which POC production is becoming more accessible. The use of automated closed systems including such as CliniMACS Prodigy© and Cocoon© has drastically reduced the need for clean rooms thereby decreasing the need for expensive infrastructure (15, 32, 60) Importantly, these automated systems comply with federal regulations requiring software that may generate electronic records involved in the manufacture of biologics. These systems can currently accommodate a lentiviral gene vector or non-viral vectors for T-cell transduction, with all subsequent steps through formulation which are conducted within this closed automated unit (61) The final product often is produced in a shorter manufacturing time (commonly 7–10 days vs. 14 days) and at a lower cost compared to centralized manufacturing, although there are not commercially available POC CAR T in the USA. Decentralized manufacturing also removes the risks and costs of transportation and may be infused fresh. The average cost of production of in-house CAR T cells can be as low as $35,000 USD if viral vector is provided by a sponsor or collaborator and is variable between $50,000- $1 million if GMP vector must be purchased by the center (29, 30) This does not include costs required for setup, staffing and maintenance.

Manufacturing platforms available for POC production

Here, we briefly describe the available bioreactors that provide a functionally closed GMP-compliant cell processing system. Various pros and cons of each platform is discussed in Table 3 (23).

(A) *CliniMACS Prodigy® system*- The CliniMACS cell system was established in 1997 by Miltenyi Biotec for enrichment and depletion of specific cell types using magnetically labeled antibodies. The Prodigy system represents a technology that automates all the necessary steps of CAR T production beyond T cell enrichment, such as activation, transduction, washing, and media feeding in one closed tubing system. An electroporation attachment is now available allowing gene editing capabilities on one device. Though this platform ensures GMP compliance and reduces strict clean room requirements, some steps are still manual and extensive training is required. Nevertheless, this system is commonly used for production in academic medical centers throughout the world (13, 30, 61, 62).

(B) *The Cocoon® platform*- The Cocoon system from Lonza is another closed manufacturing system that is based on a single use transportable cassette. The cassette internalizes all the media and reagents and can maintain the reagents in a temperature-controlled environment, although T cell enrichment currently must be performed on a CliniMACS device. The limitation again is that some steps are still manual, particularly if electroporation is needed (58, 60).

(C) *ekko™ acoustic cell processing system*- The ekko™ system from MilliporeSigma is a novel GMP-compliant platform that utilizes acoustophoresis as a method of cell processing and production. This can be utilized for separating TCR-positive from TCR-negative cells and has been used for other therapies like NK cells. The disadvantage of this system is that it has not been widely utilized for CAR T cell production and hence data is limited (62).

(D) *G-Rex® bioreactor M series*- Wilson Wolf's gas permeable rapid membrane technology allows G-Rex flasks to support

TABLE 3 Different manufacturing platforms for CAR-T production.

Manufacturing platforms	Advantages	Disadvantages	Clinical application
CliniMACS Prodigy	1. Closed system performing all steps from cell preparation and harvest to final formulation. 2. Fresh or thawed peripheral blood mononuclear cell (PBMC) products can be loaded directly. 3. Extremely flexible platform. 4. High cell output with limited processing time.	1. This system is driven by complex software that requires close monitoring and detailed training of operators. 2. High cost of installation and maintenance.	Used by Maschan and Shah et al. for CAR T production for relapsed/refractory B-cell ALL and Non-Hodgkin's lymphoma (11, 32). Used for in-house, POC CD19/20/22 CAR T production (NCT05418088).
Lonza Cocoon	1. Simple operability as it uses customized cassettes. 2. Can use reagents and multiple stimulating agents from various manufacturers. 3. Lower cost of installation and maintenance.	1. Requires stringent temperature control during processing. 2. Initial steps of T-cell enrichment and transduction remain manual and require utilization of the CliniMACS system. 3. Lower output when compared to CliniMACs.	Used in combination with CliniMACS Prodigy (11, 32). Used in production of CD-19 CAR T production for relapsed non-Hodgkin's lymphoma by Anguille et al. (60).
Wilson Wolf G-Rex (Gas permeable) bioreactor system	1. Simple, cost effective, and practical in its use for production. 2. Manufactures multiple cell types. 3. Cost-effective installation and maintenance.	1. Risk of contamination as some steps need to be performed manually e.g., electroporation and depletion of cells. 2. Higher use of man-power and increased need for training. 3. Lower cell output per unit. Requires multiple units for large-scale processes.	Being evaluated for genetically engineered T-cell expansion to treat human papilloma virus associated cancers (NCT02858310).

the production of high cell density products over short culture durations in a familiar tissue culture flask format, but without the large capital investment of fully automated systems. Several different sizes from 2 cm^2 to 500 cm^2 make scaling up a classic T-flask method straight-forward. In addition, closed system G-Rex flasks with sterile fluid paths are now available, along with a liquid handling pump, the GatheRex, that simplifies and accelerates media exchange and cell harvesting. A limitation of this system is that manual monitoring of cell density and decision-making about splitting cultures to additional vessels are required quite often during production (62, 63).

(E) Other technologies that are still under review to support widespread POC production include: the ThermoGenesis CAR TXpress™ platform, the Gibco CTS Rotea™, the Terumo Quantum® cell expansion system and the Cytiva perfusion media (62–64).

Challenges to implementation of POC production

For all the promises and advancements in POC production, the process of implementing such a manufacturing system includes many challenges that must be overcome, including personnel training, quality management, facilities design, financing, and reagent sourcing.

Automated systems often use vectors including genetically modified lentivirus for transduction of CAR T cells during their production. Limited commercial production of GMP-grade viral vectors presents a universal challenge for all institutions aiming to manufacture CAR T cells. Institutions looking to implement POC manufacturing should establish early partnerships with viral vector suppliers or consider bringing GMP vector production in house to have better control over timelines. Interestingly, vector manufacturing capability is expanding worldwide (19, 20, 65–67). It is important to note that the cost will vary depending on the amount of testing required by the client, the size of the vector batch produced, and shipping requirements. Institutions would also be wise to consider sourcing research-grade vector preparations (often at a cost of less than $50,000) to enable development work and early validation activities before transitioning to a GMP-grade preparation for final validation runs.

POC manufacturing requires a highly skilled technical team with a working knowledge of aseptic techniques, clinical-grade reagents, and a variety of release assays to prove a product is suitable for use in humans. With automated POC platforms, the challenges presented by release tests are often more difficult to overcome than the actual manufacturing. This is further complicated when fresh CAR T products are desired, as a large QC team is required to perform STAT PCR testing of vector copy number, replication competent virus and mycoplasma, STAT flow cytometric testing of percent transduced cells and other cell types, along with other release tests such as endotoxin. Unfortunately, finding, training, and retaining skilled personnel is increasingly becoming a challenge for academic institutions. Creative workforce recruitment and training approaches are needed to expand the workforce to meet the demand. Infrastructure in the form of GMP-compliant lab space, procurement of additional space and external equipment will present a significant cost burden. To help in this effort, newer manufacturing platforms aim to remove this drawback and make operation in a lower-class environment possible (39) In essence, use of a closed production platform outside of a clean room is dependent on the classification of the space and the validation performed by the manufacturing staff demonstrating that the risk of contamination is mitigated to the same degree as if the product were being processed in a clean room environment (e.g., through technologist best practices, aseptic technique, environmental monitoring). Though regulations differ between countries, to our knowledge there are no regulations that prohibit the use of closed production platforms outside of a cleanroom if manufacturing practices are properly validated and documented.

Quality control is one of the greatest challenges to implementing POC manufacturing as opposed to centralized manufacturing. While the centralized manufacturing system has a well-organized QA system, the challenge with decentralized system is that we may have facilities that are technically capable for its production but not experienced with the QA and regulatory aspects of GMP manufacturing. POC manufacturing necessitates a strong quality program at the site, particularly in the absence of quality program support from a commercial entity. For trials to move into the clinic, the manufacturing institution needs to possess professional knowledge of federal regulations and a robust team to handle information requests during the regulatory review process (39). For fresh CAR T products, a QA team that is knowledgeable and comfortable with the manufacturing process and QC tests is important to achieve a thorough but rapid batch record review and product release, as sampling, harvesting and distribution are typically performed over a 24 h time frame with a goal of infusing on first shift (68, 69). We summarize the important components required for setting up a GMP clean room in Table 4.

In the case of distributed, POC manufacturing, it would be incumbent upon the sponsor of the trial to oversee and continuously monitor (via audits and on-site visits) the operations and quality management at individual manufacturing sites. All documents would be subject to central review by the sponsoring company/entity. However, it is likely the quality agreement (which must be in place before manufacturing begins) would outsource real-time release testing results review and product sign-out to the POC manufacturing center, whose quality management system and leadership would have been thoroughly audited by the sponsor. Before trial initiation, technology transfer activities and verification studies would provide documentation of comparability in production and release testing methods across sites. Once the trial began, continuous communication and quality audits would protect against deviations and drift in critical quality attributes.

Financing POC manufacturing remains a challenge. Innovative approaches will be necessary to disseminate this approach. For

TABLE 4 Components of a GMP clean room.

1. Flush design and finish	The primary aim of a clean room is to minimize as few contaminants as possible. This is prioritized using a flush design for walls, windows, ceilings, and doors. It includes having no-edge windows, T-seals ceiling tiles, recessed light system, low air returns and flush finish sprinklers. GMP compliant doors need to be easily cleanable and resistant to cleaning agents. Sliding doors should be avoided.
2. Environmental and Microbiological monitoring	The monitoring system which includes temperature, humidity, and pressure ranges from $50,000–1 million dollars. The aim is to reduce microbial load, also known as bioburden. The design incorporates separate designated sections for specific activities such as a separate descrambling table, filling zone, stoppering zone, and a capping station.
3. Personnel and Material Air locks	Clean rooms must include personnel and material airlocks built between exit and entry points. The aim is to prevent microbial and particle contamination from protective gear.
4. Interlocking system and alarms	Interlocking and alarm systems are safe systems for opening and closing doors with minimum loss of pressure within the clean room. This prevents air exchange between the manufacturing area and the outside space.
5. At rest versus operation cleanliness level	Two separate levels of cleanliness need to be identified when workers are present within the room (*operation*) and when the clean room is unoccupied (*rest*). For example, maximum permitted number of particles at >0.5micron at rest is limited to 3,520–352,000 depending on the grade of operation.
6. HVAC system	The HVAC system plays an important role as it determines the number of air-changes required per hour depending on the dimension of the room and the products manufactured in the clean room.
7. Sinks and Drains	Sinks and drains should not be present in the clean room zone but are allowed in the gowning area. Mechanical valves should be installed between the sink and drains.

example, the Biologics License Application FDA mechanism could allow a network of centers to produce CAR T cells, under the basic assumption that the process would be verifiable and similar in all production facilities, which would in turn market the product for sale. Another intriguing possibility is to seek institutional licensing similarly to what has been done with cord blood banks in the USA.

Toxicity profiles play an important role in the feasibility of in-house developed and manufactured products. Commercially available products have extensive documentation and a detailed spectrum of possible toxicities like cytokine release syndrome and neurotoxicity. It is expected that POC manufactured products will have such extensive documentation. Most importantly, if more than one center is to provide locally manufactured CAR T cells, all participating laboratories will have to show the same standard and comparable results, including similar release tests. The almost universal use of lymphodepleting agents like cyclophosphamide and fludarabine prior to the use of CAR T cells also significantly affects the toxicity profile. These potential responses require robust clinical and quality teams to monitor reactions.

POC manufacturing of CART cells in the US system and global perspectives

Several academic institutions have started producing in-house products, like Case Western Reserve University Hospital (CWRU/UH) in Cleveland, Ohio, Medical College of Wisconsin (MCW) in Milwaukee, Wisconsin and Stanford, Palo Alto, California have utilized the CliniMACS platform using a lentiviral vector (13, 15, 32, 59). At CWRU/UH experience, 100% manufacturing success of anti-CD19 CAR T cells was achieved in 24 patients with median time from apheresis to infusion of 13 days. Similar efficacy and toxicity with other FDA approved CAR T cells products was observed (32, 59) At MCW and Stanford, POC manufacturing has been used in clinical trials of bispecific CAR T cells, including patients who had failed previous anti-CD19 CAR T cells, and they have shown feasibility with promising efficacy without safety signals concerns (13, 15) Twenty-six anti-CD19/CD20 CAR T cell products were manufactured using a fixed 14 days process in the CliniMACS Prodigy device. The target dose of CAR T cells was achieved in 85% of patients (22 of 26), with 100% successful manufacturing in CAR-naive patients (15) Similarly, the Ohio State University team is using the same platform to produce CD19/CD20/CD22 CAR T cells in 6–7 days to support a clinical trial (ClinicalTrials.gov Identifier: NCT05418088) (70).

Globally, production of CAR T cells is challenging. Different countries have different regulatory pathways, which adds to the challenge of having a single uniform international regulatory standard. Despite this, some countries have developed innovative approaches for POC production.

For instance, in Spain, a national network of hospitals was established for production of CD19-specific CAR T cells used in the treatment of relapsed ALL that resulted in approval by the European Medicines Agency (EMA). On the Spanish group pivotal clinical trial, 54 patients underwent apheresis with 87% of success in CAR T cell manufacture. The median vein-to-vein time was not yet shorter than centralized manufactured products, mostly due to 7 patients who required 2 apheresis and numerous intervening medical complications that forced delays to start lymphodepleting chemotherapy and cell infusion (38).

In China, authorities have encouraged small start-up companies to produce these products in direct collaboration with a clinical center (71). Through this partnership and decentralized CAR T cells manufacture, there are over 500 Chinese clinical trials registered aiming to improve CAR T cells manufacture efficiency and efficacy. An example is provided by Nanjing Biolegend with its anti-BCMA CAR T cell for multiple myeloma, now approved by FDA and promising data from early phase clinical trials with anti-claudin CAR T cells in gastrointestinal malignancies (4, 18, 71).

Similarly centers in Germany, Israel, India, and Brazil have started introducing the concept of POC production, with some clear challenges remaining to full access. For instance, Palani et al. in India have demonstrated production of CAR T cells using the ClinicMACS platform, but no patients have been

treated due to the limitation of financial assistance. All the above centers have laid foundation for middle income countries to begin discussions about setting up research labs, thereby enhancing the global acceptance of this novel product (24, 30, 72).

Intellectual property, research & development investment, and equitable access

Early-stage CAR T cell products were initially funded by government institutions or philanthropic societies. The next generation of CAR T products should reach all countries, regardless of national income or funding abilities. As mentioned by Lam and collaborators in the United Kingdom, decentralized manufacturing can be quite cost effective, particularly if multiple smaller units are set up in a common geographical area, thereby allowing equipment and personnel to be better utilized at a local level (29). Sharing resources between multiple hospitals could benefit a larger population by easy accessibility to these products. Likewise, partnerships with pharmaceutical suppliers would more readily facilitate hospital-based studies and clinical trials for production of genetically modified products in a cost-effective manner. Indeed, biotech companies are beginning to appreciate the advantages of POC manufacturing and are working to roll out their manufacturing and testing platforms to select academic centers with the goal of commercializing with regional manufacturing centers around the world (15, 32, 38).

Healthcare legislation

Healthcare legislation underpins all modern research and technology. To expand the use of cell and gene therapies across the world, especially in low-income areas, a uniform code of practice should be formulated to ensure adequate quality of production of these products in a safe environment. Legislation is necessary to ensure protection from unethical and harmful practices, particularly when such regulations have not been codified before. An individual's right to permit the use of his or her own cells must always be protected. The adoption of the WHO concept of Universal Health Coverage (UHC) where "all communities can use promotive, preventive, curative and rehabilitative services they need while ensuring that the use of these services does not expose the user to financial hardships" is an essential step in expanding therapeutic access and enabling high-quality research (73).

Health economics and capacity building

Given the high cost of production and delivery limiting therapeutic use in various countries, resource allocation will play an essential role in implementing a therapeutic program in emerging markets. An important concept of the ICER, or incremental cost-effective ratio, serves as a basis for considering an intervention as good value for money. Using this concept, various programs in Africa for treating hemophilia and providing cancer chemotherapy have reached a wider network of patients (74) This concept can be easily applied to cell and gene therapy by implementing POC manufacturing in targeted geographical areas and appropriate demographic areas that will provide maximum benefits for patients (75).

Current CAR T cell product prices ranges anywhere between USD $373,000 to $475,000 which is unaffordable in most nations (29, 30, 76) The use of decentralized production will be beneficial in such scenarios where pharmaceutical production will not be profitable. Also, the use of partnerships between low- and medium-income nations mat achieve a balance between cost-effectiveness and affordability. An example of this partnership is seen between South Africa and India where two biotech companies are providing services of their genetic therapies at a tenfold lower price (74, 76).

Capacity building mainly includes strengthening the workforce. The administration and production of these products need a strong local workforce. Collaborations between different countries mainly for training and education is extremely important. Various universities have increased scholarships for students from low-income nations. An initiative started by the South-African government is the BM-NHSP (Bongani Mayosi National Health Scholars Program) where students are trained in genetic and cellular therapy (74). A similar initiative was started by the Bill & Melinda Gates Foundation with the NIH (National Institutes of Health) that supports genetic therapy in under-resourced nations. To fully capitalize on decentralization, training of skilled workers in high-technology equipment production and maintenance at a local level is essential to ensure high quality therapeutic use and patient care. As enumerated above, these modifications will help in maintaining a high quality of research worldwide and will help countries reap the benefits of genetically modified agents that were once considered unavailable to them.

Conclusion

POC manufacturing is a promising approach to expand the availability and utilization of CAR T cell therapies worldwide in a both time- and cost-effective manner. While there are several obstacles to overcome (e.g., availability of vectors, personnel training, lack of facilities), several initiatives are underway to address these issues. In addition, stringent regulatory oversight is required to maintain the quality and reproducibility of these products worldwide. Likewise, academic-pharmaceutical partnerships will be necessary to promote future research and innovation in the field.

Author contributions

MS, EB, and AK- Original Concept, Writing of Manuscript, Review of literature, Review of Manuscript LO, and ML- Original

Concept, Review of Manuscript. All author contributed to the article and approved the submitted version.

Conflict of interest

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

Publisher's note

References

1. Maude SL, Laetsch TW, Buechner J, Rives S, Boyer M, Bittencourt H, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. *N Engl J Med*. (2018) 378(5):439–48. doi: 10.1056/NEJMoa1709866

2. Schuster SJ, Bishop MR, Tam CS, Waller EK, Borchmann P, McGuirk JP, et al. Tisagenlecleucel in adult relapsed or refractory diffuse large B-cell lymphoma. *N Engl J Med*. (2019) 380(1):45–56. doi: 10.1056/NEJMoa1804980

3. Neelapu SS, Locke FL, Bartlett NL, Lekakis LJ, Miklos DB, Jacobson CA, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. *N Engl J Med*. (2017) 377(26):2531–44. doi: 10.1056/NEJMoa1707447

4. Berdeja JG, Madduri D, Usmani SZ, Jakubowiak A, Agha M, Cohen AD, et al. Ciltacabtagene autoleucel, a B-cell maturation antigen-directed chimeric antigen receptor T-cell therapy in patients with relapsed or refractory multiple myeloma (CARTITUDE-1): a phase 1b/2 open-label study. *Lancet*. (2021) 398(10297):314–24. doi: 10.1016/S0140-6736(21)00933-8

5. Munshi NC, Anderson LD Jr, Shah N, Madduri D, Berdeja J, Lonial S, et al. Idecabtagene vicleucel in relapsed and refractory multiple myeloma. *N Engl J Med*. (2021) 384(8):705–16. doi: 10.1056/NEJMoa2024850

6. Abramson JS, Palomba ML, Gordon LI, Lunning MA, Wang M, Arnason J, et al. Lisocabtagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. *Lancet*. (2020) 396(10254):839–52. doi: 10.1016/S0140-6736(20)31366-0

7. Kamdar M, Solomon SR, Arnason J, Johnston PB, Glass B, Bachanova V, et al. Lisocabtagene maraleucel versus standard of care with salvage chemotherapy followed by autologous stem cell transplantation as second-line treatment in patients with relapsed or refractory large B-cell lymphoma (TRANSFORM): results from an interim analysis of an open-label, randomised, phase 3 trial. *Lancet*. (2022) 399(10343):2294–308. doi: 10.1016/S0140-6736(22)00662-6

8. Fowler NH, Dickinson M, Dreyling M, Martinez-Lopez J, Kolstad A, Butler J, et al. Tisagenlecleucel in adult relapsed or refractory follicular lymphoma: the phase 2 ELARA trial. *Nat Med*. (2022) 28(2):325–32. doi: 10.1038/s41591-021-01622-0

9. Locke FL, Miklos DB, Jacobson CA, Perales MA, Kersten MJ, Oluwole OO, et al. Axicabtagene ciloleucel as second-line therapy for large B-cell lymphoma. *N Engl J Med*. (2022) 386(7):640–54. doi: 10.1056/NEJMoa2116133

10. Jacobson CA, Chavez JC, Sehgal AR, William BM, Munoz J, Salles G, et al. Axicabtagene ciloleucel in relapsed or refractory indolent non-hodgkin lymphoma (ZUMA-5): a single-arm, multicentre, phase 2 trial. *Lancet Oncol*. (2022) 23 (1):91–103. doi: 10.1016/S1470-2045(21)00591-X

11. Shah BD, Ghobadi A, Oluwole OO, Logan AC, Boissel N, Cassaday RD, et al. KTE-X19 for relapsed or refractory adult B-cell acute lymphoblastic leukaemia: phase 2 results of the single-arm, open-label, multicentre ZUMA-3 study. *Lancet*. (2021) 398(10299):491–502. doi: 10.1016/S0140-6736(21)01222-8

12. Wang M, Munoz J, Goy A, Locke FL, Jacobson CA, Hill BT, et al. KTE-X19 CAR T-cell therapy in relapsed or refractory mantle-cell lymphoma. *N Engl J Med*. (2020) 382(14):1331–42. doi: 10.1056/NEJMoa1914347

13. Spiegel JY, Patel S, Muffly L, Hossain NM, Oak J, Baird JH, et al. CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory B cell malignancies: a phase 1 trial. *Nat Med*. (2021) 27(8):1419–31. doi: 10.1038/s41591-021-01436-0

14. Brudno JN, Lam N, Vanasse D, Shen YW, Rose JJ, Rossi J, et al. Safety and feasibility of anti-CD19 CAR T cells with fully human binding domains in patients with B-cell lymphoma. *Nat Med*. (2020) 26(2):270–80. doi: 10.1038/s41591-019-0737-3

15. Shah NN, Johnson BD, Schneider D, Zhu F, Szabo A, Keever-Taylor CA, et al. Bispecific anti-CD20, anti-CD19 CAR T cells for relapsed B cell malignancies: a phase 1 dose escalation and expansion trial. *Nat Med*. (2020) 26(10):1569–75. doi: 10.1038/s41591-020-1081-3

16. Mackensen A, Müller F, Mougiakakos D, Böltz S, Wilhelm A, Aigner M, et al. Anti-CD19 CAR T cell therapy for refractory systemic lupus erythematosus. *Nat Med*. (2022) 28(10):2124–32. doi: 10.1038/s41591-022-02017-5

17. Siddiqi T, Soumerai JD, Dorritie KA, Stephens DM, Riedell PA, Arnason J, et al. Phase 1 TRANSCEND CLL 004 study of lisocabtagene maraleucel in patients with relapsed/refractory CLL or SLL. *Blood*. (2022) 139(12):1794–806. doi: 10.1182/blood.2021011895

18. Qi C, Gong J, Li J, Liu D, Qin Y, Ge S, et al. Claudin18.2-specific CAR T cells in gastrointestinal cancers: phase 1 trial interim results. *Nat Med*. (2022) 28(6):1189–98. doi: 10.1038/s41591-022-01800-8

19. Abou-El-Enein M, Elsallab M, Feldman SA, Fesnak AD, Heslop HE, Marks P, et al. Scalable manufacturing of CAR T cells for cancer immunotherapy. *Blood Cancer Discov*. (2021) 2(5):408–22. doi: 10.1158/2643-3230.BCD-21-0084

20. Levine BL, Miskin J, Wonnacott K, Keir C. Global manufacturing of CAR T cell therapy. *Mol Ther Methods Clin Dev*. (2017) 4:92–101. doi: 10.1016/j.omtm.2016.12.006

21. Yip A, Webster RM. The market for chimeric antigen receptor T cell therapies. *Nat Rev Drug Discov*. (2018) 17(3):161–2. doi: 10.1038/nrd.2017.266

22. Tran G, Zafar SY. Financial toxicity and implications for cancer care in the era of molecular and immune therapies. *Ann Transl Med*. (2018) 6(9):166. doi: 10.21037/atm.2018.03.28

23. Ganeeva I, Zmievskaya E, Valiullina A, Kudriaeva A, Miftakhova R, Rybalov A, et al. Recent advances in the development of bioreactors for manufacturing of adoptive cell immunotherapies. *Bioeng (Basel)*. (2022) 9(12):808. doi: 10.3390/bioengineering9120808

24. Kedmi M, Shouval R, Fried S, Bomze D, Fein J, Cohen Z, et al. Point-of-care anti-CD19 CAR T-cells for treatment of relapsed and refractory aggressive B-cell lymphoma. *Transplant Cell Ther*. (2022) 28(5):251–7. doi: 10.1016/j.jtct.2022.02.017

25. Fried S, Shouval R, Varda-Bloom N, Besser MJ, Yerushalmi R, Shem-Tov N, et al. Point-of-care CAR T-cell therapy as salvage strategy for out-of-specification tisagenlecleucel. *Leuk Lymphoma*. (2022) 63(14):3385–93. doi: 10.1080/10428194.2022.2123232

26. Syed F, El Fakih R, Alahmari AD, Osman Ali AS, Aljurf M. Chimeric antigen receptor structure and manufacturing of clinical grade CAR engineered cells using different bioreactors. *Hematol Oncol Stem Cell Ther*. (2022) 15(3):137–52. doi: 10.56875/2589-0646.1048

27. Lutfi F, Holtzman NG, Kansagra AJ, Mustafa Ali M, Bukhari A, Yan J, et al. The impact of bridging therapy prior to CD19-directed chimeric antigen receptor T-cell therapy in patients with large B-cell lymphoma. *Br J Haematol*. (2021) 195 (3):405–12. doi: 10.1111/bjh.17738

28. Ortiz de Landazuri I, Egri N, Muñoz-Sánchez G, Ortiz-Maldonado V, Bolaño V, Guijarro C, et al. Manufacturing and management of CAR T-cell therapy in "COVID-19's time": central versus point of care proposals. *Front Immunol*. (2020) 11:573179. doi: 10.3389/fimmu.2020.573179

29. Lam C, Meinert E, Yang A, Cui Z. Comparison between centralized and decentralized supply chains of autologous chimeric antigen receptor T-cell therapies: a UK case study based on discrete event simulation. *Cytotherapy*. (2021) 23(5):433–51. doi: 10.1016/j.jcyt.2020.08.007

30. Palani HK, Arunachalam AK, Yasar M, Venkatraman A, Kulkarni U, Lionel SA, et al. Decentralized manufacturing of anti CD19 CAR-T cells using CliniMACS prodigy(R): real-world experience and cost analysis in India. *Bone Marrow Transplant*. (2023) 58(2):160–7. doi: 10.1038/s41409-022-01866-5

31. Benjamin R, Graham C, Yallop D, Jozwik A, Mirci-Danicar OC, Lucchini G, et al. Genome-edited, donor-derived allogeneic anti-CD19 chimeric antigen receptor T cells in paediatric and adult B-cell acute lymphoblastic leukaemia: results of two phase 1 studies. *Lancet*. (2020) 396(10266):1885–94. doi: 10.1016/S0140-6736(20)32334-5

32. Maschan M, Caimi PF, Reese-Koc J, Sanchez GP, Sharma AA, Molostova O, et al. Multiple site place-of-care manufactured anti-CD19 CAR-T cells induce high remission rates in B-cell malignancy patients. *Nat Commun*. (2021) 12(1):7200. doi: 10.1038/s41467-021-27312-6

33. Baird JH, Frank MJ, Craig J, Patel S, Spiegel JY, Sahaf B, et al. CD22-directed CAR T-cell therapy induces complete remissions in CD19-directed CAR-refractory large B-cell lymphoma. *Blood*. (2021) 137(17):2321–5. doi: 10.1182/blood.2020009432

34. Wagner DL, Fritsche E, Pulsipher MA, Ahmed N, Hamieh M, Hegde M, et al. Immunogenicity of CAR T cells in cancer therapy. *Nat Rev Clin Oncol*. (2021) 18 (6):379–93. doi: 10.1038/s41571-021-00476-2

35. Depil S, Duchateau P, Grupp SA, Mufti G, Poirot L. "Off-the-shelf" allogeneic CAR T cells: development and challenges. *Nat Rev Drug Discov*. (2020) 19 (3):185–99. doi: 10.1038/s41573-019-0051-2

36. Fesnak A, Lin C, Siegel DL, Maus MV. CAR-T Cell therapies from the transfusion medicine perspective. *Transfus Med Rev*. (2016) 30(3):139–45. doi: 10.1016/j.tmrv.2016.03.001

37. Allen ES, Stroncek DF, Ren J, Eder AF, West KA, Fry TJ, et al. Autologous lymphapheresis for the production of chimeric antigen receptor T cells. *Transfusion*. (2017) 57(5):1133–41. doi: 10.1111/trf.14003

38. Ortíz-Maldonado V, Rives S, Castellà M, Alonso-Saladrigues A, Benítez-Ribas D, Caballero-Baños M, et al. CART19-BE-01: a multicenter trial of ARI-0001 cell therapy in patients with CD19(+) relapsed/refractory malignancies. *Mol Ther*. (2021) 29 (2):636–44. doi: 10.1016/j.ymthe.2020.09.027

39. Gee AP. GMP CAR-T cell production. *Best Pract Res Clin Haematol*. (2018) 31 (2):126–34. doi: 10.1016/j.beha.2018.01.002

40. Sommermeyer D, Hudecek M, Kosasih PL, Gogishvili T, Maloney DG, Turtle CJ, et al. Chimeric antigen receptor-modified T cells derived from defined CD8+ and CD4 + subsets confer superior antitumor reactivity in vivo. *Leukemia*. (2016) 30 (2):492–500. doi: 10.1038/leu.2015.247

41. Wang X, Jinrong Q, Jolanta S, Oriana BO, Anniesha H, Qing H, et al. 459. Evaluation of miltenyi ExpAct and TransAct CD3/28 beads for CAR-T cell manufacturing. *Mol Ther*. (2016):24. doi: 10.1016/S1525-0016(16)33268-3

42. Kochenderfer JN, Dudley ME, Feldman SA, Wilson WH, Spaner DE, Maric I, et al. B-cell depletion and remissions of malignancy along with cytokine-associated toxicity in a clinical trial of anti-CD19 chimeric-antigen-receptor-transduced T cells. *Blood*. (2012) 119(12):2709–20. doi: 10.1182/blood-2011-10-384388

43. Priesner C, Aleksandrova K, Esser R, Mockel-Tenbrinck N, Leise J, Drechsel K, et al. Automated enrichment, transduction, and expansion of clinical-scale CD62l(+) T cells for manufacturing of gene therapy medicinal products. *Hum Gene Ther*. (2016) 27(10):860–9. doi: 10.1089/hum.2016.091

44. Turtle CJ, Hanafi LA, Berger C, Gooley TA, Cherian S, Hudecek M, et al. CD19 CAR-T cells of defined CD4+:cD8+ composition in adult B cell ALL patients. *J Clin Invest*. (2016) 126(6):2123–38. doi: 10.1172/JCI85309

45. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med*. (2014) 371 (16):1507–17. doi: 10.1056/NEJMoa1407222

46. Li Y, Kurlander RJ. Comparison of anti-CD3 and anti-CD28-coated beads with soluble anti-CD3 for expanding human T cells: differing impact on CD8 T cell phenotype and responsiveness to restimulation. *J Transl Med*. (2010) 8:104. doi: 10.1186/1479-5876-8-104

47. Magnani CF, Gaipa G, Lussana F, Belotti D, Gritti G, Napolitano S, et al. Sleeping beauty-engineered CAR T cells achieve antileukemic activity without severe toxicities. *J Clin Invest*. (2020) 130(11):6021–33. doi: 10.1172/JCI138473

48. Sherba JJ, Hogquist S, Lin H, Shan JW, Shreiber DI, Zahn JD. The effects of electroporation buffer composition on cell viability and electro-transfection efficiency. *Sci Rep*. (2020) 10(1):3053. doi: 10.1038/s41598-020-59790-x

49. Micklethwaite KP, Gowrishankar K, Gloss BS, Li Z, Street JA, Moezzi L, et al. Investigation of product-derived lymphoma following infusion of piggyBac-modified CD19 chimeric antigen receptor T cells. *Blood*. (2021) 138(16):1391–405. doi: 10.1182/blood.2021010858

50. Abou-El-Enein M, Römhild A, Kaiser D, Beier C, Bauer G, Volk HD, et al. Good manufacturing practices (GMP) manufacturing of advanced therapy medicinal products: a novel tailored model for optimizing performance and estimating costs. *Cytotherapy*. (2013) 15(3):362–83. doi: 10.1016/j.jcyt.2012.09.006

51. Brentjens RJ, Rivière I, Park JH, Davila ML, Wang X, Stefanski J, et al. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood*. (2011) 118(18):4817–28. doi: 10.1182/blood-2011-04-348540

52. Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA, et al. Adoptive immunotherapy for indolent non-hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood*. (2008) 112 (6):2261–71. doi: 10.1182/blood-2007-12-128843

53. Kochenderfer JN, Wilson WH, Janik JE, Dudley ME, Stetler-Stevenson M, Feldman SA, et al. Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood*. (2010) 116(20):4099–102. doi: 10.1182/blood-2010-04-281931

54. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor–modified T cells in chronic lymphoid leukemia. *N Engl J Med*. (2011) 365(8):725–33. doi: 10.1056/NEJMoa1103849

55. Nicod C, da Rocha MN, Warda W, Roussel X, Haderbache R, Seffar E, et al. CAR-T cells targeting IL-1RAP produced in a closed semiautomatic system are ready for the first phase I clinical investigation in humans. *Curr Res Transl Med*. (2023) 71(2):103385. doi: 10.1016/j.retram.2023.103385

56. Kekre N, Hay KA, Webb JR, Mallick R, Balasundaram M, Sigrist MK, et al. CLIC-01: manufacture and distribution of non-cryopreserved CAR-T cells for patients with CD19 positive hematologic malignancies. *Front Immunol*. (2022) 13:1074740. doi: 10.3389/fimmu.2022.1074740

57. Rafiq QA, Twomey K, Kulik M, Leschke C, O'Dea J, Callens S, et al. Developing an automated robotic factory for novel stem cell therapy production. *Regen Med*. (2016) 11(4):351–4. doi: 10.2217/rme-2016-0040

58. Ramakrishnan S, Kumar J, Datta SS, Radhakrishnan V, Nair R, Chandy M. Should we adopt an automated de-centralized model of chimeric antigen receptor- T cells manufacturing for low-and middle-income countries? A real world perspective. *Front Oncol*. (2022) 12:1062296. doi: 10.3389/fonc.2022.1062296

59. Jackson Z, Roe A, Sharma AA, Lopes FBTP, Talla A, Kleinsorge-Block S, et al. Automated manufacture of autologous CD19 CAR-T cells for treatment of non-hodgkin lymphoma. *Front Immunol*. (2020) 11:1941. doi: 10.3389/fimmu.2020.01941

60. Anguille S, Kuipers I, Saevels K, Beguin Y, Muyden AV, Jacques C, et al. Initial clinical results of atalanta-1, a phase I/II trial of point-of-care manufactured GLPG5102 (19CP02) in RR NHL. *Blood*. (2022) 140:10323–4. doi: 10.1182/blood-2022-163830

61. Zhu F, Shah N, Xu H, Schneider D, Orentas R, Dropulic B, et al. Closed-system manufacturing of CD19 and dual-targeted CD20/19 chimeric antigen receptor T cells using the CliniMACS prodigy device at an academic medical center. *Cytotherapy*. (2018) 20(3):394–406. doi: 10.1016/j.jcyt.2017.09.005

62. Li A, Kusuma GD, Driscoll D, Smith N, Wall DM, Levine BL, et al. Advances in automated cell washing and concentration. *Cytotherapy*. (2021) 23(9):774–86. doi: 10.1016/j.jcyt.2021.04.003

63. Wang X, Riviere I. Clinical manufacturing of CAR T cells: foundation of a promising therapy. *Mol Ther Oncolytics*. (2016) 3:16015. doi: 10.1038/mto.2016.15

64. Kaiser AD, Assenmacher M, Schröder B, Meyer M, Orentas R, Bethke U, et al. Towards a commercial process for the manufacture of genetically modified T cells for therapy. *Cancer Gene Ther*. (2015) 22(2):72–8. doi: 10.1038/cgt.2014.78

65. Schweizer M, Merten OW. Large-scale production means for the manufacturing of lentiviral vectors. *Curr Gene Ther*. (2010) 10(6):474–86. doi: 10.2174/156652310793797748

66. Chen YH, Pallant C, Sampson CJ, Boiti A, Johnson S, Brazauskas P, et al. Rapid lentiviral vector producer cell line generation using a single DNA construct. *Mol Ther Methods Clin Dev*. (2020) 19:47–57. doi: 10.1016/j.omtm.2020.08.011

67. Powers AD, Drury JE, Hoehamer CF, Lockey TD, Meagher MM. Lentiviral vector production from a stable packaging cell line using a packed bed bioreactor. *Mol Ther Methods Clin Dev*. (2020) 19:1–13. doi: 10.1016/j.omtm.2020.08.010

68. Lin-Gibson S, Lin NJ, Jackson S, Viswanathan S, Zylberberg C, Wolfrum J, et al. Standards efforts and landscape for rapid microbial testing methodologies in regenerative medicine. *Cytotherapy*. (2021) 23(5):390–8. doi: 10.1016/j.jcyt.2020.07.006

69. Kiesgen S, Messinger JC, Chintala NK, Tano Z, Adusumilli PS. Comparative analysis of assays to measure CAR T-cell-mediated cytotoxicity. *Nat Protoc*. (2021) 16(3):1331–42. doi: 10.1038/s41596-020-00467-0

70. Vasu S, Alinari L, Szuminski N, Schneider D, Denlinger N, Chan WK, et al. A phase I clinical trial of point-of-care manufactured fresh anti-CD19/20/22 chimeric antigen receptor T cells for treatment of relapsed or refractory lymphoid malignancies (non-hodgkin lymphoma, acute lymphoblastic leukemia, chronic lymphocytic leukemia, B prolymphocytic leukemia). *Blood*. (2022) 140(Supplement 1):7474–5. doi: 10.1182/blood-2022-167340

71. Hu Y, Feng J, Gu T, Wang L, Wang Y, Zhou L, et al. CAR T-cell therapies in China: rapid evolution and a bright future. *Lancet Haematol*. (2022) 9(12):e930–41. doi: 10.1016/S2352-3026(22)00291-5

72. de Macedo Abdo L, Barros LRC, Saldanha Viegas M, Vieira Codeço Marques L, de Sousa Ferreira P, Chicaybam L, et al. Development of CAR-T cell therapy for B-ALL using a point-of-care approach. *Oncoimmunology*. (2020) 9(1):1752592. doi: 10.1080/2162402X.2020.1752592

73. The World Health organization: Universal Health Coverage.

74. Edoka IP, Stacey NK. Estimating a cost-effectiveness threshold for health care decision-making in South Africa. *Health Policy Plan*. (2020) 35(5):546–55. doi: 10.1093/heapol/czz152

75. Salzman R, Cook F, Hunt T, Malech HL, Reilly P, Foss-Campbell B. Addressing the value of gene therapy and enhancing patient access to transformative treatments. *Mol Ther*. (2018) 26(12):2717–26. doi: 10.1016/j.ymthe.2018.10.017

76. Jayaraman K. Cut-price CAR-T cell therapies top India's biotech agenda. *Nat Biotechnol*. (2019) 37(12):1388–9. doi: 10.1038/s41587-019-0346-1

Frontiers in Medicine

Translating medical research and innovation into improved patient care

A multidisciplinary journal which advances our medical knowledge. It supports the translation of scientific advances into new therapies and diagnostic tools that will improve patient care.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact